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Reduced Incidence and Severity of Collagen-Induced Arthritis in Mice Lacking IL-18[1]

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We have recently reported the presence and a potential proinflammatory role of IL-18 in the synovium of patients with rheumatoid arthritis. To obtain direct evidence that IL-18 plays an influential role in articular inflammation, we investigated the development of collagen-induced arthritis in a strain of mice lacking IL-18 (IL-18$^{-/-}$) of DBA/1 background. IL-18$^{-/-}$ mice developed markedly reduced incidence of arthritis compared with heterozygous or wild-type mice. Of the IL-18$^{-/-}$ mice that developed arthritis, the severity of the disease was significantly reduced compared with the intact mice. This was accompanied by reduced articular inflammation and destruction evident on histology. IL-18$^{-/-}$ mice also had significantly reduced Ag-specific proliferation and proinflammatory cytokine (IFN-γ, TNF-α, IL-6, and IL-12) production by spleen and lymph node cells in response to bovine type II collagen (CII) in vitro compared with wild-type mice, paralleled in vivo by a significant reduction in serum anti-CII IgG2a Ab level. Treatment with rIL-18 completely reversed the disease of the IL-18$^{-/-}$ mice to that of the wild-type mice. These data directly demonstrate a pivotal role of IL-18 in the development of inflammatory arthritis and suggest that antagonists to IL-18 may have therapeutic potential in rheumatic diseases. ***The Journal of Immunology,*** **2001, 166: 517–521.**

Rheumatoid arthritis (RA)[4] is a chronic inflammatory autoimmune disease associated with destruction of cartilage and underlying bone in the joints. There is considerable evidence supporting the crucial role for proinflammatory cytokines in RA pathogenesis (1–3). We recently demonstrated that IL-18 is present in RA synovium and that synergistic combinations of IL-18, IL-12, and IL-15 may be of importance in sustaining both Th1 responses and monokine production in RA (4, 5).

IL-18 acts in both acquired and innate immunity (6–8). It costimulates Th1 cells, activates NK cells to express the Fas ligand, and acts directly as a proinflammatory cytokine by inducing CC and CXC chemokines and IL-18 itself. IL-18 synergizes with IL-12 in the induction of Th1 cells by reciprocal regulation of receptors (9, 10). It also induces production of GM-CSF, IL-2, IL-12Rα, TNF-α, PGE_2, and inducible NO synthase by mononuclear and mesenchymal cells (11, 12).

To provide direct evidence for a role of IL-18 in inflammatory arthritis, we investigated the induction of collagen-induced arthritis (CIA) in IL-18$^{-/-}$ mice of the DBA/1 background. We report here that IL-18$^{-/-}$ mice developed markedly reduced incidence and severity of CIA compared with wild-type mice. This was accompanied in vitro by significantly reduced Ag-specific T cell proliferation and proinflammatory cytokine production by lymphoid cells from the IL-18$^{-/-}$ mice compared with control wild-type mice. These results provide definitive evidence that IL-18 plays a crucial role in the development and sustenance of inflammatory arthritis.

Materials and Methods

Mice

IL-18$^{-/-}$ mice were constructed as described previously (13). To generate mice with MHC haplotype susceptible to CIA, IL-18$^{-/-}$ mice were backcrossed into the DBA/1 strain. Fifth generation mice were used for experiments. Mice were typed by PCR (13) during backcrossing. Homozygous (IL-18$^{-/-}$) mice were obtained by intercrossing IL-18$^{+/-}$ DBA/1 mice and progeny littermates were used for experiments. Both wild-type and heterozygous mice were used as controls. All mice were 8–10 wk old at the time of immunization and were maintained at the Central Animal Facilities (University of Glasgow). All animals were cared for in accordance with the guidelines of the Home Office, U.K.

Induction of CIA

Male mice received 200 μg of bovine type II collagen (CII; Sigma, Poole, U.K.) in Freund's complete adjuvant (Difco, Detroit, MI) by intradermal injection (day 0). Collagen (200 μg in PBS) was given again on day 21 by i.p. injection. Mice were monitored daily for signs of arthritis, for which severity scores were derived as follows: 0, normal; 1, erythema; 2, erythema plus swelling; 3, extension/loss function, and total score, sum of four limbs. Paw thickness was measured with a dial caliper (Kroeplin, Munich, Germany). For histological assessment, mice were sacrificed and the hind limbs from arthritic IL-18$^{-/-}$ and wild-type mice were removed and fixed in 10% neutral-buffered Formalin, and 5-μm sections were stained with hematoxylin and eosin (Sigma). The quantification of arthritis was performed by two treatment-blinded observers as previously described (14). For reconstitution experiments, IL-18$^{-/-}$ or wild-type mice were immunized (day 0) and boosted (day 21) as above. The mutant mice were injected i.p. with 100 ng/mouse/day of rIL-18 (R & D Systems, Oxon, U.K.) for 8 days, starting from day 29. Disease score was recorded as above. T cell proliferation, cytokine synthesis, and Ab production were also analyzed at the end of experiment (days 37 and 55).

Cell culture

Spleen and draining lymph node cells were cultured at 2×10^6 cells/ml for up to 96 h in medium (RPMI 1640; Life Technologies/BRL, Paisley, Glasgow, U.K.) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES buffer, and 10% FCS (all Life

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[4] Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; CII, type II collagen.

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Technologies/BRL)) at 37°C in 5% CO_2. Cells were stimulated with graded concentrations of CII. Proliferation assays were performed in triplicate in U-bottom 96-well plates (Nunc, Roskilde, Denmark) as previously described (5). Supernatants from parallel triplicate cultures were stored at −70°C until estimation of cytokine content by ELISA.

Cytokine assays

Murine TNF-α, IFN-γ (R&D Systems), IL-4, IL-6, IL-10, and IL-12 (p40 + p70) (PharMingen, San Diego, CA) were assayed by ELISA using paired Abs according to the manufacturer's instructions. Lower limits of detection were as follows: IL-4, IL-6, IL-12, and TNF-α all at 10 pg/ml and IL-10 and IFN-γ at 80 pg/ml.

Anti-collagen Ab ELISA

Serum anti-collagen II Ab titers were measured by ELISA. Briefly, 96-well plates (Maxisorb; Nunc) were coated with CII (2 μg/ml in 0.1 M NaH_2CO_3) overnight at 4°C, blocked, and serial dilutions of sera were added. Bound IgG1/2a was detected with biotin-conjugated anti-mouse IgG1 or IgG2a (PharMingen), respectively, and developed as previously described (5). Plates were read at 630 nm.

Statistical analysis

This was performed using Minitab software for Macintosh (Cupertino, CA). Clinical and histological scores were analyzed with the nonparametric Mann-Whitney *U* test. Differences between cumulative incidences at a given time point were analyzed by the χ^2 contingency analysis. Cytokine and collagen-specific IgG levels were compared using Student's *t* test. Bonferroni's correction for multiple comparison was applied to Fig. 1.

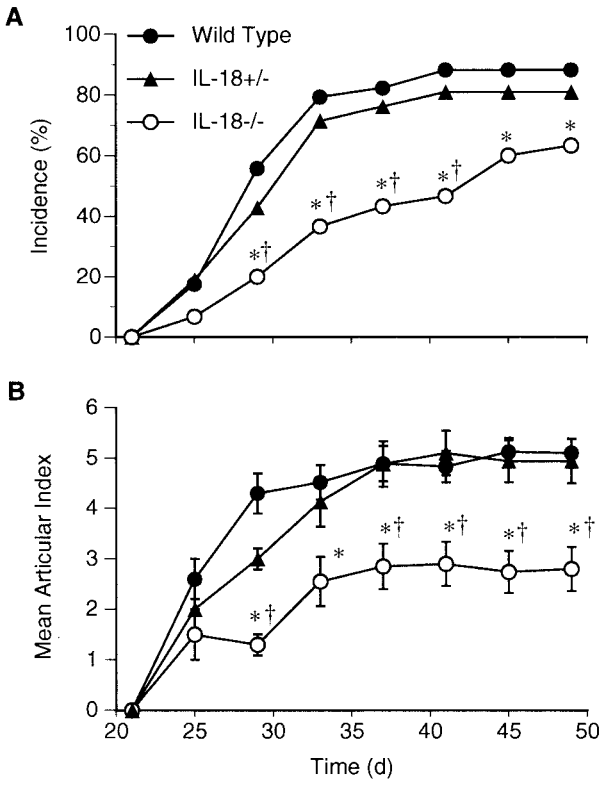

FIGURE 1. IL-18$^{-/-}$ mice developed markedly reduced incidence (*A*) and severity (*B*) of CIA. Wild-type (n = 34), heterozygous (n = 21), or IL-18$^{-/-}$ (n = 30) mice were immunized (day 0) and boosted (day 21) with CII. Mice were monitored daily for signs of arthritis. IL-18$^{-/-}$ mice developed significantly less severe disease compared with +/+ and +/− mice, which had disease pattern indistinguishable from each other. *, $p <$ 0.01 vs +/+ mice; †, $p < 0.05$ compared with +/− mice. Differences between cumulative incidences at a given time point were analysis by the χ^2 contingency analysis and the mean articular index was analyzed with the Mann-Whitney *U* test. Bonferroni's correction for multiple comparison was applied when compared between different groups of mice.

Results and Discussion

To compare the development of CIA in IL-18$^{-/-}$ and their heterozygous and wild-type littermates, mice were immunized with CII in CFA on day 0 and boosted with CII in PBS on day 21. Wild-type (n = 34) and heterozygous (n = 21) mice developed inflammatory articular disease indistinguishable from each other. Joint swelling was evident from day 25 and up to 90% of mice developed arthritis (Fig. 1). In contrast, IL-18$^{-/-}$ mice (n = 30) developed markedly reduced incidence of articular disease compared with wild-type mice (Fig. 1*A*). Of the IL-18$^{-/-}$ mice that developed articular inflammation, the severity of the disease was significantly reduced ($p < 0.01$) compared with involved wild-type mice (Fig. 1*B*). This reduced severity in the IL-18$^{-/-}$ mice was sustained throughout the chronic phase of arthritis. These results demonstrate that IL-18 is not only required for the initiation of the articular disease, it also sustains the inflammatory process characteristic of CIA.

To determine whether the lack of IL-18 altered erosive articular disease, histological examination of the hind limb joints was performed on day 37. As expected, the arthritic wild-type mice revealed extensive mononuclear and polymorphonuclear infiltration of the synovial membrane with synovial hyperplasia and adjacent cartilage and bone erosion. In contrast, the mutant mice show only a mild disease which was markedly less severe compared with that of the wild-type mice (Fig. 2).

We next investigated immunological parameters subserving differential pathological response. To compare the expression of cytokines in serum, arthritic mice were selected from homozygous and wild-type mice during the acute phase (day 37) of the disease. IL-4 and IL-5 were not detected. In contrast, high levels of IFN-γ, TNF-α, and IL-6 were detected in wild-type mice. These were present as significantly reduced concentrations in the IL-18$^{-/-}$ mice (Table I). IL-12 (p40/p70) was not detected whereas IL-10 was present only at low levels and was comparable in both groups (Table I). We also determined anti-collagen Ab production. Collagen-specific IgG2a Abs, which are typically produced during Th1 response, were significantly lower ($p < 0.02$) in the IL-18$^{-/-}$

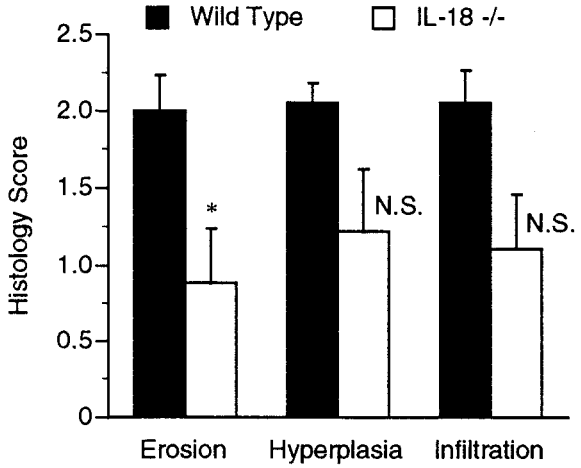

FIGURE 2. IL-18$^{-/-}$ mice developed significantly reduced articular inflammation and destruction compared with wild-type mice. Hind paws were removed (day 37) from arthritic mice, fixed, decalcified, and hematoxylin and eosin sections prepared. Histological appearance was scored for the presence of synovial bone erosion, hyperplasia, and cellular infiltration as described previously (14). The pathological changes differed significantly between IL-18$^{-/-}$ and wild-type mice. (*, $p < 0.05$, Mann-Whitney *U* test). Data are means ± SEM (n = 10).

Table I. *IL-18 $^{-/-}$ have reduced levels of proinflammatory cytokines in serum*[a]

Cytokine (pg/ml)	Wild Type	IL-18$^{-/-}$	*p*
IFN-γ	336.7 ± 64.6	121.1 ± 18.4	<0.01
TNF-α	605.3 ± 173.1	200.1 ± 29.0	<0.01
IL-6	427.1 ± 53.4	193.5 ± 51.4	<0.01
IL-10	29.6 ± 5.1	27.7 ± 8.3	NS

[a] Serum cytokine concentrations in wild type and IL-18$^{-/-}$. Sera were obtained from mice on day 37 and cytokine levels were measured by ELISA. Results are the means ± SD (six mice per group). $p < 0.01$ (wild-type vs IL-18$^{-/-}$, Student's *t* test).

mice compared with wild-type mice while IgG1 Abs remained at similar levels (Fig. 3).

We next sought further evidence for an altered immune response in mice lacking IL-18 by culturing spleen and draining lymph node cells from IL-18$^{-/-}$ and wild-type mice with equivalent articular indices at day 37 or day 55. Cells were cultured with graded concentrations of CII and T cell proliferation and cytokine production were determined. Cells from arthritic wild-type mice proliferated vigorously in response to CII in a dose-dependent manner. This response was significantly reduced in the IL-18$^{-/-}$ mice (Fig. 4). Cells from the arthritic IL-18$^{-/-}$ mice also produced markedly less IFN-γ, TNF-α, IL-6, and IL-12 compared with similarly treated cells from the arthritic wild-type mice in both the acute and the chronic phases of the disease (Fig. 4). IL-4 and IL-10 were not detectable in the culture supernatant of both groups of mice. Together, these results clearly demonstrate that IL-18 is required for the induction of the optimal production of proinflammatory cytokines during arthritic disease.

We next investigated whether the reduced articular inflammation in IL-18$^{-/-}$ mice compared with wild-type mice could be reversed by the administration of recombinant IL-18. Mice were primed and boosted with CII as above. IL-18$^{-/-}$ mice were then injected i.p. with 100 ng/mouse/day of murine rIL-18 for 8 days starting day 29, a dose which was previously shown to serve as an adjuvant to induced collagen arthritis in male DBA/1 mice (5). As expected, IL-18$^{-/-}$ mice developed significantly less severe disease compared with wild-type mice. However, this was completely reversed in both incidence and severity by the administration of rIL-18 (Fig. 5). Furthermore, IL-18$^{-/-}$ mice replenished with IL-18 had a cytokine profile (by day 37) indistinguishable from that of the wild-type mice (data not shown).

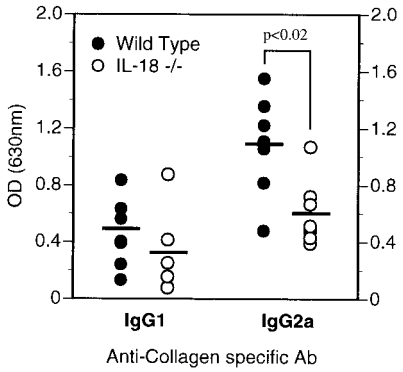

FIGURE 3. Serum anti-collagen Ab concentrations. Anti-collagen IgG1 and IgG2a Ab levels were measured on day 55 by ELISA. Data are expressed as individual serum measurement (seven mice per group) and are expressed as the mean absorbance (OD, 630 nm). Bar indicates median Ab concentrations per group, $p < 0.02$ (IL-18 wild type vs $^{-/-}$, Student's *t* test).

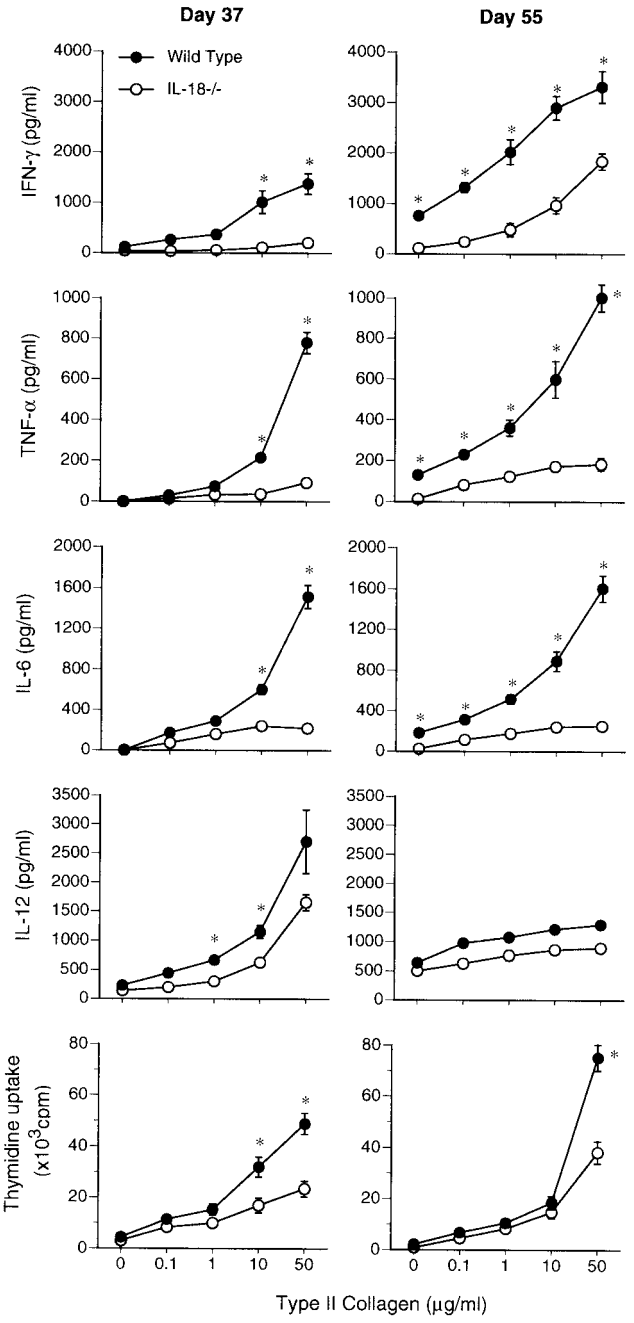

FIGURE 4. IL-18$^{-/-}$ mice produced significantly less proinflammatory cytokines and had reduced T cell proliferation to CII in vitro compared with wild-type mice. Spleen and draining lymph node cells were harvested on day 37 or day 55 and cultured with graded concentrations of CII for 96 h. Cytokine concentrations in the culture supernatant were determined by ELISA after 72 h. T cell proliferation was assayed by uptake of [^{3}H]thymidine after 96 h. *, $p < 0.05$, Student's *t* test. Data are means ± SD ($n = 3$).

Using IL-18$^{-/-}$ mice, a critical role of IL-18 in infectious diseases has been identified (13, 15). However, the role of IL-18 in autoimmune disease has yet to be clarified. Elevated levels of IL-18 message and protein have been identified in several inflammatory diseases, including inflammatory bowel disease (16, 17) and RA (4). In the animal model of diabetes (nonobese diabetic mice), IL-18 gene expression was up-regulated (18). However, administration of rIL-18 inhibited diabetes development presumably by counterregulation of Th1-mediated destructive insulitis (19).

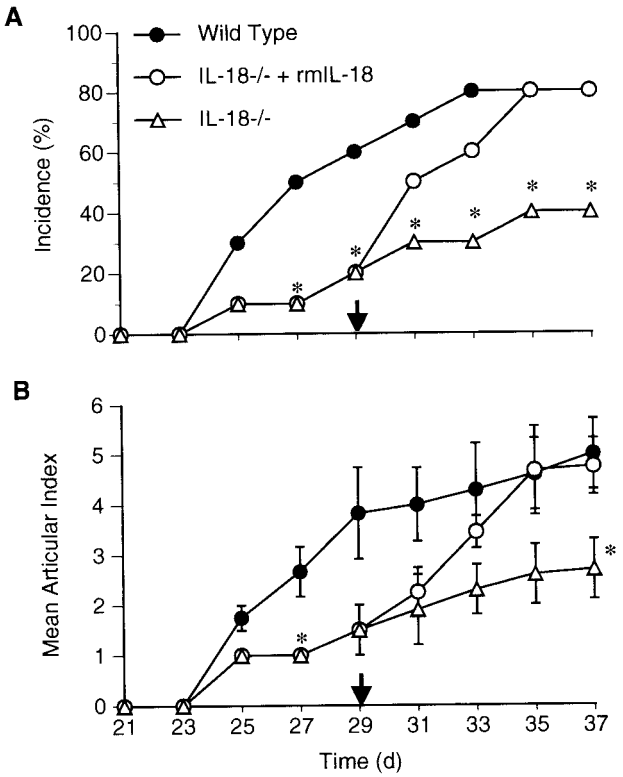

FIGURE 5. The reduced incidence (*A*) and severity (*B*) of arthritis in IL-18$^{-/-}$ mice was completely reversed to that of the wild-type mice by treatment with rIL-18. IL-18$^{-/-}$ and wild-type mice were immunized and boosted with CII as described in *Materials and Methods*. Some of the IL-18$^{-/-}$ mice were injected i.p. with 100 ng/mouse/day of IL-18 for 8 days, starting on day 29 (arrows). Differences between cumulative incidences at a given time point were analysis by the χ^2 contingency analysis and the mean articular index was analyzed with the Mann-Whitney *U* test. $*$, $p < 0.05$. Data are means ± SEM ($n = 12$).

We now provide here direct evidence that mice lacking IL-18 developed markedly reduced incidence and severity of CIA.

CIA represents an ideal system to explore the diverse inflammatory effects of IL-18 in an inducible autoimmune model. CIA has proven a useful surrogate in which to investigate the role of cytokines in RA. Th1 responses are generally implicated in the pathogenesis of RA (20). Furthermore, proinflammatory cytokines including IL-12, IL-6, TNF-α, and IFN-γ play a pathogenic role in CIA (21–24). However, the role of IL-12 and IFN-γ is complex. The levels of IL-12 and IFN-γ induced during CIA are variable and their effects on CIA development are dependent on the time of administration (21, 23). Our data clearly demonstrate that IL-18 is required for the optimal induction of Th1 cells and proinflammatory cytokines. Although we have not measured the level of IL-18 in mice with CIA, we have previously shown that IL-18R is present on macrophages of RA patients and that IL-18 can induce production of proinflammatory cytokines, particularly TNF-α in vitro (4). Hence, IL-18 likely mediates inflammatory arthritis not only by enhancing Th1 activity but also by directly inducing proinflammatory cytokine production from diverse cell types of the innate immune system. We have earlier demonstrated an important role for IL-15 in RA (25–27). IL-12 has also been implicated to contribute to the induction and manifestation of CIA (21). The fact that IL-18$^{-/-}$ mice still developed CIA, albeit at a significantly reduced level compared with intact mice, suggests that RA pathogenesis likely involves a number of proinflammatory cytokines, including IL-12, IL-15, and IL-18, which synergistically or independently activate transcription factors such and NF-κB (28).

A major feature of our finding is the sustained reduction of chronic inflammatory response in arthritic IL-18$^{-/-}$ mice compared with intact mice. IL-18 is known not to activate Th1 cell differentiation alone, but synergizes with IL-12 in the expansion of Th1 cells (29, 30). However, recent reports also indicate that IL-18 can also activate naive CD4$^+$ cells along the Th2 pathway (Ref. 31 and our unpublished data). Nevertheless, differentiated Th2 cells lost their IL-18R (9). Thus, IL-18 not only plays a crucial role in inducing proinflammatory cytokines which are likely responsible for the pathological destruction of the joint in RA, it is also required for the sustained Th1 response and hence chronic inflammation in this and other related arthritic diseases. Our results provide direct evidence for a proinflammatory role of IL-18 in arthritis. Antagonists of IL-18 are therefore likely of considerable therapeutic potential against this pervasive clinical disability.

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