Enhanced Neutrophil Extravasation and Rapid Progression of Proteoglycan-Induced Arthritis in TSG-6–Knockout Mice

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Objective. To gain insight into the mechanisms of the antiinflammatory effect of tumor necrosis factor α (TNFα)–induced protein 6 (Tnfp6) in arthritis, using Tnfip6-deficient animals.

Methods. TNFα-stimulated gene 6 (TSG-6) coding for Tnfp6 was disrupted. Tnfp6-deficient mice were backcrossed into proteoglycan-induced arthritis (PGIA)–susceptible BALB/c mice, and arthritis was induced by systemic immunization with cartilage proteoglycan (PG). Thioglycollate-induced sterile peritonitis was also assessed, to monitor the early events of neutrophil extravasation in wild-type and Tnfp6-deficient mice in the presence or absence of treatment with recombinant murine Tnfp6.

Results. The onset of PGIA was similar, but progression and severity were significantly greater, in Tnfp6-deficient mice compared with wild-type BALB/c mice. However, this was not associated with enhanced T or B cell responses to cartilage PGs, but rather, an early and more extensive infiltration of the synovium with neutrophil leukocytes was the most prominent histopathologic feature of PGIA in Tnfp6-deficient mice. This was accompanied by elevated serum levels of interleukin-6 and amyloid A, and significantly increased activities of the enzymes plasmin, myeloperoxidase, and neutrophil elastase in the inflamed paw joints of Tnfp6-null mice, when compared with that of the wild-type littermates. Loss of control over several components of inflammation resulted in extensive and rapid cartilage degradation, bone erosion, joint ankylosis, and deformities in Tnfp6-null animals. In support of the antiinflammatory effect of Tnfp6 via the inhibition of polymorphonuclear (PMN) cell efflux, neutrophil invasion during thioglycollate-induced peritonitis was 2-fold higher in Tnfp6-deficient animals than in wild-type animals, but was dramatically suppressed by intravenous injection of recombinant murine Tnfp6.

Conclusion. Tnfp6 is a multifunctional antiinflammatory protein that is produced at the site of inflammation and can be retained by the hyaluronan-rich extracellular matrix. A major effect of Tnfp6 is the inhibition of the extravasation of PMN cells, predominantly neutrophils, into the site of inflammation, most likely via a CD44/hyaluronan/Tnfip6-mediated blocking mechanism.

Tumor necrosis factor α (TNFα)–induced protein 6 (Tnfp6), the secreted product of TNFα-stimulated gene 6 (TSG-6), is a member of the hyaladherin superfamily of hyaluronan (HA) binding proteins (1,2). The constitutive expression of Tnfp6 is very low, but virtually all cell types of mesenchyme origin can produce Tnfp6 in response to proinflammatory stimuli (2–5). Tnfp6 can be detected in large quantities in synovial fluid samples from inflamed joints, but not in normal healthy joints (3). Expression of Tnfp6 is up-regulated in the inflamed synovial tissue of patients with rheumatoid arthritis or osteoarthritis (5), indicating that the in vivo production of this protein requires a milieu enriched in proinflammatory mediators.

Recombinant murine Tnfp6 (rMuTnfp6) has been shown to have a therapeutic effect on both collagen-induced arthritis (6) and proteoglycan-induced arthritis (PGIA) (7), and exhibits antiinflammatory and chondroprotective properties in antigen-induced arthritis (7). A chondroprotective effect could also be
achieved in antigen-induced arthritis by cartilage-specific constitutive expression of the Tnflp6 transgene (8).

Tnflp6 forms a stable complex with inter-α-trypsin inhibitor (IαI) (9,10), a major serine protease inhibitor in serum, and potentiates the inhibitory activity of IαI against plasmin. The inhibitory effect of the Tnflp6–IαI complex appears to be specific for plasmin, since no increase was observed in the inhibitory activity against other serine proteases (10). Since plasmin is a key activator of matrix-degrading metalloproteases, it has been postulated that Tnflp6 exerts its antiinflammatory and chondroprotective effects through the inactivation of the metalloprotease network (7,9,10).

To further elucidate the antiinflammatory properties of Tnflp6, an air-pouch model of acute inflammation was used. Injection of recombinant human Tnflp6 (rHuTnflp6) together with a stimulant (carrageenan or zymosan) into the air pouch of mice resulted in significant reductions in the number of emigrated neutrophils, as compared with air pouches injected with the stimulant only (10,11). With the use of mutant forms of rHuTnflp6, which has a reduced ability to bind HA via its Link module, it has been shown that most of the antiinflammatory activity of Tnflp6 resides within its Link module domain (11).

The primary objective of the present study was to determine whether the lack of Tnflp6 increases the predisposition of mice to develop a systemic autoimmune form of experimentally induced arthritis such as PGIA. For this purpose, we backcrossed Tnflp6-deficient mice (also referred to as Tnflp6-null, Tnflp6−/−, and Tnflp6-knockout mice) (originally created in the 129Sv/C57BL/6 mixed background) into BALB/c, a strain that exhibits genetic susceptibility to Tnflp6 deficiency in the 129Sv/C57BL/6 mixed background) into BALB/c, a strain that exhibits genetic susceptibility to Tnflp6 deficiency in the 129Sv/C57BL/6 mixed background). The Tnflp6 mutant colony has been maintained by intercrossing Tnflp6−/− females with Tnflp6−/− males, and has been expanded to generate sufficient numbers of Tnflp6-deficient (Tnflp6−/−) and wild-type (Tnflp6+/+) littermates for all experiments. The animal procedures were approved by the Animal Care and Use Committee of Rush University Medical Center. All mice were housed under standard conditions.

Enzyme-linked immunosorbent assays (ELISAs) for rat Tnflp6 and Tnflp6. Recombinant murine Tnflp6 was purified on an HA-coupled EAH-Sepharose column from supernatants of Chinese hamster ovary cells (CHO-K1; American Type Culture Collection, Manassas, VA) that were stable transfected with the Lonza pEEl14.1 vector (Lonza Biologics, Slough, Berkshire, UK) containing a full-length (1,654 bp) mouse Tnflp6 complementary DNA clone as described previously (7). Further purification was performed using reverse-phase high-pressure liquid chromatography as described for rHuTSG-6/rHuTNFIP6 (18). The purified product was then lyophilized and dissolved in phosphate buffered saline (PBS) (pH 7.4) at 1 mg/ml concentration. For in vivo treatment of mice, the rMuTnflp6 solution was sterilized by passing it through a 0.2-μm–pore-size sterile-syringe filter.

The rat monoclonal antibody (mAb), A38, recognizing the Link module domains of both human and mouse Tnflp6, has been described previously (19). A sandwich ELISA was developed using purified A38 mAb for capture, and biotinylated TSG-6–CR21 polyclonal rabbit antibody (7,8) was used for detection of Tnflp6 in serially diluted serum samples. Peroxidase-conjugated streptavidin (Zymed, San Francisco, CA) followed by o-phenylenediamine and hydrogen peroxide were used for the colorimetric detection of plate-bound Tnflp6, and rMuTnflp6 served as a reference standard.

Materials and methods

Generation of Tnflp6-null mice. Tnflp6 is a 20.3-kb–long gene on mouse chromosome 2 (4). Tnflp6-deficient mice were generated by disrupting exon 1 with the Neo-poly(A) cassette, creating a translation stop codon at 94 bp downstream of the translation start site in the Tnflp6 gene (15). As described earlier, the only phenotype abnormality in Tnflp6-deficient mice is the infertility of the Tnflp6−/− females (15). A marker-assisted speed-backcrossing protocol, heterozygous (Tnflp6+/−) males were mated with wild-type BALB/c females. Males were genotyped for the presence of the Neo gene; the heterozygous males were further tested for genomic composition using a total of 147 simple sequence-length polymorphic markers (16,17). Tnflp6+/− males with the most extensive BALB/c background were selected for subsequent backcrosses. This process was repeated 6 times until homogeneity of the BALB/c genome was achieved, and only a small region on chromosome 2 (harboring the Tnflp6 gene) was a non-BALB/c locus.

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and emulsified with 1 mg of the adjuvant dimethyldioctadecyl-
ammonium bromide (Sigma-Aldrich, St. Louis, MO) as previ-
ously described (14,21). The same doses of antigen and
adjuvant were injected on days 21 and 42, and, if necessary, on
day 63.

The paws of all immunized mice were examined twice a
week until day 21, and thereafter examined daily to record
abnormalities due to arthritic changes of the joints. The first
appearance of joint swelling was recorded as the day of the
onset of arthritis. A standard scoring system, based on the
presence of swelling and redness and having a range of 0–4 for
each paw (thus resulting in a possible maximum score of 16 for
each animal), was used for the assessment of disease severity
(12,14,22,23). Ankylosis in the peripheral joints (knee and
ankle) was defined as reduced joint mobility due to bone
deforrmities and periarticular stiffness.

Nonimmunized and PG-immunized arthritic wild-type and
Tnfip6−/− mice were killed, and the limbs were photo-
graphed and subjected to radiographic analysis using a Hewlett
Packard Faxitron X-ray System (85 KV for 18 seconds, model
43855A; Hewlett Packard, McMinnville, OR) and high-
resolution Kodak X-Omat film (Eastman Kodak, Rochester,
NY). The limbs were then dissected, fixed, decalcified, embed-
ded in paraffin, and sectioned. Tissue sections were stained
with hematoxylin and eosin for histopathologic examination.

Measurements of antigen-specific T cell responses,
antibodies, and cytokines. During the immunization period
(once a week) and at the end of the experiments, blood
samples (for serum) were collected from the retroorbital
venous plexus of immunized mice. Spleen cells were harvested
at the end of the experiments. PG-specific antibodies in serum
were measured using ELISA, as described previously
(16,17,23). Briefly, 96-well Maxisorp plates (Nunc Interna-
tional, Hanover Park, IL) were coated with human or mouse
cartilage PG (0.1 μg protein/well), the free binding sites were
blocked with 1% fat-free milk in PBS, and PG-specific anti-
bodies were measured in serially diluted serum samples of
PG-immunized mice. The total serum concentrations of anti-
PG antibodies (IgG, IgM, and IgA) were determined using
peroxidase-conjugated goat anti-mouse IgG, IgM, and IgA
antibodies (Accurate Chemical & Scientific, Westbury, NY),
and PG-specific IgG isotypes were detected with rat mAb to
mouse IgG1 or IgG2a (Zymed) as secondary reagents (16,23).
Serum antibody levels were calculated relative to the concen-
trations of the corresponding mouse IgG isotype standards (all
drawn from Zymed) or total mouse serum Ig fractions (Sigma-
Aldrich) applied to the ELISA plates (17,23).

Antigen-specific T cell responses were determined in
quaduplicate samples of spleen cells (3 × 10⁶ cells/well)
cultured in 96-well plates in the presence of 25 μg PG
protein/ml culture medium. Antigen-specific interleukin-2
(IL-2) production was measured in the culture supernatants 48
hours later, using the CTLL-2 biotssay, and T cell proliferation
was assessed on day 5 by incorporation of ³H-thymidine
(16,17,20,23). The antigen-specific T cell response was
expressed as the stimulation index, a ratio of incorporated
³H-thymidine (in counts per minute) in antigen-stimulated
spleen cell cultures relative to the cpm measured in nonstimu-
lated cultures. Antigen-specific production of interferon-γ and
IL-4 was measured in cell culture supernatants (3 × 10⁶
cells/ml) on day 4 using capture ELISA methods (BD Pharm-
genin, San Diego, CA, or R&D Systems, Minneapolis, MN) as
previously described (16,17,21). IL-1β, IL-6, and TNFα were
also measured in the serum samples of PG-immunized ani-
mals. Paired mAb and cytokine standards for ELISA were
obtained from BD Pharmingen or R&D Systems, and the
mouse serum amyloid A (SAA) ELISA kit was purchased from
BioSource International (Camarillo, CA).

Determination of the activities of enzymes (plasmin,
myeloperoxidase, and neutrophil elastase) in joint tissue
extracts. Nonarthritic or arthritic hind paws of the animals
were snap-frozen in liquid nitrogen and stored at −80°C. The
frozen paws were homogenized in PBS containing 0.5%
hexadecyl-trimethylammonium bromide, 0.1% sodium dode-
cyl sulfate, and 1% Nonidet P40 (all purchased from Sigma-
Aldrich), sonicated on ice, and cleared by centrifugation.
An assay for plasmin activity was performed using the chromo-
genic substrate tosyl-Gly-Pro-Lys-4-nitranilide acetate (Chro-
mozy PL; Roche, Mannheim, Germany) (10,11).

Neutrophil extravasation in thioglycollate-induced
peritonitis in wild-type and Tnfip6-deficient mice, with or
without treatment with rMuTnfip6. Peritonitis was induced by
intraperitoneal injection of 0.5 ml of 4% sterile thioglycollate
(Becton-Dickinson, Sparks, MD) as described previously
(26,27). Peritoneal lavage was performed 0, 2, 4, 6, 8, 12, 24, 48,
and 96 hours after thioglycollate administration. Ten
milliliters of ice-cold PBS was injected into the peritoneal
cavity of each mouse, and the abdomen was massaged for 2
minutes, after which 4 ml of the lavage fluid was collected.

Leukocytes in the fluid samples were stained with 0.2%
crystal violet (Sigma-Aldrich) in 10% acetic acid, and then
counted in a hemocytometer; total cell numbers were calcu-
lated for the 10-ml volume of PBS injected intraperitoneally.
Leukocyte differential counts were determined in Giemsa-
stained smears of the cell pellets. The cellular composition of
the peritoneal lavage fluid was confirmed by flow cytometry
using fluorochrome-labeled mAb to CD45/B220 (B cells), CD3
(T cells), and Gr-1 (granulocytes) (all from BD Pharmingen)
and mAb F4/80 to macrophages/monocytes (purchased from
Serotec, Raleigh, NC) (21). Separate groups of wild-type and
Tnfip6-deficient mice were injected intravenously with 30 μg
rMuTnfip6 in 60 μl PBS 20 minutes before intraperitoneal
thioglycollate administration, and the effect of rMuTnfip6
treatment on the cell counts, as well as the types of recruited
leukocytes in the peritoneal lavage fluid, were determined at 0,
Statistical analysis. Statistical analysis was performed using SPSS software, version 7.5 (SPSS, Chicago, IL). The Mann-Whitney and Wilcoxon tests, or the Student’s t-test, were used for comparisons between wild-type and Tnfip6-deficient mice. The level of significance was set at a P value less than 0.05.

RESULTS

Increased incidence and severity of PGIA in Tnfip6-deficient BALB/c mice. Tnfip6-deficient mice and wild-type littermates were monitored for the development of arthritis for 11–15 weeks after the primary immunization with PG. Clinical signs of arthritis first appeared between experimental days 47 and 49 (5–7 days after the third PG injection) in both groups (Figure 1). The incidence of PGIA increased more rapidly in the Tnfip6-null mice than in the wild-type mice, and by day 71 (8 days after the fourth PG injection), all of the mutant mice were arthritic, whereas the incidence of arthritis was only 62% in the wild-type group at this time point (Figure 1A). The disease progressed more rapidly and the arthritic limbs exhibited higher inflammation scores in the Tnfip6−/− mice than in the wild-type littermates throughout the entire observation period (Figure 1B). However, there were no differences in disease onset, severity, or incidence between the males and females in either group.

Gross morphology, results of histopathology, and radiographic appearance of the peripheral joints. Histopathologic examination of the hind paw joint sections prepared at the early phase of joint inflammation (2 days after onset) revealed more extensive infiltration of the synovial lining by polymorphonuclear (PMN) cells (mostly neutrophils) and a much higher number of neutrophils in the joint exudates of Tnfip6-null mice compared with wild-type mice (Figures 2A and B). On day 10 after the onset of PGIA, the histologic assessment showed evidence of inflammatory damage in only the upper zone of articular cartilage in the joints of wild-type mice (Figure 2C), whereas the cartilage had been completely destroyed in the joints of Tnfip6-deficient mice at this time point (Figure 2D).

As expected from the results of our earlier studies that demonstrated a chondroprotective effect of intraarticularly injected rMuTnfip6 (7), the articular cartilage was not protected from the inflammatory attack in mice lacking endogenous Tnfip6. There was no evidence of cartilage loss in the absence of inflammation in Tnfip6-knockout mice (e.g., in the nonarthritic joints of PG-immunized animals [results not shown]), indicating that cartilage destruction did not occur spontaneously, but was triggered by the inflammatory process. Only 10 days after the first appearance of joint swelling, Tnfip6-deficient animals showed histologic signs of reactive fibrosis at the joint margins (Figure 2D), a presumed repair response that usually occurs in wild-type BALB/c mice at a late stage of chronic PGIA, i.e., several weeks after the onset of arthritis (14).

As a result of the rapid progression and increased
severity of PGIA in Tnfip6-deficient animals, massive ankylosis developed 5–7 weeks earlier than in the wild-type littermates. The degree of ankylosis and the extent of joint deformities in Tnfip6-knockout mice far exceeded those ever observed in wild-type BALB/c mice (see Figures 3D and G). The extremely aggressive character of PGIA in Tnfip6-deficient animals was reflected in the high degree of pathologic changes that occurred in the joint shape and in the structure and density of the subchondral bone (e.g., in the metatarsophalangeal joints), as revealed by macroscopic inspection, histology, and radiographic analysis, respectively (Figure 3).

**Immune responses and cytokine profiles in PG-immunized Tnfip6-deficient and wild-type BALB/c mice.** PGIA is a systemic disease initiated and governed by autoreactive T cell responses and autoantibodies against the mouse (self) cartilage PG (13,14,20). Therefore, it was important to determine whether the dramatic differences in arthritis severity and in the rate of disease progression between wild-type and Tnfip6-deficient mice (as shown in Figures 1–3) were associated with a more aggressive autoimmune response and/or the production of related cytokines in BALB/c mice lacking Tnfip6. T and B cell responses and cytokine levels were examined at 3 different time points during disease induction and development: in the period prior to onset of arthritis (a few days after the third antigen injection) when the animals were still asymptomatic (Figure 1), at the acute phase (2–3 days after the onset of arthritis), and during the chronic phase (>4 weeks after the onset of arthritis). We did not find significant differences
between the wild-type and Tnfip6-deficient animals in PG-specific T or B cell responses at any of those time points (test results on day 81 are shown in Figure 4). Stimulation of T cells (Figure 4A) and production of antigen-specific Th1- and Th2-type cytokines (Figure 4B), heteroantibodies (against human PG) (Figure 4C), and autoantibodies (against mouse PG) (Figure 4D) were all comparable in the 2 genotypes of mice.

Enzyme activities in the joint tissues of Tnfip6-deficient and wild-type BALB/c mice. Tnfip6 binds IαI, a powerful serine protease inhibitor in serum, and this interaction has been shown to potentiate the inhibitory effect of IαI on plasmin (9). Thus, plasmin activity was expected to be elevated in the joints with inflammation. Some plasmin activity was detected in the total extracts however, serum concentrations of IL-6 (Figure 5C) and the acute-phase reactant SAA (Figure 5D) were significantly higher in the Tnfip6-deficient mice than in the wild-type mice at the acute phase of PGIA.

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of nonarthritic joints (Figure 6A), which was most likely the result of an association with the normal metabolic turnover of matrix components. After the development of arthritis, however, plasmin activity was almost 2-fold higher in the joint tissues of mice lacking Tnfip6, whereas only a moderate increase (<50%) was found in the paws of wild-type animals (Figure 6A).

As an indicator of neutrophil invasion, the activity of the neutrophil granulocyte–specific enzyme myeloperoxidase was measured in the total extracts of nonarthritic and arthritic paws of wild-type and Tnfip6-deficient animals (Figure 6B). Consistent with the histopathologic findings, myeloperoxidase activity was significantly elevated in the inflamed paws of Tnfip6-null mice when compared with the arthritic paws of wild-type animals (Figure 6B).

![Figure 4](image)

**Figure 4.** Summary of immune responses in wild-type (Tnfip6+/+) and tumor necrosis factor α (TNFα) (B), and IL-6 (C) and the acute-phase protein serum amyloid A (SAA) (D). Cytokines were measured in serum samples of wild-type and Tnfip6-deficient BALB/c mice during the acute phase (on day 53) and chronic phase (on day 81) of proteoglycan-induced arthritis. Sera were collected from the retro-orbital venous plexus of arthritic animals on day 53 (n = 15 in each group) or at the end of the experiments (day 81; n = 35 Tnfip6-/- mice and n = 32 wild-type mice, as in Figures 1 and 4). Bars show the mean and SEM. * = P < 0.01 versus wild-type mice. See Figure 4 for other definitions.

We also determined the activity of the neutrophil elastase, which could be involved in tissue damage upon release from locally activated neutrophils. The activity of this enzyme was significantly higher in the arthritic paws of Tnfip6-knockout mice compared with that in the paws of the wild-type mice (Figure 6C), and this increase was proportional to the extent of myeloperoxidase activity (as measured by neutrophil number) in Tnfip6-null joints (Figure 6B).

![Figure 5](image)

**Figure 5.** Serum concentrations of proinflammatory cytokines IL-1β (A), tumor necrosis factor (TNF) (B), and IL-6 (C) and the acute-phase protein serum amyloid A (SAA) (D). Cytokines were measured in serum samples of wild-type and Tnfip6-deficient BALB/c mice during the acute phase (on day 53) and chronic phase (on day 81) of proteoglycan-induced arthritis. Sera were collected from the retro-orbital venous plexus of arthritic animals on day 53 (n = 15 in each group) or at the end of the experiments (day 81; n = 35 Tnfip6-/- mice and n = 32 wild-type mice, as in Figures 1 and 4). Bars show the mean and SEM. * = P < 0.01 versus wild-type mice. See Figure 4 for other definitions.

Neutrophil influx into the peritoneal cavity during thioglycollate-induced peritonitis in wild-type and Tnfip6-deficient mice, and the effect of rMuTnfip6 treatment. The histopathologic examination of the joints (Figure 2) and the results of enzyme (myeloperoxidase and neutrophil elastase) assays (Figure 6) suggested that Tnfip6 deficiency could selectively facilitate the influx of neutrophil granulocytes into the site of inflammation. This was consistent with the observations of earlier studies in which it was reported that systemic or local administration of rHuTnfip6 inhibited neutrophil migration into nonspecific irritant- or cytokine-stimulated air
pouches in mice (10,11). Since a method for the accurate measurement of leukocyte infiltration in the small peripheral joints of mice is not available, we used thioglycollate-induced sterile peritonitis to compare neutrophil accumulation in wild-type and Tnfip6-deficient mice, and to quantitatively determine the effect of exogenous Tnfip6 on PMN cell extravasation.

This model is characterized by the influx of predominantly neutrophil granulocytes and monocytes/macrophages into the peritoneal cavity (26,27), from which these cells can be easily isolated. Indeed, the peritoneal exudate (lavage fluid) was dominated by PMN cells between 4 hours and 48 hours (showing a peak of influx at 24 hours) after injection of thioglycollate, whereas the number of mononuclear cells was relatively low and remained nearly constant during the 96-hour observation period in both genotypes of mice (Figure 7). At each time point between 4 hours and 96 hours, ~2–3 times more neutrophils migrated into the peritoneal cavities of Tnfip6-deficient animals than into the cavities of wild-type mice (Figure 7). A single dose (30 μg) of rMuTnfip6, injected intravenously 20 minutes before the thioglycollate challenge, reduced the neutrophil numbers in the peritoneal lavage fluid by at least 50% in both wild-type and Tnfip6-deficient mice (Figure 7, front columns), and this inhibitory effect on neutrophil influx was still detectable at 48 hours after thioglycollate administration to rMuTnfip6-injected animals.

Figure 6. Activities of myeloperoxidase and 2 serine proteases measured in tissue extracts of the nonarthritic and inflamed paws of Tnfip6-deficient and wild-type BALB/c mice with proteoglycan-induced arthritis. Tissue extracts were prepared separately from the noninflamed and the inflamed paws of the same animal 2–4 days after the onset of arthritis. Arthritis scores of the inflamed paws ranged between 1 and 4 by this time point, and therefore equal numbers (n = 12) of arthritic paws with highly comparable individual arthritis scores were selected for these assays. The overall arthritis score was a mean ± SEM 3.0 ± 0.73 in the wild-type mice and 2.91 ± 0.71 in the Tnfip6-deficient group. Enzyme activities were normalized to protein content. Myeloperoxidase activity (B) is expressed as the enzyme activity relative to the numbers of polymorphonuclear (PMN) cells (neutrophils) measured in peripheral blood of normal BALB/c mice, whereas the activity of plasminogen (C) and neutrophil elastase (C) is expressed in units, using the activity of the corresponding purified enzyme as a reference. * = P < 0.05 versus wild-type mice. See Figure 4 for other definitions.

Figure 7. Neutrophil extravasation in thioglycollate-induced peritonitis in wild-type and Tnfip6-deficient mice, with or without treatment with recombinant murine Tnfip6 (rMuTnfip6). Columns at the back represent total cell numbers in the peritoneal cavity after 0.5 ml of 4% thioglycollate injection, in which the upper areas correspond to neutrophil (polymorphonuclear [PMN]) cell number and the bottom areas are representative of the macrophage (monocyte [Mo]) numbers in the same samples. Columns at the front show the effect of rMuTnfip6 (30 μg injected intravenously 20 minutes before the thioglycollate challenge) on the peritoneal cell number. This intravenously injected rMuTnfip6, however, showed a rapid decline in the serum and was no longer detectable by enzyme-linked immunosorbent assay at 25–30 minutes after the injection (results not shown). Cells were counted in aliquots of the peritoneal lavage fluid, and differential counts were determined in May-Grunwald– and Giemsa-stained smears of the peritoneal lavage fluid and confirmed by flow cytometry. The proportion of lymphocytes (CD3+ and CD45/B220+ cells) was fewer than 2% of the total cell number at each time point (results not shown). Bars show the mean and SEM. See Figure 4 for other definitions.
DISCUSSION

In this study, we demonstrate that Tnfp6 deficiency increases the severity and accelerates the progression of PGIA in BALB/c mice. Wild-type and Tnfp6-deficient animals mount similar autoimmune responses (including antigen-specific T cell reactions, a balance between the Th1 and Th2 response, and antibody production) following immunization with cartilage PG. However, at the early (initial) phase of arthritis, there is a striking increase in the number of PMN cells in the synovium and joint exudate in mice lacking Tnfp6. Inflammatory destruction of the articular cartilage, erosion of bone, and joint deformities develop earlier and are more extensive in Tnfp6-null mice than in wild-type BALB/c mice. Serum markers of inflammation, including IL-6 and SAA, are also significantly elevated in Tnfp6-deficient mice during the acute phase of PGIA. Thus, although treatment of mice with recombinant Tnfp6 has been shown to exert a therapeutic effect in various forms of experimentally induced inflammation (6,7,10,11), the lack of endogenous Tnfp6 increases the severity of inflammation.

In vivo expression of TSG-6/Tnfip6 has been associated with inflammatory arthritis (3,5), but the protein is produced in a variety of cells exposed to proinflammatory stimuli in vitro (2,28,29), and also in a physiologic process such as matrix formation around the cumulus cell–oocyte complex (30). In fact, the relationship between impaired cumulus cell–oocyte matrix assembly and female sterility in Tnfp6-knockout mice reveals an essential role for this protein in the formation of crosslinked HA fibers around the oocyte that is necessary for the expansion of cumulus matrix and subsequent oocyte fertilization (15).

The antiinflammatory properties of Tnfp6 have been mainly attributed to its ability to potentiate the antiplasmin activity of IaI upon association with this protease inhibitor, leading to the subsequent down-regulation of the activity of a number of matrix-degrading proteases (7–10). However, a recent study (11) found that the isolated Link module domain of Tnfp6, which lacks the ability to associate with IaI, could still inhibit the influx of neutrophils to the site of inflammation. This observation suggests that Tnfp6 might exert antiinflammatory effects in an IaI-independent manner.

Although we have found elevated plasmin activity in tissue extracts prepared from thearthritic joints of Tnfp6-null mice, the most striking effects of Tnfp6 deficiency are the increases in the number of neutrophils and the activity of neutrophil-derived enzymes in the affected joints. Accelerated destruction of the articular cartilage and rapid progression of joint deformities in Tnfp6-null mice, therefore, could be associated with both enhanced neutrophil invasion and insufficient protection of cartilage matrix components from plasmin-activated proteases. We have shown that influx of PMN cells into the peritoneal cavity is also enhanced in Tnfp6-null mice in response to local injection of thioglycollate, indicating that neutrophil egress is facilitated in the absence of Tnfp6, independently of the site or type of inflammation.

Tnfp6 is an HA binding protein, and HA is known to support the CD44-dependent rolling of leukocytes (31–34), the first step toward their extravasation (35). It is a natural question, therefore, whether Tnfp6 is somehow involved in the adhesive interaction between CD44 and HA. A recent study by our group (36) demonstrated that, indeed, Tnfp6 modulates the interaction between HA and cell-surface CD44. Binding of HA to the cell surface is enhanced when CD44-positive cells are exposed to preformed Tnfp6–HA complexes, and HA in these complexes is recognized by cells expressing inactive CD44 that does not bind HA constitutively, i.e., without induction (36). Cells roll more readily on immobilized substrates consisting of Tnfp6–HA complexes as compared with substrates of HA alone, but firm adhesion to HA is not significantly facilitated in the presence of Tnfp6. Importantly, cell-surface–bound Tnfp6–HA complexes prevent CD44-mediated leukocyte adhesion to HA (36). Although these in vitro observations do not provide a full explanation for the in vivo effects of Tnfp6 (or the lack thereof), intravital videomicroscopy has currently revealed some important differences between Tnfp6-deficient and rMuTnfp6-treated mice. As shown for thioglycollate-induced peritonitis in this study, intravenously injected rMuTnfp6 almost completely abolishes leukocyte extravasation in TNFα-induced local ear inflammation (see ref. 37 and Szántó S, et al: unpublished observations).

The results of these in vivo experiments suggest that Tnfp6 plays a role in the control of leukocyte influx into arthritic joints (most likely via interference with CD44/HA-mediated adhesion events), and is involved locally in the protection of cartilage matrix from proteolytic damage through complex formation with the serine protease inhibitor IaI (7,8). The most important message of our in vitro and in vivo studies is that Tnfp6 can directly modulate cell adhesion (36,37), PMN cell extravasation (Figures 2 and 7), and perhaps also, the
production of proinflammatory mediators such as IL-6 (Figure 5C). Thus, the suppressive effects of Tnfip6 on arthritis (6,7) are exerted through more than one mechanism. The effects of Tnfip6 are not restricted to joints (and arthritis), since this protein seems to inhibit other types of inflammation as well. Inhibition of inflammatory leukocyte (neutrophil) influx into the joint could be the most important function of Tnfip6 in arthritis suppression, but its interaction with IaI might provide the cartilage matrix an enhanced protection from proteolytic damage. Therefore, in inflammatory/rheumatoid arthritis, recombinant Tnfip6 holds promise as a therapeutic agent.

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