

Research article

Open Access

A proinflammatory role for Fas in joints of mice with collagen-induced arthritisHoang Tu-Rapp¹, André Hammermüller¹, Eilhard Mix², Hans-Jürgen Kreutzer³, Roland Goerlich⁴, Hansjürgen Köhler¹, Horst Nizze³, Hans-Jürgen Thiesen¹ and Saleh M Ibrahim¹¹Department of Immunology, University of Rostock, Rostock, Germany²Department of Neurology, University of Rostock, Rostock, Germany³Department of Pathology, University of Rostock, Rostock, Germany⁴Institute of Biology VII, RWTH Aachen, Aachen, GermanyCorresponding author: Hoang Tu-Rapp, hoang.tu-rapp@med.uni-rostock.de

Received: 11 Feb 2004 Revisions requested: 3 Mar 2004 Revisions received: 30 Apr 2004 Accepted: 7 Jun 2004 Published: 19 Jul 2004

Arthritis Res Ther 2004, **6**:R404-R414 (DOI 10.1186/ar1205)© 2004 Tu-Rapp *et al.*; licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.**Abstract**

Collagen-induced arthritis (CIA) is a chronic inflammatory disease bearing all the hallmarks of rheumatoid arthritis, e.g. polyarthritis, synovitis, and subsequent cartilage/bone erosions. One feature of the disease contributing to joint damage is synovial hyperplasia. The factors responsible for the hyperplasia are unknown; however, an imbalance between rates of cell proliferation and cell death (apoptosis) has been suggested. To evaluate the role of a major pathway of cell death – Fas (CD95)/FasL – in the pathogenesis of CIA, DBA/1J mice with a mutation of the Fas gene (*lpr*) were generated. The susceptibility of the mutant DBA-*lpr/lpr* mice to arthritis induced by collagen type II was evaluated. Contrary to expectations, the DBA-*lpr/lpr* mice developed significantly milder disease than the control littermates. The incidence of disease was also significantly lower

in the *lpr/lpr* mice than in the controls (40% versus 81%; $P < 0.05$). However DBA-*lpr/lpr* mice mounted a robust immune response to collagen, and the expression of local proinflammatory cytokines such as, e.g., tumor necrosis factor α (TNF- α) and IL-6 were increased at the onset of disease. Since the contribution of synovial fibroblasts to inflammation and joint destruction is crucial, the potential activating effect of Fas on mouse fibroblast cell line NIH3T3 was investigated. On treatment with anti-Fas *in vitro*, the cell death of NIH3T3 fibroblasts was reduced and the expression of proinflammatory cytokines TNF- α and IL-6 was increased. These findings suggest that impairment of immune tolerance by increased T-cell reactivity does not lead to enhanced susceptibility to CIA and point to a role of Fas in joint destruction.

Keywords: apoptosis, Fas, rheumatoid arthritis, tolerance**Introduction**

Collagen-induced arthritis (CIA) is an animal model bearing all the hallmarks of rheumatoid arthritis (RA). CIA can be induced in susceptible strains of mice, e.g. DBA/1J, by immunization with bovine collagen type II in complete Freund's adjuvant (CFA) [1]. CIA has been extensively studied to elucidate the pathological mechanisms relevant to human RA and to identify potential therapeutic targets [2]. The development of CIA, as of RA, is known to depend on T cells, and susceptibility to the disease is linked to the MHC region [3]. Following T-cell activation, an inflammatory cascade involving T cells, macrophages/monocytes, B cells, and activated synoviocytes is triggered. The different

immune and local synovial cells produce a complex array of cytokines and other soluble mediators that are thought to be responsible for cartilage destruction and bone erosion [4-6].

One of the main features of CIA disease is synovial hyperplasia. The factors contributing to this phenomenon are unknown; however, an imbalance between rates of cell proliferation and cell death (apoptosis) has been suggested [7]. Two major pathways involved in ligand-mediated apoptosis in the immune system have been considered, namely the Fas ligand (FasL) and tumor necrosis factor (TNF) pathways. FasL and TNF are members of the TNF superfamily.

AICD = activation-induced cell death; CFA = complete Freund's adjuvant; CIA = collagen-induced arthritis; ConA = concanavalin A; ELISA = enzyme-linked immunosorbent assay; Fab = antigen-binding fragment; FACS = fluorescence-activated cell sorter; FasL = Fas ligand; FITC = fluorescein isothiocyanate; IFN = interferon; IL = interleukin; mAb = monoclonal antibody; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; PE = phycoerythrin; RA = rheumatoid arthritis; RPMI = Roswell Park Memorial Institute [medium]; TNF = tumor necrosis factor.

Both cell-death pathways have been shown to contribute to peripheral tolerance and to the maintenance of homeostasis in the immune system through activation-induced cell death (AICD) [8-11]. Additionally, FasL together with perforin and TNF are the main pathways for killer cells, and mutations in those molecules block cytotoxicity of target cells [12,13]. Thus, cell-death pathways could contribute to the pathology of arthritis in at least two ways: through promotion of autoimmunity by blocking tolerance of autoreactive lymphocytes and AICD, or through destruction of target tissues by induction of apoptosis or proliferation in susceptible cells.

A pathogenic role of TNF- α for arthritis is well documented in a number of studies and is supported by the success of anti-TNF therapy. Murine studies using TNF-receptor knockout mice and TNF transgenic mice point to a primary role in the local proliferation of synovial fibroblasts rather than to tolerance impairment of lymphocytes or death of local joint cells [14,15].

Although the exact role of Fas in arthritis remains unclear, some observations suggest an involvement of this receptor molecule in the disease process. It has been reported that a subset of T cells in patients with RA was resistant to Fas-mediated apoptosis [16,17]. Mysler and co-workers and other groups showed that T cells in systemic lupus erythematosus have an abnormal increase in surface Fas expression [18,19]. However, they showed proliferative and activating response to Fas crosslinking [20] rather than enhanced susceptibility to Fas-mediated apoptosis. Several studies demonstrated that autoreactive lymphocytes infiltrating the rheumatoid synovium are resistant to apoptosis either because of expression of the anti-apoptotic proteins bcl2 and bclxl or because of deficiency of FasL. On the other hand, conflicting evidence showing that infiltrating T cells are Fas-sensitive has been presented [16,21-24]. Synovial fibroblasts were shown to be susceptible to apoptosis induced by anti-Fas antibody, but they were shown by others to express high levels of oncogenes and bcl2 as well [24].

In this study, we attempted to evaluate the role of the Fas cell-death pathway in the pathogenesis of CIA by generating DBA/1J mice with a mutation of the Fas gene (DBA-lpr/lpr) and by examining the effect of the mutation on the immune response to collagen and on joint pathology.

Materials and methods

Mice, backcrossing, antigen, immunization, and assessment of arthritis

DBA/1J mice were obtained from Harlan-Winkelmann (Borchen, Germany) and kept under standard conditions at the animal facility of the University of Rostock. Fas mutant mice were obtained from Bomholtgard A/S (Ry, Denmark).

These mice were not available on the DBA/1J background and, therefore, were obtained as C3H-lpr. The lpr mutation was then backcrossed onto the DBA/1J background. The mice were propagated as hemizygous mutants for at least six generations and the mutation was followed by PCR analysis of tail DNA, as previously described [10]. Experimental mice were generated by brother-sister mating and homozygosity was assessed by PCR as described elsewhere [10].

Eight-week-old mice were immunized intradermally at the base of the tail with 150 μ g of bovine collagen II (Sigma, Deisenhofen, Germany) emulsified in CFA (Difco, Detroit, MI, USA). Mice were boosted with 150 μ g of collagen in incomplete Freund's adjuvant at day 21. Clinical scores were assessed immediately before immunization (day 0) and thereafter three times weekly until day 75 after immunization. Inflammation of the four paws was scored as follows: 0, no inflammation; 1, swelling/redness of one joint; 2, swelling/redness of more than one joint or mild inflammation of the whole paw; 3, severe inflammation of whole paw or ankylosis. For evaluating the susceptibility of mice to CIA, the incidence of disease (number of diseased mice divided by total number of mice), the mean score (total score of diseased mice divided by total number of mice), and the mean day of onset of disease (total days of onset divided by the number of diseased mice) were calculated. The study was approved by the appropriate authorities of the state of Mecklenburg-Vorpommern, Germany.

Cell culture, T-cell proliferation assays, and cytokine induction

Cells and cell culture

Draining lymph nodes were removed under aseptic conditions. Single-cell suspensions of mononuclear cells of pooled lymph nodes from individual mice were prepared. The cells were washed three times in culture medium before being suspended at 2×10^6 mononuclear cells per milliliter in round-bottomed, 96-well polystyrene microtiter plates (Nunc, Copenhagen, Denmark) in a total volume of 200 μ l. The culture medium consisted of RPMI 1640 with Glutamax-II (Gibco BRL, Life Technologies, Karlsruhe, Germany) supplemented with 50 IU/ml penicillin, 60 μ g/ml streptomycin, and 5% inactivated fetal bovine serum (all from Gibco BRL). For lymphocyte stimulation, 10 μ l aliquots of collagen II were added to cultures at a final concentration of 10–50 μ g/ml or 10 μ l of concanavalin A (ConA) (Difco) at a final concentration of 4 μ g/ml. These concentrations had optimal stimulatory effects as assessed in previous experiments. Cells were incubated at 37°C in humidified air with 5% CO₂ for 72 hours. Cultures were done in triplicate for proliferation assays and in duplicate for ELISA measurements of IFN- γ . NIH3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 50 IU/ml penicillin, 60 μ g/ml streptomycin, and 5%

inactivated fetal bovine serum. To determine their susceptibility to Fas-induced cell death, purified hamster antimouse Fas monoclonal antibody, clone Jo2 (Becton Dickinson, Heidelberg, Germany), was used. NIH3T3 cells were incubated with 50 ng Jo2/ml or 50 ng Jo2/ml and 1 µg protein G/ml, respectively, for 24 hours.

Proliferation assay

After 60 hours of incubation, cells were pulsed with 10 µl [³H]methylthymidine (1 µCi/ml) (Amersham Pharmacia Biotech, Freiburg, Germany) and cultured for an additional 12 hours. Cells were harvested onto fiberglass filters (Titertek, Skatron, Lierbyen, Norway). [³H]thymidine incorporation was measured in a liquid β-scintillation counter. The results were expressed as counts per minute.

ELISA for measurement of IFN-γ

After 72 hours of incubation, supernatants were collected from the lymph node cell cultures and frozen in two aliquots at -80°C. Concentrations of IFN-γ in the supernatants were determined by the Cytoscreen Immunoassay Kit (BioSource, Camarillo, CA, USA) in accordance with the manufacturer's instructions.

Anticollagen antibody assay

Sera were collected from control DBA-lpr/+ and mutant DBA-lpr/lpr mice before immunization and at days 20 and 47 after immunization and a standard ELISA was used to measure total anticollagen II IgG. In brief, ELISA plates (Greiner, Frickenhausen, Germany) were coated with 5 µg/ml collagen II and incubated overnight at 4°C. The plates were then washed three times with washing buffer (1 × phosphate-buffered saline [PBS], 1% bovine serum albumin, 0.05% Tween 20) and blocked for 1 hour at room temperature. Sera were added to the plates after washing at dilutions of 1:10, 1:50, 1:500, 1:5000, and 1:50,000. After incubation for 2 hours at 37°C, the plates were washed and biotin-conjugated AffiniPure rabbit antimouse IgG (Dianova, Hamburg, Germany), diluted 1:20,000 was added and incubated for 1 hour at 37°C. This step was followed by washing and incubation with a 1:1000 dilution of alkaline-phosphatase-conjugated streptavidin (Dianova). Plates were developed by the addition of a substrate and read at wavelength 405 nm. Negative and positive controls were washing buffer and the supernatant of the anticollagen antibody hybridoma C1C1 (a gift from Dr R Holmdahl, University of Lund, Sweden) respectively. The measurements were made in triplicate.

Histopathological analysis of joints

Histopathological features of peripheral joints were assessed in hematoxylin-stained formalin-fixed paraffin-embedded sections as described previously [25].

Flow cytometry

The following antibodies were used to study surface expression of CD4, CD8, CD45, CD90, Fas (CD95), and CD44 on lymphocytes: respectively, clone H129.19, clone 53-6.7, clone RA3-6B2, clone 30-H12, clone Jo2, and clone IM7. All antibodies were purchased from Becton Dickinson. Staining was essentially done following the manufacturer's instructions. In brief, lymph node cells were isolated as described above, washed twice in PBS, and incubated for 20 minutes on ice in 100 µl of FACS (fluorescence-activated cell sorter) buffer (1 × PBS, 0.1% bovine serum albumin, 0.1% sodium acid) in the presence of the FITC- or PE-labeled specific antibodies. Isotype controls were used at the appropriate concentrations. The dead cells were quantified by staining with propidium iodide in accordance with the instructions provided by the manufacturer (Becton Dickinson). Flow cytometric analysis was performed on the FACScan (Becton Dickinson).

RNA isolation and cDNA synthesis

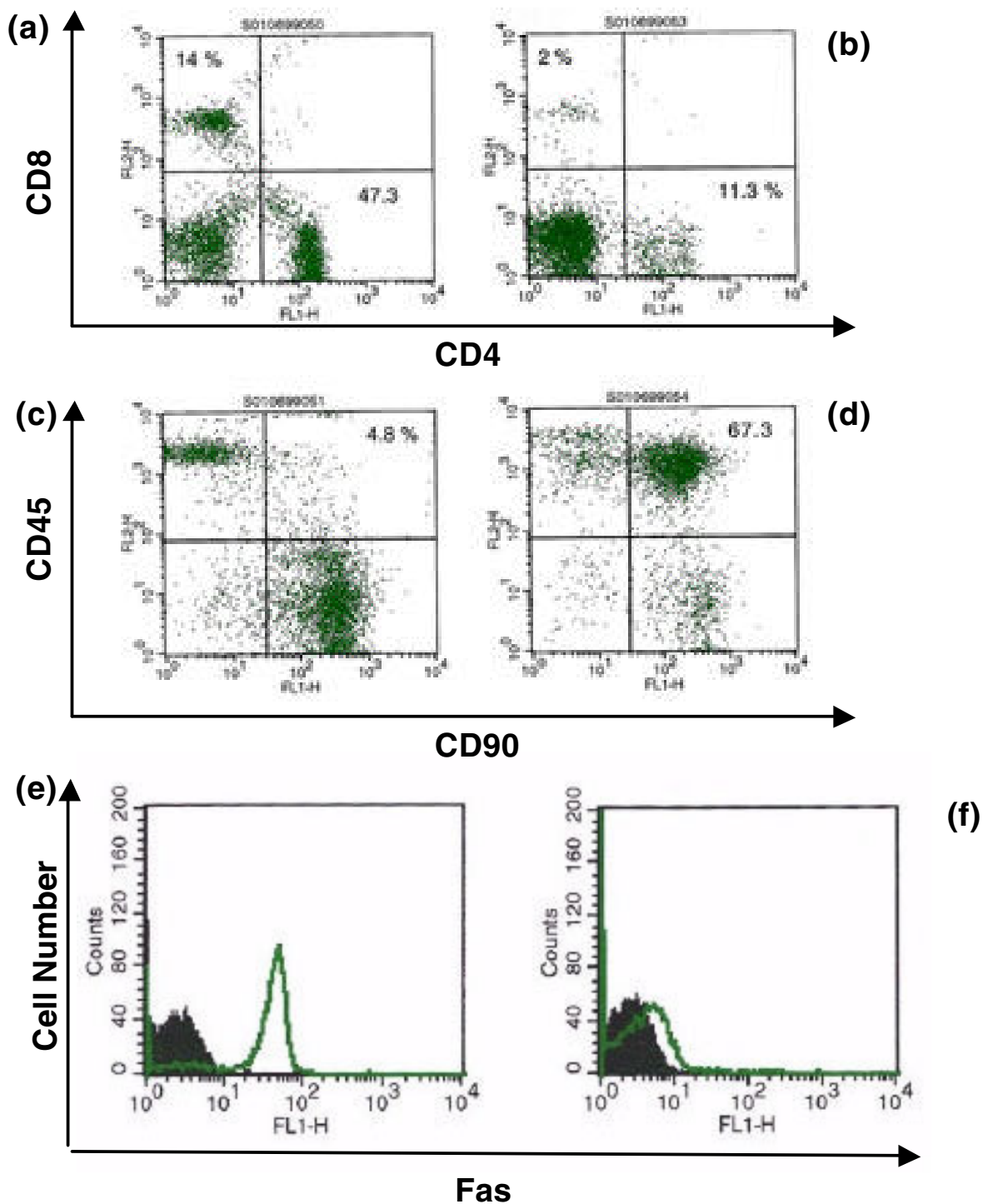
Paws were dissected at time points around the onset of disease and during its chronic stage and snap frozen in liquid nitrogen, and total RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Samples were treated with RNase-free DNase (Qiagen) on the RNeasy columns in accordance with the manufacturer's instructions. RNA was finally dissolved in 100 µl of RNase-free water.

For reverse transcription, we used 300 U of SUPERSCRIPT™ RNase H Transcriptase with the supplied buffer (Gibco BRL), 20 U of RNasin, 3 µM random hexamers (Amersham Pharmacia Biotech), deoxynucleoside triphosphate, dithiothreitol, and 2 µg of RNA sample per 25 µl reaction volume. The samples were heated for 2 hours at 42°C and rapidly cooled on ice.

TaqMan® Real-Time PCR

The TaqMan® PCR Core Reagent Kit (Applied Biosystems, Weiterstadt, Germany) was used for amplification of targets. For PCR of IL-6 and TNF-α, we used ready-made Pre-Developed TaqMan® Assay Reagents and the TaqMan® 7700 instrument (Applied Biosystems). The reaction conditions for 50 ng cDNA were as follows: 2 min at 50°C, 10 min at 95°C, 45 repeats of 15 s at 95°C, and 1 min at 60°C. For each RNA isolation, measurements of gene expression were done three times, and the mean of these values was used for further analysis. In accordance with the manufacturer's User Bulletin #2 (Applied Biosystems), the comparative C_t method and the internal control (glyceraldehyde-3-phosphate dehydrogenase) were used to normalize the expression levels of target genes.

Figure 1



The *lpr* phenotype is mild in the DBA/1J genetic background. Analysis of surface expression of CD4/CD8 (a, b), CD90/CD45 (c, d), and Fas (CD95) (e, f) on T lymphocytes purified from lymph nodes of 24-week-old DBA/1J-*lpr/lpr* mice (b, d, f) and DBA/1J littermates (a, c, e). Lymph node cells were stained with the indicated antibodies (Becton Dickinson). For analysis of CD95 (open area), an isotype control (shadowed area) is shown (e, f). Samples were analyzed on a FACScan cell sorter (Becton Dickinson).

Table 1**DBA-lpr/lpr mice are protected against collagen-induced arthritis**

	No. of mice ^a	Incidence (%) ^b	Mean score ^c at day 64	Mean day of onset ^d
DBA/1J-lpr/lpr	34	44* ^e	1.6 ± 0.75* ^f	51 ± 14
DBA/1J-lpr/+	33	81	5 ± 1.1	41 ± 6

DBA/1J-lpr/lpr and DBA/1J-lpr/+ mice were scored for arthritic lesions as described in Materials and methods. A summary of disease course in DBA/1J-lpr/lpr and their control littermates DBA/1J-lpr/+ is shown. ^a18 of the lpr/lpr mice and 16 of the lpr/+ mice are from Ma and co-workers [45]. ^bNumber of diseased mice divided by all mice. ^cScore of diseased mice divided by all mice ± SEM. ^dTotal of days of onset divided by the number of diseased mice ± SD. *^e $P < 0.05$, χ^2 test. *^f $P < 0.05$, Mann–Whitney U test.

Statistical analysis

Statistical differences between experimental and control groups were analyzed using the Mann–Whitney U test for the severity of arthritis, the χ^2 test for incidence of arthritis, and Student's t -test for day of disease onset, antibody levels, and T-cell responses. A P value of <0.05 was considered significant.

Results**The DBA/1J genetic background does not influence the lpr phenotype**

To obtain CIA-susceptible Fas mutant mice, we backcrossed the lpr mutation onto the susceptible DBA/1J background for at least six generations. Successful backcrossing to the DBA background was assessed by PCR analysis of the MHC-H2 locus (data not shown). Older (24-week-old) DBA-lpr/lpr mice showed a typical lpr phenotype of accumulation of CD4⁺/CD8⁻ doubly negative CD3⁺ T cells (Fig. 1b) and CD45⁺/CD90⁺ doubly positive cells (Fig. 1d) in the periphery; however, they did not develop spontaneous arthritis (data not shown). As expected, DBA-lpr/lpr thymocytes were resistant to anti-Fas-induced apoptosis (data not shown) and only a very low level of Fas was detected on their surface – a finding that is consistent with an earlier observation by others that lpr/lpr mice express very low levels of Fas (Fig. 1f).

Mice used for further experiments were 8 weeks old and they had a normal distribution of T-cell subpopulation, comparable to that in wild-type control DBA/1J mice.

Fas mutation does not enhance the severity or increase the incidence of disease

To assess the effect of the lpr mutation on arthritis development, we induced the disease in 8-week-old DBA-lpr/lpr mice and their heterozygous control littermates. Contrary to expectations, the lpr mutation led to a decrease in the severity and incidence of disease. Table 1 shows lower mean disease scores at day 64 after immunization in lpr/lpr mice than in control mice ($P < 0.05$). The incidence of disease was significantly lower in lpr/lpr mice than in their controls. The mean onset of disease was slightly later in the lpr/lpr mice than in their controls, but no statistical significance was achieved. Despite the mild disease in the lpr/lpr group,

individual mice of both genotypes had either severe, very mild, or no disease manifestations. Histopathological differences reflected the clinical severity of disease. No evidence of arthritic disease was observed the day before immunization (Fig. 2a,2b). At the inflamed stage of disease (score 3), both groups showed characteristic features of inflammation, such as fibroblast proliferation, cartilage degeneration, granulomatous lesions in the sublining tissues, and erosion of bone (Fig. 2c,2d); however, decreased cell proliferation and lymphocyte infiltration and erosion of cartilage and bone were generally observed in the DBA-lpr/lpr mice (Fig. 2c).

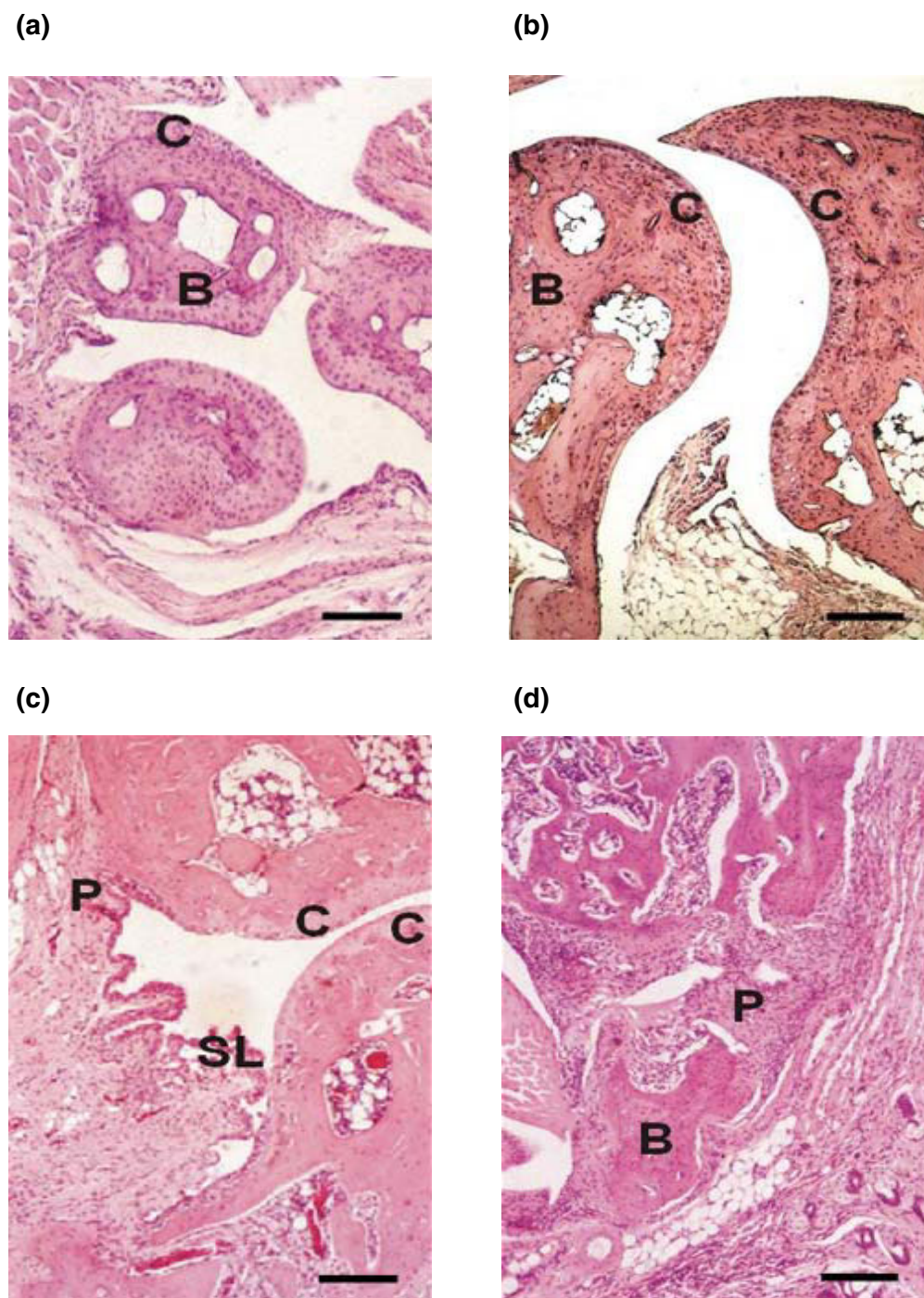
DBA-lpr/lpr mice mount a robust immune response to collagen

To determine whether the mild clinical symptoms reflected a failure to mount an adequate immune response to collagen II, we analyzed the T- and B-cell responses in homozygous DBA-lpr/lpr and heterozygous DBA-lpr/+ mice. Specifically, we analyzed the collagen-II-specific T-cell proliferation (Fig. 3a) and IFN- γ production (Fig. 3b) *in vitro* and anticollagen IgG antibody titers in sera of immunized mice (Fig. 4).

Cultured cells from draining lymph nodes were restimulated *in vitro* with collagen II or ConA 7 days after immunization. The control lymph node cells and lpr/lpr cells proliferated equally well in response to ConA (data not shown). A significantly higher proliferative T-cell response to collagen II was observed in DBA-lpr/lpr mice than in control mice (Fig. 3a). In agreement with these results, IFN- γ production after antigen stimulation was higher in DBA-lpr/lpr than in DBA-lpr/+ littermates (Fig. 3b). To show whether the enhanced immune response was due to an increased frequency of memory phenotype of lpr T cells upon stimulation with collagen, phenotypic analysis of surface expression of CD44 on T lymphocytes was performed. No change of memory cell populations after stimulation was observed (Fig. 3c).

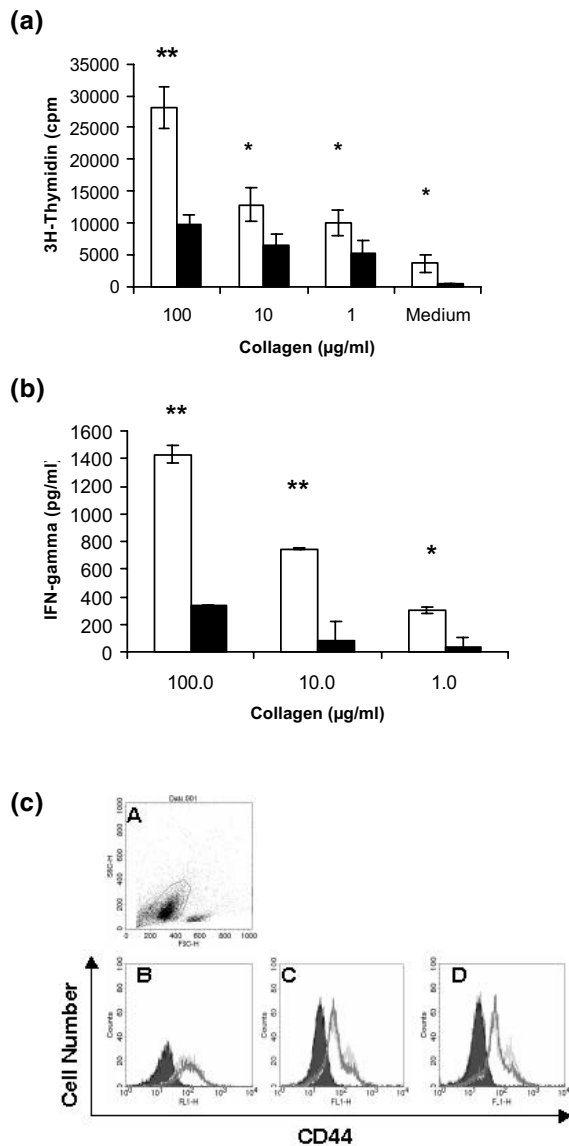
Furthermore, no significant differences were seen between the two genotypes in the levels of anticollagen II antibody titer at day 20, or in the chronic phase, at day 47. However nonimmunized DBA-lpr/lpr mice showed significantly

Figure 2



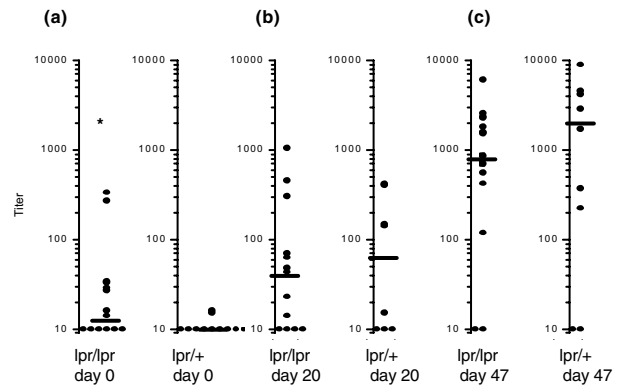
Histopathological analysis of joints from experimental and control mice before and after induction of collagen-induced arthritis. Healthy (a, b) and inflamed joints (c, d) from DBA-lpr/lpr mice (a, c) and their littermate controls (b, d). The inflamed paws had a disease score of 3. The paraffin sections were stained with hematoxylin and eosin. B, bone; C, cartilage; P, pannus; SL, synovial lining. Bars in the figure represent 200 μm (a, c) and 100 μm (b), respectively.

Figure 3



DBA-lpr/lpr mice mount a robust T-cell response to collagen. DBA-lpr/lpr lymphocytes show increased proliferation (a) and increased IFN- γ production (b) in response to *in vitro* stimulation by collagen II. Draining lymph nodes were obtained from DBA-lpr/lpr (white bars; $n = 3$) and control DBA-lpr/+ (filled bars; $n = 3$) mice 7 days after immunization with collagen II and complete Freund's adjuvant. For measurement of cell proliferation, the cells were cultured for 60 hours with collagen II at the indicated concentrations, and then pulsed with [^3H] thymidine. Concentrations of IFN- γ in the supernatant were determined by ELISA. The columns represent mean values and the error bars indicate standard deviations. Differences were statistically significant in all comparisons ($*P < 0.05$; $**P < 0.001$). The enhanced T-cell response is not due to changed subpopulations. (c) Phenotypic analysis of surface expression of CD44 on nonstimulated (cC) and stimulated (cB, cD) T lymphocytes purified from lymph nodes of homozygotes (DBA/1J-lpr/lpr) (bright line) and their heterozygote (DBA/1J-lpr/+) littermates (dark line). Lymph node cells were stimulated with concanavalin A (cB) and bovine collagen II (cD). The isotype control is shown as shadowed area. The samples were analyzed on a FACSscan (Becton Dickinson) and gated on lymphocytes (cA).

Figure 4



Changes in the development of arthritis are not due to changes in B-cell function. Titers of collagen-specific IgG antibodies were determined in sera of DBA-lpr/lpr mice ($n = 15$) and DBA-lpr/+ control mice ($n = 11$) before immunization (a) and at 20 days (b) and 47 days (c) after immunization with collagen in complete Freund's adjuvant. Horizontal lines indicate medians. Significant differences between the two groups were seen only at day 0 ($*P < 0.05$).

higher levels of anticollagen antibodies than DBA-lpr/+ control mice, in which almost no antibodies were detected (Fig. 4a).

Protection against CIA is not due to down-regulation of proinflammatory cytokines in joints

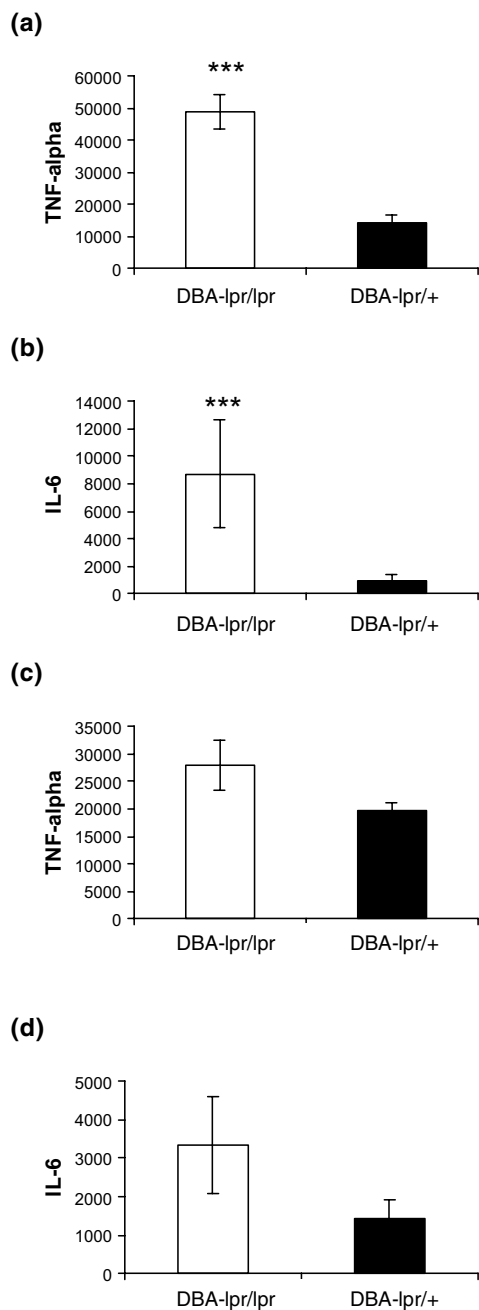
Since cytokines such as TNF- α and IL-6 are critical mediators of inflammation, we investigated the effect of Fas on the expression of proinflammatory cytokines in joints. The paws were harvested both at the onset of disease (4 and 7 weeks after immunization) and at the chronic stage of disease (10–12 weeks after immunization), and mRNA expression of cytokines was measured. In spite of mild arthritis in DBA-lpr/lpr mice, the expression of TNF- α and IL-6 was significantly higher than that in joints of DBA-lpr/+ mice at the onset of arthritis ($P < 0.001$) (Fig. 5a,5b). The mRNA expression of these cytokines was higher in joints of DBA-lpr/lpr mice than that in joints of DBA-lpr/+ mice at the chronic stage of disease, too; however no significant differences were observed (Fig. 5c,5d).

Fas ligation blocks cell death and enhances expression of proinflammatory cytokines

Since synovial hyperplasia contributes to the pathogenesis of CIA, we examined the potential stimulatory effect of anti-Fas monoclonal antibodies (mAb; clone Jo2) on synovial fibroblasts using the mouse fibroblast cell line NIH3T3. Fas is expressed in NIH3T3 (data not shown). The cells were cultured with anti-Fas mAb or protein G. Cell death was measured by staining with propidium iodide.

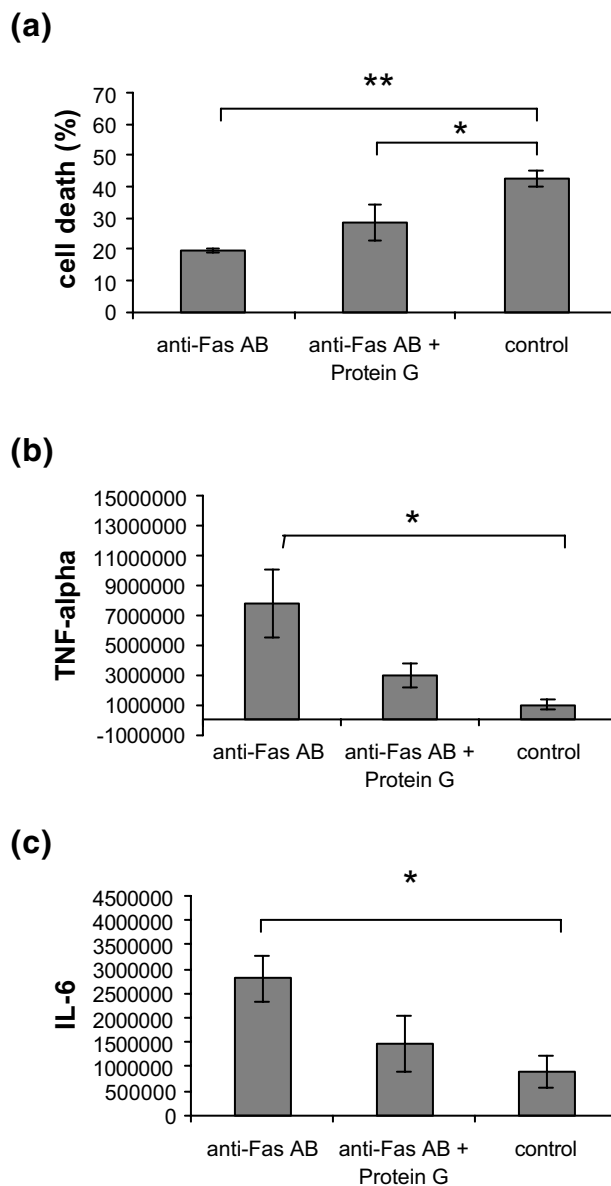
We found that anti-Fas mAb reduced cell death in NIH3T3 fibroblasts (Fig. 6a). Cell death was significantly decreased

Figure 5



Protection against collagen-induced arthritis (CIA) is not due to down-regulation of proinflammatory cytokines. Relative expression of tumor necrosis factor (TNF)- α (a, c) and IL-6 mRNA (b, d) in joints of DBA-lpr/lpr (white bars) and DBA-lpr/+ (filled bars) mice at the onset of disease (a, b) and at the chronic level of disease (c, d), as determined by real-time PCR. For measurement at the onset, the paws of the DBA-lpr/+ mice ($n = 14$) were harvested at 4 weeks. Those from DBA-lpr/lpr mice were harvested at 4 ($n = 10$) and 7 ($n = 9$) weeks; these were pooled for analysis, because they did not differ. For measurements during the chronic phase, paws of DBA-lpr/lpr ($n = 9$) and DBA-lpr/+ ($n = 10$) mice were harvested at 10–12 weeks. Significant differences were seen between the two groups at the onset of disease (***) $P < 0.001$.

Figure 6



Fas ligation blocks cell death and enhances expression of proinflammatory cytokines. Fas-induced cell death of NIH3T3 fibroblasts ($n = 3$) measured by fluorescence-activated cell sorting (a) and the relative expression of tumor necrosis factor (TNF)- α (b) and IL-6 (c) mRNA in NIH3T3 fibroblasts ($n = 3$), determined by real-time PCR. Cells (1×10^6 /ml) were stimulated with 50 ng anti-Fas antibody/ml or 50 ng anti-Fas antibody/ml and 1 μ g protein G for 24 hours. Control cells were incubated with medium only. AB, antibody.

($P < 0.01$) by treatment with anti-Fas mAb. The additional treatment with protein G causing the trimerization of Fas still resulted in significantly decreased cell death ($P < 0.05$). Furthermore, treatment with anti-Fas mAb caused a significantly ($P < 0.05$) increased expression of TNF- α and

IL-6 (Fig. 6b,6c), suggesting that Fas ligation led to stimulation and proliferation of fibroblasts.

Discussion

Numerous studies have suggested that genes regulating apoptosis are involved in the pathogenesis of autoimmune diseases, including RA [26-29]. Indeed, the success of anti-TNF therapy points to a major role for this important apoptosis pathway in arthritis development [reviewed [30]].

In this study, we show that the presence of intact Fas, another important apoptosis pathway, enhances the pathogenesis of CIA induced in DBA mice. Immunization of DBA-lpr/lpr mice and their wild-type littermates with collagen II and CFA leads to the development of CIA in both genotypes. Intact Fas is associated with the higher severity and increased incidence of arthritis but is not essential to disease induction. This is in agreement with previous studies in experimental autoimmune encephalomyelitis in C57Bl/6 mice carrying the lpr mutation. These mice had significantly milder disease than their Fas-expressing littermates [31,32].

Fas could contribute to disease in at least two ways: first, it could promote autoimmunity by blocking peripheral tolerance of autoreactive lymphocytes and inhibiting AICD. The role of the Fas molecule in autoimmunity has been well demonstrated in the MRL-lpr mice, and other animal models such as experimental autoimmune encephalitis. A minority of older MRL-lpr/lpr mice developed mild arthritis [21]. Fas mutation causes impaired T-cell tolerance and lymphadenopathy, with accumulation of abnormal cells. Thus, defects in peripheral tolerance may play an important role in the pathogenesis of RA. Secondly, Fas could contribute to disease by destroying target tissues through induction of apoptosis of chondrocytes [33]. Alternatively, Fas could contribute to synovial hyperplasia by inducing proliferation of Fas-expressing synovial fibroblasts and macrophages. Indeed, there is some evidence suggesting that fibroblasts could be activated through surface Fas [34] and that Fas expression is higher in RA synovial tissues than in osteoarthritic synovial tissues [35]. One way to clarify this matter is to examine the T-cell and B-cell responses to collagen in lpr/lpr mice. We found that the Fas-deficient T-cell response to collagen II is significantly stronger than that of normal T cells. Since no change of the collagen-II-specific T-cell precursor frequency was observed, this could reflect an increase in the intrinsic proliferative potential of lpr/lpr cells, or a defect in down-regulating the response due to impairment of AICD, or an alteration of regulatory T-cell function. It has been shown that doubly negative T cells, which are increased in lpr mice, have a regulatory function [36]. Since the suppression of aggressive T-cell responses mediated by regulatory T cells depends on interaction of Fas and Fas ligand, the Fas-defi-

cient doubly negative T cells could fail to suppress peripheral autoimmune T cells, and this failure could lead to an accumulation of aggressive T cells. The significant increase in T-cell proliferation in response to collagen in lpr/lpr mice was accompanied by significantly higher levels of IFN- γ secretion from these cells. This Th1 cytokine has been shown to be abundantly expressed in arthritic lesions both in mice and in humans [37-39]. IFN- γ together with other Th1 cytokines predominate in the acute phase of arthritis [40,41]. These results exclude the possibility that the mild clinical disease of CIA in lpr/lpr mice is caused by a lack of generation and priming of collagen-II-specific T cells.

A lack of B-cell response also does not appear to be the reason for the mild clinical arthritis in DBA-lpr/lpr mice, since we saw no significant differences in serum anticollagen II IgG antibody levels at time of onset of arthritis at day 20 or during the chronic phase of disease at day 47 between mutant mice and their wild-type littermates. This is rather surprising, as nonimmunized DBA-lpr/lpr had significantly higher levels of anticollagen antibodies than wild-type mice, which almost lacked detectable antibody levels. This indicates the existence of autoreactive collagen-II-specific B cells in DBA-lpr/lpr mice. In summary, all basic elements of a robust pathological immune response are available in DBA-lpr/lpr mice, i.e., Th1 cytokines, proliferating activated autoreactive T cells, and pathological anticollagen II antibodies. The histopathological examination of the inflamed joints from DBA-lpr/lpr and control mice with CIA reveal less inflammation/joint destruction in DBA-lpr/lpr mice in spite of the same clinical score as that of control mice.

The proinflammatory cytokines including TNF- α and IL-6 have been intensively investigated for their role in the pathogenesis of CIA. It is well known that they play a crucial role in the destruction of joints in CIA [37,42-44]. TNF- α induces synovial fibroblasts to express cytokines (such as IL-6) and other factors such as, e.g., matrix metalloproteinases, which contribute to cartilage and bone destruction.

Surprisingly, these proinflammatory cytokines were found at relatively higher levels in joints of DBA-lpr/lpr mice despite milder arthritis in comparison with the normal DBA mice. The mouse fibroblast cell line NIH3T3 is less sensitive to apoptosis induced by anti-Fas mAb and is accompanied by increased expression of TNF- α and IL-6, suggesting an activating effect by Fas ligation. Fas crosslinking may contribute to cartilage and bone destruction by activating synovial fibroblasts subsequently by production of matrix metalloproteinases, growth factors (such as granulocyte/macrophage-colony-stimulating factor), and chemokines. These results indicate that activation by proinflammatory cytokines is insufficient for full disease manifes-

tation when Fas is deficient. Similar results were obtained with synovial macrophages [45].

Taking this into consideration, one could draw the conclusion that the lack of the expected severe disease in DBA-lpr/lpr mice is due to a local attenuating effect of the Fas mutation in pathological processes involving resident joint cells. Fas ligation could also play a role in chondrocyte cell death or in activation of macrophages [45]. There is evidence indicating that antigen-specific T cells are costimulated through the Fas molecule expressed on the T-cell surface. The involvement of Fas in tissue damage has been shown in other tissue-specific autoimmune diseases, namely autoimmune thyroiditis, multiple sclerosis, and insulin-dependent diabetes mellitus. Thyroid cells obtained from patients suffering from autoimmune thyroiditis were shown to express Fas and FasL in response to cytokines and to be targets of Fas-mediated apoptosis [26]. Similarly, oligodendrocytes purified from multiple sclerosis patients were targets of Fas-mediated apoptosis [27,28]. NOD mice, an animal model of insulin-dependent diabetes mellitus with a mutation of the Fas gene (NOD/lpr mice), do not develop diabetes, pointing to a role of the Fas cell-death pathway in tissue damage in this disease as well [29].

Conclusion

Our findings, combined with conflicting reports showing that synovial T cells express Fas and FasL, that they are apoptosis-resistant or apoptosis-sensitive, and that synovial fibroblasts, chondrocytes, and osteoblasts are susceptible to anti-Fas-induced apoptosis [16,21-24], indicate an important pathogenic role for the Fas pathway in CIA. This, in addition to earlier findings on the modulation of Fas sensitivity of local joint cells by TNF- α [46], points to crosstalk between different cell-death pathways and suggests that a delicate balance between anti- and pro-apoptotic molecules exists in the rheumatoid synovium and that a pro-apoptotic shift of the balance may be partly responsible for the pathology of RA.

Competing interests

None declared.

Acknowledgements

The authors would like to thank Ms Ilona Klammfuss and Ms Eva Lorbeer for excellent technical assistance. This work is supported by grant IB24/3-2 from the DFG (German Research Foundation) and FKZ 01ZZ0108 from the BMBF (Federal Ministry for Research) to SMI.

References

- Trentham DE, Townes AS, Kang AH: **Autoimmunity to type II collagen: an experimental model of arthritis.** *J Exp Med* 1977, **146**:857-868.
- Staines NA, Wooley PH: **Collagen arthritis-what can it teach us?** *Br J Rheumatol* 1994, **33**:798-807.
- Wooley PH, Luthra HS, Stuart JM, David CS: **Type II collagen-induced arthritis in mice. I. Major histocompatibility complex (I region) linkage and antibody correlates.** *J Exp Med* 1981, **154**:688-700.
- Stuart JM, Watson WC, Kang AH: **Collagen autoimmunity and arthritis.** *FASEB J* 1988, **2**:2950-2956.
- Kinne RW, Palombo-Kinne E, Emrich F: **T-cells in the pathogenesis of rheumatoid arthritis, villains or accomplices?** *Biochim Biophys Acta* 1997, **1360**:109-141.
- Berek C, Kim HJ: **B-cell activation and development within chronically inflamed synovium in rheumatoid and reactive arthritis.** *Semin Immunol* 1997, **9**:261-268.
- Firesstein GS, Yeo M, Zvaifler NJ: **Apoptosis in rheumatoid arthritis synovium.** *J Clin Invest* 1995, **96**:1631-1638.
- Osborne BA: **Apoptosis and the maintenance of homeostasis in the immune system.** *Curr Opin Immunol* 1996, **8**:245-254.
- Nagata S, Golstein P: **The Fas death factor.** *Science* 1995, **267**:1449-1456.
- Singer GG, Abbas AK: **The Fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice.** *Immunity* 1994, **1**:365-371.
- Zheng L, Fisher G, Miller RE, Peschon J, Lynch DH, Lenardo MJ: **Induction of apoptosis in mature T cells by tumor necrosis factor.** *Nature* 1995, **377**:348-351.
- Griffiths GM: **The cell biology of CTL killing.** *Curr Opin Immunol* 1995, **7**:343-348.
- Lee RK, Spielman J, Zhao DY, Olsen KJ, Podack ER: **Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine-activated killer cells.** *J Immunol* 1996, **157**:1919-1925.
- Douni E, Akassoglou K, Alexopoulou L, Georgopoulos S, Haralambous S, Hill S, Kassiotis G, Kontoyiannis D, Pasparakis M, Plows D, Probert L, Kollias G: **Transgenic and knockout analysis of the role of TNF in immune regulation and disease pathogenesis.** *J Inflamm* 1996, **47**:27-38.
- Mori L, Iselin S, De Libero G, Lesslauer W: **Attenuation of collagen-induced arthritis in 55 kDa TNF receptor type I (TNFR1)-IgG1-treated and TNFR1-deficient mice.** *J Immunol* 1996, **157**:3178-3182.
- Salmon M, Scheel-Toellner D, Huissoon AP, Pilling D, Shamasdeen N, Hyde H, D'Andeac AD, Bacon PA, Emery P, Akbar AN: **Inhibition of T cell apoptosis in the rheumatoid synovium.** *J Clin Invest* 1997, **99**:439-446.
- Schirmer M, Vallejo AN, Weyand CM, Goronzy JJ: **Resistance to apoptosis and elevated expression of Bcl-2 in clonally expanded CD4+CD28- T cells from rheumatoid arthritis patients.** *J Immunol* 1998, **161**:1018-1025.
- Mysler E, Bini P, Drappa J, Ramos P, Friedman SM, Krammer PH, Elkon KB: **The apoptosis-1/Fas protein in human systemic lupus erythematosus.** *J Clin Invest* 1994, **93**:1029-1034.
- Amasaki Y, Kobayashi S, Takeda T, Ogura N, Jodo S, Nakabayashi T, Tsutsumi A, Fujisaku A, Koike T: **Up-regulated expression of Fas antigen (CD95) by peripheral naive and memory T cell subsets in patients with systemic lupus erythematosus (SLE): a possible mechanism for lymphopenia.** *Clin Exp Immunol* 1995, **99**:245-250.
- Sakata K, Sakata A, Vela-Roch N, Espinosa R, Escalante A, Kong L, Nakabayashi T, Cheng J, Talal N, Dang H: **Fas (CD95)-transduced signal preferentially stimulates lupus peripheral T lymphocytes.** *Eur J Immunol* 1998, **28**:2648-2660.
- Kamogawa J, Terada M, Mizuki S, Nishihara M, Yamamoto H, Mori S, Abe Y, Morimoto K, Nakatsuru S, Nakamura Y, Nose M: **Arthritis in MRL/lpr mice is under the control of multiple gene loci with an allelic combination derived from the original inbred strains.** *Arthritis Rheum* 2002, **46**:1067-1074.
- Sugiyama M, Tsukazaki T, Yonekura A, Matsuzaki S, Yamashita S, Iwasaki K: **Localization of apoptosis and expression of apoptosis related proteins in the synovium of patients with rheumatoid arthritis.** *Ann Rheum Dis* 1996, **55**:442-449.
- Cantwell MJ, Hua T, Zvaifler NJ, Kipps TJ: **Deficient Fas ligand expression by synovial lymphocytes from patients with rheumatoid arthritis.** *Arthritis Rheum* 1997, **40**:1644-1652.
- Hasunuma T, Hoa TTM, Aono H, Asahara H, Yonehara S, Yamamoto K, Sumida T, Gay S, Nishioka K: **Induction of Fas-dependent apoptosis in synovial infiltrating cells in rheumatoid arthritis.** *Int Immunol* 1996, **8**:1595-1602.
- Holmdahl R, Jonsson R, Larsson P, Klareskog L: **Early appearance of activated CD4+ T lymphocytes and class II antigen-**

- expressing cells in joints of DBA/1 mice immunized with type II collagen. *Lab Invest* 1988, **58**:53-60.
26. Giordano C, Stassi G, De Maria R, Todaro M, Richiusa P, Papoff G, Ruberti G, Bagnasco M, Testi R, Galluzzo A: **Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis.** *Science* 1997, **275**:960-963.
 27. D'Souza SD, Bonetti B, Balasingam V, Cashman NR, Barker PA, Trout AB, Raine CS, Antel JP: **Multiple sclerosis: Fas signaling in oligodendrocyte cell death.** *J Exp Med* 1996, **184**:2361-2370.
 28. Dowling P, Shang G, Raval S, Menonna J, Cook S, Husar W: **Involvement of the CD95 (APO-1/Fas) receptor/ligand in multiple sclerosis brain.** *J Exp Med* 1996, **184**:1513-1518.
 29. Chervonsky AV, Wang Y, Wong FS, Visintin I, Flavell RA, Janeway CA Jr, Matis LA: **The role of Fas in autoimmune diabetes.** *Cell* 1997, **89**:17-24.
 30. Feldmann M: **Development of anti-TNF therapy for rheumatoid arthritis.** *Nat Rev Immunol* 2002, **2**:364-371.
 31. Sabelko KA, Kelly KA, Nahm MH, Cross AH, Russell JH: **Fas and Fas ligand enhance the pathogenesis of experimental allergic encephalomyelitis, but are not essential for immune privilege in the central nervous system.** *J Immunol* 1997, **159**:3096-3099.
 32. Dittel BN, Merchant RM, Janeway CA Jr: **Evidence for Fas-dependent and Fas-independent mechanisms in the pathogenesis of experimental autoimmune encephalomyelitis.** *J Immunol* 1999, **162**:6392-6400.
 33. Kuhn K, Lotz M: **Regulation of CD95 (Fas/APO-1)-induced apoptosis in human chondrocytes.** *Arthritis Rheum* 2001, **44**:1644-1653.
 34. Aggrawal BB, Singh S, LaPushin R, Totpal K: **Fas antigen signals proliferation of normal human diploid fibroblast and mechanism its different from tumor necrosis factor.** *FEBS Lett* 1995, **364**:5-8.
 35. Chou CT, Yang JS, Lee MR: **Apoptosis in rheumatoid arthritis – expression of Fas, Fas-L, p53, and Bcl-2 in rheumatoid synovial tissues.** *J Pathol* 2001, **193**:110-116.
 36. Ford MS, Young KJ, Zhang Z, Ohashi PS, Zhang L: **The immune regulatory function of lymphoproliferative double negative T cells in vitro and in vivo.** *J Exp Med* 2002, **196**:261-267.
 37. Alonzi T, Fattori E, Lazzaro D, Costa P, Probert L, Kollias G, De Benedetti F, Poli V, Ciliberto G: **Interleukin 6 is required for the development of collagen-induced arthritis.** *J Exp Med* 1998, **187**:461-468.
 38. Joosten LA, Helsen MM, van de Loo FA, van den Berg WB: **Anti-cytokine treatment of established type II collagen-induced arthritis in DBA/1 mice. A comparative study using anti-TNF.** *Arthritis Rheum* 1996, **39**:797-809.
 39. Williams RO, Feldmann M, Maini RN: **Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis.** *Proc Natl Acad Sci USA* 1992, **89**:9784-9788.
 40. Doncarli A, Stasiuk LM, Fournier C, Abehsira-Amar O: **Conversion in vivo from an early dominant Th0/Th1 response to a Th2 phenotype during the development of collagen-induced arthritis.** *Eur J Immunol* 1997, **27**:1451-1458.
 41. Mauri C, Williams RO, Walmsley M, Feldmann M: **Relationship between Th1/Th2 cytokine pattern and the arthritogenic response in collagen-induced arthritis.** *Eur J Immunol* 1996, **26**:1511-1518.
 42. Brennan FM, Field M, Chu CO, Feldmann M, Maini RN: **Cytokine expression in rheumatoid arthritis.** *Br J Rheumatol* 1991, **1**:76-80.
 43. Klareskog L, McDevitt HO: **Rheumatoid arthritis and its animal models: the role of TNF-alpha and the possible absence of specific immune responses.** *Curr Opin Immunol* 1999, **11**:657-662.
 44. Mihara M, Moriya Y, Kishimoto T, Ohsugi Y: **Interleukin-6 (IL-6) induces the proliferation of synovial fibroblastic cells in the presence of soluble IL-6 receptor.** *Br J Rheumatol* 1995, **34**:321-325.
 45. Ma Y, Liu H, Tu-Rapp H, Thiesen HJ, Ibrahim SM, Cole SM, Pope RM: **Fas ligation on macrophages enhances IL-1R1/Toll-like receptor 4 signaling and promotes chronic inflammation.** *Nat Immunol* 2004, **5**:380-387.
 46. Asahara H, Hasunuma T, Kobata T, Inoue H, Muller-Lander U, Gay S, Sumida T, Nishioka K: **In situ expression of protooncogenes and Fas/Fas ligand in rheumatoid arthritis synovium.** *J Rheumatol* 1997, **24**:430-435.