Inflammatory Arthropathy in MRL Hematopoietic Chimeras Undergoing Fas Mediated Graft-versus-Host Syndrome

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ABSTRACT. ***Objective.*** To determine whether overexpression of the Fas ligand (FasL) on activated *lpr* T lymphocytes could induce arthritic lesions when grafted into syngeneic wild-type MRL mice expressing normal Fas receptor levels.

Methods. Lethally irradiated MRL+/+ mice were reconstituted with congenic MRL/*lpr* bone marrow cells and splenocytes overexpressing FasL. Fas-mediated cytotoxic properties of repopulating *lpr* splenic lymphocytes were evaluated *in vitro*. Simultaneously, the hind paw ankles of the hematopoietic chimeras were histologically examined.

Results. The *lpr* lymphocytes repopulating the spleen overexpressed FasL and had *in vitro* Fas-mediated cytotoxic activity. Simultaneously, *in vivo*, articular (synovitis, pannus) and periarticular (periostitis) inflammation with bone resorption were observed.

Conclusion. Arthritic lesions may be induced in Fas-expressing recipients by persistent engrafted syngeneic lymphocytes overexpressing FasL. (J Rheumatol 2001;28:956–61)

Key Indexing Terms:
FAS LIGAND FAS ANTIGEN APOPTOSIS RHEUMATOID ARTHRITIS

The autosomal recessive *lpr* mutation of the murine MRL strain is associated with the development of massive lymphadenopathy and an autoimmune syndrome[1,2] resembling human systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). This mutation corresponds to a marked decrease of the expression of the Fas receptor (FasR) that mediates apoptosis[3], as a consequence of an ETn retrotransposon insertion into the second intron of the gene[4–6], and results in the accumulation of a large number of non-malignant CD4–CD8– T lymphocytes in lymph nodes and spleen[7,8]. Owing to the massive overexpression of the Fas ligand (FasL), these double-negative (DN) cells exhibited cytotoxic activity against tumor cells bearing, either spontaneously or after transfection, the Fas molecule[9,10] or against H-2 compatible or incompatible Fas+ thymocytes or lipopolysaccharide-induced blasts[11]. This T cell population represents an activated subset that has escaped normal regulation by the Fas-mediated mechanism responsible for the activation-induced suicide (AICD) of mature T cells[12-15]. Therefore, the overexpression of FasL by activated MRL/*lpr* lymphocytes could be responsible for a chronic, non-antigen-specific autoimmune attack on organs expressing low levels of the FasR. Indeed, the *lpr* mutation is leaky, and low but measurable levels of transcription[4,5] and translation[16] of the wild-type Fas molecule have been reported in this strain. Non-negligible amounts of FasR on MRL/*lpr* hepatocytes have been observed in contrast to hepatocytes from mice rendered completely Fas-deficient by gene targeting[17].

Synoviocytes are Fas-expressing cells, particularly in arthritic mice[18] and patients with RA[19-23]. Indeed, these cells are sensitive *in vitro* to anti-Fas monoclonal antibody (Mab)-induced apoptosis[24,25] particularly in RA synovium[21]. The FasR has also been detected on human chondrocytes[26] and osteoblasts[27]. Furthermore, mononuclear T cells infiltrating the RA affected joints express FasL[23,28,29]. Soluble FasR, which may inhibit the regulation of these activated T lymphocytes by AICD, has also been observed in the synovial fluid of the inflamed joints of patients with RA[30]. These observations suggest that at least 2 mechanisms may be implicated in RA damage: first, impaired clearance of activated T cells due to a defective step in the Fas-mediated apoptotic pathway; second, overexpression of FasL on

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synovium-infiltrating lymphocytes. To test this latter hypothesis, we evaluated the consequences of an *in vivo* interaction between long-lived MRL/*lpr* lymphocytes overexpressing FasL and Fas-expressing synoviocytes of the wild-type MRL strain. These experimental conditions were met in hematopoietic chimeras created by grafting lethally irradiated wild-type MRL+/+ with MRL/*lpr* lymphoid cells (MRL/*lpr*→+/+ chimeras). These chimeras develop a graft versus host syndrome[31] because of the constitutive overexpression of FasL on lymphoid cells[9-11]. We previously showed that the livers of these chimeras were infiltrated by a polyclonal population of FasL-expressing T cells which induced liver disease through Fas-mediated apoptosis of hepatocytes[32]. We wanted to demonstrate that a chronic FasL-overexpression with a normal Fas receptor expression can induce joint damage.

MATERIALS AND METHODS

Mice. MRL/*lpr* (MRL/Mp-*lpr*/*lpr*) and MRL+/+ (MRL/Mp-+/+) originated from the Jackson Laboratory (Bar Harbor, ME, USA), and were obtained from Bomholtgard Breeding Center (Bomholtvej, Denmark). These strains were subsequently bred in our animal facilities. MRL/*lpr*→+/+ chimeras were obtained by intravenous grafting of spleen cells (5×10^7) (containing approximately 60–80% DN, 5–12% CD8+, 10–20% CD4+ T cells and about 10% B cells) plus 10^7 bone marrow cells from 5 to 8 month old MRL/*lpr* females into 2 to 3 month old MRL+/+ females lethally irradiated (850 cGy) in a ^{137}cesium irradiator. The control group consisted of irradiated MRL+/+ females injected with MRL+/+ cells (MRL+/+→+/+ chimeras).

Cytofluorometric analysis. After incubation with a monoclonal antibody (Mab) to mouse Fc receptor (clone 2.4 G2, PharMingen, San Diego, CA, USA), the expression of different cell surface antigens on erythrocyte-free spleen cells was analyzed by 2 color flow cytometry with a Facscalibur (Becton Dickinson & Co., Le Pont de Claix, France) using either fluorescein isothiocyanate or phycoerythrin conjugated Mab: rat Mab to Thy-1.2 (30-H12, PharMingen), CD4 (RM4-5, PharMingen), CD8 (53-6-7, PharMingen), or B220 (purchased from Beckman Coulter, Roissy CDG, France or produced by clone RA3-6B2, ATCC, Rockville, MD, USA) using a rat IgG2a Mab as the control (LODNP 16, Immunotech, Marseille, France). Thy-1.2-B220+ B cells were also labeled with a goat anti-mouse IgM (Sigma Chemical Co., St. Louis, MO, USA).

^{51}Cr-release cytotoxicity assay. Thymocytes ($20\text{-}30 \times 10^6$) in 0.2 ml of RPMI-10% fetal calf serum (FCS) were labeled with 100 µCi of ^{51}Cr-sodium chromate for 1 h at 37°C in a 5% CO_2 atmosphere. Splenic effectors and thymocyte targets (10^5/well) were washed 3 times and deposited in U-shaped wells of microtiter plates at the indicated effector-to-target (E:T) cell ratios in 200 µl of medium containing 5% FCS. A mixture of phorbol 12-myristate 13-acetate (final concentration: 5 ng/ml)(Sigma) plus ionomycin (final concentration: 0.5 µg/ml)(Calbiochem, San Diego, CA, USA) was added to the assay medium. Microplates were centrifuged for 1 min at 1000 rpm and incubated at 37°C for 4 h, at which time the supernatants (100 µl) were assessed for radioactivity. Specific ^{51}Cr-release from triplicate wells was calculated according to the formula:

$$\frac{\text{mean experimental release} - \text{mean spontaneous release}}{\text{mean maximum release} - \text{mean spontaneous release}} \times 100$$

The standard errors (± SE) to the means of triplicates were always < 5%. Maximum release represents the release from detergent treated targets and spontaneous release represents the release from target cells incubated with the corresponding number of cold targets instead of effector cells. This latter ranged between 10 and 20% of the maximal release in all experiments.

In some experiments, cytotoxicity was assessed in the presence of various concentrations of a fusion protein (Fas-FcIgG1) which consists of the mouse Fas extracellular domain fused to the Fc portion of human IgG1[33]. A control chimeric protein (SeAP-Fc) was obtained by replacing the mouse Fas sequence with the secreted form of human placental alkaline phosphatase[34].

Histological studies. Hind paws were fixed in 10% formalin neutral buffer solution. Tissue from the ankle joints was decalcified in RDO solution (Eurobio, Les Ulis, France), embedded in paraffin, sectioned, and stained with hematoxylin, eosin and safran.

Serological studies. Antibodies directed against collagen Type II (bovine collagen, Institut J. Boy, Reims, France) were tested by ELISA using microtiter plates coated with collagen (3 µg/ml) in phosphate-buffered saline (PBS) containing 1% ovalbumin and 0.05% Tween 20. Sera at different dilutions were incubated for 1 h at 37°C and bound antibodies were visualized by reaction with alkaline phosphatase conjugated goat anti-mouse IgG or IgM (Sigma) and p-nitrophenyl phosphate (Sigma). The serum from a high responder strain hyperimmunized with mouse collagen Type II served as the positive control[35] and pooled serum from normal non-immunized MRL+/+ mice was used as the negative control.

RESULTS

Histological examination of hind paw ankle joints. In one experimental MRL/*lpr*→+/+ chimera, macroscopic abnormalities with redness and swelling of the ankle were noted and prompted the systematic histological examination of all the chimeras' hind paws. Abnormal changes were thus detected in the ankles of 9/9 chimeras' hind paws, while those of lethally irradiated controls (8/8) reconstituted with MRL+/+ spleen plus bone marrow cells were normal (Table 1). Histological observations of the ankle joints revealed a multilayered synovial lining and replacement by pannus-

Table 1. Histopathology of MRL chimeras.

Graft Origin	Days Post Grafting	Hind Ankle	Liver
MRL+/+	29	N	N
MRL+/+	34	N	N
MRL+/+	34	N	N
MRL+/+	60	N	N
MRL/*lpr*	29	a, c, d	f, g
MRL/*lpr*	29	c, d	f, g
MRL/*lpr*	34	b, c, d, e	f, g, h
MRL/*lpr*	34	b, d, e	f, g, h
MRL/*lpr*	34	b, c, d, e	f, g, h
MRL/*lpr*	34	b, c, d, e	f, g, h
MRL/*lpr*	60	b, d, e	f, g, h
MRL/*lpr*	60	b, d, e	f, g, h
MRL/*lpr*	60	b, c	g, h

N: normal
a: paw swelling and redness
b: synovitis
c: pannus
d: bone erosion
e: periostitis
f: lymphoid infiltrations with periportal and perivascular endothelialitis
g: intralobular lymphoid infiltrations
h: hepatocyte apoptosis

like granulation tissue with striking erosion of cartilages and synovial bones (Figures 1a and b). In addition, periostitis with lymphocyte infiltrations was found in the periarticular area of 5/9 MRL/*lpr*→+/+ chimeras (Figures 2a and b). These lesions were observed from Day 29 (2 mice) to Days 34 (4 mice) and 60 (2 mice) post grafting. All experimental chimeras (9/9) presented lymphoid periportal or perivascular infiltrations of the liver with hepatocyte apoptosis, typical of the described Fas-dependent liver disease[32]. The suspected role of anticollagen antibodies in RA prompted the search for such antibodies in MRL/*lpr*→+/+ chimeras presenting articular lesions; neither IgG nor IgM antibodies to collagen were found in the sera of these animals, regardless of the time post grafting (data not shown).

Fas-dependent cytotoxic effectors in chimera spleens. The presence of infiltrating lymphoid cells in periostitis could indicate a role for circulating lymphocytes in the inflammatory lesions of chimeras undergoing Fas-mediated graft versus host reaction. Therefore, the Fas-mediated cytotoxic potential of the splenocytes repopulating irradiated recipients was tested in an *in vitro* assay against thymocytes expressing FasR. On Day 21 post grafting, splenocytes from chimeras were able to kill wild-type (MRL+/+) but not Fas-mutated (MRL/*lpr*) thymocytes. This Fas-dependent cytotoxic activity was never exerted by MRL+/+ splenocyte effector cells (Figure 3a). This result could not be attributed to a difference in a particular T cell subset in MRL/*lpr*→+/+ chimeras compared to controls. Indeed and surprisingly so, spleens from both groups presented nearly equal percentages of the different T cell subpopulations (single-positive (SP) or DN T cells) with around 2% DN T cells, even though the chimeras had been engrafted with spleen cells

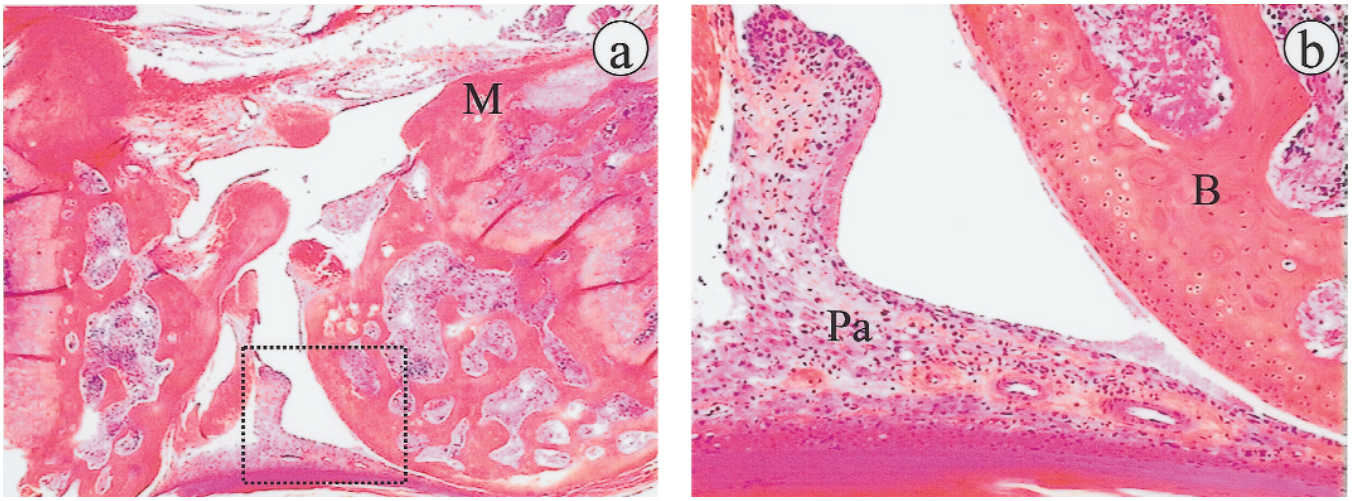

Figure 1. Histopathology of the ankle joint of a typical MRL/*lpr*→+/+ chimera (a); b: high magnification of the pannus (Pa) in the boxed area in a. M: muscle; B: bone.

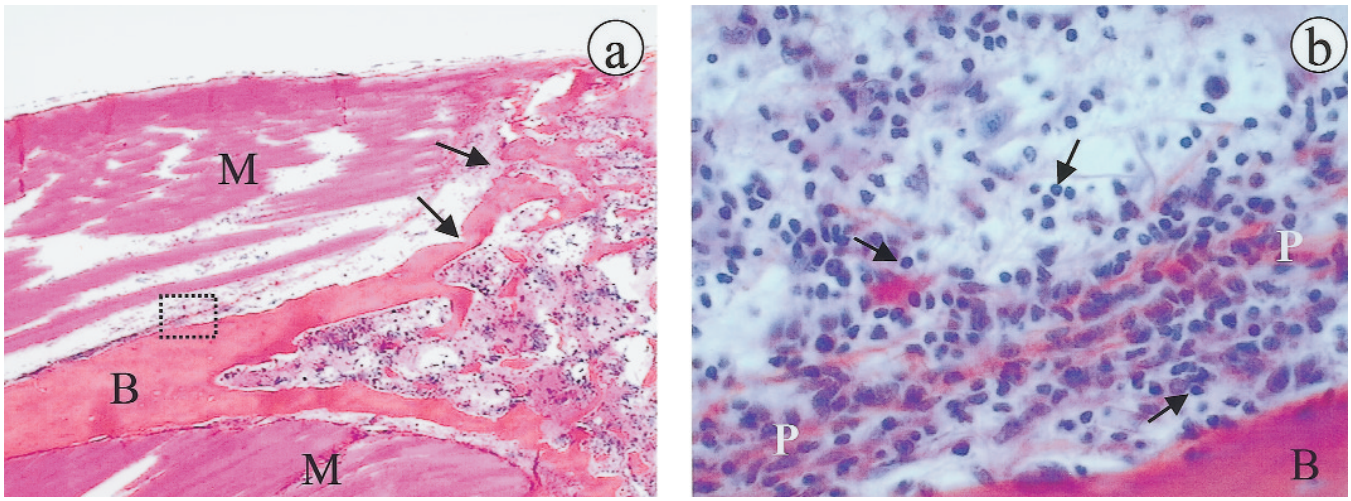

Figure 2. Histopathology of the periarticular area of a typical MRL/*lpr*→+/+ chimera. a. Mononuclear cell infiltrations in the muscle (M) with periostitis and bone (B) destruction (↑) in the tibia. b. High magnification of the boxed area showing typical lymphocyte infiltrations (↑) forcing the separation of periosteum (P) and bone (B).

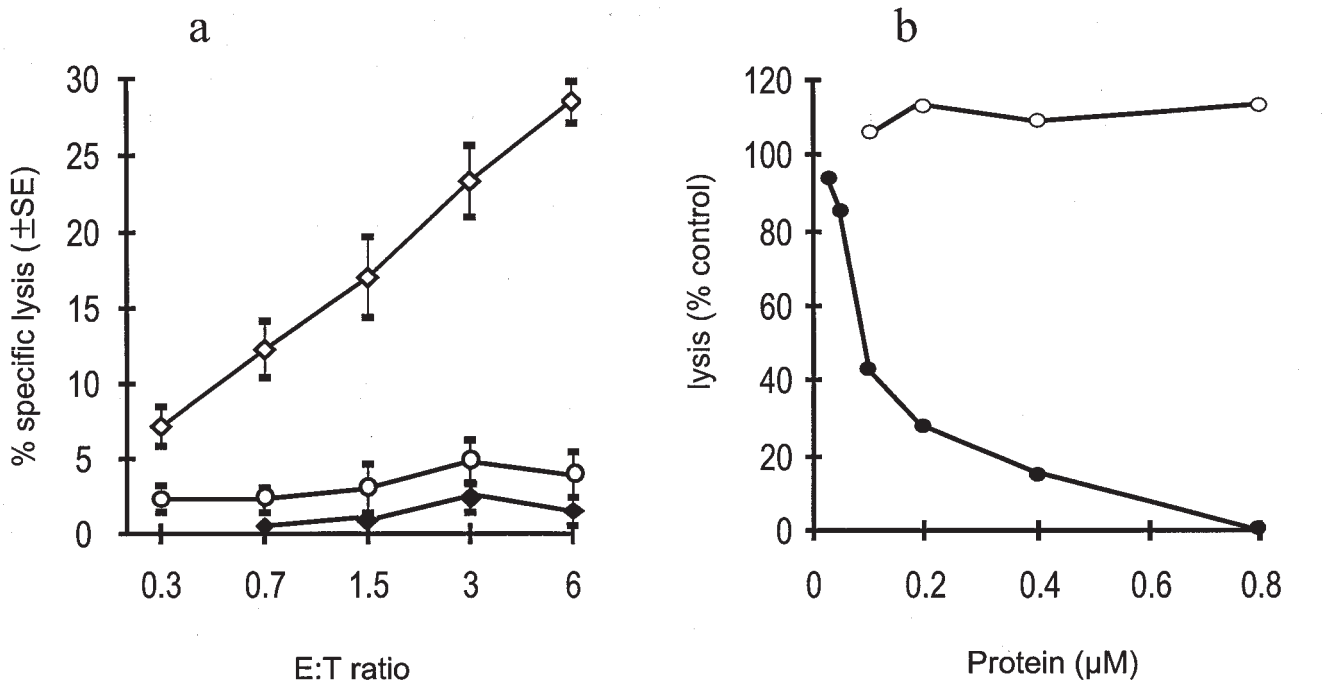

Figure 3. A. The Fas-dependent cytotoxicity of MRL/*lpr*→+/+ chimeric spleen cells on Day 21 post grafting. Values represent the means ± SE obtained from 6 individual MRL/*lpr*→+/+ chimeras (◇) or from 6 MRL/lpr+/+→+/+ control graftees (○) Target cells: MRL+/+ (open symbols ◇, ○) or MRL/*lpr* (closed symbol ◆) thymocytes. B: Inhibition of cytotoxicity against MRL+/+ thymocytes (E:T ratio of 50:1) by Fas-FcIgG1 (●) but not by control protein: SeAP-Fc (○).

containing around 60% DN T cells in the experimental group, as opposed to 2% in the control group (Table 2). The cytotoxicity of MRL/*lpr*→+/+ splenic effectors was inhibited by the recombinant Fas-IgG1 protein, indicating that they kill through the Fas pathway (Figure 3b). The *in vitro* cytotoxicity of splenocytes was evident by Day 14 post grafting (data not shown) and increased to reach a maximal level on Day 21. A semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of splenocyte RNA from chimeras and control graftees indicated that FasL expression followed the same kinetics[32]. Thereafter, histological examinations indicated that the spleens of chimeras underwent progressive cellular depletion and fibrosis as previously described[32].

DISCUSSION

The MRL/*lpr* strain is documented as a model for RA with morphological and immunological characteristics resembling the human disease. However, the joint disease develops spontaneously in only some aged animals[36-38]. In this strain, the leaky mutation in the Fas gene supports the hypothesis that the mild joint disease of the MRL/*lpr* strain might be the consequence of an interaction between the FasL-overexpressing T lymphocytes and the low levels of Fas expressed on joint cells, i.e., synoviocytes, osteoblasts, chondrocytes. In MRL/*lpr*→+/+ chimeras, the coexistence of FasL-expressing T lymphocytes (unable to undergo AICD as a consequence of their *lpr* origin) with tissues expressing normal amounts of FasR, results in the typical

Table 2. Phenotype of splenocytes from MRL/*lpr* → +/+ chimeras and control grafts.

Splenocyte origin	n	Phenotype* Thy 1.2$^+$ CD4$^+$	Thy 1.2$^+$ CD8$^+$	Thy 1.2$^+$ CD8$^-$ CD4$^-$	Thy 1.2$^-$ B220$^+$
MRL/*lpr* – splenic graft**	9	18.5 ± 5.0	11.6 ± 1.7	60.4 ± 11.5	10.0 ± 2.8
MRL/*lpr* → +/+ chimeras	9	24.5 ± 3.9	13.5 ± 1.7	2.1 ± 0.4	50.4 ± 5.0
MRL+/+ – splenic graft**	4	29.3 ± 4.9	21.5 ± 1.8	2.2 ± 0.5	33.8 ± 4.8
MRL+/+ → +/+	4	20.9 ± 2.6	18.7 ± 2.3	2.5 ± 0.3	31.5 ± 1.3

*Spleen cell subsets were determined by two-color flow cytometry. Results are expressed as mean percentages ± SE of total mononuclear cells 21–28 days post grafting.
**Splenic graft indicates the graft preparation used to repopulate the chimera specified on the following line.

Fas-mediated graft versus host syndrome previously described[31]. We also observed that these chimeras developed hepatic disease[32]. Our study shows that MRL/*lpr*→+/+ chimeras developed severe joint disease, with synovitis, pannus, bone erosion, and periostitis within a few weeks post grafting. The role of autoantibodies against collagen in the perpetuation of RA[39] was tested in MRL chimeras, but anti-collagen antibodies were never found (even in mice developing the more aggressive disease). This observation is consistent with the recent report of collagen-induced RA in mice lacking both T and B cells which indicates that collagen might have non-antigenic arthritogenic properties[40]. Splenocytes from MRL/*lpr*→+/+ chimeras, which overexpress the FasL[32], are endowed with a cytotoxic potential directed only against Fas-expressing thymocytes and specifically inhibited by soluble recombinant FasR, and are therefore dependent on the Fas pathway. Surprisingly, chimeras grafted with MRL/*lpr* spleen cells, which contain high levels of the DN T lymphocyte subpopulation, had only low percentages of this cell subtype, like control chimeras and MRL+/+ mice. Grafts with purified DN T cells gave similar results (our unpublished results). DN cells are derived from SP T cells[8,41,42] and are unable to undergo apoptosis, but they may recover their former phenotype and reexpress CD4 or CD8 molecules. This point, however, merits further investigation.

The *in vivo* model of Fas-mediated pathophysiology described here emphasizes the involvement of at least 2 variables in the initiation of joint disease. The first is the maintenance of FasL-overexpressing T cells in the periphery, which implies a defect in one step of the process by which useless activated T lymphocytes are eliminated. This defect could affect either an apoptosis receptor (illustrated by the *lpr* mutation), or one step of the signaling cascade leading to cell death. Alternatively, synovial-infiltrating lymphocytes may escape apoptosis following expression of Bcl-x after interaction with synovial stromal cells, a mechanism recently observed in synovial-infiltrating B lymphocytes[43]. It was recently reported[18] that a defect in FasL (*gld* mutation) introduced into the MRL/*gld* strain elicited a moderate arthritic syndrome (synovium proliferation in 77% and pannus in 20% of mice), indicating that RA may also be induced by alternative pathways including TNF, which stimulates synovial cell proliferation[44] and is found at elevated levels in the MRL strain[45]. The second important variable in disease induction is the presence of Fas on the target tissues which may, in turn, be enhanced by activated T cell production of IFNγ, a cytokine that is present at abnormally elevated levels in the MRL/*lpr* strain[46]. These conditions, clearly illustrated in MRL/*lpr*→+/+ chimeras, may also be at work in several previously proposed animal models of RA in which strongly and repeatedly activated T cells may initiate the inflammatory process through their FasL expression, i.e., TCR-transgenic mouse T cells that fortuitously recognize a self-antigen, escape tolerance and create a context of systemic reactivity[47]; almost half of the 1 year old female HTLV-1-transgenic mice develop an inflammatory arthropathy that can be cured by anti-Fas antibody treatment[48] and is a consequence of immunological disturbances induced by the transgene[49]; and the superantigen-induced arthritis of MRL/*lpr* mice[50].

The Fas-mediated joint disease of MRL/*lpr*→+/+ chimeras we have described provides a useful model to investigate therapeutic approaches based on blocking receptor-ligand interaction with soluble recombinant FasR.

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