Impaired Fas Signaling Pathway Is Involved in Defective T Cell Apoptosis in Autoimmune Murine Arthritis

Jian Zhang, Tamás Bárdos, Katalin Mikecz, Alison Finnegan and Tibor T. Glant

*J Immunol* 2001; 166:4981-4986; doi: 10.4049/jimmunol.166.8.4981

http://www.jimmunol.org/content/166/8/4981

**References**
This article cites 41 articles, 14 of which you can access for free at: http://www.jimmunol.org/content/166/8/4981.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Impaired Fas Signaling Pathway Is Involved in Defective T Cell Apoptosis in Autoimmune Murine Arthritis

Jian Zhang, Tamás Bárdos, Katalin Mikecz, Alison Finnegan, and Tibor T. Glant

Proteoglycan (PG)-induced arthritis (PGIA) is a novel autoimmune murine model for rheumatoid arthritis induced by immunization with cartilage PG in susceptible BALB/c mice. In this model, hyperproliferation of peripheral CD4+ T cells has been observed in vitro with Ag stimulation, suggesting the breakdown of peripheral tolerance. Activation-induced cell death (AICD) is a major mechanism for peripheral T cell tolerance. A defect in AICD may result in autoimmunity. We report in this study that although CD4+ T cells from both BALB/c and C57BL/6 mice, identically immunized with cartilage PG or OVA, express equally high levels of Fas at the cell surface, CD4+ T cells from human cartilage PG-immunized BALB/c mice, which develop arthritis, fail to undergo AICD. This defect in AICD in PGIA may lead to the accumulation of autoreactive Th1 cells in the periphery. The impaired AICD in PGIA might be ascribed to an aberrant expression of Fas-like IL-1 β-converting enzyme-inhibitory protein, which precludes caspase-8 activation at the death-inducing signaling complex, and subsequently suppresses the caspase cascade.

Copyright © 2001 by The American Association of Immunologists 0022-1767/01/$02.00

Materials and Methods

Aggs and immunization

High-density cartilage PG (aggrecan) was purified by cesium chloride gradient centrifugation, as described previously (3). Purified PG was digested...
with chondroitinase ABC and endo-β-galactosidase (both from Seikagaku America, Rockville, MD) before use for immunization to remove glycosaminoglycan side chains (21). These negatively charged glycosaminoglycan side chains have masking effects and may interfere with the Ag processing (22).

Female B6 and BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). B6 and BALB/c mice were immunized i.p. at 6 wk of age with 100 μg of arthritogenic HPG or control Ag OVA in CFA. This was followed by three identical booster injections in immunofluorescence assay (IFA) on day 7, 28, and 49. The mice were sacrificed on day 7 after final immunization, at which time most HPG-immunized BALB/c mice were arthritic.

Abs and reagents

Rabbit polyclonal anti-mouse caspase-3 (H-277), caspase-8 (T-16), Bcl-2 (C-2), and Bcl-x<sub>S</sub> (L-19) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin mAb (AC40), propidium iodide (PI), and BALB/c mice were incubated on ice for 30 min with recombinant and FasL was determined at each time point.

Activated T cells were pretreated with 50 μM 48 h with plate-bound anti-CD3 and anti-CD28 mAbs. Splenic T cells from BALB/c mice were activated for 72 h in 24-well plates precoated with anti-CD3 mAb (10<sup>5</sup> cells/ml). The cells were then washed twice with ice-cold PBS (pH 7.4), followed by centrifugation at 200 × g for 5 min. The cells were lysed in ice-cold lysis buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EGTA, 50 mM β-glycerophosphate, 2 mM Na<sub>2</sub>VO<sub>3</sub>, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin.

Electrophoresis and immunoblotting

Protein concentrations in the cell lysates were determined using a bicinchoninic acid assay kit (Pierce, Rockford, IL). Thirty micrograms of proteins from cell lysates was loaded onto each lane of 10 or 15% SDS-PAGE gel, separated, and then blotted to nitrocellulose membrane (Amersham, Piscataway, NJ). The membranes were blocked in 2% BSA and 0.1% Tween 20 in PBS for 2 h at room temperature. Anti-caspase 3 and anti-actin Abs were used at 1/500, and the other Abs at 1/1000 dilutions. After overnight incubation at 4°C with agitation, membranes were washed three times with PBS and 0.1% Tween 20 (PBS-T). The HRP-coupled goat anti-rabbit Ab or rabbit anti-mouse Ab was used at 1/5000 or 1/3000 dilution for 2 h at room temperature. Membranes were washed (10 min) with PBS-T, and the specific proteins were identified using the ECL system (Amersham).

Cytokine assays

CD4<sup>+</sup> T cells from HPG- or OVA-immunized B6 and BALB/c mice were cultured in 96-well plates precoated with anti-CD3 (10 μg/ml). Supernatants collected after 72 h were assayed for their IFN-γ and IL-4 concentrations by capture ELISA method using recombinant mouse IFN-γ and IL-4.

**Preparation of cell lysates**

CD4<sup>+</sup> T cells were collected by centrifugation at 200 × g for 5 min at 4°C. The cells were then washed twice with ice-cold PBS (pH 7.4), followed by centrifugation at 200 × g for 5 min. The cells were lysed in ice-cold lysis buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EGTA, 50 mM β-glycerophosphate, 2 mM Na<sub>2</sub>VO<sub>3</sub>, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin.

**Electrophoresis and immunoblotting**

Protein concentrations in the cell lysates were determined using a bicinchoninic acid assay kit (Pierce, Rockford, IL). Thirty micrograms of proteins from cell lysates was loaded onto each lane of 10 or 15% SDS-PAGE gel, separated, and then blotted to nitrocellulose membrane (Amersham, Piscataway, NJ). The membranes were blocked in 2% BSA and 0.1% Tween 20 in PBS for 2 h at room temperature. Anti-caspase 3 and anti-actin Abs were used at 1/500, and the other Abs at 1/1000 dilutions. After overnight incubation at 4°C with agitation, membranes were washed three times with PBS and 0.1% Tween 20 (PBS-T). The HRP-coupled goat anti-rabbit Ab or rabbit anti-mouse Ab was used at 1/5000 or 1/3000 dilution for 2 h at room temperature. Membranes were washed (10 min) with PBS-T, and the specific proteins were identified using the ECL system (Amersham).
IL-4 standards. Cytokine standard curves were linear in the range of 20–20,000 pg/ml.

Statistical analysis
A two-way ANOVA and Student t tests were performed to determine statistical significance using StatView software (Abacus Concepts, San Francisco, CA).

Results and Discussion

CD4⁺ T cells from HPG-immunized B6 and BALB/c mice display activated phenotype

AICD results from repeated stimulation through the TCR and is believed to be dependent upon Fas-FasL interaction (13–20). To assess the AICD susceptibility, the expression of CD25 and Fas in CD4⁺ T cells from HPG- or OVA-immunized B6 and BALB/c as well as unimmunized naive BALB/c mice was determined using flow cytometry. Interestingly, levels of CD25 and Fas expressed by CD4⁺ T cells from immunized animals were higher than by those from unimmunized naive animals (Fig. 1A). This finding suggests that T cells are activated during immunizations and are ready to undergo AICD. In contrast, AICD in naive T cells requires repeated stimulations through the TCR to induce high levels of Fas and FasL expression. As shown in Fig. 1B, naive CD4⁺ T cells from BALB/c and B6 mice expressed very low levels of Fas and FasL. Primary stimulation with plate-bound anti-CD3 and anti-CD28 mAbs significantly up-regulated Fas but not FasL expression in T cells (Fig. 1B). The expression of FasL required TCR restimulation, which processes down-regulated Fas expression (Fig. 1B). The down-regulation of Fas expression could be due to the internalization of Fas receptor upon ligation with FasL. Similar kinetics of Fas and FasL expression in BALB/c and B6 CD4⁺ T cells during induction of AICD suggest that these T cells are equally susceptible to AICD. To confirm that AICD is mediated by Fas-FasL interaction in our experimental system, activated T cells were preincubated with Fas-Fc chimeric protein, which blocks Fas-FasL interaction, and then stimulated with anti-CD3 to induce AICD. As shown in Fig. 1C, Fas-Fc chimeric protein significantly abrogated TCR ligation-induced AICD.

AICD is defective in CD4⁺ T cells in PGIA

Fas-mediated AICD is an important mechanism of peripheral T cell tolerance (14–17, 20). Mice or human individuals lacking functional Fas or FasL display profound lymphoproliferative reactions associated with (auto)immune disorders (23–26). In PGIA, CD4⁺ T cells proliferate at a high rate in response to PG stimulation (7), and exhibit a Th1-type response (5–7). These observations suggest that a defect in AICD of autoreactive Th1 cells may contribute to the pathogenesis of the disease. To test this hypothesis, CD4⁺ T cells from HPG- or OVA-immunized arthritis-resistant B6 and arthritis-susceptible BALB/c mice were activated with plate-bound anti-CD3 mAb for 48 h, and the levels of AICD in these T cells were determined by flow cytometry using FITC-labeled annexin V and PI (27). Note that the protocols used in this study were different from that described in the literature (28). In our experimental system, T cells from immunized animals were activated and expressed very high levels of Fas at the cell surface, suggesting that these cells are ready to undergo Fas-mediated AICD. However, TCR-induced AICD was significantly lower for CD4⁺ T cells from HPG-immunized BALB/c mice than from HPG-immunized B6 mice or OVA-immunized BALB/c and B6 mice (Fig. 2A). Since naive T cells might undergo Fas-independent apoptosis if cultured in the absence of Ag or costimulation (29), we

FIGURE 2. AICD is impaired in PGIA. A, CD4⁺ T cells from HPG- or OVA-immunized B6 (HPG- or OVA-B6) and BALB/c mice (HPG- or OVA-B/c) were stimulated with plate-bound anti-CD3 mAb (10 μg/ml; ) for 48 h or left unstimulated (NS, ■). Apoptotic cells were determined by FITC-labeled annexin V and PI staining. B, Splenic CD4⁺ T cells from HPG-immunized B6 mice were pretreated with 50 μg/ml Fas-Fc protein and stimulated for 48 h with plate-bound anti-CD3 mAb. Apoptotic cells were determined as above. C, splenic CD4⁺ T cells (2 × 10⁵/ml) from HPG- or OVA-immunized B6 and BALB/c mice were cultured for 48 h in round-bottom plates precoated with anti-CD3 mAb in the presence of 50 μg/ml Fas-Fc protein. Alternatively, CD4⁺ T cells (1 × 10⁵/ml) from HPG-immunized BALB/c and B6 mice were cultured in the presence of 20 μg/ml HPG and APCs (2500 rad-irradiated syngenic spleen cells, 2 × 10⁵/ml), and T cell proliferation was determined by [³H]thymidine incorporation (***, p < 0.001).

FIGURE 3. The up-regulation of FasL expression in CD4⁺ T cells in PGIA is normal. A, CD4⁺ T cells from HPG- or OVA-B/c and B6 mice were stimulated with plate-bound anti-CD3 mAb for 48 h and stained with PE-labeled anti-FasL mAb. B, CD4⁺ T cells from HPG- or OVA-immunized B6 and B6 mice were preincubated for 30 min at 4°C with recombinant mouse FasL (0.8 μg/ml), followed by cross-linking for 24 h at 37°C with anti-6X histidine mAb (10 μg/ml; ■) or left unstimulated (NS, ■), and apoptotic cells were determined. The results are representative of two independent experiments.
then tested whether cell death observed in immunized control animals was Fas dependent. To this end, CD4\textsuperscript{+} T cells from HPG-immunized B6 mice were stimulated with plate-bound anti-CD3 mAb, and AICD was determined as above. As shown in Fig. 2B, TCR-induced AICD in HPG-immunized B6 mice was blocked by a Fas-Fc chimeric protein, suggesting that AICD observed in immunized B6 mice is Fas dependent. Moreover, the defective AICD in PGIA correlated with hyperproliferation of CD4\textsuperscript{+} T cells induced by TCR ligation and HPG stimulation (Fig. 2C). Hyperproliferation of CD4\textsuperscript{+} T cells and defective AICD was only observed in HPG-immunized BALB/c mice (Fig. 2, A and C), indicating that defective AICD observed in PGIA was not a general phenomenon of immunization with Ags or a strain-dependent phenomenon.

A recent study suggests that cross-linking Fas with recombinant FasL could provide a costimulatory signal to T cells and induce a higher rate of proliferation (30). FasL ligation-induced costimulation of T cells is mediated by FLIP, which is possibly involved in NF-κB and extracellular signal-regulated kinase pathways (30). Therefore, it is possible that hyperproliferation of CD4\textsuperscript{+} T cells from arthritic animals might result from a stronger costimulation from Fas receptor. To test this hypothesis, splenic CD4\textsuperscript{+} T cells from HPG- and OVA-immunized B6 and BALB/c mice were stimulated with plate-bound anti-CD3 mAb in the presence or absence of a mouse Fas-Fc chimeric protein, and T cell proliferation was determined. As shown in the left panel of Fig. 2C, Fas-Fc chimeric protein significantly inhibited TCR-induced CD4\textsuperscript{+} T cell proliferation from all groups, especially from arthritic BALB/c mice. These findings are consistent with a role of Fas signaling in costimulation (30).

**Impaired AICD in PGIA does not result from defective FasL expression**

The next question was whether this defective AICD was due to the impaired FasL expression on the cell surface. To test this possibility, CD4\textsuperscript{+} T cells from HPG- or OVA-immunized B6 and BALB/c mice were stimulated with plate-bound anti-CD3 for 48 h and FasL expression was determined by flow cytometry. After CD3 ligation in vitro, FasL expression in CD4\textsuperscript{+} T cells from HPG- or OVA-immunized B6 and BALB/c mice was up-regulated in a similar fashion (Fig. 3A). This observation suggests that there might be a defect in the Fas-mediated signaling pathway rather than a decrease in the expression of either Fas or FasL itself. In support of this notion, CD4\textsuperscript{+} T cell death induced by cross-linked FasL stimulation is impaired in HPG-immunized BALB/c mice (Fig. 3B).

**Defective AICD is not due to a high expression of memory T cell phenotype in PGIA**

Recent studies suggest that memory T cells are more resistant to AICD than naive T cells are (28, 31). Defective AICD could also be explained by a higher proportion of memory cells in HPG-immunized BALB/c to B6 mice. To test this hypothesis, the profiles of memory vs naive T cells from HPG-immunized B6 and BALB/c mice were examined using CD44 and CD62L as markers. Memory T cells express high levels of CD44 and low levels of CD62L, whereas naive T cells express low levels of CD44 and high levels of CD62L (28, 32). As shown in Fig. 4, CD4\textsuperscript{+} T cells in both groups identically expressed high levels of CD44 and low levels of CD62L, suggesting that CD4\textsuperscript{+} T cells were primed in both experimental groups. Thus, the resistance of CD4\textsuperscript{+} T cells to AICD observed in HPG-immunized BALB/c mice could not be ascribed to an increased expression of memory phenotype. It is noteworthy that although immunizations were used to generate memory T cells (28), the immunization protocol used was different from the one we used in our study in which CFA and IFA were used and mice were immunized with Ag/CFA or IFA four times.

**Defective AICD in PGIA is not due to differential expression of Th1 vs Th2 cells**

It has been shown that Th1 cells are susceptible, whereas Th2 cells are resistant to AICD (33). BALB/c mice carry a genetic predisposition toward a Th2-type response (34). Immunization of BALB/c mice with HPG, however, can override this genetic inclination, and a significant shift toward Th1 dominance was observed in arthritic animals (Fig. 5). This shift in Th1/Th2 balance

---

**FIGURE 4.** CD4\textsuperscript{+} T cells from B6 and BALB/c (B/c) mice immunized with HPG or OVA showed similar memory cell phenotype. Freshly isolated splenic CD4\textsuperscript{+} T cells from unimmunized naive BALB/c and B6 mice as well as HPG- or OVA-immunized B6 and BALB/c mice were stained with FITC-labeled anti-CD44 and PE-labeled anti-CD62L mAbs, respectively, and analyzed by flow cytometry. The results are representative of two independent experiments.

**FIGURE 5.** The resistance of CD4\textsuperscript{+} T cells to AICD in PGIA is not due to increased expression of Th2 cells. CD4\textsuperscript{+} T cells (2 × 10\textsuperscript{6}/ml) from HPG-B6, HPG-BALB/c (B/c), OVA-B/c, and OVA-B6 mice were cultured for 72 h in 24-well plate in the presence of plate-bound anti-CD3 mAb, and IFN-γ and IL-4 concentrations in the culture supernatants were determined by ELISA.
was even more evident when cytokine (IL-4 and IFN-γ)-producing spot-forming cells or serum IgG1/IgG2a autoantibody levels were compared (5). It is important to note CD4+ T cells from HPG- or OVA-immunized B6 and BALB/c mice exhibit a Th1-type response (Fig. 5). This may be due to the B6 genetic background that favors Th1 cell differentiation (35). Nevertheless, arthritis cannot be induced in the absence of HPG in BALB/c mice, and B6 mice do not develop differentiation (35). Nevertheless, arthritis cannot be induced in the absence of HPG in BALB/c mice, and B6 mice do not develop arthritis when immunized with HPG. Furthermore, a significantly higher level of AICD was observed in HPG-immunized B6 CD4+ T cells than in BALB/c CD4+ T cells, even though HPG-immunized BALB/c CD4+ T cells also displayed a Th1 phenotype. Taken together, these observations suggest that immunization of BALB/c, but not B6, mice with HPG promotes T cell (more likely Ag-specific autoreactive Th1 cells) escape from peripheral deletion. Hyperproliferation of CD4+ T cells from arthritic animals, in response to in vitro TCR or HPG stimulation (Fig. 2C), further supports this hypothesis.

Aberrant expression of FLIP in PGIA may mediate the resistance of AICD

The caspase cascade initiated by Fas-FasL interaction is central for Fas-mediated cell death, and this process can be inhibited by FLIP (36–38). Our observations suggested that there might be a defect in the Fas-mediated signaling pathway in PGIA (Fig. 2A). Therefore, we investigated whether FLIP, an important inhibitor of the Fas-mediated signaling pathway, played a role in the inhibition of AICD in PGIA. The proteins from CD4+ T cells, either unstimulated or stimulated for 48 h with plate-bound anti-CD3, were blotted with anti-FLIP, anti-caspase-8 (casp-8), and anti-caspase-3 (casp-3) Abs, respectively. Equal loading of proteins was confirmed by anti-actin blotting. B, CD4+ T cells from HPG- or OVA-immunized B6 and BALB/c mice were incubated for 30 min on ice with recombinant mouse FasL mAb, cross-linked for 10 min at 37°C with anti-6x histidine mAb, and lysed. Cell lysates were immunoprecipitated with anti-Fas mAb, and DISC components were determined by immunoblotting with anti-Fas, anti-caspase-8, and anti-FLIPL Abs, respectively. The results are representative of three independent experiments.

FIGURE 6. Aberrant expression of FLIP may mediate the resistance of CD4+ T cells to AICD in PGIA. A. Splenic CD4+ T cells from HPG- or OVA-immunized B6 and BALB/c (B/c) mice were stimulated with plate-bound anti-CD3 mAb for 48 h in the presence of 30 U/ml IL-2. Cells were collected at day 2, lysed, and blotted with anti-FLIP, anti-caspase-8 (casp-8), and anti-caspase-3 (casp-3) Abs, respectively. Equal loading of proteins was confirmed by anti-actin blotting. B, CD4+ T cells from HPG- or OVA-immunized B6 and BALB/c mice were incubated for 30 min on ice with recombinant mouse FasL mAb, cross-linked for 10 min at 37°C with anti-6x histidine mAb, and lysed. Cell lysates were immunoprecipitated with anti-Fas mAb, and DISC components were determined by immunoblotting with anti-Fas, anti-caspase-8, and anti-FLIPL Abs, respectively. The results are representative of three independent experiments.

References


