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

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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 B6 mice, identically immunized with human 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-1β-converting enzyme-inhibitory protein, which precludes caspase-8 activation at the death-inducing signaling complex, and subsequently suppresses the caspase cascade initiated by Fas-Fas ligand interaction. Moreover, this aberrant expression of Fas-like IL-1β-converting enzyme-inhibitory protein may also mediate TCR-induced hyperproliferation of CD4⁺ T cells from arthritis BALB/c mice. Our data provide the first insight into the molecular mechanism(s) of defective AICD in autoimmune arthritis.

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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\(_{\text{L}}\) (L-19) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin mAb (AC40), propidium iodide (PI), HRP-coupled goat anti-rabbit IgG, and rabbit anti-mouse IgG Abs were obtained from Sigma (St. Louis, MO). Anti-FLIP\(_{\text{L}}\) was purchased from Serotec (Kidlington, OX, U.K.). The following reagents were purchased from BD PharMingen (San Diego, CA): FITC-labeled anti-Fas (Jo-2), PE-labeled anti-FasL (MFL-3), FITC-labeled anti-CD44 (IM7), PE-labeled anti-CD62L (MEL-14), anti-CD3 (145-2C11), PE-labeled anti-CD25 (PC16) mAbs, and FITC-labeled annexin V. ELISA kits for mouse IFN-γ and IL-4, mouse Fas\(^+\) T cell subset isolation column kits, mouse Fas-Fc chimeric proteins, recombinant mouse FasL, which consists of aa residues 132–279 of mouse FasL, the signal peptide of human CD3 and six histidine residues, and anti-6X histidine mAb were purchased from R&D Systems (Minneapolis, MN). Goat anti-hamster IgG Ab was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

**Cell activation and detection of AICD**

Freshly isolated splenic CD4\(^+\) T cells (2 x 10^6/ml) from HPG- or OVA-immunized B6 and BALB/c mice (purity ≥95% as determined by FACS analysis of CD4 cell surface expression) were suspended in RPMI 1640 medium containing 10% heat-inactivated FCS, 10 mM HEPES, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 0.05 mM 2-ME, and 2 mM glutamine (all from Life Technologies, Grand Island, NY). The cells were cultured for 72 h in 24-well plates precoated with anti-CD3 mAb (10 μg/ml), and then restimulated with plate-bound anti-CD3 for 24 h. Apoptotic cells were determined by annexin V and PI staining. The results are representative of three independent experiments.

**Cytokine assays**

CD4\(^+\) 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 standards, respectively.
HPG-immunized BALB/c and B6 mice were cultured in the presence of 20Bion in T cells (Fig. 1). The expression of FasL required TCR CD28 mAbs significantly up-regulated Fas but not FasL expression. Primary stimulation with plate-bound anti-CD3 and anti-CD28 mAbs significantly up-regulated Fas but not 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...
then tested whether cell death observed in immunized control animals was Fas dependent. To this end, CD4+ 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+ T cells induced by TCR ligation and HPG stimulation (Fig. 2C). Hyperproliferation of CD4+ 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 co-stimulation 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+ T cells from arthritic animals might result from a stronger costimulation from Fas receptor. To test this hypothesis, splenic CD4+ 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+ 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+ 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+ 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+ 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+ T cells in both groups identically expressed high levels of CD44 and low levels of CD62L, suggesting that CD4+ T cells were primed in both experimental groups. Thus, the resistance of CD4+ 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...
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 (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-FLIPα, 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.

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-FLIPα Ab. FLIP protein was constitutively expressed in unstimulated cells, and disappeared following CD3 ligation in CD4+ T cells from HPG-immunized B6 or OVA-immunized BALB/c mice, but remained unchanged in CD4+ T cells from HPG-immunized BALB/c mice (Fig. 6A). Consistent with these results, reduced cleavage of pro-caspase-8 and pro-caspase-3 was found in CD4+ T cells from HPG-immunized BALB/c animals (Fig. 6A). These data indicate that the defective AICD in PGIA may be the result of aberrant FLIP expression, subsequently leading to impaired activation of caspase-8 and caspase-3. In support of our hypothesis, it has been reported that overexpression of FLIP in lymphocytes can result in accumulation of autoreactive T and B cells in the periphery and autoimmunity (39). Our data also support the previous report in which FLIP has been shown to potentiate TCR signaling pathways that are required for T cell proliferation (30).

Following ligation of Fas receptor, Fas, Fas-associated death domain protein, and caspase-8 form a DISC. FLIP is also recruited to the DISC, which can determine the cell’s susceptibility to Fas-mediated apoptosis (38, 40). We investigated whether enhanced recruitment of FLIP in the DISC precludes the caspase-8 activation in CD4+ T cells from HPG-immunized BALB/c mice. To test this possibility, CD4+ T cells from different groups were stimulated with cross-linked recombinant mouse FasL for 10 min, and then lysed. Cell lysates were immunoprecipitated with anti-Fas and FLIPα Abs, respectively. As shown in Fig. 6B, although the amounts of Fas did not change in CD4+ T cells from HPG-immunized B6 and BALB/c mice, significantly higher amounts of FLIP were recruited to the DISC in HPG-immunized BALB/c than B6 mice following Fas ligation. Consistent with a role of FLIP in Fas-mediated cell death, the amount of pro-caspase-8 in the DISC was lower in CD4+ T cells from HPG-immunized BALB/c mice than from control mice.

In conclusion, our results indicate that although CD4+ T cells from HPG-immunized BALB/c mice expressed equally high levels of Fas at the cell surface as did CD4+ T cells from control mice, they failed to undergo AICD. This defective AICD is associated with hyperproliferation of CD4+ T cells and a dominant Th1-type response. Furthermore, defective AICD in PGIA may be mediated by the aberrant expression of FLIP, which inhibits caspase-8 recruitment at the DISC. Accordingly, high levels of FLIP may also be responsible for the hyperproliferation of CD4+ T cells from arthritic animals. These observations suggest that autoreactive Th1 cells may escape from peripheral deletion in PGIA, thus leading to, or at least contributing to, the development of autoimmune disease.

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References


