Impaired apoptosis, extended duration of immune responses, and a lupus-like autoimmune disease in IEX-1-transgenic mice

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Susceptibility of activated T cells to apoptosis must be tightly regulated to ensure sufficient T cell progeny for an effective response, while allowing a rapid depletion of them at the end of the immune response. We show here that a previously isolated, NF-κB/rel target gene IEX-1 (Immediate Early response gene X-1) is highly expressed in T cells at early stages of activation, but declines with a prolonged period of activation time, coincident with an increased susceptibility of T cells to apoptosis during the late phases of an immune response. Transgenic expression of IEX-1 specifically in lymphocytes impaired apoptosis in activated T cells, extended a duration of an effector-phase of a specific immune response, and increased the accumulation of effector/memory-like T cells and the susceptibility to a lupus-like autoimmune disease. Our study demonstrated an antiapoptotic effect of IEX-1 on T cell apoptosis triggered by ligation of Fas and T cell receptor (TCR)/CD3 complex. The ability of extending life expectancy of T effectors, in line with a decrease in its expression following prolonged T cell activation, suggests a key role for IEX-1 in regulating T cell homeostasis during immune responses.

antiapoptosis | autoimmunity | NF-κB | T cells

Immune responses are initiated when resting T cells are triggered by antigen/MHC complexes to undergo clonal expansion and differentiation into effector populations. This response is followed by a rapid depletion of T effectors through apoptotic pathways to reduce the risk of autoimmunity (1–3). Therefore, lifespans of T effectors are critical not only in determining the duration of an immune response, but also in preventing from autoimmunity. Compelling evidence suggests that NF-κB/rel transcription factors play a pivotal role in linking between an innate and an adaptive immune response (4). However, the underlying mechanisms are not completely understood, one of which may be to extend the lifespan of T effectors by activation of NF-κB/rel transcription factors as a result of copious proinflammatory cytokines released during innate immune responses (5). In support, considerable studies indicate that activation of NF-κB/rel transcription factors promotes the survival of a variety of cells both *in vitro* and *in vivo* (6–9).

IEX-1 (Immediate Early response gene X-1), also named IER3, p22/PRG1, Dif-2, or mouse homology *gly96*, was isolated by several groups, but its function remains controversial (10–13). IEX-1 is a NF-κB/rel target gene and its promoter region contains a regulation site for NF-κB transcription factor (14, 15). It can be rapidly up-regulated by various activators of the NF-κB/rel transcription factors such as TNF-α, IL-1β, PMA (phorbol 12-myristate 13-acetate), growth factors, viral infection, lipopolysaccharide (LPS), irradiation, etc. (10–13, 16–18). IEX-1 was shown to inhibit cell proliferation in some cells, but it appeared to accelerate cell cycle progression in others (11, 19–21). It was also reported to promote apoptosis in 293 and HeLa cells under serum deprivation (20, 22). In contrast to these *in vitro* studies, we show here that IEX-1 protects activated T cells from apoptosis *in vivo* during an immune response using IEX-1-transgenic mice. Transgenic mice that target expression of IEX-1 to lymphocytes show a decrease in apoptosis of activated T cells and an increase in a duration of an immune response effector-phase, in the accumulation of effector/memory-like T cells, and in the susceptibility to a lupus-like autoimmune disease. In accordance with a physiological role for IEX-1 in regulating susceptibility of activated T cells to apoptosis, its expression levels inversely correlate with the susceptibility of T cells to apoptosis during T cell activation. Our studies thus demonstrate contribution of IEX-1 to the survival of responding T lymphocytes and to the maintenance of self-tolerance.

Materials and Methods

Animals. To generate IEX-1-transgenic (Tg) mice, human IEX-1 coding sequence was subcloned into the *Sal*I/*Bam*HI sites in a pHSE-3′ expression cassette (23). The cassette contains an H-2K^b promoter at the 5′ end and an Ig heavy chain (μ) enhancer at the 3′ end that directs transgene expression specifically in lymphocytes (23). Linearized pHSE-IEX-1 was microinjected into the male pronucleus of fertilized eggs derived from (C57BL/6 × DBA) F2 embryos. Transgenic founders were identified by Southern blotting analysis of tail DNA samples by using human IEX-1 cDNA as a probe or PCR as described (16), and subsequently bred with C57BL/6 mice. A mixed cohort of F1 and F2 generations of two independent lines of IEX-1-Tg mice derived from founders #2 and #4, respectively, were used. MRL+/+ and MRL/*lpr*/*lpr* mice were purchased from The Jackson Laboratory. All mice were maintained at the animal facility of the Baylor College of Medicine in accordance with college's guidelines.

Flow Cytometric Analysis. To immunostain human peripheral blood T (PBT) cells, PBT cells were isolated by immunomagnetic negative selection as described (24) and stimulated with 1 μg/ml immobilized anti-CD3 antibody (Ab) (OKT3) in the presence of 5% accessory cells. Cells collected at the indicated days were either left unstimulated or restimulated with 10 μg/ml immobilized anti-CD3 Ab for 2 h. They were then stained with FITC-conjugated anti-CD2 Ab and R-phycoerythrin (PE)-conjugated anti-human Fas Ab (7C11) following fixation in 1% paraformaldehyde. To detect intracellular levels of IEX-1, fixed cells that were prestained with FITC-anti-CD2 Ab were permeabilized by 1% Nonidet P-40, incubated with 20% normal goat serum at 4°C for overnight to block nonspecific binding, and then reacted with rabbit anti-IEX-1 Ab followed by staining with PE-conjugated goat anti-rabbit Ab (16).

For immunophenotyping of mouse lymphocytes, single-cell

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Abbreviations: Tg, transgenic; DTH, delayed-typed hypersensitivity; SEB, staphylococcal enterotoxin B; PBT, peripheral blood T; LN, lymph nodes.

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suspensions were prepared from the thymus, spleen, and lymph nodes (LN) and fixed in 1% formaldehyde. Abs specific for mouse antigens including FITC-conjugated anti-CD4, anti-CD8, anti-CD62L, and anti-CD3; PE-conjugated anti-B220, and anti-CD8; biotin-conjugated anti-CD44, anti-CD25, and anti-CD19; and cy-Chrome (CyC)-conjugated streptavidin were all purchased from PharMingen. Abs specific for human lymphocyte markers were all obtained from Coulter. The stained cells were analyzed using a FACScan cytometer (Becton Dickinson) with Cellquest or an Epics Elite (Coulter).

***In Vitro* T Cell Assays.** For proliferation assays, single-cell suspensions prepared from mouse LN and spleens were treated with a mixture of rat anti-mouse Abs against CD19, CD32, and CD16 followed by depletion of Ab-bound cells with BioMag goat anti-rat IgG (Polysciences) as per the manufacturer's instruction. The resulting T cells of about 90% purity were stimulated in triplicate with varying concentrations of ConA or phytohemagglutinin (PHA) or immobilized anti-CD3 Ab (2C11). [^{3}H]Thymidine (ICN) of 0.5 μCi per well (1 Ci = 37 GBq) was added 3 days later for ConA and PHA stimulation or 5 days later for anti-CD3 Ab stimulation. [^{3}H]thymidine incorporation was measured after 16 h by using a microplate scintillation counter (TopCount, Packard).

For apoptosis assays in human PBT cells, PBT cells activated as above were collected at indicated days, and incubated for 24 h with anti-human Fas Ab (7C11, IgM). Apoptotic cell death of a gated CD2+ population was determined by a multiparameter flow cytometric assay after staining with anti-CD2-FITC, Hoechst 33342 vital dye, and propidium iodide (24).

To assay apoptosis in activated mouse T cells, LN T cells were treated with 5 μg/ml ConA (Sigma) for 48 h followed by incubation with 10 μg/ml α-methyl α-D-mannoside to neutralize residual ConA. The activated cells were continuously cultured with replenishment of IL-2 every 2 days to predispose the cells to apoptosis. At day 5–7, viable T cells were collected after passage through a Percoll density gradient, and cultured in triplicate for 48 h at 5×10^4 per well in 96-well plates that were precoated with 10 μg/ml anti-CD3 Ab (2C11) or 15 μg/ml anti-mouse Fas Ab (Jo2, PharMingen) in the presence or absence of 30 ng/ml cycloheximide (CHX). To reduce background cell death, 100 units/ml rIL-2 was included in the media at all times. The percentages of viable cells were quantified by a MTT cell viability detection kit (Promega) that incorporates a colorimetric method for determining the number of viable cells in the culture. The percentage of apoptosis was calculated as $100 \times (1 -$ average absorbance from triplicate cultures obtained in the presence of anti-CD3 or anti-Fas Ab/average absorbance of triplicate cultures obtained in the absence of anti-CD3 or anti-Fas Ab).

***In Vivo* Assays.** Peripheral depletion of T cells was carried out by i.p. administration of 30 μg staphylococcal enterotoxin B (SEB) (Sigma). Spleens were collected at the indicated days following SEB administration. Percentages of $CD4^+V_\beta8^+$ T cells or $CD4^+V_\beta6^+$ were assessed by three-colored flow cytometry analysis using Abs directed against CD4, CD8 or CD3, and $V_\beta8$ or $V_\beta6$ antigens. To induce a delayed-typed hypersensitivity (DTH) response, mice at 6–8 weeks of age were primed by s.c. administration into two sites on the dorsum of each mouse with 0.1 ml of 10 mg/ml heat-killed *Mycobacterium tuberculosis* (H37Ra from Difco) emulsified in incomplete Freunds's adjuvant. DTH responses were induced in the primed mice three weeks later by s.c. injection into the right hind footpad with 25 μl of the same *Mycobacterium* suspended in PBS. The mice received a third antigen challenge in the left hind footpad 1 month after the second antigen challenge. Footpad swelling responses were monitored with a dial caliper daily for 2 weeks.

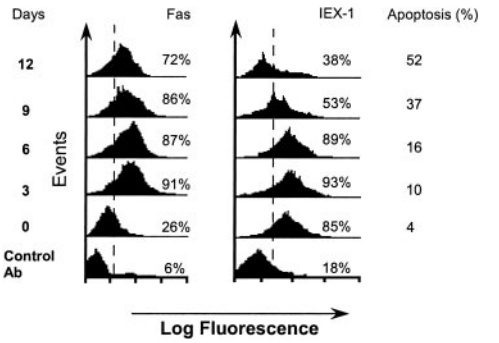

Fig. 1. Down-regulation of IEX-1 expression following T cell activation. PBT cells were stimulated with anti-CD3 Ab and collected at 2 h (day 0) or indicated days. The cells were either left unstimulated (day 0) or restimulated with anti-CD3 Ab for another 2 h, and stained with FITC-anti-CD2 Ab and phycoerythrin (PE)-conjugated-anti-Fas Ab (*Left*) or anti-IEX-1 specific polyclonal Ab (*Right*). Aliquots of cells were treated with anti-Fas Ab in the presence of 100 units/ml of IL-2. The percentages of apoptotic cell death were determined on a gated $CD2^+$ T cell population and indicated next to the FACS panels on the right. One representative result of two (Fas expression and Fas-induced apoptosis) and four (IEX-1 expression) experiments performed is shown.

Measurement of Serum Igs, Anti-Double-Stranded DNA Ab, and Proteinuria. Serum Ig levels were determined by ELISA using an Ig isotyping kit (Southern Biotechnology Associates) as per the manufacturer's protocol. Ig concentrations were calculated from the plotted standard curve of serial dilutions of the mouse standard control Abs. Autoantibodies directed against double-stranded DNA in sera from individual mice were also measured by ELISA as described (25). Urine was collected from each mouse by manually applying pressure on the bladder or using a metabolic cage. Proteinuria was determined by a Vitro 250 Chemistry Analyzer (Ortho-Clinical Diagnostics, Johnson and Johnson, Raritan, NJ).

Histological Examination. Tissues were fixed with 10% formalin, embedded in paraffin, and cut at 4 microns. Sections were stained with hematoxylin and eosin (H&E) by standard methods. For immunofluorescent studies of Ig deposition, the tissues were embedded in OCT, flash-frozen in liquid nitrogen, and sectioned with a cryostat. Four-to-five micrometer sections were air-dried and fixed with cold acetone for 15 min. The cryosections were pretreated with 20% of normal goat serum overnight to block nonspecific binding followed by staining the sections with FITC-conjugated goat anti-mouse IgG (1:250) (Sigma).

Statistical Analysis. A Student's two-tailed *t* test was used unless otherwise indicated.

Results

Inverse Correlation of IEX-1 Expression and Susceptibility of T Cells to Fas-Induced Apoptosis. Resting T cells or T cells at the early stages ($\leq$3 days) of activation were largely resistant to Fas-induced apoptosis ($\leq$10%; Fig. 1), as has also been shown by earlier studies (26–28). Such resistance was not ascribed to the level of Fas or Fas-ligand expression, because early activated T cells coexpressed high levels of Fas (Fig. 1 *Left*) and Fas-ligand (26, 27). T cells acquired susceptibility to Fas-induced cell death with increasing days of stimulation (Fig. 1; ref. 26), presumably resulting from an altered ratio of antiapoptotic to proapoptotic factors in these cells (5, 28–30). We show here that IEX-1 is highly expressed in T cells stimulated for 2 h with anti-CD3 Ab and its expression is further increased in more activated T cells (day 3) following restimulation (Fig. 1 *Right*). The high levels of IEX-1 expression were also induced until day 6, but declined gradually thereafter. Interestingly, the decline in IEX-1 expression coincides with an enhanced suscepti-

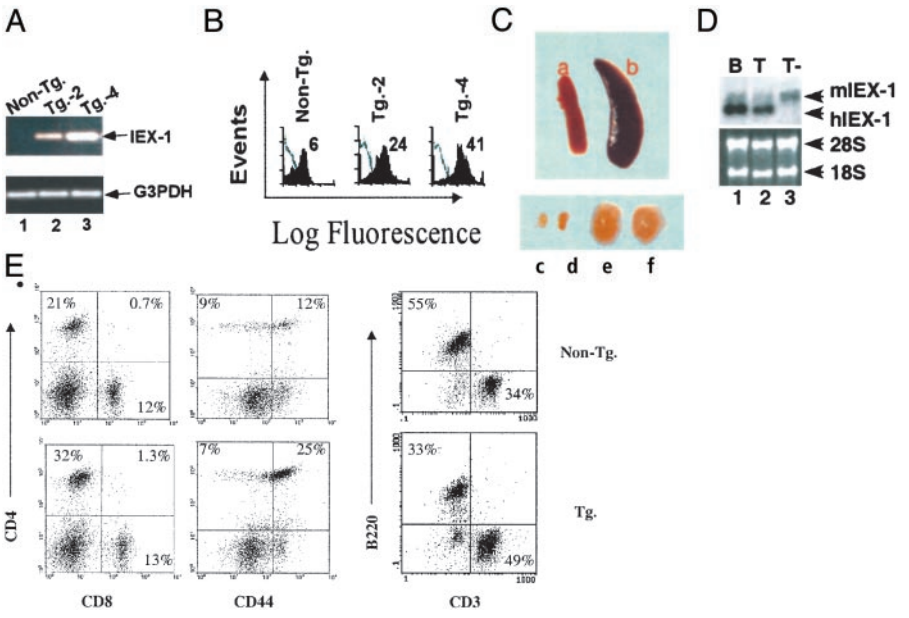

Fig. 2. Splenomegaly, lymphadenopathy, and accumulation of effector/memory-like T lymphocytes in IEX-1-Tg mice. (*A*) RT-PCR analysis of IEX-1 mRNA in IEX-1-Tg mice. Total RNA was isolated from LN T cells of non-Tg mice (lane 1), and IEX-1-Tg mice Tg-2 (lane 2) and Tg-4 (lane3), derived from founders 2 and 4, respectively, and assayed by RT-PCR using primers that amplified human IEX-1 only. *Lower* is amplification of G3PDH (glyceraldhyde-3-phosphate dehydrogenase) from the same RT-reaction samples for controls. (*B*) Relative IEX-1 protein expression in IEX-1-Tg mice. LN T cells isolated from above mice were labeled with FITC-conjugated anti-CD3 Ab followed by staining with anti-IEX-1 polyclonal Ab (solid) or preimmunized normal rabbit Ab (outlined) as in Fig. 1. The mean immunofluorescence values of anti-IEX-1 Ab staining on gated $CD3^+$ cells are indicated in each profile. (*C*) Splenomegaly and lymphadenopathy in IEX-1-Tg mice. Shown are the spleen and LN (superficial inguinal) from 9–10-month-old IEX-1-Tg mice (b, d, e, and f) and non-Tg age-matched control mice (a and c). (*D*) Northern blotting analysis of IEX-1 expression in T and B cells. Trangenic or human IEX-1 (hIEX-1) and endogenous mouse IEX-1 (mIEX-1) expressed in B (lane 1) and T (lane 2) cells are indicated by arrows on the right of the upper panel. T cells from non-Tg mice (T-) expressed mIEX-1 only following stimulation with anti-CD3 Ab for 2 h as a positive control (lane 3). RNA loading control is given in the low panel. (*E*) A comparison of splenocyte phenotypes between IEX-1-Tg (Tg) and non-Tg (Non-Tg) mice. Splenocytes were stained with indicated Abs and analyzed by three-colored flow cytometry. One representative result of eight IEX-1-Tg and six non-Tg mice analyzed is shown; the data obtained from all mice are summarized in Table 1.

bility of T cells to apoptosis induced by ligation of Fas. A 2- to 3-fold increase in Fas-induced apoptosis (37–52%) is seen at days 9–12 as compared with the apoptosis (10–16%) obtained at 3–6 days. A similar expression pattern of IEX-1 expression was also obtained in mouse T cells following stimulation with anti-CD3 Ab (data not shown).

Splenomegaly and Lymphadenopathy in IEX-1-Tg Mice. An inverse correlation between IEX-1 expression levels and resistance of T cells to Fas-induced apoptosis implicates a role for IEX-1 in the survival of T cells during T cell activation, because our *in vitro* studies demonstrated that IEX-1 inhibited apoptosis in some cells (unpublished data). To directly test this, we generated transgenic mice that targeted IEX-1 expression to peripheral lymphocytes. As endogenous IEX-1 expression is down-regulated following a prolonged period of T cell activation, and transgenic expression of IEX-1 is independent of T cell activation, we reasoned that constitutive, high levels of IEX-1 expression should protect activated T cells from apoptosis during the late phase of an immune response. Two independent lines of IEX-1-Tg mice were obtained from two IEX-1-Tg founders #2 and #4. They expressed high but varying levels of IEX-1, as shown by reverse transcription (RT)-PCR and flow cytometric analysis using IEX-1 specific polyclonal Ab (Fig. 2 *A* and *B*). However, the variation in the levels of IEX-1 expression appeared to have little impact on the observed phenotypes that were statistically indistinguishable between these two transgenic lines. Perhaps constitutive IEX-1 expression is essential for its influence on the lifespan of activated T cells. The expression level of transgenic IEX-1 is about 10-fold higher than that of endogenous IEX-1 observed in T cells in the absence of stimulation (Fig. 2*D*, lane 2).

Most IEX-1-Tg animals showed no gross developmental abnormalities early in life, but starting at 8 weeks, about 10% of them developed splenomegaly, the incidence increasing to 42% at 9–10 months of age. The spleens of IEX-1-Tg mice were two to three times larger, as assessed by both weight and size, than those from control littermates (Fig. 2*C*, b vs. a, and Table 1, $P < 0.01$). Although splenomegaly became evident at 8 weeks of age, its severity remained similar out to 9–10 months of age while its incidence increased. The increased spleen weights correlated with elevated cellularity (Table 1). Analysis of lymphocyte phenotypes in the spleens of 9–10-month-old IEX-1-Tg mice revealed a 50% increase in $CD4^+$ T cells as compared with non-Tg littermates (Fig. 2*E* and Table 1, $P < 0.01$). In particular, percentages of $CD3^+CD4^+$ T cells expressing high levels of cell surface CD44 antigen were remarkably increased compared with non-Tg mice (Fig. 2*E* and Table 1, $P < 0.005$). In contrast, alterations in the levels of CD25, CD62L, and CD69 molecules in these $CD4^+$ T cells were not statistically significant when IEX-1-Tg and non-Tg mice were compared (data not shown), suggesting that the accumulated T cells may not be true memory T cells. We refer to these T cells as effector/memory-like T cells. In addition, an unusual population of

Table 1. Splenomegaly, lymphadenopathy, and accumulation of effector/memory-like T cells in IEX-1-Tg mice

	Total number of cells (10^7)			Spleens					Lymph nodes				
	Thymus	Spleens	LN	Weights, mg	$CD3^-$ $B220^+$, %	$CD3^+$ $B220^-$, %	$CD4^+$, %	$CD4^+$ CD44hi, %	Weights, mg	$CD4^+$, %	$CD4^+$ CD44hi, %	$CD8^+$, %	$CD8^+$ CD44hi, %
Non-Tg ($n = 6$)	12.2 ± 2.3	11.3 ± 1.2	0.7 ± 0.1	84 ± 5	55 ± 6	34 ± 4	20 ± 2	11 ± 1	3.3 ± 0.2	25 ± 3	12 ± 2	20 ± 2	11 ± 1
IEX-1-Tg ($n = 8$)	14.1 ± 3.6	20.7 ± 6.2	4.4 ± 1.7	177 ± 54	33 ± 8	49 ± 9	31 ± 5	24 ± 4	21.5 ± 5.5	37 ± 7	27 ± 3	28 ± 5	21 ± 6

Data are expressed as means ± SD. Cells from the spleens and LN were stained and analyzed as in Fig. 2*E*. Mice were 9–10 months old.

$CD3^+CD4^+CD8^+$ T cells (8–13%) was also seen in the spleens of some IEX-1-Tg mice (4/15), the source of which remains to be determined.

Unlike the differences seen in the spleens, degrees of lymphadenopathy observed varied from mild (2-fold) to severe (12-fold) in IEX-1-Tg mice (Fig. 2*C*, d vs. e and f, and Table 1) as compared with non-Tg littermates (Fig. 2*C*, c). Lymphadenopathy was seen mostly in the mice after 8 months of age. In younger mice, although this trend was apparent, it was not statistically significant, suggesting that IEX-1-induced lymphadenopathy became evident depending on age. A remarkable increase in $CD4^+$ T cells and to a lesser extent, $CD8^+$ T cells, was also seen in the LN of 9–10-month-old IEX-1-Tg mice as compared with age-matched non-Tg mice (Table 1, $P < 0.01$ for $CD4^+$ T cells and $P < 0.05$ for $CD8^+$ T cells). Similar to splenocytes, the number of $CD4^+CD44^{hi}$ and $CD8^+CD44^{hi}$ T cells in the LN also increased significantly (Table 1). Analysis of splenic B cells showed no significant alteration in the number of B cells in IEX-1-Tg mice as compared with non-Tg littermates (Fig. 2*E*, Table 1), although B cells expressed transgenic IEX-1 at levels comparable to T cells (Fig. 2*D*, lane 1 vs. lane 2). Thymi from IEX-1-Tg mice and the negative littermate controls were also similar in size and in cell numbers (Table 1), suggesting that constitutive expression of IEX-1 did not significantly affect thymocyte or B cell development.

Impaired Apoptosis in T Cells from IEX-1-Tg Mice. The observed increase in the number of T cells in the secondary lymphoid tissues could be the result of either an increase in the proliferation of T cells or a decrease in apoptosis. Given that IEX-1 had been previously implicated in the promotion of cell cycle progression (11, 13), we evaluated the effect of IEX-1 on T cell proliferation. Splenic and LN T cells were isolated from IEX-1-Tg mice and negative littermates at 6 weeks of age, before the development of any overt signs of splenomegaly and lymphadenopathy, and assayed for T cell proliferation. We failed to detect any significant effect of IEX-1 on T cell proliferation following activation with 0.1–1 μg/ml of anti-CD3 Ab or 0.5–5 μg/ml ConA or 0.5–4 μg/ml phytohemagglutinin (PHA) (data not shown). Further, IEX-1 appeared not to affect proliferation of B cells challenged with anti-IgM or anti-CD40 Ab in the presence of IL-4, suggesting that both T and B cells proliferative responses were within normal limits.

We next assessed the apoptotic response of activated T cells from IEX-1-Tg mice. As shown in Fig. 3*A*, the average level of apoptosis induced by anti-CD3 Ab was 42.8% ($n = 8$) in T cells obtained from non-Tg control mice, which was significantly higher than the 29.0% ($n = 10$) observed in T cells from IEX-1-Tg mice. A significant decrease in Fas-induced apoptosis was also seen in T cells from IEX-1-Tg mice as compared with non-Tg mice (19% in non-Tg mice vs. 9.5% in IEX-1-Tg mice, $n = 5$, $P < 0.01$, Fig. 3*B*). In contrast, Fas-induced apoptosis of B cells was similar in the presence vs. absence of constitutive IEX-1 expression when the cells were stimulated with anti-CD40 Ab and IL-4 for 5–7 days followed by treatment with anti-Fas Ab (data not shown). IEX-1 thus appeared not to affect B cell apoptosis under these conditions, in agreement with little increase in the number of B cells in the mice.

To determine whether IEX-1-induced resistance of activated T cells to apoptosis also took place *in vivo*, IEX-1-Tg mice and non-Tg littermates were injected i.p. with SEB superantigen (SAg). The SAg leads to the elimination of SEB-responsive $V_\beta8^+$ T cells and is a commonly used model system for studying peripheral T cell depletion *in vivo* (31). As shown in Fig. 3*C*, SAg-activated peripheral $CD4^+V_\beta8^+$ T cells doubled in number within a 48 h period in both IEX-1-Tg mice and non-Tg littermates, as measured by flow cytometric analysis using Abs specific for CD3, CD4, and $V_\beta8$ antigens. This expansion was followed by a profound depletion of $CD4^+V_\beta8^+$ T cells in non-Tg mice, to levels lower than that found prior to SEB administration. In contrast, SEB-mediated depletion

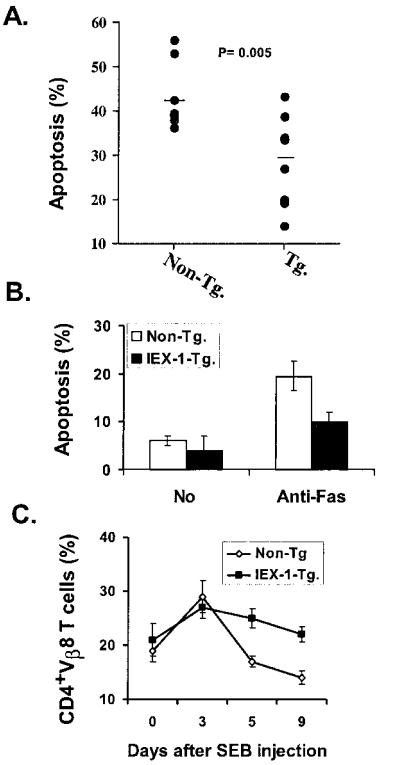

Fig. 3. Impaired apoptosis in T cells from IEX-1-Tg mice. T cells from IEX-1-Tg (Tg) and non-Tg (Non-Tg) littermates at 6–8 weeks of age underwent apoptosis triggered by anti-CD3 Ab (*A*) or anti-Fas Ab (*B*) following activation. (*A*) The means from triplicate samples of each mouse with a total of ten IEX-1-Tg and eight non-Tg mice performed. Standard deviation (SD) of the triplicate cultures was less than 3% (data not shown) and each symbol represents an individual mouse. (*B*) The means ± SD of Fas-induced apoptosis in T cells ($n = 5$), which represents one result of three experiments performed in triplicate. (*C*) A defect in superantigen-mediated depletion of T cells in IEX-1-Tg mice. IEX-1-Tg and non-Tg littermates were administrated with 30 μg of SEB. Percentages of $CD4^+V\beta8^+$ T cells relative to total $CD4^+$ T cells in the spleens were determined at the indicated days by flow cytometric analysis. Data are shown as means ($n = 6$) ± SD of three independent experiments in which two mice per time point were used for each experiment.

of T cells was significantly delayed in IEX-1-Tg mice. Neither significant expansion nor depletion of non-SEB responsive $V_\beta6^+$ T cells was detected in these mice (data not shown). The ability of IEX-1 to attenuate T cell elimination *in vivo* in an antigen-specific fashion supports the notion that it has a role in the survival of activated T cells during an immune response.

Elevated and Extended DTH Responses in IEX-1-Tg Mice. To investigate how IEX-1-induced resistance of activated T cells to apoptosis affected inflammation, we assessed DTH responses in the presence as compared with absence of constitutive expression of IEX-1. As is shown in Fig. 4, a DTH response was evident within a day after the antigen challenge similarly in both IEX-1-Tg and non-Tg littermates, as measured by a dramatic increase in footpad thickness due to severe swelling (*Left*). By day 6, footpad swelling began to ameliorate in non-Tg mice, consistent with re-establishment of lymphocyte homeostasis, in part secondary to apoptosis of activated T cells (1–3). However, little amelioration in the footpad swelling was observed in IEX-1-Tg mice even at day 10. The maximal DTH response was thus sustained significantly longer in IEX-1-Tg mice (9 days) than in non-Tg mice (4 days). An extended DTH effector-phase response was even more significant in IEX-1-Tg mice following a second boost immunization in their left hind footpads (*Right*). We noticed, at day 3 following the antigen

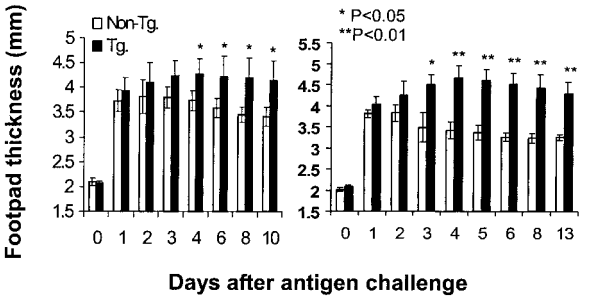

Fig. 4. An elevated and extended DTH response in IEX-1-Tg mice. A DTH response was induced in IEX-1-Tg (Tg) mice and non-Tg (Non-Tg) littermates by intracutaneous injection of *Mycobacterium tuberculosis* into the right hind footpad (*Left*). The mice were immunized again with the same antigen in the left hind footpads one-month later (*Right*). The footpad thickness (mm) was measured daily by a caliper. Dada are shown as means ($n = 7$) ± SD of footpad thickness. One representative result of three experiments performed is shown.

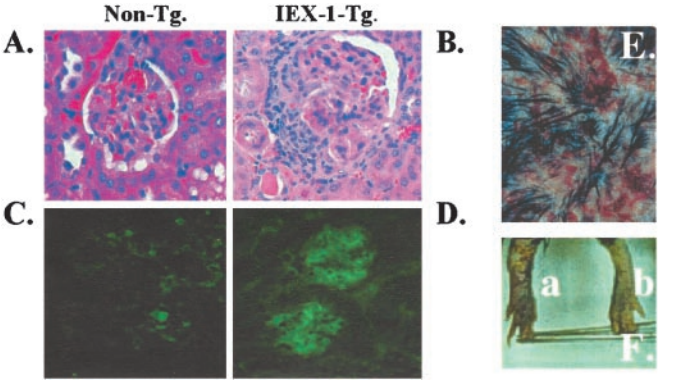

Fig. 5. A lupus-like autoimmune disease in IEX-1-Tg mice. (*A* and *B*) Hematoxylin and eosin (H&E)-stained kidney sections: a glomerulus from a 9-month-old non-Tg mouse and an age-matched IEX-1-Tg mouse, respectively. (*C* and *D*) Immunofluorescent staining of kidney sections with FITC-conjugated goat anti-mouse IgG Ab. Note that fluorescence in glomeruli of a 9-month-old IEX-1-Tg mouse (*D*), but not in glomeruli of a non-Tg age-matched mouse (*C*). (*E* and *F*) Erythema with alopecia in the skin (*E*) and swelling of joint (*F*, a) in IEX-1-Tg mice; b in *F*, normal foot control.

challenge, that the left footpad swelling decreased by more than 10% in non-Tg mice, in agreement with that repeated stimulation with the same antigen expedites the depletion of peripheral T cells (1–3). In sharp contrast to the expeditious decrease in the footpad swelling in non-Tg mice, the left hind footpad swelling in IEX-1-Tg mice attained a higher maximum and persisted for a longer period. The left footpad thickness of IEX-1-Tg mice was about 185% of that measured in non-Tg mice at day 4 following the second antigen boost. Our results clearly demonstrate an elevated and, perhaps more importantly, an extended DTH response in IEX-1-Tg mice. This extended duration of a DTH response may be a direct reflection of the ability of IEX-1 to prevent responding T cells from apoptosis and thus to prolong their survival at the local sites.

A Lupus-Like Autoimmunity in IEX-1-Tg Mice. In several murine model systems, a lupus-like autoimmune response has been detected in animals with defects in T cell apoptosis (32, 33). To determine whether this was the case in IEX-1-Tg mice, serum Ig levels in a cohort of 21 IEX-1-Tg mice and 17 non-Tg controls were measured to assess global immune system activation. Our ELISA results showed that serum IgM and total IgG levels were similar between IEX-1-Tg mice and non-Tg littermates (data not shown). Analysis of individual IgG isotypes also revealed similar levels of IgG1, IgG2b, IgG3, and IgA in these animals. However, an elevated level of serum IgG2a by more than 30-fold as compared with that in non-Tg littermates was obtained in 67% of IEX-1-Tg mice after 9 months of age ($P < 0.001$; Table 2). This result is highly relevant to the immunopathogenesis of a lupus-like syndrome, because murine IgG2a activates complement better than all other IgG subclasses except for IgG3 (34). In agreement with development of autoimmune responses in the animals, serum autoantibodies directed against double-stranded DNA were also increased significantly in ≈54% of IEX-1-Tg mice (Table 2).

Histological examination of representative mice with increased levels of anti-double-stranded DNA autoantibodies identified glomerulonephritis with thickened basement membranes, mesangial hypercellularity, and lymphoplasmacytic infiltration in the kidney as shown in Fig. 5*B*. We also observed positive immunofluorescent staining in kidney sections prepared from IEX-1-Tg mice but not in non-Tg mice when the tissues were stained with FITC-conjugated goat anti-mouse IgG Ab, indicating IgG immune complex deposits in the kidney (Fig. 5 *C* and *D*). Concomitant renal damage was the significantly higher amount of proteinuria measured in 61% of IEX-1-Tg mice as compared with non-Tg mice, although it was lower than that measured with MLP/*lpr* mice (Table 2). As evidence of more widespread inflammation, about 30% (12/40) of IEX-1-Tg mice spontaneously developed erythema with alopecia of the skin (Fig. 5*E*), and swelling in the foot joints (Fig. 5*F*, a). The syndrome was noted in IEX-1-Tg mice only after 7 months of age. There was no evidence of the disease in young IEX-1-Tg mice or in non-Tg mice even after prolonged observation (0/26). Systemic lupus erythematosus (SLE) autoimmune disease is characterized by the production of pathogenic autoantibodies causing systemic tissue injury, most prominently glomerulonephritis, skin lesions, and arthritis. Our studies indicate a lupus-like autoimmune disease developed in IEX-1-Tg mice as they aged, clearly suggesting that IEX-1 can enhance the susceptibility of a given host to a lupus-like disease, because no such disease developed in non-Tg littermates maintained under similar conditions.

Discussion

Our studies clearly demonstrate that constitutive expression of IEX-1 in lymphocytes decreases apoptosis in activated T cells, resulting in a prolonged effector-phase of an immune response, the accumulation of effector/memory-like T cells, and the development of a lupus-like autoimmune disease in the animals. In keeping with its possible role in regulating susceptibility of activated T cells to apoptosis *in vivo*, IEX-1 is highly expressed in T cells after stimulation, but declines during the late phases of T cell activation. The decreased expression of IEX-1 is accompanied by an increased

Table 2. Elevated levels of IgG2a, anti-ds DNA autoantibodies, and proteinuria in IEX-1-Tg mice

Mice	IgG2a	Anti-ds DNA (titer)	Proteinuria
IEX-1-Tg ($n = 21$)	1 ± 0.5 mg/ml (67%)	1:200–1:1,600 (54%)	39 ± 17 mg/dl (61%)
Non-Tg ($n = 17$)	0.03 ± 0.05 mg/ml	<1:100	<5 mg/dl
MLR/*lpr* ($n = 4$)	ND	>1:3,200	94 ± 77 mg/dl

ND, not done. The data are expressed as means ± SD for IgG2a and proteinuria. IEX-1-Tg and non-Tg mice were 9–10 months old and MRL/*lpr* mice were 4 months old.

susceptibility of activated T cells to apoptosis. These findings underscore a role for IEX-1 in regulating the lifespan of responding T cells and duration of an immune response.

IEX-1 expression can be rapidly induced by proinflammatory cytokines such as TNF-α and IL-1 as well as byproducts of bacterial and viral infections, apparently through activation of NF-κB/rel transcription factors (10, 12, 13, 16–18). These proinflammatory cytokines are critical for the survival of responding T lymphocytes and for the linkage between the innate and adaptive immune responses (35–39). A recent study using gene microarray to compare gene expression in T cells activated by antigen in the presence vs. absence of adjuvant revealed that up-regulation of NF-κB transcription factors including Bcl-3, p65 (RelA), but not Bcl-2 and Bcl-xL antiapoptotic genes, might be responsible for the adjuvant-mediated boost of T cell responses, by increasing life expectancy of activated T cells during and after the initial proliferative phase of immune responses (5, 40, 41). It is thus very likely that IEX-1 expression levels can be sustained in activated T cells in the presence of proinflammatory cytokines or byproducts of bacterial and viral infections, contributing to NF-κB/rel-mediated prevention of apoptosis of responding T cells during a productive immune response under physiological conditions. The finding of a longer duration of an immune response in IEX-1-Tg mice than in non-Tg mice in response to antigen stimulation implicates a potential strategy to improve vaccine responses by administration of vaccines, along with stimuli that induce the expression of survival genes in lymphocytes.

IEX-1 appears to exert apparently contradictory effects, depending on the type of cells and/or stimuli *in vitro* studies. At present, we do not understand the molecular basis for its diverse effects on different cells. One possibility is that the down stream target of IEX-1 is subject to the control of other cellular signals. Our ongoing study shows that IEX-1 binds to a sulfhydryl-rich (>30% cysteines) protein, suggesting that IEX-1 may participate in the regulation of redox status of cells.

Recently, genome-wide linkage studies in both humans and mice reveal that the strongest linkage to SLE disease is within or near the MHC locus (42–44). This, on one hand, emphasizes a critical role for MHC molecules in the development of SLE disease. On the other hand, it does not adequately explain the multigenic nature of the disease. It is thus reasonable to predict that other genes encoded within or near the locus, in addition to the MHC allele, may also contribute to the disease. Interestingly, IEX-1 is mapped within the MHC locus [human 6p21.3 that is syntenic with a region on mouse 17(18.65)] (15). This finding, coupled with impaired apoptosis in activated T cells and an increased susceptibility of IEX-1-Tg mice to a lupus-like autoimmune disease, suggests that IEX-1 may be one of the genes within the MHC locus, in conjugation with MHC, predisposing to the susceptibility of SLE disease. This possibility is consistent with other studies demonstrating that a defect in apoptosis of T cells predisposes a susceptible host to autoimmune disease in both human and murine systems (32, 33, 45–48).

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