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Ultraviolet Light-Induced Immune Tolerance Is Mediated via the Fas/Fas-Ligand System[1]

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Hapten sensitization through UV-exposed skin induces tolerance that is mediated via the induction of hapten-specific T suppressor cells. However, the detailed mechanisms underlying tolerance induction remain unclear to date. We show here that the apoptosis-related surface Ag Fas (APO-1, CD95) and its ligand, Fas ligand (FasL) are critically involved, since Fas-deficient *lpr* mice and FasL-deficient *gld* mice do not develop UV-induced tolerance. Adoptive transfer experiments revealed that the mediation of tolerance does not require the expression of Fas or FasL by the T suppressor cells but does require the expression of both molecules by the cells of mice receiving the T suppressor cells. To identify the mechanisms involved, the effect of suppressor cells on Ag-presenting dendritic cells (DC) was studied. Coincubation of hapten-pulsed DC with T cells that were obtained from UV-tolerized mice resulted in an enhanced death rate of DC, and this cell death was dependent upon Fas expression. The addition of IL-12, which recently was found to break established tolerance in vivo, prevented DC death. Moreover, IL-12 did not only rescue DC from T suppressor cell-induced death but also from apoptosis induced by rFasL, suggesting that IL-12 may interfere with the Fas/FasL system. Together, these data indicate a crucial role for the Fas/FasL system in UV-induced tolerance, and suggest that UV-induced T suppressor cells may act by inducing the cell death of APCs via the Fas pathway. The ability of IL-12 to break established tolerance may be due to the prevention of DC death induced by T suppressor cells. ***The Journal of Immunology,*** **1998, 160: 4262–4270.**

UV light represents one of the most significant environmental factors influencing humans, especially with regard to its hazardous health effects, which include premature skin aging, skin cancer, and exacerbation of infectious diseases (1–3). Several of these effects are mediated by the immunosuppressive properties of UV, which are best demonstrated by the inhibition of cellular immune reactions such as contact hypersensitivity (CHS)[3] (4, 5). Accordingly, cutaneous exposure to UV impairs sensitization to haptens applied directly to the irradiated skin surface (4, 5). In addition and most likely as a separate event, individuals sensitized through UV-exposed skin develop hapten-specific tolerance (4, 5), which is due to the development of hapten-specific T suppressor cells (6). Although the existence of T suppressor cells has been appreciated for more than a decade, the mechanisms by which T suppressor cells mediate tolerance have remained unclear.

There is increasing evidence that apoptosis plays an important role in the homeostasis of the immune system (7). The Fas Ag (APO-1, CD95) is a type I membrane protein (8, 9) that mediates apoptotic cell death upon interaction with its natural ligand, Fas ligand (FasL) (CD95L) (10). Mutation of the *fas* or *fasL* gene causes abnormal lymphoproliferation and generalized autoimmune disease in *lpr* and *gld* mice, respectively (11, 12). Recently, it was shown that the Fas/FasL system is crucial for the existence of immune-privileged organs such as the testis (13). Testis grafts from mice expressing FasL survive indefinitely when transplanted into allogeneic animals, while testis derived from *gld* mice lacking functional FasL are rejected. There is also recent evidence for a relationship between Fas/FasL-mediated cell death and tolerance induction following Ag injection into the anterior chamber of the eye (14), another immune-privileged organ which expresses high levels of FasL (15).

Since tolerance can be considered a form of immune privilege, we addressed whether UV-induced tolerance is mediated via the Fas/FasL system. Here, we show that 1) *lpr* and *gld* mice that lack expression of functional Fas and FasL, respectively, are resistant to UV-induced tolerance; 2) adoptive transfer experiments revealed that Fas and FasL expression is crucial for development of UV-mediated tolerance in the recipient but not in the donor; 3) T cells from UV-tolerized donors induce the cell death of hapten-pulsed bone marrow (BM)-derived dendritic cells (DC); 4) DC obtained from *lpr* and *gld* mice are resistant to cell death induced by T suppressor cells; and 5) the addition of IL-12, which was recently found to break UV-induced tolerance (16–18), can rescue DC from cell death induced by either suppressor cells or rFasL. Taken together, these data provide evidence for an important link between the apoptosis-associated Fas/FasL system and UV-induced immune tolerance. Furthermore, they demonstrate for the first time that T suppressor cells exert their suppressive activity via the Fas/

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[3] Abbreviations used in this paper: CHS, contact hypersensitivity; DC, dendritic cells; BM, bone marrow, DNFB, 2,4-dinitrofluorobenzene; DNBS, 2,4-dinitrobenzenesulfonic sodium salt; FasL, Fas ligand; FSC, forward light scatter; PI, propidium iodide; SSC, side light scatter.

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FasL system and show that IL-12 might break tolerance by interfering with the Fas/FasL system.

Materials and Methods

Reagents

For detection of Fas expression, a rabbit polyclonal IgG (M-20, Santa Cruz Biotechnology, Santa Cruz, CA) was used. FasL was detected using an FITC-conjugated anti-FasL mAb (Alexis, Grünberg, Germany). Murine rIFN-γ was obtained from Genzmye (Cambridge, MA), rFasL was kindly provided by K. Schulze-Osthoff (Department of Internal Medicine, University of Tübingen, Tübingen, Germany), and murine rIL-12 was a generous gift from S. Wolf (Genetics Institute, Cambridge, MA).

Contact hypersensitivity

C3H/HeN, C3H/HeN-*lpr*, and C3H/HeN-*gld* mice were obtained from Bomholtgard Breeding and Research Center (Ry, Denmark). Mice at an age of 8 to 10 wk were used for the experiments. Mice (referred to as C3H, *lpr*, and *gld*, respectively) were sensitized on day 0 by painting 25 μl of 0.5% 2,4-dinitrofluorobenzene (DNFB) onto the shaved back. After 5 days, mice were challenged by painting 20 μl of 0.3% DNFB onto the left ear. Ear swelling was quantitated 24 h later using a spring-loaded micrometer. CHS was determined as the amount of swelling of the hapten-challenged ear compared with the thickness of the vehicle-treated ear in sensitized animals and expressed in centimeters $\times 10^{-3}$ (mean $\pm$ SD). After 2 weeks, mice were resensitized through nonirradiated abdominal skin and challenged on the right ear after 5 days. Data were analyzed by Student's *t* test, and differences were considered significant at $p < 0.05$. Each group consisted of at least seven mice, and each experiment was performed at least three times.

UV irradiation

Mice were exposed to 1000 J/min UV on the shaved back daily for 4 consecutive days. FS-20 fluorescent lamps (Westinghouse Electric, Pittsburgh, PA), which emit most of their energy within the UVB range (290–320 nm), were used. DNFB was applied to the surface of the irradiated area 24 h after the last UV exposure.

Adoptive transfer

Donor mice were exposed to UV and sensitized with DNFB through UV-exposed skin. At 10 days after sensitization, spleens and regional lymph nodes were removed, and single-cell suspensions were prepared. The cell number was adjusted to 2.5×10^8 cells/ml, and 200 μl were injected i.v. into naive recipient mice. Recipients were sensitized 24 h later by epicutaneous application of DNFB on the shaved back. After 5 days, mice were challenged on the left ear, and ear swelling was evaluated 24 h later. For control purposes, identical numbers of cells obtained from untreated or DNFB sensitized mice were injected.

Isolation of T cells

Peripheral lymph nodes were obtained, single cell suspensions were prepared, and lymphocytes were depleted of RBCs. T cells were purified using a two step procedure consisting of a nylon wool column passage and the subsequent depletion of remaining contaminating cells with Ab-coated glass columns (Cellect Mouse T cell kit, Tebu, Edmonton, Canada), resulting in a >99% pure T cell preparation.

Generation of DC

DC were generated by culture of BM cells as previously described (19), although with some modifications. BM was collected from tibias, erythrocytes were lysed, and the remaining cells were cultured in petri dishes at a density of 0.5×10^6 cells/cm^2 for 4 h. Nonadherent cells were collected, and 1×10^6 cells/ml were placed in 24-well plates in RPMI 1640 medium (5% FCS, 50 μM 2-ME, 1% nonessential amino acids, 20 μg/ml gentamicin), 150 U/ml granulocyte macrophage CSF, and 100 U/ml IL-4. Two-thirds of the medium was replaced every 2 days, and nonadherent cells were harvested on days 7 or 8. DC aggregates were purified by 1 g sedimentation and subcultured in 6-well plates. The expression of surface molecules characteristic for DC was determined by flow cytometry. DC cultures of >70% brightly I-A$^+$ cells were used.

Flow cytometry

Aliquots of 1×10^5 DC were incubated with mAbs against I-A^{b,d,q}, I-E^{d,k} (M5/114), B7-1 (1G10), B7-2 (Gl-1) (PharMingen, San Diego, CA), CD11c (N418) (Endogen, Boston, MA), Thy-1.2 (30 H-12) (American Type Culture Collection, Rockville, MD), and normal rat IgG2b (PharMingen) as isotype controls for 45 min on ice. After washing, cells were incubated with FITC-conjugated goat anti-rat IgG (Boehringer Mannheim, Mannheim, Germany). Propidium iodide (PI) was added, and cells were washed twice and subsequently analyzed in a flow cytometer (Epics XL, Coulter, Miami, FL).

Coculture of DC and lymphocytes

DC (1×10^6) were incubated for 48 h with 5×10^6 purified T cells from the regional lymph nodes of sensitized, UV-tolerized (i.e., UV-exposed and DNFB-sensitized), or naive mice. Cultures were supplemented with 2,4-dinitrobenzenesulfonic sodium salt (0.1 mM, DNBS). In some cultures, rIL-12 (200 ng/ml) was added at the start of coculture. Cells were harvested and assessed by flow cytometry. DC were identified in forward light scatter (FSC)/side light scatter (SSC) dot plots by their larger size and granularity, and the DC- or T cell-specific gates were subsequently set. Cell viability was evaluated by flow cytometry using PI incorporation and scatter characteristics (lower FSC).

Detection of cell death

The murine DC line XS52 (kindly provided from A. Takashima, Department of Dermatology, University of Dallas, Dallas, TX) (20) was treated with 50 ng/ml of human rFasL. Cells were evaluated for apoptosis using a cell death detection ELISA (Cell Death Detection ELISA^{PLUS}, Boehringer Mannheim) 16 h later. The basis of this test is the detection of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates using biotinylated anti-histone- and peroxidase-coupled anti-DNA Abs. The amount of nucleosomes is photometrically quantified by the peroxidase activity retained in the immunocomplexes.

Northern blot analysis

Cells (2×10^5) were washed twice with PBS, lysed in 4 M guanidine thiocyanate (0.5 ml)/300 mM sodium acetate (pH 4.8)/1% lauroylsarcosine/100 mM 2-ME for 10 min, vortexed for 1 min, and frozen at −20°C until used. Cell lysates were extracted once with acidic phenol (pH 4.8) and twice with chloroform. Precipitation was performed with equal volumes of isopropanol and centrifugation at 14,000 revolutions per minute for 10 min. The pellet was rinsed with 70% ethanol, residual solvent was evaporated in a vacuum desiccator, and the pellet was dissolved in double distilled water. The amount of total RNA was determined photometrically at 260 nm. For Northern blot analysis, total cellular RNA was denatured in 50% formamide, 6% formaldehyde, and 1× MOPS (0.002 M 3-(*N*-morpholino)propane-sulfonic acid, 0.05 M sodium acetate (pH 7.0), and 0.01 M Na_2/EDTA) at 65°C for 5 min. Total RNA (10 μg) was separated by gel electrophoresis using 1% agarose gels containing 2.2% formaldehyde and 1× MOPS. Gels were blotted onto nylon membranes (Hybond-N, Amersham, Buckinghamshire, U.K.) and baked at 80°C for 2 h. Membranes were then prehybridized in 50% formamide, 10% dextran sulfate, 1% SDS, and 1 M NaCl for at least 15 min. Hybridization was conducted using deoxyadenosine-5′-[^{32}P]triphosphate-labeled cDNA probes encoding murine Fas (kindly provided by S. Nagata, Department of Genetics, Osaka University, Osaka, Japan) at 42°C for at least 12 h in a hybridization oven (Hybaid, London, U.K.). After hybridization, filters were washed in 2× SSC at room temperature for 10 min, in 2× SSC containing 1% SDS at 60°C for 1 h, and finally in 0.1× SSC for 1 h. Filters were exposed to x-ray films at −70°C.

Results

Development of UV-induced tolerance requires Fas and FasL

To address whether UV-induced tolerance is mediated via the Fas/FasL system, we used *lpr* and *gld* mice, which are defective in functional Fas and FasL expression, respectively (11, 12). For better comparison with other data, a genetic background was chosen which is known to be UV-susceptible and which is routinely used in photoimmunologic studies (C3H/HeN) (21). UV-induced immunosuppression was analyzed by determining CHS responses after sensitization with the contact sensitizer DNFB through UV-exposed skin. Both *lpr* and *gld* mice exhibited a profound UV-induced suppression of CHS that was indistinguishable from that of C3H mice (Fig. 1*A*). UV-induced specific immune tolerance was analyzed by resensitizing the mice through non-UV-irradiated

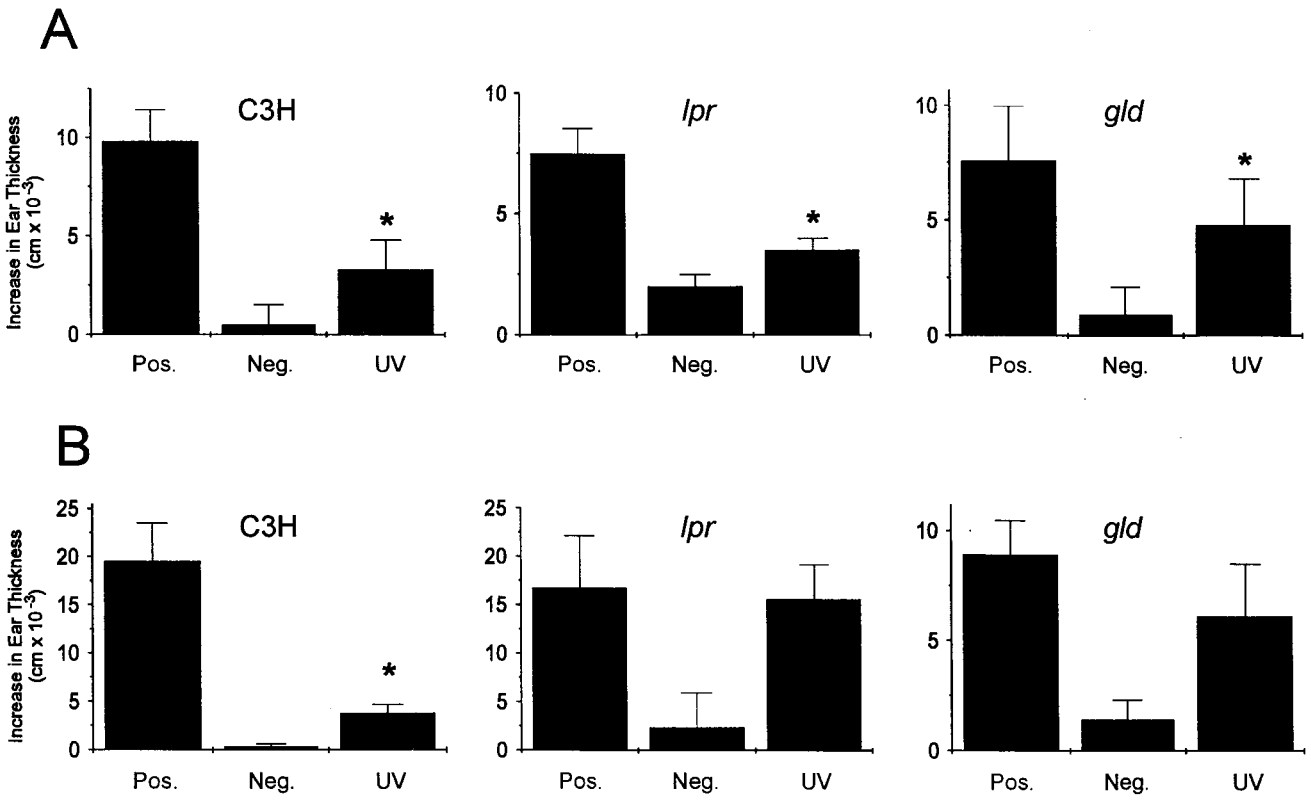


FIGURE 1. Development of UV-induced tolerance requires Fas and FasL. *A*, UV-induced inhibition of CHS in *lpr* and *gld* mice is shown. C3H, *lpr*, and *gld* mice were sensitized with DNFB on UV-exposed (UV) or untreated (Pos.) back skin and challenged on the ear after 5 days. Ear swelling response was measured 24 h later. *B* shows the lack of development of tolerance in *lpr* and *gld* mice. Mice were sensitized with DNFB on UV-exposed (UV) or untreated (Pos.) back skin, resensitized 2 wk later with DNFB through abdominal skin, and challenged on the ear 5 days later. In all experiments, negative control mice (Neg.) were ear-challenged without prior sensitization. Ear swelling response is expressed as the difference (centimeter $\times 10^{-3}$, mean $\pm$ SD) between the thickness of the challenged and the untreated ear. *, $p < 0.05$ vs positive control.

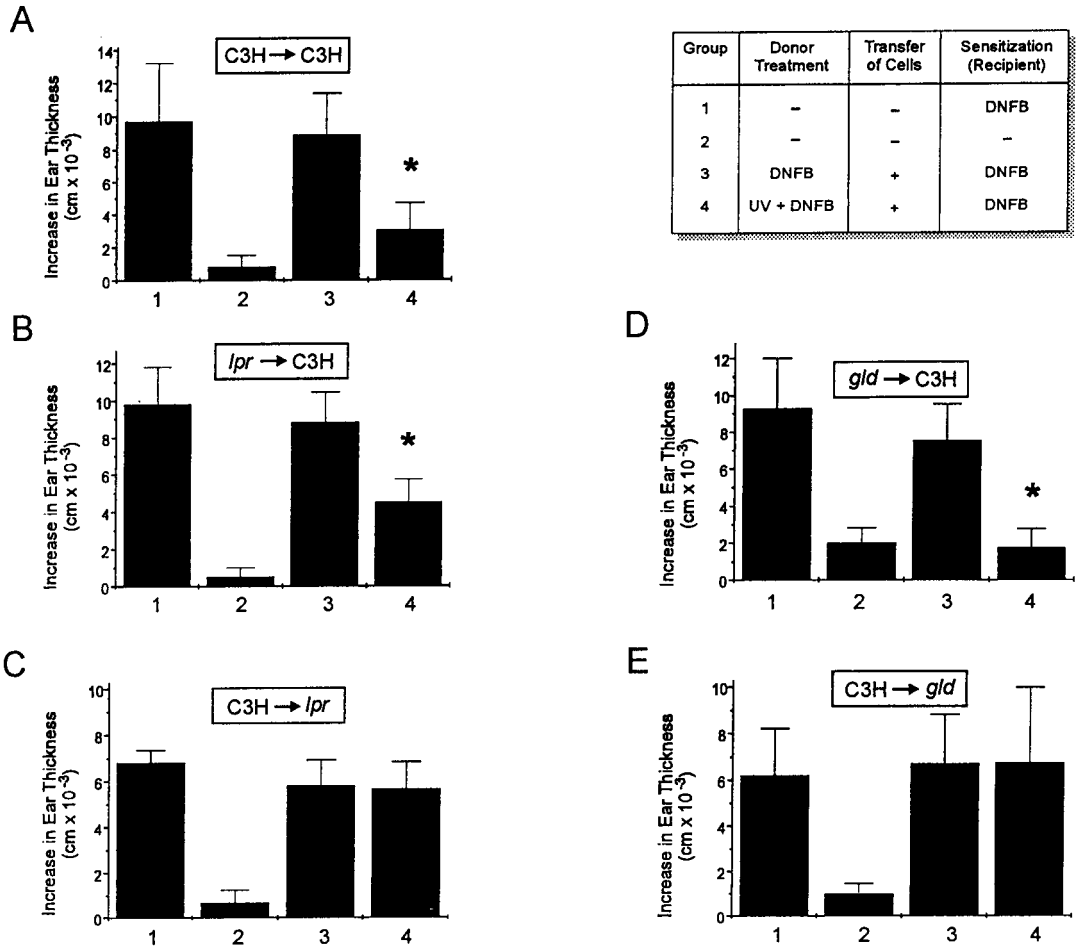
skin 2 wk after the initial UV exposure. Whereas C3H mice became tolerant to the hapten DNFB, neither *lpr* nor *gld* mice developed tolerance (Fig. 1*B*). These data support the hypothesis that the Fas/FasL system is essential for the generation of UV-induced tolerance. Furthermore, they indicate that the suppression of CHS and the induction of tolerance by UV light are separate events and mediated by different pathways; this observation supports previous findings (22).

Transfer of tolerance requires Fas and FasL expression in the recipient

Tolerance can be transferred to naive, nonirradiated mice by adoptive transfer of T cells from tolerized mice, indicating the existence of hapten-specific T suppressor cells (6). To test the role of Fas/FasL in suppressor cell function, we performed adoptive transfer experiments using different combinations of C3H, *lpr*, and *gld* mice as donors or recipients. The transfer of lymph node and spleen cells from UV-exposed and DNFB-treated C3H mice into naive C3H mice inhibited the subsequent DNFB sensitization of the recipients (Fig. 2*A*), confirming the presence of suppressor cells within the transferred cell population. In contrast, the transfer of cells from UV-exposed and hapten-sensitized *lpr* donors into naive *lpr* mice did not inhibit subsequent DNFB sensitization; similar findings were obtained with *gld* mice (data not shown). To determine whether the expression of Fas and FasL, respectively, by the recipient or by donor-derived cells is required in mediating tolerance, cross-transfer experiments were performed. Suppression was observed when cells from UV-exposed and hapten-treated *lpr* donors were injected into naive C3H mice (Fig. 2*B*), and also when cells from UV-exposed and hapten-treated *gld* mice were transferred into naive C3H mice (Fig. 2*D*). However, both naive *lpr* and naive *gld* recipients of T suppressor cells from UV-exposed and hapten-treated C3H mice exhibited normal sensitization against the specific hapten (Fig. 2, *C* and *E*). Together, these findings show that the transfer of tolerance does not require Fas or FasL expression on the suppressor cells but does require both molecules on the cells of the recipient.

T cells from UV-tolerized mice enhance DC death in a Fas/FasL-dependent manner

The present findings indicate that the Fas/FasL system is essential for the mediation of UV-induced tolerance but not for the generation of those T cells transferring suppression. Thus, one might suspect that these T cells mediate tolerance by killing immunocompetent cells which are important during (re)sensitization. However, since the transfer experiments (Fig. 2) clearly showed that the transfer of tolerance does not require Fas or FasL expression on the suppressor cells but does require both molecules on the cells of the recipient, we surmised that T cells from UV-tolerized mice might not kill their potential targets via the expression of FasL, but instead may induce recipient cells that are critical during sensitization to undergo cell death in a Fas/FasL-dependent manner. Thus, we hypothesized that APCs may undergo apoptosis when presenting the hapten to T suppressor cells and that this action is mediated via the Fas/FasL system. To test this hypothesis, coincubation experiments of DC and T suppressor cells were performed. However,



Group	Donor Treatment	Transfer of Cells	Sensitization (Recipient)
1	–	–	DNFB
2	–	–	–
3	DNFB	+	DNFB
4	UV + DNFB	+	DNFB

FIGURE 2. Adoptive transfer. At 24 h after the injection of lymphoid cells obtained from donors, which were sensitized through UV-exposed (4) or unirradiated (3) skin, recipients were sensitized and ear-challenged 5 days later. As controls, mice were either conventionally sensitized and ear challenged (1) or challenged without prior sensitization (2). Ear swelling was measured 24 h after challenge. Donor and recipient strains in the different experiments are indicated. *, $p < 0.05$ vs positive control (1).

since UV-induced T suppressor cells have not been phenotypically characterized, and specific markers do not exist, it was obligatory to use bulk T cell suspensions from UV-tolerized (i.e., UV-exposed and DNFB-sensitized) mice as a source for T suppressor cells. Therefore, it is important to note that the term "T suppressor cells" used throughout this manuscript refers to such bulk T cell suspensions. T cells from naive, DNFB-sensitized, or UV-tolerized C3H mice were coincubated with syngeneic BM-derived DC in the presence of the water soluble DNFB-analogue, DNBS, for 48 h. Cell viability was evaluated by flow cytometry using PI incorporation and scatter characteristics (Fig. 3 shows a representative FACS profile). In the absence of growth factors, DC gradually die in culture, and this death is largely independent of coculture with T cells in the absence of hapten. However, in the presence of hapten, a strikingly higher percentage of DC died upon coculture with T cells obtained from UV-tolerized mice (Table I, group c) as compared with those from naive mice (group a). In contrast, the increase in DC death was much less pronounced in cocultures with T cells obtained from sensitized mice (group b). Enhanced DC death was not observed in the absence of the specific hapten (group k) and when using allogeneic DC (group l). Among T cells, activation-induced cell death was clearly evident in primed (group b) as opposed to naive T cells (group a); however, no additional increase was observed in T cells obtained from UV-tolerized mice (group c). These data suggest that DC death during hapten presentation is strongly increased in the presence of hapten-specific suppressor cells.

Because the in vivo experiments indicated that Fas and FasL expression is necessary for mediation of tolerance, we also used DC from *lpr* and *gld* mice, respectively. Unlike C3H DC, *lpr* DC were much less sensitive to cell death in the presence of T cells from UV-tolerized mice (group g), and similar findings were observed when using *gld* DC (group j). The resistance of both *lpr* and *gld* DC against T suppressor cell-induced death suggests that both Fas and FasL apparently need to be expressed on DC, implying the induction of autocrine suicide. However, when DC from *lpr* and *gld* mice were mixed, enhanced DC death was again observed in the presence of T suppressor cells (% dead DC: *lpr*, 15.3; *gld*, 18; *lpr* plus *gld*, 50; C3H, 65). These data suggest that DC in this system die via the Fas/FasL system not only in an autocrine manner but also in a paracrine manner.

T cells from UV-tolerized mice up-regulate Fas expression on DC

Since the coculture experiments revealed that T cells obtained from UV-tolerized mice enhance DC death, and that this may be mediated via the Fas/FasL system, we postulated that these T cells might induce Fas expression on DC. To address this issue, DC obtained from C3H mice were again cocultured in the presence of DNBS with T cells obtained from naive, DNFB-sensitized or

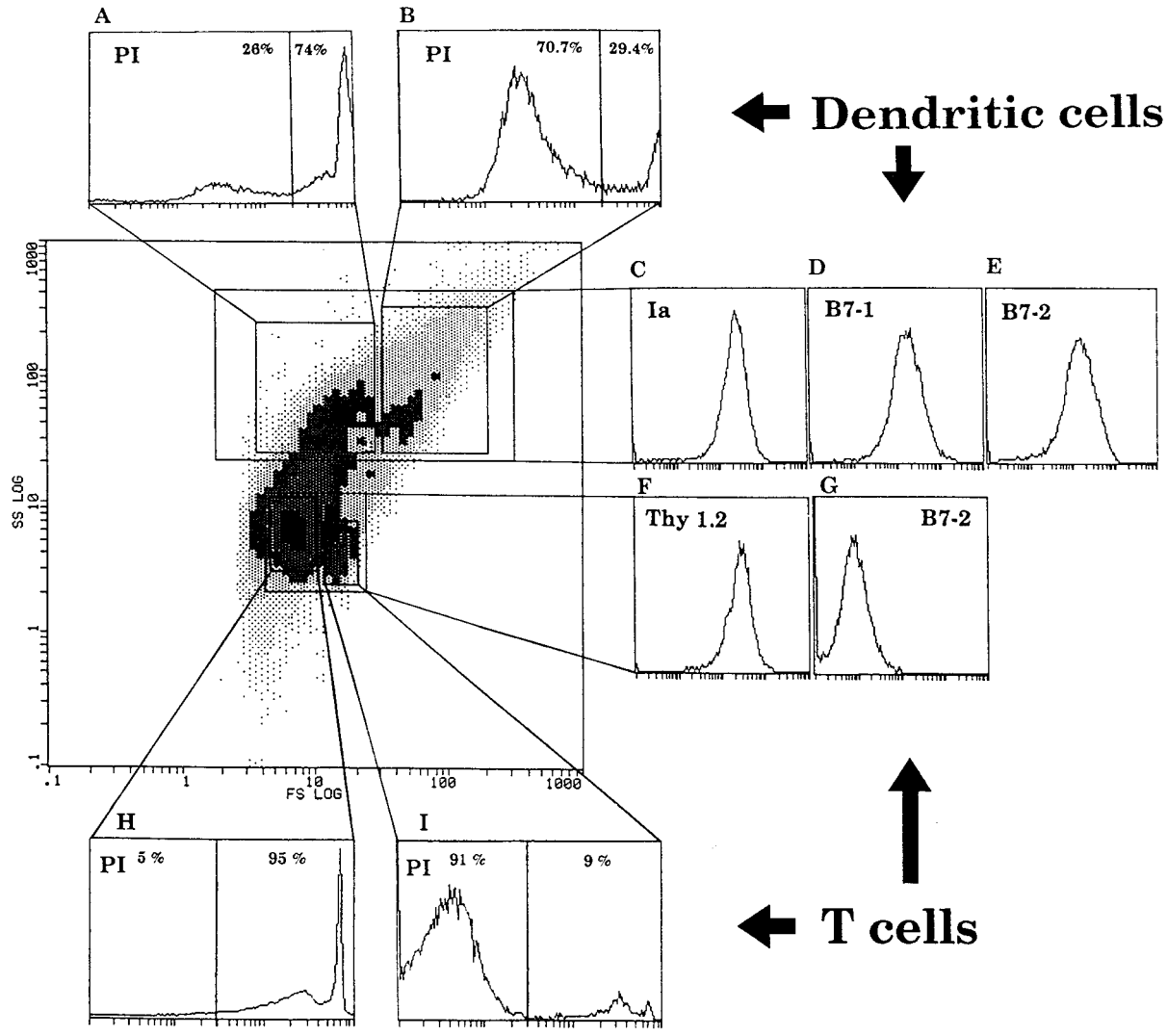


FIGURE 3. Differentiation of cell type (DC vs T cells) and viability (viable vs dead cells) by flow cytometry in coculture systems. BM-derived DC (1×10^6) were coincubated for 48 h with 5×10^6 purified T cells obtained from DNFB-sensitized mice, harvested, and analyzed by flow cytometry using PI to stain dead cells. In FSC/SSC dot plots, DC can be distinguished from T cells by their larger size and granularity (*A–E* are DC, *F–I* are T cells). Within both populations, dead cells can be distinguished from viable cells by their smaller size (lower FSC) as well as by PI uptake.

-tolerized C3H mice. Cells were stained 48 h later with an anti-Fas Ab, and FACS analysis was performed. DC were gated, and Fas expression on DC was measured. DC constitutively expressed Fas, and this expression was not affected by the coincubation of DC with T cells from either naive or sensitized T cells, while up-regulation of Fas expression was found upon coincubation with T cells obtained from UV-tolerized mice (Fig. 4). Therefore, these data demonstrate that T cells from UV-tolerized mice enhance Fas expression on DC.

IL-12 prevents killing of DC induced either by FasL or by T cells from UV-tolerized mice

Recently, we and others reported that IL-12 can break established UV-induced tolerance (16–18), although the underlying mechanism remains unclear. Based on the data obtained from the coincubation experiments, we hypothesized that IL-12 could counteract the inhibitory activity of suppressor cells by preventing suppressor cell-mediated DC death. Therefore, T cells from UV-tolerized mice and DC were coincubated in the presence of rIL-12. The addition of IL-12 reduced the number of dead DC (Table I, d). This suggests that IL-12 may break tolerance by preventing DC from undergoing cell death induced by T suppressor cells. Since the in vivo data indicated a crucial role of the Fas/FasL system in the mediation of tolerance, we wondered whether IL-12 could rescue DC from FasL-induced killing. Therefore, the DC line XS52 was incubated with rFasL, and apoptosis was measured 16 h later using a cell death ELISA. Treatment of XS52 cells with rFasL resulted in pronounced cell killing (Fig. 5). In contrast, when XS52 cells were preincubated with rIL-12 for 8 h before the addition of rFasL, fewer cells died.

IL-12 down-regulates Fas expression

Since T suppressor cells induced Fas expression on DC, and since IL-12 prevented DC from T suppressor cell-induced killing, we postulated that IL-12 might rescue DC by down-regulating Fas expression on DC. This appeared to be the case, as the addition of IL-12 to DC coincubated with T cells from tolerized mice resulted in down-regulation of Fas expression on DC (Fig. 4, *bottom panel*). To further confirm this effect of IL-12, XS52 cells were cultured in the presence of IL-12 or left untreated and evaluated for Fas expression. FACS analysis revealed constitutive Fas expression that could be additionally enhanced by IFN-γ. Preincubation

Table I. *T cells from UV-tolerized mice enhance DC death*

DC[a]	T cells[b]	Culture[c]	% Dead Cells[d] DC PI	DC Scatter	T cells PI	T cells Scatter
a) C3H	naive	DNBS	29	30	37	32
b) C3H	sensitized	DNBS	47	43	78	79
c) C3H	UV-tolerized	DNBS	74	72	80	82
d) C3H	UV-tolerized	DNBS + IL-12	30	22	22	15
e) *lpr*	naive	DNBS	22	17	21	11
f) *lpr*	sensitized	DNBS	37	26	27	16
g) *lpr*	UV-tolerized	DNBS	32	25	24	14
h) *gld*	naive	DNBS	20	17	8	15
i) *gld*	sensitized	DNBS	27	39	6	20
j) *gld*	UV-tolerized	DNBS	23	24	9	20
k) C3H	UV-tolerized	0	9	13	11	13
l) BALB/c	UV-tolerized	DNBS	13	17	38	33

[a] DC were obtained from BM of untreated mice.
[b] Purified T cells were obtained from naive, DNFB-sensitized, or UV-tolerized (DNFB sensitization through UV-exposed skin) mice.
[c] DC (1×10^6) were pulsed with DNBS (0.1 mM, 10 min at 37°C) and coincubated with T cells (5×10^6) for 48 h. IL-12 was added at a concentration of 200 ng/ml.
[d] The percentage of dead cells was evaluated by flow cytometry. DC and T cells were gated electronically and analyzed separately for relative numbers of dead cells using PI incorporation as well as FSC and SSC characteristics. Data are from experiments with superimposable results.

with IL-12 significantly down-regulated both constitutive and IFN-γ-induced Fas expression on XS52 cells (Fig. 6).

To gain an insight into the mechanism by which IL-12 down-regulates Fas expression, XS52 cells were stimulated with IL-12 for 2 h and then treated with IFN-γ for the next 2 h. For control purposes, XS52 cells were either exposed to IFN-γ or IL-12 or left untreated. RNA was extracted, and Northern blot was analysis performed using a cDNA probe encoding murine Fas. IFN-γ significantly induced Fas mRNA expression, which was reduced upon treatment with IL-12 (Fig. 7).

Discussion

Based on the recent observations that the apoptosis-related Fas/FasL system is responsible for the existence of immune-privileged organs such as the testis and the anterior chamber of the eye (13, 15), and since tolerance can be considered as a type of immune privilege, we were interested in whether the Fas/FasL system is involved in the mediation of UV-induced tolerance. For this purpose, *lpr* mice that lack functional Fas (11) and *gld* mice that lack functional FasL (12) were tested for their ability to develop UV-induced immune tolerance. Similar to control C3H mice, both *lpr* and *gld* mice could not be sensitized by epicutaneous application of the hapten DNFB onto UV-exposed skin, excluding the possibility that the *lpr* or the *gld* mutations interfere with UV-susceptibility. However, both *lpr* and *gld* mice developed a vigorous CHS response upon DNFB resensitization, clearly indicating that, in contrast to C3H mice, tolerance had not developed in these strains. Thus, the Fas/FasL system appears to be critically involved in UV-induced tolerance but not in UV-mediated suppression of the induction of CHS. Since both *lpr* and *gld* mice failed to develop tolerance, it is unlikely that additional potential gene defects in these mouse strains account for this effect. Both *lpr* and *gld* mice have altered immune responses and show autoimmune features including lymphadenopathy, splenomegaly, lymphocytosis, hyperimmunoglobulinemia, and autoantibody production. However, it is unlikely that these changes have an impact on the present tolerance experiments, because the autoimmune features usually begin to manifest around week 14 (23). Therefore, we used 8- to 10-wk-old animals, an age at which the mice do not show major pathologic changes.

Since UV-induced tolerance is mediated by transferable hapten-specific T suppressor cells (6), we also investigated whether suppressor cells had developed in Fas- and FasL-deficient mice, respectively. Whereas transfer of lymphocytes from UV-irradiated *lpr* or *gld* mice into unirradiated, naive, syngeneic recipients failed to inhibit subsequent sensitization, transfer of these cells into unirradiated, naive C3H mice caused immune tolerance in the recipients. Likewise, transfer of lymphocytes from UV-irradiated C3H mice into naive, unirradiated *lpr* or *gld* mice failed to induce hapten-specific tolerance (Fig. 2). Thus, 1) suppressor cells are generated in Fas- and FasL-deficient mice; and 2) to exert their immunomodulatory function, the expression of Fas and FasL is required on the cells of the recipient but not on the suppressor cells themselves. In a different experimental system, Griffith et al. (14) recently showed that tolerance is induced via Fas/FasL-dependent apoptosis of lymphocytes upon Ag injection into the anterior chamber of the eye. The study demonstrated that Fas expression on lymphoid cells and FasL expression on eye cells is required for the induction of tolerance. In contrast, our data show that in the UV model, the Fas/FasL system is not necessary for the induction of tolerance-mediating suppressor cells, since these cells, obtained from either *lpr* or *gld* mice, are functionally active provided they are injected into a Fas/FasL-expressing donor. This observation implies that T suppressor cells need the Fas/FasL system to exert their inhibitory activity. However, since neither Fas nor FasL need to be expressed on the suppressor cell for mediating tolerance (Fig. 2, *B* and *D*), T suppressor cells apparently do not induce apoptosis directly via Fas/FasL interaction but rather via an indirect mechanism, e.g., by up-regulating Fas and FasL expression on cells in the recipient.

This view is supported by the cross-transfer experiments shown in Figure 2, which suggest that UV-induced T suppressor cells do not kill their targets via the expression of FasL, as suppression is still observed upon transfer of suppressor cells obtained from *gld* mice which lack FasL. These data imply that suppressor cells induce other cells in the recipient to undergo cell death via the Fas/

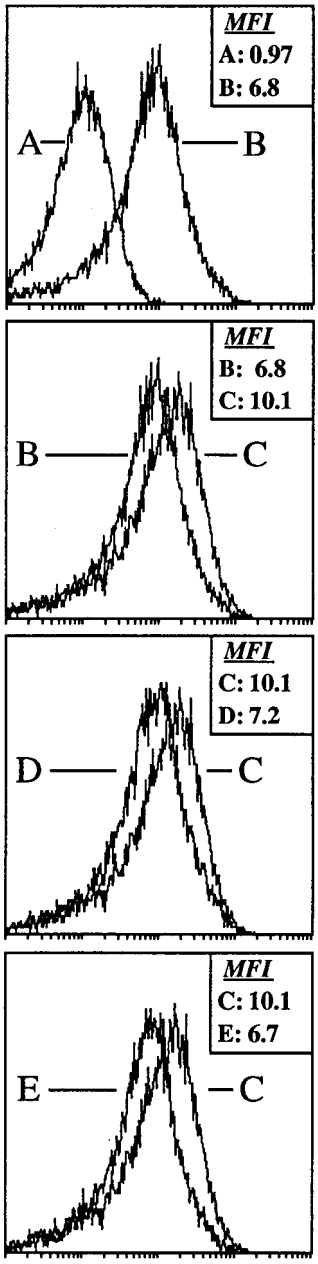


FIGURE 4. T cells from UV-tolerized mice up-regulate Fas expression on DC. DC from C3H mice were coincubated with T cells obtained from either naive (*B*), sensitized (*D*), or UV-tolerized (*C*) C3H mice. In the bottom panel, DC were coincubated with T cells from UV-tolerized mice in the presence of IL-12 (E). Cells were stained 48 h later with an anti-Fas Ab. DC were gated and evaluated for Fas expression by FACS analysis. Rabbit IgG was used as an isotype control (*A*). Histograms show fluorescence intensity (*x*-axis) vs cell number (*y*-axis); the mean fluorescence intensity for each sample is indicated on the graphs.

FasL pathway. Since APCs and hapten-specific T cells are critical during sensitization, these cells would be potential targets. We assumed that Th cells are a less likely candidate, since Glass et al. (24) observed that suppressor cells fail to impair the CHS response of cotransferred immune lymph node cells, indicating that hapten-specific T suppressor cells do not exhibit the capacity to suppress effector cells which are already primed against the relevant hapten. Thus, we hypothesized that APCs may undergo cell death when presenting the hapten in the presence of T suppressor cells.

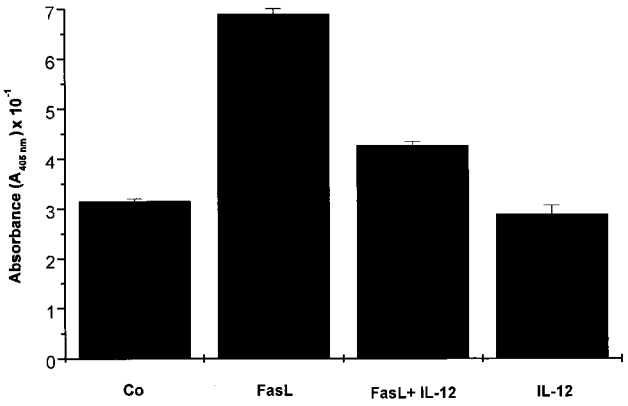


FIGURE 5. IL-12 inhibits FasL-induced apoptosis. XS52 cells (1×10^6/ml) were incubated with rFasL (50 ng/ml), and apoptosis was evaluated 16 h later. One sample was incubated with rIL-12 (200 ng/ml) for 8 h before the addition of rFasL. Control cells were left untreated (Co) or treated with IL-12 alone. Apoptosis was examined by determining nucleosomal DNA fragmentation using an apoptosis determination kit. The rate of apoptosis is reflected by the increase of absorbance shown on the *y*-axis.

To address this issue, T cells were isolated from C3H mice 5 days after sensitization through UV-exposed skin. Since suppression can be transferred at this time, T suppressor cells must already be present in these T cell suspensions. Since UV-induced T suppressor cells have not been phenotyped, and specific markers do not exist, it is not possible to isolate and purify the hapten-specific T suppressor cells. Therefore, we had to use bulk T cell suspensions obtained from UV-tolerized mice (i.e., hapten-treated through UV-exposed skin), and thus the term "T suppressor cells" refers to such bulk T cell suspensions. To investigate whether APCs undergo cell death in the presence of these T suppressor cells, DC were obtained from BM of C3H mice, coincubated with the T cell suspensions in the presence of the water soluble DNFB analogue, DNBS, and analyzed by flow cytometry. As this study revealed, a strikingly higher percentage of DC died upon coculture with T cells obtained from UV-tolerized mice as compared with those from naive or sensitized mice. This event is hapten-specific and MHC-dependent, because it is not observed in the absence of hapten or when DC obtained from allogeneic BALB/c mice were used. DC death appears to be due to apoptosis, because dead DC stained positively for annexin V (data not shown), a specific surface marker for apoptosis (25).

In analogy to our in vivo experiments, which indicated that the transfer of tolerance is dependent upon the Fas/FasL system, DC from *lpr* mice were much less sensitive to cell death in the presence of T cells from UV-tolerized mice. Similar findings were observed when using DC from *gld* mice, implying that, to undergo suppressor cell-induced death, both Fas and FasL apparently need to be expressed on DC. Although in many systems the death of Fas- expressing cells is induced by other cells carrying FasL, T cells have been shown to undergo programmed cell death due to Fas/FasL interaction in a cell-autonomous manner (26, 27). Similarly, in Hashimoto's thyroiditis, thyrocytes undergo programmed cell death due to cell-autonomous coexpression of Fas/FasL (28). Because neither Fas nor FasL is required on the suppressor cells (as demonstrated by the adoptive transfer experiments shown in Fig. 2), it is likely that the two molecules become coexpressed on DC, thereby inducing an autocrine suicide. This possibility is supported by previous reports indicating that DC can express CD95 and CD95L (29–31). Accordingly, FACS analysis revealed that Fas expression on DC is induced in the presence of T suppressor

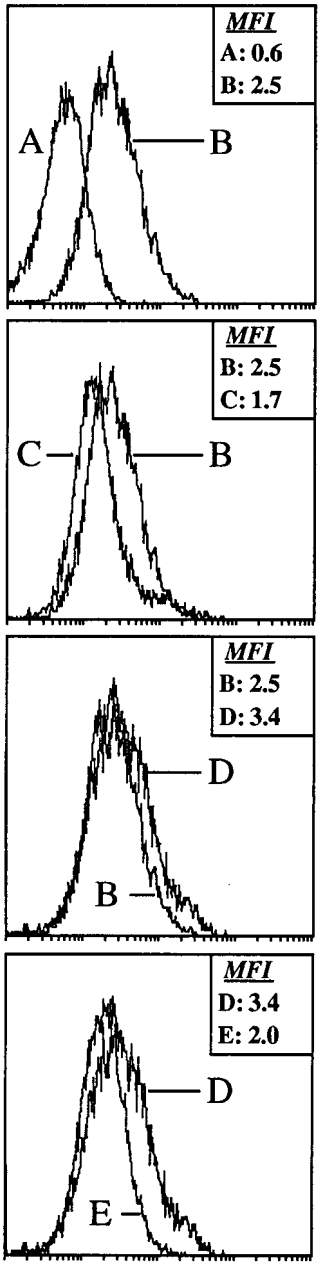


FIGURE 6. IL-12 down-regulates Fas expression by XS52 cells. XS52 cells were stimulated with 200 ng/ml murine rIL-12 (*C*), 50 ng/ml murine rIFN-γ (*D*), IL-12 plus IFN-γ (*E*), or left untreated (*B*). Cells were stained 16 h later with an anti-Fas Ab and subjected to FACS analysis. Rabbit IgG was used as an isotype control (*A*). Histograms show fluorescence intensity (*x*-axis) vs cell number (*y*-axis); the mean fluorescence intensity for each sample is indicated on the graphs.

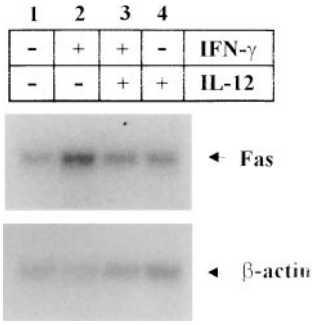


FIGURE 7. IL-12 down-regulates IFN-γ-induced Fas mRNA expression in XS52 cells. XS52 cells were stimulated with murine rIL-12 (200 ng/ml) and murine rIFN-γ (50 ng/ml) was added 2 h later (3). As controls, cells were either left untreated (1) or stimulated with IFN-γ (2) or IL-12 (4). RNA was extracted, and Northern blot analysis was performed 4 h later with a cDNA probe encoding murine Fas. Blots were rehybridized with a cDNA probe encoding for β-actin.

cells, whereas no major differences were observed in FasL expression (data not shown). However, FasL data must be interpreted with caution, since the evaluation of FasL expression by FACS analysis can be problematic due to the rapid shedding of FasL (32). While DC obtained from *gld* or *lpr* mice were clearly resistant to T suppressor cell-induced apoptosis, DC death was observed when DC from *gld* and *lpr* mice were mixed. This observation implies that not only autocrine but also paracrine suicide may be relevant. On the other hand, it is important to mention that changes in the death rates were observed not only for DC but also for T cells. In particular, T cells obtained from either sensitized or tolerized C3H mice died to a greater extent upon coincubation with DC in the presence of the specific hapten. Although we did not pursue this phenomenon, we interpreted it to be compatible with activation-induced cell death (7). However, when similar experiments were performed with DC from either *gld* or *lpr* mice, reduction of the T cell death rate was observed, as was indeed unexpected. While reduced T cell death upon coincubation with DC from *gld* mice is explainable by the fact that the FasL expressed on DC is involved in activation-induced T cell death, we do not as of yet have an explanation as to why the same resistance of T cells was observed when DC from *lpr* mice were used.

We are aware that the in vitro system we used is in some ways artificial and has a variety of limitations: 1) we cannot formally exclude that the suppressor cells may induce FasL expression of a third-party cell type, representing a minor contaminant in the DC preparation; 2) the major disadvantage is the use of bulk T cell suspensions as a source for T suppressor cells; and 3) although DC death was strikingly enhanced upon coculture with T cells from tolerized mice in the presence of the hapten, it is important to note that a certain degree of DC death was also observed in the control coculture experiments. In particular it appears that untolerized, Ag-specific T cells can also induce DC death to some extent, implying more of a quantitative and rather than a qualitative difference between suppressor and activating T cells. Nevertheless, despite these disadvantages, these data provide evidence as to how UV-induced T suppressor cells might act and also give an explanation for our in vivo data, which clearly show that the apoptosis-related Fas/FasL system is essential for UV-induced tolerance.

To further pursue this issue, we studied the effect of IL-12 on DC death in the coculture experiments. Recently, we and others have reported that IL-12 is able to prevent UV-induced inhibition of the induction of CHS when injected i.p. between UV-exposure and hapten sensitization (16–18). More importantly, it was observed that the injection of IL-12 at a later time point, when T suppressor cells have already developed, enables sensitization against the specific hapten. However, the mechanism by which IL-12 breaks established tolerance is still unclear. Due to its ability to counteract the activity of suppressor cells, we were interested in determining whether IL-12 interferes with T suppressor cell-induced DC death. Indeed, the addition of IL-12 to cocultures of DC and T suppressor cells significantly reduced the number of dead DC. Since experiments using DC from *lpr* and *gld* mice, respectively, indicated that Fas/FasL expression appears essential for the induction of DC death, we also asked whether IL-12 could interfere with Fas-mediated apoptosis of DC. For this purpose, the DC

line XS52 was treated with rFasL, and the induction of apoptosis was measured. While rFasL induced apoptosis of XS52 cells, preincubation of XS52 with rIL-12 before the addition of rFasL significantly reduced the rate of apoptosis. Moreover, T suppressor cells induced Fas surface expression on DC (Fig. 4), whereas IL-12 down-regulated Fas expression on DC. Since Northern blot analysis revealed reduced levels of Fas-specific transcripts upon IL-12 treatment, the inhibitory effect may be regulated at the mRNA level. Although we regard down-regulation of Fas expression in DC as an important event, by which IL-12 may protect DC from Fas-mediated cell death, other mechanisms may also be involved, since IL-12 treatment caused a reduction but not a complete suppression of Fas expression on DC. In this respect, the induction of antiapoptotic genes such as *bcl-2*, *bcl-*x_L, FLICE-inhibitory protein, Fas-associated phosphatase-1 (31, 33–36) must be considered as an additional possibility.

Together, these data provide evidence for an important link between the apoptosis-associated Fas/FasL system and immune tolerance by demonstrating that the Fas/FasL system is crucially involved in mediating UV-induced tolerance. The data further indicate that T suppressor cells can exert their suppressive activity in vivo only in the presence of a functionally active Fas/FasL system. Based on our in vitro data, we speculate that UV-induced T suppressor cells may cause the cell death of APCs in the presence of the specific hapten via the Fas/FasL system. IL-12 can rescue APCs from suppressor cell-induced cell death, a mechanism possibly responsible for the unique ability of IL-12 to break established immune tolerance.

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