

Newly discovered role for Fas ligand in the cell-cycle arrest of CD4⁺ T cells

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Fas Ligand (FasL) can induce apoptosis of Fas-bearing cells. It is expressed on the cell surface of many tumor cells, immune-privileged tissues and activated lymphocytes. We report here that FasL can itself transduce signals, leading to cell-cycle arrest and cell death in CD4⁺ T cells. *In vitro*, FasL engagement inhibited CD4⁺ T-cell proliferation, cell-cycle progression, and IL-2 secretion. *In vivo*, FasL engagement prevented superantigen-mediated CD4⁺, but not CD8⁺, T-cell expansion. These findings demonstrate that FasL engagement regulates cell-cycle progression, and show that FasL engagement *in vivo* has a potent anti-inflammatory effect specific for CD4⁺ T cells.

Fas ligand (FasL, CD95L) is a cell surface molecule belonging to the tumor necrosis factor/nerve growth factor family¹. FasL is constitutively expressed in immune-privileged tissues such as eye, brain and testis, as well as on some tumors, and is inducibly expressed on T, B and NK cells during activation of the immune system²⁻⁵. Engagement of Fas by FasL can result in apoptotic death of the Fas-bearing cell⁶⁻⁸. Thus, Fas/FasL interactions downregulate immune responses by inducing T-cell apoptosis, as activated T cells express both Fas and FasL⁶⁻⁹. Mice with mutations in Fas (ref. 10; *lpr* or *lpr^{cs}*) or FasL (ref. 11; *gld*) develop massive lymphadenopathy and are susceptible to autoimmune disease as a consequence of disrupted T-cell homeostasis. Fas/FasL interactions can contribute to the pathology of infectious, autoimmune and neoplastic disease. Organs that express Fas, such as liver, thyroid and pancreas, are susceptible to Fas/FasL-mediated organ damage during infectious and autoimmune diseases¹²⁻¹⁵. FasL expression on tumor cells can prevent immune-mediated tumor rejection by triggering apoptosis of the attacking T cells¹⁶⁻¹⁹. Paradoxically, FasL renders other tumors susceptible to immune clearance^{20,21}.

FasL has been viewed as mediating its functions wholly through Fas engagement, in its role as a ligand, without necessarily affecting the cell on which it is expressed. However, we observed that engaging FasL on T cells in culture resulted in growth arrest and eventually apoptosis of these cells, indicating that FasL was itself transducing signals. Here we demonstrate that the proliferation of wild-type and Fas-defective¹⁰ *lpr* and *lpr^{cs}* CD4⁺ T cells was inhibited by FasL engagement *in vitro* and *in vivo*. FasL-defective¹¹ *gld* T cells were not affected by FasL engagement. These findings demonstrate a newly discovered, FasL-mediated mechanism for growth control of CD4⁺ T cells, with the potential for regulating CD4⁺ T cell inflammatory responses *in vivo*.

FasL engagement inhibits CD4⁺ T-cell proliferation

It has been observed that in a population of activated T cells, those expressing FasL were predominantly dead (ref. 9 and J.D. and M.K.N., unpublished observations). This indicated that FasL

might directly transduce signals involved in cell death, and prompted us to examine the effects of engaging FasL during *in vitro* activation of CD4⁺ T cells. To deliver physiological signals through FasL, we used FasFc, a dimeric molecule consisting of Fas attached covalently to the Fc portion of human IgG. Soluble FasFc has been shown to block Fas-mediated killing by binding to FasL and interfering with Fas/FasL interactions⁶. For many receptors, soluble ligand suffices to block an interaction, but oligomeric engagement, provided by antibodies or ligands immobilized on tissue culture wells, is required to deliver a signal. Therefore, we cultured freshly isolated CD4⁺ T cells in wells coated with anti-CD3 to provide an activating stimulus, and FasFc to engage FasL. Human IgG or CTLA4-Fc (ref. 22) were used as control proteins for FasFc. We found that CD4⁺ T-cell proliferation was substantially inhibited in the presence of immobilized FasFc, but was unaffected by immobilized control proteins (Fig. 1a). The inhibition of proliferation by FasL engagement was dose-dependent and specific, as the control proteins did not affect the CD3-induced proliferative response, demonstrating that FasL itself transduced a signal to the T cell.

There were salient differences in T-cell morphology between the treatment groups. The unstimulated cells were predominantly shriveled and dead after culture. The stimulated cells treated with control proteins (human IgG or CTLA4-Ig) were enlarged, clumped and numerous. In contrast, the stimulated cells treated with FasFc remained small and had not proliferated, but were not dead. To confirm that blastogenesis was inhibited by FasL engagement, we analyzed the cells by flow cytometry (Fig. 1b). FasFc treatment inhibited activation-induced blastogenesis (as determined by cell size), but did not result in increased apoptosis after 2 days in culture (Fig. 1b). Thus, FasL engagement inhibited CD3-induced blastogenic and proliferative responses in CD4⁺ T cells.

FasL engagement arrests cell-cycle progression

Cell-cycle analysis demonstrated that FasL engagement caused the CD3-stimulated T cells to remain as resting G₀/G₁ cells, and

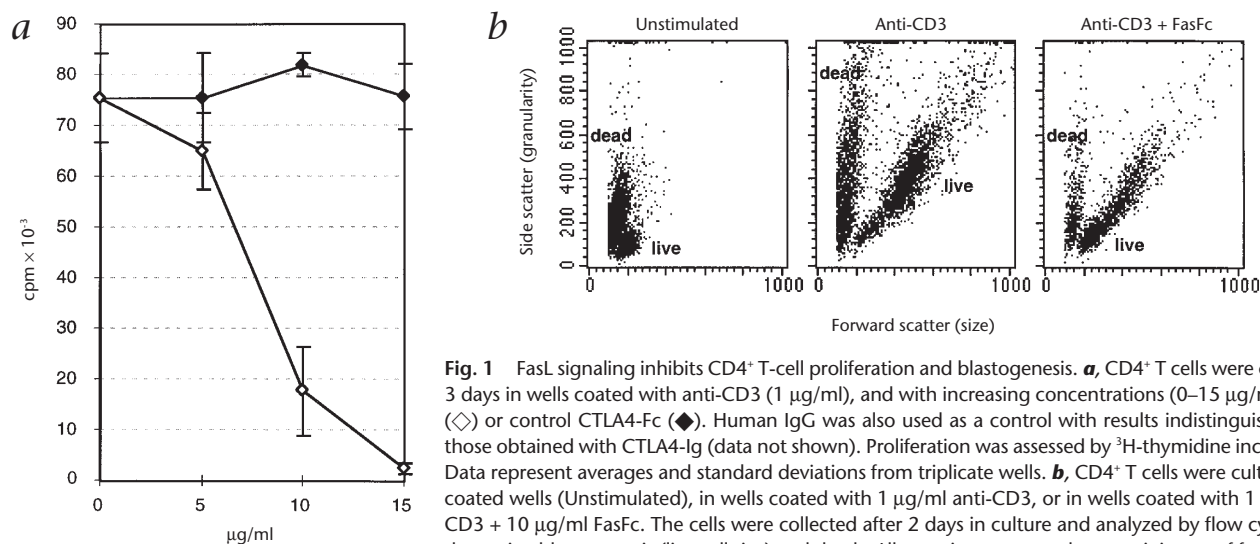


Fig. 1 FasL signaling inhibits CD4⁺ T-cell proliferation and blastogenesis. **a**, CD4⁺ T cells were cultured for 3 days in wells coated with anti-CD3 (1 µg/ml), and with increasing concentrations (0–15 µg/ml) of FasFc (◇) or control CTLA4-Fc (◆). Human IgG was also used as a control with results indistinguishable from those obtained with CTLA4-Ig (data not shown). Proliferation was assessed by ³H-thymidine incorporation. Data represent averages and standard deviations from triplicate wells. **b**, CD4⁺ T cells were cultured in uncoated wells (Unstimulated), in wells coated with 1 µg/ml anti-CD3, or in wells coated with 1 µg/ml anti-CD3 + 10 µg/ml FasFc. The cells were collected after 2 days in culture and analyzed by flow cytometry to determine blastogenesis (live cell size) and death. All experiments were done a minimum of four times.

inhibited their entry into G₂/S phase (Fig. 2a). FasL engagement did not increase the percentage of apoptotic cells during the first 2 days in culture, when growth arrest was visible (Fig. 2a). After 3 days in culture, apoptotic cells began to accumulate more rapidly in FasFc-treated wells than in control wells, and after 4 days, FasL engagement resulted in a substantial increase in cell death (Fig. 2b). Thus, FasL engagement of CD3-stimulated cells resulted in growth arrest, followed 24–48 hours later by apoptosis.

We assessed the effect of FasL engagement on the expression of cell surface activation molecules and intracellular cell-cycle regulatory proteins in stimulated CD4⁺ T cells. The upregulation of T-cell activation molecules, including CD69, Fas and the IL-2 receptor α (IL-2Rα, CD25), was substantially inhibited by FasL engagement (Fig. 3). The retinoblastoma protein, an intracellular molecule implicated in cell-cycle control, is upregulated in T cells undergoing IL-2-dependent proliferation²³, and is degraded in human T cells undergoing Fas-induced death²⁴. We found that the retinoblastoma protein was present in considerably lower amounts in FasFc-treated cells (Fig. 3). Taken together, these results indicate that FasL-mediated signals arrest CD4⁺ T-cell activation and prevent cell-cycle progression.

FasL signals inhibit IL-2 production

IL-2 is produced by CD4⁺ T cells as an autocrine growth factor. We reasoned that FasL might mediate cell-cycle arrest by inhibiting the production of IL-2. We found that FasL engagement prevented IL-2 secretion (Fig. 4a), and that the addition of exogenous recombinant IL-2 to the cultures reversed the FasL-mediated antiproliferative effect (Fig. 4b). Furthermore, FasL expression, induced by CD3-stimulation, was not reduced by FasL engagement or by treatment with exogenous IL-2 (Fig. 4c). These data demonstrate that IL-2 does not act by downregulating FasL or by stimulating the outgrowth of FasL⁻ T cells. Thus, FasL engagement may prevent CD4⁺ T cells from producing sufficient IL-2 for activation and proliferation.

FasL does not inhibit proliferation of Fas-deficient cells

To rule out the possibility that immobilized FasFc was blocking Fas-induced death, and confirm that immobilized FasFc was acting directly through FasL engagement, we assessed the effect of immobilized FasFc on CD4⁺ T cells from mice with mutations of

Fas or FasL (Fig. 5). In CD4⁺ cells from *lpr* mice, which express FasL but little or no Fas (ref. 25), and those from *lpr^{cs}* mice, which express FasL and non-functional Fas (ref. 10), proliferation was inhibited in a dose-dependent manner (Fig. 5). In fact, *lpr* T cells, which express no Fas and reportedly have increased FasL expression²⁶, were reproducibly more sensitive to FasL engagement (Fig. 5). In contrast, *gld* T cells, which express nonfunctional FasL and normal Fas (ref. 11), do not respond to treatment with FasFc (Fig. 5). Thus, FasL-mediated CD4⁺ T-cell inhibition is independent of Fas expression and requires functional FasL.

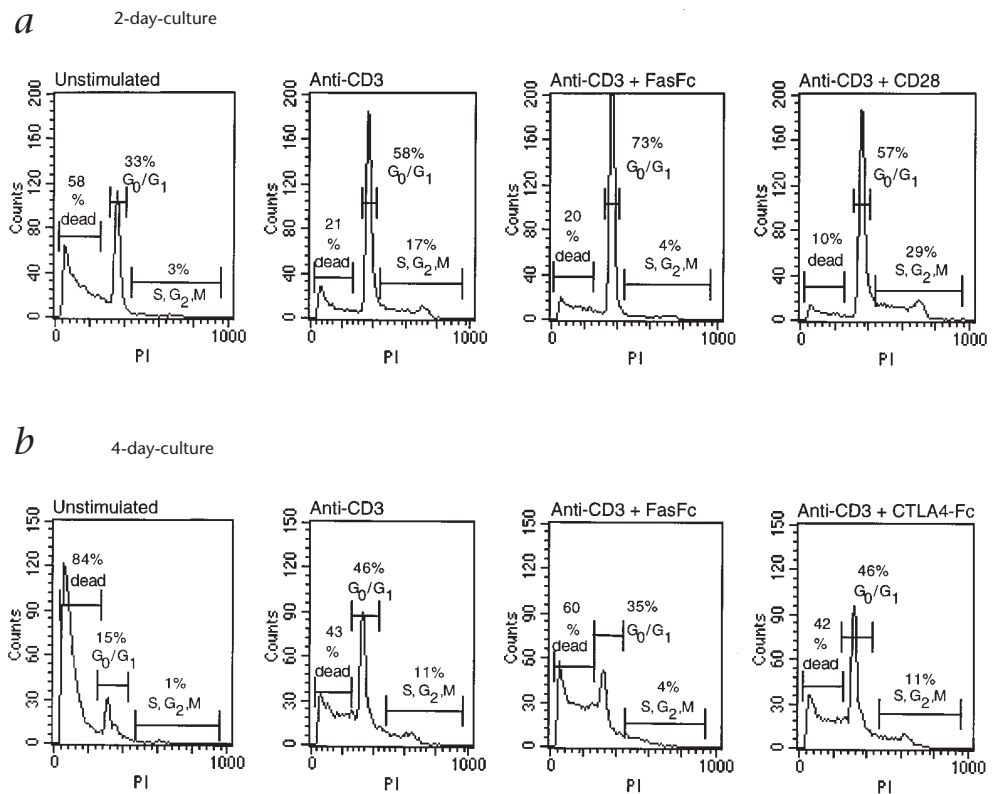
Blocking Fas/FasL interactions enhances T cell proliferation

Activated T cells express both Fas and FasL, and can therefore undergo cell-autonomous Fas-induced death^{6–8}, which can be blocked by soluble FasFc^{6–8}. Soluble FasFc should therefore also block Fas/FasL interactions leading to FasL signal transduction. We added soluble FasFc to cultures of wild-type, *lpr*, *lpr^{cs}* and *gld* cells. As expected, soluble FasFc had no effect on the proliferative responses of *lpr* and *gld* T cells (Fig. 6). However, soluble FasFc enhanced the proliferative response of wild-type T cells, which could be interpreted as a block of Fas-mediated death, a block of FasL-mediated inhibition, or both. Interestingly, however, proliferation of *lpr^{cs}* T cells was also enhanced by soluble FasFc. The *lpr^{cs}* Fas molecule is expressed normally at the cell surface, but bears a point mutation in the intracellular death domain, which prevents it from transducing a death signal. Soluble FasFc cannot affect Fas-mediated death in *lpr^{cs}* T cells, and thus must be acting through FasL. These data show that FasL engagement by endogenous Fas exerts an inhibitory influence on CD4⁺ T-cell proliferation.

FasL inhibits superantigen-driven CD4⁺ T-cell expansion *in vivo*

We determined whether FasFc-mediated FasL engagement *in vivo* also inhibited T-cell proliferative responses. We chose to analyze superantigen-driven T-cell expansion for several reasons. Superantigens, products of gram-positive bacteria, induce potent and well-characterized T-cell inflammatory responses in humans and mice²⁷. Superantigens bind to MHC class II antigens and engage specific T-cell receptor (TCR) Vβ chains, resulting in expansion of a significant percentage of the peripheral T-cell repertoire, which can be assessed with specific anti-Vβ antibodies²⁸.

Fig. 2 FasL engagement results in early cell-cycle arrest followed by apoptosis. CD4⁺ T cells were cultured in uncoated wells (Unstimulated), in wells coated with 1 μ g/ml anti-CD3, or in wells coated with 1 μ g/ml anti-CD3 + 10 μ g/ml FasFc. Control CTLA4-Fc or anti-CD28 were used at the same concentration as FasFc (10 μ g/ml). Anti-CD28 antibody was used to provide co-stimulation for comparison with this system. After 2 days (**a**) or 4 days (**b**) in culture, the cells were collected, permeabilized, and stained with propidium iodide for DNA content analysis. Gates were set to assess the percentages of dead (<2n DNA), G₀/G₁ (2n DNA), and S+G₂+M (>2n DNA) cells.



Superantigens induce a rapid proliferative expansion of reactive T cells, followed by deletion of the responding cells^{27,28}. We injected mice with an optimal dose (100 μ g, which maximizes the proliferative response) of the superantigen staphylococcal enterotoxin B (SEB), and followed the response of TCR-V β 8⁺ cells, which are SEB-reactive, and TCR-V β 6⁺ cells, which do not react to SEB and serve as an internal control for each mouse. Co-injection of FasFc, but not of control human IgG, prevented the expansion phase of the V β 8⁺CD4⁺ T-cell response in wild-type mice (Fig. 7a). The deletion phase was not prevented by FasFc, indicating that FasL engagement allows specific tolerance to develop despite effective suppression of the inflammatory response (Fig. 7a). FasFc blocked the expansion phase of the V β 8⁺CD4⁺ T cells in *lpr* mice (Fig. 7b), but not in *gld*

mice (Fig. 7c), demonstrating that FasFc acts by signaling through FasL and not by blocking Fas ligation. FasFc had no effect on CD8⁺ V β 8⁺ T-cell expansion in any of the mice (Fig. 7d-f), nor on V β 6⁺ (SEB-unreactive) T cells (Fig. 7a-f), indicating that FasL engagement selectively suppressed SEB-reactive CD4⁺ T-cell responses *in vivo*. Mice injected with SEB and control IgG appeared listless and systemically ill, whereas mice that received SEB with FasFc were normally active and well.

Discussion

Here we have demonstrated that FasL engagement results in cell-cycle arrest and cell death of CD4⁺ T cells. FasL signals can partially overcome activation signals, and prevent CD4⁺ T-cell clonal expansion *in vitro* and *in vivo*. The mechanism of FasL-induced inhibition of T-cell proliferation is, at least in part, the result of inhibited IL-2 production. Our data on *lpr*^g mice indicates that FasL engagement may tonically inhibit normal T-cell proliferative responses, perhaps in an analogous manner to CTLA-4 engagement, which also inhibits T-cell activation, IL-2 production and cell-cycle progression²⁹⁻³¹.

Implications for *in vivo* regulation of CD4⁺ T cell

We have shown here that FasL engagement *in vivo* can selectively prevent the potent CD4⁺ inflammatory response induced

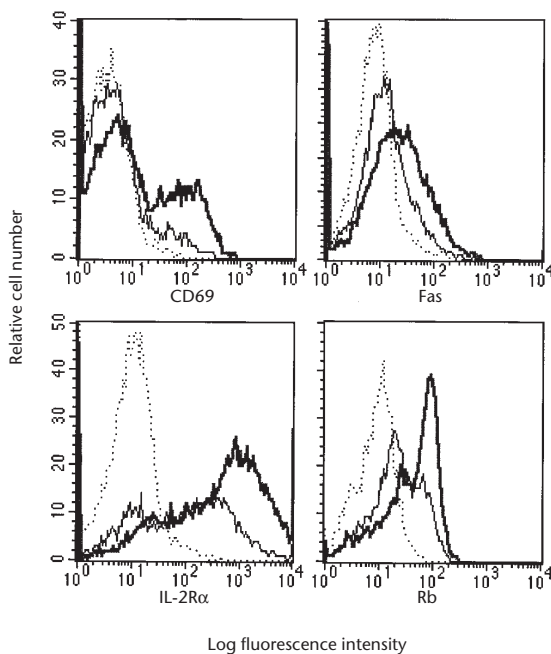


Fig. 3 FasL engagement inhibits the upregulation of activation molecules and cell-cycle regulatory proteins. CD4⁺ T cells were cultured in uncoated wells (unstimulated), in wells coated with 1 μ g/ml anti-CD3, or in wells coated with 1 μ g/ml anti-CD3 + 10 μ g/ml FasFc. After 2 days in culture, cells were collected and analyzed by flow cytometry. The histograms show the expressions of CD69, Fas, IL2R α (CD25) and the retinoblastoma protein (Rb) on unstimulated CD4⁺ T cells (dotted line) or CD4⁺ T cells treated with anti-CD3 (thick gray line) or anti-CD3 + FasFc (thin black line).

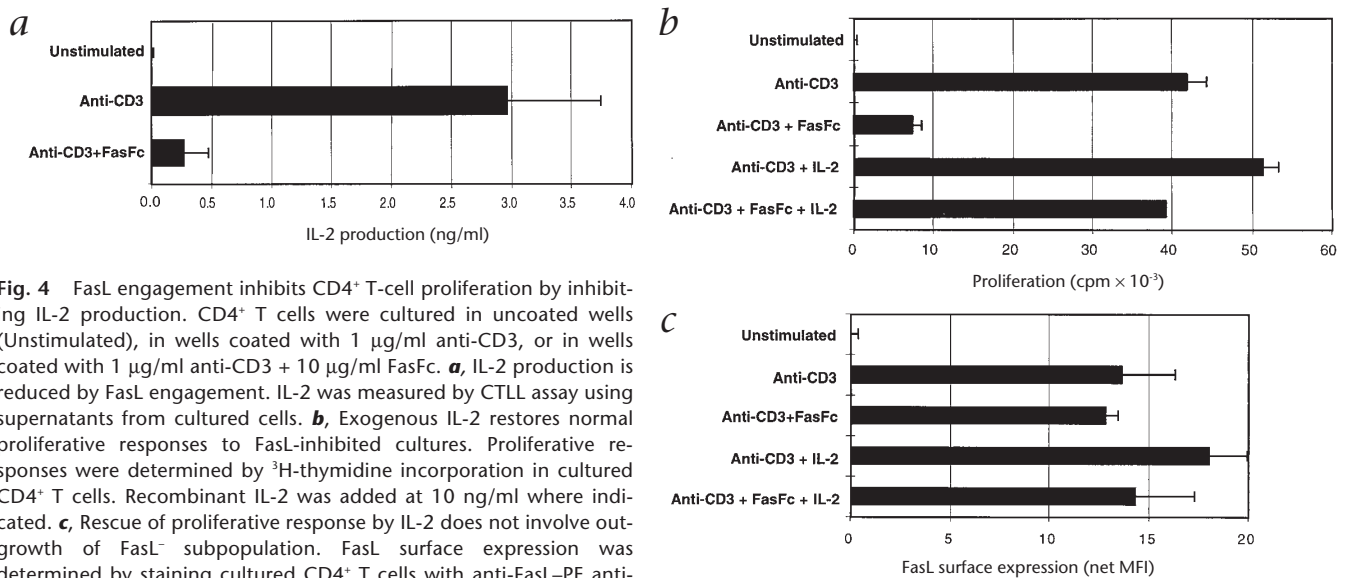


Fig. 4 FasL engagement inhibits CD4⁺ T-cell proliferation by inhibiting IL-2 production. CD4⁺ T cells were cultured in uncoated wells (Unstimulated), in wells coated with 1 μ g/ml anti-CD3, or in wells coated with 1 μ g/ml anti-CD3 + 10 μ g/ml FasFc. **a**, IL-2 production is reduced by FasL engagement. IL-2 was measured by CTLL assay using supernatants from cultured cells. **b**, Exogenous IL-2 restores normal proliferative responses to FasL-inhibited cultures. Proliferative responses were determined by ³H-thymidine incorporation in cultured CD4⁺ T cells. Recombinant IL-2 was added at 10 ng/ml where indicated. **c**, Rescue of proliferative response by IL-2 does not involve outgrowth of FasL⁻ subpopulation. FasL surface expression was determined by staining cultured CD4⁺ T cells with anti-FasL-PE antibodies. Net mean fluorescence intensity (MFI) was determined by subtracting MFI (obtained by flow cytometry) of the isotype control stained samples from the MFI of the corresponding anti-FasL-stained sample for each treatment. Error bars represent standard deviations of data from three independent experiments.

by superantigen administration, without affecting non-superantigen-reactive CD4⁺ T cells. Furthermore, CD8⁺ responses were not suppressed by FasL engagement, consistent with a report showing that FasL can co-stimulate suboptimally activated CD8⁺ cytotoxic T-lymphocyte clones *in vitro*³². Many autoimmune diseases, as well as allograft rejection, are driven by CD4⁺ T-cell proliferation^{33,34}. Thus, selective suppression of antigen reactive CD4⁺ T cells, without compromising CD8⁺ anti-viral immunity, may be important therapeutically. In addition, FasL engagement does not block the deletion phase of the CD4⁺ T-cell response to superantigens, and therefore may be allowing some mechanisms of peripheral tolerance to operate, even in the absence of a

strong proliferative response. Immunosuppressive agents widely used in clinical transplantation, such as cyclosporine, can trigger autoimmune syndromes if drug treatment is stopped, indicating that they disrupt physiological mechanisms of tolerance induction³⁵. Our data indicate that FasL engagement may prevent clonal expansion while simultaneously facilitating activation-driven clonal deletion, thus potentially tolerizing the host to the activating antigens.

Implications for tumor biology

Many tumors express FasL¹⁶⁻²⁰. Although FasL expression protects some tumors from T cell-mediated rejection, other FasL⁺Fas⁻ tumors are efficiently killed *in vivo*^{20,21}. This seemingly paradoxical observation indicates that some tumor cells, like CD4⁺ T cells, may undergo growth arrest in response to FasL engagement. Such tumors may be responsive to *in vivo* treatment with FasFc or antibodies against FasL.

Several groups have expressed FasL by transfection or transgenically in a variety of tissues, in an attempt to provide protection from T cell-mediated rejection^{12,36-40}. However, unexpectedly, most of these tissues either were not protected or underwent destruction. This occurred even when the tissue itself did not express Fas, but required Fas expression on the infiltrating cells^{38,39}, implying a Fas-dependent but not Fas-mediated death-inducing signal. This result may perhaps be explained by a destructive FasL signal, analogous to what we describe in CD4⁺ T cells, indicating that FasL signal transduction may be the rule rather than the exception.

The idea that FasL is not only a ligand for Fas but also transduces inhibitory signals to CD4⁺ T cells (and potentially to other

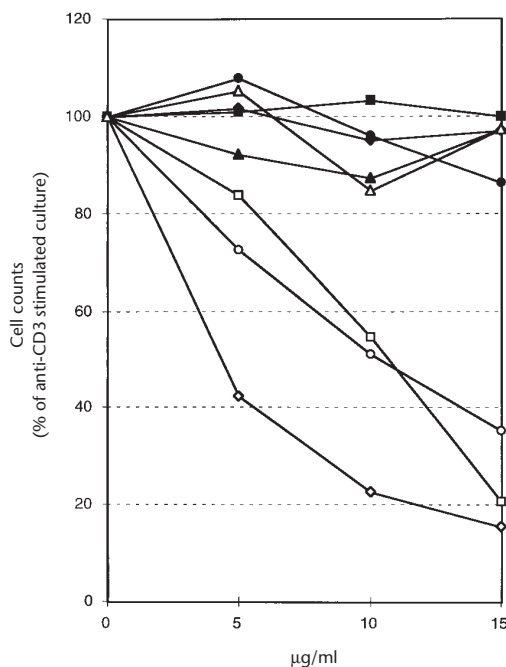


Fig. 5 FasL engagement inhibits the proliferation of wild-type, *lpr* and *lpr^g*, but not *gld*, CD4⁺ T cells. CD4⁺ T cells were cultured for 3 days in wells coated with anti-CD3 (1 μ g/ml), and with increasing concentrations (0 to 15 μ g/ml) of FasFc (open symbols) or control CTLA4-Fc (filled symbols). Cells were then collected and counted. Data were normalized as a percentage of anti-CD3 stimulated wells for each strain. Squares, B6 cells; diamonds, *lpr* cells; circles, *lpr^g* cells; triangles, *gld* cells. The net cell numbers recovered in the anti-CD3 stimulated wells (considered 100% for each strain) were: B6, 12.0×10^4 ; *lpr*, 14.5×10^4 ; *lpr^g*, 10.0×10^5 ; and *gld*, 8.9×10^4 . The data shown are representative of three independent experiments.

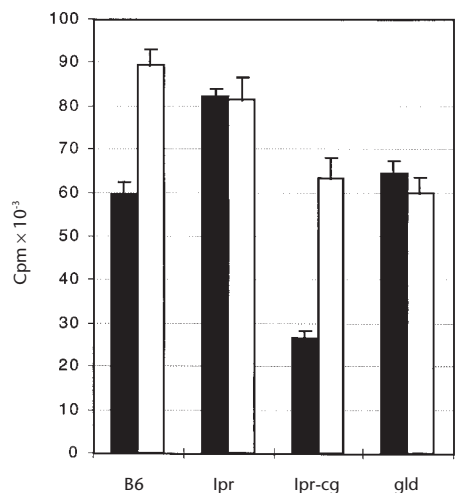


Fig. 6 Soluble FasFc blocks endogenous FasL-mediated inhibition of proliferation in *lpr^{cg}*, but not *lpr* and *gld*, cells. CD4⁺ T cells were cultured for 3 days in wells coated with anti-CD3 (1 μ g/ml), in medium containing soluble control CTLA4-Fc (10 μ g/ml; filled bars) or soluble FasFc (10 μ g/ml; open bars). Proliferation was determined by ³H-thymidine incorporation. Data represent averages and standard deviations from triplicate wells.

cell types) may contribute to our understanding of the complex regulation of T-cell immune responses. It also indicates new strategies in the treatment of autoimmune diseases, transplantation and tumors.

Methods

Mice. C57BL/6 (B6), B6.MRL-Fas^{lpr} (*lpr*) and B6Snm.C3H-Fas^{gld} (*gld*) mice 5–8 weeks old obtained from Jackson Laboratories (Bar Harbor, Maine) were used for *in vitro* studies. Female C3H/HeJ (C3H), C3H.MRL-Fas^{lpr} (*lpr*) and C3H/HeJ-Fas^{gld} (*gld*) mice 5–6 weeks old obtained from Jackson Laboratories (Bar Harbor, Maine) were used for *in vitro* studies. CBA-Fas^{lpr-cg} (*lpr^{cg}*) mice were provided by K.B. Elkon (Hospital for Special Surgery, New York, New York) and were bred and maintained in our colony in compliance with state, federal and institutional guidelines. All mice were age-matched by experiment.

Cell preparation. CD4⁺ T cells were purified from single-cell suspensions of murine spleens by negative selection on CD4 T-cell columns (Biotex,

Edmonton, Canada), using the manufacturer's directions, and supplementing the antibody cocktail with anti-B220 antibody (supernatant from clone RA36B2, diluted 1:5), to remove any abnormal CD4⁺CD8⁺B220⁺ T cells that accumulate in *lpr*, *lpr^{cg}* and *gld* lymphoid tissues. This purification procedure resulted in a population that was more than 90% CD4⁺B220⁻ T cells.

Antibodies and Ig constructs. Anti-CD3 was prepared by protein A purification of culture supernatants from hybridoma 145-2C11, and was used at 1 μ g/ml. FasFc, a chimeric, dimeric molecule consisting of Fas attached covalently to the Fc portion of human IgG, was obtained either as a gift from S.-T. Ju or was obtained from Alexis Corporation (San Diego, California), and was used at the concentrations indicated in the figure legends. Human IgG (Caltag Laboratories, Burlingame, California) and the Ig construct CTLA4-Fc (ref. 22)(provided by P. Linsley, Bristol Myers Squibb, Seattle, Washington) were used as controls for FasFc, at the same concentrations as the FasFc.

Cell culture. The wells of 96-well flat-bottomed tissue culture plates were coated with antibodies and/or Ig constructs by incubating in each well 100 μ l PBS containing 1 μ g/ml anti-CD3, with or without FasFc or control proteins as indicated, for 1 hour at 37 °C. The wells were then washed three times with PBS + 5% FCS. The freshly isolated T cells were plated into the coated wells, at 5 \times 10⁴ cells per well, in complete RPMI medium (with glutamine, β -mercaptoethanol and antibiotics) supplemented with 5% FCS. Recombinant mouse IL-2 (PharMingen, San Diego, California), was added with the cells where indicated.

Proliferation assays and cell counts. Cells were cultured for 3 days as described above, then were 'pulsed' with ³H-thymidine (1 μ Ci/well), cultured for an additional 18 hours, collected, and ³H-thymidine incorporation was assessed using a Packard scintillation counter. For cell counts, cells were collected after a 3-day culture, stained with trypan blue and counted using light microscopy.

Flow cytometry. Cell cycles were analyzed by permeabilizing and staining the cells with Bauer DNA staining solutions containing 0.1% Triton X-100 and 50 μ g/ μ l propidium iodide. Fresh cell phenotypes were determined by staining with anti-Fas-FITC, anti-IL-2R α (CD25)-FITC, anti-CD69-PE or anti-FasL-PE (clone KAY-10) antibodies (PharMingen, San Diego, California). Backgrounds were established by staining with standard isotype controls (PharMingen, San Diego, California) matched by species, fluorochrome and isotype. Cells were prepared for intracellular staining by fixation in 2% paraformaldehyde and permeabilization with 0.03% saponin, followed by staining with anti-retinoblastoma antibody (clone G3-245, PharMingen, San Diego, California), goat-anti-mouse-biotin secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania), and streptavidin-TRI-COLOR[®] (Caltag Laboratories,

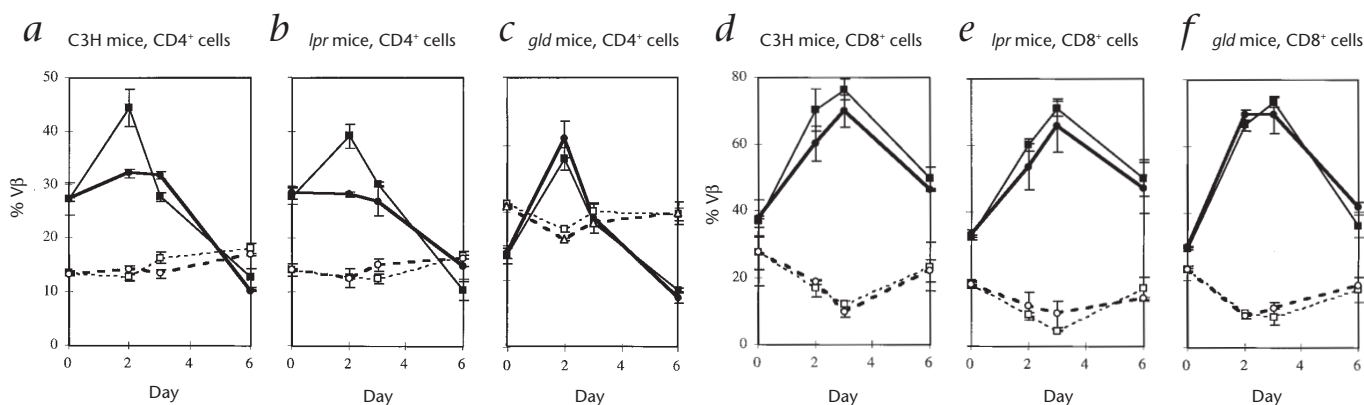


Fig. 7 FasL engagement prevents CD4⁺ T cell expansion in response to superantigens *in vivo*. Mice were injected with 100 μ g SEB plus control human IgG or FasFc on day 0. Blood samples were obtained from mice on days 0, 2, 3 and 6, and the percentages of V β 8⁺ (SEB-reactive) and V β 6⁺ (SEB-unreactive; internal control) cells in the CD4⁺ (**a-c**) and CD8⁺ (**d-f**)

subpopulations were determined by flow cytometry at each time point. (—■—), V β 8; (—●—), V β 8, FasFc-treated; (---□---), V β 6; (---○---), V β 6, FasFc-treated. V β 8⁺CD4⁺ cell expansion was prevented in wild-type (**a**) and *lpr* (**b**), but not *gld* (**c**) mice. Data points and error bars represent the means and standard deviations of two to four individual mice per group.

Burlingame, California). Data were acquired on a Coulter Elite Epics flow cytometer (Coulter, Hialeah, Florida) and analyzed with CellQuest software (Becton Dickinson, San Jose, California).

IL-2 assay. Supernatants from cells cultured as described above were collected after 2 days, serially diluted, and used to stimulate CTLL-2 (ref. 41) cells (5,000 cells per well). Plates were incubated at 37 °C for 24 hours, then 'pulsed' with 20 µl Alamar Blue per well, incubated overnight, and 'read' by spectrophotometer at 570 and 590 nm. IL-2 concentrations for each well were determined by the preparation of a standard curve with recombinant mouse IL-2 (PharMingen, San Diego, California).

In vivo studies. Mice were injected intraperitoneally with 100 µg SEB mixed with 500 µg human IgG (Sigma) or 500 µg FasFc, a gift from C. Dinarello (University of Colorado Health Sciences Center, Denver, Colorado). The day of injection was called day 0. Peripheral blood (50 µl) was collected from the tail vein on days 0 (before SEB injection), 2, 3 and 6. Red blood cells were depleted from the samples using two successive treatments with standard Geys' solution. Peripheral blood leukocytes were then stained with anti-CD4-PE, anti-CD8-TC, and either anti-Vβ8-FITC or anti-Vβ6-FITC (PharMingen, San Diego, California). Data were acquired by flow cytometry and analyzed by gating on CD4⁺ or CD8⁺ T-cell subsets to determine the percentages of Vβ8⁺ or Vβ6⁺ cells within each subset at each time point. Data were obtained for individual mice, and two to four mice per treatment group and per strain were bled for each experiment at each time point.

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