

## Fas ligand-dependent suppression of autoimmunity via recruitment and subsequent termination of activated T cells

Jaime F. Modiano,<sup>a,b,c,1</sup> Juan Sun,<sup>b,d,1</sup> Julie Lang,<sup>a</sup> Guido Vacano,<sup>e</sup> David Patterson,<sup>e</sup> Daniel Chan,<sup>c,f</sup> Alex Franzusoff,<sup>c,d,g</sup> Roberto Gianani,<sup>h</sup> Sandra J. Meech,<sup>d</sup> Richard Duke,<sup>a,c,d</sup> and Donald Bellgrau<sup>a,c,d,\*</sup>

<sup>a</sup>Integrated Department of Immunology, University of Colorado Health Sciences Center, Denver, CO 80262, USA

<sup>b</sup>AMC Cancer Research Center, Denver, CO 80214, USA

<sup>c</sup>University of Colorado Cancer Center, Denver, CO 80010, USA

<sup>d</sup>GlobeImmune, Inc., Denver, CO 80010, USA

<sup>e</sup>Eleanor Roosevelt Institute, Denver, CO 80206, USA

<sup>f</sup>Division of Medical Oncology, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262, USA

<sup>g</sup>Department of Cellular and Structural Biology, University of Colorado Health Sciences Center, Denver, CO 80262, USA

<sup>h</sup>Department of Pathology, University of Colorado Health Sciences Center, Denver, CO 80262, USA

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### Abstract

Signals transmitted by binding of Fas ligand (FasL) to the Fas receptor (CD95/Apo-1) have pleiotropic effects on cellular function that present opportunities for therapeutic applications. For example, depending on the circumstances, overexpression of FasL can enhance, prevent, or reverse growth of spontaneous or transplantable tumors. Furthermore, local administration of FasL into a single paw in susceptible mice protects from or reduces the severity of collagen-induced arthritis (CIA) in all paws. Here, we define mechanisms that mediate systemic protection induced by locally delivered FasL. Protection is not solely dependent on local interactions between Fas and FasL, but rather requires induction of a paradoxical inflammatory response that not only destroys Fas-resistant tumors, but also recruits motile, activated, Fas-bearing T cells that are Fas sensitive. We demonstrate by following the antigen-specific recruitment and subsequent termination of transgenic T cells that activated T cells, including autoreactive cells responsible for CIA, are eliminated within this inflammatory environment through the overexpressed FasL. The nature of the inflammatory response, which depends on the Fas ligand being cell bound and not soluble, and the magnitude of FasL expression within the inflammatory milieu are essential for this effect, as arthritogenic inflammation alone resulting from CIA induction is insufficient to ameliorate the disease or eliminate antigen-specific T cells, even upon systemic delivery of soluble FasL. These data show that gene delivery of membrane-bound FasL can effectively recruit and eliminate autoreactive T cells.

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**Keywords:** Arthritis; Fas ligand; Inflammation; Activation-induced cell death

### Introduction

Lpr and gld mice have lymphoproliferative diseases and autoimmunity mediated by dysfunctional Fas (CD95) receptor–ligand interactions [1,2]. The lpr–gld models

have been widely interpreted to indicate that Fas receptor–ligand interactions are important in the control of clonal expansion of activated lymphocytes. This has led to numerous explorations of the role of Fas and its ligand (Fas ligand or FasL) in the control or generation of autoimmunity.

Murine collagen-induced arthritis (CIA) is one example. Murine CIA resembles human rheumatoid arthritis [3,4] and continues to be a useful model to determine the preclinical efficacy of potential therapies. The synovitis and erosion of cartilage are caused by an immune-

\* Corresponding author. Integrated Department of Immunology, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Box B188, Denver, CO 80262. Fax: +1-303-315-3304.

E-mail address: [Don.Bellgrau@UCHSC.edu](mailto:Don.Bellgrau@UCHSC.edu) (D. Bellgrau).

<sup>1</sup> These authors contributed equally to the paper.

mediated inflammatory response dominated by activated T lymphocytes that includes both cellular and humoral components. CIA can be ameliorated by direct injection of a recombinant, replication-defective adenovirus carrying the FasL gene into the inflamed joint [5]. The observation that treatment of the hind feet afforded protection for the untreated front feet was particularly intriguing and suggested that local administration of FasL had systemic effects. However, the mechanisms that mediated the protective systemic effect of local FasL administration were not formally established.

For this study, we sought to define the mechanisms responsible for systemic protection from CIA following local administration of FasL. To explore these mechanisms, we took advantage of previous observations that document FasL can delay tumor growth or promote tumor regression [6–10] to confirm that this effect requires induction of a robust inflammatory response that can be achieved only by the membrane-bound form of FasL. We then explored the relationship between this inflammation and the therapeutic effect on CIA. Consistent with previous reports [5], our results show that FasL not only ameliorates CIA, but also prevents the disease from developing altogether. Here we show that the mechanisms of this FasL-dependent protection involve induction of a somewhat paradoxical inflammatory response that “baits” motile, Fas-bearing activated T cells (including autoreactive cells) that are then “trapped” and destroyed within this inflammatory milieu by cells that overexpress FasL.

## Materials and methods

### Animals

Male DBA/1 *lacJ* (H-2<sup>d</sup>) mice aged 7–8 weeks and female C57BL/6 (B6, H-2<sup>b</sup>) mice aged 6–8 weeks were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free animal satellite facility of the University of Colorado Health Sciences Center. All protocols and procedures involving live animals were reviewed and approved by the UCHSC IACUC.

### Cells and cell lines

G10 (FL+) and F10 (FL–) are derivatives of the human K562 chronic myelogenous leukemia cell line that are stably transfected with plasmids encoding human Fas ligand (FasL) and neomycin phosphotransferase, respectively [11]. L1210-Fas is a mouse lymphocytic leukemia cell line that expresses high levels of Fas receptor (CD95) and is exquisitely sensitive to FasL-dependent apoptosis. Lewis lung (LL) is a pulmonary carcinoma cell line from B6 mice [12] that expresses Fas but is resistant to FasL-mediated apoptosis. Primary mouse spleen cells and

lymph node cells were prepared as described [13]. Cells were grown in RPMI 1640 media (Gibco Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 10  $\mu$ M HEPES, 50  $\mu$ M 2-ME, and 10  $\mu$ g gentamicin in an atmosphere of 5% CO<sub>2</sub> at 37°C.

### Generation of recombinant adenoviruses

Viruses were prepared from mammalian expression plasmids encoding mutant forms of human FasL. The following forms of FasL were generated by RT-PCR: intracellular truncated (ict)—lacking amino acids 8–69 encompassing the proline-rich signaling domain; soluble (sol)—lacking intracellular and transmembrane regions from amino acids 1 to 133. These forms were compared with wild-type (wt) FasL. FasL and enhanced green fluorescent protein (EGFP) were inserted into the left end of the Ad5 replacing the E1 region and driven by the human ubiquitin promoter in all constructs (thus the designations Ad.u.G.hFL-wt, Ad.u.G.hFL-ict, Ad.u.G.hFL-sol, and Ad.u.EGFP). For simplicity, we have further abbreviated these designations in the text as FasL-wt, FasL-ict, FasL-sol, and Ad-GFP. The recombinant viruses were generated by using the AdEasy System, confirmed by PCR, and amplified in FasL-resistant 293-crmA cells as described [5,14].

### Cytotoxicity assays

As one measure to ensure the biological activity of FasL in the various adenovirus constructs, cytotoxicity assays were performed *in vitro* comparing stably transfected tumors with cells infected with recombinant adenoviruses as described previously [14]. LL cells were infected with various forms of adenoviral-delivered FasL and used as effectors in cytotoxicity assays, L1210-Fas cells were used as targets, and FL+ and FL– K562 cells were used as positive and negative controls, respectively. Results are presented as the mean  $\pm$  SD for triplicate samples.

### Flow cytometry

To assess expression of Fas and FasL on LL cells, 4  $\times$  10<sup>6</sup> cells were suspended in 400  $\mu$ l PBS buffer and stained with a biotinylated anti-Fas antibody (Jo2 hamster IgG, BD/PharMingen, San Diego, CA) and a PE-conjugated anti-FasL antibody (Nok-1, PharMingen). Analyses were performed using FACSCAN. Determination of the extent of antigen-specific recruitment involved the use of antibodies that distinguish Thy 1.1+/CD8+ T cells of the B6.PL recipients from Thy 1.2+/CD8+ transgenic donor OT-1 T cells. The antibodies used to the adoptively transferred cells from the recipient cells included anti-Thy1.2 (53-2.1) conjugated to phycoerythrin (PE), anti-CD8 (53-6.7) conjugat-

ed to biotin or fluorescein isothiocyanate (FITC), anti-V $\beta$ 5 (MR9-4, specific for OT-1) conjugated to biotin, and anti-CD90.1 (OX-7) conjugated to biotin. Antibodies used to measure activation of adoptively transferred cells included anti-CD69 (H1.2F3) conjugated to FITC, anti-CD44 (IM7) conjugated to PE, and anti-CD62L (MEL-14) conjugated to biotin. Streptavidin-allophycocyanin (SA-APC) was used for the detection of biotinylated antibodies. Gates were set to include only Thy1.2+/CD8+ T cells to determine the percentage of “activated” cells. At least 50,000 cells per sample were collected for analysis.

#### *Tumor induction*

For orthotopic tumor growth,  $5 \times 10^5$  unmodified LL cells or  $5 \times 10^5$  LL cells infected with Ad-FasL constructs were injected intravenously (tail vein) to B6 mice. Mice were humanely sacrificed after 19 days, and the tumor burden in the lungs was determined at necropsy. For subcutaneous tumor growth, injected  $5 \times 10^5$  unmodified LL cells or  $5 \times 10^5$  LL cells infected with Ad-FasL constructs were injected under the skin on the right and left flanks of DBA/1 mice. Mice that showed tumor growth were humanely sacrificed after 22 days; the tumor burden was determined at necropsy. Six mice per group were used for each experiment.

#### *Induction of CIA*

CIA induction was performed as described [3–5]. Briefly, DBA/1 mice were anesthetized with 125–250 mg/kg of Avertin and then immunized in the base of the tail with 100  $\mu$ g bovine type II collagen emulsified in complete Freund’s adjuvant (CFA). After 21 days, the mice were boosted with the same amount of collagen in incomplete adjuvant (ICFA). The mice were inspected once a week for approximately 6 weeks for the development of arthritis.

#### *Clinical assessment of arthritis*

Starting 3 days after the second immunization, mice were examined for development of arthritis using two measurement systems. The first was paw swelling defined by footpad volume with a water replacement system [15]. The entire paw was placed in water and the displacement measured. The second approach was the use of the widely used clinical arthritis index [4,16]. In this setting, arthritis was assessed using an established macroscopic scoring system ranging from 0 to 4: 0, normal; 1, detectable arthritis with erythema; 2, significant swelling and redness; 3, severe swelling and redness from joint to digit; and 4, maximal swelling with ankylosis. The macroscopic score was expressed as mean  $\pm$  SD. We performed three independent, blinded measurements to compare paw volume and arthritis index measurement systems for the assessment of arthritis.

The water displacement approach was as sensitive and consistently more objective than the arthritis index, and therefore all the data used for efficacy analysis of FasL suppression of arthritis are presented using the paw swelling measuring system.

#### *Treatment of the CIA with adenoviruses*

Adenovirus treatment was performed 1 and 3 weeks, respectively, after the second immunization with ICFA using  $0.5 \times 10^{10}$  particles of recombinant, replication-defective adenovirus in 20  $\mu$ l of PBS, or PBS alone injected periarticularly to the tarsal joint of the left rear foot.

#### *Adoptive transfer of transgenic T cells to assess antigen-specific recruitment and its regulation*

T cells ( $1-2 \times 10^7$ ) purified from the lymph node of ovalbumin-specific T cell receptor transgenic OT-1 mice on B6 background (Thy 1.2) were injected intravenously into MHC-matched B6PL (Thy 1.1) congenic recipients. Twenty-four hours later, the recipients were immunized at the base of the tail with  $2 \times 10^7$  yeast cells expressing ovalbumin (OVAX) [17]. After an additional 2–5 days, draining inguinal lymph nodes (DLN), cervical, axillary, and mesenteric lymph nodes (LN), and spleens were isolated and single cell suspensions were made to determine the frequency of CD8+/Thy1.2+ vs. CD8+/Thy1.1+ lymphocytes. Once a reproducibly increased frequency of OT-1 (Thy1.2+CD8+) T cells was documented (>3.0% vs. <0.5% at day 5), the experiments were repeated with FasL-producing adenoviruses injected periarticularly to the tarsal joint of one rear foot at day 2 after OVAX administration, that is, 3 days after the start of the experiment. The inguinal lymph nodes or lymphocytes from other sources were again isolated and examined at days 4, 5, and 6 post-OVAX administration to determine the frequency of OVA antigen-specific transgenic T cells. Controls for the specificity of antigen-specific recruitment of ovalbumin-specific OT-1 T cells by antigen-expressing yeast included animals challenged with yeast lacking any additional gene insert or expressing the HIV *gag* antigen instead of ovalbumin.

#### *Soluble FasL protein*

To ensure soluble FasL was synthesized appropriately, H1334 (Fas-negative human non-small cell lung cancer) cells were infected with 300 pfu of FasL-sol. The supernatants from infected cells were collected 3 days later and used to perform a sandwich ELISA by using a kit from MBL (Naka-Ku, Nagoya, Japan) to measure soluble human FasL protein as per the manufacturer’s instructions. Soluble FasL protein was determined to be 11.5 ng/ml in the supernatant of H1334 cell cultures infected with 300 pfu

of Ad-FasL-sol. Soluble FasL production on other versions of FasL was under the 0.1 ng/ml sensitivity of the assay.

### *Histopathologic examination*

Skin biopsy samples were collected from adenovirus injection sites, fixed in O.C.T. Tissue-TEK medium, and stored at  $-80^{\circ}\text{C}$ . Tumor samples were fixed in 10% neutral-buffered formalin. Tissues were sectioned in 5- $\mu\text{m}$  slices and stained with hematoxylin and eosin for microscopic examination.

### *Statistical analyses*

Results were compared using Student's *t* test and by analysis of variance.  $P < 0.05$  was considered statistically significant.

## **Results**

### *Fas ligand activity in recombinant adenoviruses*

The biological effects of FasL may be mediated through its pro-apoptotic effects or its ability to initiate inflammatory responses upon binding Fas receptors on target cells [7,8,18,19], or alternatively, through FasL-mediated signals in effector cells [20]. We initially examined the capacity of three forms of FasL (wild-type, soluble, and signaling-deficient intracellular truncated) to promote apoptosis and inflammation *in vitro* and *in vivo*. Like the murine EL-4 thymoma line used by us in a previous study [14], Lewis Lung carcinoma (LL) cells [12,21] are resistant to Fas-dependent apoptosis (J. Sun and D. Bellgrau, unpublished observation). Therefore, they can be infected with, but not killed, by FasL-expressing adenovirus, providing a suitable system to explore the activity of adenoviral-delivered, recombinant FasL constructs. LL cells were infected with adenovirus (200 pfu) encoding GFP alone (Ad-GFP), or with adenoviruses encoding wild-type FasL (FasL-wt), soluble FasL (FasL-sol), or intracellular-truncated FasL (FasL-ict) for 72 h, and used to determine cytotoxicity against L1210-Fas target cells. Fig. 1a shows that control FasL-bearing effector cells (FL+) killed L1210-Fas cells, but the same targets were resistant to control FasL-negative effector cells (FL-), demonstrating the FasL dependence of the assay. LL cells infected with Ad-GFP showed no cytotoxic activity against L1210-Fas target cells, whereas LL cells infected with adenoviruses encoding each of the three forms of FasL killed the target cells as or more efficiently than the FL+ controls. Therefore, all three FasL-infected tumor lines lysed Fas-bearing target cells *in vitro* (Table 1).

Next, we examined the pro-inflammatory potential of these FasL constructs *in vivo*. Adenovirus constructs were delivered to mice by subcutaneous injection in the flank.

Injection of vehicle or Ad-GFP produced minimal inflammatory changes. Similarly, injection of Ad-FasL-sol promoted only mild inflammatory responses. Thus, the soluble version of FasL failed to induce an inflammatory response *in vivo* even though it could mediate cell-mediated cytotoxicity *in vitro*. In contrast, injection of either Ad-FasL-wt or Ad-FasL-ict led to the appearance of inflammatory infiltrates that were initially dominated by neutrophils at the site of injection and eventually led to extensive necrosis (data not shown). This FasL-dependent inflammatory response is reported to mediate therapeutic effects of FasL such as tumor rejection [7–10,22]. To verify this, we examined how the FasL constructs affected establishment and growth of LL tumors. LL cells were injected into the tail vein or subcutaneously in the right flank of C57BL/6 mice (the syngeneic strain) or DBA/1 mice (the strain used to examine the therapeutic effects of FasL expression in CIA). The results in both strains of mice were indistinguishable. Tumors appeared to grow at the same rate, and tumor rejection following Fas ligand administration appeared to follow similar kinetics in both strains, indicating that the slow tumor growth in DBA/1 mice were not due to allogeneic rejection. All mice inoculated with unmodified LL cells intravenously developed multiple nodular lung tumors and had to be humanely sacrificed after 19 days (Fig. 1b). Similarly, all mice inoculated with unmodified LL cells subcutaneously developed single, large, rapidly growing tumor masses and had to be humanely sacrificed after 22 days (Fig. 1c). Transduction of LL cells with Ad-GFP had no protective effects on tumor growth in the lungs or under the skin. Tumor growth was similarly not affected when LL cells were transduced with Ad-FasL-sol. In contrast, LL cells transduced with Ad-FasL-wt (Figs. 1b–c) or Ad-FasL-ict (not shown) failed to form tumors at either site. This inhibition of tumor growth was not due to the reduced viability of the transduced LL cells per se, as tumor cells were identifiable microscopically in sections obtained from the site of tumor inoculation (Fig. 1d). However, a robust suppurative inflammatory infiltrate was associated with the tumor cells, which exhibited frequent chromatin condensation and margination (Fig. 1d), suggesting that the failure of tumors to grow was secondary to tumor cell killing by inflammatory cells (Table 1).

### *Suppression of arthritis development by FasL-wt treatment*

Having confirmed a biologic activity for the various Fas ligand constructs, we explored their therapeutic effect in the CIA model. For this purpose, we compared the disease course in mice induced with collagen and adjuvant (collagen plus CFA at day 0 followed by collagen and ICFA at day 21) with similarly induced animals that were treated with the FasL constructs either once (7 days after ICFA) or twice (7 and 21 days after ICFA). Fig. 2 shows that paw volume in mice that did not receive the arthritogenic treatment was unchanged through the obser-

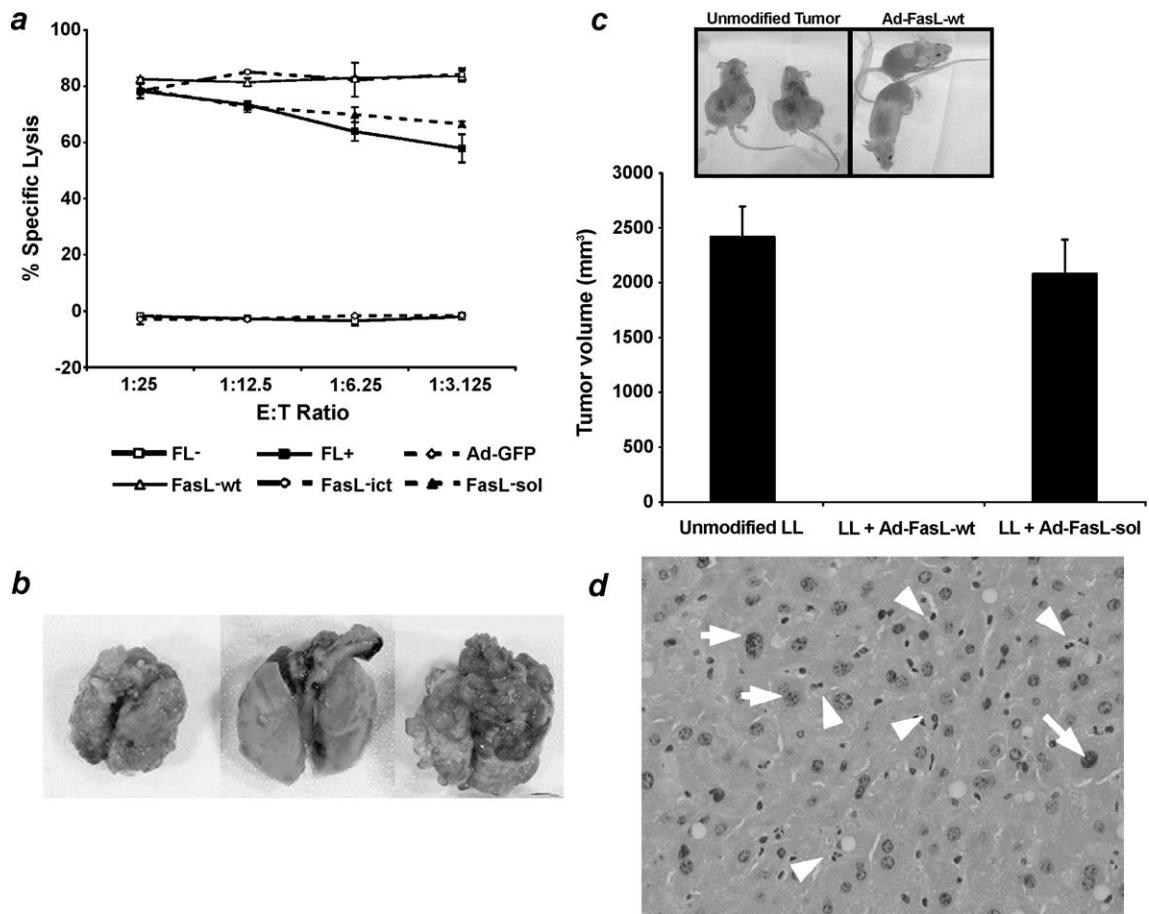


Fig. 1. Differential effects on arthritis reduction and protection from tumors of membrane-bound and soluble forms of FasL. (a) Cytotoxicity of LL cells infected with 300 pfu of adenoviruses encoding with wild-type (wt), intracellular truncated (ict), or soluble (sol) forms of FasL was examined using L1210-Fas cells as target cells in a chromium release assay. FL+ and FL- are derivatives of the human K562 cell line engineered to overexpress FasL or neomycin phosphotransferase, respectively. Effector to target (E/T) ratios were 25:1, 12.5:1, 6.25:1, and 3.125:1. Each value represents the mean  $\pm$  SD of triplicate samples. Spontaneous  $^{51}\text{Cr}$  release was 24% for L1210-Fas. (b) Representative heart–lung mounts from C57BL/6 mice 14 days after intravenously inoculation with unmodified LL cells (left) or LL cells transduced with Ad-FasL-wt (middle) or Ad-FasL-sol (right). (c) Tumor volume ( $\text{mm}^3 \pm \text{SD}$ ) from groups of six DBA/1 mice 21 days after subcutaneously injection into the flank with  $5 \times 10^5$  unmodified LL cells, LL cells transduced with Ad-FasL-wt, or Ad-FasL-sol. Insets show the appearance of mice that received unmodified LL cells or cells transduced with Ad-FasL-wt immediately before sacrifice. (d) Photomicrograph of a representative section of the remaining tumor excised from a mouse receiving Ad-FasL-wt (panel c). Note the neutrophils infiltrating the mass (arrowheads) and evidence of chromatin margination (an early indicator of cell death) in tumor cells (arrows). Staining H&E, magnification =  $\times 200$ .

vation period. In contrast, in the groups that received the arthritogenic stimulus, arthritis was characterized by swelling of all four paws, even though the induction site where collagen and adjuvant were administered was at the base of the tail (Fig. 2a). Periarticular administration of Ad-FasL-wt in a single paw 1 week after arthritis initiation reproducibly slowed the progression of the disease (Fig. 2a), and when Ad-FasL was administered 1 week and again 3 weeks after arthritis initiation, arthritis was reversed to levels comparable to the uninduced controls (Fig. 2a). Administration of the Ad-FasL-ict construct was as effective as administration of the Ad-FasL-wt construct (Fig. 2b). However, treatment of mice with Ad-FasL-sol was ineffective to reverse or delay the progression of arthritis, suggesting that only membrane-bound forms of FasL, and possibly the inflammatory response associated with them, have therapeutic applications in the treatment

of arthritis. Therefore, while soluble FasL could mediate cell-mediated cytotoxicity in vitro, it failed to suppress experimentally induced autoimmunity, and this failure correlated with a failure to produce an inflammatory response (Table 1).

#### *Mechanisms of FasL-mediated amelioration of arthritis*

CIA is characterized by synovitis and erosion of cartilage caused by an immune-mediated inflammatory response involving activated T lymphocytes. It was shown previously that periarticular treatment with FasL could reverse or delay arthritis in this model [5], but the mechanisms responsible for this effect were not identified. The observation that the effect was systemic (i.e., FasL afforded therapeutic benefit even to paws not treated with FasL) suggested that the FasL might be distributed systemically, or that the activated,

Table 1  
Pleiotropic effects and outcomes of treatment with adenoviruses expressing FasL

Model	Experimental procedure	Effect	Interpretation
In vitro cytotoxicity	Use of tumor cells overexpressing FasL as effectors in chromium release killing assays	Death of Fas+ L1210 target cells	Apoptosis induced by interaction of membrane-bound or soluble FasL with Fas on the cell surface of target cells
Transplantable Lewis lung carcinoma (resistant to Fas-dependent apoptosis)	Enforced expression of FasL in tumor cells by adenovirus transduction	Tumor growth or tumor regression	Tumor inhibition correlates with membrane-bound (but not soluble) FasL
Inflammation defined by pathology	Enforced expression of FasL in tumor cells by adenovirus transduction	Substantial neutrophilic infiltrate with all but soluble version	Correlation between infiltrate and tumor regression
Collagen-induced arthritis (CIA)	Local delivery of adenovirus FasL to a single paw	Protection or reversal of CIA in all paws	Systemic protection when membrane-bound (but not soluble) FasL is expressed
Systemic antigen-specific recruitment of activated T lymphocytes	Effect of local delivery of adenovirus FasL on antigen-specific recruitment	Ablation of antigen-specific T cells in peripheral lymphoid organs	Antigen-specific recruitment to and destruction of motile, activated T cells to site where membrane-bound (but not soluble) FasL is expressed

arthritogenic T cells might be recruited to the site of FasL delivery and eliminated. To address the possibility that FasL was distributed systemically, we took advantage of the GFP tag encoded by the adenovirus constructs. Visualization of GFP following injection into the rear paw 2 days earlier showed that the majority of the GFP remained localized at the site of injection (Fig. 3, arrow), but some GFP could also be observed radiating medially from the paw and anteriorly along the flank of the animal (Fig. 3, arrowheads). A similar distribution of GFP was observed when adenoviruses encoding FasL were injected. Although these observations could not distinguish between distribution of the intact virus through hematogenous or lymphatic routes from distribution of recombinant protein released from cells infected at the site of administration that subsequently died, they nevertheless indicate that some of the adenoviral-delivered FasL can reach distant sites and possibly contribute to the amelioration of disease.

Given the small levels of GFP detectable distant to the site of administration and the magnitude of the therapeutic effect, we surmised that another mechanism must be operative. We hypothesized that activated Fas-bearing T cells could be recruited to the site of inflammation initiated by membrane-bound FasL distal to the site of antigen delivery, where these cells might be efficiently terminated by the same FasL. To test the hypothesis, we used the OT-1 model to explore the antigen-specific recruitment of lymphocytes in a process that involves lymph node swelling associated with trapping of antigen-specific T lymphocytes. This approach was designed to mimic CIA in that the antigen was provided at a distal site (the base of the tail) while the FasL was delivered into a rear paw. We have shown

previously that OVAX, a vaccine that consists of *Saccharomyces cerevisiae* engineered to express chicken ovalbumin (OVA), induces MHC Class I-dependent, T cell-mediated destruction of OVA-expressing tumors in vivo [17]. The mechanism involves uptake of yeast by dendritic cells with subsequent presentation of yeast antigens, including OVA, to MHC Class I-restricted T cells. We took advantage of this system to recruit MHC Class I-restricted T cells in an antigen-specific manner. OVAX was used as the “bait” for OT-1 transgenic T cells that are specific for OVA presented in the context of MHC Class I. To monitor OT-1 T cells, they were adoptively transferred to congenic B6 mice that differed from OT-1 in the expression of Thy1. The results show that the frequency of CD8+/Thy1.2+ OT-1 cells in the draining lymph nodes of adoptively transferred mice that were mock vaccinated was less than 0.3%, and even with vaccination with soluble ovalbumin they were less than <0.5% (Fig. 4, top). This confirms that soluble ovalbumin is not presented via the Class I pathway and has no significant impact on the recruitment of MHC Class I-restricted, OVA-specific OT-1 T cells in this setting. Vaccination with OVAX routinely led to a 10-fold increase in the frequency of CD8+/Thy1.2+ OT-1 T cells in draining nodes (Fig. 4, gate R8 top vs. bottom), supporting previous observations that yeast vaccines promote antigen presentation via the Class I pathway [17]. Predictably, the frequency of OT-1 T cells did not increase when unmodified yeast or yeast engineered to express an antigen other than OVA was used for vaccination (data not shown). The OT-1 T cells in the draining lymph nodes expressed CD69 (reproducibly 30% vs. less than 5% without immunization; Fig. 5, left panel) and downregulated the CD62 ligand on CD44+ T

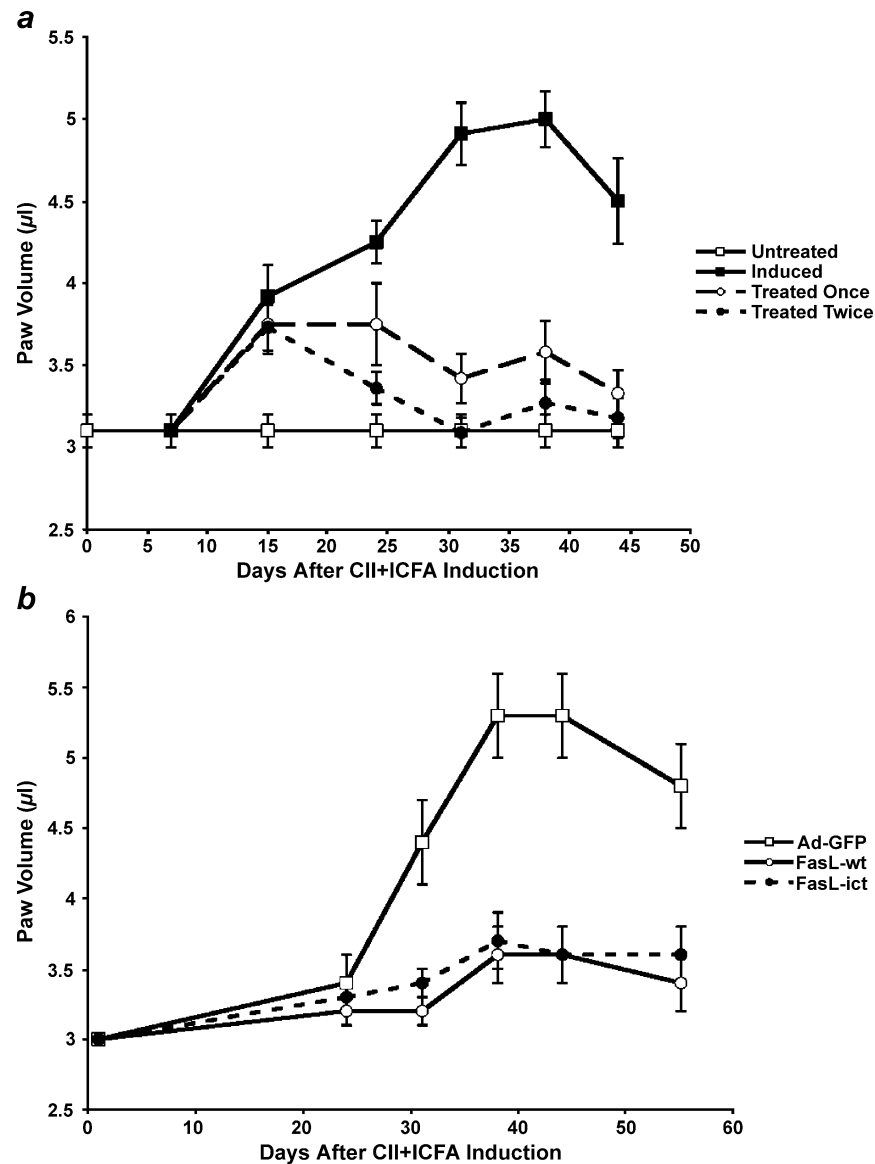


Fig. 2. Effect of wild-type FasL gene transfer on collagen-induced arthritis. (a) Four groups of 12 DBA/1 mice were used to examine the effect of FasL on collagen-induced arthritis. One group received sham treatments (saline injections). The remaining three groups were induced to develop arthritis with collagen + CFA followed by collagen with ICFA injected on the base of the tail as described in Materials and methods. One of these groups received sham injections for the adenovirus treatments. One group was injected with  $0.5 \times 10^{10}$  particles of Ad-FasL-wt periarticularly in the left tarsal joint 1 week after the ICFA treatment, and the last group was injected with  $0.5 \times 10^{10}$  particles of Ad-FasL-wt periarticularly in the left tarsal joint 1 and 3 weeks after ICFA treatment. Mice were examined for signs of inflammation and paw volume was measured using a water displacement system as described. (b) Three groups of 14 mice DBA/1 mice were induced to develop arthritis. One and 3 weeks after the second immunization,  $0.5 \times 10^{10}$  particles of Ad-FL-GFP, Ad-FL-wt, or Ad-FasL-ict were injected periarticularly to the left tarsal joint. Mice were examined for signs of inflammation and paw volume was measured as described.

cells (Fig. 5, right panel; reproducibly >50% vs. less than 15% without immunization), indicating they were indeed activated. After 4 days, reproducible antigen-specific accumulation of OT-1+ T cells could also be detectable in the spleen although the frequency was not as high as in the draining lymph nodes (routinely 10–20% of that observed in the draining node; Fig. 6, left panel). An observed decrease in frequency of OT-1+ T cells from days 4 to 6 in the spleen was consistent with the interpretation that some of these splenic T cells appeared to be migrating to other peripheral sites after activation.

These data validated the potential use of the model to explore recruitment and termination of activated, antigen-specific T cells by FasL delivered at a distant site. For this purpose, OT-1 T cells were adoptively transferred to B6 mice on day 0 of the experiment as described above, and mice were vaccinated with OVAX at the base of the tail (the same site used for arthritis induction) 1 day later. On day 2, mice received Ad-FasL-wt in the rear paw (the same site used for arthritis treatment) at a dose that showed anti-arthritis effects. The frequency of OT-1 cells was then monitored in draining lymph nodes, other peripheral lymph

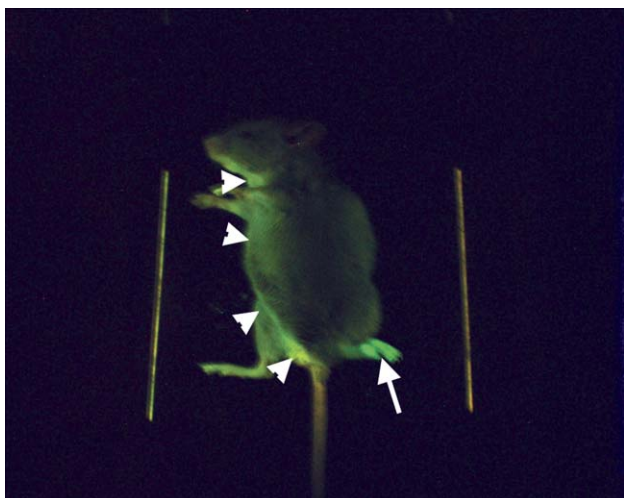


Fig. 3. Dispersal of recombinant adenovirus beyond the local site of injection. Ad-GFP was injected into the footpad of three mice and the animals were monitored for the presence of GFP distal to the injection site (arrow). GFP in this representative animal can be visualized as it spreads proximally through the leg and up through the flank (arrowheads) 2 days after administration.

nodes, and spleen. Mice that were adoptively transferred with OT-1 T cells, but were not vaccinated with OVAX, and mice that received OT-1 T cells and OVAX, but no Ad-FasL-wt, treated in an identical fashion as those shown in Fig. 4, were used as controls. While the effects of FasL treatment on the frequency of OT-1+ T cells in the draining node could be observed between days 4 and 6, the effect of Ad-FasL-wt to completely terminate OT-1 T cells was most thoroughly and reproducibly observed after 6 days in the spleen. At this time, there was profound reduction in the frequency of OT-1+ T cells in animals treated with FasL; specifically, 0.5–0.8% OT-1 cells remained on day 4, and these were reduced to background levels (<0.1%) by day 6. In the draining lymph node, these numbers were 2.5–4.9% vs. 0.1–0.2%. In contrast, OT-1 T cells in animals that did not receive FasL or those treated with the control Ad-GFP never decreased by more than 4-fold from day 4 to 6 (0.41–0.11% in Fig. 6 top vs. 0.79–0.02% bottom), indicating the observed reduction in OT-1 T cells was not simply due to mobilization of activated cells out of the splenic lymphoid compartment (Table 1). However, the frequency of activated T cells in the draining lymph nodes was lower in animals

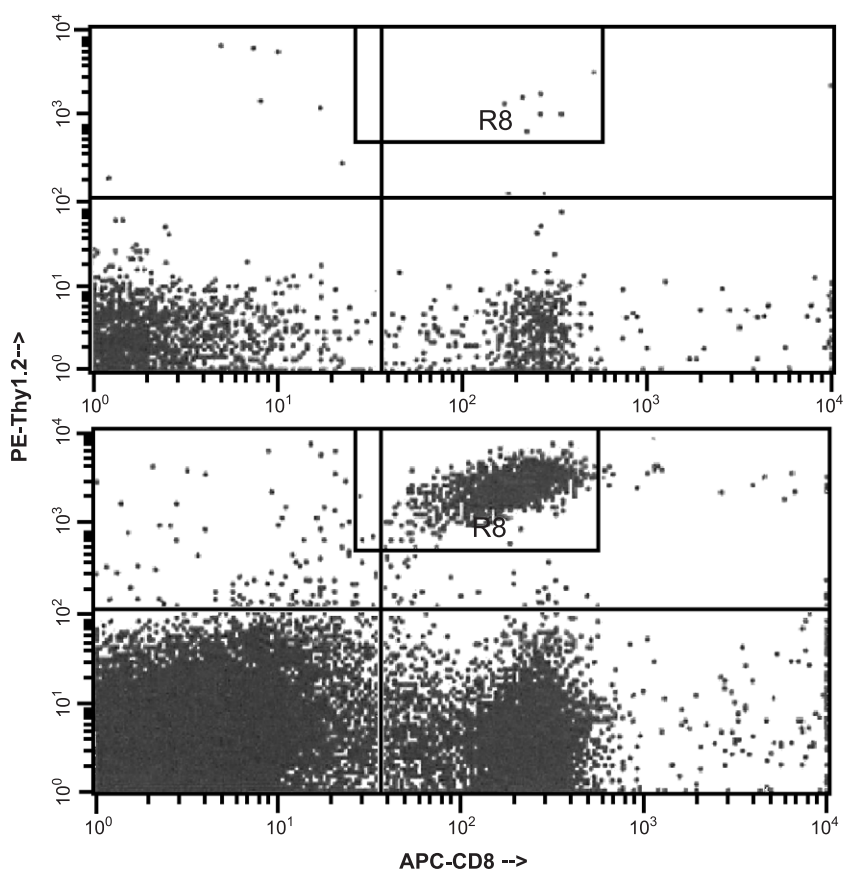


Fig. 4. Antigen-specific recruitment of Class I MHC + OVA restricted transgenic T cells by recombinant yeast-expressing ovalbumin. Ten to 20 million purified T cells from the Class I MHC + Ovalbumin specific T cell receptor transgenic OT-1 mice (Thy1.2+) were injected intravenously into B6.PL recipient mice (Thy1.1+). Two days later the animals were challenged at the base of the tail with 200  $\mu$ g of whole ovalbumin protein (top panel) or with ovalbumin expressing yeast (OVAX) (bottom panel). OT-1-positive T cell recruitment was followed in the draining node by gating for CD8/Thy1.2 double-positive cells. The figure demonstrates that Thy1.2+CD8+ double-positive cells in gate R8 represent less than 0.4% positive cells when ovalbumin without benefit of yeast delivery (or no antigen) was injected (top panel) vs. 4% double-positive cells (bottom panel).

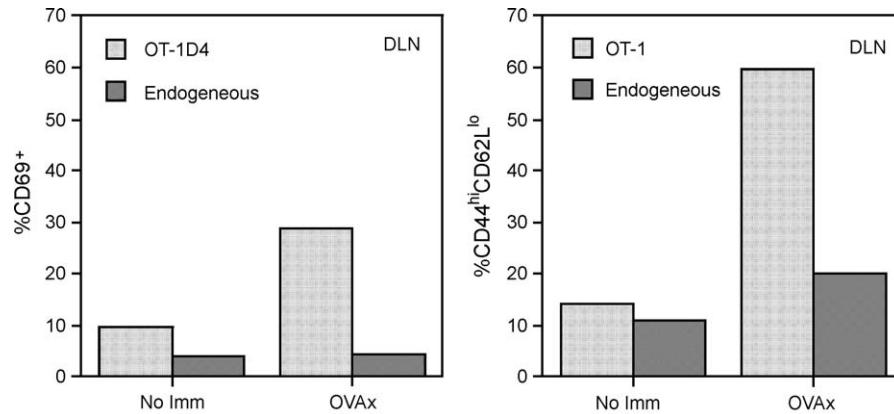


Fig. 5. Antigen specific recruitment induces an activation phenotype. Cells were triple-stained with PE-Thy1.2/FITC-CD69, CD62L, or CD44/APC-CD8, gated on Thy1.2/CD8 double-positive cells or Thy1.2<sup>-</sup>/CD8<sup>+</sup> endogenous cells, and analyzed for frequency of “activated” T cells defined as CD69<sup>+</sup>, CD62L<sup>-</sup>, and CD44<sup>+</sup>. In addition, cells were stained with PE-CD44/FICT-Thy1.2/APC-CD62L combinations and gated on Thy1.2<sup>+</sup> cells to measure “memory” CD44<sup>hi</sup>, CD62L<sup>-</sup> cells. The results show that OT-1+ T cells in the draining node of OVAX-treated animals express phenotypes consistent with activation, defined by these markers. This is a representative experiment of five, where in each case, cells from an individual mouse were analyzed and where the antigen-immunized animal always had a positive frequency of >30% for CD69 and >50% for CD44<sup>hi</sup>/CD62L<sup>lo</sup>. In each experiment, the comparison between non-immunized vs. immunized mice showed that the values for the latter were at least 3-fold those for the former.

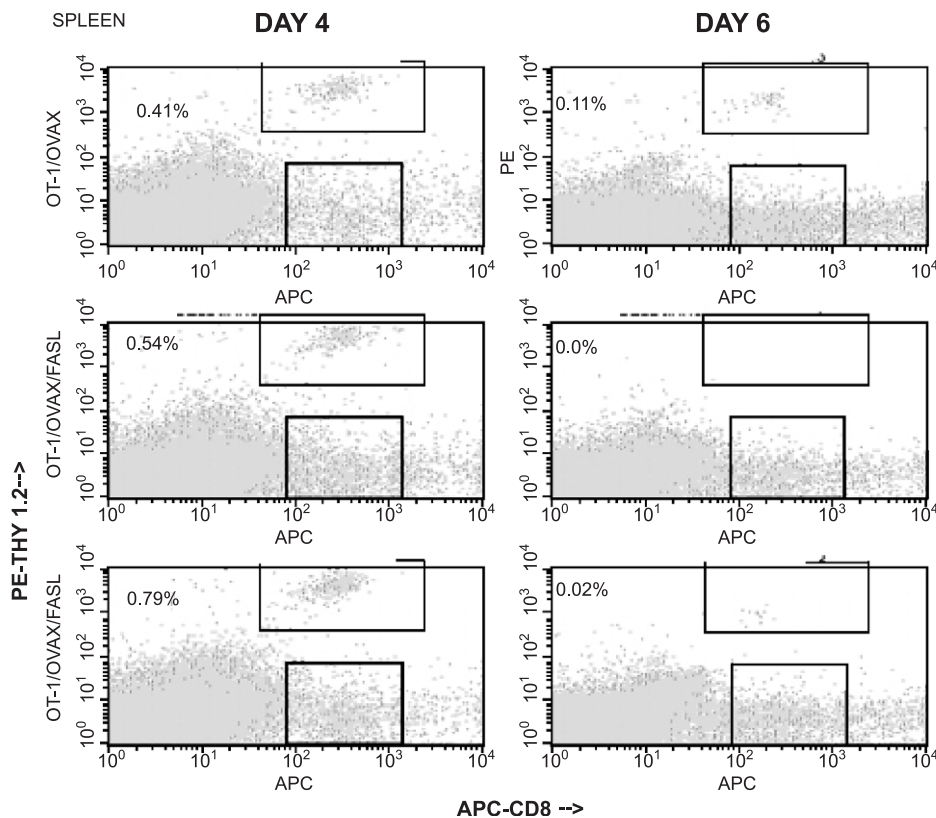


Fig. 6. Termination of antigen-specific recruitment following administration of FasL. The experiments in Fig. 4 were repeated with animals treated with FasL-expressing adenovirus 2 days after OVAX administration as described in Materials and methods. Note that OVAX was administered in the base of the tail while FasL was delivered into a rear paw to mirror the arthritis treatment protocol. Results show spleen cells from a control animal that received Ad-GFP (top) and two animals that received Ad-FasL-wt (middle and bottom), where FasL treatment terminated OT-1+ T cells (0.0% and 0.02%, respectively). The experiment was repeated as in Fig. 3 using one animal per time point per group (except in the experiment shown where two animals immunized with OVAX and treated with FasL were analyzed simultaneously for each time point). The differences between the two groups (with and without FasL) at day 6 were statistically significant ( $P < 0.01$ ) using a two-tailed Student's *t* test for independent samples.

that did not receive FasL, suggesting that trafficking out of the lymph nodes may have also contributed to this effect. These experiments indicate that both activation and mobilization to sites distal to the original site of antigen exposure were important for the successful termination of activated T cells by the locally administered FasL. In addition, as would be predicted from the data in the arthritis treatment model, Ad-FasL-sol did not reduce the frequency of OT-1 T cells (not shown).

#### *Summary of results regarding the pleiotropic therapeutic applications of Fas ligand*

Engagement of Fas by FasL can promote apoptosis of susceptible cells, but other mechanisms may be responsible for the possible clinical benefit of FasL gene therapy for cancer and CIA. Above, we describe the capacity of different forms of FasL (wild-type, soluble, and intracellular truncated) to promote apoptosis of susceptible cells *in vitro*, to retard or prevent tumor growth, and to afford protection from CIA. In addition, we begin to define the immunological mechanisms responsible for protection from CIA. A summary description of each experimental model used, the results, and the interpretation of the data is provided in [Table 1](#).

## **Discussion**

A therapeutic benefit of FasL gene transfer in the treatment of CIA was documented previously [5], but the mechanisms responsible for its systemic effects were not defined. It is apparent that not all forms of FasL have equivalent therapeutic potential, as a soluble form is far less effective in preventing or suppressing CIA. In parallel experiments, we and others showed that wild-type, but not soluble forms of, FasL could protect mice from various tumors [7,9–11,22–24], indicating that, while many tumors are resistant to Fas-mediated apoptosis, they are sensitive to the *in vivo* protective effects of FasL that restrict tumor expansion and are associated with its ability to promote inflammation at the site of tumor growth. Engagement of Fas receptors on inflammatory cells by FasL might trigger the activating event, or this could be mediated through signals transmitted by FasL itself, leading, for example, to secretion of pro-inflammatory cytokines by FasL-bearing cells. To distinguish between these two mechanisms, we examined the efficacy of an intracellular truncated form of FasL (FasL-ict), which is signaling deficient, in the treatment of CIA. FasL-ict prevented the onset of the disease as effectively as the FasL-wt. Therefore, signals transmitted by FasL-bearing cells, as opposed to Fas-bearing cells that they target, would appear to be excluded. Neither hepatotoxicity nor death was seen in any of the mice treated with FasL-ict (or the Ad-GFP controls), suggesting this molecule may be most well suited for therapeutic development.

The question of how local administration of FasL accounted for the systemic elimination of arthritogenic cells remained. To address this, we used adoptive transfer of OT-1 transgenic T cells to congenic recipients as a means to follow the fate of OVA-specific transgenic T cells in the presence or absence of locally delivered FasL. We observed that these antigen-specific T cells rapidly moved to the site where antigen was presented (the draining lymph node), but eventually they also migrated to other sites (the spleen). Upon administration of FasL, the activated OT-1 T cells disappeared from the spleen ([Fig. 6](#)), even though the FasL was delivered to a site (a rear paw) distal to the source of antigen-specific recruitment (the base of the tail). Therefore, while it is intuitively obvious that delivery of FasL to the site where antigen-specific T cells are recruited, that is, injection of FasL directly into an arthritis paw or into a node where antigen drained, might result in their termination, the elimination of T cells that are activated distal to where FasL is delivered requires that either FasL travel to the site of T cell activation, or that the activated T cells move to the site of FasL delivery.

Both mechanisms are probably active. While FasL delivered in the rear foot led only to a local inflammatory response, GFP encoded by the adenovirus was detectable distal to the injection site. There were no pathologic effects of this systemic distribution as evidenced by the absence of gross or microscopic changes in the liver and the absence of systemic inflammation. At present, we also cannot rule out the possibility that the GFP seen distal to the injection site was released locally from damaged cells and carried elsewhere by lymphatic or hematogenous spread. Thus, we feel it is improbable that this is the only operative mechanism that leads to the termination of Fas positive, activated T cells. It is here where the data regarding antigen-specific recruitment are informative: while OVAX administration to the base of the tail provokes antigen-specific recruitment of transgenic OT-1 T cells to the draining lymph node, there is a less pronounced, but still significant, increase in OT-1 T cells in other peripheral lymph nodes and in the spleen. The kinetics of this response and the activation status following antigen exposure suggest that not only were T cells activated in the draining lymph node, they are also present elsewhere and traveled to distant sites as a consequence of signals provided by the inflammatory response. In effect, the inflammatory response provided a “bait” to recruit activated T cells where they could then undergo FasL-induced cell death.

The mechanism that initiates inflammation *in vivo* is of great interest. This effect is not mediated by adenovirus *per se*, as adenoviruses expressing GFP or the soluble version of FasL did not exhibit pro-inflammatory activity. Nor is there a requisite sensitivity to Fas-mediated apoptosis by the tumor. However, it has been reported that the host must bear functional Fas receptors in order for inflammation to occur [9,25], suggesting that targeting Fas on neutrophils is an essential aspect of this process. Engagement of Fas by FasL on neutrophils may enhance the oxidative burst, which

recruits additional inflammatory cells (or kills tumors). Alternatively, overexpression of FasL by virus-infected cells may kill Fas-positive neutrophils in such a violent fashion that the ensuing necrosis (rather than a quiet apoptotic death) sets up a wave of antigen presenting cell activation that induces more inflammation. This latter mechanism would not necessarily require that the neutrophils express Fas—even though under normal circumstances they do—as there are other cellular targets that can execute FasL-mediated cell death [25].

In the case where FasL is delivered via T cells that can recirculate, the potential interactions between receptor and ligand in whole organisms are more obvious. For example, when diabetes-prone NOD mice are bred to Fas-defective *lpr* background, the animals do not develop diabetes [26]. One obvious reason for this is that the islets lack Fas as a target, but a more compelling explanation is that the *lpr* compensates for the Fas deficiency by overexpressing FasL on its T cells [27]. In fact, it has been shown that these FasL-overexpressing T cells can protect against diabetes upon adoptive transfer via an associative termination of Fas-bearing autoreactive T cells [28].

In summary, we confirm and extend the findings showing that local administration of FasL offers systemic protection and therapeutic benefit in a model of CIA, and we further define a potential mechanism for this effect, where a somewhat paradoxical inflammatory response “baits” motile, Fas-bearing autoreactive T cells that are then “trapped” and destroyed within this inflammatory milieu by cells that overexpress FasL. These findings illustrate the potential applications for this molecule under the appropriate conditions as an effector to treat autoimmunity.

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