

FAS ligand gene transfer for cancer therapy[#]

Review Article

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Abbreviations: activation-induced cell death, (AICD); cyclin dependent kinase, (CDK) death-inducing signaling complex, (DISC); Fas ligand, (FasL); Fas-associated death domain, (FADD); IL-1-converting enzyme, (ICE); Lewis Lung carcinoma, (LL); Lung Kruppel-like factor, (LKL or KLF-2); tumor necrosis factor, (TNF); TNF-related apoptosis inducing ligand, (TRAIL);

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Summary

Fas ligand (FasL) gene therapy has been explored in various clinical settings. This versatility can be traced to pleiotropic effects elicited by interactions of Fas and FasL in different tissues, to the context within which FasL is expressed, and to the persistence of FasL in the system. When used to treat cancer, FasL expression is achieved on tumor cells by exogenous gene delivery. In susceptible tumors, ectopic FasL expression leads to Fas-dependent apoptosis, in Fas-resistant tumors, it initiates robust, local inflammatory responses that result in tumor cell death. This minimizes persistence of FasL in the system, but along with the inflammation, tumor cell death promotes specific, protective antitumor immune responses. In this review, we discuss mechanisms that underlie the ability of FasL gene therapy to promote or sustain antitumor responses, as well as its potential as a contemporary clinical tool to treat minimal residual disease and microscopic metastases.

I. Introduction: Rationale to use Fas ligand gene transfer for cancer therapy

Advances in early detection and in conventional tumor therapies have made many cancers treatable, chronic diseases. However, suffering and death rates remain unacceptable for cancers of the lung, breast, prostate, bone, malignant melanoma, and others that are metastatic or inaccessible to conventional treatments. Immunotherapy is an appealing modality to treat cancer because many tumors that are resistant to conventional treatment such as radiation and chemotherapy or that are inaccessible to a surgical approach can be treated using immunologic approaches. One such approach that has shown preclinical promise is the use of Fas ligand (FasL) gene transfer. When expressed in the context of tumor

cells, FasL promotes tumor cell killing directly and indirectly, and induces reliable antitumor immune responses that can protect animals against subsequent tumor challenge (**Figure 1**). This approach could be applicable to most cancers, since it relies on the native tumor to induce a systemic antitumor immune response. Conceptually, this may allow development of tumor vaccines without the need to identify or enrich specific tumor antigens.

II. Apoptosis, Fas, and Fas Ligand

Apoptosis is an evolutionarily conserved, innate cell death program that can be secondary to withdrawal of trophic growth factor signals or to signals mediated by death receptor pathways (Boise and Thompson, 1996).

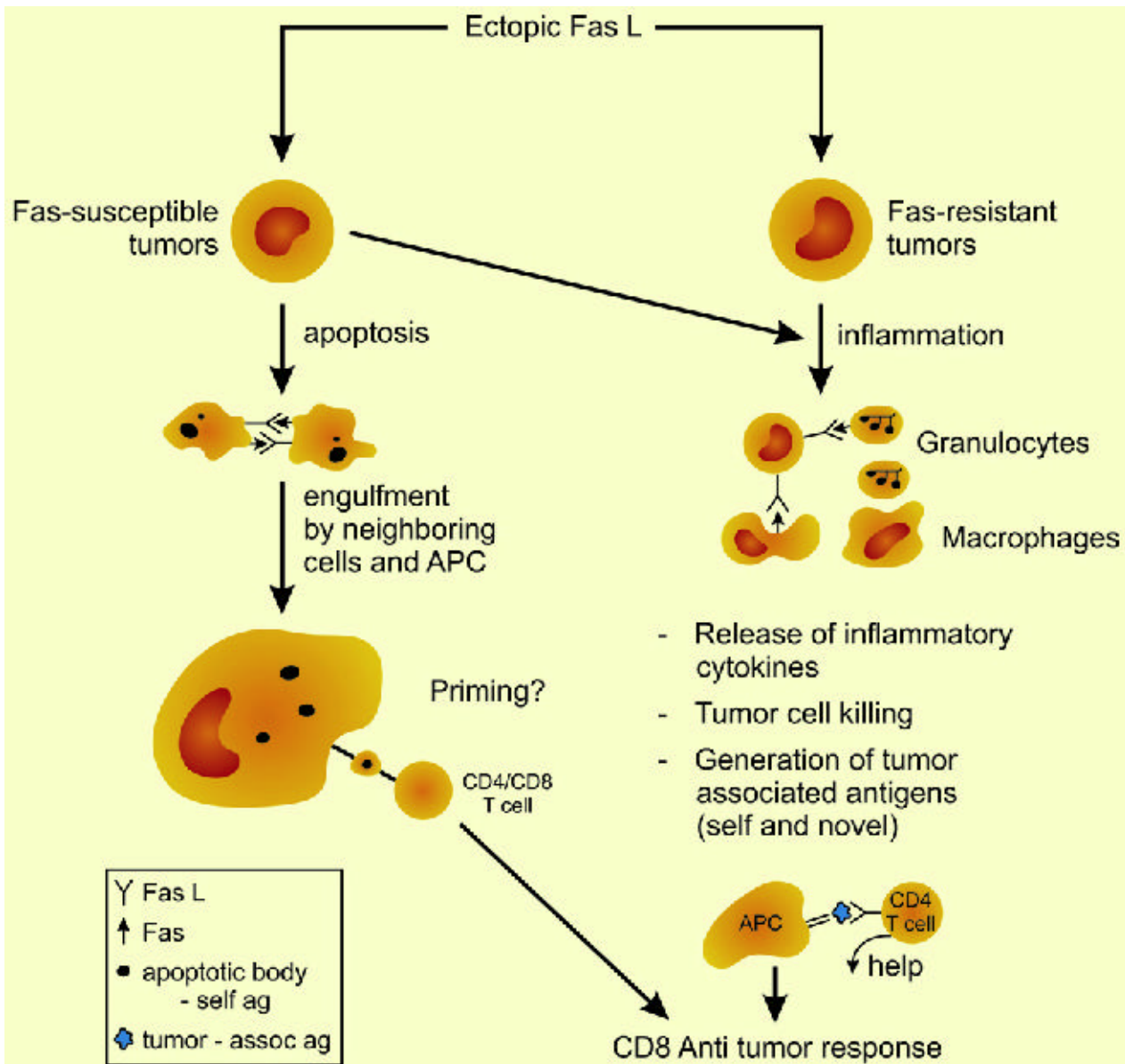


Figure 1. Antitumor effects of FasL gene transfer. Ectopic expression of FasL can induce antitumor immunity against both Fas-sensitive and Fas-resistant tumors. In Fas sensitive tumors, the interaction between Fas and FasL leads to apoptosis, which in turn can provide priming antigens for an immune response (Bianco et al, 2003). In both cases, ectopic expression of FasL promotes inflammation via interaction with Fas on host leukocytes (neutrophils and macrophages). The inflammatory response kills the tumor cells, making tumor antigens available to prime the immune system.

Death due to trophic factor withdrawal is an evolutionary adaptation seen in multicellular organisms where “death of the one benefits the whole” (Plas et al, 2001; Vander Heiden et al, 2001). The mechanisms involve glucose sensors and the energy producing machinery of the mitochondria. Death receptor-dependent cell death occurs upon ligation of the receptors by their cognate ligands. Death receptors are closely related proteins of the tumor necrosis factor (TNF) receptor family and include TNF receptors I and II, Fas (CD95 or Apo-1) and TNF-related apoptosis inducing ligand (TRAIL) receptors (DR4 and DR5) (Schulze-Osthoff et al, 1998). In particular, Fas is a 45-kDa cell surface glycoprotein that is expressed widely in tissues, although its expression is highest in the liver and in cells of the immune system. Its natural ligand, FasL, is a 55-kDa, TNF-related type II transmembrane protein (Nagata and Golstein, 1995; Krammer, 2000). In

contrast to Fas, expression of FasL is largely restricted to activated T cells and natural killer (NK) cells, although it also is present in some tumors (Nagata and Golstein, 1995; Krammer, 2000; Restifo, 2000; O’Connell et al, 2001). The roles of Fas and FasL in immune homeostasis were first revealed by the *lpr* and *gld* mice, which show pathological lymphoproliferation and autoimmunity that is mediated by dysfunctional Fas receptor/ligand interactions (Watanabe-Fukunaga et al, 1992; Suda et al, 1993). The *lpr/gld* models have been widely interpreted to indicate that Fas receptor/ligand interactions are important to contract clonally expanded populations of activated lymphocytes. This ‘activation-induced cell death’ (AICD) mediated by the interaction of Fas and FasL is essential to terminate immune responses and eliminate unneeded and potentially hostile lymphocytes in normal animals (Nagata

and Golstein, 1995; Schulze-Osthoff et al, 1998; Holtzman et al, 2000).

Most studies on Fas and FasL have focused on their pro-apoptotic functions, although it is noteworthy that these proteins transduce proliferative and activating signals through pathways that remain incompletely understood (Wajant, 2002; Desbarats et al, 2003). Fas-mediated cell death can result by a self-propagating caspase cascade (in so called "type I" cells), or by mitochondrial amplification (in "type II" cells). The mechanisms controlling Fas-dependent apoptosis have been reviewed extensively elsewhere (Nagata and Golstein, 1995; Schulze-Osthoff et al, 1998; Krammer, 2000; Savill and Fadok, 2000; Wajant, 2002) and will only be discussed here briefly. In type I cells, ligation of Fas by FasL or by anti-Fas antibodies that promote Fas receptor multimerization leads to formation of a death-inducing signaling complex (DISC) that contains the Fas-associated death domain (FADD) protein and procaspase 8 (FLICE). Procaspase 8 is activated proteolytically in the DISC to form active caspase-8, which in turn cleaves and activates caspase-3 and caspase-9 to culminate in apoptosis (Krammer, 2000). Type II cells are refractory to DISC formation; in these cells, caspase-8 cleaves the BH3 protein Bid, which in turn promotes release of Cytochrome c and SMAC/DIABLO from the mitochondria with consequent activation of downstream caspases. The apoptotic effects of FasL can be propagated locally by cleavage of the protein into a soluble form (FasL-sol) by the action of matrix metalloproteases.

Tumors that express Fas receptor and retain intact downstream signaling pathways are susceptible to FasL-induced apoptosis. In such cells, ectopic FasL is an efficient inducer of apoptosis *in vitro* (Hedlund et al, 1999; Bianco et al, 2003), even in tumor cells that are resistant to Fas ligation with anti-Fas antibodies. *In vivo*, ectopic expression of FasL can prevent tumor growth and induce tumor regression in both transplantable and naturally occurring tumors (Hedlund et al, 1999; Bianco et al, 2003). More importantly, FasL gene therapy has been shown to be safe in tumor-bearing dogs that approximate human cancer patients more closely than experimental rodents, and the disease-free interval and survival in dogs that received adjuvant FasL was equal to or greater than that seen in historical controls treated with standard-of-care (Bianco et al, 2003).

While FasL-induced tumor cell death and regression in Fas-sensitive tumors was predictable, a more curious observation was that FasL could produce similar effects in Fas-resistant tumors. For example, we and others showed that murine EL-4 thymoma cells (Leon et al, 1998), Lewis Lung carcinoma (LL) cells (Modiano et al, 2004), CT26 human colon carcinoma cells (Arai et al, 1997), and some naturally occurring canine melanomas (Bianco et al, 2003) are resistant to Fas-dependent apoptosis, but remain sensitive to the antitumor effects of ectopic FasL expression *in vivo*. These and other studies underscore that one or more downstream effects of FasL that are independent of its pro-apoptotic function are important for its antitumor activity.

III. Inflammation induced by Fas ligand

A hallmark of apoptosis is cell death in the absence of inflammation (Savill and Fadok, 2000). Especially in higher vertebrates, this benefits the organism by allowing organogenesis, removal of effete cells and tissue remodeling without triggering "danger signals" that activate innate immune responses and damage tissues. This concept of quiescent cell death led to the hypothesis that FasL might contribute to peripheral tolerance by destroying activated T cells that ventured into sites of "immune privilege", such as the anterior chamber of the eye and other tissues (Bellgrau et al, 1995; Griffith et al, 1995; Uckan et al, 1997). In fact, several reports suggested that ectopic expression of FasL by solid and hematopoietic tumors contributed to tumor growth and survival by making the tumor an immune privileged site (Hahne et al, 1996; O'Connell et al, 1996; Greil et al, 1998; Houston et al, 2003). This notion of immune evasion and tumor counterattack mediated by FasL remains controversial, as it has not been universally reproducible (Chappell et al, 1999; Restifo, 2000, 2001). Rather, a large number of studies showed that transplantation of tissues expressing ectopic FasL led to vigorous suppurative inflammation at the transplant site, with consequent tissue rejection (Restifo, 2000). It remains possible that, in some cases, FasL mediates immune privilege when it is expressed in tissues that produce large amounts of anti-inflammatory cytokines such as transforming growth factor- (TGF-) (O'Connell et al, 2001), and that inducing inflammation that overcomes immune privilege is a quantitative, rather than a qualitative feature of FasL expression (Gregory et al, 2002).

As noted above, a series of experiments designed to test the concept of immune privilege conferred by ectopic expression of FasL showed, somewhat surprisingly, that ectopic expression of FasL had proinflammatory properties and unanticipated effects (Allison et al, 1997; Kang et al, 1997). This is attributable to release of chemotactic factors and inflammatory cytokines upon apoptosis of macrophages or neutrophils (see below). However, it now appears that the magnitude of the proinflammatory properties also is dependent on the context of FasL expression. In the case where ectopic FasL expression occurs endogenously (i.e., as a transgene), the organism can mitigate its inflammatory effects. For example, one study showed that transgenic expression of FasL in heart muscle led to mild leukocyte infiltration with increased levels of TNF- , IL-1 , IL-6, and TGF- , but no tissue destruction (Nelson et al, 2000). On the other hand, the inflammatory effects of ectopic expression of exogenous FasL such as that seen when the gene is expressed in tumors by DNA delivery or adenovirus transduction, are unmitigated. This could be because the gene is delivered exogenously, or because higher levels of expression are achievable in this context. Many laboratories, including our own (Modiano et al, 2004) showed that ectopic expression of FasL in transplanted tumors caused neutrophils to infiltrate the tumor injection site (Arai et al, 1997; Seino et al, 1997;

Shimizu et al, 1999, 2001; Behrens et al, 2001; Shudo et al, 2001; Gregory et al, 2002; Simon et al, 2002).

The nature of the inflammatory response elicited by ectopic FasL expression is becoming clearer. It appears to begin with activation of a caspase cascade, including caspase-1, which is also called IL-1-converting enzyme (ICE). In addition to IL-1, ICE also cleaves and activates IL-18; these cytokines act to recruit neutrophils and macrophages (O'Connell et al, 1996; Restifo, 2000; Shudo et al, 2001; Hohlbaum et al, 2002). High levels of FasL expression in a concentrated area, such as a tumor mass, lead to extensive apoptosis of neutrophils and macrophages (Hohlbaum et al, 2001; Shimizu et al, 2001), which in turn perpetuates the inflammatory response by recruiting additional leukocytes. Although adenoviruses themselves activate innate immunity and promote inflammation, tumors transduced with control adenoviruses (e.g., Ad-GFP) do not elicit either tumor cell killing or protective antitumor responses. This indicates that the inflammatory response that promotes antitumor immunity is specifically initiated by FasL. This can be advantageous in the context of adenoviral gene therapy, given the concerns of persistent adenovirus transduction of human or animal patients. Replication deficient adenoviruses can persist in the body for weeks to months (>70 days), but the adenovirus-mediated expression of FasL is extinguished in a relatively short time (~14 days) because the transduced cells are killed as a consequence of the inflammatory response (Regardsoe et al, 2004). However, the possibility that adenovirus DNA, and hence the ectopic gene, persists in the system and help maintain the immune response cannot not be discounted.

IV. FasL gene transfer in cancer therapy – mechanisms that generate protective antitumor immune responses

It is useful to understand the properties of FasL-mediated antitumor responses before we can clarify their underlying mechanisms. First, the generation of protective antitumor responses in experimental animals is wholly dependent on the initial inflammatory response (Shimizu et al, 1999), and it can be blocked by administration of neutralizing anti-FasL antibodies that prevent neutrophils from infiltrating the tumor site. The response is also dependent on the interaction of ectopic FasL with endogenous Fas, as evidenced by the observation that *lpr* mice do not reject FasL-expressing tumors, nor do they mount protective antitumor responses (Shimizu et al, 1999). Conversely, this response is independent of the tumor cells' susceptibility to apoptosis mediated by death receptors, and it does not require secretion of perforin granules (Behrens et al, 2001).

The kinetics of the protective antitumor immune response generated by FasL gene transfer resembles those seen in primary immune responses to antigen. **Figure 2** shows that, in the transplantable Lewis Lung carcinoma model of B6 mice, systemic protective antitumor immunity against wild type, FasL-negative tumors arises within ~1 week after the first exposure to tumor antigens (generated by tumor destruction during the initial rejection of the FasL-expressing tumor). After 14 days, 100% of mice reject a wild type tumor challenge. The kinetics of the response probably reflect the time required to produce sufficient tumor-specific T cells, as immunization of mice with FasL-expressing tumors on the contralateral flank at the same time or subsequent to wild type tumor challenge can delay, but not completely prevent growth of wild type tumors (**Figure 3**).

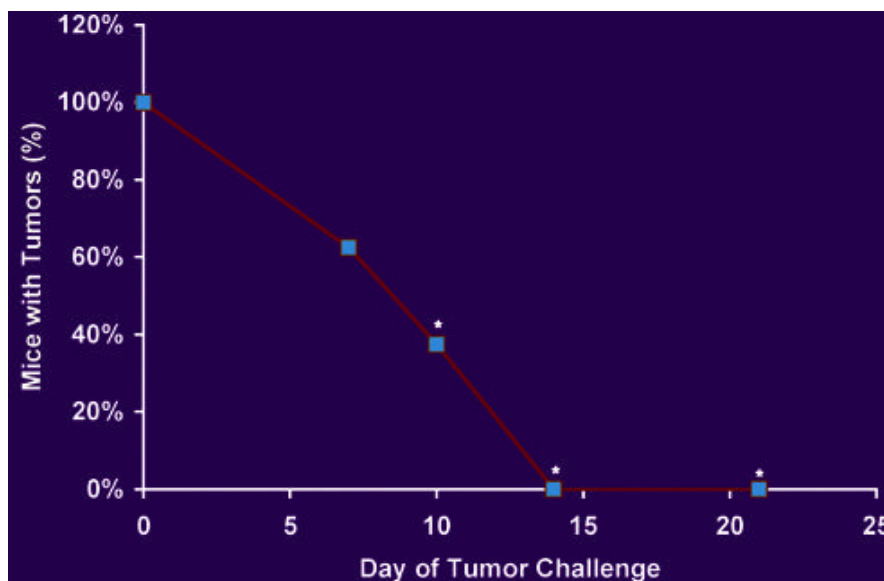


Figure 2. Kinetics of FasL-Mediated Antitumor Responses. Five groups of 8 mice were vaccinated with LL cells engineered to express FasL by adenovirus transduction. Groups were challenged with unmodified, wild type LL cells on the day of vaccination, or 7, 10, 15, or 21 days later. The data show the percent of mice from each group that developed tumors after challenge. Asterisks indicate that protection was statistically significant ($P < 0.01$) when mice were challenged at day +10 or later; and trend was present as early as day +7 ($P = 0.07$).

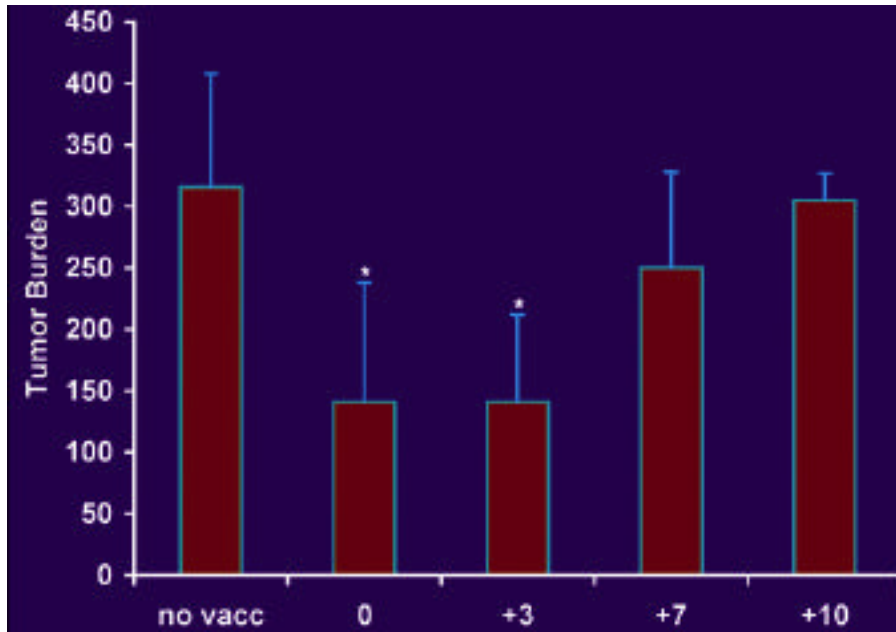


Figure 3. FasL Gene Transfer Delays Growth of Transplantable Tumors. Groups of mice were challenged with unmodified, wild type LL cells in the lateral flank, and vaccinated with LL cells engineered to express FasL by adenovirus transduction in the contralateral flank on the same day, or 3, 7, or 10 days later. All of the mice developed tumors, but the tumor burden in mice vaccinated on Day 0 and Day +3 was significantly smaller than controls ($P < 0.005$, asterisks).

Protective antitumor immunity is only achieved with ectopic expression of membrane-bound isoforms of FasL (Hohlbaum et al, 2001, 2002; Shudo et al, 2001; Gregory et al, 2002; Simon et al, 2002). In fact, ‘outside-in’ signaling by FasL is not necessary as evidenced by the observation that an intracellular truncated form of FasL (Modiano et al, 2004) can induce a protective antitumor response that is as effective as wild type FasL and follows similar kinetics (**Figure 4**). Rejection of FasL-expressing tumors can occur in the absence of T cells, although it is delayed in athymic nude mice (Shimizu et al, 1999) and in lymphocyte-deficient SCID mice (our unpublished results). On the other hand, the generation of protective antitumor immunity requires CD⁸ T cells as evidenced by the fact that depletion of CD⁸ T cells prevents the development of such a response (Shimizu et al, 1999) and by our observation that this response also does not occur in MHC class-I knockout mice (that lack peripheral CD⁸ T cells). The protective antitumor response generated by FasL gene transfer is specific, as it cannot protect mice from challenge with a distinct tumor (Shimizu et al, 1999).

The data presented above indicate that antitumor immunity induced by ectopic FasL expression in tumors is similar to any other immune response to antigen. However, the reasons why most tumors by themselves do not induce robust antitumor responses remain the subject of intense investigation. A few cases are documented where tumor-associated antigens are generated *de novo* through mutational events, producing proteins that the immune system recognizes as foreign (“non-self”) (Wolfel et al, 1995). However, most tumor-associated antigens are, in fact, normal “self” proteins to which the immune system is tolerant (Sotomayor et al, 1996; Ganss et al, 1999), and although T cells that recognize these tumor

antigens can undergo limited clonal expansion *in vivo*, these cells are largely ineffective to mount antitumor responses (Lee et al, 1999). Thus, antitumor immune responses seem to be subject to the same set of rules that maintain autoimmune responses in check, that is, the immune system may be largely tolerant to tumor antigens.

When we consider that robust inflammation induced by ectopic FasL induces protective antitumor immunity despite the fact that lymphocytes see cancer cells as “self”, it raises a series of testable, non-mutually exclusive hypotheses. The first is that “autoimmune prone” cells that recognize tumor cells are present in normal naïve animals, but their activation is repressed by intrinsic and/or extrinsic negative regulatory pathways that are overcome by the consequences of ectopic FasL expression (increased antigen load and inflammation). The second is that the inflammatory response initiated by the interaction of FasL with host neutrophils and macrophages provides ‘danger signals’ that break self-tolerance. And the third is that proteolysis of tumor-derived antigens within the inflammatory milieu formed in response to ectopic FasL expression generates novel ‘non-self’ peptides that can be recognized by T cells. Specifically, peptides produced by extracellular proteolysis would be distinct from both native “self” peptides and peptides contained within apoptotic bodies. Peptides generated extracellularly would likely be processed through Class II MHC pathways, whereas peptides contained within apoptotic bodies could be processed through either Class I or Class II MHC pathways (Bellone et al, 1997; Albert et al, 1998; Henry et al, 1999). Tumors that are resistant to FasL-dependent apoptosis, but that are rejected upon ectopic expression of FasL offer viable systems to distinguish among these possibilities (**Table 1**).

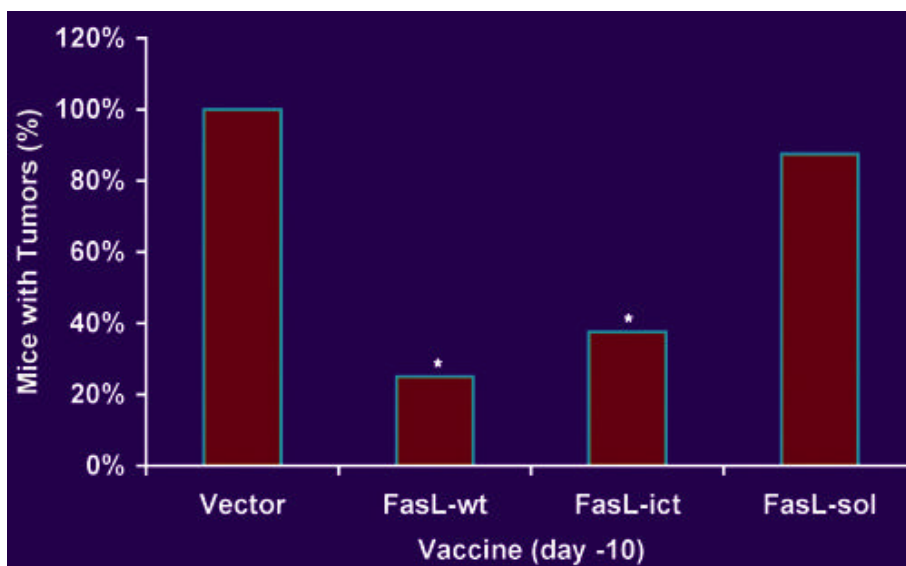


Figure 4. Outward Signaling by Membrane-Bound FasL is Responsible for Antitumor Response. Four groups of mice were vaccinated as in Figure 2 with LL cells transduced using a replication-defective adenovirus encoding wild type FasL (FasL-wt), a membrane-bound, intracellular truncated FasL mutant (FasL-ict), or a soluble form of FasL (FasL-sol) (Modiano et al, 2004). Groups were challenged with wild type LL cells 10 days after vaccination and followed for tumor growth. The data show the percent of mice from each group that developed tumors after challenge. Asterisks indicate that FasL-wt and FasL-ict afforded significant protection ($P < 0.02$) as compared to the control group.

Table 1. Possible mechanisms for antitumor responses initiated by ectopic FasL gene transfer and consequent inflammation

1.	Release of lymphocytes from negative regulation	<ul style="list-style-type: none"> • Increased antigen presentation upon tumor cell death overcomes threshold of intrinsic negative regulation • Cytokines produced by phagocytic cells signal to disable negative regulatory molecules • Expansion of self-reactive tumor-specific cells, or non-self reactive cells with low affinity for antigen promotes antitumor responses
2.	Danger signals that break tolerance	<ul style="list-style-type: none"> • Maturation of dendritic cells • Increased antigen load with recognition by B cells (self-antigen) and presentation to T cells • T cell help leads to antitumor responses (humoral, cellular)
3.	Generation of novel peptides	<ul style="list-style-type: none"> • Primary tumor cell killed by inflammatory cells • Inflammatory milieu promotes proteolytic digestion of tumor-derived antigens • Novel (non-self) tumor-associated peptides generated from proteolytic digestion are presented to T cells • T cell help leads to cellular antitumor responses

A. Negative regulation and inhibition of antitumor immune responses

The possibility that “autoimmune prone” cells that recognize tumor cells are present in normal naïve animals is supported by the observation that, under some experimental conditions, homeostatic proliferation is sufficient to overcome tolerance and reject transplanted tumors without a need for a priming response (Hu et al, 2002). In these studies, expansion of naïve cells transferred into Rag1-deficient mice generated specific, type I CD⁴ and CD⁸ tumor-specific T cells when mice were challenged with B16 melanoma cells. It has been shown that naïve T cells undergoing homeostatic

proliferation resemble memory cells (Goldrath et al, 2000), and that their requirements for activation are quite distinct from those of naïve T cells, as they are less sensitive to the influence of negative regulation. This suggests that in normal animals (absent homeostatic proliferation), tumor-specific cells might be restrained by the tolerogenic effects of negative regulation.

Peripheral tolerance can be mediated by regulatory T cells (extrinsic) (Shevach 2002), as well as by intrinsic negative regulatory factors (Buckley et al, 2001; Tzachanis et al, 2001; Yusuf and Fruman 2003; Lang et al, 2004). A growing body of evidence supports an important role for regulatory T cells to suppress antitumor responses (Sakaguchi et al, 2001; Gavin and Rudensky 2003).

Therefore, it is possible that antitumor responses initiated by FasL selectively eliminate regulatory T cells. Yet, this is unlikely as CD⁴⁺, CD²⁵⁺ regulatory T cells are largely resistant to Fas-dependent apoptosis (Banz et al, 2002). Hence, the alternative possibility is that the inflammatory response initiated by FasL provides impetus for signals that disable intrinsic negative regulation. At least four interrelated pathways of lymphocyte negative regulation have been described. These include transcriptional repression by Lung Kruppel-like factor (LKLf or KLF-2) (Kuo et al, 1997; Buckley et al, 2001), Tob (Tzachanis et al, 2001), Forkhead Box proteins (Yusuf and Fruman 2003), and NFATc2 (Baksh et al, 2002; Frazer-Abel et al, 2004). These proteins all share the functional maintenance of proteins such as the cyclin dependent kinase (CDK) inhibitor p27Kip-1 (p27), which prevent cell cycle entry and consequently increase the threshold of T cell activation. Intriguingly, the phenotypes of mice harboring targeted deletion of these molecules are distinct. Homozygous deletion of LKLf resulted in embryonic lethality; however, conditional deletion of LKLf in the lymphoid compartment using the *Rag*-deficient model led to viable mice whose peripheral T lymphocytes had a spontaneously activated cell surface phenotype and underwent premature apoptosis (Buckley et al, 2001). Tob-deficient mice are viable (Tzachanis et al, 2001), but have hyper-responsive T cells that exist in a state of partial activation. Mice deficient in FoxP3 lack regulatory T cells and develop autoimmunity (Fontenot et al, 2003). Finally, NFATc2-deficient mice have marked splenomegaly and hyper-responsive T cells (Xanthoudakis et al, 1996; Baksh et al, 2002; Frazer-Abel et al, 2004). These models will be useful tools to explore the relationship between immune activation that follows FasL-dependent inflammatory responses and disengagement of intrinsic negative T cell regulation, as well as the significance of these events in the generation of antitumor immune responses.

B. Danger signals that break tolerance to self-antigens promote antitumor immunity

The observations that naïve cells can recognize tumors when activation thresholds are reduced by homeostatic proliferation or by inactivation of negative regulatory factors do not distinguish if these cells are self-reactive cells restrained by tolerance, or simply non-self reactive cells with low affinity for antigen. In a series of elegant experiments using FasL-expressing B16 melanoma cells, Simon et al showed that the generation of protective antitumor immunity required inflammation associated with FasL, and that the response was directed against self-antigens (Simon et al, 2002). The mechanisms responsible for protective immunity appeared to require traditional T cell dependent responses: FasL-induced inflammation led to maturation of dendritic cells that not only expressed higher levels of co-stimulatory molecules (CD80, CD86, MHC Class II), but also were more efficient antigen presenting cells; depletion of CD⁴ T cells abrogated the response. Intriguingly, depletion of CD⁸ T cells did not affect the response and adoptive transfer of these cells to naïve mice did not protect them from tumor challenge, but the response was transferable by antibody. The difference

between these experiments and others showing protection that was mediated by T cells is unclear, but it may be peculiar to the growth patterns and resistance to T cell lysis by B16 melanoma rather than due to the route of inoculation (subcutaneous) or other experimental conditions. Most importantly, the antibodies that mediated protective antitumor responses in these animals were clearly self-reactive, directed against melanocyte differentiation antigens, and non cross-reactive with antigens derived from other tumors.

These results show that FasL can mimic the 'danger signals' that promote dendritic cell maturation into antigen presenting cells, and that these cells can then initiate T cell-dependent, antitumor responses against self-antigens. That is, ectopic expression of FasL in a tumor environment can break peripheral tolerance and produce antitumor responses that resemble autoimmunity. Yet, a fine balance may remain between this 'autoimmune' antitumor response and unregulated autoimmunity, as the authors of this study reported the occurrence of a predictable autoimmune response against normal melanocytes (depigmentation) in only 20% of their experimental animals (Simon et al, 2002).

C. Tumor cell death and antigen load in antitumor immune responses

A limitation of tumor vaccines and treatments directed against specific tumor antigens is that expression of such antigens can be variable in cancer patients. To circumvent this limitation a treatment approach can be used that relies on inducing immunity against self-antigens or novel antigens generated from the patient's own tumor. In principle, the 'danger signals' associated with ectopic FasL expression that result in tumor cell death and inflammation should enhance the load of intact tumor antigens that are processed and presented by antigen presenting cells, therefore leading to greater recognition of endogenous tumor antigens. On the other hand, proteolytic activity at the site also could generate novel antigens that are distinct from self-antigens. Immunologic priming by apoptosis has been documented in various systems, including uptake and presentation of viral antigens (Bellone et al, 1997; Albert et al, 1998) and tumor antigens (Henry et al, 1999). We showed that apoptotic cell priming increased the capacity of peripheral lymphocytes to kill viable melanoma cells in the presence of IL-2, indicating that there was recruitment of additional IL-2-responsive cells upon presentation of apoptotic cell antigens (Bianco et al, 2003). This was true *in vivo*, as apoptosis induction also offered a remarkable advantage to prime cytolytic activity of autologous cells from patients with naturally occurring tumors.

Adoptive transfer experiments such as those described above could be used to formally distinguish if protective antitumor responses initiated by ectopic expression of FasL-priming require generation of distinct antigens that cannot be recognized by naïve cells even if negative regulation is disengaged and tolerance is broken. If this were the case, such experiments would show that the only mice that acquired protective immune responses were those adoptively transferred with T cells from mice

that were originally immunized with FasL-expressing tumors for >1 week. A failure of these adoptive transfer experiments to offer protection from challenge with wild type tumor, would suggest that additional cells (e.g., dendritic cells or B cells) are required for the effect, or that other essential events required for the response occur during presentation of tumor antigens in the host that is vaccinated with the FasL-expressing tumor. A similar experiment could be used to test the efficacy of this therapy to treat minimal residual disease and metastasis, by evaluating protection afforded by the adoptively transferred cells to reject transplantable tumors that are inoculated systemically and seed distant sites.

V. Conclusion

In this review, we present a preponderance of evidence to support the generation of specific, protective antitumor responses by ectopic expression of FasL in distinct tumor types. Understanding the mechanisms that mediate these responses will allow us to continue developing this therapy as an integral component of the armamentarium to manage cancer patients and improve their outcomes.

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