Fas ligand breaks tolerance to self-antigens and induces tumor immunity mediated by antibodies

Anna Katharina Simon,[1,5] Awen Gallimore,[1,3,5] Emma Jones,[1] Birgit Sawitzki,[1,2] Vincenzo Cerundolo,[1] and Gavin R. Screaton[1,4]

[1]MRC Human Immunology Unit and Nuffield Department of Medicine, Institute of Molecular Medicine
[2]Nuffield Department of Surgery
John Radcliffe Hospital, Headington, Oxford OX3 9DS, United Kingdom
[3]Medical Biochemistry, UWCM, Heath Park, Cardiff CF14 4XN, United Kingdom
[4]Correspondence: screaton@molbiol.ox.ac.uk
[5]These authors contributed equally to this work.

Summary

The role of Fas ligand (FasL) in programmed cell death via interaction with its receptor Fas is well characterized. It has been proposed that expression of FasL can confer immune privilege to some organs, allowing them to kill infiltrating lymphocytes and inflammatory cells. However, a number of studies have shown that when tumors or transplants express FasL, rejection often occurs as a consequence of proinflammatory functions of FasL. Here we demonstrate that FasL elicits tumor immunity in a murine melanoma model with weak immunogenicity and low expression of major histocompatibility complex (MHC) class I. We show that protected mice recognize melanocyte differentiation self-antigens. Importantly, tumor immunity is mediated by antibodies, as it can be transferred by serum from protected mice.

Introduction

Immune surveillance by both the innate and acquired arms of the immune system plays a part in eliminating some transformed cells, although the scale of the protection afforded is debated (Lanier, 2001). The frequent appearance of tumors in humans illustrates the fact that many tumor cells are ignored, develop mechanisms to escape, or at least tip the balance of an immune response, preventing their elimination. Fas ligand (FasL), a member of the TNF family of proteins, is well characterized for its role in triggering apoptosis. Expression of FasL by activated lymphocytes allows them to kill target cells expressing Fas (Krammer, 2000). However, expression of FasL by these cells is a two-edged sword, as activated lymphocytes themselves express Fas and become susceptible to death. The Fas/FasL axis thus limits an immune response and is a major player in the process of activation-induced cell death (Janssen et al., 2000).

FasL expression is normally tightly restricted to activated lymphocytes. However, it has been demonstrated that some nonlymphoid tissues such as the eye and testis can express FasL (Bellgrau et al., 1995; Griffith et al., 1995; Saas et al., 1997). This has been proposed to underlie the immune privilege enjoyed by these organs, in effect allowing them to kill infiltrating lymphocytes (i.e., "kill the killers"). Shortly after these initial reports, some tumors were demonstrated to express FasL, and this was proposed to be a mechanism used to escape an immune response (O'Connell et al., 2001; Walker et al., 1998). These findings are now the subject of much debate, as forced expression of FasL on tumors or transgenic expression in organs such as the pancreas often leads to a brisk neutrophil infiltration and elimination of tumor or damage to the transgenic organ (Green and Ferguson, 2001).

We have used the B16F10 melanoma model to study the effects of FasL expression. This tumor is poorly immunogenic and thus a good model to test strategies to enhance tumor immunity. We find that mice reject tumor cells transfected with FasL and go on to develop tumor immunity. Unlike previous studies where immunity has been shown to be mediated by CD8 lymphocytes, this immunity can be transferred by serum. Here we show that immune responses directed to melanocyte differentiation antigens are indeed induced by FasL-expressing tumor cells.

Results

Fas binding, and not the cytoplasmic chain of Fas, mediates tumor rejection of FasL-expressing tumors

Counter to initial observations, a number of investigators have now demonstrated that FasL transfected tumor cells are more

SIGNIFICANCE

Tumors often evade immune responses despite expressing a number of potentially specific target molecules. Eliciting effective responses to these is a major challenge in the field of tumor immunology. In this study, we show that the molecule Fas ligand behaves as an adjuvant to generate an antitumor response. The immunity is mediated by tumor-specific antibodies, which can recognize and lyse the tumor cell line. This demonstration of the involvement of FasL in the generation of antitumor antibodies may help in the definition of new tumor antigens and in the design of better vaccines and therapies for melanoma.

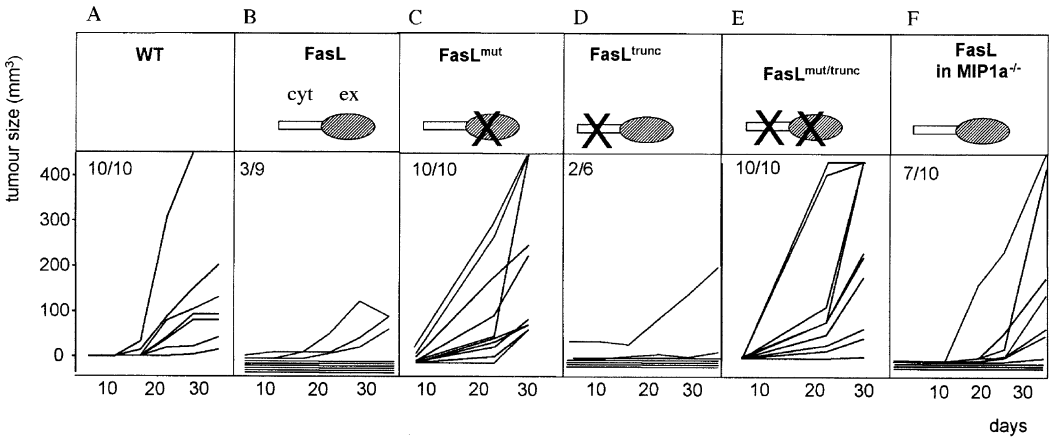

Figure 1. Rejection of FasL-expressing tumors

C57BL/6 mice were injected sc with 5×10^5 tumor cells. Cells were either untransfected B16WT (**A**) or stably transfected with full length FasL (**B**), full length FasL with a mutation in the Fas binding site ($FasL^{mut}$; **C**), truncated FasL ($FasL^{trunc}$ **D**), or truncated FasL with no binding to Fas ($FasL^{mut/trunc}$; **E**). Mip1α-deficient mice were given B16F10 transfected with full length FasL (**F**). Tumor growth was measured twice a week over a period of 5 weeks. Numbers represent tumor-bearing mice/total.

efficiently rejected than FasL negative cell lines upon their injection in vivo. The brisk tumor rejection seems to be mediated in the main by infiltrating neutrophils. Although it seems clear that once present, neutrophils can kill the tumor cells, little is known about how the neutrophils are attracted in the first place. In a first series of experiments, we established a model of FasL-mediated tumor rejection using the well-characterized murine melanoma cell line B16F10. Stable cell lines expressing FasL were established (B16FasL) and injected subcutaneously (sc) into C57BL/6 mice.

Examination of the site of injection revealed a small but clearly palpable swelling at the injected site up to 2 weeks after injection. All 10 mice injected with the parental or wild-type B16F10 (B16WT) developed tumors (Figure 1A), whereas 6 of 9 rejected the FasL-expressing tumor (Figure 1B), confirming previous studies using the B16 model and other murine tumor models (Walker et al., 1998). This is probably due to a direct interaction between Fas and FasL, since a mutant form of FasL ($FasL^{mut}$), which contains a single point mutation in the extracellular domain that prevents Fas binding (Y218R) (Schneider et al., 1997), abolished rejection, and tumors developed in all 10 mice (Figure 1C). This is consistent with previous observations that FasL can activate neutrophils via Fas and that tumor rejection is prevented in lpr mice that express a mutated nonsignaling Fas (Hohlbaum et al., 2000; Shimizu et al., 2001). FasL has a long and conserved cytoplasmic domain rich in proline residues (Hane et al., 1995; Suda et al., 1993), which could potentially allow "reverse signaling" via FasL to the tumor cell, perhaps causing the secretion of neutrophil chemoattractants. However, deletion of this domain ($FasL^{trunc}$ Figures 1D and 1E) has no influence on rejection, implying that it is the engagement of Fas, rather than FasL reverse signaling, that leads to tumor rejection.

Further support for role of neutrophils was obtained when tumors were injected into Mip1α deficient mice. Mip1α has previously been shown to act as a neutrophil chemoattractant (Alam et al., 1994; Lira et al., 1994). In agreement with these observations, the ability to reject B16FasL was impaired in Mip1α deficient mice. Although the kinetics of tumor growth were slightly slower for the B16FasL tumor versus the B16WT control, 7/10 mice injected with the B16FasL developed tumors, suggesting a possible role for this neutrophil chemoattractant in FasL-mediated tumor rejection (Figure 1F).

FasL-expressing melanoma induces tumor immunity

Of 123 mice vaccinated with a large dose of live B16FasL, 56% remained tumor-free (Table 1). When tumor-free vaccinated mice were challenged with 5×10^5 B16WT, about half of the 69 mice (54%) rejected the second tumor. These results were confirmed using two independent sublines of B16WT. For subsequent experiments, it was established that 10^7 irradiated B16FasL could protect against a second tumor. This has the advantage of preventing the growth of the FasL-transfected tumors occurring in approximately 44% of mice given live B16FasL. All of the mice given irradiated B16FasL remained tumor-free. 70% of these mice treated with irradiated B16FasL developed tumor immunity (n = 90) and rejected tumor when challenged with B16WT. As expected, irradiated B16WT did not

Table 1. The primary B16FasL is rejected and induces tumor immunity

	% mice tumor-free	
Pretreatment/primary challenge (tumor type)	Primary challenge[a]	Secondary challenge with B16WT[b]
B16WT 5×10^5 live	6% (n = 34)	N/A
B16FasL 5×10^5 live	56% (n = 123)	54% (n = 89)
B16WT 10^7 irradiated	N/A	10% (n = 50)
B16FasL 10^7 irradiated	N/A	70% (n = 90)

[a] Mice were either injected with 5×10^5 B16FasL or 10^7 irradiated B16FasL.
[b] Four to eight weeks later, tumor-free mice were challenged with 5×10^5 B16F10 wild type tumor.

Table 2. CD8 lymphocytes do not confer protection in B16FasL-immunized mice

		Tumor-free mice	
		Experiment 1	Experiment 2
A:	Isotype control depletion	4/4	5/5
	$CD4^+$ depletion	4/4	6/6
	$CD8^+$ depletion	4/4	6/6
B:	Transfer of $CD8^+$	0/3	0/4
	Transfer of $CD4^+$ and $CD8^+$	0/3	ND
	Transfer of $CD4^+$-depleted splenocytes	ND	0/4

A: Protected mice were treated twice with a pair of αCD4 or αCD8-depleting antibodies, then challenged the following day with 5×10^5 B16WT. Tumor growth was monitored over a period of 5 months.
B: Lymphocytes were transferred from protected mice into naïve recipients, then challenged with 5×10^5 B16WT. Tumor growth was monitored over a period of 8 weeks.

give rise to tumor immunity (Table 1), confirming results of other studies (Dranoff et al., 1993). Similar results were obtained using several different clones expressing FasL (data not shown).

Tumor immunity can be transferred by serum, but not by $CD4^+$ or $CD8^+$ T cells

Mice were vaccinated with B16FasL and challenged with B16WT tumor. Animals that rejected the tumor challenge were then selected for further study and are subsequently referred to here as "protected mice." To determine which cells are critical for tumor immunity, protected mice were depleted of $CD4^+$ or $CD8^+$ T cells by administration of depleting antibodies in vivo. Mice were then rechallenged with B16WT. Both $CD4^+$ and $CD8^+$ T cells depleted mice were still able to reject the tumor (Table 2). Similarly, the transfer of purified $CD8^+$ or $CD8^+/CD4^+$ lymphocytes from protected mice did not prevent tumor growth. We also tested fresh ex vivo killing of B16WT or targets infected with vaccinia viruses encoding melanocyte differentiation antigens. At a variety of effector target ratios, we detected no lytic activity from splenocytes or purified $CD8^+$ lymphocytes (data not shown).

The lack of role of CD4 or CD8 T cells in mediating the tumor immunity prompted us to test for the development of a humoral antitumor response. Serum was collected from protected mice leaving a minimum period of 7 weeks after injection of B16FasL. This was pooled and then injected into naïve mice, which were subsequently challenged with 5×10^5 B16WT. 14/17 mice given serum were protected from challenge, whereas all of 17 given normal mouse serum developed tumor (Figure 2A). Serum was still protective when obtained 6 months after vaccination, and serum from unvaccinated tumor-bearing mice failed to protect (data not shown).

To show that the tumor protection was mediated by antibody, the immunoglobulin fraction of the serum from B16FasL-treated or control mice was purified over a Protein L column, known to bind a high proportion of murine IgG and IgM. 1.2 mg of purified Ig was injected intravenously, followed by challenge with 2×10^5 B16WT. In this experiment, all four mice given control Ig developed tumor at day 26, while 3/4 of mice given serum from protected mice remained tumor-free (Figure 2A).

To determine whether tumor immunity is established in a T cell-dependent manner, mice were depleted of CD4 or CD8 T cells before vaccination with B16FasL. Depletion of CD8 did not affect the ability of protected mice to reject a secondary challenge with B16WT. However, the majority of protected mice depleted of CD4 developed tumor when challenged (Figure 2B). This was confirmed in a second experiment where mice were thymectomised and treated with depleting antibodies for CD4 and CD8 T cells. These profoundly T cell-deficient mice were injected with B16FasL and subsequently challenged with B16WT. Ten control, nondepleted mice rejected the tumor, while 3/10 T cell-deficient mice rejected tumor. Thus, although mediated by antibody, the immune response to B16WT requires CD4 T cell help.

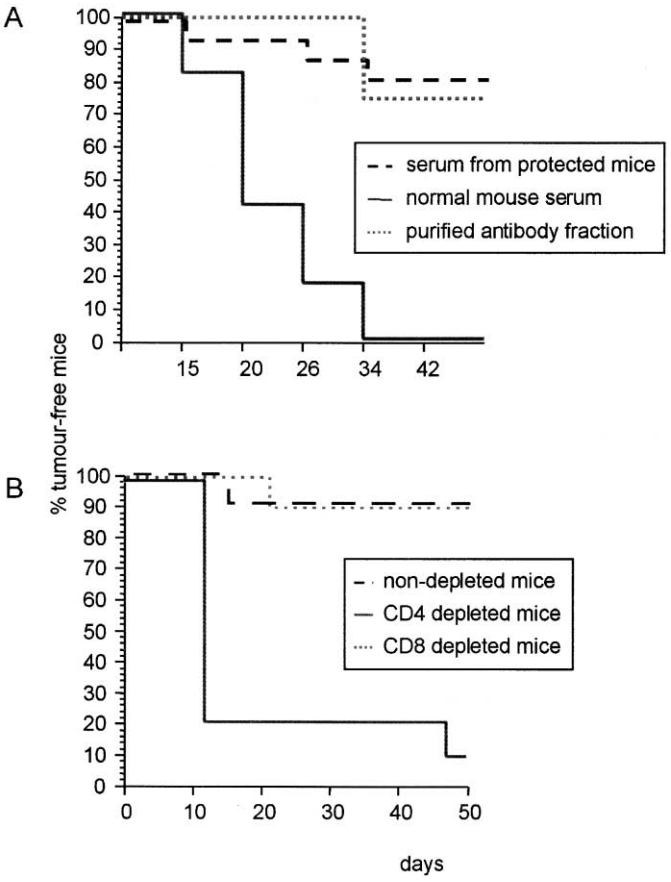

Figure 2. Serum from protected mice transfers tumor immunity, and protection is dependent on CD4 T cells

A: 200 μl of serum or 1.2 mg purified antibodies from protected mice was transferred into naïve C57BL/6 mice, and mice were challenged the following day with 5×10^5 or 2×10^5 B16WT, respectively.
B: C57BL/6 mice were depleted of CD4 or CD8 T lymphocytes, injected with 10^7 B16FasL, and then challenged with 5×10^5 B16F10.

The serum from immunized mice reacts with melanoma antigens and can trigger complement-mediated lysis of B16F10

B16WT (melanoma line from C57BL/6 mouse strain), K1735 (melanoma line from the C3H mouse strain), MC57 (methylcholanthrene-induced fibrosarcoma cell line from C57BL/6 mice), 293T cells (human embryonal kidney fibroblast), HuTK-143B

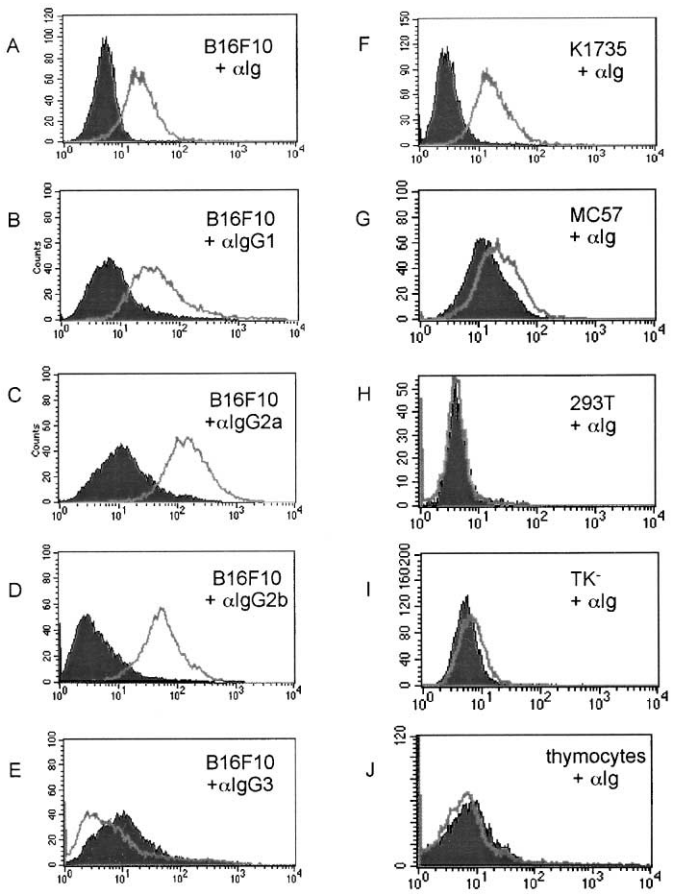

Figure 3. Serum from protected mice surface stains B16F10 and includes the isotypes IgG1, IgG2a, IgG2b

Tumor and primary cells were stained by indirect immunofluorescence using the indicated secondary antibodies and analyzed by FACS. Dark shading-control serum, open line-serum from protected mice.

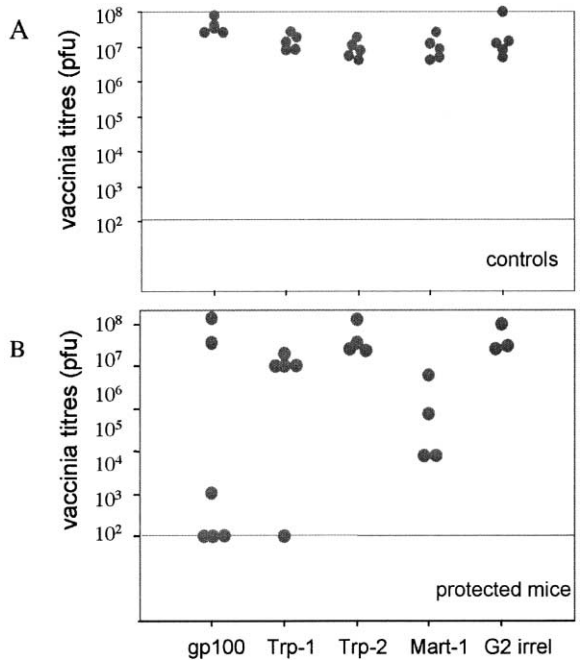

Figure 4. Protected mice show responses to melanocyte antigens

Mice were injected with vaccinia virus expressing the melanocyte differentiation antigens gp100, Trp-1, Trp-2, Mart-1, or an irrelevant antigen, G2. Five days later, viral titers were determined in ovaries.

A: Naïve mice.

B: Protected mice.

This experiment was repeated twice with similar results. The dashed line represents the limit of sensitivity of the assay.

(TK$^-$) cells, (human osteoblastoma cell line) and thymocytes from C57BL/6 mice were stained by indirect immunofluorescence using serum from protected, tumor-bearing, or control mice. FACS analysis showed no staining with either control serum or serum from tumor-bearing mice, whereas strong staining of B16WT was seen using the serum from protected mice (Figure 3A). Serum also stained the other melanoma cell line K1735 (Figure 3F). There was a small amount of staining of MC57 cells but not of 293T, TK$^-$, or thymocytes from C57BL/6 (Figures 3G–3J). All cells were cultured under the same conditions and the negative results on some of these indicate that antigens recognized by the polyclonal serum are not due to contaminants present in the culture medium. Seven weeks after injection of B16FasL, antibodies were of the IgG1, IgG2a, and IgG2b isotypes (Figures 3B–3D). No specific antibodies of the IgG3 (Figure 3E) or IgE or IgM isotypes were detected in the serum of protected mice, although specific IgM antibodies were present in the earlier phase of the immune response (less than seven weeks after injection of B16FasL).

Tumor-immune mice react to melanocyte differentiation antigens

In a search for antigen-specific responses induced by vaccination with B16FasL, we took advantage of a panel of vaccinia viruses expressing the melanocyte differentiation antigens gp100, Trp-1, Trp-2, Melan-A/Mart-1, or an irrelevant antigen (G2, a glycoprotein from lymphocytic choriomeningitis virus) (Overwijk et al., 1998). When injected into naïve mice, these viruses replicate, and this can be assayed by counting plaque-forming units from ovaries infected with vaccinia virus. If the mouse has preexisting immunity against melanocyte antigens carried by the virus, then replication will be inhibited and the ovary plaque count reduced. Vaccinia titers measured in ovaries were consistently high in control mice (Figure 4A). The majority of vaccinated mice infected with vac-gp100 cleared the virus or had reduced viral load (4 out of 6, Figure 4B), one vaccinated mouse cleared vac-Trp-1, and viral titers to Mart-1/MelanA were lowered in 2 mice by a factor of 10^3 as compared to controls (Figure 4B). It is possible that B16FasL will also break tolerance to other tumor-associated and tumor-specific antigens expressed by B16, and that the protection we observe may well be improved by such a polyspecific response. The observation that approximately 20% of mice exhibited depigmentation at the site of tumor inoculation (B16FasL) further supports the findings that immune responses to melanocyte antigens were induced in these mice.

B16FasL can mature dendritic cells

To gain more insight into how FasL expression might help prime tumor specific responses, we examined whether B16FasL could mature dendritic cells (DC). Bone marrow-derived DCs were cultured in vitro for 4 days and matured in the presence of

irradiated B16WT, B16FasL, or soluble FasL-FLAG fusion protein. FasL was incubated either alone or crosslinked with anti-FLAG mAb, then washed and added to allogeneic splenocytes in a 4-day proliferation assay using tritiated thymidine. Activation of splenocytes was improved significantly when DCs were matured in the presence of B16FasL or soluble crosslinked FasL (Figures 5A and 5B). The maturation markers MHC class II, CD83, and CD86 were also upregulated on DCs cultured with FasL (Figure 5C).

Antibody-mediated tumor immunity is dependent on Fc Receptors (FcRs)

As complement and Fc receptor mediate effector functions of antibodies, we sought to determine the role of these two components during the tumor rejection in the protected mice. In an in vitro complement lysis assay, protected but not preimmune serum gave significant lysis of B16WT (Figure 6A). Next, we depleted complement in protected mice by repeated injections (30 μg/mouse each injection) of Cobra venom factor (CVF), which depletes C3 (Shapiro et al., 2002). The mice were then challenged with B16WT. In two separate experiments, 4/5 controls and 7/8 CVF-treated mice rejected tumor.

To look for a role of antibody-dependent cell-mediated cytotoxicity (ADCC), we injected protected serum into Fc receptor γ chain-deficient mice (FcRγ$^{-/-}$, which lack FcγRI, FcγRIII, and FcεRI) (Takai et al., 1994). Mice were given serum from protected or control mice and then challenged with B16WT. All control mice were protected from tumor when injected with protected serum, whereas in the FcRγ$^{-/-}$, the same serum no longer provided protection (Figure 6B).

Discussion

FasL has an interesting pedigree in the field of tumor immunology. Although Fas is expressed on a number of nonlymphoid tissues, such as the liver, FasL expression is tightly regulated, being found predominantly on activated lymphocytes. The role of FasL in the immune system is well studied; it is one of the mechanisms used by lymphocytes to kill targets expressing Fas (Krammer, 2000), and it has also been suggested that Fas can deliver an activating signal to T cells (Kennedy et al., 1999). In addition, activated lymphocytes that coexpress Fas and FasL become susceptible to apoptosis (Janssen et al., 2000). The importance of this for the control of peripheral T cell populations is illustrated by the lymphoproliferation and autoimmunity developed by humans or mice with mutations in Fas or FasL (Matiba et al., 1997).

A few extralymphoid tissues including the brain, eye, and testis have been shown to express FasL. Expression of FasL at such sites is proposed to limit inflammation and confer immunoprivilege by allowing them to kill infiltrating lymphocytes expressing Fas (Bellgrau et al., 1995; Griffith et al., 1995; Saas et al., 1997). These observations incited much interest in several fields, most notably transplantation and tumor immunology. Initial observations demonstrated expression of FasL on some tumors, where it was proposed to allow them to escape the immune response. Following these observations, a number of investigators tested the effects in tumor cells or transgenic organs overexpressing FasL. Often the results were disappointing (from the tumor's standpoint); tumor cells or transgenic tissues fared worse than their nontransfected counterparts (Green and

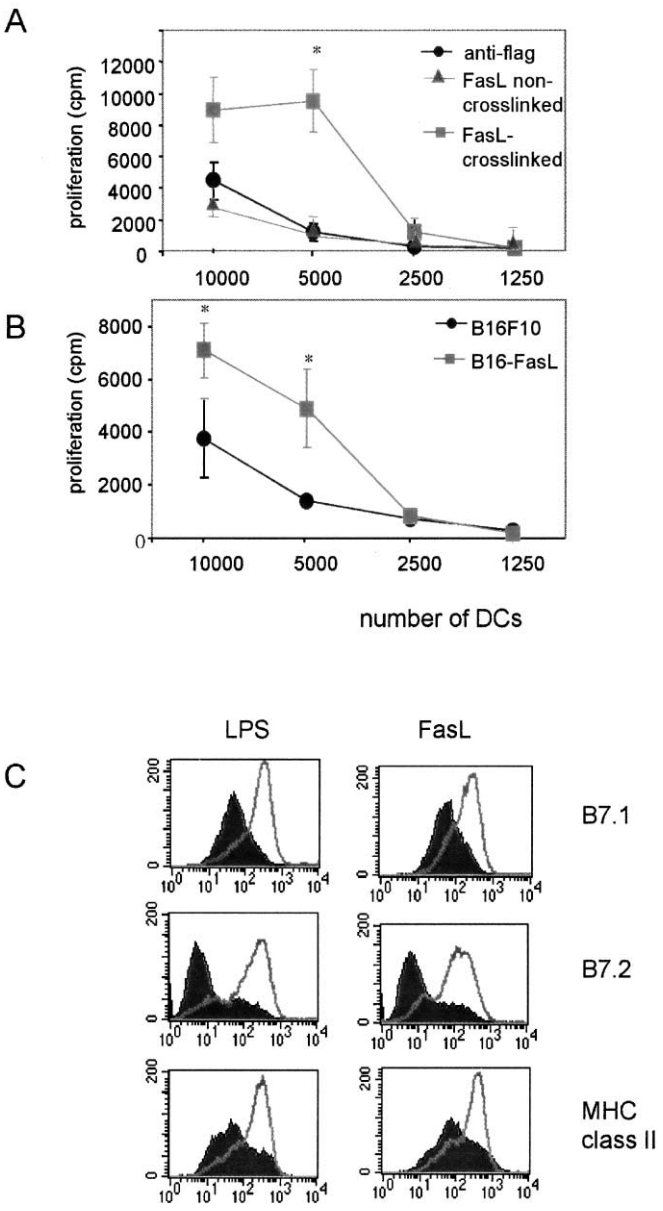

Figure 5. Dendritic cells mature in the presence of B16FasL

A and B: After culture in GM-CSF and IL-4, bone marrow dendritic cells from C57BL/6 mice were matured overnight with anti-FLAG mAb as control, or FasL noncrosslinked or crosslinked with anti-FLAG (**A**), or irradiated B16WT or B16FasL (**B**). BALB/c spleen cells were added to increasing numbers of dendritic cells, and proliferation was measured by ^{3}H thymidine incorporation. Background proliferation in the absence of spleen cells was subtracted. This experiment was repeated three times with similar results. * $P < 0.05$, Student's T test. Maturation markers of dendritic cells were stained by mAb after maturation with recombinant FasL overnight.

C: Dark shading, anti-FLAG alone; open line, maturation with LPS or recombinant FasL crosslinked.

Ferguson, 2001). The expression of FasL in a number of instances provoked an intense neutrophil infiltrate (Allison et al., 1997; Seino et al., 1997). Neutrophils express Fas and can be activated by FasL to become cytotoxic (Chen et al., 1998). It is believed that this neutrophil activation leads to tumor rejection, and blocking with soluble Fas-Fc fusion protein reduced neutro-

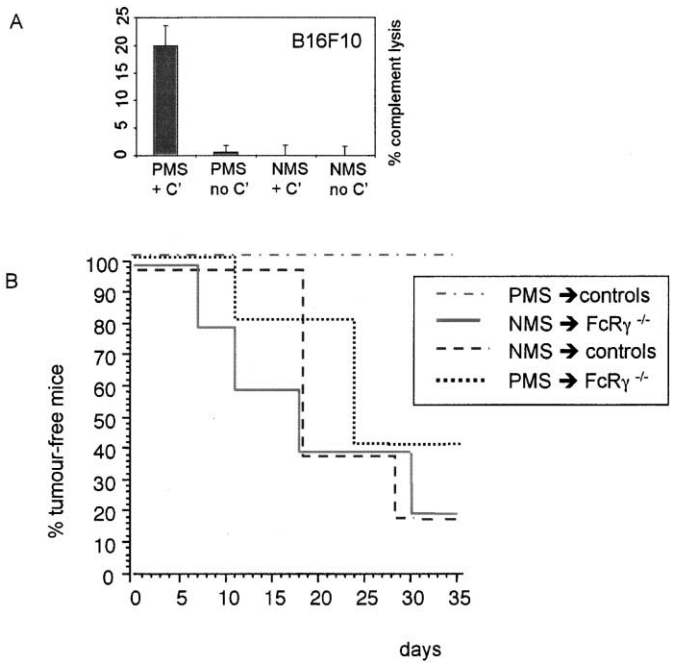

Figure 6. The serum mediated tumor immunity is FcR-dependent

A: Serum from protected mice (PMS) was used in a complement lysis assay. Normal mouse serum (NMS) and wells without complement were included as controls. This panel is representative of three separate experiments.
B: NMS or PMS was injected into FcRγ-deficient mice or age-matched C57BL/6 controls, which were then challenged with 1×10^5 B16WT.

phil activation and killing of FasL-transfected tumor cells (Chen et al., 1998). Our results using B16F10 transfected with a FasL mutant which fails to bind Fas confirm these results.

However, it is not entirely clear why FasL-transfected tumors induce such a brisk neutrophil infiltrate. Previous studies have suggested a role for IL1β, as FasL-transfected fibrosarcoma was not rejected in IL1β-deficient mice (Miwa et al., 1998). In addition, soluble FasL can also attract neutrophils, although expression of a soluble form of FasL in tumor cells did not lead to their rejection, or to a neutrophil infiltrate (Hohlbaum et al., 2000; Ottonello et al., 1999; Seino et al., 1998). In this study, we tested rejection in mice deficient in MIP1α. These mice were also less efficient at rejecting the tumor, suggesting a possible role for MIP1α as a neutrophil chemoattractant in FasL-induced tumor rejection (Lee et al., 2000). Consistent with this finding is the recent demonstration that membrane-bound FasL leads to increase of mRNA expression of MIP1α and other proinflammatory mediators (Hohlbaum et al., 2001). As soluble FasL does not induce neutrophil infiltration, we examined the role of the cytoplasmic domain of FasL. This proline-rich domain of around 70 amino acids is highly conserved between species and can bind the src kinase fyn in vitro (Hane et al., 1995). Previous studies have shown a role for FasL in the proliferation of $CD8^+$ cells and also in cell cycle arrest of $CD4^+$ cells (Desbarats et al., 1998; Suzuki and Fink, 1998). Thus, it has been suggested that FasL may deliver a "reverse signal" via the cytoplasmic domain. In our experiments, deletion of the cytoplasmic domain had no effect on the kinetics of tumor growth ex vivo (data not shown), and likewise had no effect on tumor rejection in vivo.

Although the initial rejection of FasL-expressing tumor has been studied extensively, little is known about how this affects long-term tumor immunity. In a previous study, a CD8-dependant protective response to lymphoma was induced by treatment with tumor transfected with FasL (Seino et al., 1997; Shimizu et al., 1999). In our experiments, depletion of $CD8^+$ cells had no effect, perhaps because B16F10 expresses low levels of MHC class I. Similarly, depletion of $CD4^+$ cells from protected animals did not reduce tumor rejection, although transfer of $CD4^+$ cells and splenic B cells prevented tumor growth in 2/4 animals while slowing growth in the other 2/4 (data not shown).

The B16F10 melanoma model has recently been shown to belong to the type of tumors that are weakly immunogenic because they grow as nodules "walled off" from the immune system, and in addition it expresses low levels of MHC class I, preventing activation of antitumor immunity mediated by $CD8^+$ lymphocytes (Ochsenbein et al., 2001). Thus, the B16F10 is a particularly difficult tumor model in which to raise an adaptive immune response, and might be representative of many human tumors (Pardoll, 2001).

To our surprise, serum provided almost complete protection against a secondary tumor challenge. The serum from protected mice can recognize surface determinants on B16F10 and the unrelated melanoma cell line K1735. It is impossible at this stage to say how broad the response is or which antigens on the melanoma are responsible for the protection afforded by serum transfer. We are planning to produce monoclonal antibodies from the protected animals to define some of these antigens. However, our screen of melanocyte differentiation antigens expressed in recombinant vaccinia virus has identified some antigens. gp100 and Trp-1 responses were detected in the protected mice. Trp-1 can be expressed at the cell surface, and passive transfer of antibodies against Trp-1 has been shown to induce rejection of melanoma in vivo (Hara et al., 1995; Naftzger et al., 1996).

How FasL is able to elicit these responses is at present not entirely clear. It seems likely that the effect is multifactorial. First, the recruitment and activation of neutrophils by FasL is likely a crucial event, as it not only allows early innate killing of the tumor, but also leads to the secretion of proinflammatory cytokines and further immune activation (Fuchs and Matzinger, 1996). Dendritic cells may be recruited by this milieu and induced to take up tumor cells/antigen. Furthermore, FasL expressed on the tumor can help mature the DCs, leading to increased expression of costimulatory molecules and consequent priming (Rescigno et al., 2000) of CD4 T cells, which are clearly needed to establish this immune response (Figure 2). The isotypes of the specific antibodies are IgG1, IgG2a, and IgG2b, indicating a mixed TH1/TH2 pattern. The use of other proinflammatory cytokines expressed on tumor cells such as GM-CSF, B7-1, MHC class II, or cytokines may well work in a similar fashion by inducing effective DC maturation and crosspriming (Smyth et al., 2001). Recent evidence suggests that the innate antitumor response mediated by natural killer cells modulates the development of the adaptive immune response (Kelly et al., 2002). Similarly, it is possible that the innate immune response initiated by FasL induces the humoral antitumor response found in this study. Our experiments suggest a role for ADCC in tumor destruction, as the tumor rejection was much reduced in $FcR\gamma^{-/-}$ mice.

Several monoclonal antibodies, such as anti-CD20 and anti-HER2, are already used in the treatment of cancer, and a larger number are currently undergoing clinical trials (Houghton and Scheinberg, 2000). Antibodies are attractive for the treatment of cancer, as they have many modes of action: blocking or stimulation of a cell surface receptor, or cytotoxicity via complement or via ADCC. We show here that ADCC can account for some protection in this system. Finally, if inactive on these counts, conjugated antibody can be used to deliver drugs or toxins to cancer cells. Single monoclonals will have their limitations, as they put a huge selective pressure on the tumor to lose antigen expression, as do therapies inducing $CD8^+$ T cells, where loss of MHC class I will abolish responses against all antigens. In order to produce a truly effective therapeutic tumor vaccine/immunotherapy in humans, it will probably be necessary to induce responses against a number of antigens using multiple effector mechanisms. Our observations here may help in the definition of new cell surface tumor markers and may provide an additional route to stimulate a broader antitumor response.

Experimental procedures

Tumor cells and mice

Tumor cells were maintained in RPMI (Sigma) supplemented with 10% fetal calf serum (FCS), L-glutamine, penicillin-streptomycin. FasL was cloned C-terminal to GFP in pEGFP-C1 (Clontech). Mutants lacking the 72AA cytoplasmic chain and/or a point mutation that abolishes binding to Fas (Y218R) were created by PCR. B16F10 were transfected with DMRIE (Gibco) and selected on 1.5 mg/ml G418; single cells were cloned by FACS and expression of FasL checked using Nok-1 (Pharmingen). The in vivo results are representative of several different clones. 5×10^5 live or 1×10^7 irradiated tumor cells were injected into C57BL/6 mice and tumor growth measured. C57BL/6 mice were bred in our animal facility, and the $FcR\gamma^{-/-}$ were obtained from Taconic (Germantown, NY).

Depletion/transfer of $CD4^+$ and $CD8^+$ lymphocytes

Pairs of rat IgG2b hybridomas secreting anti-CD4 (YTS191.1.2, YTS33.1.2) and anti-CD8 (YTS169.4.2.1, YTS156.7.7) were used for depletions as described previously (Qin et al., 1987). 100 μg of antibody was injected intraperitoneally 1 and 3 days prior to injection of 5×10^5 B16WT or 10^7 B16FasL. One day after the last injection, less than 1% of $CD4^+$ or $CD8^+$ were detected in peripheral blood by FACS.

Transfer of $CD4^+$, $CD8^+$, B cells, and serum

$CD4^+$ and $CD8^+$ lymphocytes from spleen and inguinal lymph nodes were purified by positive selection using directly conjugated beads to anti-CD4 or anti-CD8 (Miltenyi Biotec), or using antibodies against CD4 (Pharmingen), CD8 (Caltag), or B220 (Pharmingen) followed by anti-rat beads. Purity of CD4 and CD8 was between 70% for $CD8^+$ and 95% for $CD4^+$ cells. 10^7 CD4 and 5×10^6 CD8 per mouse were reinjected intravenously (i.v.) the day of purification. To obtain serum, blood was clotted at 37°C for 1 hr, then kept at 4°C for at least 1 hr, and spun at 20,000 g for 10 min. 200 μl of serum per mouse was reinjected i.v. Purification of the Ig fraction of serum was performed with a protein L column (Pierce) according to the manufacturer's protocol. Protein L binds to the κ light chain and allows purification of IgM and IgG and possibly other murine antibody isotypes (Svensson et al., 1998). 1.2 mg of the Ig fraction was reinjected per mouse. For all transfer experiments, mice were challenged the day following transfer with 5×10^5 B16WT.

Proliferation assay with dendritic cells

Murine dendritic cells were isolated from bone marrow. Erythrocytes were depleted with lysis buffer (Flowgen). Cells were cultured with RPMI 5% FCS (Hyclone) with 500 U/ml IL-4 (Peprotech) and 1000 U/ml GM-CSF (Peprotech). Cultures were fed on day 2 and day 4 by aspirating off medium and adding fresh medium. In some experiments, DCs were fed only on GM-CSF (200 U/ml), and medium was changed on days 2 and 3 by discarding adherent and loosely adherent cells. Using this protocol, murine DCs have an immature phenotype on day 4. On day 4, soluble FasL-flag was added alone or crosslinked with 5 μg/ml of anti-flag. Alternatively, DCs were incubated with irradiated (12,000 rad) B16F10 FasL overnight. Allogeneic proliferation was set up on day 5 by culturing 10^5 BALB/c spleen cells with 10^4 C57BL/6 dendritic cells that had been matured previously in the presence or absence of FasL. Four days later, proliferation was assayed ^{3}H-thymidine incorporation.

Vaccinia assay

Mice were infected intraperitoneally with 2×10^6 PFU of vaccinia expressing different melanocyte antigens or control antigens. Both ovaries were harvested at 5 days, and the vaccinia titers were determined on TK^- monolayers as described previously (Binder and Kundig, 1991).

Flow cytometry and complement lysis assay

FACS staining with serum was performed at a concentration of 1/100, and control serum was obtained from untreated or nonimmunized mice with tumors. Second layer antibodies included anti-mouse Ig (Dako), anti-IgG1, IgG2a, IgG2b, and IgG3 (Serotec). For the complement assay, B16F10 cells were labeled for 90 min with ^{51}Cr, washed thoroughly, and plated out at 2.5×10^4 per well. Pooled serum from protected mice or normal mouse serum was added at 1:5 dilution for 1 hr, washed off, then rabbit complement (Low-Tox; Cedarlane, Canada) was added at 1:10 dilution for 3 hr. Chromium release into the supernatant was determined by liquid scintillation.

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