Decreased Tumor Surveillance in Perforin-deficient Mice

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Summary

Immune surveillance against tumors usually depends on T cell recognition of tumor antigens presented by major histocompatibility complex (MHC) molecules, whereas MHC class I$^-$ tumors may be controlled by natural killer (NK) cells. Perforin-dependent cytotoxicity is a major effector function of CD8$^+$ MHC class I–restricted T cells and of NK cells. Here, we used perforin-deficient C57BL/6 (PKO) mice to study involvement of perforin and Fas ligand in tumor surveillance in vivo.

We induced tumors in PKO and normal C57BL/6 mice by (*a*) injection of different syngeneic tumor cell lines of different tissue origin in naive and primed mice; (*b*) administration of the chemical carcinogens methylcholanthrene (MCA) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) plus 7,12-dimethylbenzanthracene (DMBA), or (*c*) by injection of acutely oncogenic Moloney sarcoma virus. The first set of models analyzes the defense against a tumor load given at once, whereas the last two sets give information on immune defense against tumors at the very moment of their generation. Most of the tumor cell lines tested were eliminated 10–100-fold better by C57BL/6 mice in an unprimed situation; after priming, the differences were more pronounced. Lymphoma cells transfected with Fas were controlled 10-fold better by PKO and C57BL/6 mice when compared to untransfected control cells, indicating some role for FasL in tumor control. MCA-induced tumors arose more rapidly and with a higher incidence in PKO mice compared to C57BL/6 or CD8-deficient mice. DMBA+TPA–induced skin papillomas arose with similar high incidence and comparable kinetics in both mouse strains. C57BL/6 and PKO mice have a similar incidence of Moloney murine sarcoma and leukemia virus–induced sarcomas, but tumors are larger and regression is retarded in PKO mice.

Thus, perforin-dependent cytotoxicity is not only a crucial mechanism of both cytotoxic T lymphocyte– and NK-dependent resistance to injected tumor cell lines, but also operates during viral and chemical carcinogenesis in vivo. Experiments addressing the role of Fas-dependent cytotoxicity by studying resistance to tumor cell lines that were stably transfected with Fas neither provided evidence for a major role of Fas nor excluded a minor contribution of Fas in tumor surveillance.

Tumor cells arise in individuals as a result of exposure to mutagenic chemicals, radiation, viruses, or spontaneous mutations (1). Whether or not an individual succumbs to the tumor depends on the balance between growth kinetics and metastatic capacity of the tumor versus the ability of the host to control the tumor. For this, the host has two major mechanisms at its disposal: innate and acquired immunity. Innate immunity (e.g., NK cells) is not antigen specific and is able to act quickly and without priming. Acquired immunity depends on priming, is therefore relatively slow but highly specific, and has a memory. A tumor can prime acquired immunity if it presents tumor-specific antigens in the context of MHC class I or class II molecules to CD8$^+$ or CD4$^+$ T cells, respectively (1–3). Furthermore, expression of costimulatory molecules (e.g., B7, intracellular adhesion molecule-1, and LFA-3) has been shown to dramatically increase the efficiency of priming in certain instances (4–7). Under selective pressure, however, tumor escape variants that lack MHC class I or II molecules, costimulatory molecules, or tumor-specific antigens (8–11)

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may arise. Some escape variants that had lost MHC class I expression are efficiently controlled in vivo by NK cells (12, 13) in a strictly perforin-dependent way (13).

Perforin-deficient (PKO)[1] mice have been generated on a C57BL/6 genetic background (H-2^b) by homologous recombination and are healthy under specific pathogen-free conditions (14). PKO mice completely lack perforin-dependent cytotoxicity, whereas the Fas (CD95)/FasLigand (FasL)–dependent cytotoxic pathway is unimpaired (14–22). Therefore, PKO mice allow the assessment of the relative contribution of each of the two major cell-mediated cytotoxic pathways to a variety of immune responses in vivo and in vitro (14, 15, 17–22). We have shown previously that the defense against infection with the noncytopathic lymphocytic choriomeningitis virus (LCMV) or, to a lesser extent, with the intracellular bacterium *Listeria monocytogenes* depends on perforin-dependent cytotoxicity (14, 17, 23). Control of syngeneic MC57G fibrosarcoma cells was 10-fold better in C57BL/6 mice compared to PKO mice (14). Also, the development of insulin-dependent diabetes mellitus in a transgenic model (24) is perforin dependent. On the other hand, PKO mice were normal with respect to control of infection with cytopathic viruses like vaccinia virus (VV) or Semliki forest virus (25). Finally, a role for perforin-dependent cytotoxicity was demonstrated in rejection of MHC class I–disparate allografts such as heart (26) and allogeneic tumor cell lines (27), as well as in mixed lymphocyte reactions in vitro (14, 15), and in GVHD (28).

Here, we evaluate the role of perforin- and Fas-dependent cytotoxicity in the control of a variety of established, transplantable syngeneic MHC class I^+ tumor cells in vivo and assess the susceptibility of PKO mice to primary individually arising tumors induced by chemical carcinogens and by an oncogenic virus.

Materials and Methods

Mice

Inbred C57BL/6 mice were obtained from the Institute für Zuchthygiene (University of Zürich, Switzerland). PKO (14), CD8-deficient mice ($CD8^{-/-}$; 29), and RAG-2–deficient mice ($RAG2^{-/-}$; 30) have been described before and were bred under specific pathogen-free conditions in our own facilities. PKO mice had a C57BL/6 (H-2^b) background. Mice at least 7 wk old were used in all experiments that were performed according to cantonal and federal laws on animal protection, which requires use of minimal numbers of experimental animals.

Cell Lines, Antibodies, and Viruses

RMA is a mutagenized RBL-5 (Rauscher virus–induced T lymphoma [12]) cell line. EL-4 is a 9,10-dimethyl-1,2-benzanthracene-induced T lymphoma (American Type Culture Collection [ATCC], Rockville, MD). B16 is a spontaneous melanoma, MC57G is a methylcholanthrene-induced fibrosarcoma (31), and MBL-2 is Moloney virus–induced lymphoma. N1 are EL-4 cells stably transfected with the nucleoprotein (NP) of vesicular stomatitis virus (VSV), and V1 is a mock-transfected control (N1 and V1 were originally obtained from L. Lefrancois, Division of Rheumatology, Connecticut Health Center, Farmington, CT; 32). SR23B adenovirus type 5 early region 1 (Ad5E1) + E*Jras* transformed tumor cell line was generated from mouse embryonic cells as described previously (33) and contains an Ad5E1B-encoded CTL epitope. All cell lines were of C57BL/6 origin and were maintained in IMDM supplemented with 5% FCS and antibiotics.

Monoclonal rat anti–mouse CD4 (YTS191.1) and monoclonal rat anti–mouse CD8 (YTS169.4) were originally obtained from Dr. H. Waldmann (Dunn School of Pathology, Oxford, UK; 34). Treatment involved injection of 2 mg i.p. of the appropriate antibody. Efficiency of depletion was checked by FACS® analysis (Becton Dickinson & Co.). Purified monoclonal hamster anti–mouse TNF-α TN3-19.12 has been described (35) and was kindly provided by Dr. R. Schreiber (Washington School of Medicine, St. Louis, MO); TN3-19.12 was used in vivo at 0.25 mg/wk (two injections maximal) to neutralize TNF-α.

VSV Indiana (Mudd-Summer isolate) was originally obtained from Dr. D. Kolakofsky (University of Geneva, Geneva, Switzerland) and were grown on BHK21 (baby hamster kidney, ATCC CRL 8544) cells infected with low multiplicity of infection (moi) and plaqued on Vero cells. VV expressing the NP of VSV (VV-VSV-NP) was kindly given by Dr. B. Moss (National Institutes of Health, Bethesda, MD; 36). VV expressing FasL has been described (37). All VV stocks were made on BSC40 cells. LCMV, isolate WE (LCMV-WE), was initially obtained from Dr. F. Lehmann-Grube (Heinrich-Pette Institüt für Experimental Virologie und Immunologie, Universität Hamburg, Germany; 38) and was propagated in L929 fibroblast cells. Moloney murine sarcoma and leukemia virus (MoMSV) complex was prepared and titered as described previously (39).

Transfection of Cell Lines with Fas

MBL-2 cells were stably transfected with a construct carrying the mouse Fas gene and the neomycin resistance gene (kindly provided by Dr. P. Golstein, Centre d'Immunologie INSERM-CNRS, Marseille, France; 22) by electroporation and were subsequently selected with 0.9 mg/ml geneticin (G418; Gibco BRL, Basel, Switzerland). G418-resistant cells were checked for Fas expression by FACS® analysis: 1 million cells were incubated with 10% normal hamster serum (15 min at 4°C) followed by biotin-labeled hamster anti–mouse Fas (Jo-2; PharMingen, San Diego, CA) mAb. Cells were stained with streptavidin-FITC or streptavidin-PE. Fas^+ cell lines were subcloned by limiting dilution. MBL-2 and MBL-2.Fas cells were insensitive to TNF-mediated cytotoxicity in vitro (data not shown).

Fas-dependent Cytotoxicity Assay

To test whether the transfected Fas was functional, Fas-transfected (Fas^+) and control (Fas^-) cell lines were labeled with $Na^{51}CrO_4$ and were used as targets for $FasL^+$ effectors. $FasL^+$ effectors were generated by infecting MC57G fibroblasts with VV-FasL (37) for 2 h with a multiplicity of infectin (moi) of 2. Effectors and targets (10^4 per well) were incubated for 5 h at 37°C in a standard ^{51}Cr release assay. In addition, PKO and control

[1] *Abbreviations used in this paper:* DMBA, 7,12-dimethylbenzanthracene; FasL, Fas ligand; LCMV, lymphocytic choriomeningitis virus; LCMV-WE, LCMV isolate WE; MCA, methylcholanthrene; moi, multiplicity of infection; MoMSV, Moloney murine sarcoma virus, NP, nucleoprotein; PKO, perforin-deficient (mice); TPA, 12-O-tetradecanoylphorbol-13-acetate; VSV, vesicular stomatitis virus; VV, vaccinia virus.

Table 1. *C57BL/6-derived Tumor Cell Lines*

	Tumor	Class I*	Class II*	Fas*	Fas-mediated lysis‡
MC57G	MCA fibrosarcoma	330	3	8	0
MBL-2	Moloney B lymphoma	1,100	3	20	0
MBL-2.Fas	Moloney B lymphoma, Fas transfected	1,200	3	250	54
EL-4	DMBA T lymphoma	1,500	3	28	4
RMA	Rauscher T lymphoma	1,300	3	33	6
B16	Melanoma	40	3	10	0
SR23B	Adeno-transfected embryonic cells	75	3	10	0

*Cells were stained with biotin-labeled anti-MHC class I, anti–class II or anti-Fas mAb, followed by streptavidin-PE. Surface expression of all cell lines was measured by FACS® (CellQuest software) in one experiment and given in arbitrary units with the fluroescence of second stage antibody alone set at 3.

‡Percentage specific killing by VVFasL–infected MC57G effector cells at an E/T ratio of 10 in a 5-h ^{51}Cr release assay.

C57BL/6 mice were infected intravenously with 200 PFU LCMV-WE. 8 d later, their spleens were tested in a CTL assay for LCMV-specific cytotoxicity on ^{51}Cr-labeled, LCMV peptide (GP33, immunodominant CTL epitope in H-2^b mice)-loaded targets (40). Percentage of specific ^{51}Cr release = [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100%.

Tumor Induction In Vivo by Injection of Tumor Cell Lines

Normal Untreated Mice. PKO and C57BL/6 mice were injected intraperitoneally with different amounts of viable tumor cells (Table 1). Mice were monitored daily for tumor growth by determining their weight; they were killed if the weight increased to more than 15% compared to noninjected controls. In case of SR23B cells, subcutaneous tumor volume was determined with a caliper measuring two perpendicular diameters.

Effect of Priming with Viral Antigens. PKO and C57BL/6 mice were primed intravenously with 2×10^6 PFU VSV, with 2×10^6 PFU VV-VSV-NP, or were left unprimed. After 14 d, mice were injected intraperitoneally with different numbers of N1 (EL-4 thymoma expressing VSV-NP) or V1 (control EL-4), and tumor growth was monitored.

Effect of Priming with Tumor Antigens. PKO and C57BL/6 mice were primed intraperitoneally with 5×10^6 irradiated (100 Gy) MBL-2 cells in 0.2 ml PBS on days 0 and 7. On day 14, mice were injected intraperitoneally with different numbers of viable MBL-2 or MBL-2.Fas cells and were monitored for tumor growth. Alternatively, mice were primed subcutaneously with 10^7 irradiated (25 Gy) SR23B cells in 0.1 ml PBS on days 0 and 14 and challenged subcutaneously on day 28 with the same number of SR23B cells.

Male mice were used for all experiments to avoid possible tumor rejection caused by T cells recognizing the male antigen H-Y.

Tumor Induction by Tumorigenic Agents

Carcinogenic Chemicals. Groups of 10 C57BL/6, PKO, and $CD8^{-/-}$ mice were injected subcutaneously in the left hind leg with 0.1 ml corn oil containing 25, 100, or 400 μg methylcholanthrene (MCA), and mice were monitored weekly for fibrosarcoma development.

Alternatively, 100 μl acetone containing 10 nmol 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and 3 nmol 7,12-dimethylbenzanthracene (DMBA) was applied twice weekly on the shaved skin of the back to groups of eight C57BL/6 and PKO mice. Skin application was continued during the course of the experiment, and mice were observed twice per week for the development of papillomas (41).

MoMSV. Groups of 5–10 C57BL/6 and PKO (untreated, anti-TNF–treated, CD4- or CD8-depleted) or $CD8^{-/-}$ mice were injected in the left thigh with 100 μl PBS i.m. containing 10^3 focus forming units of MoMSV complex, as has been described (39). Mice were monitored for tumor growth, and tumor size was determined using a caliper measuring two perpendicular diameters.

Isolation and Culture of MCA-induced Sarcomas

When MCA-induced sarcomas had reached a volume of ~0.5 cm^3, mice were killed and tumors were removed aseptically. Tumors were cut into small pieces and were trypsinized for 5 h at room temperature, clumps were removed, and single cells were cultured in IMDM supplemented with 10% FCS, glutamine, and antibiotics. Cells were split when they reached confluency. All sarcomas could be kept in culture for at least 3 mo. The expression of MHC class I, class II, and Fas was determined by FACS® analysis.

Statistical Analysis

Results were compared statistically using the unpaired Mann-Whitney *U* test.

Results

Perforin Plays a Role in Surveillance against Most Established Tumor Cell Lines

PKO mice and C57BL/6 mice were injected with syngeneic tumor cell lines of different tissue origins, and tumor growth was monitored.

Tumors of Nonlymphoid Origin. B16 melanoma cells grew unrestricted in both C57BL/6 and PKO mice, even when

Table 2. *Control of Syngeneic Tumor Cell Lines by C57BL/6 and PKO Mice*

Tumor	C57BL/6		PKO	
	Primed*	Unprimed	Primed	Unprimed
MC57G	ND	10^6 (4/4)	ND	10^5 (4/4)
MBL-2	$>10^6$ (6/6)	10^4 (6/6)	10^5 (6/6)	$<10^2$ (6/6)
MBL-2.Fas	$>10^6$ (4/4)	10^5 (6/6)	10^6 (2/4)	10^3 (6/6)
RMA	$>10^6$ (4/4)	10^4 (7/7)	$>10^6$ (4/4)	10^4 (6/6)
RMA-S	ND	10^4 (8/8)	ND	10^2 (8/8)
EL-4	$>10^6$ (6/6)	10^4 (6/6)	$<10^2$ (6/6)	$<10^2$ (6/6)
B 16	ND	10^2 (3/3)	ND	10^2 (3/3)

The cell numbers in the table represent the lowest number of tumor cells that could not be controlled in vivo. The numbers in brackets are the number of mice with tumors out of the total number tested which were obtained in two to three independent experiments with groups of two to three mice.

*Mice were primed with tumor cells (MBL-2, RMA) by injection of 5×10^6 irradiated tumor cells (MBL-2, MBL-2 Fas, RMA) at days −14 and −7 before tumor challenge at day 0 or with viral antigens (VSV, VV-VSV-NP) by injection of 2×10^6 PFU at day −14 before tumor (VSV-NP–transfected or mock-transfected EL-4) challenge at day 0.

as little as 100 cells were injected. MC57G fibrosarcoma cells were controlled 10-fold better by C57BL/6 mice than by PKO mice (Table 2) (14). In contrast, 10^7 adenovirus + *ras*-tranformed mouse embryonic cells (SR23B) were rejected by both unprimed mouse strains (Fig. 1). The tumors were smaller in PKO mice, suggesting even a better control by these mice. After priming with irradiated SR23B cells, both mouse strains were equally protected against tumor growth (data not shown).

Tumors of Lymphoid Origin. EL-4 and MBL-2 cells were controlled 100–1,000-fold better by naive C57BL/6 mice when compared to naive PKO mice (Table 2 and Fig. 4). Priming with tumor cells (MBL-2) even increased the resistance against subsequent challenge 100-fold in C57BL/6 and PKO mice (Table 2). As previously shown (42), EL-4 tumor cells expressing a viral antigen derived from VSV (N1), but not their mock-transfected counterparts (V1) were readily controlled by mice that had been previously primed with the virus (Fig. 2). In PKO mice, however, both N1 and V1 showed uncontrolled growth, indicating that this form of antitumor immunity is essentially mediated by perforin. RMA cells, which are related to MBL-2 cells, however, were controlled to a similar extent in both naive and primed C57BL/6 and PKO mice. The MHC class I^- derivative of RMA cells, RMA-S (12), was controlled considerably better by C57BL/6 mice (13).

The data shown in Table 2 were obtained using two to three mice per group in two to three independent experiments in which established tumor cell lines were "titrated" into mice with 10-fold differences in cell number injected. If a certain tumor dose could not be controlled, then this

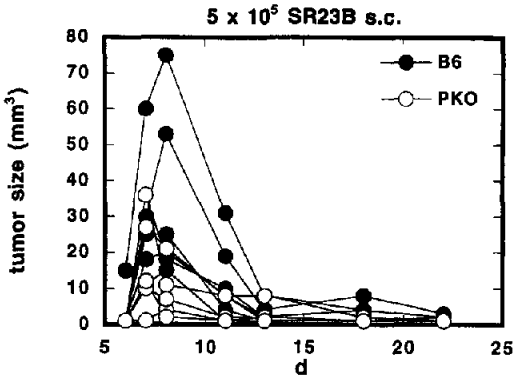

Figure 1. Adenovirus + *ras* transformed mouse embryonic cells are controlled by perforin-deficient mice. C57BL/6 (●) and PKO (○) mice were given 5×10^5 SR23B cells (expressing Ad5E1 and E*ras*) subcutaneously on day 0, and tumor development was monitored. Each line represents an individual mouse.

was the case in 100% of the mice tested and, in addition, a 10-fold lower load of the same tumor cell line was always controlled. An exception to this was formed by MBL-2.Fas cells in primed PKO mice: in both experiments, one out of two mice controlled 10^6 cells, and one out of two controlled 10^5 cells, but died after 10^6 cells, albeit with a delay of 2 wk compared to the time mice usually died in these experiments (Fig. 4).

Stable Transfection of MBL-2 Lymphoma Cells with Functional Fas

MBL-2 cells were transfected with an expression vector encoding mouse Fas and G418 resistance by electroporation, and G418-resistant MBL-2 cells were subcloned twice. One of the selected subclones (MBL-2.Fas9) displayed a homogeneous expression pattern of Fas (Fig. 3 *A*) and was killed by $FasL^+$ MC57G cells (Fig. 3 *B*). Spleno-

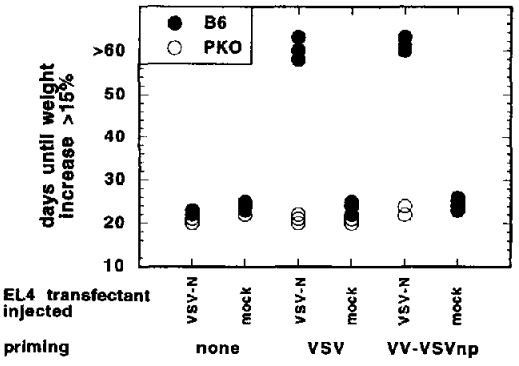

Figure 2. Growth of syngeneic EL-4 thymoma cells in primed and unprimed C57BL/6 and perforin-deficient mice. Mice were primed at day 0 with 2×10^6 PFU live VSV, 2×10^6 PFU live recombinant VV-expressing VSV-NP, or were left unprimed. At day 14, mice were challenged intraperitoneally with 10^6 live VSV-NP–transfected or mock-transfected EL-4 cells. Mice were observed daily for tumor growth by monitoring the weight of the mice. Mice were killed if the weight increased >15% compared to uninjected mice. Each point represents one mouse.

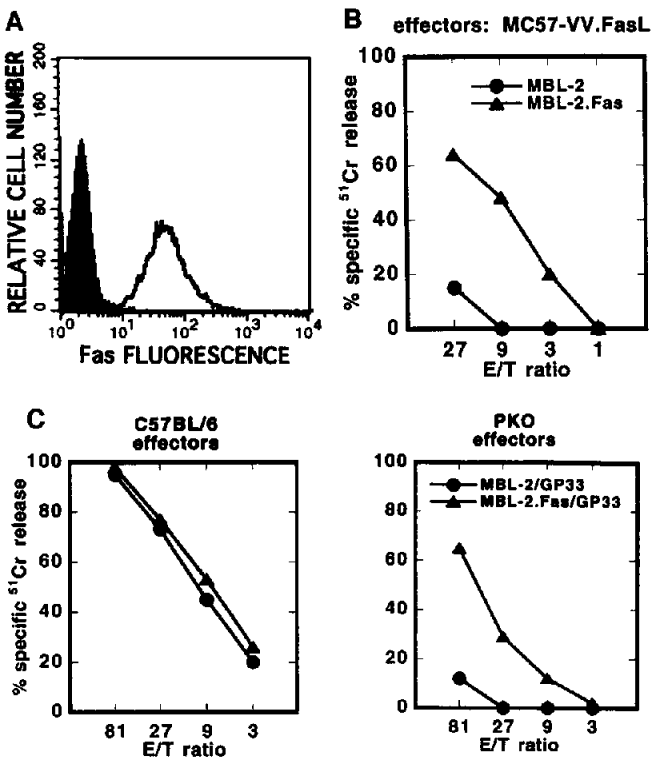

Figure 3. Lysis of Fas-transfected MBL-2 lymphoma cells by perforin-deficient effectors. (*A*) Fas-expression of Fas- and mock-transfected MBL-2 cells. 10^6 cells were stained with a biotinylated monoclonal hamster anti-mouse Fas antibody (Jo-1) followed by streptavidin-FITC. Viable cells were gated by a combination of forward and 90° side light scatter, and were analyzed with a FACScan® cytofluorometer using logarithmic scales. *Filled curve*, MBL-2 cells; *open curve*, MBL-2 Fas cells. (*B*) Enhanced lysis of Fas^+ MBL-2 cells compared to Fas^- MBL-2 cells by FasL-expressing effectors in vitro. MC57G cells were infected with VV-FasL (moi = 2) for 3 h and were used as effectors to kill ^{51}Cr-labeled MBL-2 and MBL-2.Fas target cells in a standard 5 h ^{51}Cr release assay. The spontaneous release of MBL-2 and MBL-2.Fas target cells was 17 and 14%, respectively. (*C*) Fas^+ MBL-2 cells are lysed by perforin-deficient effectors in vitro. PKO and control C57BL/6 mice were infected intravenously with 200 PFU LCMV-WE. After 8 d, splenocytes (effectors) were analyzed for ex vivo cytotoxic activity using LCMV-specific peptide (GP33)-loaded MBL-2 or MBL-2.Fas target cells in a standard ^{51}Cr release assay. Lysis of MBL-2 and MBL-2.Fas without peptide was <5% at the highest E/T ratio, and spontaneous release of targets was <18%

cytes of PKO and C57BL/6 mice that had been infected with LCMV 8 d previously killed LCMV glycoprotein (GP)33-loaded MBL-2.Fas cells to a similar extent (Fig. 3 *C*). As expected, untransfected MBL-2 cells (virtually Fas^-, Fig. 3 *A*) were killed by C57BL/6 effectors, but only to a low extent by perforin-deficient effectors (14); this suggested a major role for the perforin-dependent cytolytic pathway against Fas^- or Fas^{low} targets. Thus, transfection of MBL-2 cells with Fas generated a cell line with a high, stable, and functional expression of Fas.

We also transfected MC57G and B16 cells with Fas and obtained clones with high expression. In vitro, however, these cells remained insensitive to Fas/FasL-mediated apoptosis (data not shown), probably because of a lack of crucial intracellular signalling molecules downstream of Fas (43). Therefore, no in vivo experiments were performed with MC57G.Fas and B16.Fas cells.

Resistance against Fas-transfected MBL-2 Cells

To evaluate whether growth characteristics of MBL-2 cells were influenced by Fas, MBL-2 and MBL-2.Fas cells were titrated intraperitoneally in $RAG2^{-/-}$ mice. In addition, growth kinetics in vitro were compared. We did not observe differences in tumor take or tumor growth between MBL-2 and MBL-2.Fas cells at the doses tested (10^2–10^4–10^6, $RAG2^{-/-}$ mice could not control the tumor) in $RAG2^{-/-}$ mice or of proliferation in vitro (data not shown). The potency of Fas/FasL interactions in tumor surveillance in vivo was investigated in naive or primed mice. Naive C57BL/6 mice controlled 10^3 MBL-2 cells and 10^4 MBL-2.Fas cells, but could not control an inoculum of 10^4 MBL-2 or 10^5 MBL-2.Fas. As little as 10^2 MBL-2 cells grew in an uncontrolled fashion in PKO mice, whereas 10^2 MBL-2.Fas cells were rejected (Fig. 4). This suggests that in an unprimed situation, Fas/FasL interactions may contribute to tumor control.

In a subsequent experiment, mice were primed intraperitoneally with 4 million irradiated MBL-2 cells. Mice were subsequently challenged with different numbers of viable MBL-2 or MBL-2.Fas tumor cells, and tumor growth was monitored. Primed C57BL/6 mice controlled the growth of $>10^6$ MBL-2 or MBL-2.Fas cells; on the other hand, primed PKO mice exhibited tumor growth after injection of 10^5 MBL-2 cells. Two out of four PKO mice could eliminate 10^5 MBL-2.Fas cells, whereas the other one displayed delayed tumor growth (Table 2). Thus, priming with irradiated MBL-2 cells induced resistance

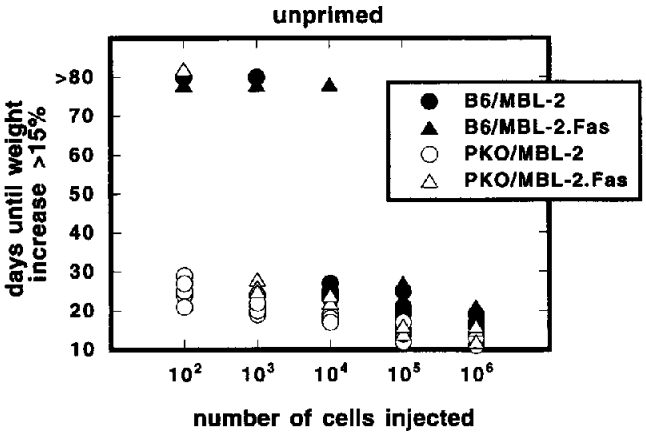

Figure 4. Growth of syngeneic MBL-2 and MBL-2.Fas lymphoma cells in naive C57BL/6 and PKO mice. PKO or control C57BL/6 mice were injected intraperitoneally with different amounts of viable MBL-2 or MBL-2.Fas lymphoma cells in 0.25 ml PBS. Mice were observed daily for tumor growth by monitoring the weight of the mice. Mice were killed if the weight increased >15% compared to uninjected control mice. *Circles*, MBL-2 cells, *triangles*, MBL-2.Fas cells; *filled symbols*, C57BL/6; *open symbols*, PKO. Each point represents one mouse. Cumulative data of three similar experiments (six mice per group total) are shown.

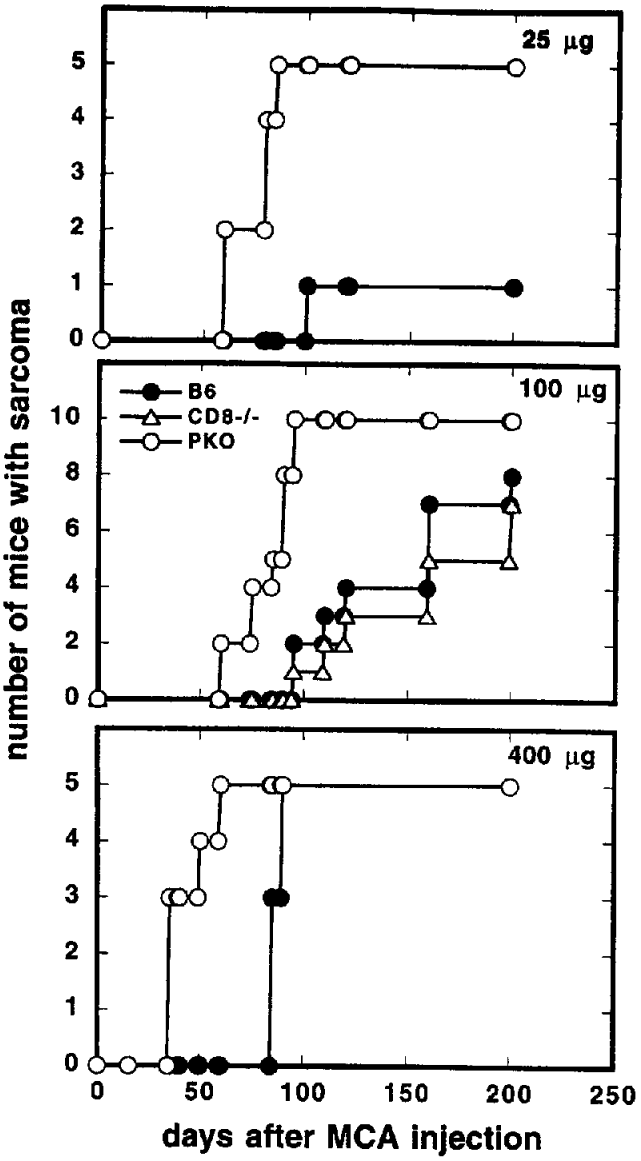

Figure 5. Control of MCA-induced carcinogenesis by perforin. PKO (○), $CD8^{-/-}$ (△) and control C57BL/6 (●) mice were injected subcutaneously in the left flank with 25, 100, or 400 µg MCA in 0.1 ml corn oil. Mice were observed every 5 d for palpable tumors. If tumors were >15 mm in diameter, mice were killed. Experimental groups consisted of 5 (25 µg, 400 µg) or 10 (100 µg) mice. Differences between PKO and C57BL/6 mice were statistically significant (unpaired Mann-Whitney *U* test), whereas differences between $CD8^{-/-}$ and C57BL/6 mice were not

against a $>10^4$- and a $>10^3$-fold higher dose of MBL-2 in C57BL/6 and PKO mice, respectively. In C57BL/6 a $>10^3$-fold and in PKO an 10^3-fold higher protection was induced against MBL-2.Fas.

Tumors Induced by Chemical Carcinogens

Subcutaneous injection of MCA induced fibrosarcomas in a dose-dependent fashion. Injection of 25 µg (Fig. 5, *top*) induced tumors in 100% of the PKO, but only in 20% of the C57BL/6 mice. Administration of 100 µg MCA

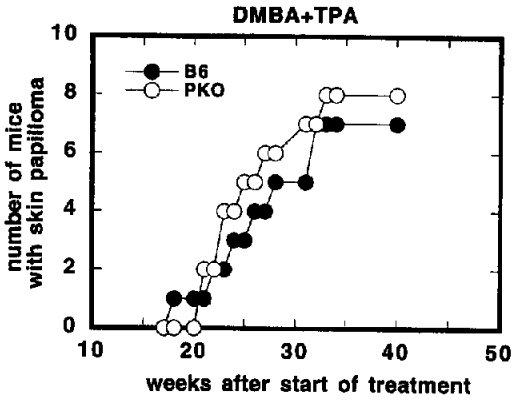

Figure 6. Skin papilloma induction by DMBA+TPA: independence of perforin. Groups of eight perforin-deficient and seven control C57BL/6 mice were treated twice weekly with 3 nmol DMBA + 10 nmol TPA in acetone. The time of the appearance of the first papilloma was registered for each mouse.

(Fig. 5, *middle*) or 400 µg (Fig. 5, *bottom*) induced sarcomas in C57BL/6 mice with an incidence of 80 and 100%, respectively. At all doses, a significant accelerated onset of tumor (Fig. 5) and tumor size (data not shown) was observed in PKO mice. Administration of 100 µg MCA to $CD8^{-/-}$ mice induced sarcomas with comparable kinetics and incidence as those seen in C57BL/6 mice. Experimental groups were statistically compared using the unpaired Mann-Whitney *U* test, and showed that tumor development in PKO mice and C57BL/6 mice was statistically different after injection of 100 µg MCA ($P < 0.0005$) or 400 µg MCA ($P < 0.009$). The difference between PKO and $CD8^{-/-}$ mice was also statistically significant ($P < 0.0008$), whereas there was no difference between C57BL/6 and $CD8^{-/-}$ mice ($P = 0.9$). Statistical analysis was not performed on groups injected with 25 µg MCA, because only one out of five C57BL/6 mice, but five out of five PKO mice, developed a sarcoma. FACS® analysis performed within 10 d after isolation of different MCA-induced sarcomas showed that the sarcomas were MHC class $I^{low/-}$, MHC class II^-, and Fas^- (not shown). The expression of the above-mentioned surface molecules was similar in the three different strains of donor mice.

The first DMBA+TPA–induced skin papillomas developed in C57BL/6 and PKO mice after 18 wk and 21 wk, respectively, and 100% incidence was reached after 31.5 and 34 wk, respectively (Fig. 6). The incidence and kinetics of DMBA+TPA–induced papillomas were similar in PKO and C57BL/6 mice, arguing against a role for perforin-mediated cytotoxicity (CTL and/or NK) in the control of DMBA+TPA–induced papillomas.

Virally Induced Fibrosarcomas

Intramuscular administration of MoMSV to PKO and C57BL/6 mice led to tumor induction in all mice with similar kinetics. In addition, occurrence of regression was not dependent on perforin (Fig. 7). Nevertheless, the tumors were larger on average and regression was retarded in PKO mice, suggesting involvement of perforin in the acute

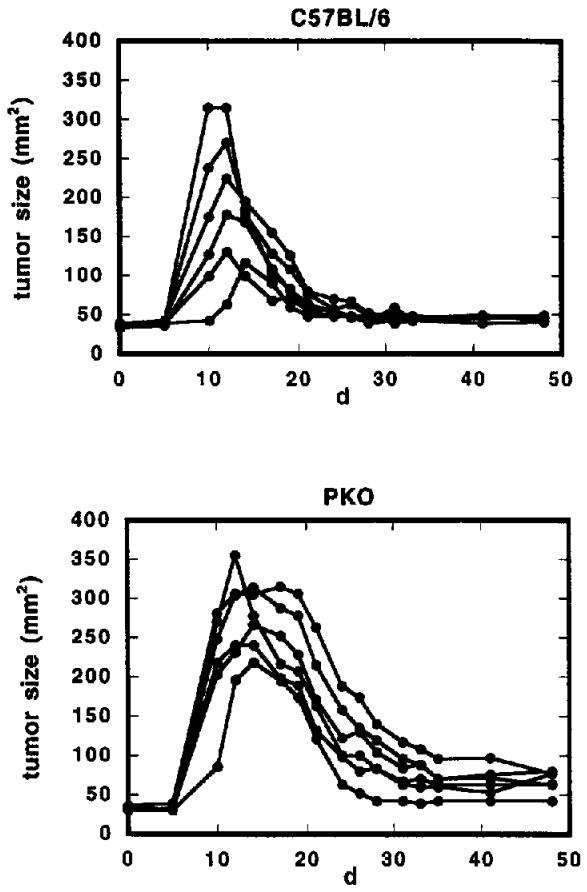

Figure 7. Control of virally induced tumors by PKO mice PKO and control C57BL/6 mice were given 10^3 focus forming units MoMSV intramuscularly in 100 μl PBS in the left hind leg. Mice were monitored regularly for palpable tumors. Each line represents an individual mouse

effector phase of tumor rejection. CD8-depleted C57BL/6 mice displayed similar tumor size and regression kinetics as PKO mice (not shown), indicating that $CD8^+$ T cells, and not NK cells, exert the perforin-mediated effect. CD4-depleted C57BL/6 mice, on the other hand, developed large tumors that could not be eliminated (data not shown). We observed that PKO and C57BL/6 mice were sick and lost weight when the tumor reached its peak size. Treatment with anti–TNF-α antibody abolished this sickness. Tumor size and kinetics of regression, however, were not affected by this treatment.

Discussion

The perforin dependence of the eradication of several established syngeneic tumor cell lines was investigated. Most cell lines showed a difference in tumorigenic dose between naive PKO and C57BL/6 mice, indicating that even without previous priming, perforin-mediated cytotoxicity is a crucial effector mechanism in tumor control. In primed mice, the difference between PKO and wild-type mice was even more clear. This suggests that priming of $CD8^+$ CTLs rather than priming of $CD4^+$ T cells is responsible for resistance to high tumor loads in C57BL/6 mice.

Two tumor cell lines formed an exception to this rule. RMA cells showed equal growth in both naive (10^4) and primed (10^6) PKO and C57BL/6 mice, which may be explained by the fact that control is mediated by perforin-independent mechanisms, probably by $CD4^-$ cells using an unidentified effector mechanism (1). Because RMA and MBL-2 are related lymphoma lines and express similarly high levels of MHC class I and no MHC class II, and both cell lines are Fas^{low}, the observation that the control of RMA is not dependent on perforin, whereas the control of MBL-2 cells is, was unexpected. This may be explained by a difference in efficiency of priming $CD4^+$ and/or $CD8^+$ T cells, or by a different growth rate of RMA vs. MBL-2 cells. The second exception is formed by SR23B cells: this adenovirus + *ras*-transformed cell line induced larger tumors in C57BL/6 mice than in PKO mice. This unexpected finding may be explained by the fact that PKO mice may have increased basal levels of macrophage activation and/or cytokines such as interferon-γ and TNF-α as compensatory mechanisms, all of which are known to possess antitumor activity.

Stable transfection of MBL-2 cells with Fas rendered them susceptible to Fas/FasL–dependent apoptosis in vitro. To investigate the contribution of the Fas pathway to in vivo tumor surveillance, control of MBL-2.Fas and MBL-2 cells was compared in naive and primed PKO and C57BL/6 mice. In unprimed PKO and C57BL/6 mice, Fas^+ cells were controlled 10-fold better compared to Fas^- cells. Priming of PKO mice increased the number of tumor cells needed for uncontrolled growth by 100-fold for both MBL-2 (10^4 cells) and for MBL-2.Fas (10^5 cells), suggesting priming of perforin-independent mechanisms. Priming of C57BL/6 mice, however, resulted in control of $>10^6$ MBL-2 or MBL-2.Fas cells, stressing the need for a collaboration between perforin-dependent and -independent mechanisms for full immune protection against MBL-2 cells. Thus, our experiments failed to detect a major involvement of Fas-dependent cytotoxicity in tumor control, but cannot exclude a minor role.

To investigate the influence of perforin on the induction of primary tumors in vivo, we analyzed different models in which tumors are induced by chemicals (MCA, DMBA+TPA) or virus (MoMSV). The major difference of this approach compared to injection of syngeneic cell lines is that the immune system is not confronted with a considerable tumor load at once, but that immunosurveillance is tested at the very moment a tumor arises. Moreover, in contrast to injection of homogeneous cell lines, each mouse develops its own tumor that may have individually different tumor antigens, even if the same chemical is injected in the same animal (44). Subcutaneous injection of MCA is known to induce sarcomas at the site of injection (44) that usually do not regress once they developed. C57BL/6 and $CD8^-$ mice developed sarcomas with a high incidence but with much slower kinetics than PKO mice. This suggests that NK cells or $CD4^+$ T cells may act

directly or indirectly as important effectors controlling MCA-induced sarcomas. Ex vivo analysis of MHC class I, class II, and Fas expression of MCA-induced sarcomas, however, showed that sarcomas were Fas^- and MHC class II^- and were MHC class $I^{-/low}$, independent of whether they were isolated from PKO, C57BL/6, or $CD8^{-/-}$ mice. Although MHC class I expression could be upregulated to high levels by exposure to IFN-γ in vitro (not shown), it remains unclear whether this occurs in vivo. Because four out of four MCA-induced sarcomas tested were sensitive to NK-mediated lysis in vitro, this suggests that NK-mediated control may be an important pathway in vivo. In a second set of experiments in which DMBA+TPA–induced skin papillomas were studied, no differences in incidence, kinetics, or size of papillomas were observed between PKO and C57BL/6 mice, indicating that perforin does not play a key role. Because the latter model is immunologically not characterized (susceptibility of SCID or nu/nu mice is not known), it is not possible at the present time to be more specific about possible effector mechanisms.

Sarcomas induced by inoculation of mice with MoMSV are known to be rejected within 3 wk by immunocompetent mice in which a prominent H-$2D^b$–restricted CTL response is observed (45–48), whereas lethal tumors develop in nu/nu mice (Ossendorp, F., and C.J.M. Melief, unpublished data). Furthermore, CD4 depletion results in uncontrolled tumor growth (Ossendorp, F., and C.J.M. Melief, unpublished data). CD8 depletion, on the other hand, did not alter the incidence or kinetics of tumor growth, but led to a significant delay in tumor rejection (data not shown). PKO mice displayed a phenotype similar to CD8-depleted mice after inoculation with MoMSV, suggesting a distinct, perforin-dependent role for $CD8^+$ CTLs in tumor control, although the tumor could be rejected without this pathway. It is not known at the present time which effector mechanism is used by $CD4^+$ T cells to reject MoMSV-induced sarcomas, and this question is subject to further studies. Apparently, $CD4^+$ T cells do not eradicate tumors through TNF-α because treatment with neutralizing anti-TNF antibodies in vivo improved the general well-being of the mice at the peak of the tumor growth, but did not interfere with tumor rejection (data not shown) independent of mouse strain or treatment.

Finally, during the 3 yr we now have bred PKO mice and have kept some (~100) for >12 mo, we have not observed spontaneous tumors of any kind. This does not necessarily call into question the in vivo relevance of perforin for tumor control because (*a*) a mouse facility is a relatively protected environment in which mice are not exposed to irradiation, toxins, chemicals, or exogenous oncogenic viruses; and (*b*) spontaneous tumor development may be too rare for meaningful observation in the few mice that were not used for experiments when young and reached an age of >1 yr in our facilities.

Taken together, our data illustrate that several mechanisms may control tumor growth; perforin-mediated cytotoxicity by $CD8^+$ CTLs (for MHC class I^+ tumors) and NK cells (for tumors expressing low levels of MHC class I) are of major importance; Fas/FasL interactions can contribute to tumor control in vivo, albeit to a lesser extent than perforin-dependent lysis, at least in tumor models using several established tumor cell lines or primarily induced types of tumors studied here.

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