Brief Definitive Report

Coupling and Uncoupling of Tumor Immunity and Autoimmunity

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Summary

Self-antigens, in the form of differentiation antigens, are commonly recognized by the immune system on melanoma and other cancers. We have shown previously that active immunization of mice against the melanocyte differentiation antigen, a tyrosinase-related protein (TRP) $gp75^{TRP\text{-}1}$ (the *brown* locus protein) expressed by melanomas, could induce tumor immunity and autoimmunity manifested as depigmentation. In this system, tumor immunity and autoimmunity were mediated by autoantibodies. Here, we characterize immunity against another tyrosinase family glycoprotein TRP-2 (the *slaty* locus protein), using the same mouse model and method of immunization. As observed previously for $gp75^{TRP\text{-}1}$, immunity was induced by DNA immunization against a xenogeneic form of TRP-2, but not against the syngeneic gene, and depended on $CD4^+$ cells. Immunization against TRP-2 induced autoantibodies and autoreactive cytotoxic T cells. In contrast to immunization against $gp75^{TRP\text{-}1}$, both tumor immunity and autoimmunity required $CD8^+$ T cells, but not antibodies. Only autoimmunity required perforin, whereas tumor immunity proceeded in the absence of perforin. Thus, immunity induced against two closely related autoantigens that are highly conserved throughout vertebrate evolution involved qualitatively different mechanisms, i.e., antibody versus $CD8^+$ T cell. However, both pathways led to tumor immunity and identical phenotypic manifestations of autoimmunity.

Key words: melanoma • melanocyte • tyrosinase-related protein • T cell • perforin

Self-antigens on human cancer are the most prevalent antigens recognized by the immune system (1). This reflects the fact that cancers arise from the hosts' own tissues, and are not truly foreign. Thus, in some respects, immune recognition of cancer appears to be different from immune recognition of viruses or bacteria, and typically more akin to autoimmunity. Recognition of self-antigens on cancer presents problems. First, immunity to cancer may not develop because of immune tolerance. Second, even when the immune system can recognize and respond to self-antigens, immunity may not be sufficient to reject cancers. Finally, if immunity to self-antigens develops, there are potential autoimmune sequelae.

Differentiation antigens form one prototype of self-antigens on cancer (2–4). A differentiation antigen distinguishes a cell lineage from other cell types, and is typically expressed at specific stages of differentiation (5). Immune recognition of human cancer has been intensively investigated in melanoma, a cancer arising from melanocytes (1). Studies of melanoma have surprisingly shown that the immune system commonly recognizes products of genes that are specifically expressed by melanocytes, particularly genes that are involved in synthesis of pigment (1, 6). Examples include tyrosinase, the critical enzyme required for synthesis of the pigment melanin, and tyrosinase-related proteins (TRPs) that determine the type of melanin synthesized (TRP-2 and $gp75^{TRP\text{-}1}$) [3, 4, 7]). These melanoma/melanocyte differentiation antigens can be recognized by antibodies and by $CD8^+$ and $CD4^+$ T cells, and thus can be broadly seen by the immune system (3, 4, 7–18).

We have reported previously that immunization of C57BL/6 mice against the tyrosinase family antigen $gp75^{TRP\text{-}1}$ induces tumor immunity and autoimmunity that is mediated by autoantibodies (13, 15–17). Using DNA immunization strategies, we have investigated immunity induced against another homologous tyrosinase family member, TRP-2. The mouse $gp75^{TRP\text{-}1}$ and TRP-2 proteins are closely related, having 52% identity and 67% homology, and these genes have been highly conserved throughout vertebrate evolution (e.g., the identity of mouse $gp75^{TRP\text{-}1}$ and chicken TRP-2 is also 52%).

W.B. Bowne and R. Srinivasan share first authorship on this paper.

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Materials and Methods

Mice. C57BL/6 mice (6–8-wk-old females) were acquired through the National Cancer Institute breeding program. Homozygotic mice genetically deficient for β2-microglobulin ($\beta 2m^{-/-}$), MHC II ($Abb^{-/-}$), and perforin ($B6.pfp^{-/-}$), all in a C57BL/6 background, were obtained from Taconic Farms, Inc. Immunoglobulin μ chain ($\mu MT^{-/-}$) and *gld*/*gld* (B6.*gld*) mice were acquired from The Jackson Laboratory. These mice were bred and kept in a pathogen-free Memorial Sloan-Kettering Cancer Center vivarium according to National Institutes of Health Animal Care guidelines. All mice entered the study between 7 and 10 weeks of age.

Cell Lines and Tissue Culture. B16F10/LM3 (15) is a pigmented mouse melanoma cell line of C57BL/6 origin, derived from the B16F10 line, provided by Dr. Isaiah Fidler (M.D. Anderson Cancer Center, Houston, TX). The EL-4 cell line was derived from a C57BL/6 mouse lymphoma, and SK-MEL-188 is a human melanoma cell line. Tumor cell lines were cultured as described (15).

Plasmid Constructs. The human TRP-2 (hTRP-2) and mouse TRP-2 (mTRP-2) expression vectors (supplied by Drs. S.A. Rosenberg and J.C. Yang, National Cancer Institute, Bethesda, MD) were previously described (9). These genes were cloned into the PCR3 vector, which was used as a control vector without inserts. The mouse GM-CSF gene, provided by PowderJect Vaccines Inc., was cloned into the WRGBEN vector (13).

DNA Immunization. The method of DNA immunization has been reported (13). In brief, plasmid DNA encoding hTRP-2, mTRP-2, or GM-CSF was coated onto 1.0 μm gold bullets. Animals were immunized by delivering gold–DNA complexes using a helium-driven gun (Accell®; PowderJect Vaccines Inc.) into each abdominal quadrant (1 μg plasmid DNA/quadrant) for a total of four injections.

Depletion and Depigmentation Studies. NK cell depletion was performed as described (15). Animals were injected intraperitoneally with mAb PK136 (anti–NK-1.1) 3 d before immunization, and every 4 d thereafter. Depigmentation experiments were performed as described (13, 15). In brief, after the final immunization, mice were shaved and depilated over the posterior flank and observed for 8 wk. Scoring of depigmentation was performed by dividing the abdomen into four equal quadrants. Quadrants were recorded as positive when they had estimated >50% depigmented hairs. Depigmentation was scored 0–4+ according to the number of quadrants that were depigmented in each mouse (e.g., 3+ if three of four quadrants are depigmented >50%).

Antibody Responses to TRP-2. Antibody responses to syngeneic mTRP-2 were measured by immunoprecipitation, followed by Western blot assay as described (13). B16F10/LM3 melanoma cells were lysed, followed by immunoprecipitation with anti–PEP-8, a rabbit polyclonal antisera raised against a carboxyl-terminal peptide of TRP-2 (from Vincent Hearing, National Cancer Institute).

CTL Assay. Lymphocytes (2×10^7) from draining inguinal lymph nodes were cocultured for 5 d with naive irradiated (3,000 rads) splenocytes (2×10^7), mitomycin C (100 μg/ml, 120 min at 37°C)-treated human SK-MEL-188 cells (4×10^5) as a source of antigen, and pristane-induced macrophages (2×10^6) from C57BL/6 mice. H-$2K^b$–restricted lymphocyte lysis of EL-4 tumor cells pulsed for 1 h at 37°C with 4 μg $mTRP\text{-}2_{181–188}$ peptide (sequence VYDFFVWL) was determined by a 4-h standard ^{51}Cr release assay (16).

Mouse Tumor Studies. All mice were injected intravenously via the tail vein with 2×10^5 B16F10/LM3 melanoma cells. Tumor challenge was performed 5 d after the final immunization, unless otherwise indicated. Mice were killed at 14–22 d after tumor challenge, all lobes of both lungs were dissected, and surface lung metastases were scored and counted for all lobes under a dissecting microscope. Statistical analysis of tumor growth was performed using the two-sided Student's *t* test, assuming unequal variances and Wilcoxon Scores (Rank Sums).

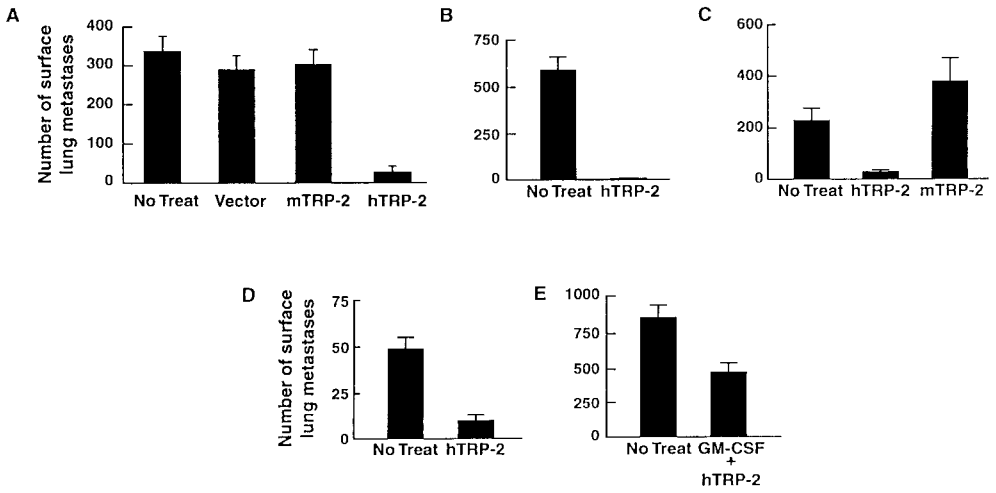

Figure 1. Protection and rejection of mouse melanoma B16F10/LM3 after immunization with human TRP-2 (hTRP-2), but not mouse TRP-2 (mTRP-2) DNA. C57BL/6 mice, 10–12 per group, were immunized cutaneously with hTRP-2 or mTRP-2 DNA by particle bombardment. Mice were challenged with B16 melanoma cells intravenously and scored for surface lung metastases after 14–17 d. (A and B) Mice were immunized three times at weekly intervals with hTRP-2, mTRP-2, or PCR3 control vector (vector), and compared to untreated mice. Mice were challenged with B16F10/LM3 melanoma 5 d after the last immunization. B is a repeat of the experiment in A, without the vector control and mTRP-2 groups. Significant tumor protection was observed in both experiments ($P < 0.0001$). (C) Mice were immunized five times at weekly intervals with hTRP-2, or mTRP-2, or were untreated. Significant protection was observed with hTRP-2 compared with no treatment ($P = 0.001$) or with mTRP-2 ($P = 0.001$). The difference between mice treated with mTRP-2 and untreated mice was not significant ($P = 0.16$). (D) Immunization with hTRP-2 DNA started 4 d after tumor challenge, or mice remained untreated. A significant therapeutic effect was observed ($P < 0.001$). (E) Immunization with hTRP-2 plus GM-CSF DNA started 10 d after tumor challenge. Significant therapeutic effect was observed compared with other treatment and control groups ($P < 0.01$). GM-CSF treatment alone yielded 692 ± 69 metastases, mTRP-2 gave 902 ± 65 metastases, hTRP-2 gave 783 ± 75 metastases, and control null vector gave 705 ± 61 metastases (not shown in figure). Results are shown as mean number of lung metastases ± SEM.

Results

Xenogeneic, but Not Syngeneic, TRP-2 DNA Induces Tumor Rejection. hTRP-2 has 90% homology and 83% identity to the amino acid sequence of C57BL/6 mTRP-2. DNA immunization with xenogeneic hTRP-2 decreased B16F10/LM3 lung metastases by ≥90% ($P < 0.0001$) in tumor protection experiments (Fig. 1, A–C). There was no significant evidence of tumor immunity after immunization with syngeneic mTRP-2 DNA compared with untreated mice or mice injected with control null vector (Fig. 1, A and C).

To assess the potency of DNA immunization using xenogeneic hTRP-2 DNA, mice were immunized 4 d after tumor challenge or 10 d after tumor challenge, when lung metastases were numerous and macroscopic. Immunization at 4 d decreased metastases by >80% ($P < 0.001$; Fig. 1 D). Therapeutic effects were observed 10 d after tumor challenge using immunization with hTRP-2 DNA plus recombinant mouse GM-CSF DNA as an immune adjuvant. Vaccination significantly decreased lung metastases by approximately half ($P = 0.004$; Fig. 1 E). No significant decrease in lung metastases was observed after treatment with hTRP-2 or mTRP-2 DNA, or GM-CSF DNA alone (Fig. 1 E, see legend), although there was a trend towards decreased metastases with GM-CSF alone that did not reach significance ($P > 0.05$). These results showed a requirement for xenogeneic antigen and the adjuvant effect of GM-CSF in the treatment of established tumors.

Xenogeneic hTRP-2 DNA Vaccination Induces Autoantibodies and Autoreactive CTLs. We next determined whether immunization with mTRP-2 or xenogeneic hTRP-2 generated antibody and $CD8^+$ T cell responses against syngeneic mTRP-2. 6 of 12 mice immunized with hTRP-2 had detectable IgG antibodies (IgG1 and IgG2b isotype) against mTRP-2 (data not shown). No autoantibodies against syngeneic mouse TRP-2 were generated after immunization with mTRP-2 (0/12). Generation of autoantibodies after immunization with hTRP-2 required both $CD4^+$ and $CD8^+$ T cells, because no autoantibodies were detected in mice deficient in MHC class I (0/11) or II molecules (0/10).

CTL responses against TRP-2 were detected after immunization with xenogeneic hTRP-2, but not syngeneic mTRP-2 DNA. Specifically, $CD8^+$ CTL from draining lymph nodes (supraclavicular nodes), stimulated in vitro for 5 d, recognized an MHC class I H-$2K^b$–restricted peptide of mTRP-2 after immunization with hTRP-2 DNA (Fig. 2 [9]). Interestingly, the H-$2K^b$–restricted peptide of mTRP-2, mTRP-$2_{181–188}$, is identical between mouse and human TRP-2, including the immediate flanking amino acid residues. Thus, this self-peptide in the context of self–TRP-2 DNA does not induce CTL responses, but presentation of the same peptide in the context of xenogeneic hTRP-2 is immunogenic.

Tumor Rejection Requires $CD4^+$ and $CD8^+$ Cells, but Not B Cells or NK Cells. These results suggested that either antibody or CTL responses, or both, mediated tumor rejection. Roles for critical cell types were investigated by immunizing $\beta 2m^{-/-}$ mice deficient in MHC class I and $CD8^+$ T cells, MHC $II^{-/-}$ mice deficient in MHC class II and $CD4^+$ T cells, $Ig\mu^{-/-}$ mice deficient in mature B cells, and mice depleted of $NK1.1^+$ cells, including NK cells (Fig. 3). Both MHC class I and II molecules were required for tumor rejection, supporting a central role for both $CD8^+$ and $CD4^+$ T cells. Neither NK cells nor B cells were necessary for tumor immunity. Noticeably, mice deficient in B cells developed fewer baseline metastases compared with wild-type C57BL/6 mice, and were completely free of any detectable tumor after treatment with hTRP-2 (12 of 12 mice). This phenomenon of enhanced T cell–dependent tumor rejection associated with B cell deficiency has been reported previously (10). These results showed that T cell immunity, including both $CD8^+$ and $CD4^+$ T cells, was required for tumor rejection, but antibodies were not.

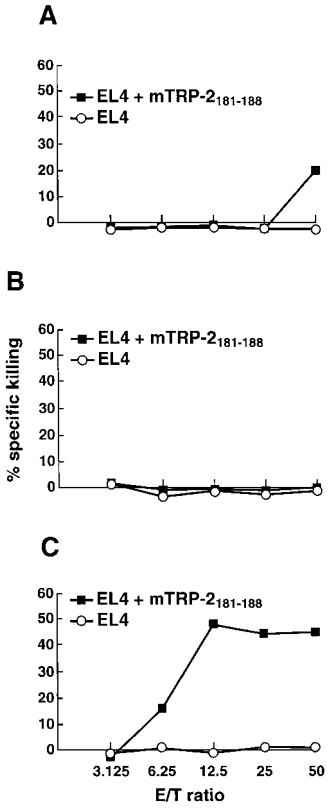

Figure 2. CTL response to TRP-$2_{181–188}$ peptide in mice that were not immunized (A), or immunized with mTRP-2 (B) or hTRP-2 (C). C57BL/6 mice (2 or 3 per group) were immunized as described in the legend to Fig. 1. 7 d after the last immunization, draining lymph nodes were pooled and stimulated as described in Materials and Methods for 5 d and tested for cytotoxicity against EL-4 target cells, either pulsed with TRP-2 peptide or unpulsed. Results are representative of two experiments.

Xenogeneic Immunization Induces Autoimmunity That Also Requires T Cells. Signs of autoimmunity, manifested as depigmentation, were observed in mice immunized with hTRP-2, but not generally in mice immunized with syngeneic mTRP-2 (Fig. 4). Depigmentation appeared 4–5 wk after starting immunization over depilated and shaved areas of the mouse coat, spreading to unshaved areas in most mice. Autoimmunity also required T cells, but not antibodies or NK cells, showing that tumor immunity and autoimmunity were coupled by a requirement for class I and II MHC expression leading to a requirement for T cells.

Requirement for Perforin in T Cell–dependent Autoimmunity, but Not Tumor Immunity. CTLs have been proposed to be critical effector cells that mediate tumor rejection. Cytotoxicity of T cells can be mediated by exocytic granules in-

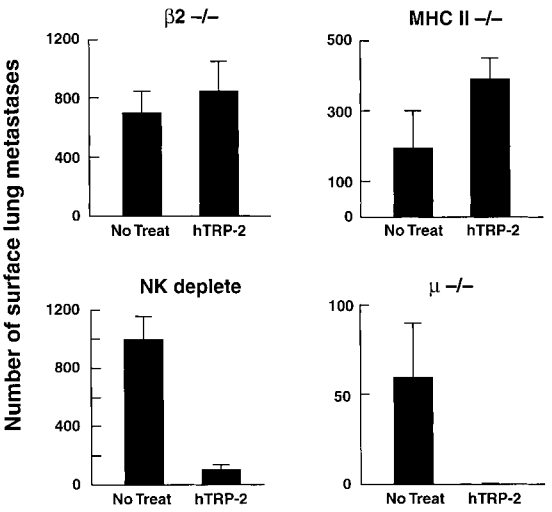

Figure 3. Tumor rejection in C57BL/6 mice deficient in immune molecules and cells. Mice, 10–12 per group, were immunized cutaneously with hTRP-2 DNA as described in the legend to Fig. 1. Lung metastases were evaluated after 18 d. Groups included mice deficient in β2 microglobulin (β2−/−), MHC II, immunoglobulins (μ−/−), and depleted of NK1.1$^+$ cells (NK deplete). All mice were treated in the same experiment, and differences in lung metastases in the no treatment groups reflect tumorigenicity in different mouse strains or under different conditions (e.g., NK deplete). Significant protection was observed in NK depleted ($P < 0.0001$) and μ−/− ($P = 0.03$), but not β2−/− or MHC II−/− ($P > 0.10$) mice. This experiment is representative of two experiments. Results are shown as mean ± SEM.

volving perforin, or by cell membrane molecules that induce death of target cells. Tumor immunity proceeded in the complete absence of perforin ($P = 0.0002$) in *pfp*$^{-/-}$ mice (Fig. 5), whereas autoimmunity was mostly inhibited (Fig. 4). Fas ligand was not necessary for either autoimmunity (Fig. 4) or tumor immunity (Fig. 5). These results are consistent with perforin-mediated killing of normal melanocytes in hair follicles playing a central role in autoimmunity. However, tumor immunity could proceed in a perforin-independent manner.

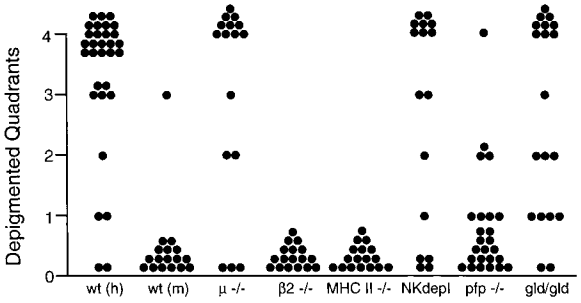

Figure 4. Depigmentation in mice treated with hTRP-2 or mTRP-2 DNA. Groups of mice (12–15 per group) were immunized with hTRP-2 or mTRP-2 DNA as described in the legend to Fig. 1, or remained untreated. Each dot represents a separate mouse. Groups include C57BL/6 (wt) mice treated with hTRP-2 (h) or mTRP-2 (m) DNA. In addition, immunoglobulin-deficient (μ−/−), β2 microglobulin-deficient (β2−/−), MHC II–deficient (MHC II−/−), NK-depleted, and perforin-deficient (perf−/−) mice were immunized with hTRP-2.

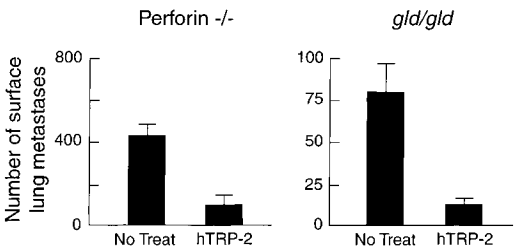

Figure 5. Tumor rejection in perforin-deficient (*pfp*−/−) mice and *gld/gld* mice (deficient in fas ligand) treated with hTRP-2, compared with mice not treated. Mice (9 or 10 per group) were immunized cutaneously with hTRP-2 DNA as described in the legend to Fig. 1. Significant tumor protection was observed in *pfp*−/− ($P = 0.0002$) and *gld/gld* ($P < 0.0001$) mice.

Discussion

TRP-2 is recognized by CTLs of patients with melanoma (7, 11, 12), and has also been defined as a potential tumor-rejection antigen in C57BL/6 mice (9). Thus, TRP-2 provides a model for a differentiation antigen with relevance to a human cancer. Xenogeneic DNA vaccination is one strategy to immunize against potentially weak self-antigens. The approach using a xenogeneic source of antigen is well known to produce autoimmunity, but has also been used to induce tumor immunity against gp75^{TRP-1} and another melanocyte/melanoma differentiation antigen gp100 (13, 14, 16–18). Other strategies have shown that syngeneic mouse gp75^{TRP-1} expressed in insect cells (16) or by vaccinia virus (14) can also induce tumor immunity and trigger depigmentation. Expression of the syngeneic protein in the context of xenogeneic cells that may package the antigen in insoluble inclusion bodies (e.g., insect cells) or with strongly immunogenic viral proteins are alternative strategies, at least for inducing antibody-dependent immunity against tyrosinase family antigens.

As noted above, the CTL response directed against the H-2K^b-restricted peptide of mTRP-2, mTRP-2$_{181–188}$, is remarkable because this peptide is identical between mouse and human TRP-2, including the immediate flanking amino acid residues. It is possible the distant amino acid residues alter processing of this peptide in hTRP-2, allowing more efficient presentation. Alternatively, amino acid differences in other peptides of hTRP-2 may provide T cell help, which in turn is sufficient to trigger CTL responses to the mTRP-2$_{181–188}$ self-peptide. The observation that only hTRP-2 DNA (but not mTRP-2 DNA) induced CD8$^+$ T cell responses suggests that T cell tolerance was broken by xenogeneic DNA immunization, although we recognize this could reflect differences in efficiency of processing.

Tumor Immunity and Autoimmunity: Two Means to the Same End. Immunity against gp75^{TRP-1} led to tumor protection and to depigmentation that was indistinguishable from autoimmunity induced by immunization against TRP-2 (13). Similar results were observed by Naftzger et al. (16) and Overwijk et al. (14) after immunization with syngeneic gp75^{TRP-1} expressed by baculovirus in insect cells or in vaccinia virus, respectively. In gp75^{TRP-1} systems, tumor immunity and autoimmunity were mediated by autoanti-

bodies, and tumor immunity depended on NK cells and functional Fc receptors; neither tumor immunity nor autoimmunity required $CD8^+$ T cells (13–17). In contrast, tumor immunity and autoimmunity against TRP-2 required MHC class I molecules, and by implication $CD8^+$ T cells, without a requirement for antibodies or NK cells. Thus, either autoantibodies or T cells can provide specificity for tumor immunity and autoimmunity.

Antibody Versus $CD8^+$ T Cells: Both Need $CD4^+$ Cells. The final phenotype of tumor immunity and autoimmunity can be dependent either on antibodies or $CD8^+$ T cells, but in both cases $CD4^+$ cells are required. Vaccination against TRP-2 as late as 10 d after tumor challenge induced substantial decreases in tumor burden, whereas active immunization against $gp75^{TRP\text{-}1}$ at these later time points was ineffective (13). We propose that this reflects in part the kinetics of T cell versus antibody responses, where effective T cell immunity can be generated over days, whereas effective antibodies may require weeks. Passive transfer of antibodies against $gp75^{TRP\text{-}1}$ had significant effects 4 and 7 d after tumor challenge (15), consistent with this view.

Tumor Immunity and Autoimmunity. The potential coupling of tumor immunity with autoimmunity has been suggested by the clinical observation that patients with metastatic melanoma who develop vitiligo have a better prognosis and are more likely to respond to therapy (19–22). The differences in mechanisms underlying tumor immunity and autoimmunity could be a consequence of fundamental differences in effector mechanisms used to kill tumor cells versus normal melanocytes. Alternatively, the differences could reflect the different tissue sites of melanocytic cells. In this scenario, the effector mechanisms in the skin require perforin, whereas other mechanisms are used in the lung. Mechanisms other than perforin or fas ligand may be involved in tumor rejection, but it is also possible that perforin and fas ligand provide redundant mechanisms for effector functions against tumors. Finally, the uncoupling of tumor immunity from autoimmunity in this model shows that one can inhibit autoimmunity and still be permissive for tumor immunity. This opens strategies for inducing immunity to treat cancer where autoimmunity can be inhibited while cancer immunity proceeds.

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