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Peripheral CD8+ T Cell Tolerance Against Melanocytic Self-Antigens in the Skin Is Regulated in Two Steps by CD4+ T Cells and Local Inflammation: Implications for the Pathophysiology of Vitiligo

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Experimental evidence has suggested a role for CD8+ cytotoxic T lymphocytes (CTL) in the pathophysiology of vitiligo, a pigmentation disorder with focal loss of melanocytes in the skin. The discovery of tyrosinase-related protein 2 (TRP2) as a model melanocytic self-antigen recognized by CD8+ CTL in C57BL/6 mice allowed us to analyze the requirements for CD8+ T cell-mediated autoimmune destruction of melanocytes in an experimental model. Using two different genetic methods for the induction of cellular immunity *in vivo*, gene gun bombardment of the skin and injection of recombinant adenovirus, we show that peripheral tolerance of CD8+ T cells recognizing a single TRP2-derived H2-K^b-binding peptide is regulated in two steps. In the induction phase, stimulation and expansion of TRP2-specific CD8+ T cells *in vivo* depend on CD4+ T cell help. In the effector phase, autoimmune destruction of melanocytes in the skin depends on local inflammation. Our results suggest that accidental stimulation of CD8+ CTL recognizing major histocompatibility complex class I-binding peptides derived from melanocytic proteins in the context of an inflammatory skin disease may play an important role in the pathophysiology of vitiligo.

Key words: CD8+ T cells/immune tolerance/melanocytes/TRP2/vitiligo
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Vitiligo is a common pigmentation disorder where melanocytes are focally destroyed in the skin. The association with other autoimmune diseases suggests immunological pathomechanisms in vitiligo (Berd *et al,* 1996; Dittmar and Kahaly, 2003). To support this hypothesis, autoantibodies to melanosomal proteins such as the tyrosinase family of enzymes have been detected in the serum of patients with vitiligo (Song *et al*, 1994; Cui and Bystryn, 1995; Baharav *et al*, 1996; Fishman *et al*, 1997; Kemp *et al*, 1997). More recently, autoreactive CD8+ cytotoxic T lymphocytes (CTL), which specifically recognize melanocytic differentiation antigens, were demonstrated in perilesional skin and in the peripheral blood (Ogg *et al*, 1998; Lang *et al*, 2001; Le Gal *et al*, 2001; Palermo *et al*, 2001; Mandelcorn-Morson *et al*, 2003). Melanocyte-specific CTL have been identified in patients with melanoma where vitiligo may occur during immunotherapeutic intervention (Scheibenbogen *et al*, 1994; Rosenberg and White, 1996; Rosenberg, 1997; Okamoto *et al*, 1998). Importantly, adoptive transfer of melanoma antigen-specific cytotoxic T lymphocytes may be associated with the regression of melanoma metastases and the appearance of vitiligo, thus providing direct evidence of T cell-mediated vitiligo in humans (Yee *et al*, 2000; Dudley *et al*, 2002).

Abbreviations: Ad, recombinant adenovirus; EGFP, enhanced green fluorescent protein; TRP2, tyrosinase-related protein 2

Experiments in murine models were also able to demonstrate that CD8+ CTL recognizing shared lineage-specific melanocytic self-antigens such as tyrosinase or the tyrosinase-related protein 2 (TRP2) can cause autoimmune destruction of melanocytes leading to vitiligo-like fur depigmentation (Bowne *et al*, 1999; Overwijk *et al*, 1999; Colella *et al*, 2000; Steitz *et al*, 2000). The induction and regulation of melanocyte-specific CTL are not well understood. Clearly, mechanisms maintaining peripheral self-tolerance must control potentially autoreactive, melanocyte-specific CTL *in vivo*. This could be directly demonstrated by immunization of tyrosinase gene knockout albino and wild-type mice against the enzyme tyrosinase where strong CTL responses could be stimulated in tyrosinase-deficient mice, whereas only very weak reactivity was observed in wild-type mice (Colella *et al*, 2000). Our group previously reported that the *in vivo* induction of TRP2-specific CD8+ T cells using novel genetic immunization techniques was only successful when TRP2 was linked to foreign helper determinants (Steitz *et al*, 2002).

In this study, we wished to analyze the requirements for CD8+ T cell-mediated autoimmune destruction of melanocytes in C57BL/6 mice in greater detail. Using the gene gun and recombinant adenoviruses—two fundamentally different genetic approaches for the induction of cellular immunity *in vivo*—we provide evidence that peripheral tolerance of CD8+ CTL recognizing melanocytic self antigens is regulated in two steps: (1) the primary stimulation of

potentially autoreactive CD8+ T cells in the lymphoid system depends on CD4+ T cell help in the induction phase and (2) the local autoimmune destruction of melanocytes in the skin requires a strong inflammatory stimulus in the effector phase.

Results

Construction of expression plasmids encoding fusion proteins between enhanced green fluorescent protein (EGFP) and defined amino acid sequences of murine TRP2 We previously showed that effective stimulation of $TRP2_{aa180–188}$ peptide-specific CD8+ T cells with genetic immunization strategies required linkage of the weakly immunogenic TRP2 with strong helper determinants. Following bombardment of the abdominal skin with plasmid DNA encoding a fusion protein between TRP2 and the immunogenic marker protein EGFP using the gene gun, we observed *in vivo* stimulation and expansion of $TRP2_{aa180–188}$ peptide-specific CD8+ T cells associated with vitiligo-like fur depigmentation (Steitz *et al*, 2002). In contrast, immunization with unmodified murine TRP2 only very rarely induced significant T cell reactivity or coat color changes. But in these experiments we could not establish a direct relationship between $TRP2_{aa180–188}$ peptide-specific CD8+ T cells and vitiligo because full-length TRP2 encoding aa30–519 fused in frame to EGFP was used for immunization. To specifically address the role of CD8+ T cells recognizing the $TRP2_{aa180–188}$ peptide for the induction of autoimmune vitiligo, we then constructed plasmid DNA encoding fusion proteins between EGFP and the truncated sequences aa30–188, aa30–179, or aa180–188 of murine TRP2 (Fig 1*A*). These fragments of TRP2 were generated by PCR, sequenced to exclude mutations, joined in frame to the C-terminal end of EGFP, and inserted into an expression plasmid containing a CMV immediate-early promotor and an SV40 polyadenylation signal. Expression of EGFP by all constructs was confirmed in transiently transfected 293 cells *in vitro* by fluorescence microscopy. Additionally, the size of the fusion proteins was verified by western blot analyses of lysates from transiently transfected 293 cells (Fig 1*B*).

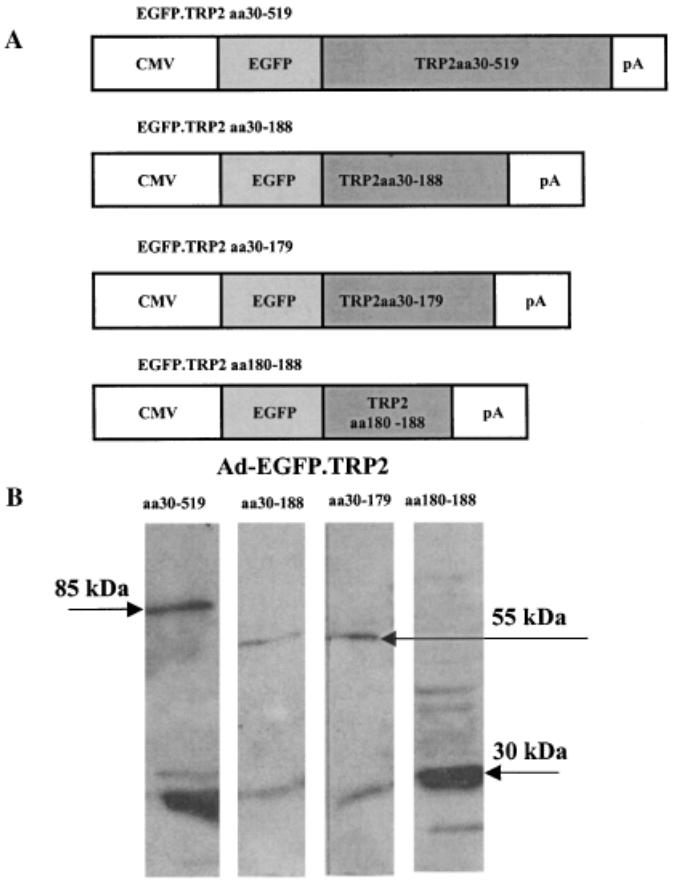
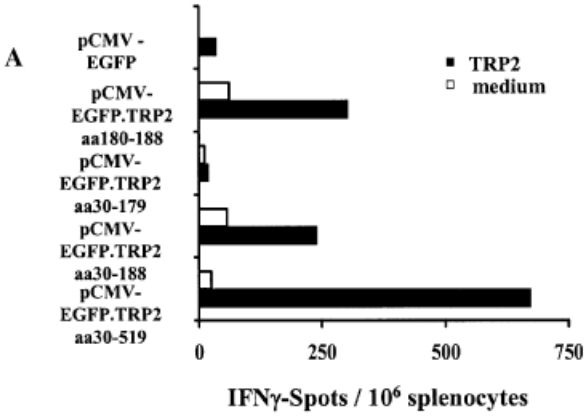


Figure 1
Expression plasmids and recombinant adenoviruses expressing C-terminal fusion proteins between enhanced green fluorescent protein (EGFP) and defined amino acid sequences of murine tyrosine-related protein 2 (TRP2). (*A*) The construction schemes of the expression plasmids pCMV-EGFP.$TRP2_{aa30–519}$, pCMV-EGFP.$mTRP2_{aa30–188}$, pCMV-EGFP.$mTRP2_{aa30–179}$, and pCMV-EGFP.$mTRP2_{aa180–188}$ used in this study are depicted. (*B*) Additionally, recombinant adenoviruses were generated expressing these fusion proteins and transgene expression verified by probing lysates of infected 293 cells in western blots with EGFP-specific antibodies.

Stimulation of CD8+ T cells *in vivo* and induction of autoimmune vitiligo following gene gun immunization with plasmid DNA containing a single H2-K^b-binding peptide derived from the melanocytic protein TRP2 In subsequent experiments, we tested the newly constructed expression plasmids encoding the various fusion proteins for their ability to stimulate $TRP2_{aa180–188}$ peptide-specific CD8+ T cells *in vivo* and induce autoimmune vitiligo in the skin. Groups of 6 C57BL/6 mice were shaved on the abdomen and bombarded with the expression plasmids pCMV-EGFP.$TRP2_{aa30–519}$, pCMV-EGFP.$mTRP2_{aa30–188}$, pCMV-EGFP.$mTRP2_{aa30–179}$, pCMV-EGFP.$mTRP2_{aa180–188}$, or pCMV-EGFP using the gene gun on a weekly basis for 5 wk. Two mice of each group were sacrificed 1 wk after the fifth immunization to analyze induction of antigen-specific CTL *in vivo*. To this end, we tested splenocytes in interferon (IFN)γ-ELISPOT assays for recognition of the synthetic H2-K^b-binding peptides $TRP2_{aa180–188}$ and medium control. As expected, immunization with plasmid DNA encoding the truncated fusion proteins EGFP.$mTRP2_{aa30–188}$ or EGFP.$mTRP2_{aa180–188}$ stimulated $TRP2_{aa180–188}$ peptide-specific CD8+ T-cells *in vivo* as effective as immunization with plasmid DNA encoding full-length EGFP.$TRP2_{aa30–519}$ (Fig 2*A*). The remaining four mice of each group were monitored for the appearance of fur depigmentation. All mice that had been immunized with plasmid DNA containing the immunogenic EGFP and the $TRP2_{aa180–188}$ peptide eventually developed autoimmune vitiligo on the site of gene gun bombardment on the abdomen within a few weeks after the last immunization (Fig 2*B* and *C*). Importantly, bombardment of the skin with the plasmid expressing the $TRP2_{aa180–188}$ peptide epitope attached to the C-terminus of EGFP effectively induced vitiligo-like coat color changes, demonstrating that CD8+ T cells specific for a single and apparently dominant H2-K^b-binding peptide epitope derived from TRP2 are able to destroy melanocytes in the epidermal layers of the hair follicle. Presumably, CD4 helper T cells recognizing the immunogenic marker protein EGFP provide linked help for the induction of $TRP2_{aa180–188}$-specific CD8+ cytotoxic T cells. To support this hypothesis, we



B

	vitiligo
pCMV-EGFP	0/8
pCMV-EGFP.TRP2 aa180-188	13/15
pCMV-EGFP.TRP2 aa30-179	0/11
pCMV-EGFP.TRP2 aa30-188	9/11
pCMV-EGFP.TRP2 aa30-519	10/11

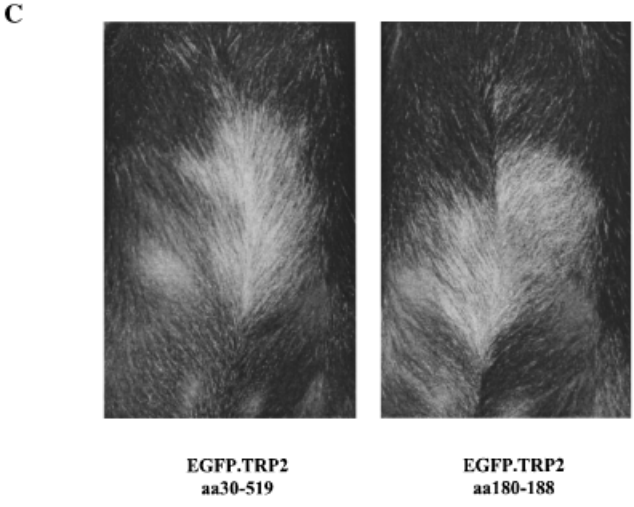


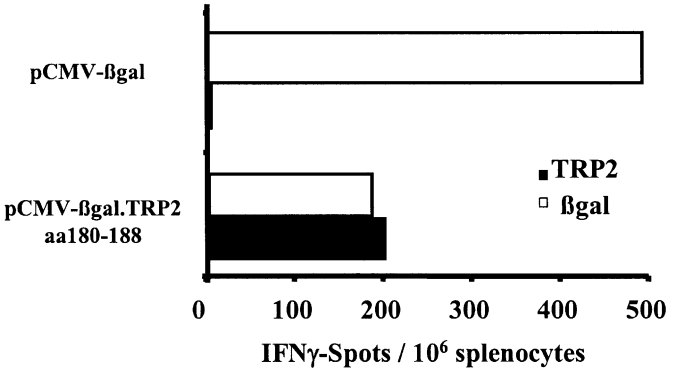
Figure 2
Induction of autoreactive CD8+ T cells specific for the H2-K^b-binding peptide SVYDFFVWL corresponding to aa180–188 of tyrosine-related protein 2 (TRP2) using the gene gun leads to autoimmune destruction of melanocytes resulting in vitiligo-like fur depigmentation. Groups of C57BL/6 mice were immunized by particle-mediated bombardment of the skin with the indicated plasmid DNA. (*A*) Splenocytes were harvested after immunization and release of IFNγ in response to the H-2K^b-binding TRP2$_{aa180–188}$ peptide tested using ELISPOT assays. Results are expressed as mean number of spot forming cells per 10^6 splenocytes. These data are representative of three to five independent experiments. (*B*) Alternatively, mice were followed for the appearance of vitiligo-like fur depigmentation. The cumulative number of mice developing coat color changes as a result of the immunization with the indicated plasmid DNA are shown. (*C*) Representative pictures of mice immunized as indicated are shown.

genetically immunized groups of CD4-deficient C57BL/6 mice with the expression plasmids pCMV-EGFP.mTRP2$_{aa180–188}$ or pCMV-EGFP. None of the 8 CD4-deficient mice investigated in two independent experiments developed vitiligo-like coat color changes, indicating the need for CD4+ T cell help during the induction phase of the immune response. Furthermore, depletion of CD4+ T cells during the priming phase completely prevented the induction of TRP2$_{aa180–188}$-specific CD8+ T cells *in vivo* (data not shown).

Influence of a competitively H2-K^b-binding epitope in the helper determinant It is conceivable that competitively H2-K^b-binding epitopes in the helper determinant may influence the immunogenicity of the weakly immunogenic self-epitope derived from TRP2. Therefore, we constructed plasmid DNA encoding for the TRP2$_{aa180–188}$ peptide attached in frame to the C-terminus of the immunogenic marker protein β-galactosidase using PCR techniques and inserted this sequence into the CMV-driven expression plasmid pAdlox as described above. *Escherichia coli* β-galactosidase contains the strong H2-K^b-binding peptide epitope βgal$_{aa497–504}$. Expression of β-galactosidase by pCMV-βgal.TRP2$_{aa180–188}$ was confirmed *in vitro* by X-gal staining of transiently transfected 293 cells. Groups of 6 C57BL/6 mice were shaved on the abdomen and bombarded with the expression plasmids pCMV-βgal.TRP2$_{aa180–188}$ or pCMV-βgal as a control using the gene gun on a weekly basis for 5 wk. Again, two mice of each group were sacrificed 1 wk after the third immunization to analyze induction of antigen-specific CTL *in vivo* using the IFNγ-ELISPOT technique. Mice immunized with pCMV-βgal.TRP2$_{aa180–188}$ showed reactivity for both the TRP2$_{aa180–188}$ and the βgal$_{aa497–504}$ peptide (Fig 3*A*). The remaining mice were again monitored for coat color changes. As expected, all mice immunized with plasmid DNA expressing the fusion protein βgal.TRP2$_{aa180–188}$ developed vitiligo-like fur depigmentation (Fig 3*B*). This result provides some experimental evidence that a strong competitively H2-K^b-binding epitope in an immunogenic protein such as β-galactosidase does not interfere with the ability to stimulate CD8+ T cells *in vivo* specific for the attached TRP2$_{aa180–188}$ peptide and precipitate autoimmune vitiligo. Thus, any foreign protein that accidentally contains a major histocompatibility complex (MHC) class I peptide sequence with similarity to a melanocytic self-antigen and is presented to the immune system in an immunogenic way may stimulate cross-reactive cytotoxic T cells that are potentially able to destroy melanocytes in the skin.

Local inflammation in the skin is required for autoimmune destruction of melanocytes in the effector phase We previously observed that the injection of recombinant adenoviruses encoding for the fusion protein EGFP.TRP2$_{aa30–519}$ only very rarely led to vitiligo-like fur depigmentation despite strong stimulation of TRP2$_{aa180–188}$ peptide-reactive CD8+ T cells *in vivo*, which were associated with protective immunity against experimentally induced melanoma metastases in the lungs (Steitz *et al*, 2002; Fig 4*A*). In subsequent experiments, we combined the injection of recombinant adenovirus with gene gun bombardment of the skin. Groups of six wild-type mice were injected

A



B

	vitiligo
pCMV-ßgal	0/6
pCMV-ßgal.TRP2 aa180-188	7/7

Figure 3
The presence of a strong competitively H2-K^b-binding epitope in the fusion protein between tyrosine-related protein 2 (TRP2) and *Escherichia coli* β-galactosidase did not impair the ability to stimulate TRP2-specific CD8+ T cells and induce vitiligo. Groups of C57BL/6 mice were immunized by particle-mediated bombardment of the skin with the indicated plasmid DNA. (*A*) Splenocytes were harvested after immunization and release of IFNγ in response to the H-2K^b-binding TRP2$_{aa180–188}$ peptide tested using ELISPOT assays. Results are expressed as mean number of spot forming cells per 10^6 splenocytes. These data are representative of three to five independent experiments. (*B*) Alternatively, mice were followed for the appearance of vitiligo-like fur depigmentation. The cumulative number of mice developing coat color changes as a result of the immunization with the indicated plasmid DNA are shown.

intraperitoneally with 5×10^8 p.f.u. of the recombinant adenoviruses Ad-EGFP.TRP2$_{aa180–188}$ or Ad-βgal followed after 1 and 2 wk with gene gun bombardment of the shaved abdomen with the plasmids pCMV-EGFP.TRP2$_{aa180–188}$ or pCMV-βgal. Surprisingly, we observed very rapid appearance of coat color changes in mice immunized with the EGFP.TRP2$_{aa180–188}$ fusion constructs. Depigmented fur could be detected shortly after the second gene gun bombardment (Fig 4*B*). Interestingly, Induction of CD8+ T cells recognizing the TRP2$_{aa180–188}$ peptide *in vivo* using the recombinant adenovirus Ad-EGFP.TRP2$_{aa180–188}$ and gene gun bombardment with the irrelevant expression plasmid pCMV-βgal also efficiently promoted fur depigmentation, indicating that local trauma and the associated inflammatory response is sufficient to cause autoimmune destruction of melanocytes in the skin. In subsequent experiments, we verified this hypothesis by experimentally inducing a contact allergy against Fluovio-2,6-dinitro-benzene (DNFB) in combination with the injection of recombinant adenovirus. Groups of mice were sensitized to DNFB 1 wk before the injection with Ad-EGFP.TRP2$_{aa180–188}$ or Ad-βgal. One week later, mice were again treated with DNFB on the ears and the abdomen to elicit the effector phase of contact allergy.

A

	vitiligo
Ad-EGFP.TRP2 aa180-188	0/10
Ad-EGFP	0/10

B

1. immunization	2. immunization	vitiligo
Ad-EGFP.TRP2 aa180-188	pCMV-EGFP.TRP2 aa180-188	10/12
	pCMV-ßgal	10/10
Ad-ßgal	pCMV-EGFP.TRP2 aa180-188	9/10
	pCMV-ßgal	0/6

C

1. immunization	skin treatment	vitiligo
Ad-EGFP.TRP2 aa180-188	DNFB	12/14
	acetone : oil	0/14
Ad-ßgal	DNFB	0/14
	acetone : oil	0/14

Figure 4
Local inflammation in the skin is required for autoimmune destruction of melanocytes by autoreactive CD8+ T cells specific for the tyrosine-related protein 2 (TRP2)$_{aa180–188}$ peptide. Groups of C57BL/6 mice were treated as indicated and followed for the appearance of vitiligo-like fur depigmentation. (*A*) The cumulative number of mice developing vitiligo-like fur depigmentation in response to intraperitoneal injection with the indicated recombinant adenoviruses are shown. (*B*) The cumulative number of mice developing vitiligo-like fur depigmentation in response to a combined immunization with the indicated recombinant adenoviruses followed by particle-mediated bombardment of the skin with the indicated plasmid DNA are shown. (*C*). The cumulative number of mice developing vitiligo-like fur depigmentation in response to immunization with recombinant adenovirus combined with the experimental induction of contact allergy against DNFB are shown.

Again, depigmentation of fur could be detected within 2 wk in areas of strong inflammation in the skin (Fig 4*C*).

Discussion

The involvement of potentially autoreactive antibodies and cytotoxic T lymphocytes in the pathophysiology of vitiligo is not completely understood. Recently, the application of

novel genetic immunization strategies for the induction of antigen-specific immune responses against melanosomal enzymes of the tyrosinase family has provided some insight into the mechanisms regulating immunity and tolerance to this category of antigens. Stimulation of antibodies as well as CD4 + helper T cells specific for the brown locus protein gp75/TRP1 has been associated with vitiligo-like fur depigmentation in C57BL/6 mice (Bowne *et al*, 1999; Overwijk *et al*, 1999). Coat color changes were also observed following induction of CD8 + cytotoxic T cells specific for the slaty locus protein TRP2 (Bowne *et al*, 1999; Steitz *et al*, 2000). However, vitiligo was not observed with several immunization strategies including the use of cultured dendritic cells as biological adjuvant despite strong induction of protective immunity against B16 melanoma cells (Bronte *et al*, 2000; Schreurs *et al*, 2000; Steitz *et al*, 2001).

In our experiments, we directly addressed the role of CD8 + CTL recognizing melanocytic self-antigens for the induction of vitiligo. We constructed an expression plasmid and a recombinant adenovirus that only encode the nine amino acids SVYDFFVWL corresponding to the H2-K^b-binding peptide epitope derived from aa180–188 of the melanosomal enzyme TRP2 attached to the immunogenic marker protein EGFP. This avoids the potential induction of CD4 + Th cells and antibodies specific for TRP2. Several lines of evidence demonstrate that the *in vivo* stimulation of $TRP2_{aa180–188}$-specific CD8 + CTL requires EGFP-specific CD4 + T helper cells during the induction phase of the immune response. Firstly, immunization of CD4-deficient mice did not lead to vitiligo-like depigmentation. Secondly, injection of recombinant adenovirus encoding autologous murine TRP2 followed by bombardment of the skin with plasmid DNA encoding autologous murine TRP2 did not stimulate $TRP2_{aa180–188}$-specific CD8 + T cells *in vivo* (Steitz *et al*, 2000) and never led to autoimmune vitiligo in any of the mice. In contrast, we consistently observed autoimmune vitiligo in almost every mouse when we intraperitoneally injected a recombinant adenovirus encoding fusion proteins between EGFP and fragments of TRP2 containing the $TRP2_{aa180–188}$ epitope and subsequently bombarded the skin with the gene gun. Thirdly, we could demonstrate that the depletion of CD4 + T cells immediately before the injection of recombinant adenoviruses abrogated *in vivo* stimulation of $TRP2_{aa180–188}$-specific CD8 + T cells.

Since $TRP2_{aa180–188}$-specific CD8 + CTL precursors obviously escape central deletion they must be regulated in the peripheral immune organs. One possibility is that they simply ignore their antigen in the skin under normal circumstances. This has been directly demonstrated in mice transgenically expressing a TCR derived from CD8 + CTL recognizing an altered peptide ligand to aa25–33 of the silver locus protein pmel17/gp100 (Overwijk *et al*, 2003). It is also conceivable, however, that $TRP2_{aa180–188}$-specific CTL are actively controlled by regulatory T cells. Recently, it has been shown that such regulatory cells may be stimulated by immature dendritic cells (Hawiger *et al*, 2001). Indeed, immature Langerhans cells from the epidermis constantly carry melanosomal proteins to the lymph node (Stoitzner *et al*, 2002). Alternatively, tolerance mechanisms may also be acquired during the neonatal period where CD8 + T cells have been demonstrated to access the epidermis and become functionally tolerant to peripherally expressed self-antigens (Alferink *et al*, 1998). To overcome peripheral tolerance, naïve $TRP2_{aa180–188}$-specific CTL need to be stimulated by activated antigen-presenting dendritic cells. Interactions with CD4 + Th cells mediated by additional signals such as CD40–CD40L signaling leading to full maturation of dendritic cells subsequently control the expansion and the effector function of $TRP2_{aa180–188}$-specific CTL.

Similar to other investigators, we found that effective induction of $TRP2_{aa180–188}$-specific CD8 + CTL *in vivo* is not necessarily associated with vitiligo-like fur depigmentation. Rather, autoimmune destruction of melanocytes appeared to depend on the immunization strategy. Bombardment of the skin with the expression plasmid encoding EGFP.$TRP2_{aa180–188}$ always led to vitiligo, whereas mice immunized by intraperitoneal injection with the corresponding recombinant adenovirus only rarely developed coat color changes. Subsequent experiments combining both gene transfer techniques revealed that autoimmune destruction of melanocytes by activated $TRP2_{aa180–188}$-specific CD8 + CTL largely depended on the inflammatory stimulus in the skin provided by the particle bombardment. Lane *et al* (2004) recently also reported that vaccination-induced autoimmune vitiligo is initiated by some form of trauma in the skin. In contrast to our experiments, however, they immunize with full-length human TRP2 protein and therefore cannot exclude responses by CD4 + Th cells and antibodies.

It is currently not exactly known how inflammation and trauma in the skin promote autoimmune destruction of melanocytes by vaccine-induced $TRP2_{aa180–188}$-specific CD8 + CTL. We hypothesize that the production of proinflammatory cytokines and chemokines by activated keratinocytes following local trauma or inflammation upregulates adhesion molecules on capillary venules, thereby facilitating lymphocyte homing to the skin and providing access to antigen-expressing melanocytes in the hair follicles. The inflammatory response might also expose melanocytes to cytotoxic destruction by upregulating their MHC expression, which is normally low to absent. Thus, activation of self-reactive T cells can potentially cause autoimmunity if there is a subsequent inflammatory event in the target tissue. This issue is of great importance for the experimental development of melanoma vaccines, since it implies that melanoma cells may also be completely ignored by activated pigment cell-specific CD8 + CTL unless an inflammatory microenvironment is established in the tumor tissue.

Our results suggest that immunity and tolerance of $TRP2_{aa180–188}$-specific CD8 + CTL in mice are regulated in two steps. In a first general step, stimulation and expansion of melanocyte-specific CD8 + CTL in secondary lymphoid tissue are controlled by CD4 + Th cells and dendritic cells. In a second tissue-specific step, an inflammatory stimulus is required to cause overt autoimmune destruction of melanocytes in the skin. A similar observation was reported in an experimental model of transplant tolerance where local inflammation also controlled the rejection of skin grafts carrying a defined MHC class I alloantigen recognized by transgenic CD8 + CTL (Limmer *et al*, 1998). We envision the following possible pathophysiologic scenario for the development of vitiligo: an infectious pathogen happens to express a protein containing an MHC class I-binding

peptide sequence with similarity to a melanocyte protein. This has already been described in the human system, where an HLA-A2-binding peptide epitope derived from the Herpes simplex virus protein Glycoprotein C is very similar to a HLA-A2-binding peptide epitope derived from the melanocytic self-antigen MART1/MelanA. Antigen-specific CD8+ T cells not only recognize infected cells but also cross-react with melanocytes and melanoma cells (Loftus *et al*, 1996). During active infection, the viral protein containing the self-peptide is presented in an immunogenic form and stimulates virus- and pigment cell-specific CD8+ T cells. Subsequently, these T cells may be drawn into the skin by an additional inflammatory stimulus. This can be a minor form of trauma that could provide an explanation for the clinical observation that vitiligo is frequently observed on the hands. Alternatively, a strong sunburn that is not infrequently reported by patients during the beginning and progression of vitiligo could precipitate the autoimmune destruction of melanocytes.

We are aware of the fact that our experimental model only focuses on the role of pigment cell-specific CTL in the pathogenesis of autoimmune vitiligo. Our experiments may only in part represent the human condition where not only cellular but also humoral immune responses to melanocytic proteins have been extensively described in the serum of many patients (Song *et al*, 1994; Cui and Bystryn, 1995; Baharav *et al*, 1996; Fishman *et al*, 1997; Kemp *et al*, 1997). Humoral immune responses have also been described to induce autoimmune vitiligo with the melanocytic antigen TRP1 in mice (Bowne *et al*, 1999). Thus the predominant type of the immune response apparently depends on the melanocytic self-antigen studied. Future investigations will help to clarify this very interesting and important issue.

Materials and Methods

Animals, expression plasmids, and recombinant adenoviruses Six to 12 wk-old wild-type and CD4-deficient C57BL/6 mice (H-2^b) were bred at the Central Animal Facility of the University of Bonn. Experiments were approved by the Rheinische Friedrich Wilhelm University and the government of NRW Germany. The institutional and national guide for the care and use of laboratory animals was followed. Plasmids expressing fusion proteins between the immunogenic helper determinants EGFP or β-galactosidase and parts of murine TRP2 were constructed using standard PCR-techniques, sequenced to exclude mutations introduced by PCR and inserted into the adenoviral shuttle vector pAdlox containing a CMV immediate-early promotor and a SV40 polyadenylation signal. Plasmids were grown in *E. coli* strain DH5α and purified using Qiagen Plasmid Maxi Kits (Qiagen, Hilden, Germany). E1- and E3-deleted adenoviral vectors expressing the fusion proteins were generated through Cre-lox recombination with reagents generously provided by Dr S. Hardy (Somatix, Alameda, California; Hardy *et al*, 1997). Adenoviruses were propagated on 293 cells, purified by cesium chloride density gradient centrifugation and subsequent dialysis according to standard protocols, and stored at −70°C.

Verification of antigen expression Antigen expression was verified by fluorescence microscopy and immunoblotting. 293 cells were either transfected with plasmid DNA using a standard $CaPO_4$ precipitation in the presence of 25 μM of chloroquine for the first 10–16 h or infected with recombinant adenoviruses. The expression of EGFP could be directly confirmed in living cells by fluorescence microscopy. Alternatively, cells were solubilized after 48 h in lysis buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA (ethylenediaminetetraacetic acid), 0.5% NP40, pH 7.5) containing protease inhibitors (Complete Protease Inhibitor Cocktail, Boehringer, Mannheim, Germany), subjected to a 10% SDS-PAGE, and electroblotted onto PVDF membranes. Membranes were blocked with 5% non-fat dry milk in T-PBS (phosphate-buffered saline) (0.1% Tween 20 in PBS) for 2 h and incubated with either a polyclonal rabbit anti-TRP2 antibody (Pep8, kindly provided by Dr Vincent Hearing, NIH, Bethesda, Maryland) or a polyclonal rabbit anti-EGFP antibody (BD Biosciences/Clontech, Heidelberg, Germany) at a 1:400 dilution in T-PBS + 2% non-fat dry milk for 1 h. Bound antibodies were detected with 1:5000 dilutions of a peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson Immunoresearch, Westgrove, Pennsylvania) in T-PBS + 2% non-fat dry milk for 1 h and visualized using chemiluminescence on X-ray film (Amersham, Freiburg, Germany).

Genetic immunization, induction of contact allergy, and evaluation of vitiligo Genetic immunization using the gene gun has been performed as described previously (Steitz *et al*, 2000). Briefly, plasmid DNA was precipitated onto 1.6 μm gold particles at a density of 2 μg of DNA per mg of particles. For immunizations, shaved skin of the abdomen was bombarded *in vivo* by two shots with the Helios gene gun (BioRad, Munich, Germany) at a pressure of 400 psi resulting in the delivery of approximately 1 mg gold carrying 2 μg plasmid DNA. Immunizations were repeated weekly for 5 wk. Genetic immunization with recombinant adenovirus was performed by one intraperitoneal injection of 5×10^8 p.f.u. recombinant adenovirus resuspended in 0.2 mL PBS. In prime-boost experiments, mice were first immunized with recombinant adenovirus and subsequently bombarded with plasmid DNA on day 7 and 14 as described above. For the experimental induction of a contact allergy mice were sensitized on day 0 and 1 with 25 μL of a 0.5% DNFB in an acetone:oil (4:1) solution (Sigma, Munich, Germany) painted on the shaved skin of the abdomen and ear. On day 7 sensitized mice were immunized recombinant adenovirus as described above. On day 14 mice were challenged with 10 μL of a 0.2% DNFB solution in acetone:oil (4:1) painted on the shaved skin of the abdomen and ear. The appearance of vitiligo-like fur depigmentation as a sign of autoimmune destruction of melanocytes was evaluated weekly for 4 wk after the last immunization.

Peptides, ELISPOT assays The H-2K^b-binding peptide SVYDFFVWL (TRP2$_{aa180–188}$) derived from the murine melanosomal protein TRP2 (Bloom *et al*, 1997) and the H-2K^b-binding peptide ICPMYARV (βgal$_{aa497–504}$) derived from *E. coli* β-galactosidase (Brossart *et al*,1997) were purchased from GENOSPHERE (Paris, France). Peptides were dissolved at 10 μg per mL in PBS containing 10% DMSO and stored at −20°C. The induction of peptide-specific T cells was measured using the ELISPOT technique. Briefly, splenocytes were harvested 1 wk after the last immunization and red blood cells depleted. 10^6 splenocytes per well were restimulated in 200 μL of CM containing 1 μg per mL synthetic peptide and 25 IU per mL rhIL-2 in Millipore HA plates, which were coated overnight with 10 μg per mL (50 μL per well) of anti-mIFNγ mAb (R4-6A2, Pharmingen, Heidelberg, Germany) in PBS. After 22 h cells were washed out of the plates and bound cytokines visualized by incubation with 2.5 μg per mL (50 μL per well) of biotinylated anti-mIFNγ mAb (XMG1.2, Pharmingen) for 1.5 h at 37°C, followed by 100 μL per well streptavidin–peroxidase (Roche, Manheim, Germany, 1:1500 dilution in PBS containing 1% BSA, and 0.05% Tween 20) for 1/2 h at RT, and premixed peroxidase substrate kit DAB (Vector Laboratories, Heidelberg, Germany). The number of spots was counted using the bioreader system (AIP, Straβberg, Germany) and expressed as mean number of spots of repeated duplicate or triplicate determinations.

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