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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*).

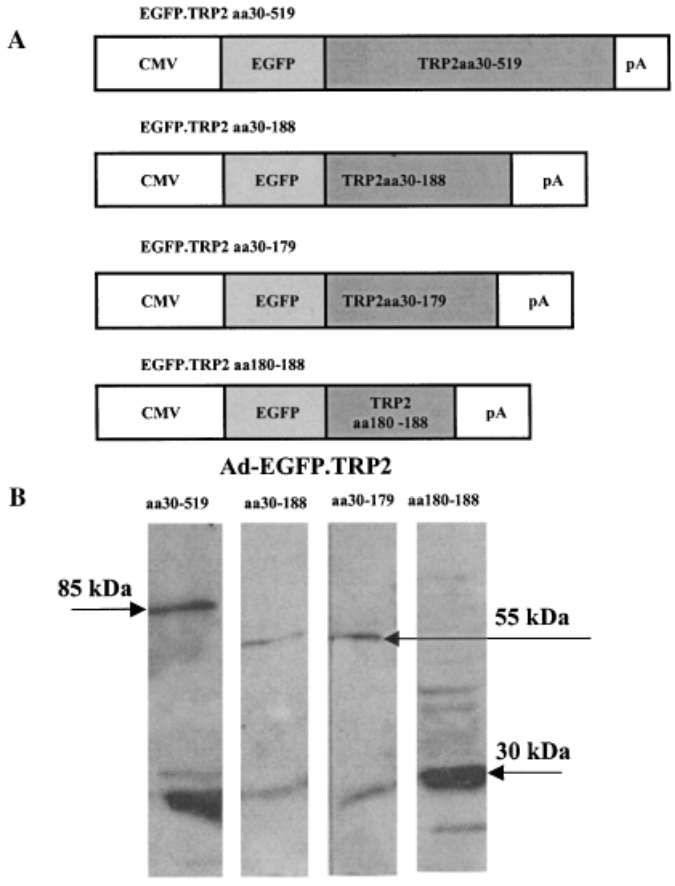

Figure 1
Expression plasmids and recombinant adenoviruses expressing C-terminal fusion proteins between enhanced green fluorescent protein (EGFP) and defined amino acid sequences of murine tyrosine-related protein 2 (TRP2). (*A*) The construction schemes of the expression plasmids pCMV-EGFP.$TRP2_{aa30–519}$, pCMV-EGFP.m$TRP2_{aa30–188}$, pCMV-EGFP.m$TRP2_{aa30–179}$, and pCMV-EGFP.m$TRP2_{aa180–188}$ 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.m$TRP2_{aa30–188}$, pCMV-EGFP.m$TRP2_{aa30–179}$, pCMV-EGFP.m$TRP2_{aa180–188}$, 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.m$TRP2_{aa30–188}$ or EGFP.m$TRP2_{aa180–188}$ 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