

Induction of GVHD-Like Skin Disease by Passively Transferred CD8⁺ T-Cell Receptor Transgenic T Cells into Keratin 14-Ovalbumin Transgenic Mice

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To understand the mechanisms involved in immunological tolerance to skin-associated antigens, we have developed transgenic (Tg) mice that express a model self-antigen, membrane-bound chicken ovalbumin (OVA), under the control of a keratin 14 (K14) promoter. K14-mOVA Tg mice express OVA mRNA in the epidermis, and appear normal. K14-mOVA Tg mice failed to mount T cell and delayed type hypersensitivity reactions to OVA, suggesting that the Tg mice were tolerant to OVA. Skin dendritic cells, including Langerhans cells, may contribute to the tolerance induction because migratory skin DC derived from K14-mOVA efficiently activated CD8⁺ T cells from OVA-specific T-cell receptor (Va2/Vb5) Tg (OT-I) mice. OT-I cells expanded and accumulated in skin-draining lymph nodes after intravenous injected into K14-mOVA mice and exhibited activation markers. Graft-versus-host disease-like skin lesions appeared in K14-mOVA mice by day 7 after injection of OT-I cells. These studies demonstrate that K14-mOVA Tg mice are susceptible to an autoimmunelike skin disease induced by passively transferred naïve CD8⁺ OVA T-cell receptor Tg T cells, and serve as a good model for understanding self-tolerance and for the investigation of the pathogenesis, treatment and potential prevention of cell-mediated autoimmune reactions in skin.

Key words: autoimmune disease/GVHD/Langerhans cells/tolerance
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Induction and maintenance of immunological tolerance to self-antigens is critical for the prevention of autoimmune diseases. Although the majority of T cells that react with self-antigens are deleted during thymic differentiation (Kappler *et al*, 1987; Kieselow *et al*, 1988; Pircher *et al*, 1989), some T cells reactive with peripheral and/or developmentally regulated self-antigens may escape clonal deletion and persist in the peripheral circulation. Therefore, additional regulatory mechanisms must be operative in order to prevent the activation of such auto-reactive T cells in peripheral tissues.

Regulatory mechanisms that maintain peripheral tolerance include anergy (Schwartz, 1990), immunological ignorance (Ohashi *et al*, 1991), and immune deviation (Zheng *et al*, 1999). The relative contributions of each of these to the maintenance of tolerance to skin-associated antigens are unknown. Recently, transgenic (Tg) mouse models in which model self-antigen are expressed in specific tissues have been developed to investigate the mechanisms underlying peripheral tolerance. Kurts *et al* (1996), used a rat insulin promoter (RIP) to induce tissue-specific expression of a membrane-bound form of ovalbumin (OVA). In these mice, OVA expression was induced only in pancreatic cells,

kidney proximal tubular cells, thymus, and testis. In another study, Vezys *et al* (2000), used an intestinal fatty acid binding promoter (iFABP) to induce intracellular OVA expression in small intestinal enterocytes. The advantage of studies using OVA as a model self-antigen is the availability of OVA-specific T-cell receptor (TCR) Tg mice. If CD8⁺ OVA TCR Tg T cells were passively transferred into RIP-OVA or iFABP-OVA Tg mice, Tg T cells were activated and proliferated in lymph nodes (LN) draining OVA expressing tissues, however, tissue destruction was not observed. Furthermore, these activated TCR Tg T cells were deleted after initial activation. Interestingly, if tissue destruction was induced in these OVA Tg mice by injection with activated cytotoxic T lymphocytes or infection with vesicular stomatitis virus, the passively transferred TCR Tg CD8⁺ T cells attack OVA expressing tissue, resulting in autoimmune reactions.

Skin is the tissue that covers most of the body surface and works as a barrier against the external environment. Therefore, immunocompetent cells in the skin (Langerhans cells (LC), skin homing T cells, etc.) must simultaneously subserve their roles of inducing protective immunity against harmful environmental stimuli and in preventing autoimmune reactions in the skin. To understand the development and maintenance of peripheral tolerance to skin-associated antigens, we developed Tg mice (K14-mOVA) that express a model self-antigen (OVA) under the control of the keratin 14 promoter. After passive transfer of OT-I CD8⁺ OVA TCR Tg

Abbreviations: CFA, complete Freund's adjuvant; GVHD, graft-versus-host disease; K14, keratin 14; LC, Langerhans cells; LN, lymph node; OVA, ovalbumin; mOVA, membrane bound OVA; TCR, T-cell receptor; Tg, transgenic

T cells (without any further activation), these mice develop graft-versus-host disease (GVHD)-like skin reactions.

Results

Expression of the OVA transgene in K14-mOVA Tg mice Five Tg mouse lines were established in which mOVA was expressed under the control of K14 promoter. Both Southern blot and PCR analysis of genomic DNA confirmed the integration of the transgene (not shown). Expression patterns of mRNA in different tissues were analyzed by RT-PCR. OVA mRNA was detected in skin, but not in other tissues such as brain, thymus, LN, heart, liver, spleen, kidney, intestine, skeletal muscles (Fig 1B), or esophagus (data not shown).

K14-mOVA Tg mice recognize mOVA as a self-antigen To determine whether K14-mOVA Tg mice were tolerant to OVA, these mice were immunized with OVA emulsified with complete Freund's adjuvant (CFA) by footpad injection on day 0, and draining LN cells were harvested on day 7 and assessed for proliferative responses to OVA using a standard *in vitro* proliferation assay. When control littermate mice were immunized and challenged with OVA, concentration-dependent T-cell proliferation was observed (Fig 2). In contrast, K14-mOVA Tg mice demonstrated significantly lower proliferative responses to OVA. No significant proliferation was observed either in the Tg or non-Tg mice if the unrelated antigen, pigeon cytochrome c, was used for immunization. Tolerance to OVA in K14-mOVA Tg mice was further assessed by *in vivo* analysis of their DTH reactions. Immunization of littermate control mice with OVA emulsified in CFA resulted in significant footpad swelling if these animals were challenged with OVA 7 d after priming (Fig 3). In contrast, K14-mOVA Tg mice immunized with OVA/CFA developed no significant change in their footpad thickness (similar to the results in unimmunized K14-mOVA Tg or littermate control mice). These results indicate that K14-mOVA mice are tolerant to the model self-antigen OVA.

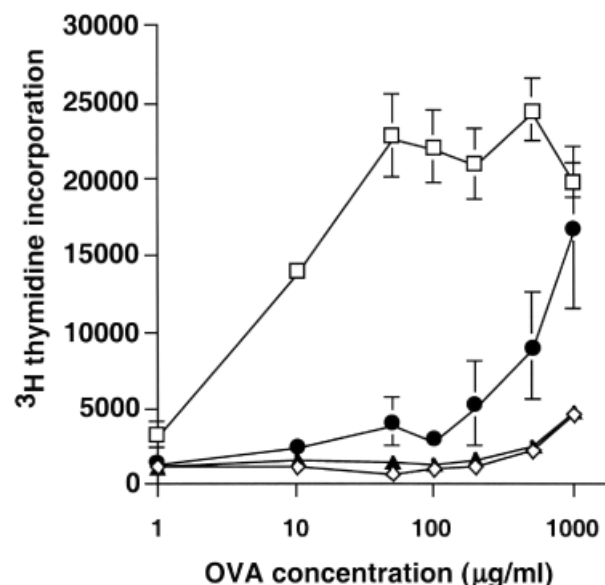


Figure 2

K14-mOVA Tg mice are tolerant to OVA. Priming with OVA + CFA failed to induce proliferative response of draining LN cells recovered from K14-mOVA Tg mice. Both K14-mOVA Tg mice and their littermate control mice were immunized with OVA emulsified with CFA by footpad injection on day 0, draining LN cells were harvested on day 7, and the proliferative response of LN cells to OVA was assessed by a standard *in vitro* proliferation assay. Concentration-dependent proliferation was observed in control littermate mice immunized and challenged with OVA (open squares). In contrast, K14-mOVA Tg mice demonstrated significantly lower proliferative responses to OVA (closed circles). If unrelated antigen, pigeon cytochrome c (CC), was used for immunization, no significant proliferation was observed in either the K14-mOVA Tg mice (closed triangles) and or in the littermate mice (open diamonds). K14, keratin 14; mOVA, membrane-bound OVA; Tg, transgenic; CFA, complete Freund's adjuvant; LN, lymph nodes.

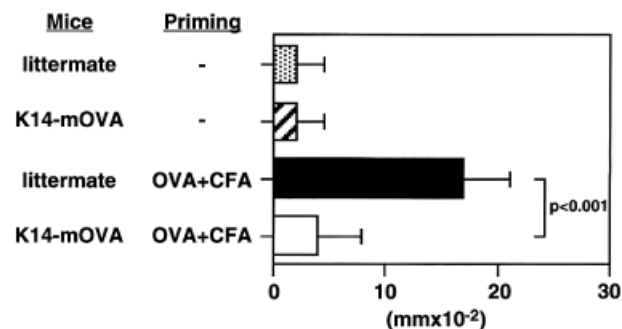


Figure 3

K14-mOVA Tg mice failed to induce DTH response to OVA. Both K14-mOVA Tg mice and their littermate control mice were subcutaneously injected with OVA emulsified with CFA. Seven days later, mice were challenged with phosphate-buffered saline with or without OVA into left hind footpads. Significant footpad swelling was observed in littermate controls immunized with OVA + CFA (closed bar). In contrast, K14-mOVA Tg mice immunized with OVA + CFA demonstrated no significant footpad swelling (open bar), which was similar to the result in non-immunized K14-mOVA mice (hatched bar) or non-immunized littermate controls (gray bar). Data indicates an average and standard deviation of ten animals in each group. K14, keratin 14; mOVA, membrane-bound OVA; Tg, transgenic; DTH, delayed-type hypersensitivity; CFA, complete Freund's adjuvant.

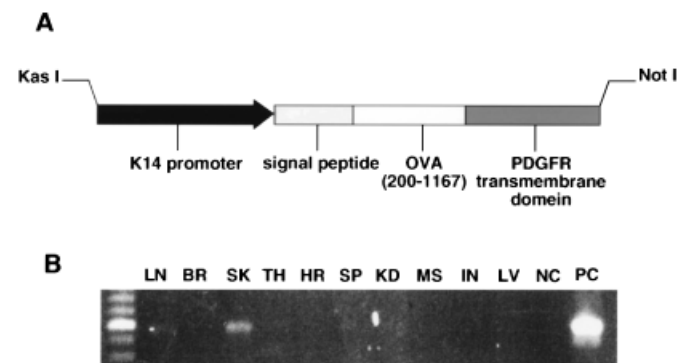


Figure 1

Tg construct used in generation of K14-mOVA Tg mice and tissue-specific expression of mOVA mRNA. (A) A truncated OVA cDNA (200–1167) and transmembrane domain of PDGFR were placed downstream of 2.1 kb human K14 promoter region in frame. (B) RT-PCR analysis demonstrated mOVA mRNA expression in skin. LN, lymph node; BR, brain; SK, skin; TH, thymus; HR, heart; SP, spleen; KD, kidney; MS, muscle; IN, intestine; LV, liver; NC, negative control without template; PC, positive control with a plasmid containing Tg construct; K14, keratin 14; mOVA, membrane-bound OVA; PDGFR, platelet-derived growth factor receptor; Tg, transgenic.

Homing and activation of passively transferred OT-I T cells CD8⁺ T cells were prepared from pooled LN and spleen cells of OT-I TCR Tg mice, and various numbers of CD8⁺ cells were injected via the tail vein into K14-mOVA Tg

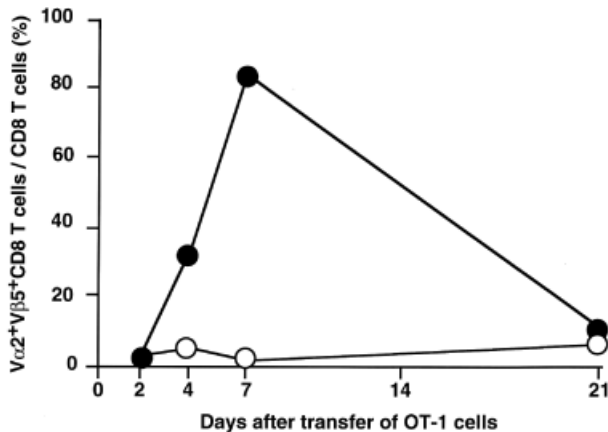


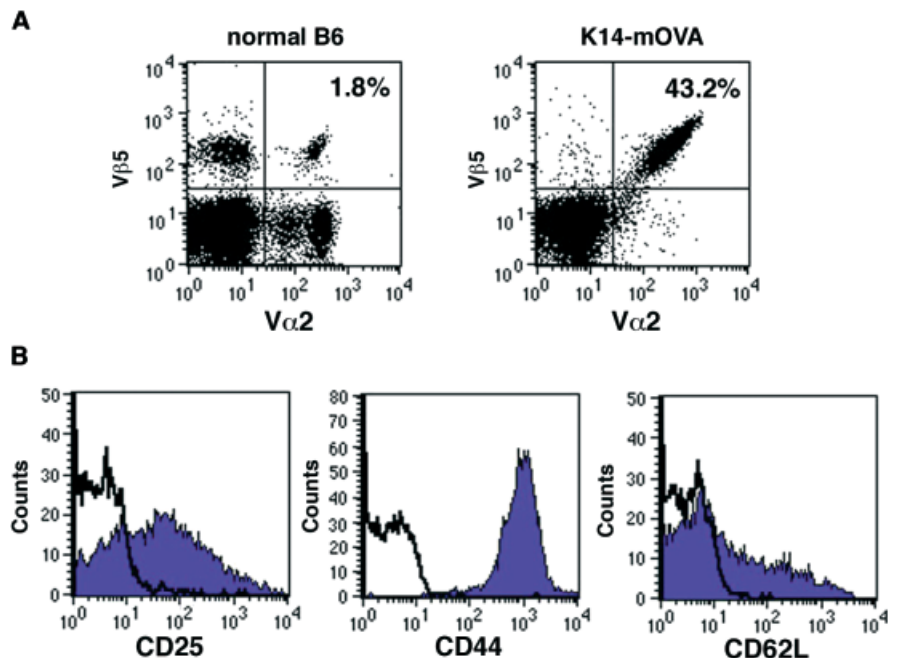
Figure 4
Kinetics of accumulation of OT-I cells in lymph node (LN) after passive transfer. At 2, 4, 7, and 21 d after passive transfer, draining superficial LN cells were analyzed for the accumulation of OT-I cells. At 7 d after the transfer, 90% of CD8 T cells were OT-I cells in LN of K14-mOVA mice (closed circles). In all, 2%–3% of CD8 T cells were OT-I cells in littermate mice that were infused with OT-I cells (open circles).

mice or into littermate control mice (Kurts *et al*, 1996). At 2, 4, 7 and 21 d after passive transfer of 5×10^6 , draining superficial LN cells were harvested and analyzed for the presence of OT-I cells. As early as 4 d after the transfer, $V\alpha 2^+ V\beta 5^+$ Tg T cells accumulated in the LN draining skin (Figs 4 and 5A). When the phenotype of $V\alpha 2^+ V\beta 5^+$ Tg T cells in K14-mOVA Tg LN was assessed transferred Tg T cells exhibited a $CD25^+ CD44^{bright} CD62L^{dim}$ activated phenotype (Fig 5B). In contrast, OT-I Tg T cells transferred into littermate control mice showed $CD25^- CD44^{dim} CD62L^{bright}$ resting phenotype (data not shown). A time course analysis demonstrated that OT-I T cells in K14-mOVA Tg mice started to accumulate in regional LN on day 4, further expanded on day 7, and returned to a baseline level (as in littermate control mice), resulting in no significant

accumulation of OT-I T cells in LN on day 21 (Fig 4). Thus, transferred OVA-specific OT-I cells recognize OVA antigen in K14-mOVA Tg mice, but not in their littermate control mice.

Development of GVHD-like skin disease following OT-I T-cell transfer Following the activation of OT-I T cells in regional LN, K14-mOVA Tg mice developed acute inflammatory skin lesions starting on day 5 (Fig 6C and D); 5×10^6 cells regularly induced GVHD-like reactions, whereas 1×10^6 cells induced lesser changes and 5×10^5 cells did not induce GVHD-like reactions. Footpads and ear skin became erythematous and swollen. Footpad swelling was most intense from days 7 to 11, and started to wane on day 12 (Fig 6E). Histopathological examination of the dorsum of the feet on day 7 revealed severe dermal edema and mononuclear cell infiltration in K14-mOVA Tg mice (Fig 6D). A thickened epidermis, exocytosis, dyskeratotic cells, and dermal inflammation were also seen when footpads were examined (Fig 7). A similar inflammatory reaction was also observed in the esophagus (Fig 8A and B). In addition to the skin and esophageal lesions, the spleen was slightly enlarged, and the thymus was markedly decreased in size. No significant inflammatory reaction was observed in other tissues including brain, lung, heart, liver, intestine, kidney, and skeletal muscles (data not shown). Control littermate mice transferred with OT-I T cells did not exhibit inflammatory skin lesions after transfer of cells (Fig 6A and C). K14-mOVA mice progressively lost body weight resulting in about 20%–30% loss by day 14 (Fig 9A). The majority of the K14-mOVA Tg mice, but not their littermate controls, died between 14 and 21 d after passive transfer (data not shown). Death is probably due to the inflammation that is seen around the mouth and in the esophagus, which makes it difficult or impossible for the mice to eat and drink. If K14-mOVA Tg mice were examined 21 d after transfer, these animals showed erythematous scaly macules and less hair

Figure 5
OT-I T cells passively transferred into K14-mOVA Tg mice expanded and expressed activation markers. FACS profiles showing: (A) Draining superficial lymph node cells from K14-mOVA Tg and littermate control mice were analyzed for the presence of OT-I cells ($V\alpha 2^+ V\beta 5^+$ CD8 T cells). At 4 d after the cells transfer, OT-I cells accumulated in the LN draining skin in K14-mOVA mice (43.2% positive for $V\alpha 2^+ V\beta 5^+$), but not in littermate controls (1.8% positive for $V\alpha 2^+ V\beta 5^+$). (B) When the phenotype of $V\alpha 2^+ V\beta 5^+$ CD8 T cells in the superficial LN of OVA Tg mice was analyzed, OT-I cells demonstrated a $CD25^+ CD44^{bright} CD62L^{dim}$ activated phenotype. In contrast, when the phenotype of $V\alpha 2^+ V\beta 5^+$ CD8 T cells in the superficial LN of littermate mice was analyzed, these T cells showed a $CD25^- CD44^{dim} CD62L^{bright}$ resting phenotype (data not shown). K14, keratin 14; mOVA, membrane-bound OVA; Tg, transgenic; LN, lymph node.



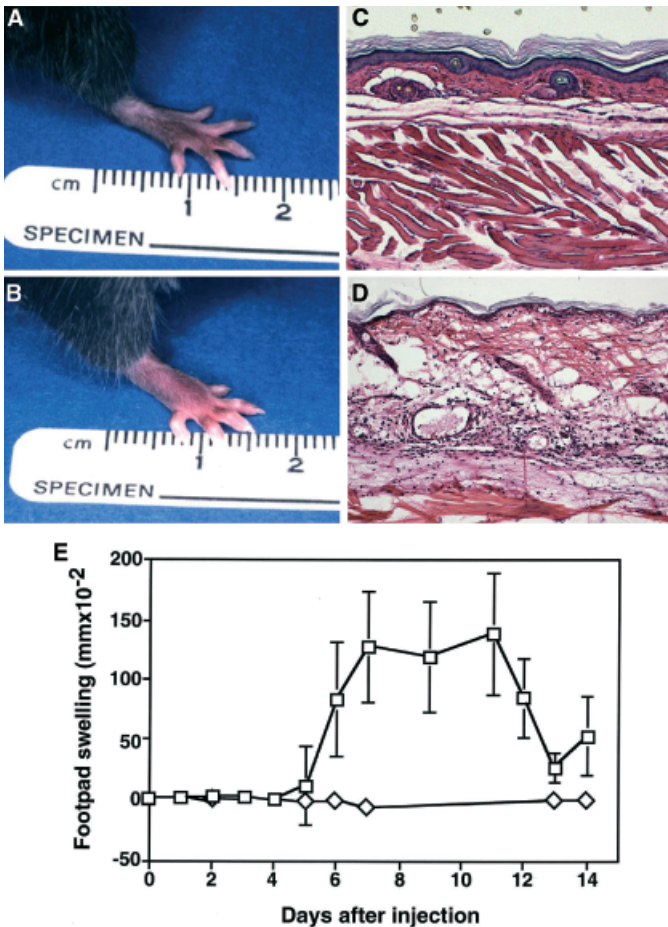


Figure 6
K14-mOVA Tg mice, but not their littermate control mice, developed acute inflammatory skin lesions after OT-I T cell transfer. OT-I cells were injected via the tail vein into K14-mOVA Tg mice or into littermate control mice. Footpad swelling developed as early as 5 d after transfer, became maximum on days 7–11 (compare (A) littermate control with (C) K14-mOVA mouse) and then decreased on day 12 (E). Histopathologic analysis of dorsum of the feet of the K14-mOVA Tg 7 d after i.v. injection of OT-I cells showed vasodilation, mononuclear cell infiltration, and severe dermal edema (D) as compared with littermate control mice (after injection of OT-I cells) where no significant pathologic reaction was observed (B). K14, keratin 14; mOVA, membrane-bound OVA; Tg, transgenic.

on their face and feet (Fig 9B). Histologically, K14-mOVA mice demonstrated hyperkeratosis, scattered dyskeratotic cells, mild mononuclear cell infiltration, and marked sclerotic change in the dermis, similar to chronic GVHD skin lesions (Fig 9D).

Migratory DC from K14-mOVA Tg mice induce proliferation of OT-I T cells To determine whether LC from K14-mOVA mice were capable of presenting OVA to OT-I CD8⁺ T cells, migratory dendritic cells were obtained 3 d after culture of ear skin. Unpulsed LC derived from normal B6 skin explant failed to induce proliferation of OT-I T cells (Fig 10). In contrast, unpulsed LC derived from K14-mOVA Tg skin explant induced significant concentration-dependent proliferation of OT-I T cells. These data indicate that OVA protein produced by K14-mOVA Tg keratinocyte is delivered to LC for the cross-presentation of OVA peptide in the context of class I major histocompatibility complex (MHC) molecules. Thus, tolerance observed in K14-mOVA Tg mouse may be due to the presentation of OVA antigen by LC migrating to the regional LN during the development of their immune systems.

Discussion

In this study, we demonstrated that skin-directed expression of a model self-antigen, OVA, is induced in Tg mice by using human K14 promoter and GVHD-like inflammatory skin disease developed by transferring OVA-specific OT-I CD8⁺ T cells.

Tg mouse models have been used as tools to investigate the mechanisms involved in peripheral tolerance. Early studies using tissue-specific expression of allogeneic MHC class I (Miller *et al*, 1990; Schonrich *et al*, 1991) and class II (Lo *et al*, 1988; Sarvetnick *et al*, 1988; Bohme *et al*, 1989; Lo *et al*, 1989; Miller *et al*, 1990) molecules demonstrated functional peripheral tolerance *in vivo*. Mice expressing model self-antigens such as lymphocytic choriomeningitis virus (LCMV) nucleoprotein, LCMV glycoprotein (Oldstone *et al*, 1991; Lo *et al*, 1992; Ohashi *et al*, 1993; Morgan *et al*, 1999) or influenza virus hemagglutinin (Roman *et al*, 1990) under the control of an insulin promoter did not exhibit autoimmunity, unless they were infected with the respective virus. In accordance with these previous studies, no significant inflammatory reaction was observed in K14-mOVA mice even after the immunization with OVA + CFA. Furthermore, lymphocytes recovered from OVA immunized K14-mOVA mice demonstrated significantly reduced proliferative responses to OVA. These data indicate that antigen-specific tolerance is induced in OVA reactive lymphocytes either by clonal deletion during development or by the regulatory mechanisms operating in peripheral tissues (Oldstone *et al*, 1991; Ohashi *et al*, 1993). Interestingly,

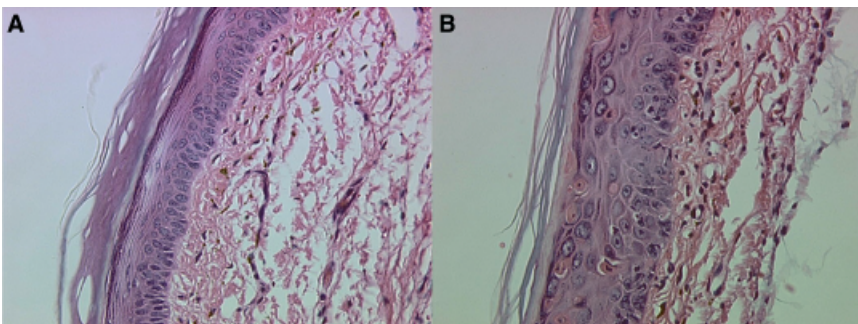
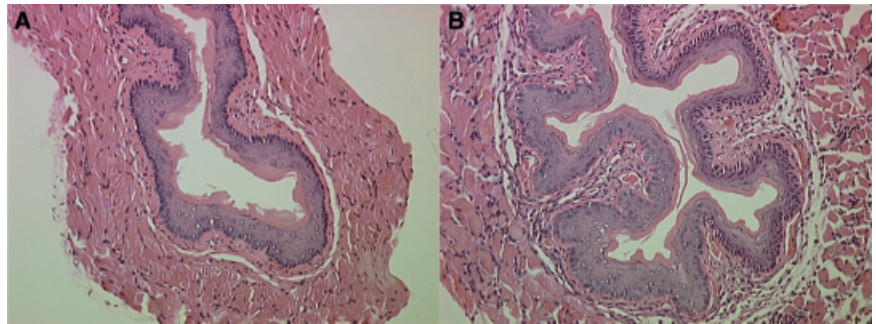


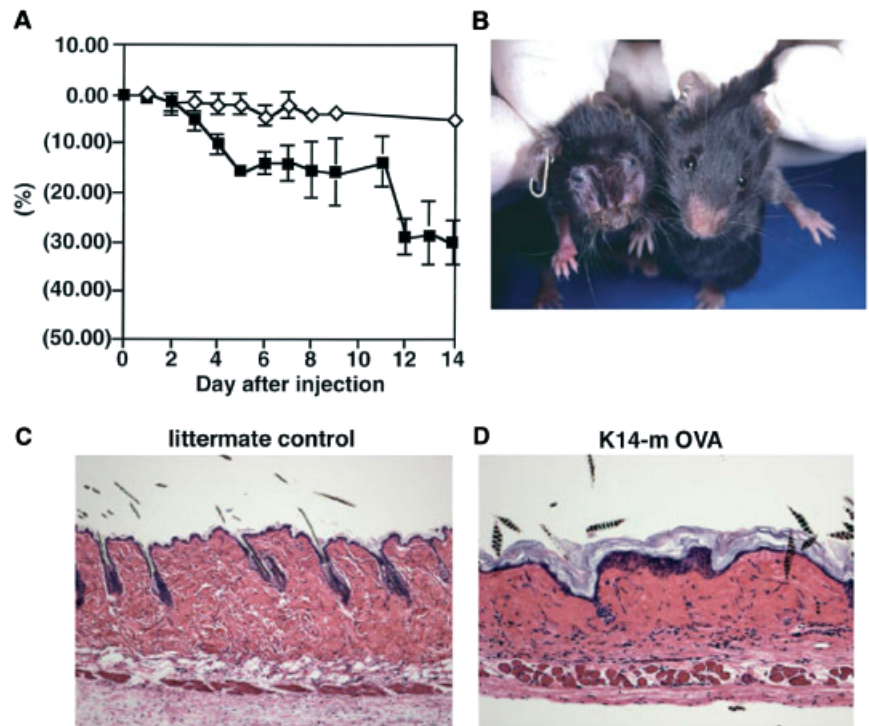
Figure 7
Graft-versus-host disease-like histology in the footpads of K14-mOVA mice after i.v. injection of OT-I cells. Histology of the footpad of normal C57BL/6 mice (A) and of K14-mOVA mice (B) 14 d after injection of 5 × 10⁶ OT-I cells. The K14-mOVA mice show a thickened epidermis, exocytosis, dyskeratotic keratinocytes, and dermal inflammation. K14, keratin 14; mOVA, membrane-bound OVA.

Figure 8

Esophagus of K14-mOVA mice show submucosal inflammation 7 d after i.v. injection of OT-I T cell transfer. Marked inflammation of the esophagus of K14-mOVA mice (B) ensues 7 d after OT-I T cell transfer (compare with (A) esophagus of normal C57BL/6 mice after i.v. injection of OT-I T cells). K14, keratin 14; mOVA, membrane-bound OVA.

**Figure 9**

Progressive weight loss and chronic graft-versus-host disease-like skin lesions developed in K14-mOVA Tg mice after OT-I T cell transfer. (A) K14-mOVA mice, but not their littermate control mice, progressively lost their body weight after transfer of the OT-I cells, resulting in about 20%–30% loss on day 14. (B) Gradually K14-mOVA Tg mice lost hair with mechanical rubbing and showed scaly lesions and were small compared with littermate mice. (C) littermate control and (D) K14-mOVA mouse. Histopathology showed marked hyperkeratosis, dyskeratosis, and severe dermal fibrosis in K14-mOVA Tg mice 21 d after OT-I T cell transfer. K14, keratin 14; mOVA, membrane-bound OVA; Tg, transgenic.



inflammatory skin reactions and tissue destruction were induced by simply transferring OT-I T cells without any further activation in our model. In prior studies using OVA as a model self-antigen (Kurts *et al*, 1996; Vezys *et al*, 2000), passively transferred OT-I T cells accumulated in the OVA expressing tissues and draining LN, and demonstrated an activated phenotype and proliferate in the LN, however, no pathogenic inflammatory reaction developed in OVA expressing tissues (Kurts *et al*, 1996; Vezys *et al*, 2000). Furthermore, transferred OT-I T cells were deleted (Kurts *et al*, 1997) or became anergic (Vezys *et al*, 2000) in these studies. Although we do not know the reason for this discrepancy, the presence of antigen-laden LC may result in the development of the inflammatory reactions seen in our K14-mOVA Tg mouse model. LC are bone marrow-derived antigen-presenting cells uniquely located within the epidermis (Katz *et al*, 1979). LC work as sentinels in skin and subserve an important role in T-cell-mediated skin diseases, such as contact dermatitis (Tamaki *et al*, 1981) and atopic dermatitis (Taylor *et al*, 1991). In GVHD, it is well known that epidermal LC disappear from the skin lesion (Lampert *et al*, 1982; Aractingi *et al*, 1997), suggesting their activation and migration to the regional LN. In our model system, expansion of OT-I cells began on day 4 and then the

swelling of the footpads and ears and histological changes occurred starting on days 6–7 and LC decreased in density from the epidermis when these K14-mOVA Tg mice were analyzed 7–10 d after OT-I T-cell transfer (data not shown). It has been previously demonstrated that DC can internalize soluble proteins or acquire antigen from apoptotic cells, and subsequently can activate class I restricted CD8⁺ cytotoxic T lymphocytes via a mechanism termed cross-priming (Brossart and Bevan, 1997; Albert *et al*, 1998). Consistent with these previous studies, migratory LC derived from K14-mOVA skin explants efficiently activated CD8⁺ OT-I T cells. Activated OT-I T cells may then attack OVA expressing epidermis and further enhance activation and migration of LC to the regional LN. Importantly, when we injected OT-I cells locally into the ears or footpads of K14-mOVA mice, no significant skin reaction ensued as determined histologically (data not shown). This supports the concept that cells need to be activated through interaction with antigen-presenting cells in the LN.

In summary, we have established a new Tg mouse model system in which T-cell-mediated autoimmune skin reaction can be induced by transferring TCR Tg mouse T cells. Although we do not know the underlying mechanisms by which autoimmune skin disease could be induced in our

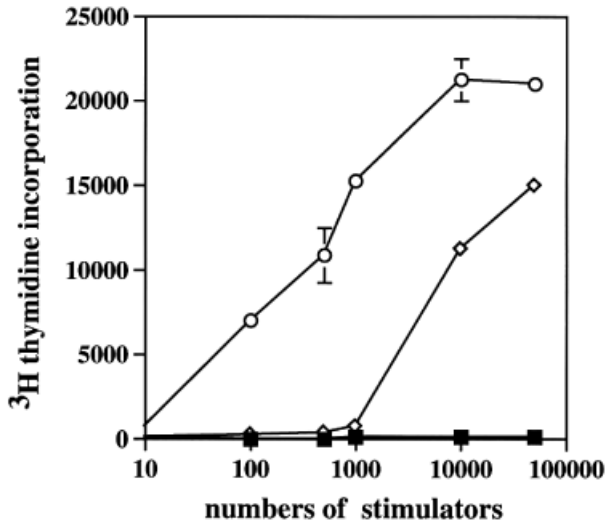


Figure 10

Migratory LC prepared from K14-mOVA Tg skin explants induce expansion of OT-I T cells. Migratory LC were prepared from normal B6 or K14-mOVA Tg mice and used as stimulator cells. Unpulsed LC derived from normal B6 skin explant failed to induce proliferation of OT-I T cells (closed squares). In contrast, unpulsed LC derived from K14-mOVA Tg skin explants (open diamonds) and LC derived from normal B6 mice that are OVA-peptide pulsed (open circles) induced significant concentration-dependent proliferation of OT-I cells. LC, Langerhans cells; K14, keratin 14; mOVA, membrane-bound OVA; Tg, transgenic.

system, this Tg mouse model will be useful to investigate both the induction and loss of peripheral tolerance, and to test potential therapeutic agents for the treatment of autoimmune diseases.

Materials and Methods

Mice All mice were obtained from the National Cancer Institute Animal Production Program (Frederick, Maryland), housed in a clean (not pathogen-free) facility that does not allow MHV or pinworms, bred, and used in accordance with institutional guidelines. OVA-specific class I restricted TCR Tg mice (OT-I) (Hogquist *et al*, 1994) were obtained from Dr Judy Kapp (Emory University, Atlanta, Georgia).

Construction of transgene and generation of Tg mouse lineages The plasmid pK14-mOVA was constructed as follows. OVA cDNA fragment (200–1167) was amplified from the TC-OVA plasmid (Lim *et al*, 1998) by PCR, using primers containing unique *Bgl*III and *Sac*II sites in their 5' upstream and 3' downstream sequences, respectively. The amplified PCR product was cloned into unique *Bgl*III and *Sac*II sites of the pDisplay expression vector (Invitrogen, Carlsbad, California) in frame to make a fusion protein that consisted of the murine immunoglobulin κ chain V-J2-C signal peptide, the OVA fragment (200–1167), and the transmembrane domain of platelet-derived growth factor receptor (PDGFR) (Fig 1A). To induce transgene expression under the control of the K14 promoter, the cytomegalovirus promoter of a pCMB β mammalian reporter vector (Clontech, Palo Alto, California) was excised with *Eco*R1 and *Xho*I (New England Biolabs, Beverly, Massachusetts), and replaced with a 2.1 kb human K14 promoter. Then, the vector sequence encoding β galactosidase was excised with *Not*I (New England Biolabs), and replaced with the membrane-bound OVA (mOVA) fusion protein construct, which was excised by digestion with *Eag*I (New England Labs). The construct was sequenced to verify authenticity of the mOVA fusion protein open reading frames and the junction of the K14 promoter to the mOVA sequence. For microinjection of the transgene, the Tg vector sequence was

excised with *Kas*I and *Hind*III (New England Biolabs), purified by gel electrophoresis, and microinjected into the pronuclei of C57BL/6 fertilized eggs. Transgene integration in founder mice was screened by Southern blot and PCR analysis of genomic DNA prepared from tail biopsies.

RT-PCR analysis of transgene expression Total RNA was prepared from various tissues including brain, thymus, lung, heart, liver, intestine, kidney, muscle, skin, esophagus, spleen, and LN using TriZol (Lifetechnology, Rockville, Maryland) according to the manufacturer's protocol. The total RNA preparation was reverse transcribed using a random hexamer (Roche, Foster City, California), and used as a template for the PCR reaction. Contaminating genomic DNA was digested with DNase I (Lifetechnology) before reverse transcription. 5' (TTCCAGGATTCGGA-GACAGTATTG) and 3' (TCATCAGGCAACAGCAAC) primers were used to amplify the mOVA fragment, which was detected on a 1.2% agarose gel containing ethidium bromide. All reagents for RT-PCR were purchased from Lifetechnology unless otherwise indicated.

Proliferation assays K14-mOVA Tg mice and littermate control mice were injected with 50 μ L (2 mg per mL) of OVA (Sigma, St Louis, Missouri) emulsified with complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Michigan) into one footpad. Seven days later, single cell suspensions were prepared from draining LN with complete RPMI (RPMI 1640) supplemented with 10% FCS (Biologicals, Rockville, Maryland), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 1% antibiotic-antimycotic, 10 mM HEPES (Lifetechnologies, Grand Island, New York) and 50 μ M 2-ME (Sigma Chemical Co., St Louis, Missouri) using standard techniques. A total of 3×10^5 cells per well were added to 96-well flat-bottom tissue culture plates with or without various concentrations of OVA and cultured for 4 d. A measure of 1 μ Ci [^{3} H]thymidine was added to each well during the last 18 h of culture period. At the end of the culture period, cells were harvested and cell-associated radioactivity was counted by direct β counting using a gas ionization counter (Packard, Meriden, CN).

Delayed-type hypersensitivity (DTH) reaction K14-mOVA Tg mice and littermate control mice were subcutaneously injected with 50 μ L of OVA emulsified with CFA onto the both lateral aspects of the lower abdominal area. Seven days later, mice were challenged with a subcutaneous injection of 40 μ L of phosphate-buffered saline (PBS) with, or without, OVA (2 mg per mL) into the left hind footpad and changes in footpad thickness at 24 and 48 h after challenge were determined using the technique of Matsu *et al*, 1999.

Preparation and passive transfer of OT-I T cells Single cell suspensions were prepared from pooled spleens and LN of OT-I mice, and resuspended in complete RPMI. CD8 $^+$ T cells were purified using CD8 columns (R&D Systems, Minneapolis, Minnesota) according to the manufacturer's protocol. FACS analysis of purified samples demonstrated greater than 98% purity of CD8 $^+$ cells. In all, $5\text{--}10 \times 10^6$ cells were resuspended in PBS and injected intravenously (i.v.) into K14-mOVA or control littermate mice. Body weight and footpad swelling were measured in both groups at various times after injection.

FACS analysis of K14-mOVA Tg mice after passive transfer At various time points, single cell suspensions were prepared from thymus, spleen, and LN of K14-mOVA Tg mice or littermate control mice that were injected i.v. with OT-I CD8 $^+$ T cells. Expression of surface molecules was quantified by flow cytometry using the following monoclonal antibodies (mAb); FITC-conjugated anti-V α 2, biotin-conjugated anti-V β 5, PE-conjugated anti-CD25, anti-CD44, anti-CD62L, or appropriate isotype-matched control mAb (Pharmingen). APC-conjugated streptavidin (Pharmingen, San Diego, California) was used for the visualization of biotin-conjugated mAb. Staining was performed according to standard techniques as

previously described and cells were analyzed with a FACSCalibur using Cell Quest software.

Mixed lymphocyte reaction Single cell suspensions were prepared from pooled spleens and LN of OT-I mice, and resuspended in complete RPMI. CD8⁺ T cells were purified using CD8 columns (R&D Systems). LN DC were isolated using a modification of a previously described method (Vremec *et al*, 1992). Pooled LN were teased and treated with collagenase (1 mg per mL) and DNase (0.02 mg per mL) (Sigma) at room temperature for 30 min with EDTA treatment during the last 5 min. Light density fractions were selected by centrifugation with 14.5% metrizamide (Sigma) at 1700 G. Interface cells were recovered and resuspended in complete RPMI for further use; 1.5×10^5 OT-I cells were co-cultured with various numbers of stimulator cells in 96-well flat-bottom tissue culture plates in complete RPMI for 4 d. Skin-derived DC (migratory LC) were isolated by methods published previously (Larsen *et al*, 1990) after 3 d of culture in complete RPMI. In all, 1.5×10^5 OT-I T cells were co-cultured with various numbers of stimulator cells in 96-well flat-bottom tissue culture plates in complete RPMI for 2 d and 16 h. During the last 16 h of culture period, [³H]thymidine was added (1 μ Ci per well) and cell-associated radioactivity was counted by direct β counting as described.

Histological analysis Tissue samples were prepared from K14-mOVA Tg mice at various times after passive transfer of OT-I cells and fixed in 10% neutral-buffered formalin. Paraffin embedded tissues were sectioned and stained with hematoxylin and eosin using standard techniques (American Histolabs, Rockville, Maryland).

Statistical analysis The Student t test was used. Differences were considered significant if $p < 0.05$.

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