Skin Inflammation Is Not Sufficient to Break Tolerance Induced against a Novel Antigen

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Skin Inflammation Is Not Sufficient to Break Tolerance Induced against a Novel Antigen

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Depending on the cellular and molecular microenvironment, immune responses generated by skin-associated lymphoid tissues can lead to protective immunity against pathogens or to tolerance. In this study, we investigated immune responses to an Ag expressed de novo in adult skin under homeostatic conditions by generating transgenic mice producing the Ag Ova in a Cre-inducible manner in keratinocytes. Expression of Ova was induced in adult mice with a tamoxifen-inducible K5-CreER transgenic line. Although Ova was efficiently expressed by keratinocytes and presented by Langerhans cells after Cre-mediated transgene recombination, adult transgenic mice did not develop any obvious autoimmune disease symptoms like hair or weight loss. Ag-specific T cells were activated after Ova expression as indicated by up-regulation of CD44 and CD69. After in vitro restimulation Ova-specific T cells showed reduced IFN-γ production suggesting induction of tolerance after Ova expression in the skin. After transfer into Ova-expressing mice, naïve OT-1 T cells transiently proliferated in skin-draining lymph nodes, infiltrated the skin but did not cause disease. Topical application of danger signals at the time of Ova induction did also not induce autoimmune disease. The unresponsiveness of Ag-specific T cells after induction of Ova expression could only be circumvented by simultaneous priming with CpG-matured, bone marrow-derived dendritic cells. Our data suggest that low amount of Ag expressed in the induction phase of the immune response results in tolerance even in the presence of danger signals and thereby helps to preserve homeostasis in the skin under normal and pathologic conditions.

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were excluded from all experiments. Mice were kept in the animal facility of

Ligand presentation assay

Skin explant culture

LC populations were isolated as previously described (25). Epidermal sheets were prepared from ears and body skin of three mice by Dispase digestion (Roche) and cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS and 5 X 10^{-5} M 2-mercaptoethanol in 24-well tissue culture plates to allow LC to migrate out of the epidermis. LC were collected autoclaved, enriched on a Nycodenz gradient (Sigma-Aldrich) and positively sorted by immunomagnetic bead procedure (Miltenyi Biotec). The skin explant culture was performed with LiveCycle software (BD Biosciences) (Roche) as previously described (19, 22) by using the porphobilinogen deaminase (PBGD) gene as an internal loading control. Primer sequences can be obtained upon request.

Isolation of dermal and epidermal cell suspensions

Mice were killed by cervical dislocation. After shaving, the total skin and the dorsal and ventral halves of the ears were placed on 0.8% trypsin (Sigma-Aldrich) for 45 min at 37°C. The epidermis was separated from the dermis and both tissues incubated separately at 37°C for 40 min in medium containing 250 μg/ml DNasel (Sigma-Aldrich) with gentle stirring. Cells were filtered through a 70-μm nylon mesh and centrifuged. Epidermal cells were cultured onto nitrogen-fibronectin coated dishes in low calcium MEM (Sigma-Aldrich) containing 8% chelated FCS for 4–5 days as previously described (19). To separate Langerhans cells (LC) and keratinocyte populations, cultured epidermal cell suspensions were labeled with biotinylated anti CD11c Ab (BD Pharmingen) or anti I-A^β Ab followed by incubation with streptavidin-microbeads and positive sorting over Large Cell columns (Miltenyi Biotec).

Analysis of peptide presentation by ELISA

Flow cytometric analysis

Cell suspensions from isolated LN were filtered through a 70-μm nylon mesh, counted, and stained with mAbs for 30 min at 4°C. Dermal and epidermal cell suspensions were isolated as described above and stained with fluorescently labeled Abs after blocking with Fc-block (BD Pharmingen). The following mAbs were used: anti CD3e-PE (clone H1.2F3), anti CD80-PE (clone 16-10A1), anti CD86-FITC (clone GL-1), anti MHCII-FITC (clone 2G9), anti Vα2–PE (clone B20.1), anti Vβ3,1/2.5-biot. (clone MR9-4), all BD Pharmingen and anti CD4-TC (clone RM4-5), anti CD8α-Alexa 647 (clone 5H10), both Caltag Laboratories. Biotinylated Abs were further identified with incubation with Streptavidin RPE-Cy5 (DakoCytomation). Data were acquired on a FACSCalibur or LSRII flow cytometer (BD Biosciences) and analyzed by using CellQuest software (BD Biosciences).

ELISPOT assay

The number of Ova specific, IFN-γ-producing CD^8^ T cells was determined by ELISPOT as described (24). In brief, whole LN cells of primed TX-treated and untreated K5-Ova/K5-CreER and control mice were plated in duplicates at the indicated cell number in multiscreen 96-well assay plates (Millipore) that had been precoated with the anti-IFN-γ Ab R4-6A2 (5 μg/ml). Cells were stimulated with the H-2Kb-restricted Ova peptide SIINFEKL (100 μg/ml) for 18 h at 37°C/5% CO₂. After extensive washing, the biotinylated anti-IFN-γ Ab AN18.17.24 (2 μg/ml) was added for 2 h. Detection was conducted with POD conjugated to Streptavidin (Boehringer Mannheim; dilution: 1/5000 for 1 h) followed by the addition of 100 μl substrate (0.8 mg/ml 3,3’-diaminobenzidine (Sigma-Aldrich)/0.4 mg/ml Nicl, Sigma-Aldrich)/0.0095% H₂O₂ in 0.1 M Tris (pH 7.5). Spots were counted using the Bioreader-2000 (Biosys).

Skin explant culture

LC populations were isolated as previously described (25). Epidermal sheets were prepared from ears and body skin of three mice by Dispace digestion (Roche) and cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS and 5 X 10^{-5} M 2-mercaptoethanol in 24-well tissue culture plates to allow LC to migrate out of the epidermis. LC were collected autoclaved, enriched on a Nycodenz gradient (Sigma-Aldrich) and positively sorted by immunomagnetic bead procedure (Miltenyi Biotec). Routinely, purity was >85%. For responder cell isolation, spleens and LN from 2 OT-1 mice were pooled and negatively depleted with streptavidin microbeads (Miltenyi) to obtain a pure CD8α^- T cell population using a mixture of the following biotinylated mAbs: anti Ter119 (clone Ter119), anti CD4 (clone GK1.5), anti CD19 (clone 1D3), anti CD45R (clone RA3–6B2), anti CD11b (clone M1/70), anti CD11c (clone HL3), anti-Ly6C/G clone RB6/8C5), and anti-NK1.1 (clone PK136; all BD Pharmingen). Purity was routinely >95%. DC were then titrated against
0.5 × 10^6 OT-1 T cells up to a DC/T ratio of 2:1 and cultured in NCM (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, t-glutamine (2 mM), HEPES (25 mM), and 5 × 10^{-5} M 2-ME) for 72 h in round-bottom 96-well plates (Nunc). Eighteen hours before harvesting, cells were labeled with 1μCi [³H]Thymidine/well. For positive control, DCs were incubated with 100 μg/ml SIINFEKL peptide and washed in medium before coculture with OT-1 T cells. Radioactivity was measured on a Microbeta liquid scintillation counter (Wallac). As an alternative approach to assess Ag presentation of isolated DCs, NaCt, OT-1 T cells were labeled with 5 μM CFSE in PBS for 10 min at room temperature and washed with NCM. Two hundred thousand OT-1 T cells were incubated with the indicated numbers of APCs. Proliferation was analyzed by flow cytometric analysis after staining with anti CD69 Ab (BioLegend) as described above.

**Brdu incorporation assay**

Mice were injected i.p. with 250 μg Brdu (Roche) and Brdu incorporation in CD8α- cells was analyzed after 20 h by flow cytometry. Skin draining LN were minced, cells were stained for CD8α, resuspended in ice-cold 95% Ethanol/0.15 M NaCl and kept on ice for 30 min. Cells were fixed in 1% PFA/0.01% Tween 20 in PBS at 4°C overnight. Cells were then incubated in 1 ml DNaseI solution (50 Kunitz U/ml DNaseI, 0.15 M NaCl, 4.2 mM MgCl2, 10 mM HCl), washed, and incubated with anti Brdu-FITC (BD Pharmingen) or mouse-IgG FITC as isotype control Ab and analyzed by flow cytometry.

**Adoptive transfer**

Suspensions of LN and spleen from CD45.1 congenic OT-1 mice were prepared and negatively depleted by immunomagnetic beads procedure to obtain pure CD8α+ T cells as described above. Cells were subsequently labeled with 5 nM CFSE for 20 min at room temperature and washed twice with PBS (Life Technologies). Cells (3 × 10^6) in 200 μl PBS were injected into the tail vein of each mouse. At each indicated time point, skin-draining and mesenteric LN were analyzed for the presence of CD45.1+ cells by flow cytometry using biotinylated anti CD45.1 mAb (clone A20, BD Pharmingen) followed by SA-PE Cy5 staining. Skin samples were collected and separated into dermis and epidermis. Pooled samples from three mice were analyzed by flow cytometry.

**In situ apoptosis after adoptive transfer**

Suspensions of OT-1 T cells were prepared as described above. BM-DC were prepared from the femurs and tibiae of C57BL/6 mice. After RBC lysis, cells were cultured in NCM in the presence of 100 U/ml GM-CSF for 7 days. Half of the medium was replaced every other day. On day 7, cells were incubated with 100 ng/ml LPS overnight and thereafter pulsed with 20 μg/ml Ova peptide SIINFEKL for 1 h. BM-DC (2 × 10^5) were then cultured with 2 × 10^5 OT-1 cells for at least 10 days. IL-2 (40 U/ml) was added to the culture every other day. After 1 wk, cells were restimulated with Ova-peptide pulsed BM-DC. After 10 days, OT-1 cells were harvested, washed, and resuspended in PBS. Cells (5 × 10^6) were injected intradermally into one ear of mice that had been treated with TX for about 3 wk. Frozen sections of skin from TX-treated mice were stained with anti-activated Caspase 3 Ab (clone A20, BD Pharmingen) followed by SA-PE Cy5 staining. Skin samples were collected and separated into dermis and epidermis. Pooled samples from three mice were analyzed by flow cytometry.

**Topical immune stimulation in the skin**

Mice were treated with 7.5 μg of 12-O-tetradecanoylphorbol-13-acetate (TPA) in acetone/sunflower seed oil emulsion or acetone/sunflower seed oil emulsion alone on the inner halves of both ears on day 0 and 3. Ova expression was induced by TX injection on days 1 to 3 and then weekly. After 4 wk, mice were restimulated with 5 μg of TPA or acetone/sunflower seed oil emulsion on the inner halves of the ears and mice were killed for analysis after 24 h. For 2,4-dinitro-1-fluorobenzene (DNFB) treatment, mice were sensitized with 50 μl of 0.5% DNFB (SIGMA-Aldrich) in acetone/sunflower seed oil emulsion or acetone/sunflower seed oil emulsion alone on shaved belly skin. After 7 days, mice were challenged with 0.3% DNFB solution on inner ears and tail to induce a delayed type hypersensitivity response. At the same time, TX injection was started. Mice were rechallenged every 10 days or 1 mo and analyzed 24 h later. For TLR ligations mice were induced to express Ova and simultaneously injected intradermally with 48 μg of CpG-ODN 1668 (TCCATGCGGTTCCTGAT GCT, Purimex) or 48 μg of CpG-ODN 1720 (TCCATGAGCTTCCCTGA TGCT) or treated topically with Imiquimod (Aldara 5% cream, 3M, France) or vehicle on the skin every other day and observed for weight changes and skin alterations for at least 3 wk. To analyze T cell activation, mice were killed after 1 or 3 wk and skin draining LN suspensions were restimulated in vitro with increasing doses of SIINFEKL peptide. Cells were cultured for 48 (K5-Ova/K5-CreER OT-1) or 72 h (K5-Ova/K5-CreER) before [³H]Thymidine was added for 18 h. Proliferation was analyzed by flow cytometry as described above.

**In vivo treatment with bone marrow-derived mature DC**

BM-DC were generated as described above. On day 7, CpG 1668 (Purimex) was added to the culture. On day 9, loosely adherent mature DC were harvested, pulsed for 1 h with 10 μg/ml SIINFEKL peptide, and washed twice with PBS. Cells (5 × 10^6) were injected into each ear of recipient mice. At the same time, mice were injected with TX to induce expression of OVA. Eight days later, ear draining retroauricular LN were...
FIGURE 2. Cre-recombination leads to Ova-expression in epidermal cells. A. Epidermal cell suspensions were isolated from mice of transgenic lines 21, 22, 26, and 29 that were either left untreated or treated with TX. Freshly prepared epidermal cell suspensions were plated in triplicates and incubated with 1 × 10^4 4B10 hybridoma T cells that recognize presentation of SIINFEKL peptide on MHC-I H2-Kb. After 24 h, supernatants were collected and analyzed for secreted IL-2 by ELISA. Only epidermal cells from TX-treated mice induced IL-2 production. For positive control, nontransgenic keratinocytes were pulsed with 100 μg/ml Ova peptide SIINFEKL and then incubated with 4B10 cells. One representative experiment of four is shown. B. Epidermal cells isolated from skin of mice of line 26 were depleted of MHC-II^+ and CD11c^+ cells before coculture with 4B10 cells. Results show the relative difference of IL-2 production normalized to control cells as a summary of three experiments. *** p < 0.001; n.s. not significant. C. LC and keratinocytes were isolated from cultured epidermal cell suspensions by magnetic activated cell sorting with biotinylated anti-I-Ab followed by streptavidin microbeads and titrated numbers were incubated for 2 days with 2 × 10^5 CFSE labeled OT-1 T cells in triplicates. The percentage of divided (CFSE diluted), CD69^+ OT-1 T cells is depicted. D. Representative histogram blots of OT-1 cells stimulated by 10^5 CFSE-labeled OT-1 T cells. For restimulation in vitro cell numbers were normalized according to the percentage of V/H9251 isolated from cultured epidermal cell suspensions by MACS to identify the cell type presenting the Ova peptide. E. Quantitative real-time PCR showed comparable low expression of Ova in keratinocytes of the lines 26 and 29 whereas Ova expression was slightly higher in line 21 (Fig. 1D). Ova expression in keratinocytes of line 26 (26K5-Ova/K5-Cre mice), where Cre is constitutively active, was similar to the respective inducible mice (Fig. 1D). All K5-Ova/K5-Cre mice derived from the three independent founders were healthy and did not show any skin alterations after induction of Ova expression. F. Ova-derived peptides are presented by epidermal cells in vitro. We next analyzed whether Ova-derived peptides were presented on the surface of keratinocytes after TX-induced transgene recombination (Fig. 2). Freshly prepared epidermal cell suspensions were cocultured with the T cell hybridoma 4B10, which secretes IL-2 when specifically recognizing the Ova peptide SIINFEKL presented on H2-K^b. As shown in Fig. 2A, epidermal cells from transgenic lines 26, 21, and 29 induced the production of IL-2 only after TX-induced Ova-expression. In line 22, where no transgene recombination occurred after TX-induced Cre activation, no IL-2 production was observed (Fig. 1C). Quantitative real-time PCR showed comparable low expression of Ova in keratinocytes of the lines 26 and 29 whereas Ova expression was slightly higher in line 21 (Fig. 1D). Ova expression in keratinocytes of line 26 (26K5-Ova/K5-Cre mice), where Cre is constitutively active, was similar to the respective inducible mice (Fig. 1D). All K5-Ova/K5-Cre mice derived from the three independent founders were healthy and did not show any skin alterations after induction of Ova expression.

isolated and cell suspensions analyzed by flow cytometry for the percentage of Ag-specific CD8^+ T cells. For restimulation in vitro cell numbers were normalized according to the percentage of Vo2^+ Vβ5.2^+ T cells and cultured with increasing concentrations of SIINFEKL peptide. After 2 days, 0.5 Ci [3H]thymidine/well was added for 16 h. Proliferation was analyzed as described above. Two to five mice per group were analyzed. The experiment was repeated in 26K5-Ova/K5-CreER mice in C57BL/6 background with comparable result.

Statistical analysis
The statistical significance of the data was determined by applying the two-tailed Student's t test. The difference was considered statistically significant at p < 0.05.

Results
Inducible expression of Ova in the epidermis
We have generated four independent transgenic mouse lines (lines 21, 22, 26, and 29) expressing Ova from the keratin 5 promoter (K5-Ova mice) in a Cre-inducible and time-controllable manner (Fig. 1A). The K5 promoter targets expression of Ova to basal cells of stratified epithelia and thymic epithelial cells (20). A truncated form of Ova lacking the signal-sequence but including all major MHC-I and MHC-II-restricted peptides was used to selectively induce cellular immune responses. Expression of Ova could be induced after Cre-mediated removal of the YFP cassette, which is located between the promoter and the Ova gene and is flanked by two loxP sites (Fig. 1A). In an uninduced state, YFP was detected in a fraction of epidermal cells of all four lines (Fig. 1B and data not shown). To induce Ova-expression in epidermal cells, all K5-Ova lines were crossed with a TX-inducible K5-CreER line (K5-Ova/K5-CreER mice) (21). As a positive control for Cre-mediated recombination we crossed K5-Ova mice to K5-Cre mice (K5-Ova/K5-Cre) expressing Cre constitutively from embryonic day 14.5 on (20). After TX injection, Cre-mediated recombination occurred in keratinocytes of the transgenic lines 21, 26, and 29 (Fig. 1C). Quantitative real-time PCR showed comparable low expression of Ova in keratinocytes of the lines 26 and 29 whereas Ova expression was slightly higher in line 21 (Fig. 1D). Ova expression in keratinocytes of line 26 (26K5-Ova/K5-Cre mice), where Cre is constitutively active, was similar to the respective inducible mice (Fig. 1D). All K5-Ova/K5-CreER mice derived from the three independent founders were healthy and did not show any skin alterations after induction of Ova expression.

Ova-derived peptides are presented by epidermal cells in vitro
We next analyzed whether Ova-derived peptides were presented on the surface of keratinocytes after TX-induced transgene recombination (Fig. 2). Freshly prepared epidermal cell suspensions were cocultured with the T cell hybridoma 4B10, which secretes IL-2 when specifically recognizing the Ova peptide SIINFEKL presented on H2-K^b. As shown in Fig. 2A, epidermal cells from transgenic lines 26, 21, and 29 induced the production of IL-2 only after TX-induced Ova-expression. In line 22, where no transgene recombination occurred after TX-induced Cre activation, no IL-2 production could be detected. Interestingly, IL-2 production was slightly higher in line 21, thus reflecting the higher expression levels observed by quantitative real-time PCR. Because the goal of this study was to investigate the effect of a de novo expressed Ag in adult skin under homeostatic conditions, we decided to focus our analysis on line 26.

Epidermal cells consist not only of keratinocytes but also of LC, therefore we depleted MHC-II^+/CD11c^+ cells from epidermal cell suspensions by MACS to identify the cell type presenting the
SIINFEKL peptide. Depletion of MHC-II⁺/CD11c⁺ cells from TX-treated K5-Ova/K5-CreER epidermal suspensions reduced IL-2 production by the hybridoma to control levels, indicating that LCs are primarily responsible for peptide presentation (Fig. 2B).

To verify, that indeed LC are cross-presenting Ova expressed in keratinocytes we isolated LC by two different methods, either by trypsinization of epidermis and purification with anti-MHC-II and MACS beads (Fig. 2, C and D) or skin explant culture (Fig. 2, E and F). LC isolated from TX-treated K5-Ova/K5-CreER mice by both methods presented Ag and were able to stimulate OT-I T cells, although not to the same extent as LC isolated from mice constitutively expressing Ova. Interestingly, keratinocytes isolated in the same experiment were not able to stimulate proliferation of OT-I T cells when used at similar numbers (Fig. 2C), most probably because they are lacking costimulatory molecules necessary to activate naive OT-I T cells. These results show that Ova is presented by epidermal cells of the K5-Ova/K5-CreER transgenic lines 21, 26, and 29 and that Ova-derived peptides can be cross-presented by epidermal LC after TX-induced Cre activation.

Cross-presentation of Ova in skin-draining lymph nodes

We have shown that Ova peptide can be cross-presented by MHC class II⁺/CD11c⁺ cells such as LC (Fig. 2, C and D). To investigate the effects of Ova expression on Ag-specific T cells, 26K5-Ova/K5-CreER mice were crossed with OT-I transgenic mice, in which almost all T cells should recognize the Ova derived peptide SIINFEKL. We then analyzed the phenotype of Ag-specific T cells in the periphery. CD8α⁺/Vα2⁺ T cells of TX-treated K5-Ova/K5-CreER-OT-I mice of all three lines (21, 26, and 29) expressed high levels of CD44 in skin-draining LN and in peripheral blood 3 wk after induction of Ova-expression (Fig. 3A and data not shown). However, none of the mice developed disease symptoms like hair or weight loss at any time-point after induction of Ova expression.

In addition, 6 days after Ova induction by TX-induced transgene recombination T cells had acquired an effector-phenotype as evidenced by up-regulation of CD69 and down-regulation of CD62L on CD8α⁺ T cells (Fig. 3D). DC from skin-draining LN of TX-treated K5-Ova/K5-CreER mice of line 26 (26K5-Ova/K5-CreER + TX), 26K5-Ova/K5-CreER mice constitutively expressing Ova and control mice not expressing Ova were incubated with CD8α⁺ T cells from OT-I mice (Fig. 3, C–E). These results suggest that T cell priming by Ova had occurred in the periphery and T cells had acquired an activated and effector-phenotype. We therefore assessed whether epidermis-derived DC are directly involved in the presentation of Ova-derived peptides in skin-draining LN. Isolated CD11c⁺ DC from skin-draining LN of TX-treated K5-Ova/K5-CreER mice of line 26 (26K5-Ova/K5-CreER + TX), 26K5-Ova/K5-CreER mice constitutively expressing Ova and control mice not expressing Ova were incubated with CD8α⁺ T cells from OT-I mice (Fig. 3, C–E). Surprisingly, Ag-specific T cell proliferation could not be detected after stimulation with DC from TX-treated 26K5-Ova/K5-CreER mice when using [3H]Thymidine incorporation as a read out (Fig. 3C). However, DC from constitutively Ag-expressing 26K5-Ova/K5-CreER mice efficiently presented Ova (Fig. 3D). We repeated those experiments, this time using CFSE dilution as a read out. Again, only DC from 26K5-Ova/K5-CreER mice efficiently induced activation and proliferation of OT-I T cells as evidenced by up-regulation of CD69 and dilution of CFSE (Fig. 3E).

Central tolerance mechanisms after induction of Ova expression

The K5 promoter is active in stratified epithelia but also in cortical thymic epithelial cells. It has been reported that constitutive expression of soluble Ova peptide in cortical epithelial cells of OT-1 transgenic mice results in reduction of CD4 and CD8 expression on DP thymocytes as well as partial deletion and TCR editing of Ag specific thymocytes (26). We therefore crossed K5-Ova/K5-CreER mice into the OT-I background and analyzed thymus, LN, and spleen for changes in T cell numbers and the TCR pattern of Ag specific T cells after induction of Ova expression.
TX-treated 26K5-Ova/K5-CreER-OT1 mice exhibited a reduced percentage of Vα2+CD8α− thymocytes and lymphocytes (Fig. 4, A and B). A comparable reduction in Ag-specific T cells was found in lymphocytes of line 29 but not line 21 (data not shown). As previously reported, this reduction is likely to be due to rearrangement of the TCR α-chain, since we detected reduced levels of Vα2 on DP thymocytes, and lymphocytes showed increased numbers of Vβ3.2/Vα2 CD8α− cells (Fig. 4, C and D). However, DP dull cells, which have previously been described by McGargill et al. (27), could not be detected. The percentage of CD8α SP thymocytes was reduced, suggesting a partial block in thymocyte maturation (Fig. 4E), whereas the percentage of CD4+ T cells was increased in LN, peripheral blood and spleen compared with OT-1 mice (Fig. 4, F and G and data not shown). Thus, Ova expression induces central tolerance by altered selection of Ag specific thymocytes and secondary TCR rearrangement.

Peripheral tolerance in Ova-expressing mice

Although Ag-specific CD8α+ T cells in the periphery of Ova-expressing mice had an activated phenotype, mice did not show any signs of disease. We therefore investigated whether peripheral T cells from TX-treated K5-Ova/K5-CreER mice were tolerant to Ova. Cytokine production and proliferation in response to Ova were analyzed in T cells isolated from skin-draining LN. Lymphocytes from uninduced 26K5-Ova/K5-CreER mice primed with Ova in the presence of CFA produced IFN-γ in a dose-dependent manner when restimulated with SIINFEKL peptide in vitro 14 days after vaccination. Lymphocytes from TX-treated 26K5-Ova/K5-CreER mice were impaired in their capacity to produce IFN-γ in response to Ova peptide suggesting that they were tolerant against Ova (Fig. 5A). The proliferative capacity of Ova Ag-specific T cells was investigated in 26K5-Ova/K5-CreER mice in an OT-1 background. Freshly isolated T cells from skin-draining LN of control OT-1 and K5-Ova OT-1 mice proliferated strongly when re-stimulated in vitro with SIINFEKL peptide (Fig. 5B). However, proliferation of T cells was reduced when LN suspensions from TX-treated 26K5-Ova/K5-CreER-OT1 mice were restimulated under the same conditions (Fig. 5B). Moreover, proliferation of endogenous Ag specific CD8α+ T cells in vivo was also slightly reduced after induction of Ova expression (Fig. 5, C and D). Together, these results indicate that Ova expression had induced tolerance in 26K5-Ova/K5-CreER transgenic mice. This conclusion was supported by the observation that the percentage of T cells in the skin of 26K5-Ova/K5-CreER-OT1 mice was not increased after TX-induced Ova expression (Fig. 5E).

Migration and activation of adoptively transferred OT-1 T cells

In K5-Ova/K5-CreER transgenic mice induced to express Ova in the epidermis Ag-specific T cells seem to be tolerant to Ova even when they are present at high numbers. We next investigated, whether tolerance could be broken by adoptive transfer of naive OT-1 T cells into 26K5-Ova/K5-CreER mice. Intravenously injected naïve, CFSE-labeled Ly5.1+ CD8α+ OT-1 T cells could be found in skin-draining and mesenterial LN of all injected mice. As early as 2 days after transfer, proliferation of transferred, CD45.1+ T cells could be detected in skin-draining LN of Ova-expressing but not of control mice (Fig. 6, A and B). Low proliferation was detected in the mesenterial LN of Ova-expressing mice, perhaps reflecting expression of Ova in gut epithelia of the upper intestinal tract (Fig. 6B). Proliferation of transferred T cells peaked at day 7 in the LN and declined thereafter reaching control levels at day 21 (Fig. 6B). Homeostatic proliferation could be excluded as a reason for the observed T cell expansion because endogenous Ag-specific T cells did not proliferate more in Ova-expressing mice compared with control after induction of Ova expression (Fig. 5D). CD45.1+ transferred T cells in Ova-expressing mice showed an activated phenotype as evidenced by increased surface CD44 expression whereas there were no signs of activation in control mice at any time point analyzed (Fig. 6, C and D). Nevertheless, mice did not show any signs of disease during the whole time period after adoptive transfer. When the experiment was repeated under the same conditions in the OT-1 background, the kinetics of migration and proliferation were comparable (data not shown). These results indicate that Ova is efficiently cross-presented in the skin-draining LN of TX-treated 26K5-Ova/K5-CreER OT1 mice with the indicated mice were isolated and stained for CD4, CD8α, Vα2, and Vβ5.2. Blots are representative for at least five mice/group. C–F, Thymocytes and lymphocytes from the indicated mice were isolated and stained for CD4, CD8α, Vα2, and Vβ5.2. Blots are representative for at least five mice/group. C–F, Thymocytes and lymphocytes from the indicated mice were isolated and stained for CD4, CD8α, Vα2, and Vβ5.2. Blots are representative for at least five mice/group. C–F, Thymocytes and lymphocytes from the indicated mice were isolated and stained for CD4, CD8α, Vα2, and Vβ5.2. Blots are representative for at least five mice/group.
dermis and epidermis to investigate, whether T cells migrated into the skin after priming in the regional LN after adoptive transfer. Whereas the percentage of total T cells in the dermis remained constant and comparable to control mice, an increase in the percentage of Vα2+Vβ5.2+ T cells was observed in the dermis of TX-treated 26K5-Ova/K5-CreER mice over the observed time period (Fig. 6, F and H). In the epidermis, the percentage of hematopoietic cells was not changed except for a mild increase on day 7 in Ova-expressing mice (data not shown). Within the hematopoietic cell population we could detect increasing numbers of CD8α+ T cells in the epidermis (Fig. 6, G and H). This shift in the composition of the T cell repertoire was not sufficient to induce disease in the skin of Ova-expressing mice, even when observed up to 3 mo after adoptive transfer (data not shown) suggesting that T cells were depleted over time.

The data presented so far suggest that induction of Ova expression in adult mice results in T cell unresponsiveness due to tolerance. However, it is possible that T cells, although efficiently primed, cannot recognize Ova on keratinocytes because of too low Ova production and presentation by keratinocytes. To rule out this possibility, we investigated whether in vitro-activated Ag-specific OT-1 T cells would be able to kill Ova-expressing keratinocytes in vivo. Staining for activated caspase 3 revealed that increased numbers of apoptotic cells were present in ears of Ova-expressing mice that were injected with in vitro activated OT-1 T cells compared with contra lateral ears injected with PBS or ears of nontransgenic controls injected with activated OT-1 T cells (Fig. 7, A and B). Caspase 3 positive cells were likely keratinocytes and not LC because they were negative for MHC-II. In some cases, apoptotic keratinocytes were found in close proximity to MHC-II positive LC (Fig. 7C) but not to CD3εhigh γδ T cells (Fig. 7B). These data show that keratinocytes of TX-treated 26K5-Ova/K5-CreER mice are presenting sufficient amounts of Ova-derived peptides in vivo to be recognized and killed by in vitro activated OT-1 T cells thereby excluding that tolerance is not broken because of clonal ignorance related to low Ova expression.

**Priming with CpG-treated BM-DC can overcome tolerance induction**

We next investigated whether the application of danger signals at the time of Ova induction would affect Ova-specific immune responses. As shown in Fig. 5A, the strong Th-1 inducing adjuvant CFA was not able to break established tolerance in 26K5-Ova/K5-CreER mice. We then tested the effect of the contact sensitizers DNFB and 2,4,6-trinitrochlorobenzene by treating mice on both ears while inducing Ova expression (28). No Ag-specific T cell proliferation occurred after restimulation in vitro and no induction of skin disease was detected up to 2 mo after induction of Ova expression when compared with Acetone treated controls (Fig. 8A and data not shown). Similar results were obtained when using the contact irritant TPA (Fig. 8B), which is known to induce erythema, edema, proliferation, and infiltration of leukocytes into the skin (29).

It has been reported that ligation of various TLR enhances the ability of DC to induce efficient immune responses (13). We therefore treated 26K5-Ova/K5-CreER OT1 mice at the time of TX treatment either topically with Imiquimod or injected them intradermally with CpG1668. However, no major changes in the T cell response against Ova in vivo or in vitro could be detected (Fig. 8, C and D). We speculated that this might be due to the low amount of Ag cross-presented on migrating skin DC and therefore tested whether direct presentation by mature BM-DC pulsed with Ova peptide was more efficient. We injected 5 × 10⁶ peptide-pulsed, CpG-matured DC into each ear of 26K5-Ova/K5-CreER OT1 mice and at the same time induced Ova expression (28E). By this regimen, the in vitro proliferative response of OT-1 T cells isolated 8 days later was enhanced when compared with TX-treated 26K5-Ova/K5-CreER OT1 mice and comparable to control OT-1 mice suggesting that tolerance could be broken by...
injection of mature DC presenting antigenic peptides (Fig. 8E). Together, these findings indicate that tolerance cannot be broken by addition of danger signals in situations of low levels of Ag expression during the induction phase of immune responses.

**Discussion**

The T cell repertoire is shaped in the thymus to avoid the development of potentially autoreactive T cells in a process called negative selection (30). Nevertheless, additional tolerance mechanisms are necessary to control T cells that either escape central tolerance or recognize environmental or newly expressed Ags (31). Tissue-resident DC have the opportunity to induce and maintain peripheral tolerance by acquiring Ags which, after DC migration and Ag processing, are presented to T cells in draining LN (15, 32). We were interested in analyzing if and how the immune system reacts to a previously unknown Ag expressed at low levels in skin-draining LN after adoptive transfer. Dermal cells from three mice were pooled. In Ova-expressing mice a higher percentage of CD3ε+ cells express the Ag-specific Vα2 chain of the TCR. G, Analysis of the infiltrate in the epidermis at 2, 4, 7, and 14 days after adoptive transfer. Epidermal cells from three mice were pooled. The percentage of CD8α+ hematopoietic cells (CD45+) was transiently increased, peaking on day 7 after transfer into Ova-expressing mice. H, Representative dot plots of day 4 after adoptive transfer of the results shown in F and G, gated on CD3ε+ and on CD45+ cells, respectively.

As previously described, thymic cortical expression of Ova in OT-1 mice resulted in altered selection of Ag specific thymocytes (26). Central tolerance in this model induced a strong reduction in the number of Ag specific T cells whereas mice still developed skin disease (16). In our inducible mice, we found reduced maturation of CD4/CD8 DP to CD8 SP thymocytes. However, we observed only minimal recombination of the endogenous α-chain and no signs of activation in OT-1 transgenic thymocytes. As a consequence we still found substantial numbers of Ag specific T cells that were activated in blood, LN and spleen of Ova-expressing mice. Nevertheless, disease was not induced in the OT-1 background.

Adoptive transfer of Ag-specific T cells has been used in several models to study autoimmunity (6, 8, 16, 17). When we transferred naive OT-1 T cells into K5-Ova/K5-CreER mice no autoimmune disease was observed. These results are in line with findings from Azukizawa et al., who used a membrane-bound, truncated version of Ova, but are contradictory to the findings of Shibaki et al. and McGargill et al., who expressed a membrane-bound form of Ova or a MHC-I restricted SIINFEKL peptide in the epidermis, respectively (8, 16, 17). In the latter case, mice developed autoimmune disease in the OT-1 background.
The lack of autoimmune disease against Ova in our inducible K5-Ova/K5-CreER model could be explained by several mechanisms, e.g., ignorance or tolerance. Ignorance has been described as avoiding recognition of self-proteins by T cells with low TCR avidity (33, 34). In our case, this can be excluded, because OT-1 T cells recognize the Ova-derived peptide SIINFEKL on MHC-I with high affinity. Another possibility is ignorance due to the low amount of Ag expressed (9). Although the amount of Ag expressed in K5-Ova/K5-CreER mice was low, it is very unlikely that ignorance is responsible for the lack of autoimmune disease in our model. First, we detected presentation of Ova-derived peptides in epidermal cell suspensions and efficient Ag presentation by epidermal-derived LC in vitro. Second, adoptively transferred OT-1 T cells proliferated selectively in skin-draining LN when Ova was expressed. Third, peripheral OT-1 T cells displayed increased levels of CD44 and CD69 on their surface after induction of Ova-expression. Moreover, these CD69+ cells expressed low levels of CD62L, indicating that these CD8α+ T cells had gained effector function. Fourth, in vitro-activated OT-1 T cells were able to kill keratinocytes in situ after intradermal injection, confirming that Ova-peptides are presented on epidermal keratinocytes. All these observations suggest that there is induction of tolerance.

Three forms of tolerance can be distinguished: either depletion, induction of T cell anergy, or active suppression by regulatory T cells. It has been shown that radiosensitive cells like T cells are playing a role in maintaining tolerance in the skin of Ova-expressing mice (17). Regulatory T cells (Treg) are mainly generated in the thymus, however there is increasing evidence that Ag-specific Treg cells can be generated in peripheral tissues by activated DC (35, 36). It has recently been reported that overexpression of Receptor Activator of NFκB Ligand (RANKL) in the epidermis of transgenic mice resulted in an LC-triggered increase in the number of CD4+ CD25+ Treg cells in the skin and protection from autoimmune disease (37). We therefore analyzed whether CD4+ CD25+ FoxP3+ Treg cells were induced in K5-Ova/K5-CreER mice. However, we could not detect any difference in the number of Treg cells in the skin-draining LN of TX-treated K5-Ova/K5-CreER mice. Furthermore, depletion of CD4+ or CD25+ cells before induction of Ova-expression in K5-Ova/K5-CreER OT-1 mice did not result in disease induction (data not shown).

Continuous presentation of Ova-derived peptides on DC in the skin-draining LN could have resulted in induction of T cell anergy. It has been described for viral infections that persistence of viral Ag favors development of anergy in T cells rather than their depletion (38, 39). A similar mechanism seems to be operating in our model with Ag expressed in the adult skin. The T cell numbers and memory phenotype observed months after induction and continuous expression of Ova were comparable to those detected 3 wk after induction. Furthermore, we did not find indications for ho- 

![Image](http://www.jimmunol.org/)
endogenous and on transferred Ag-specific T cells. We did not, for example, detect TCR α-chain recombination on transferred CD45.1+/Vα2+ T cells (data not shown). Thus, it may well be that several mechanisms act in concert to avoid induction of an autoimmune disease against self Ag.

Danger signals occurring during the induction phase of the Ova-specific immune response might result in a shift from tolerance induction to autoimmunity (41). However, treatment with DNFB, 2,4,6-trinitrochlorobenzene, or TPA during induction of Ova expression did not change the outcome of the immune response against Ova. Alternatively, innate activation of DC by pattern recognition receptors like TLRs is an important pathway for the induction of adaptive immune responses (42). Mice deficient for MyD88 show reduced CTL responses indicating that TLR play an important role in cross-priming (43). Ligation of TLR has been shown to be able to replace CD4 T cell help in CTL responses (44). We therefore tested whether TLR ligation during T cell priming or in already tolerized animals would alter the outcome of the immune response. Treatment of K5-Ova/K5-CreER OT-1 mice in vivo with Imiquimod, CpG, or both at the time of TX treatment and Ova induction did not result in disease development. Furthermore, neither treatment with polyI:polyC nor with Imiquimod could break established tolerance in K5-Ova/K5-CreER mice (data not shown). This was surprising because we and others have previously shown that Imiquimod and CpG are potent immune stimulators capable of inducing efficient immune responses against melanoma in the skin (45–48). We speculated that this lack of immune stimulation was due to weak cross-presentation, as indicated by the poor Ag presentation in skin draining LN of TX-immune stimulation was due to weak cross-presentation, as melanoma in the skin (45–48). We speculated that this lack of


