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27. The complete coding region of the rat CREB cDNA (*7*) was amplified by the polymerase chain reaction (PCR) and fused in frame to GAL4 (1 to 147) encoded in the plasmid pSG424 (*9*) to form GAL4-CREB. The Ser^{133} to Ala mutation (M1) was subcloned (as an Aat II–Kpn I restriction fragment) into GAL4-CREB from the plasmid RSV-CREBfM1 (*6*) to generate GAL4-CREBM1. GAL4-CREBΔLZ and GAL4-CREBΔLZM1 were constructed by amplifying the corresponding regions from GAL4-CREB and GAL4-CREBM1 by PCR.
28. The two bands slightly above c-fos^H that are also detected in the ribonuclease (RNase) protection assay represent transcripts initiating from within the vector and from a cryptic mRNA start site in the TATA region of the c-*fos* construct (*4*).
29. CREB was purified by DNA-affinity chromatography [J. T. Kadonaga and R. Tjian, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5889 (1986)] on columns (40 μg of DNA per milliliter) prepared from oligonucleotides of the following sequences: CRE column, 5′-CCCG*TGACGTCA*ACAC-3′ and 5′-*TGACGTCA*CGGGGTGT-3′; AP-1 column, 5′-CCCG*TGAGTCA*ACAC-3′ and 5′-*TGACTCA*CGGGGTGT-3′ (consensus elements in italics). Nuclear extracts (200 mg of protein) were prepared from 2×10^{11} mouse L cells by the chloroquine DNA intercalation method [H. Schroter, P. E. Shaw, A. Nordheim, *Nucleic Acids Res.* **15**, 10145 (1987)]. We purified CREB by sequential passage through a serum response element column [R. Treisman, *EMBO J.* **6**, 2711 (1987)], two CRE columns, one AP-1 column to remove AP-1 proteins, and a final CRE column. CRE-binding activity was followed by gel shift assay (*4*). Purified CREB (~0.5 μg, as estimated by in vitro DNA-binding activity) was concentrated in a Centricon ultrafiltration device (Amicon, Danvers, MA) and dialyzed overnight against 10 mM Hepes (pH 7.4) and 10% glycerol.
30. In vitro phosphorylation of CREB by purified rat brain CaM kinase II (1 to 5 μg/ml) was performed in 50 mM Pipes (pH 7), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), calmodulin (5 μg/ml), bovine serum albumin (100 μg/ml), 10 μM adenosine triphosphate (ATP), 1.5 μM [γ-^{32}P]ATP, and either 0.3 mM $CaCl_2$ or 0.2 mM EGTA. In vitro phosphorylation by CaM kinase I was performed as described (*17*) with 1 mM EGTA or 1 mM EGTA and 1.5 mM $CaCl_2$. In vitro phosphorylation by PKA catalytic subunit (20 to 40 μg/ml) (Sigma) was performed in 10 mM Hepes (pH 7.2), 10 mM $MgCl_2$, 1 mM DTT, 10 μM ATP, and 1.5 μM [γ-^{32}P]ATP.
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32. Two-dimensional tryptic analysis of CREB B phosphorylation in vitro by CaM kinase I, CaM kinase II, and PKA reveals that the major phosphopeptide labeled by these enzymes comigrates exactly with phosphopeptide 1 labeled in CREB (Fig. 4) (*10*), suggesting that CREB and CREB B may possess a conserved phosphorylation site. Hai *et al.* (*8*) have identified a protein, ATF1, that contains a sequence that is identical to the CREB peptide that contains Ser^{133}. ATF1 may therefore be identical to CREB B.
33. We thank H. Schulman and N. Waxham for purified CaM kinase II; A. Nairn for CaM kinase I; I. Sadowski for pSG424; M. Carey for pG9E4T; and M. Montminy for antiserum W39 to CREB and RSV-CREBfM1. Supported by NIH grant R01 CA 43855, grant R01 NS 28829 from the National Institute of Neurological Disorders and Stroke, an American Cancer Society Faculty Research Award (FRA-379) (M.E.G.), and a Scholar's Award from the McKnight Endowment Fund for Neuroscience (M.E.G.).

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Recognition of Self Antigens by Skin-Derived T Cells with Invariant γδ Antigen Receptors

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Thy-1$^+$ dendritic epidermal T cells (dECs) express invariant γδ antigen receptors and are found in intimate contact with keratinocytes in murine epidermis—thus raising the possibility that keratinocytes express a ligand for the antigen receptor of these T cells. Thy-1$^+$ dECs were stimulated to produce lymphokines by interaction with keratinocytes in vitro. This stimulation was mediated through the dEC antigen receptor and did not appear to be restricted by the major histocompatibility complex. Thus, dECs can recognize self antigens and may participate in immune surveillance for cellular damage rather than for foreign antigens.

IN MICE, MOST T CELLS IN THE LYMphoid tissues express diverse antigen receptors that consist of α and β chains and recognize antigens bound to self major histocompatibility complex (MHC) proteins. A minority of lymphoid T cells express clonally diverse γδ T cell receptors (TCRs) (*1*) and recognize a wide array of antigens, including MHC class I, II, and Ib gene products, as well as bacterial heat shock proteins (*2–7*). In contrast to the lymphoid organs, several epithelial tissues contain mostly γδ T cells. The epidermis contains a unique population of cells, the Thy-1$^+$ dECs (*8–10*), which express invariant γδ TCRs composed of $V_\gamma 3/J_\gamma 1\text{-}C_\gamma 1$ and $V_\delta 1/D_\delta 2/J_\delta 2\text{-}C_\delta$ chains (*11, 12*) and have not been found elsewhere in the adult mouse (*13*). Similarly, the intraepithelial T cells of the female reproductive tract and the tongue express γδ TCRs composed of an invariant $V_\gamma 4/J_\gamma 1\text{-}C_\gamma 1$ chain and an invariant δ chain identical to that found in the dECs (*14*). The absence of clonal diversity in the TCRs of these epithelium-associated T cells suggests that they might perform immunological functions that are distinct from those of T cells with clonally diverse antigen receptors. These nondiverse T cells might recognize damage-induced self antigens in a mechanism of trauma signal surveillance (*11, 15*). Here, we provide evidence in support of this concept by demonstrating that dECs can specifically recognize self antigens produced by skin-derived keratinocytes. This information and the physical interactions observed in situ lend support to a potential role of dECs in surveillance of skin keratinocytes in vivo for damage induced by stress, infection, or other means.

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Initial studies established that dECs secrete interleukin-2 (IL-2) and proliferate in response to activation with antibodies to the TCR (*16*). Thy-1$^+$ dECs were isolated from normal BALB/c mouse skin and cultured in the presence of a variety of potential antigen presenting cells (APCs) to determine if a specific antigen-mediated interaction could be detected. Enriched keratinocytes were obtained by depleting epidermal cell suspensions of Thy-1$^+$ dECs and MHC class II$^+$ Langerhans cells by treatment with antibody and complement. Lymphokine production was measured in supernatants obtained after 24 hours of culture of the dECs with stimulator cells, and T cell proliferation was measured at 72 hours. The dECs were stimulated to produce lymphokines and to proliferate (*17*) on contact with freshly isolated skin keratinocytes or a cultured keratinocyte cell line, PAM (*18*) (Fig. 1A). Stimulation of dECs could not be detected after interaction with freshly isolated fibroblasts, splenocytes, peritoneal exudate cells (PECs), or the 3T3 fibroblast cell line, all of BALB/c origin. Similar results were obtained on incubation of a dEC clone, 7-17, from AKR mice (*19*), with the various BALB/c stimulator cells. Freshly isolated keratinocytes, as well as the PAM cell line, stimulated secretion of IL-2 by the 7-17 cells (Fig. 1B). Thus, dECs can respond to skin keratinocytes but not to the other cells tested.

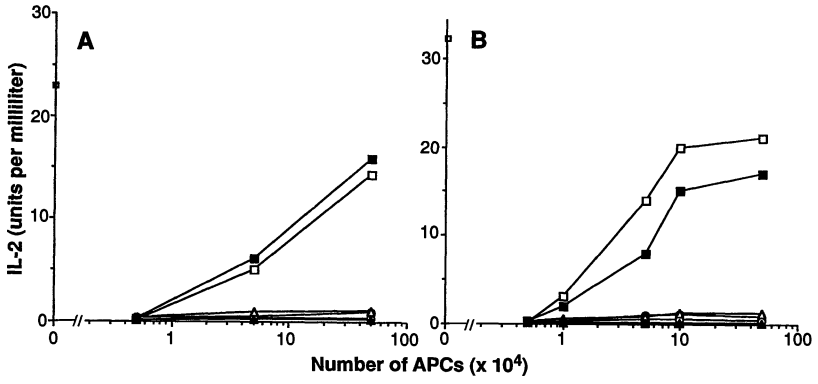

Fig. 1. Keratinocytes stimulate Thy-1$^+$ dECs to secrete IL-2. The dECs were either freshly isolated Thy-1$^+$ dECs from BALB/c mice (**A**) or a Thy-1$^+$ AKR-derived dEC clone, 7-17 (**B**). The dECs (1×10^5) were added to triplicate wells containing irradiated skin keratinocytes (■), fibroblasts (●), peritoneal exudate cells (○), splenocytes (△), 3T3ca31 cells (▲), or PAM keratinocyte cells (□), all of BALB/c origin. The response to immobilized monoclonal antibody 500A2 to CD3 is shown as a square on each vertical axis. Supernatants were collected after 24 hours, and IL-2 activity was measured as relative uptake of 3-(4,5-α-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the IL-2–dependent cell line CTLL-20 (*25*) in a 24-hour assay. Units were calculated by comparison to a standard provided by the National Cancer Institute Biological Response Modifiers Program. Epidermal sheets were dissociated by trypsin digestion, as described (*26*). Epidermal cells were stained with fluorescein isothiocyanate–conjugated antibodies to Thy-1, and dECs were isolated by fluorescence-activated cell sorting. Keratinocytes were obtained by treating epidermal cells with antibodies to Thy-1 and complement to remove dECs and antibodies to class II MHC molecules and complement to remove Langerhans cells. Spleen cells were depleted of T cells with antibodies to Thy-1 and complement. Stimulator cells were added to microtiter wells and cultured overnight. Plates were irradiated (16 Gy) and culture medium was removed before adding T cells. Data are representative of greater than six separate experiments. Stimulation of the 7-17 dEC clone by PAM, but not by 3T3 cells, has been demonstrated in greater than 35 separate experiments. Results are the mean of triplicate cultures with SEM values being <10% of the means.

Several approaches were taken to determine whether the stimulation of dECs by keratinocytes was due to a specific interaction with the TCR. First, a panel of T cell clones, tumors, and hybridomas with known TCR gene usage was examined for reactivity with the keratinocyte line PAM (Fig. 2A). All cells that expressed TCRs with $V_\gamma3$ and $V_\delta1$ chains responded to PAM cells by secreting lymphokines. Data are shown for the dEC clone 7-17, dEC hybridoma IC6.F11, and the fetal $V_\gamma3$-expressing hybridoma IM82.7. Ten T cell lines that expressed other V_γ and V_δ or α and β chains did not secrete detectable amounts of IL-2 after exposure to PAM cells, but could produce IL-2 after stimulation with antibodies to the CD3 complex. DN12.1 and DN7.1 cells express TCRs composed of $V_\gamma4$ chains paired with a δ chain that is identical to that found in the dEC receptor (*20*); these cells did not secrete IL-2 in response to keratinocytes. Thus, the presence of the $V_\gamma3/V_\delta1$ TCR is required for productive interaction with the skin keratinocytes.

We also examined the effect of antibodies to the TCR complex on keratinocyte recognition (Fig. 3). Nonstimulatory Fab fragments of antibodies that recognize the $V_\gamma3$ TCR [monoclonal antibody (MAb) 536] or the CD3ε chain (MAb 500A2) effectively blocked the interaction between dEC clone 7-17 and the keratinocyte line PAM. Antibodies to other T cell surface structures, including Thy-1 and Ly-6C, did not inhibit lymphokine secretion (*17*). Finally, we examined the reactivity of a cell line derived from the human T cell leukemia Jurkat, which had been transfected with rearranged

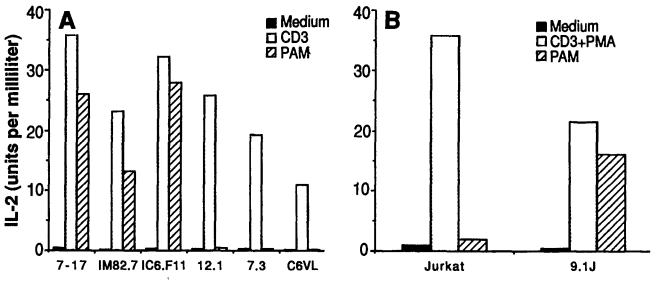

Fig. 2. $V_\gamma3^+$ T cells recognize keratinocytes. (**A**) Fifteen T cell clones, hybridomas, and tumor cell lines were analyzed for the ability to respond to the PAM keratinocyte cell line by secretion of IL-2. Representative cell lines are shown. The 7-17, IM82.7, and IC6.F11 cells express $V_\gamma3$ and $V_\delta1$ chains. The DN12.1 (12-1) and DN7.1 cell lines express $V_\gamma4$ associated with the dEC Vδ1 chain. The DN7.3 (7.3) cell line expresses Vγ2 and $V_\delta5$ chains and is representative of the six cell lines tested expressing γδ TCRs other than $V_\gamma3$ and $V_\delta1$. The C6VL tumor line is representative of the four αβ TCR$^+$ cell lines tested. (**B**) Wild-type human Jurkat tumor cells and Jurkat cells transfected with Vγ3 and Vδ1 (9.1J) were analyzed for reactivity with PAM keratinocytes. Experiments were performed as in Fig. 1. Each cell line was tested in at least six separate experiments. Results shown represent the mean of triplicate cultures with SEM values being <10% of the means. Antibodies to mouse CD3 (MAb 500A2) or antibodies to human CD3 (MAb Leu 4) plus phorbol myristate acetate (PMA) (10 ng/ml) were included to demonstrate that all T cells tested could secrete IL-2 when stimulated. The results of control incubations of cells with culture medium are also shown.

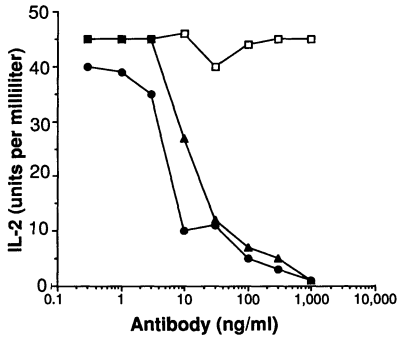

Fig. 3. Antibodies to the TCR complex block dEC stimulation by keratinocytes. The 7-17 dEC cell clone was cultured with the PAM keratinocyte cell line plus medium (□), Fab fragments of MAb 500A2 to CD3 (▲), or Fab fragments of MAb 536 to $V_\gamma3$ (●). IL-2 was measured as in Fig. 1. Results shown are the means of triplicate cultures with SEMs of <10% of the means. Similar results were obtained in four separate experiments. Other antibodies tested had no effect on IL-2 release (*24*).

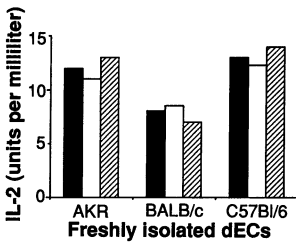

Fig. 4. Freshly isolated dECs respond similarly to keratinocytes isolated from mice of different MHC haplotypes. Fresh dECs and keratinocytes were isolated and assayed as in Fig. 1. Keratinocytes were enriched from AKR (solid bars), BALB/c (open bars), and C57Bl/6 (striped bars) skin. Results shown are the means of triplicate cultures with SEMs of <10% of the means. Results are representative of four separate experiments with all three mouse strains. Experiments with freshly isolated AKR and C57Bl/6 cells were performed greater than twelve times.

$V_\gamma 3$ and $V_\delta 1$ genes from a dEC clone (*21*) (Fig. 2B). The $V_\gamma 3/V_\delta 1$ TCR transfectant, but not the parental $\alpha\beta$ TCR^+ Jurkat, responded to keratinocytes by secretion of IL-2. Thus, expression of the dEC TCR is sufficient to confer reactivity to keratinocytes.

Our results thus indicate that the TCR of dECs can recognize antigens expressed by skin-derived keratinocytes. However, there is no evidence that dECs are activated in situ in normal skin; perhaps the antigen is induced by stress that is associated with dissociation of the tissue or by in vitro tissue culture. Antigens derived from endogenous heat shock proteins are potential targets for recognition. Most mammalian cells in tissue culture can express increased levels of heat shock proteins (*22, 23*). Although the antigen recognized by the Thy-1^+ dECs remains to be identified, our data demonstrate that $\gamma\delta$ TCR^+ cells present in the skin of mice recognize keratinocytes and may function in a form of self surveillence, providing protection against cell damage.

The ability of the BALB/c-derived PAM cells (H-2^d) to stimulate both BALB/c dECs (H-2^d) and the AKR-derived 7-17 dEC clone (H-2^k) suggests that the recognition of keratinocyte antigen by dECs may not be MHC-restricted. This suggestion is also supported by the fact that freshly isolated dECs from three strains of mice with distinct MHC haplotypes recognized and responded equally well to allogeneic and syngeneic keratinocytes (Fig. 4). To determine if the MHC participates in the recognition of antigen by dECs, we tested antibodies to MHC proteins for their ability to block the interaction of PAM cells with 7-17 cells (*24*). Antibodies reactive with classical MHC molecules as well as products of the Qa region had no effect on the stimulation observed. Together, these results indicate that antigen recognition by dECs is not restricted by classical MHC molecules, but they do not rule out a role for MHC gene products or other molecules in antigen presentation to dECs.

The skin routinely encounters a variety of insults as a result of constant exposure to the environment and must support a number of mechanisms for protection. In the adult, dECs and T cells expressing nondiverse $\gamma\delta$ TCRs that are resident in other epithelial tissues may provide a form of immune surveillance in which self-reactive T cells provide a first line of defense against infection or malignancy by the recognition of, and response to, damaged or infected neighboring cells. Such a form of surveillance for conserved epitopes of stress-related proteins on damaged cells would allow resident invariant $\gamma\delta$ T cells to respond to a variety of deleterious agents without the need for diverse TCRs that have specificity for foreign antigens. This type of immune recognition may have arisen as one of the earliest forms of protection against damage and disease.

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