Overexpression of CD1d by Keratinocytes in Psoriasis and CD1d-Dependent IFN-γ Production by NK-T Cells

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The MHC class I-like protein CD1d is a nonpolymorphic molecule that plays a central role in development and activation of a subset of T cells that coexpress receptors used by NK cells (NK-T cells). Recently, T cells bearing NK receptors were identified in acute and chronic lesions of psoriasis. To determine whether NK-T cells could interact with epidermal cells, we examined the pattern of expression of CD1d in normal skin, psoriasis, and related skin disorders, using a panel of CD1d-specific mAbs. CD1d was expressed by keratinocytes in normal skin, although expression was at a relatively low level and was generally confined to upper level keratinocytes immediately beneath the lipid-rich stratum corneum. In contrast, there was overexpression of CD1d in chronic, active psoriatic plaques. CD1d could be rapidly induced on keratinocytes in normal skin by physical trauma that disrupted barrier function or by application of a potent contact-sensitizing agent. Keratinocytes displayed enhanced CD1d following exposure to IFN-γ. Combining CD1d-positive keratinocytes with human NK-T cell clones resulted in clustering of NK-T cells, and while no significant proliferation ensued, NK-T cells became activated to produce large amounts of IFN-γ. We conclude that CD1d can be expressed in a functionally active form by keratinocytes and is up-regulated in psoriasis and other inflammatory dermatoses. The ability of IFN-γ to enhance keratinocyte CD1d expression and the subsequent ability of CD1d-positive keratinocytes to activate NK-T cells to produce IFN-γ, could provide a mechanism that contributes to the pathogenesis of psoriasis and other skin disorders. The Journal of Immunology, 2000, 165: 4076–4085.

Psoriasis is a common, chronic, inherited skin disorder triggered when activated bone marrow-derived immunocytes infiltrate the epidermis and establish a Th1-type cytokine network producing keratinocyte hyperplasia and angiogenesis (1). The precise mechanism responsible for immunocyte activation in skin is not known; however, injection of IFN-γ can trigger the appearance of psoriatic lesions in genetically susceptible individuals (2). Most studies of the genetic factors associated with psoriasis point to chromosome 6 and the class I MHC as disease-associated loci, including a specific association with HLA-Cw6 (3, 4). Typically, APCs present peptide Ags in the context of such MHC class I molecules to CD8⁺ T cells (5), but definitive pathological roles for CD8⁺ and CD4⁺ T cells in psoriasis remain uncertain.

Recently, we demonstrated the presence of NK-T cells in the epidermis of acute and chronic psoriatic plaques (6–8). A hallmark of NK-T cells is their expression of certain C-type lectin NK cell receptors (NKR)s such as CD94 and CD161 (9–11). Classical NK-T cells may play an immunoregulatory role for recognition of both self and foreign Ags and are implicated in the pathogenesis of autoimmune and inflammatory diseases (12–19). An important clue to the function of NK-T cells was provided by their interaction with professional APCs via CD1d (20–22). CD1d has some similarities in structure to MHC class 1 molecules (23), but it is not encoded by the MHC complex (the gene for CD1d is located on chromosome 1 in humans) and, in contrast to MHC class 1 molecules, is not polymorphic (20). While initially CD1d was believed to bind and present peptide Ags to T cells (24), more recent studies highlight its ability to present glycolipids and GPI-linked proteins (22, 25–29).

NK-T cells can become activated in a CD1d-restricted fashion with subsequent proliferation and cytokine production, including IFN-γ and IL-4. This CD1d-dependent activation can be enhanced by the addition of specific glycolipid Ags, most notably the synthetic α-galactosylceramide (29–32). The ligand recognized by the NK-T cells in psoriasis and the functional consequences of such interactions are unknown. Such glycolipid-reactive NK-T cells, besides expressing CD94 and CD161, have a rather specific TCR rearrangement (8), but the precise relationship between CD1d-reactive NK-T cells and T cells bearing NKRs is currently unclear. Thus, in addition to documenting the presence of

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Abbreviations used in this paper: NKR, NK cell receptor; NN skin, normal skin; PN skin, symptomless skin from patients with psoriasis elsewhere; PP skin, active untreated psoriatic plaques; DN, double negative; TPA, 12-O-tetradecanoyl phorbol-13-acetate; TBS, Tris-buffered saline, pH 7.6; PNGase F, peptide N-glycosidase F.
CD1d in psoriasis and other skin disorders, we also searched for NK-T cells bearing Vα24 and Vβ11 as well as CD94 and CD161.

Previous investigators focused on CD1d expression in the gastrointestinal tract, exploring its role in epithelial immunity and inflammatory bowel disease (33–35). This report documents patterns of CD1d expression by keratinocytes in vitro and in human skin and psoriasis in vivo. We also observed in psoriatic lesions the presence of intraepidermal lymphocytes expressing a variety of markers shared by classical NK-T cells. To explore the functional significance of these in vivo findings, NK-T cell clones were used to explore the potential for CD1d expressed on keratinocytes to trigger CD1d mediated immunologic responses.

Materials and Methods

Human tissue samples

Three-millimeter punch biopsy specimens of normal adult human skin (NN skin; n = 16), symptomless skin from patients with psoriasis elsewhere (PS skin; n = 18), and active untreated psoriatic plaques (PP skin; n = 16) were obtained under local anesthesia after informed consent was given. Similarly, punch biopsy specimens were obtained from volunteers in which previous investigators focused on CD1d expression in the gastrointestinal mucosa and skin. Tissue samples were snap-frozen in isopentane chilled in liquid nitrogen and stored at −80°C until cryostat sections (5 μm thick) were cut and placed on glass slides. Some of these biopsies were performed during the course of an earlier investigation (36).

Cell culture

Multipassaged normal human keratinocytes and an immortalized human keratinocyte cell line (HaCaT) were grown in a low calcium, serum-free medium (KGM, Clonetics, San Diego, CA) as previously described (37, 38). Three different NK-T cell clones were examined (DN2.D5, DN2.D6, and DN2.B9) that were generated as previously described (29, 31). Briefly, a panel of double-negative (DN) Vα24+ Vβ11+ human peripheral blood-derived NK-T cell clones was established by sequential negative magnetic bead (Dynal, Lake Success, NY) isolation and positive FACS sorting, followed by stimulation with PHA (Difco, Detroit, MI) and IL-2 (70 U/ml) in the presence of irradiated (5000 rad) peripheral blood mononuclear cells. HeLa cells or CIR cells were transfected with the expression vector pSRa-Neo containing a CD1a insert encoding CD1d as previously described (31).

Immunohistochemical staining

Cryostat sections were fixed using cold acetone followed by an avidin-biotin peroxidase staining procedure as previously described (39). Briefly, primary Abs (10 μg/ml) were incubated for 30 min at room temperature followed by subsequent steps according to the manufacturer’s instructions (Vectastain, Vector, Burlingame, CA). Positive detection was accomplished using 3-amino-4-ethylcarbazole as the chromagen producing a red reaction product with 1% hematoxylin as the counterstain (39).

Abs and cytokines

Primary murine Abs included anti-CD1d (clone NOR3.2 IgG, BioSource, Camarillo, CA), anti-Vα11 (IgG, Becton Dickinson, Mountain View, CA), and anti-Vα24; anti-CD161 and anti-CD94 were purchased from Coulter (Hialeah, FL). Anti-ICAM-1 was revealed by chemiluminescence with the ECL kit (Amersham, Piscataway, NJ) on ECL film.

Proliferation studies and cytokine assay

NK-T cells (5 × 10^4/well) were cultured in triplicate in RPMI and 10% FCS in 96-well round-bottom tissue culture plates together with either keratinocytes or HeLa cells (2.5 × 10^4/well). NK-T cell proliferation was measured after 72 h by [3H]thymidine incorporation (1 μCi/well) using target cells pretreated with mitomycin C (0.1 mg/ml) for 1 h at 37°C (Sigma, St. Louis, MO) as previously described (29). The synthetic glycolipid (α-galactosylceramide) used was provided by the Pharmaceutical Research Laboratory/Kirin Brewery (Gunma, Japan) as previously described (29, 31). To induce CD1d, keratinocytes were pretreated with IFN-γ (10 U/ml) for 48 h, washed, lifted with trypsin/EDTA followed by mitomycin treatment, and washed three times by repeated centrifugation. No residual IFN-γ was detected in these keratinocyte suspensions by ELISA. Stimulation of NK-T cells was performed in the presence of 1 ng/ml 12-O-tetradecanoyl phorbol-13-acetate (TPA), as previously described (29). Released cytokine levels for IFN-γ and IL-4 were measured in duplicate after 48 h of coincubation between NK-T cells with or without keratinocytes and HeLa cells by ELISA with matched Ab pairs in relation to cytokine standards (R&D Systems, Minneapolis, MN). In some experiments, Ficoll-Hypaque interface mononuclear cells were stimulated using staphylococcal enterotoxins SEB and SEC2 (1 μg/ml each; Toxin Technologies, Sarasota, FL) as previously described (7).

Flow cytometry

Cells were incubated with the indicated primary and secondary Abs, washed, and resuspended in PBS containing 2% FCS. Cells were analyzed using a Coulter XL flow cytometer. The mAb CD1d 27 was used to detect CD1d, and anti-ICAM-1 was employed to compare with CD1d as well as isotype-matched control (Coulter). Data were analyzed with Elite software from Coulter.

RT-PCR

Detection of CD1d mRNA was performed using RT-PCR. Sequences of the primers designed to specifically detect CD1d were: exon 2, sense, 5'-CTC CAG ATC TCG TTC TCT GCC AAC TAT-3'; and exon 3, antisense, 5'-TTG AAT GGC CAA CTT GTT CCA AAG-3'. These primers amplify an ~400-bp fragment using an annealing temperature of 53°C and 35 cycles of PCR. Control studies using a variety of different templates demonstrated the reaction to be specific for CD1d, with no detectable amplification of CD1a, -b, or -c transcription under these conditions (data not shown).

Western blot and immunoprecipitation analysis

For Western blot analysis, lysates were performed on subconfluent cultures of keratinocytes and HaCaT cells (or CD1d-transfected CIR cells serving as a positive control) by incubating 75-cm2 flasks with 1 ml of lysis buffer (25 mM Tris-HCl, pH 7.5; 5% 2-ME; 200 μM PMSF, and 65 mM Tris-HCl, pH 6.8) for 30 min at 4°C followed by harvesting with a cell scraper, sonication, and centrifugation at 100,000 g for 30 min. Aliquots of the supernatant were applied to 10% SDS-PAGE gels under reducing conditions and transferred to nitrocellulose. After blocking nonspecific protein binding using Tris-buffered saline, pH 7.6 (TBS)-milk (10 mM NaCl and 5% fat-free milk), the strips were incubated with mAb CD1d overnight at 4°C, followed by a secondary Ab (goat anti-mouse Ig G, Dako, Carpinteria, CA) for 1 h at room temperature. After washing with TBS-Tween, binding of secondary Ab was revealed by chemiluminescence with the ECL kit (Amersham, Piscataway, NJ) on ECL film.

For immunoprecipitation/Western blot analysis HaCaT cells were grown in T-75 flasks, and lysates were prepared as described above. Lysates were centrifuged (10,000 × g) to remove nuclei and debris and were precleared three times at 4°C with protein G-coupled Sepharose beads (Pharmacia). Equal volumes of precleared lysates were then incubated with 5% (v/v) protein G-coupled Sepharose beads to which nonbinding control (MPC-11; IgG2b) or CD1d-specific (CD1d51; IgG2b) mouse Abs had been chemically cross-linked using dimethylsuberimidate as previously described (41). Forty-one lysates were incubated with mAb-coupled beads at 4°C for 6 h and then washed with TBS and split into two aliquots (i.e., with and without peptide N-glycosidase F (PNGase) F samples). Beads from each aliquot were suspended in 12 l of denaturation buffer (0.5% SDS and 1% 2-ME) and heated in a boiling water bath for 10 min, followed by addition of 21 of 10% Nonidet P-40 and 1 of 0.5 M sodium phosphate (pH 7.5 at 25°C). PNGase F (New England Biolabs, Beverly, MA) was then added and the sample and imidazole (100X) incubated for 18 h at 37°C. Samples were then brought to 31 with gel loading buffer (3% SDS, 0.02% bromophenol blue, 10% glycerol, and 62.5 mM Tris, pH 6.8) and electro-phoresed on a 12% polyacrylamide-SDS slab gel. Western blotting to a
polyvinylidene fluoride (NEN Life Science Products, Boston, MA) membrane was performed at 100 V for 1 h at 4°C. The membrane was blocked using 5% nonfat milk in TBS for 18 h, and then probed using mAb CD1d75 (5 μg/ml) in TBS with 0.1% Tween 20 and 0.05% NaN₃ for 1 h at room temperature. The membrane was washed three times with TBS with 0.1% Tween 20 without NaN₃ and developed using HRP-protein A and enhanced chemiluminescence (SuperSignal West Dura Extended Duration Substrate, Pierce, Rockford, IL) according to the manufacturer’s instructions.

Results

Expression of CD1d in thymus and normal and prepsoiratic (PN) human skin

Because previous studies suggested that CD1d is expressed in thymus, we first assessed the staining pattern using a commercially available anti-CD1d mAb (i.e., NOR3.2) on frozen sections of thymic tissue. While CD1d staining was observed in cortical thymocytes, dendritic cells, and endothelial cells with this mAb, a more striking surface expression on Hassel’s corpuscles was noted, producing concentric rings of positive immunoreactivity (Fig. 1). This marked CD1d positivity of an epithelial element of thymic tissue prompted an examination of the skin for CD1d expression. Normal skin biopsy specimens were stained with NOR 3.2 mAb, which revealed CD1d to be consistently present in the three or four outermost layers of keratinocytes in a distinct plasma membrane staining pattern (Fig. 1B). Occasional staining of the basal cell layer or midlayers of keratinocytes in the stratum spinosum was also seen in some normal skin samples. Thus, most of the keratinocytes in the stratum granulosum extending up to the lipid-rich stratum corneum were CD1d positive. There was no apparent CD1d expression noted on Langerhans cells, but weak, focal CD1d was present on dermal dendritic cells and endothelial cells. A similar immunohistochemical staining pattern, emphasizing CD1d expression by upper level keratinocytes, was also present in prepsoiratic (PN) skin taken from psoriatic patients (Fig. 1C). For both normal skin and PN skin, CD1d was present focally on eccrine duct epithelium (Fig. 1C, inset) and acrosyringium (data not shown). The dermal papillae and hair matrix were CD1d negative (Fig. 1D), but the inner root sheath epithelium was diffusely positive, with some anagen follicles displaying outer root sheath epithelium and sebocytes being CD1d positive (Fig. 1E). No CD1d was discerned on dermal mast cells or fibroblasts or in s.c. tissue sites (data not shown).

Increased expression of CD1d in psoriatic skin

In contrast to normal skin and symptomless skin from psoriasis patients, chronic psoriatic plaques had more extensive keratinocyte CD1d expression (Fig. 1, F and G). Because the granular cell layer is generally absent in a psoriatic lesion (42), CD1d expression...
began just beneath the large amount of lipid-rich scale and extended to include 8–12 layers beneath the stratum corneum to suprabasilar layers (Fig. 1G). CD1d was primarily confined to plasma membrane, producing a broad “chicken-wire” appearance of the plaques. There was focal CD1d expression in the basal cell layer in several of the plaques, as well as more diffuse and intense CD1d on dermal dendritic cells and endothelial cells (Fig. 1, F and G). Because mild trauma to the skin (i.e., repeated tape stripping) can trigger psoriasis (43), we examined tissue samples for CD1d expression. Within 72 h of barrier perturbation accomplished by repeated tape stripping, symptomless (PN) skin acquired diffuse epidermal CD1d expression in juxtaposition to the overlying parakeratotic scale (Fig. 1H).

Because early lesions of psoriasis can resemble evolving allergic contact dermatitis reactions (44) skin biopsies obtained from sensitized individuals exposed epicutaneously to the hydrophobic wax-containing oil of the poison ivy leaf (i.e., urushiol) were examined (36). Eight hours after exposure, no changes in CD1d expression were noted, but as early as 24 h following exposure, biopsy samples revealed increased CD1d expression by epidermal keratinocytes. At 48 h as mononuclear cells appeared in the upper dermis and epidermis, keratinocytes in the mid and superficial epidermal layers were CD1d positive (Fig. 1I).

To confirm and extend the pattern of CD1d expression in psoriatic plaques as revealed by the NOR3.2 mAb, a panel of 15 different Abs raised against CD1d was used for immunohistochemical analysis. Four of these Abs did not produce any positive staining using cryostat sections of psoriatic plaques (CD1d8, CD1d34, CD1d40, and CD1d60), but the other Abs produced distinctive patterns that clustered into three groups. One set of Abs (CD1d19 and CD1d37) against CD1d produced rather faint staining of all cell layers of the epidermis (data not shown). The second set of Abs (CD1d27 and CD1d42) produced a pattern most closely resembling NOR3.2 staining, in that the basal cell layer had low to no immunoreactivity, but there was strong and diffuse staining of all suprabasilar keratinocytes of the plaque up to and including the stratum corneum. In general, there was greater cytoplasmic reactivity compared with NOR3.2 staining, but plasma membrane expression was detectable with this set of anti-CD1d Abs (data not shown). The third group of Abs (six different Abs; CD1d44, CD1d51, CD1d55, CD1d59, CD1d68, CD1d69, and CD1d75) produced strong and diffuse staining of all cell layers of the psoriatic epidermis (including the basal cell layer) with cytoplasmic greater than plasma membrane intensity (data not shown). To portray a representative profile of staining besides NOR3.2, a different anti-CD1d mAb (CD1d27) is portrayed. Fig. 2 displays NN, PN, and PP skin reactivity as well as thymic epithelium stained with CD1d27 mAb. Overall, the staining results with this large panel of mAbs suggest that there may be multiple epitopes on CD1d that are differentially expressed in psoriatic plaques. The biochemical characterization of keratinocyte CD1d expression in vitro and in vivo are further defined in a later section.

Expression of CD161, CD94, Va24, and Vβ11 in normal skin and psoriasis

Only rare CD161−, Va24−, or Vβ11-positive lymphocytes were present in normal and symptomless (i.e., PN) skin (data not shown). A positive control specimen was an invasive squamous cell carcinoma that had prominent CD1d expression by the malignant cells accompanied by numerous Va24-positive lymphocytes (data not shown). Compared with the sporadic presence of Va24- or Vβ11-positive lymphocytes in normal skin, CD94− and CD161-positive T cells were more consistently and more frequently found in skin biopsies of psoriatic plaques with prominent CD1d expression. In the psoriatic plaques examined, a striking abundance of CD161-positive T cells within CD1d-positive keratinocyte layers was observed (Fig. 3, A–C). Beside CD161 expression by psoriatic lesional intraepidermal T cells, CD94+ lymphocytes were also present in the hyperplastic epidermis (Fig. 3D).

Modulation of CD1d and biochemical characterization of keratinocyte CD1d expression

Normal multipassaged human keratinocytes or HaCaT cells grown using a low calcium, serum-free medium constitutively expressed CD1d at a relatively low level with primarily cytoplasmic localization, as revealed by immunohistochemical staining with either NOR3.2 (Fig. 4, upper panels) or CD1d27 mAbs (Fig. 4, lower panels). However, following 48 h of exposure to IFN-γ, enhanced plasma membrane CD1d expression by keratinocytes was observed (Fig. 4). Similarly, while proliferating keratinocytes did not consistently express ICAM-1 (Fig. 4, upper panels), after IFN-γ

**FIGURE 2.** CD1d expression revealed by CD1d27 immunostaining of thymic epithelium (A, inset), NN skin (A), PN skin (B), and PP skin (C). Note the concentric rings of staining of Hassal’s corpuscle in the thymus (A, inset). There was focal CD1d detected by this mAb in normal skin (A), which was more conspicuous in PN skin (B). Psoriatic plaques had extensive cytoplasmic as well as plasma membrane staining on keratinocytes above the basal cell layer all the way to the surface of the skin (C).
exposure plasma membrane ICAM-1 expression was detected. Immortalized HaCaT cells (passages 134–135) produced similar immunohistochemical staining profiles in response to IFN-γ (data not shown).

To quantitate surface expression of CD1d by keratinocytes before and after IFN-γ treatment, flow cytometry was performed (Fig. 4). While the untreated keratinocytes had little or no surface CD1d expression or ICAM-1 expression, exposure to IFN-γ produced marked expression of both CD1d and ICAM-1, as measured by the peak shift for the mean channel fluorescence.

To further characterize and extend these immunostaining results, RT-PCR was performed as well as Western blot analysis. RT-PCR using RNA extracted from untreated and IFN-γ-treated normal keratinocytes and HaCaT cells revealed the expected 400-bp product consistent with CD1d mRNA transcripts, whereas omission of RT produced negative results (data not shown). Western blot analysis revealed at least two distinct immunoreactive bands with apparent Mr of 45–47 kDa for both HaCaT cells and normal human keratinocytes as well as in CD1d-transfected CIR cells that served as a positive control (Fig. 5, upper panel). Western blot analysis revealed that IFN-γ enhanced keratinocyte CD1d levels (Fig. 5, upper right panel). Immunoprecipitation/Western blot analysis of HaCaT cells revealed enhanced expression of a 47-kDa form of CD1d by IFN-γ treatment (Fig. 5, lower panels). The Mr of CD1d was reduced from 47 kDa to two species of 32 and 30 kDa after PNGase F treatment in HaCaT cells before and after exposure to IFN-γ. This enzymatic treatment indicates the presence of glycosylated forms of CD1d. By Western blot analysis the overexpression of CD1d in PP compared with NN skin involved both 47- and 30-kDa species of CD1d (Fig. 5, lower right panels).

In vitro responses of NK-T cells to CD1d-positive keratinocytes and CD1d-transfected HeLa cells

The ability of CD1d molecules expressed by keratinocytes to stimulate human CD1d-restricted NK-T cell proliferation and IFN-γ production (Fig. 6) was assessed using previously described procedures (29) All three NK T cell clones (i.e., DN2.D5, DN2.D6, and DN2.B9) express canonical TCR α-chain rearrangements as well as CD161, and variable levels of other NK receptors, such as CD94 (data not shown). All these NK-T cell clones responded with vigorous proliferation when mixed with equal numbers of CD1d-transfected HeLa cells, but not mock-transfected HeLa cells. In a typical experiment the NK-T cell clones alone incorporated <10^3 cpm of [3H]thymidine when cultured with mock-transfected HeLa cells, which was increased 10- to 100-fold by coculture with CD1d-transfected HeLa cells plus TPA with or without the glycolipid α-galactosylceramide. For DN2.D5 and DN2.D6, only TPA addition was necessary, while DN2.B9 cells required both TPA and the synthetic CD1d-presented lipid Ag α-galactosylceramide for optimal proliferation with CD1d-HeLa cells. None of these NK-T cell clones significantly increased their [3H]thymidine incorporation when combined with either CD1d-negative or CD1d-positive keratinocytes (data not shown). However, there was cluster formation noted in cultures in which the NK-T cell clones were mixed with IFN-γ-pretreated keratinocytes (to induce CD1d), but not when untreated keratinocytes were added (Fig. 6). This led us to explore the possibility that NK-T cells were being activated to

**FIGURE 3.** CD161-positive lymphocytes in diseased skin samples with enhanced CD1d expression. A, Psoriatic plaque contained numerous CD161-positive lymphocytes in the hyperplastic epidermis, with few positive cells in dermis. B, A different psoriatic plaque with CD161-positive lymphocytes in the epidermis and rare cells in underlying perivascular dermal infiltrate. C, High power of B with CD161-positive lymphocytes in epidermis. D, Psoriatic plaque also contained CD94-positive lymphocytes.
secrete cytokines rather than proliferate in response to CD1d-positive keratinocytes. Initial experiments explored the relative levels of IFN-γ and IL-4 produced by these three NK-T cell clones in the absence of keratinocytes as well as when either untreated or IFN-γ-pretreated keratinocytes were added. Typically, the NK-T cell clones spontaneously produced 5 pg/ml of IFN-γ, and when TPA/lipid was added, this did not exceed 20 pg/ml. These IFN-γ levels were only slightly increased (between 20 and 40 pg/ml of IFN-γ) when untreated keratinocytes were added (Fig. 6). However, the NK-T cell clones responded to the addition of IFN-γ-pretreated and washed keratinocytes did not contain any detectable IFN-γ, with a lower limit of detection of 3 pg/ml (data not shown).

Compared with the high induction of IFN-γ levels, none of the three NK-T cell clones responded to either untreated or IFN-γ-pretreated keratinocytes by increasing their IL-4 levels (Fig. 6) by >25% above their constitutive levels in the absence of keratinocytes (~32 pg/ml of IL-4). While these same NK-T cell clones can produce high levels of IL-4 following cross-linking of their TCRs (29), combining the NK-T cell clones with keratinocytes did not trigger similar levels of IL-4 production.

To further explore functional interactions between NK-T cells and keratinocytes, the DN2.B9 NK-T cell clone was more extensively studied. DN2.B9 cells were induced to proliferate and secrete high levels of IFN-γ when combined with CD1d-positive, but not CD1d-negative, HeLa cells (Figs. 7 and 8). This reaction required the copresence of suboptimal amounts of TPA (1 ng/ml).
and α-galactosylceramide (50 ng/ml). While no significant proliferation of DN2.B9 cells was induced by either untreated or IFN-γ-pretreated keratinocytes, IFN-γ production by DN2.B9 cells was strongly elevated with the CD1d-positive keratinocytes. As with the CD1d-positive HeLa cells, the production of IFN-γ was enhanced by addition of TPA and glycolipid. When IL-4 levels were measured in these wells, the relatively low levels made constitutively by DN2.B9 cells were not consistently or significantly increased when either CD1d-positive HeLa cells or keratinocytes with or without IFN-γ pretreatment were added (Fig. 6 and data not shown). It should be noted that high levels of IFN-γ were produced when DN2.B9 cells were combined with CD1d-transfected HeLa cells and TPA that did not require any pretreatment with IFN-γ, further excluding the possible effects of residual IFN-γ as contributing to the Th1 polarization.

**FIGURE 7.** Proliferative response of DN2.B9 NK-T cells. Note the relative lack of proliferation of DN2.B9 NK-T cells when cocultured with either untreated or IFN-γ-pretreated keratinocytes. However, cluster formation occurred when NK-T cells were mixed with IFN-γ-pretreated keratinocytes or CD1d⁺ HeLa cells (inset). DN2.B9 NK-T cells proliferated vigorously when mixed with CD1d⁺ HeLa cells, which was reduced by addition of anti-LFA-1 (IgG) and anti-CD1d blocking mAb (IgM), whereas a control mixture of irrelevant IgG and IgM Abs did not significantly inhibit proliferation.

**FIGURE 8.** IFN-γ production by DN2.B9 NK-T cells. Under the same conditions as those described in Fig. 7, the NK-T cell clone secreted IFN-γ when combined with either keratinocytes pretreated with IFN-γ or CD1d⁺ HeLa cells. The importance of the CD1d recognition was confirmed using a specific Ab against CD1d (IgM), and this reaction was also inhibited by Ab against LFA-1 (IgG), but not by control, irrelevant IgG/IgM Abs.

*IFN-γ production by NK-T cells was blocked by anti-CD1d Ab*

To determine the molecular basis for recognition by the DN2.B9 cells of target cells induced to express CD1d, various blocking Abs were added (Figs. 7 and 8). A blocking Ab against CD1d as well as an anti-LFA-1 Ab (but not control IgG/IgM Abs) significantly inhibited the proliferation of DN2.B9 cells stimulated by CD1d-positive HeLa cells. In addition, these same Abs reduced the production of IFN-γ stimulated by CD1d-positive HeLa cells as well as CD1d-positive keratinocytes. The anti-CD1d Ab did not inhibit...
SEB/SEC2 or PHA-stimulated Ficoll-Hypaque mononuclear cell proliferation (data not shown).

Discussion

In this study we demonstrated expression of CD1d by normal human skin and its pronounced overexpression in psoriatic skin lesions. Keratinocytes in vitro and in vivo synthesized and expressed CD1d, which was capable of triggering CD161+ NK-T cells to produce high levels of IFN-γ, but not IL-4. Previously, we observed that cross-linking the TCR in these NK-T cell clones led to enhancement of both IFN-γ and IL-4 levels (29), suggesting that a selective stimulatory event was being mediated by CD1d-expressing keratinocytes, leading to preferential IFN-γ production. The failure to trigger significant NK-T cell proliferation despite enhanced IFN-γ release has been previously observed with other NK-T cell clones via recognition of class I MHC molecules (45). The stimulation by CD1d of T cells bearing NK receptors preferentially induces a cytokine switch to IFN-γ (46, 47). Moreover, the differential induction of IFN-γ production, but not IL-4, after the NK-T cell clones recognized CD1d on keratinocytes has potentially important implications for psoriasis. Not only is there overexpression of CD1d by psoriatic epidermal keratinocytes and the presence of NK-T cells bearing CD94 and CD161, but the cytokine IFN-γ has been shown to trigger psoriatic lesions (2). We therefore postulate that a positive feedback loop could be established in skin due to the presence of NK-T cells being activated to produce IFN-γ upon contact with CD1d-positive keratinocytes, leading to further CD1d expression and subsequent NK-T cell release of more IFN-γ. The lack of a proliferative response by NK-T cells to CD1d+ keratinocytes is also consistent with the general number and distribution of CD94- and CD161-positive NK-T cells in psoriasis. Thus, the NK-T cells are never observed in tight clusters or in very large numbers as might be expected if they were undergoing a local proliferative response; rather, they are found as more evenly distributed single cells throughout a psoriatic plaque.

In normal human skin CD1d was generally restricted to the outermost keratinocyte layers in the stratum granulosum just beneath the lipid-rich stratum corneum. It appeared that the plasma membrane staining for CD1d was juxtaposed directly with the stratum corneum, although CD1d could also be seen in some specimens to be present in the basal cell layer. In addition to epidermal keratinocytes, CD1d was detected on upper dermal dendritic cells, corneum, although CD1d could also be seen in some specimens to be present in the basal cell layer and extending to the outermost keratinocytes imme-

Siderely beneath the parakeratotic layer juxtaposed to the stratum corneum. CD161-positive T cells were frequently observed in di-

rect contact with keratinocytes expressing CD1d in psoriatic plaques. Given this anatomical juxtaposition, it is possible for various types of glycolipids in the psoriatic scale to be directly exposed to the abundant keratinocyte cell surface CD1d. Moreover, given the large hydrophobic binding pockets in CD1d, the presence of CD1d on the outer layers of epidermis in psoriatic plaques opens up the possibility that various glycolipids present in the stratum corneum could play a role in triggering a response by NK-T cells or other T cell subsets capable of recognizing such glycolipids in the context of CD1d. During epidermal differentiation keratinocytes produce different amounts and types of various glycolipids, including glucosylceramides (49). Alterations in these glycolipids in the stratum corneum can have a significant impact on the barrier function of skin. Previously investigators have focused on the direct proliferative effect of glucosylceramides on epidermal keratinocytes (50). However, it is also clear that barrier perturbation can initiate cytokine cascades and thus influence inflammatory and mononuclear cell activation (51, 52). The rapid and diffuse up-regulation of CD1d expression seen in the symptomless skin following repeated tape stripping demonstrates the highly inducible nature of CD1d expression by keratinocytes. Furthermore, a reciprocal interaction appears likely, because T cells can also influence barrier function (53). Because cytokines such as IL-1 and TNF-α are released after tape stripping (52), these and other cytokines are being studied to determine whether they can also induce CD1d expression by keratinocytes. With regard to psoriasis, abnormalities in barrier function have been well documented (54, 55), with both quantitative and qualitative changes, including alterations in glycolipids, that may not be similarly present in other forms of scaly dermatitis such as atop dermatitis (56). Thus, a cycle can be envisioned in which pathogenic NK-T cells initiate barrier abnormality, which, in turn, would generate glycolipids that could be presented by keratinocyte CD1d and further activate CD161+ T cells in psoriasis and allergic contact dermatitis (57).

With regard to the triggering of NK-T cells, it is likely to be important that these T cells bear both TCRs as well as NKR.

There is precedent for synergistic interactions between these two classes of receptors (58), and there is a possibility that such synergistic activation of NK-T cells may have relevance to psoriasis. The relative paucity of Vα24- or Vβ11-positive lymphocytes in psoriasis indicates that the T cells in psoriasis with NKR may not represent classical invariant TCR-bearing NK-T cells. Our recent results characterizing a psoriatic pathogenic T cell line that was also lacking a canonical TCR rearrangement but was CD1d reactive (8) suggest greater heterogeneity of CD1d-reactive T cells subsets beyond Vα24-positive NK-T cells (59). We observed that when this NK-T cell line was injected into engrafted autologous PN skin using a SCID mouse model, a psoriatic plaque was created (8). This acute lesion was characterized by the juxtaposition of NKR-bearing T cells in the epidermis containing CD1d-positive keratinocytes (8). Taken together, these in vivo findings along with the current in vitro findings support the idea that NK-T cells may be playing an important pathophysiological role in psoriasis (6–8, 60, 61).

Besides the ability of keratinocytes to initiate (48), perpetuate (62), and terminate (63) immune reactions involving conventional T cell responses to nominal Ags and superantigens, CD1d expression may also imbue the keratinocyte with the capacity to interact with NKR-bearing T cells. As a member of a nonclassical, MHC-independent, Ag-presenting system, CD1d expression as seen in psoriasis provides a novel opportunity for therapeutic targeting and for understanding the immunologic and genetic basis of psoriasis as well as the potential role for innate immunity in skin disease (60, 61). At least one group has identified familial linkage of psoriasis to chromosome 1 near the CD1d locus (64). Furthermore, as suggested by the absence of CD1d in the basal cell layer and its expression in suprabasalar keratinocytes of psoriatic plaques, it appears that CD1d is influenced by the differentiation status of the keratinocytes, much like other neighboring genes on chromosome
1q21 also implicated in psoriasis (65). It will be important to determine whether mutations in CD1d are found in psoriatic patients, particularly as amino acid residues that may influence recognition by T cells expressing NKRs (66). In conclusion, CD1d is more widely distributed throughout various body sites than originally observed, and skin-derived keratinocyes expressing CD1d can be recognized by T cells triggering IFN-γ production rapidly, which does not require preceding T cell proliferation.

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