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Costimulation of Dendritic Epidermal γδ T Cells by a New NKG2D Ligand Expressed Specifically in the Skin[1]

Michael I. Whang, Nadia Guerra, and David H. Raulet[2]

Dendritic epidermal T cells (DETCs) are a highly specialized population of γδ T cells that resides in the murine skin and participates in wound healing and tumor surveillance. Despite the expression of other stimulatory receptors on these cells, mechanisms involving activation have focused primarily on the invariant Vγ3-Vδ1 TCR expressed by DETCs. All DETCs also express the activating NKG2D receptor, but the role of NKG2D in DETC activation remains unclear, as does the identity of NKG2D ligands that are functionally expressed in the skin. In this study, we document the cloning of an NKG2D ligand H60c that is expressed specifically in the skin and in cultured keratinocytes and demonstrate its role in the activation of DETCs and NK cells. The ligand is unique among NKG2D ligands in being up-regulated in cultured keratinocytes, and its interaction with NKG2D is essential for DETC activation. Importantly, it is shown that engagement of NKG2D is not sufficient to activate DETCs, but instead provides a costimulatory signal that is nevertheless essential for activating DETCs in response to stimulation with keratinocytes. ***The Journal of Immunology,*** **2009, 182: 4557–4564.**

Skin γδ T cells, also called dendritic epidermal T cells (DETCs),[3] express a canonical invariant TCR that recognizes an Ag expressed by stressed, damaged, or transformed keratinocytes. Mice deficient in γδ T cells have increased sensitivity to carcinogen-induced skin carcinogenesis as well as a defect in skin wound repair, supporting a role for DETCs in surveillance of the epidermis (1–3). The ligand recognized by the DETC TCR has not yet been identified. It is known that DETCs require positive selection within the thymus for thymic egress and homing to the epidermis (4). Whereas it has been shown that the *Skint1* gene is necessary for this process (5), it remains uncertain whether Skint1 interacts directly with the DETC TCR.

The activating NKG2D immunoreceptor is expressed by NK cells, activated CD8 T cells, and subsets of CD4 T cells, NKT cells, and γδ T cells, including the aforementioned DETC γδ subset (2, 6–8). Several human and mouse ligands for NKG2D have been identified, all of which are related to MHC class I molecules (7, 9, 10). Current evidence indicates that NKG2D ligands are generally expressed poorly by normal cells, but are up-regulated in diseased cells in response to disease-associated stress (11–14). Cell surface expression of NKG2D ligands is induced as a result of tumorigenesis or infection with certain pathogens, which may lead to NKG2D-mediated activation of lymphocytes, target cell lysis, cytokine production, and protection from the tumor or pathogen (6, 7, 11). Genetic ablation of NKG2D (13) results in a higher incidence of tumors in some models of spontaneous cancer, and blockade of the receptor with Ab in some cases impairs viral immunity (15). As an indication of the interplay of NKG2D with herpesviruses during evolution, some of these viruses have incorporated evasins that block the expression of NKG2D ligands (16–19). Furthermore, many advanced tumors in humans shed high levels of soluble MICA ligand, which desensitizes the receptor and is believed to enable evasion of the immune system (20, 21).

There exist a surprisingly large number of different ligands for NKG2D, with eight identified thus far in humans and up to nine in mice (8, 22). A family of ligands in mice comprised of several retinoic acid early-inducible 1 (Rae1) isoforms, murine UL-16-binding protein-like transcript 1 (Mult1) and histocompatibility 60 (H60), is orthologous to a comparable family of human ligands that are called UL-16 binding proteins (ULBPs) and/or RAET1 proteins (8). The MICA/MICB ligands represent a separate family of NKG2D ligands, which is present in humans but not in mice (7). It remains unclear why each individual has the potential to express so many different NKG2D ligands. The various ligands might function essentially identically, providing a level of redundancy to a system that depends on sustained ligand expression for effective immune surveillance. Alternatively, some ligands may exhibit unique functions or regulation that provide specific forms of immune protection. However, studies to date show that despite having varied affinity for NKG2D (8), the various ligands, when expressed in transfected cells, usually provoke similar functional outcomes such as target cell lysis, cytokine production, and tumor cell rejection (9, 23–25). An exception to this is a study suggesting a distinct, NKG2D-independent, function of the H60 ligand in mice (26). Differences in regulation or localization of expression remain possibilities, although the ligands studied to date are often coexpressed on tumor cell lines. Furthermore, cell surface expression of several different NKG2D ligands, including Rae1, Mult1, and some of the ULBPs, can be enhanced by exposing cultured cells to genotoxic agents (12).

A controversial issue with respect to NKG2D function is whether it provides a stimulatory or costimulatory signal to responding cells. Most stimulatory NK cell receptors pair with DAP12, FcεRIγ, or CD3ζ, which are signaling adaptor proteins

Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley, CA 94720

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[2] Address correspondence and reprint requests to Dr. David H. Raulet, Department of Molecular and Cell Biology, University of California, Berkeley, 142 Life Sciences Addition 3200, Berkeley, CA 94720-3200. E-mail address: raulet@berkeley.edu

[3] Abbreviations used in this paper: DETC, dendritic epidermal T cell; Mult1, murine UL-16-binding protein-like transcript 1; Rae1, retinoic acid early inducible 1; H60/60a/60c, Histocompatibility 60/60a/60c;ORF, open reading frame; For, forward; Rev, reverse; ULBP, UL-16 binding protein.

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bearing ITAMs that can potently activate lymphocyte functions when phosphorylated (27–29). In all cell types that express it, NKG2D pairs with DAP10, a signaling subunit that lacks an ITAM, and instead contains a different tyrosine- based motif (YxxM) that binds to the p85 subunit of PI3K (30). Because YxxM motifs are also found in the cytoplasmic domain of costimulatory molecules such as CD28, NKG2D has often been attributed with costimulatory rather than primary stimulatory activity.

Evidence indicates, however, that NKG2D can exhibit full activating activity in several contexts, either because the receptor can, in some cases, associate with the ITAM-containing DAP12 signaling subunit (as in mouse NK cells) (31–33) or because DAP10 can in some cells provide a sufficient signal to induce cytotoxicity and possibly even cytokine production (34–37). In conventional CD8 T cells, NKG2D is believed to associate only with DAP10 (31, 37, 38), and engagement of the receptor is usually capable of enhancing TCR-dependent activation of the cells (6, 11, 31, 39), but is not sufficient for activation. However, when human CD8 T cells and certain $\gamma\delta$ T cells are cultured in IL-15, NKG2D engagement in the absence of TCR signaling may be sufficient for triggering cytotoxicity (38). In human cells, NKG2D cannot pair with DAP12 (36–38), suggesting that DAP10 may provide a sufficient signal for cellular activation in certain contexts. Reportedly, NKG2D engagement without TCR engagement was also sufficient for activating mouse DETCs, but by a distinct mechanism wherein DETCs, unlike most T cells, were reported to express DAP12 that can associate with NKG2D (40).

In this study, we describe a ligand for the mouse NKG2D receptor that is expressed specifically in the epidermis and document its capacity to activate both NK cells and DETCs. Consistent with stress-mediated ligand induction, the ligand is potently up-regulated by culturing keratinocytes in vitro and, significantly, engagement of NKG2D by the new ligand provides potent costimulatory activity but is not sufficient for activating DETCs.

Materials and Methods

Mice

C57BL/6J (B6) mice and all other strains were bred in the University of California, Berkeley Animal Facility from mice obtained from The Jackson Laboratory. CD1(ICR) mice were obtained from Charles River. *Klrk*$^{-/-}$ (NKG2D-deficient) mice were generated in our laboratory on the B6 genetic background (13). All animal experiments were performed according to the guidelines of the Office of Laboratory Animal Care, University of California, Berkeley.

Molecular cloning of H60c

The H60c transcript was detected in the National Center for Biotechnology Information database (XM_136905) as the most significant hit to H60a using the "blastn" algorithm. The hit, "*Mus musculus* similar to histocompatibility Ag H60," was listed as a predicted computational sequence derived from an annotated genomic sequence (NT_039490) using a gene prediction method: Genomescan. Based on this sequence, we designed primers for 5′ rapid amplification of cDNA ends (Invitrogen), which enabled us to identify the start codon of the open reading frame (ORF). Putative 3′ exons were identified in the genomic sequence and confirmed by RT-PCR. The H60c ORF was amplified from PDV cDNA following reverse transcription using the following primers: forward (For), ctcgagcc accATGGTCTCTGGGCACTGCAGTCACG and reverse (rev), gcggccgc CTAGAGGATGTAGATGAGCAATATC (restriction sites in small letters and 5′ consensus Kozak sequence underlined). The predicted ORF sequence was confirmed by sequencing. The H60c ORF sequence was subcloned into the pMSCV vector for stably transducing H60c into mammalian cells.

Cell lines and cell preparations

The Pam 212 (Pam) epidermal cell line and the PDV squamous cell carcinoma line were gifts from Dr. W. Havran (The Scripps Research Institute, La Jolla, CA). Pam, Pam-H60c, PDV, and PDV-H60c cell lines were maintained in DMEM (Invitrogen) supplemented with 10% (v/v) heat-inactivated FCS, 2 mM glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 1× penicillin and streptomycin, 1× vitamins, and 50 μM 2-ME (DMEM/c). The FcγR$^+$ P815 mastocytoma cell line, the FcγR$^+$ Daudi B lymphoblast cell line, and the RMA mouse T lymphoma cell line and its derivatives, RMA-H60c, RMA-Rae1β, and RMA-Rae1ε, were cultured in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated FCS, 2 mM glutamine, 25 mM HEPES, 50 μM 2-ME, and 1× penicillin and streptomycin (RPMI/FCS). IL-2-activated NK cells were prepared by incubating B6 splenocytes for 4 days in RPMI/FCS supplemented with 1000 U/ml rIL-2. Epidermal cells were prepared by enzymatically dissociating skin preparations from B6 wild-type and NKG2D-deficient mice as described elsewhere (41). Daudi, RMA, Pam, and PDV cells were transduced with replication-deficient retroviruses encoding H60c (pMSCV-H60c) or Rae1ε (pMSCV-Rae1ε), as described previously (23). The generation of the RMA-Rae1β transductant was previously reported (23). All cell lines and cell preparations were maintained at 37°C in 5% CO_2.

Preparation of DETCs

TCRδ^+ cells were sorted from skin dissociations (see above) and cultured in RPMI/FCS supplemented with 1.25 μg/ml Con A and 20 U/ml rIL-2 for 4 days, followed by culture for several weeks in the same medium without Con A. After 3 wk of culture, cells were restimulated with 2.5 μg/ml Con A for 18 h. To prepare DETCs with optimal cytotoxic activity, 6 days before the assay DETCs were preplated on terminally differentiated keratinocyte feeder cells. The differentiated feeder cells were prepared from primary keratinocyte cultures (see below) by replacing the keratinocyte medium with DMEM/c and culturing for 1 day. After 2 days of culturing with feeder cells, DETCs were repurified by magnetic sorting using Thy1.2 Ab according to the manufacturer's instructions (Miltenyi Biotec) and returned to culture in RPMI/FCS supplemented with 20 U/ml IL-2 for 4 days before performing the ^{51}Cr release assays. DETCs used for all experiments were >99% Vg3$^+$.

Keratinocyte lines

Keratinocyte lines were generated as described elsewhere (42). In short, epidermal cells were dissociated with a solution containing 0.3% trypsin and cultured in Defined Keratinocyte-Serum-free Medium (Invitrogen) supplemented with 10 ng/ml epidermal growth factor (PeproTech) and 10^{-10} M cholera toxin (Calbiochem) and plated on collagen I (catalog no. 354236; BD Biosciences)-coated plates.

Northern blotting

Total cellular RNA was prepared from the indicated tissues and cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Northern blots were preformed as described elsewhere (25). Blots were hybridized with a [α-^{32}P]CTP-labeled probe corresponding to the first 348 nt of the ORF, which is mostly divergent between H60c and the other H60-like sequences. The blots were washed at 65°C in 0.5× SSC/1% SDS. Under these conditions of stringency, the probe is not expected to hybridize to H60a or H60b, which are each less than 64% identical at the nucleotide level in this region. After autoradiography, the blot was stripped and rehybridized with a ^{32}P-labeled GAPDH probe as a loading control.

Abs, tetramers, and flow cytometry

The generation of the NKG2D tetramer and the staining protocol for this reagent were described previously (9). FITC-conjugated Vγ3 TCR Ab (F536) and CD104 Ab (346-11A) were purchased from BD Pharmingen. Purified NKG2D Ab (MI-6), PE-conjugated anti-NKG2D (CX5), PE-conjugated CD107a Ab (eBio1D4B), and rat IgG2a isotype control Ab (eBR2a) were purchased from eBioscience. The following Abs specific for NKG2D ligands were purchased from R&D Systems: pan-Rae1 mAb (186107), MULT1 mAb (237104), and biotinylated H60a polyclonal Ab (catalog no. BAF1155). The latter Ab, which cross-reacts with H60c, was used to detect H60c in the B6 strain. Because the *H60a* gene is a pseudogene in the B6 strain and H60b is not expressed in the skin (22), the anti-H60a Ab is specific for H60c in this strain.

Cytotoxicity assay

^{51}Cr-labeled target cells were cocultured with IL-2- activated NK cells or short-term DETC lines in a standard 4 or 5 h ^{51}Cr release assay in RPMI/FCS. Blockade of the NKG2D receptor with the MI-6 mAb has been described elsewhere (6). Data represent the mean ± SD of triplicate measurements. For redirected lysis assays, effector cells were preincubated with 50 μg/ml NKG2D Ab for 30 min before adding target cells, without washing out the Ab.

Stimulation assays

For stimulating cells with plate-bound Abs, CD3ε and/or NKG2D Abs were immobilized to 96-well, high-binding, flat-bottom plates (Corning) overnight at 4°C. DETCs were then added to the wells. For degranulation assays, PE-conjugated CD107a Ab and GolgiStop (BD Pharmingen) were also added at the initiation of the cultures, which were conducted for 4 h. The cells were lifted by repeated pipetting, washed in PBS supplemented with 3% FCS, and analyzed by flow cytometry. For IL-2 production assays, GolgiPlug (BD Pharmingen) was added at the initiation of the cultures. After culturing the cells for 4 h, the cells were harvested, permeabilized, and stained with IL-2 Abs according to the manufacturer's (BD Pharmingen) instructions before flow cytometry. Data represent the mean ± SD of triplicate measurements.

For analysis of IFN-γ secretion by NK cells, RMA, RMA-Rae1e, or RMA-H60c stimulator cells were cocultured with IL-2- activated NK cells in 96-well plates for 6 h in the presence of GolgiPlug (BD Pharmingen). Permeabilization and staining of the cells was performed according to the manufacturer's instructions (BD Pharmingen). Data represent the mean ± SD of triplicate measurements.

RNA preparation from wounded skin

Mice were anesthetized with isoflurane. The mouse backs were shaved, back skin was pulled up, and a sterile 2.5-mm punch tool was used to create three sets of wounds as previously described (1). Mice were caged individually and wounds were left uncovered. Wounded mice (two per time point) were euthanized 1–4 days after wounding and the skin was excised, including a 2-mm border around the wound. The excised skin was flash frozen with liquid nitrogen and dispersed with mortar and pestle before total cellular RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions.

Quantitative real-time PCR

cDNA was prepared using the Superscript III Kit (Invitrogen) and quantitative RT-PCR was performed with SYBR GreenER (Invitrogen) using either the Applied Biosystems 7300 Real-Time PCR System or Applied Biosystems StepOnePlus Real-Time PCR System. The following oligonucleotides were used for amplification: H60c For, AGATTTCAGTTGCT GCCTCA and H60c Rev, ACATGTGCAGCAGTGGTTG; Itgb4 For, CCAGAGGCCTGAGAACAGAG and Itgb4 Rev, CCCCTTGGTCT TCTTTAGCC; and GAPDH For, GAAGGTCGGTGTGAACGGA and GAPDH Rev, GTTAGTGGGGTCTCGCTCCT. Data represent the mean ± SD of triplicate measurements.

Results

Identification of a cDNA encoding a protein related to H60

A database query for sequences with homology to murine NKG2D ligand H60 identified a novel expressed sequence tag (National Center for Biotechnology Information accession number XM_36905). In an initial study, no cell surface expression was obtained after transfecting an expression vector that used the 5′-most ATG codon in the coding region of the expressed sequence tag as the presumptive initiator codon (data not shown). This observation prompted a 5′ rapid amplification of cDNA end experiment using RNA from squamous cell carcinoma cell line PDV, which identified an additional ATG codon in the expressed sequence, 30 codons upstream. Based on predicted RNA splice sites in the genomic sequence, primers were designed to amplify a cDNA containing the entire ORF of 684 nt, including the newly identified initiator codon. Transfection of Chinese hamster ovary cells with an expression vector containing this sequence yielded robust cell surface expression.

The H60c ORF was 684 nt and encoded a mature predicted membrane protein of 18.4 kDa, including class I-like domains that are highly related to those of H60 (73% amino acid identity). The remaining portion of the ectodomain was shorter than and only 16% identical to that of H60 (now called H60a). In contrast to H60a, which is a type I transmembrane protein, prediction programs suggested the new protein was attached to the plasma membrane via a GPI anchor, which was confirmed by enzymatic digestion with glycosylphosphatidylinositol-specific phospholipase C (data not shown).

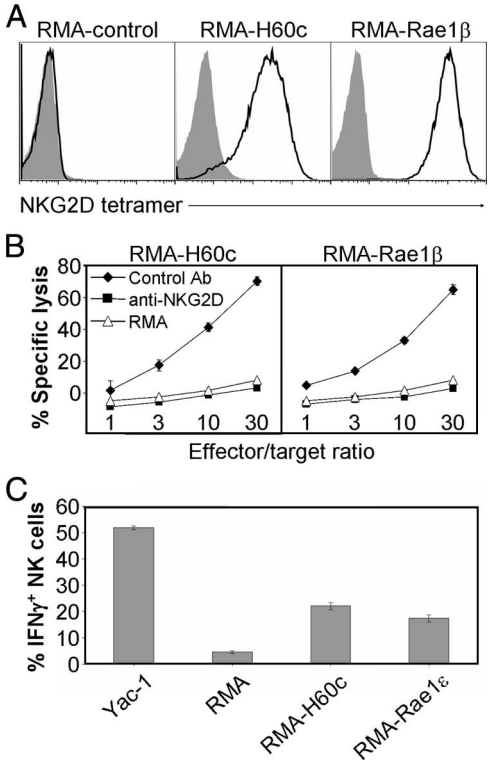

FIGURE 1. Activation of NK cells by novel NKG2D ligand. *A*, The T cell lymphoma RMA was transduced with retroviruses encoding the indicated proteins or with empty vector (RMA-control). Cells were stained with NKG2D tetramers (open histogram) or streptavidin (shaded histogram). *B*, IL-2-stimulated NK cells were used as effector cells against the indicated target cells in a standard 4-h ^{51}Cr release assay. NKG2D Ab (MI-6) or isotype control Ab (each at 50 μg/ml final concentration) was added as indicated. *C*, IL-2-activated NK cells were stimulated for 6 h with the indicated RMA transductants. Accumulation of IFN-γ in NK cells was determined by intracellular cytokine staining and electronic gating on NK1.1$^+$CD3$^-$ cells. The results are representative of three or more experiments.

H60c is located near the telomere of chromosome 10, in the band A1. *H60a*, in contrast, is located 14 Mb away in the A3 region. Thus, groups of related NKG2D ligands are clustered in two locations, including H60c and Mult1 in the A1 region and Rae1ε and H60a in the A3 region, suggesting that the A1 and A3 regions represent the products of a gene duplication event.

During the course of this work, another report appeared describing two new H60-related cDNAs, called H60b and H60c (22). The H60c amino acid sequence deduced by the authors corresponded to the truncated sequence mentioned above (see *Discussion*). Considering the identity of the rest of their sequence to ours, we will use the term "H60c" in this manuscript to describe the new ligand.

H60c is a ligand for NKG2D and functionally activates NK cells

To test the functions of H60c, the full-length cDNA was inserted in a retroviral expression vector, which was transduced into the ligand-negative RMA T cell lymphoma line, followed by selection of positive cells by flow cytometry. The transduced cells were used to test the functions of H60c.

H60c-transduced RMA cells, like control Rae1β-transduced RMA cells, were stained brightly with tetramers of the extracellular domain of NKG2D, whereas control RMA cells did not stain above background (Fig. 1*A*). Furthermore, H60c-transduced RMA cells, like Rae1β-transduced RMA cells, were lysed efficiently by IL-2-activated NK cells, whereas control transductants were not.

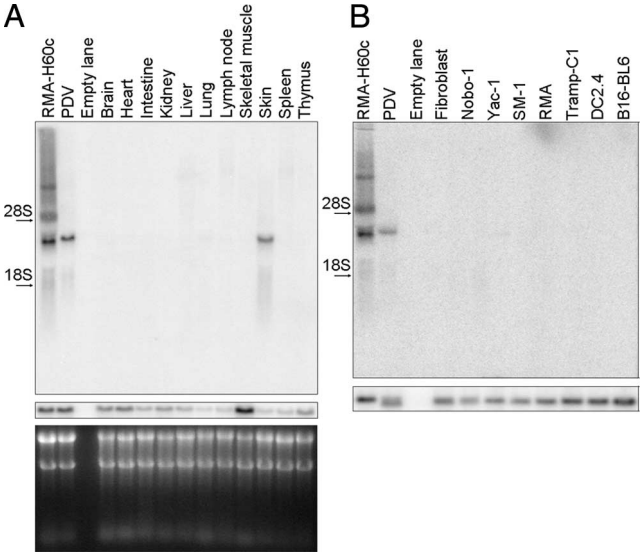

FIGURE 2. Expression of H60c mRNA in tissues and in tumor cell lines. *A* and *B*, Total RNA (30 μg) from the indicated tissues (*A*) or cell lines (*B*) was electrophoresed on agarose gels and immobilized on a charged nylon membrane. The membrane was hybridized with a ^{32}P-labeled H60c probe (*top panel*). After the final exposure, the membranes were stripped and rehybridized with a GAPDH probe to control for loading of all lanes (*middle panel*). Ethidium bromide staining of ribosomal RNA in the gel lanes before transfer is shown at the *bottom* of *A*. As determined in other studies (Ref. 25 and our unpublished data), most of the cell lines examined in *B* expressed one or more of the other known NKG2D ligands (Rae1 (R), Mult1 (M), and/or H60a (A)), including fibroblast (R and M), Nobo-1 (R and M), Yac-1 (R, M, and A), SM-1 (R and M), RMA (no ligands), Tramp-C1 (R), DC2.1 (R and M), and B16-BL6 (no ligands). The results are representative of three experiments.

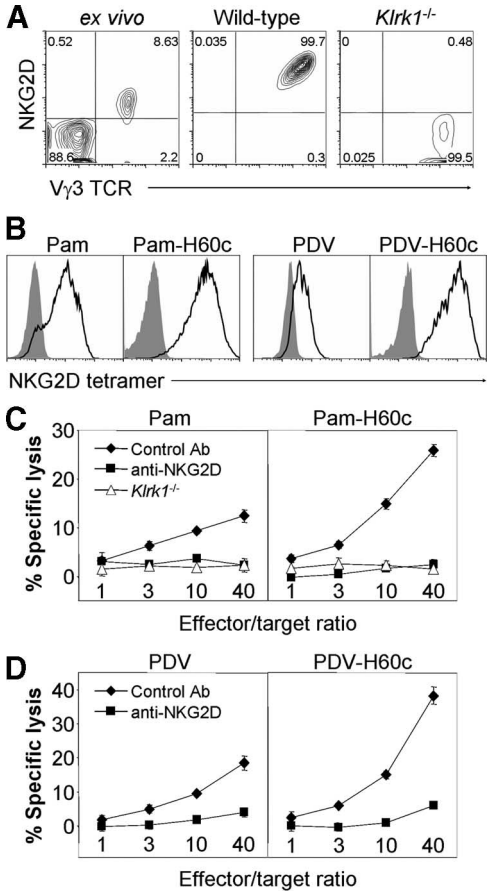

FIGURE 3. H60c functionally activates epidermal γδ T cells. *A*, Staining of dissociated skin cells shows that Vγ3$^+$ epidermal γδ T cells express NKG2D. Contour plots show NKG2D and Vγ3 TCR expression on ex vivo cells (18-h after dissociation; *left panel*) or on in vitro-expanded (for 6 wk) wild-type DETCs (*middle panel*) or DETCs from *Klrk1*$^{-/-}$ (*Klrk1* is the mouse NKG2D gene; *right panel*). *B*, The indicated keratinocyte tumor cell lines and their H60c transductants were stained with NKG2D tetramers (open histograms) or streptavidin (shaded histograms). *C* and *D*, In vitro-expanded wild-type and *Klrk1*$^{-/-}$ DETCs were used as effector cells against the indicated target cells in a standard 5-h ^{51}Cr release assay. NKG2D mAb (MI-6) or isotype control Ab was used to block interactions. The results are representative of three or more experiments.

Lysis of H60c-transduced cells, or Rae1β-transduced cells, was entirely abrogated by the addition of an NKG2D Ab that blocks ligand recognition, confirming the role of NKG2D in H60c recognition (Fig. 1*B*). RMA cells expressing H60c, like those expressing Rae1ε, were also potent stimulators of IFN-γ production by IL-2-activated NK cells, whereas control RMA cells were not (Fig. 1*C*). These data demonstrated that H60c engages NKG2D, resulting in target cell cytotoxicity and IFN-γ production mediated by activated NK cells.

H60c mRNA is detected exclusively in the skin

Northern blot analysis showed that the natural H60c transcript is relatively large, ~3 kb (Fig. 2*A*). Examination of RNA from multiple tissues demonstrated significant H60c expression in the normal skin, but not in any of the other tissues examined (Fig. 2*A*). Expression in the skin was confirmed by RT-PCR and Northern blot analysis of tissues from several other mouse strains including 129/J, BALB/cJ, B10.D2/J, DBA/2J, and CD1(ICR) (data not shown). Furthermore, H60c transcripts were undetectable in numerous tumor cell lines of diverse origin, which variously express Rae1, Mult1, and H60a, the exception being a keratinocyte cell line, PDV (Fig. 2*B*). These data show that H60c is expressed specifically in skin cells and in a keratinocyte cell line, suggesting that H60c has a specific function in the skin.

Function of H60c in the activation of epidermal γδ T cells

DETCs, the γδ T cells resident in the murine epidermis, uniformly express NKG2D (Fig. 3*A*) (2, 6). To address whether H60c plays a role in DETC activation, the Pam 212 keratinocyte cell line was used as a target cell for short-term lines of cultured DETCs (3). The Pam cells cultured in our laboratory lacked H60c transcripts, but naturally expressed H60a (data not shown) and stained well with the NKG2D tetramers (Fig. 3*B*). Despite the expression of H60a, Pam cells were lysed only modestly by DETCs (Fig. 3*C*). Transduction of Pam cells with H60c resulted in strongly enhanced staining with NKG2D tetramers, as well as substantially enhanced sensitivity of the cells to lysis by DETCs (Fig. 3*C*). Similarly, the PDV keratinocyte cell line, which expresses low amounts of Rae1ε and H60c (data not shown), were poor targets for DETCs, whereas lysis was enhanced when the cells were transduced to express high levels of H60c (Fig. 3*D*). Strikingly, lysis of both untransduced and H60c-transduced Pam cells and PDV cells was completely dependent on NKG2D engagement, as shown by the failure of DETCs from *Klrk1*$^{-/-}$ (*Klrk1* is the mouse NKG2D gene) mice to lyse the cells (as tested in the case of Pam cells) and the complete blockade of lysis by NKG2D Abs (shown for both cell lines) (Fig. 3, *C* and *D*). These results established the role of NKG2D in H60c recognition by DETCs and suggested that NKG2D recognition is crucial for significant DETC activation in response to keratinocytes.

The results with established keratinocyte cell lines were informative but complicated by the expression of NKG2D ligands other

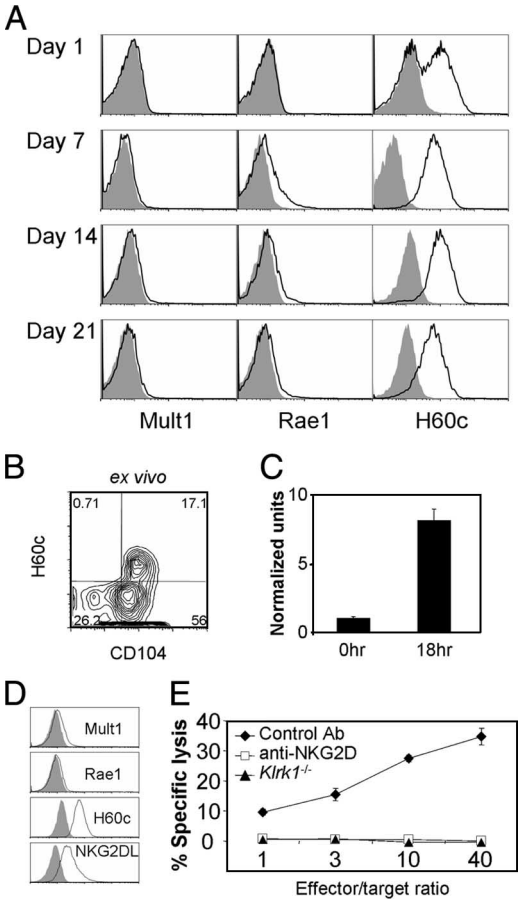

FIGURE 4. Cultured primary keratinocytes express H60c and activate DETCs. *A*, Dissociated C57BL/6 skin cells were cultured for the indicated period before analysis by flow cytometry. Cells were stained with Abs that recognize Rae1, MULT1, or H60c (open histogram) or control Abs (shaded histogram). *B*, Two-color contour plot shows H60c and β_4 integrin expression on ex vivo keratinocytes (18-h after dissociation). *C*, Increased H60c transcripts in keratinocytes after in vitro culture for 18 h, determined by quantitative RT-PCR. The data were normalized to GAPDH transcript amounts. *D*, Target cells used in *E* were stained with Abs that recognize Rae1, MULT1, or H60c or with NKG2D tetramers (open histograms) or control Abs or streptavidin (shaded histograms). *E*, In vitro-expanded DETCs from wild-type or *Klrk1*$^{-/-}$ mice were used as effector cells for cytotoxicity assays against keratinocytes harvested from primary cultures. NKG2D Ab (MI-6) or isotype control Ab was used to block interactions. Keratinocytes were in culture for 30 days. The results are representative of three or more experiments.

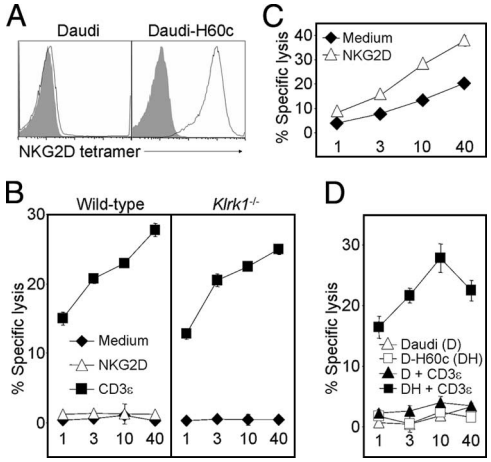

FIGURE 5. NKG2D stimulation costimulates DETC-mediated killing. *A*, FcγR$^+$ Daudi cells and Daudi cells transduced with H60c (Daudi-H60c) were stained with NKG2D tetramers (open histogram) or streptavidin (shaded histogram). *B*, In vitro-expanded DETCs from wild-type or NKG2D-deficient mice precoated with NKG2D Ab (MI-6) or CD3ε Ab (145-2C11) were used as effector cells against Daudi target cells. *C*, IL-2-activated NK cells precoated with NKG2D Ab were used as effector cells against Daudi target cells. *D*, DETCs precoated with a limiting dose of anti-CD3ε Ab (4 ng/ml) were used as effector cells against Daudi and Daudi-H60c target cells. The results are representative of three experiments.

than H60c by these cells. In the course of establishing primary cultures of keratinocytes using published procedures (42), we observed that short-term cultures of keratinocytes spontaneously expressed H60c but not any of the other ligands (Fig. 4, *A* and *B*). In these studies, CD104 expression was used to identify keratinocytes (43). Whereas only a subset of keratinocytes expressed H60c on day 1 of culture, all keratinocytes expressed H60c after a few days in culture (Fig. 4, *A* and *B*). Expression was maintained until at least day 21 of culture. The kinetics of expression suggested that cell surface expression of H60c protein by keratinocytes is induced in cell culture, despite the presence of transcripts in the cells. This conclusion was supported by the absence of detectable H60c protein in normal skin as determined by immunofluorescent staining of skin sections (data not shown). Furthermore, the amount of H60c transcripts in skin cells increased sharply in cultured skin cells (Fig. 4*C*). Note that it was not possible to assess H60c protein by flow cytometry on freshly isolated keratinocytes, because the protein is enzymatically cleaved by the proteases used to dissociate keratinocytes from skin samples (data not shown). For analysis of cultured cells, cells were lifted without the use of proteases by pipetting after incubation in EDTA solution.

Cells from primary keratinocyte cultures were used to assess whether lysis of keratinocytes by DETCs occurs when the keratinocytes express endogenous H60c in the absence of other NKG2D ligands. The primary cells, which only expressed H60c (Fig. 4*D*), were lysed efficiently by DETCs, and killing was completely dependent on NKG2D, as shown by testing NKG2D-deficient DETCs or by blocking the killing with NKG2D Abs (Fig. 4*E*). Therefore, recognition of H60c by NKG2D was essential for cytolysis of primary cultures of keratinocytes by DETCs.

Signaling via NKG2D is necessary but not sufficient for DETC killing

Having shown that NKG2D engagement is essential for activation of DETCs, it was important to address whether it provides a sufficient signal for activation or works primarily as a costimulatory receptor in conjunction with signals from other receptors such as the TCR. As one approach to address whether engagement of NKG2D triggers cytolysis by DETCs independently of TCR engagement, redirected lysis experiments were performed in which Fc receptor-bearing, human Daudi cells, bound with NKG2D Abs and/or TCR Abs, were used as target cells for DETCs. The choice of a human B lymphoblast cell line for these studies was based on the supposition that these cells were unlikely to express a putative ligand for the DETC TCR, which is thought to be restricted to keratinocytes and fetal thymocytes (3, 4, 44). In addition, Daudi cells do not express ligands that bind mouse NKG2D tetramers (Fig. 5*A*). Indeed, unmodified Daudi cells were not lysed by DETCs (Fig. 5*B*). Daudi cells precoated with CD3ε Abs were lysed efficiently, even by DETCs from NKG2D-deficient mice, demonstrating that strong TCR engagement triggers DETC-mediated lysis in the absence of NKG2D engagement (Fig. 5*B*). In contrast, Daudi cells precoated with NKG2D Abs were not lysed by DETCs (Fig. 5*B*), but were lysed by IL-2-activated NK cells

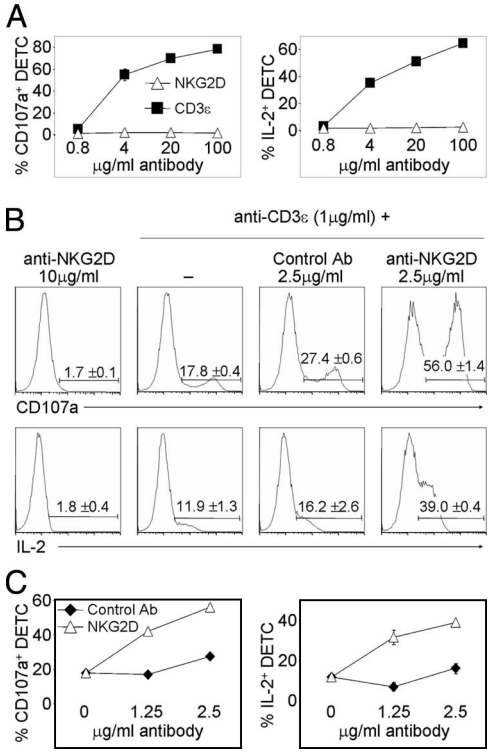

FIGURE 6. NKG2D stimulation costimulates DETC degranulation and IL-2 production. *A* and *B*, In vitro-expanded DETCs were stimulated with the indicated concentrations of either plate-bound CD3ε Ab (145-2C11) or NKG2D Ab (MI-6) for 4 h. Degranulation (*left* of *A* and *top* of *B*) or intracellular IL-2 accumulation (*right* of *A* and *bottom* of *B*) were determined by flow cytometry. *C*, DETCs were stimulated with a suboptimal concentration of plate-bound CD3ε Ab (1 μg/ml) plus increasing concentrations of either NKG2D Ab or control Ab. The results are representative of three or more experiments.

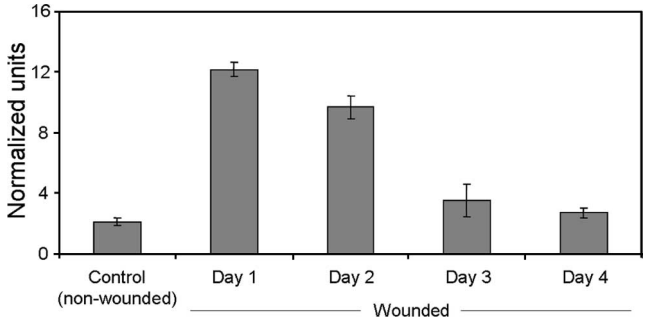

FIGURE 7. H60c mRNA is up-regulated in wounded skin. C57BL/6 mice received full-thickness wounds in their back skin. Relative levels of H60c mRNA were analyzed by quantitative RT-PCR in nonwounded and wounded skin. There was a dramatic increase in overall cellularity in the wounded tissue immediately after injury as a result of infiltrating lymphohematopoietic cells, which prevented the use of conventional housekeeping transcripts for normalization when comparing unwounded skin and wounded skin. Instead, given our finding that only CD104$^+$ cells expressed H60c, we normalized the samples based on CD104 (integrin β_4) transcript amounts (Itgb4 gene). The results are representative of three experiments.

(Fig. 5*C*), suggesting that NKG2D engagement, by itself, is not sufficient to trigger cytolysis by DETCs but may be sufficient to trigger lysis by NK cells. Similar results were obtained using the FcγR$^+$ P815 mouse mastocytoma cell line, which expresses a low amount of endogenous NKG2D ligands (data not shown). Taken together, these data suggested that NKG2D engagement was not sufficient for target cell lysis by DETCs and was not required for lysis when potent stimulation through the TCR was provided in the redirected lysis assay.

To extend these studies, Daudi cells were transduced with H60c and cells expressing relatively high levels were selected for analysis (Fig. 5*A*). H60c-transduced Daudi cells were no more sensitive to DETCs than untransduced Daudi cells (Fig. 5*D*), consistent with the redirected lysis experiments performed with NKG2D Ab. To determine whether TCR and NKG2D signaling activate DETC in a cooperative fashion, redirected lysis experiments were performed with cells that had been precoated with a limiting dose (4 ng/ml) of CD3ε Ab. This dose of Ab was insufficient to elevate lysis of untransduced Daudi cells over the level observed with no Ab, but resulted in substantial cytolysis when used in conjunction with H60c-transduced cells (Fig. 5*D*). These data demonstrated cooperative signaling by the TCR and NKG2D.

As another approach to this question, DETC degranulation and IL-2 production were assessed after stimulation of the cells with plate-bound Abs. Whereas stimulation of DETCs with high doses of immobilized CD3ε Ab resulted in massive degranulation, as shown by strong externalization of the CD107a protein that marks granule exocytosis, stimulation with high doses of NKG2D Ab did not by itself induce degranulation over the control level in any of the short-term DETC lines (Fig. 6*A*). Similarly, high doses of CD3ε Ab, but not NKG2D Ab, stimulated intracellular accumulation of IL-2 by DETCs (Fig. 6*A*). To determine whether NKG2D signaling is costimulatory, plates were coated with a limiting dose of CD3 Ab (1 μg/ml) and increasing doses of either NKG2D Ab or isotype control Ab. NKG2D Ab resulted in a substantial elevation of the degranulation response and IL-2 production over the response observed with limiting CD3ε Ab alone, whereas the isotype control Ab resulted in only a minimal elevation of the response (Fig. 6, *B* and *C*). Taken together, these experiments support the conclusion that NKG2D engagement is insufficient to induce degranulation or IL-2 production by DETCs, but strongly enhances both of these functional responses.

H60c mRNA is up-regulated during wounding

One of the key roles attributed to epidermal γδ T cells is the ability to accelerate wound healing by producing keratinocyte growth factors (1). In light of the evidence that H60c, the only ligand expressed by keratinocytes ex vivo, contributes to DETC activation, we asked whether H60c is induced during injury. B6 mice received full-thickness wounds, and the levels of H60c mRNA were analyzed from wounded skin 1–4 days after wounding. Strikingly, the amounts of H60c mRNA increased substantially as a result of wounding, with peak mRNA levels observed 1–2 days after wounding (Fig. 7). These results suggest a possible role for H60c in wound repair.

Discussion

The data reported herein document the cloning of H60c, a new ligand for the mouse NKG2D immunoreceptor, and its function in the activation of NK and epidermal γδ T cells. During the course of this work, the same gene was reported by another group (22). The AUG start codon identified in the published report did not, however, support cell surface expression of H60c in our hands (unpublished data). This finding does not contradict the published data, because Takada et al. (22) used a chimeric protein in which the H60c sequences were fused to a heterologous leader peptide (22). Our further analysis identified a distinct in-frame ATG codon 90 bases upstream in the H60c cDNA sequence, which is likely to represent the initiator codon. It is known that the first AUG codon in a mRNA is usually used for translation and, typically, such

translation inhibits the use of downstream AUG codons as start codons (45). Furthermore, the extended H60c cDNA sequence used in our expression vector encoded a longer signal sequence containing a track of 12 nonpolar residues flanked by charged residues. Most definitive was our finding that transduction of a construct containing this upstream sequence resulted in robust cell surface expression of H60c, while transfection of the shorter sequence did not. These considerations suggest that the published sequence directed the synthesis of an incomplete protein, lacking 30 aa at the N terminus.

For unknown reasons, evolution has equipped each individual with several distinct NKG2D ligands. Each cell could have the potential for expression of all of these ligands, providing extensive redundancy. Alternatively, or in addition, the various ligands may be regulated by distinct signals in specific cell types, providing tissue-specific protection from the infections or cancers that occur in that tissue. In support of the latter possibility, the data herein document that H60c is expressed specifically in the skin and is up-regulated at the mRNA and protein level in cultured keratinocytes. Furthermore, only H60c and not other NKG2D ligands were up-regulated in these primary cultures. The relative roles of H60c vs other NKG2D ligands in suppression of skin tumors in mice treated with carcinogens remains to be tested. Whereas H60c was expressed in several keratinocyte lines, including the PDV skin tumor cell line, H60c was not expressed in any of the other types of tumor cell lines tested, which represented diverse tissue types and expressed other NKG2D ligands, nor was H60c up-regulated in cultured fibroblasts (data not shown). These findings strongly suggest a specific role of H60c in keratinocytes. Engagement of NKG2D by H60c on cultured keratinocytes was essential for activation of skin resident $\gamma\delta$ T cells. Numerous studies show that cell culture imparts a form of stress called "culture shock" (46, 47). In some respects, culture shock mimics oncogene-induced stress as occurs in early tumorigenesis, although the specific events that induce H60c in cultured keratinocytes remain to be determined (46, 47). Interestingly, the human NKG2D ligand ULBP4 has also been reported to have a skin-specific expression pattern, although its role in skin immunity and the signals that regulate it are unknown (48). Whether other NKG2D ligands have specialized regulation remains to be established, but appears likely given the diverse patterns of mRNA expression observed in normal tissues documented in several reports (49, 50).

Cytolysis of keratinocytes expressing H60c by DETCs was dependent on NKG2D. This was demonstrated by using DETCs prepared from gene-targeted NKG2D-deficient mice and independently by the addition of an NKG2D mAb that is known to block ligand binding. By both methods, DETC lysis of keratinocytes was severely compromised, showing that NKG2D is an essential receptor for induction of cytotoxicity. The obligatory role of H60c in lysis of keratinocytes raised the possibility that engagement of NKG2D is sufficient to trigger DETCs. Indeed, a recent study reported that NKG2D ligation without TCR engagement triggers cytotoxicity and cytokine production in DETCs (40). We were, however, unable to demonstrate a sufficient role for NKG2D-mediated cytotoxicity or cytokine production. DETC effectors were unable to redirect lysis against two different FcγR$^+$ target cell lines (Daudi and P815) coated with NKG2D Ab. In addition, H60c-transduced Daudi cells were not lysed by DETCs. Furthermore, Ab-mediated cross-linking of NKG2D on DETCs was insufficient for degranulation and cytokine production. These results demonstrate unambiguously that NKG2D engagement, by itself, is not sufficient to trigger cytotoxicity or cytokine production by DETCs that are fully capable of lysing cultured keratinocytes. The basis for the discrepancy with the earlier report is not known, but one possibility is that the procedure used to generate DETC cultures in the previous report induced a promiscuous cytotoxicity program in the cells.

Multiple studies have suggested a costimulatory function of NKG2D for both mouse and human CD8$^+$ $\alpha\beta$ T cells and, in certain instances, NKG2D may be sufficient to trigger cytotoxicity in IL-15-primed CTLs, independent of TCR signaling. Whether engagement of the DETC TCR is required for keratinocyte lysis is still unclear. Redirected lysis of Daudi targets coated with CD3ε Ab revealed that signaling through the TCR complex was sufficient for degranulation, but this may reflect the strong signaling emanating from the TCR under these conditions. Consistent with this notion, when limiting doses of anti-CD3ε Abs were coated on plates, NKG2D Ab enhanced DETC activation. In addition, transduction of H60c dramatically enhanced redirected lysis of FcR$^+$ cells by Daudi cells when limiting doses of CD3ε Ab were used. In another attempt to address the role of the TCR in DETC activation, we tested TCR$\gamma\delta$ (GL3) Abs for their capacity to block DETC activation by cultured keratinocytes (data not shown). Although TCR$\gamma\delta$ Ab did not block the response, the experiment cannot be interpreted because it is not known whether the Ab can block binding of the DETC TCR to its putative ligand, which has not been identified.

Given the synergy between TCR and NKG2D engagement in activating DETCs and the demonstrated requirement for NKG2D in keratinocyte lysis, it appears likely that DETC activation normally requires engagement of both the TCR and NKG2D by ligands on target cells. Based on these considerations, the TCR signal resulting from recognition of the TCR ligand on cultured keratinocytes is probably too weak to trigger the DETCs by itself, leading to the requirement for NKG2D engagement. Although it has been proposed that up-regulation of the TCR ligand on keratinocytes is responsible for lysis of stressed keratinocytes, our data show that the up-regulation of H60c can also play a critical role in this process. Indeed, it is possible that normal keratinocytes constitutively express sufficient amounts of TCR ligand to support a strong DETC response when NKG2D ligands are induced by cell stress. This possibility is supported by the finding that experimental induction of transgene-encoded Rae1 led to a strong immune response in the skin in the absence of other known cell stresses (51). Conversely, our evidence that strong TCR engagement triggers DETCs in the absence of NKG2D suggests that strong up-regulation of the TCR ligand on keratinocytes may also be sufficient to trigger the cells. Hence, different forms of stress may independently stimulate responses by inducing H60c or the TCR ligand separately, providing multifunctionality. In addition to these signals, additional costimulatory receptor/ligand pairs also participate in the response and may exhibit additional forms of regulation.

In response to injury, epidermal $\gamma\delta$ T cells have been shown to promote wound healing (1). In light of the evidence that H60c is the only NKG2D ligand expressed by keratinocytes ex vivo and contributes to DETC activation, we asked whether H60c is induced during injury. A marked increase in H60c mRNA was detected within 1 day of wounding. These data raise the possibility that induction of DETC activity in wounded skin may be partly due to induction of H60c in keratinocytes as a result of wounding.

The identification of a cell stress-regulated NKG2D ligand expressed specifically in the skin and the evidence that NKG2D engagement is critical for costimulating DETC activation in response to cultured keratinocytes open new avenues to examine the role of specialized T cell subsets, as well as the NKG2D receptor, in immunity.

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Disclosures

The authors have no financial conflict of interest.

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