CD161 (NKR-P1A) Costimulation of CD1d-dependent Activation of Human T Cells Expressing Invariant V\(\alpha 24\)J\(\alpha Q\) T Cell Receptor \(\alpha\) Chains

By Mark Exley,*,§ Steven Porcelli,*,§ Margo Furman,*,§ Jorge Garcia,*,§ and Steven Balk*§

From the *D epartment of C ancer B iology, H ematology/O ncology, B eth Irael- D eaconess M edical C enter, B oston, M assachusetts 02215; the ‡ D ivision of R heumatology, I mmunology, and A llergy, B righam and W omen’s H ospital, B oston, M assachusetts 02215; and the § H arvard M edical School, B oston, M assachusetts 02115

Summary

A population of human T cells expressing an invariant V\(\alpha 24\)J\(\alpha Q\) T cell antigen receptor (TCR) \(\alpha\) chain and high levels of CD161 (NKR-P1A) appears to play an immunoregulatory role through production of both T helper (Th) type 1 and Th2 cytokines. Unlike other CD161\(^+\) T cells, the major histocompatibility complex–like nonpolymorphic CD1d molecule is the target for the TCR expressed by these T cells (V\(\alpha 24\)inv\(t\) T cells) and by the homologous murine NK1 (NKR-P1C)\(^+\) T cell population. In this report, CD161 was shown to act as a specific costimulatory molecule for TCR-mediated proliferation and cytokine secretion by V\(\alpha 24\)inv\(t\) T cells. However, in contrast to results in the mouse, ligation of CD161 in the absence of TCR stimulation did not result in V\(\alpha 24\)inv\(t\) T cell activation, and costimulation through CD161 did not cause polarization of the cytokine secretion pattern. CD161 monoclonal antibodies specifically inhibited V\(\alpha 24\)inv\(t\) T cell proliferation and cytokine secretion in response to CD1d\(^+\) target cells, demonstrating a physiological accessory molecule function for CD161. However, CD1d-restricted target cell lysis by activated V\(\alpha 24\)inv\(t\) T cells, which involved a granule-mediated exocytotic mechanism, was CD161-independent. In further contrast to the mouse, the signaling pathway involved in V\(\alpha 24\)inv\(t\) T cell costimulation through CD161 did not appear to involve stable association with tyrosine kinase p56\(Lck\). These results demonstrate a role for CD161 as a novel costimulatory molecule for TCR-mediated recognition of CD1d by human V\(\alpha 24\)inv\(t\) T cells.

Key words: CD1d • CD161 • costimulation • V\(\alpha 24\)J\(\alpha Q\) • T cells

\[^{1}\text{Abbreviations used in this paper: CHO, Chinese hamster ovary; DN, CD4/CD8 double negative; V\(\alpha 24\)inv, V\(\alpha 24\)J\(\alpha Q\) TCR-expressing.}\]
sential for all Th2 responses, since β2-microglobulin-deficient mice, which lack detectable NK1.1+ as well as most CD8+ T cell populations, can still mount such responses (23, 24). CD1d knockout mice, which similarly lack NK1+ T cells, are also able to generate model Th2 responses such as nonspecific production of IgE (25–27). Murine NK1+ T cells have also been shown to have NK-like cytotoxic activity (8, 9, 12, 28). This NK-like activity is induced by IL-12 (29) and appears to play a role in IL-12–mediated tumor rejection, a Th1-like cell-mediated response (30). Although the precise functions of human Vα24+ T cells remain to be defined, quantitative and qualitative defects in these T cells or the corresponding murine population are predictive of progression in certain human and murine autoimmune conditions (28, 31–35).

It has been established that NK locus-encoded C-type lectins can mediate NK cell activation, and that rodent NK1, but not human CD161, acts as an autonomous NK cell stimulatory structure (3, 4, 36–38). Direct stimulation of murine NK1+ T cells through NK1 rather than the TCR results in a cytokine switch to IFN-γ (13, 39, 40), suggesting that precisely how these cells are activated may contribute to determining the composition of the immune response. In this study, the role of the human NK1 homologue CD161 and other candidate accessory molecules in regulation of human DN Vα24+ T cell responses to CD1d was assessed. The results demonstrated that CD161 functions as a costimulatory receptor for CD1d recognition by Vα24+ T cells. However, in contrast to murine NK1+ T cells, ligation of human CD161 on Vα24+ T cells did not directly activate cytokine secretion, and CD161 costimulation did not result in the selective production of IFN-γ. Our results identify CD161 expressed by Vα24+ T cells as a costimulatory molecule for this unique T cell population.

Materials and Methods

T Cell Clones and Cell Lines. Vα24+ T cell clones were derived and phenotypic analysis was performed as described (17, 19). In brief, a panel of DN Vα24+ Vβ11+ human peripheral blood T cell clones was established by sequential negative magnetic bead (Dynal, Inc., Lake Success, NY) and positive FACScs sorting of human peripheral blood T cells followed by stimulation with PHA-P (Difco Laboratories Inc., Detroit, MI) and IL-2 (1.5 nM, equivalent to ~70 IU/ml; Ajinomoto, Yohkohama, Japan) in the presence of irradiated (5,000 rads) peripheral blood mononuclear cells. Vα24+ T cell clones were then established by limiting dilution. CD4+ Vα24+ TCR− Vα24+ Vβ11+ control T cells were established in a similar manner. Human CD1d-transfected Chinese hamster ovary (CHO) cells and human HLA-A,-B negative C1R B cells (41) were generated as described (19). The murine Vα14+ TCR+ CD1d-specific T-T hybridoma DN32.D3 (7) was provided by Dr. A. Bendelac (Princeton University, Princeton, NJ).

Antibodies. Antibodies used were anti-Vα24 (C15B2) and anti-Vβ11 (C21D2), both provided by Dr. R. A. Lanzavecchia (Institute for Immunology, Basel, Switzerland); anti-TCR αβ (BMA031; gift of Dr. R.G. Kurrie, Boehringer Werke, Marburg, Germany); anti-CD3 (SPV-T3b [provided by Dr. H. Spits, Netherlands Cancer Center, Amsterdam, Netherlands] and OKT3 [American Type Culture Collection, Rockville, MD]; anti-CD4 (OKT4; American Type Culture Collection); anti-CD8α (OKT8; American Type Culture Collection); anti-CD1β (25T8-5H7; provided by Dr. E. Reinherz, Dana-Farber Cancer Institute, Boston, MA); anti-CD28 (9.3 [gift of Dr. J. Hansen, Hutchinson Cancer Center, Seattle, WA] and CD28.2 [PharMingen, San Diego, CA]); anti-CD69 (FN50; PharMingen); anti-CD94 mAb (DX-22 [gift of Dr. L. Lanier, DNAX, Palo Alto, CA], HP-3D9 [PharMingen], HP-381 [Coulter Corp., Miami, FL], and IgA NK93 [provided by Drs. M. Robertson, Indiana University Medical Center, Indianapolis, IN, and J. Ritz, Dana-Farber Cancer Institute, Boston, MA]); anti-CD161 (DX-1 and DX12 [also provided by Dr. Lanier], HP-3G0L [provided by Dr. M. Lopez-Botet, Hospital de la Princesa, Madrid, Spain], and 191.88 [gift of Dr. A. Poggi, Instituto Nazionale per la Ricerca sul Cancro, Genoa, Italy]); anti-p40 (NKTA255; provided by Dr. A. Poggi); p38 (C1.7; Coulter Corp.), Fifth Leukocyte Workshop, NK Section, mAb against killer inhibitory receptors p58 (GL1B3, EB6, CH-L, and HP-3E4) and p70 (DX-9; provided by Dr. Lanier); anti-MHC class I (W6/32; American Type Culture Collection), anti-CD1b (4A7.6.5, IgG2a; gift of Dr. D. Olive, Institut National de la Sante et de la Recherche Medicale, Marseille, France), and isotype control mAbs (P3, IgG2a; MPC-11, IgG2b; American Type Culture Collection); rat anti-murine NK1.1 (PK136; PharMingen); and normal mouse and rat sera. p56Lck was detected with a mixture of antibodies (#42 rabbit serum [provided by Drs. B. Krise and J. Rose, Yale University, New Haven, CT] and 3A5 mAb [Santa Cruz Biotechnology, Inc., Santa Cruz, CA]), p56Lck-specific mAbs were raised using CD1d-IgG fusion protein as immunogen (reference 19, and S. Porcelli and S. Bak, unpublished). CD1d mAbs were purified from culture supernatants of hybridomas grown in medium supplemented with ultra-low IgG fetal bovine serum (Hyclone, Logan, UT) by protein G (Amersham Pharma- cia Biotech, Inc., Piscataway, NJ) chromatography. Fluorescein-conjugated goat anti-murine IgG antibody was obtained from DAKO Corp. (Carpinteria, CA) and Biosource International (Camarillo, CA).

Functional Analysis of T Cells. For activation of T cells (105/well), anti-CD3 mAb OKT3 was bound overnight in PBS (50 μg/well) to 96-well flat-bottomed tissue culture plates, and unbound antibody was washed off. Coating mAb concentrations were 1 μg/ml OKT3 for subsequent incubations with no PMA and 0.1 μg/ml for incubations with PMA (Sigma Chemical Co., St. Louis, MO) at 1 ng/ml, unless otherwise indicated. Plate-bound (50 μg/well) or soluble costimulatory mAbs at 10 μg/ml or indicated concentrations were then added for at least 4 h. Subsequently, rested T cells at 2–4 wk after PHA stimulation were incubated with plate-bound mAb and IL-2 at 0.3 nM. In the case of soluble mAbs, an equal amount of cross-linking anti-murine IgG antibody was added after the T cells had been allowed to settle on the limiting plate-bound anti-CD3 mAb. For CD1d responses, equal numbers of CD1d+ human C1R B cell transfectants or control mock-transfected C1R cells were incubated with the rested T cells, PMA (1 ng/ml unless otherwise stated), and IL-2 at 0.3 nM, as described previously (19).

R eleased cytokine levels at 48 h were determined in triplicate by ELISA with matched antibody pairs in relation to cytokine standards (PharMingen; Endogen, Inc., Cambridge, MA) and converted to nanograms or picograms per milliliter using the Softmax program (Molecular Devices Corp., Sunnyvale, CA).
Similarly, T cell proliferation between 48 and 72 h was determined by [3H]thymidine incorporation (1 μCi/well), using target cells pretreated with mitomycin C (0.09 mg/ml) for 1 h. Results shown are with SEM.

Cytolytic activity of Vα24inv T cells was assessed by conventional 51Cr-release assays as described previously (42, 43). The assay was performed during the T cell growth phase 7–14 d after PHA stimulation. Spontaneous, specific, and total (Triton X-100) 51Cr released at 4 h were measured.

Assessment of Protein Interactions of NK Locus Molecules. Interaction of membrane and cytosolic proteins was assayed as described previously (44). In brief, Vα24inv T cell clone DN2.B9 or the murine Vα14inv TCR + CD1d-reactive T-T hybridoma DN 32.D3 was lysed with 1% Triton X-100 in Tritis-buffered saline with protease inhibitors. Specific and associated proteins were precipitated with Con A agarose beads (Amersham Pharmacia Biotech, Inc.) or antibodies prebound to protein A/G bead mixture (Pierce Chemical Co., Rockford, IL). After washing, bound material was eluted under nonreducing conditions for C-type lectin Western blotting. Protein analyzed by SDS-PAGE was blotted onto nitrocellulose (Schleicher & Schuell, Keene, NH) and probed with antibodies. mAb H-3G10 reacted in Western blotting with nonreduced CD161 (80-kD band). No activity was detected with the reduced antigen or with other CD161 mAbs in Western blots. p56Lck was immunoblotted with #42 serum and 3A5, followed by second antibody–peroxidase conjugates (Promega Corp., Madison, WI) and chemiluminescence detection (Amersham Pharmacia Biotech, Inc.).

**Results**

Potent Costimulation of Vα24inv T Cells by CD161 mAbs. Human Vα24inv T cells express high levels of CD161 and variable levels of other members of the NK locus C-type lectin family (14–20). FACS® profiles of two representative Vα24inv T cell clones, DN2.D5 and DN1.10B3, are shown in Fig. 1. CD161 (NKR-P1A) was strongly expressed by these two clones derived from two different donors (Fig. 1), as well as by all of six additional CD1d-reactive Vα24inv T cell clones (19). CD69, another C-type lectin encoded in the NK locus, was also expressed by all of the Vα24inv T cell clones (Fig. 1; reference 19). Although transiently expressed after activation of conventional T cells, CD69 showed prolonged expression on Vα24inv T cell clones for at least several months after PHA stimulation (data not shown). CD94, a third NK locus-encoded C-type lectin, was expressed by seven out of eight Vα24inv T cell clones (not DN1.10B3; Fig. 1). Analysis of other potential Vα24inv T cell accessory molecules showed that p40 (45) and p38 C.1.7 proteins (46), both previously found on NK cells and some cytolytic T cells, were expressed by some Vα24inv T cell clones. However, these molecules were also found on several control Vα24+ noninvariant cells and other T cell clones not belonging to this subset (data not shown). Vα24inv T cells had variable expression of CD28, from barely detectable on some clones to levels comparable to conventional T cells (Fig. 1, and data not shown). Finally, as shown previously, the Vα24inv T cells did not express the NK cell–associated p58 or p70 killer inhibitory receptors or the other NK cell markers CD16, CD56, and CD57 (19). Thus, established CD1d-reactive Vα24inv T cell clones were consistently CD161+CD69+, with more variable expression of other candidate accessory molecules.

Ligation of murine NK1 (NKR-P1C) alone activates murine NK1+ T cells and, in contrast to TCR stimulation, results in an exclusively IFN-γ–secreting Th1-biased phenotype (13, 39, 40). Therefore, we examined the effect of direct ligation of CD161 on stimulation and costimulation of human Vα24inv T cells. Proliferative responses were measured in the presence of appropriate suboptimal concentrations of immobilized CD3 mAb. Proliferation of all Vα24inv T cell clones tested (DN2.D5, DN2.D6, DN2.D7, and DN1.10B3) and the Vα24inv T cell line DN2.Vb11+ was substantially augmented by CD161 mAbs 191.B8 (47) and DX-1 (36) in the absence of PMA (Fig. 2 A, and data not shown). With the addition of phorbol ester, which is required for activation of these cells by CD1d+ targets in vitro (19), similar costimulation by CD161 ligation was also observed (Fig. 2 B). PMA lowered the concentrations of CD3 mAb required ~10-fold (Fig. 2, A and B). Under both conditions, costimulation of Vα24inv T cells by CD161 was readily seen over a 25-fold range of anti-CD3 mAb concentrations (Fig. 2, A and B). Optimal costimulation via plate-bound CD161 required >1 μg/ml 191.B8 coating mAb and was not seen with soluble 191.B8, even at up to 10 μg/ml in the presence of a soluble anti-linking secondary antibody (Fig. 2 C). In no experiment was pro-

![Figure 1](image_url)
liferation by human Vα24inv T cell clones observed in response to plate-bound CD161 mAb in the absence of CD3 mAb (Fig. 2, A, B, and D; Table 1; and data not shown). Similar lack of direct stimulation was observed using two different CD161 mAbs (DX-1 and 191.B8) at concentrations up to 20 μg/ml (Fig. 2, Table 1, and data not shown).

As with proliferative responses, and unlike in the mouse, there was no IL-4 or IFN-γ secretion by human Vα24inv T cell clones in response to plate-bound CD161 mAb in the absence of CD3 mAb, either in the presence or absence of PMA (Fig. 2 D, Table 1, and data not shown). However, both IL-4 and IFN-γ production by Vα24inv T cell clones induced by limiting anti-CD3 mAb were substantially augmented by CD161 mAbs 191.B8 and DX-1 in both the presence and absence of PMA (Table 1, and data not shown). Antibody-mediated CD161 ligation did not alter the pattern of cytokines produced by suboptimal TCR stimulation. IL-4 to IFN-γ secretion ratios between the CD161-costimulated and CD1d-specific responses varied by only approximately threefold, and indicated that there was no polarization of cytokine secretion toward IFN-γ production induced by CD161 costimulation of the human Vα24inv T cells (Table 1).

Similarly to CD161 mAb, CD94 mAb HP-3D9 also produced significant costimulation of Vα24inv T cell proliferation (Fig. 2, A and B) and IFN-γ and IL-4 secretion.

| Stimulus | Proliferation | IFN-γ | IL-4 | IL-4/IFN-γ |
|----------|---------------|-------|------|-----------|
| None     | 8,951         | <100  | <100 | —         |
| CD3      | 35,077        | 3,320 | <100 | —         |
| CD161    | 6,930         | <100  | <100 | —         |
| CD3/CD161| 89,525        | 10,670| 734  | 0.069     |
| CD94     | 8,199         | <100  | <100 | —         |
| CD3/CD94 | 73,020        | 8,963 | 497  | 0.055     |
| CD161/CD94| 9,824        | <100  | <100 | —         |
| CD3/CD161/CD94| 93,072 | 11,250| 626  | 0.056     |
| C1R CD1d | 27,133        | 41,080| 916  | 0.022     |

CD161+ Vα24inv T cell clone DN2.D6 (10⁶ cells/well) was stimulated with limiting quantities of plate-bound CD3 mAb (0.1 μg/ml; 1 ng/ml PMA) and plate-bound (10 μg/ml) accessory mAb (CD161 191.B8 or CD94 HP-3D9) as in Figs. 2 and 3. In the same experiment, additional DN2.D6 cells were stimulated with live CD1d+ C1R cell transfectants (10⁷/well; 1 ng/ml PMA) as in Fig. 4. Results shown are representative of three independent experiments where both plate-bound and CD1d responses were measured in parallel. [3H]Thymidine incorporation was determined in triplicate at 72 h, shown as cpm. IL-4 and IFN-γ cytokine detection limits were <100 pg/ml. —, Cytokine ratios not calculable due to undetermined cytokine levels below detection limits.
also costimulatory for Vα24Invt T cell proliferation or cytokine secretion through CD94 alone or in combination with CD161, using several different antibodies (Fig. 2, A, B, and D; Table 1; and data not shown). In no case was synergistic or even additive costimulation of proliferation by CD161 and CD94 mAbs seen (Fig. 2 D, and Table 1), and in no case was significant alteration of cytokine secretion observed with simultaneous addition of both mAbs (Table 1). This was true even at lower levels of CD3 mAb and suboptimal levels of costimulation of proliferation by more recently activated Vα24Invt T cells (Fig. 2 E). Anti-CD28 mAb (CD28.2), which potently costimulated control conventional T cell clones (not shown), showed only weak costimulation of the proliferation and cytokine secretion of the CD28− Vα24Invt T cell clones, and only in the absence of PMA (Fig. 2, A and B). Anti-p40 mAb NKTA255 was also costimulatory for Vα24Invt T cells, but only in the absence of PMA (not shown). CD69 mAb (Fig. 2, A and B), p38 C1.7 mAb (not shown), HLA class I mAb, and isotype-matched nonbinding control mAb had no costimulatory or direct stimulatory activity (Fig. 2, A and B, and data not shown). Therefore, human Vα24Invt CD1d-reactive T cells differed from their murine counterparts in lack of direct activation in response to CD161, or indeed, other C-type lectin ligation, whereas both CD161 and CD94 mAbs were specifically costimulatory.

Role of CD161 in CD1d-dependent Activation of Vα24Invt T Cells. Since CD1d is a natural ligand of Vα24Invt T cells, it was important to determine whether CD161 or other molecules contributed to T cell activation in response to CD1d+ target cells. Vα24Invt T cells were incubated with CD1d transfectants, and proliferation and cytokine responses were measured. Recognition of CD1d+ human B cell transfectants by Vα24Invt T cells, measured as proliferation, or IFN-γ or IL-4 cytokine secretion, was inhibited by CD1d mAbs 51.1 (Fig. 3, A–C) and 42.1 (not shown). Proliferation in response to CD1d was comparably inhibited with CD1d mAb DX-1 (Fig. 3 A). Similarly, secretion of both IFN-γ and IL-4 was inhibited by CD1d mAb DX-1 (Fig. 3, B and C). Each of three different CD1d mAbs tested inhibited proliferative and cytokine secretion responses to CD1d recognition, with HP-3G10 consistently the most potent, followed by 191.B8, and then DX-1 (Fig. 3, D and E, and data not shown). Inhibition of proliferation and cytokine secretion in response to CD1d by CD161 mAb was seen over a wide range of PMA concentrations (0.05–5 ng/ml; Fig. 3, and data not shown) and not just under suboptimal conditions (<1 ng/ml PMA).

In contrast to these results with CD161 mAb, the costimulatory CD94 mAb HP-3D9 did not inhibit T cell proliferation or cytokine secretion in response to CD1d (Fig. 3, A–C). Other mAbs specific for CD94 (DX-22 and HP-3B1), CD69 (FN50), p38 (C1.7), and the weakly costimulatory p40 mAb (NKTA255) had no consistent inhibitory effect on CD1d-dependent T cell proliferation or cytokine secretion (not shown). The HLA class I control mAb (W6/32) had a small inhibitory effect (Fig. 3, D and E).

Figure 3. CD1d and CD161 mAbs specifically inhibit Vα24Invt T cell response to CD1d. Vα24Invt T cells (10^5/well) were stimulated with mitomycin C-treated CD1d+ C1R cell transfectants (10^5/well) and 1 ng/ml PMA. Control, CD1d-specific, and other mAbs were included in incubations at 10 μg/ml. Representative results from five independent experiments are shown. (A) DN2.D6 T cell proliferation (cpm) was determined in triplicate at 72 h. (B) IL-4 cytokine ELISA was determined at 48 h from the same experiment as in A. (C) IL-4 determined as for IFN-γ. Control mock C1R-containing wells had background proliferation of 6,000 cpm, 6.4 ng/ml IFN-γ, and 2.4 ng/ml IL-4. In a further experiment with two Vα24Invt T cell clones, three different CD1d mAbs were used. (D) DN2.D6. (E) DN2.D5.
which was also seen using CD1d1 CHO cells as targets (not shown). Since the class I mAb does not bind to cells of hamster origin, this result appears to reflect mAb binding directly to the T cells in the assay.

Involvement of molecules other than CD1d on the target cell and CD161 on the T cell was tested by incubation with mAbs against various molecules preferentially expressed on resting and activated B and/or T cells. mAbs against CD19, CD20, CD22, CD23, CD24, CD25, or CD28 did not affect activation of invariant TCR + T cells by CD1d1 B cells, whether measured as proliferation, or IFN-γ or IL-4 secretion (not shown). Consistent with lack of effect of CD28 mAb, CTL-associated antigen 4 (CTLA4)-Ig fusion protein, which blocks both B7-1 and B7-2 costimulation, had no significant effect on CD1d-dependent T cell stimulation (S.B. Wilson, personal communication). Therefore, of the molecules studied, only CD1d itself and CD161 were found to contribute to Vα24 Inv T cell responses to CD1d1 target cells.

Lack of CD161 dependence of CD1d-specific cytolytic activity by Vα24 Inv T Cells. Recently activated Vα24 Inv T cell clones displayed potent and specific cytolytic activity against C1R CD1d1 transfectants (Fig. 4). Vα24 Inv T cell clones induced 20–70% of maximal stable release from CD1d1 C1R cells at E/T ratios of 10:1 (Fig. 4A, and data not shown). Cytotoxicity of the same T cell clones against C1R mock transfectants was <10% at these E/T ratios, demonstrating CD1d1 specificity of cytolytic activity. As seen for proliferative and cytokine secretory responses above, the CD1d1-specific cytolytic effector response of the T cell clones was inhibited in a dose-dependent manner by CD1d1-specific mAbs 42.1 and 51.1. These CD1d1 mAbs had an IC50 of ∼1 μg/ml (Fig. 4A) and could reduce cytolytic to nearly background levels at higher concentrations of mAb (Fig. 4A and B). This confirmed that cytolytic activity, like proliferation and cytokine secretion, was a response to the intact CD1d molecule. The cytolytic activity of Vα24 Inv T cells was abolished by EGTA, indicating a Fas-independent mechanism requiring release of cytolytic granules (Fig. 4B).

To determine the role of CD161 in cytolytic activity, CD161 mAbs were also included. No effects of any of the three CD161 mAbs on CD1d-specific cytolytic activity were seen at up to 10 μg/ml. This was true even when a limiting amount of CD1d1 mAb (0.08 μg/ml) was included to amplify any inhibition (Fig. 4B), after preliminary experiments showed no inhibition by CD161 alone. Cytolytic responses were also PMA-independent. These results demonstrated that stimulatory pathways activated by CD161 ligation and PMA were not required for CD1d-specific cytolytic activity of Vα24 Inv T cells. These observations parallel conventional CTLs, for which stimulatory molecules such as CD28 are not required to induce cytolytic activity by recently activated T cells.

Lack of Association of Vα24 Inv T Cell p56Lck and Human CD161. Because human Vα24 Inv T cells lack CD4 and CD8αβ, which are essential for physiological activation of conventional T cells through p56Lck, an association between Vα24 Inv T cell p56Lck and certain accessory molecules might be expected. Association between murine NK1 and p56Lck has been described (48), but human CD161 (36) does not contain the cytoplasmic tail p56Lck binding motif found in CD4 and CD8 (49) and in all of the murine NKR-P1 molecules (1) (see Fig. 5A). Therefore, we directly tested for

**Figure 5.** Association of p56Lck with murine NK1, but not human Vα24 Inv T cell CD161. (A) Comparison of human (reference 36) and murine NKR-P1 (references 1 and 2) amino acid sequences around the functional p56Lck binding motif (reference 47) found in murine NKR-P1 (Fig. B) p56Lck immunoblot of nonreduced murine p56Lck, NK1.1, and control immunoprecipitations from Vα24 Inv T-T hybridoma DN32.D3. (C) p56Lck immunoblot of CD3, CD161, and Con A precipitations from Vα24 Inv T cell clone DN2.89. (D) CD161 (HP-3G10) immunoblot of nonreduced p56Lck, CD161 (DX-1), or control mAb (Cont.) immunoprecipitations from Vα24 Inv T cell clone DN2.89.
interaction of CD161 with V\(\alpha 24\)neg T cell p56Lck by immunoprecipitation and subsequent Western blotting.

In preliminary experiments, it was confirmed that murine NK1.1\(^+\) T cell hybridoma DNY32.D3 (7) did show association of CD161 with p56Lck (Fig. 5 B). Human p56Lck was also expressed by DN V\(\alpha 24\)neg T cell, and Con A precipitation of Triton X-100 lysates followed by Western blot showed that p56Lck was constitutively associated with glycoprotein(s) (Fig. 5 C). However, CD161 immunoprecipitated did not contain detectable p56Lck (Fig. 5 C). Furthermore, in the reciprocal experiment in which Triton X-100 lysates were immunoprecipitated with p56Lck antibody and immunoblotted with CD161 mAb, there was also no detectable association of CD161 with p56Lck (Fig. 5 D). We conclude that p56Lck was not stably associated with CD161 in V\(\alpha 24\)neg T cells. Taken together, the results presented support the model that human CD161 functions as a novel costimulatory molecule for human V\(\alpha 24\)neg T cells.

Discussion

CD161- V\(\alpha 24\)neg T cells are likely to play an important immunoregulatory role (28, 31–35), presumably through interactions with CD1d\(^+\) target cells (19). However, it is unclear how activation and effector functions of this T cell population in response to CD1d recognition are regulated. By analogy with conventional MHC-restricted T cells, it appears likely that activation of V\(\alpha 24\)neg T cells is regulated by the engagement of accessory molecules on the T cell surface by specific ligands expressed by appropriate target cells. In the absence of CD4 and CD8\(\alpha\) and with highly variable levels of CD28, therefore, CD161 and other related molecules were investigated as potential costimulatory or accessory molecules for V\(\alpha 24\)neg T cells. The results reported here indicate that CD161–ligand interactions positively regulate CD161- V\(\alpha 24\)neg T cell activation.

CD161, the single known human NKR-P1 molecule, which was first characterized on NK cells and some T cell populations (36), is expressed at high levels by V\(\alpha 24\)neg T cells and some T cell populations (37). It was shown that p56Lck was constitutively associated with glycoprotein(s) (Fig. 5 C). However, CD161 immunoprecipitated did not contain detectable p56Lck (Fig. 5 C). Furthermore, in the reciprocal experiment in which Triton X-100 lysates were immunoprecipitated with p56Lck antibody and immunoblotted with CD161 mAb, there was also no detectable association of CD161 with p56Lck (Fig. 5 D). We conclude that p56Lck was not stably associated with CD161 in V\(\alpha 24\)neg T cells. Taken together, the results presented support the model that human CD161 functions as a novel costimulatory molecule for human V\(\alpha 24\)neg T cells.

The primary effector function associated with V\(\alpha 24\)neg T cells has been production of Th1 and Th2 cytokines. Murine hepatic NK1.1\(^+\) T cells have also been shown to have NK-like cytolytic activity (29), but whether they can mediate CD1d-restricted cytolyis has not been determined. Furthermore, murine NK1.1\(^+\) T cells directly mediate antitumor effects through a cytotoxic mechanism that appears to be CD1d-independent (32). This report demonstrates that an additional effector function for human V\(\alpha 24\)neg T cells is direct CD1d-restricted cytosis. The major mechanism of this effector activity appears to be cytolytic granule release, based on Ca\(^{2+}\) dependence. Significantly, this activity was PMA-independent and was not affected by CD161 mAb. These results likely reflect the less stringent requirements for triggering of the cytolytic effector func-
tion of activated T cells than for full activation of resting cell. The inability of CD161 mAb to block cytolytic activity provides evidence against CD161 functioning as a coreceptor for CD1d recognition, analogous to the role of CD4 and CD8, since CD8 mAb routinely inhibits conventional cytolytic T cells. In contrast, this suggests a parallel with other costimulatory molecules such as CD28, which are not required for cytotoxic T cell lysis of target cells. Alternatively, the Vα24inv TCR could have very high affinity for CD1d, which can eliminate the need for coreceptor ligand for cytolysis, as has been described for some CD8-independent cytolytic T cells (52).

To further assess how CD161 contributes to activation of Vα24inv T cells, association between CD161 and p56ck was assessed. Rodent NK R-P1C (NK1) is directly stimulatory for both NK cells and NK1+ T cells (3, 13, 39, 40) and can associate with p56ck via a cytoplasmic tail motif CCXP/S/T (47), as used by CD4 and CD8 (48). However, human CD161 does not contain this motif, and mAbs against this molecule do not directly activate nor do they block classical human NK cell cytolysis (36). Human Vα24inv T cell CD161 did not detectably associate with p56ck using detergent conditions (1% Triton X-100), which readily confirmed the murine NK1.1-p56ck interaction. Based on lack of association of human CD161 with Vα24inv T cell p56ck, and by functional analogy with the classical costimulatory molecule CD28, we propose that human CD161 ligation results in activation of another signaling molecule. Interestingly, the response of Vα24inv T cells to CD1d transfectants in vitro is PMA A-dependent, and CD161 can still provide a costimulatory signal in the presence of PMA, suggesting that the CD161 costimulatory signal does not depend solely on classical protein kinase C molecules. Murine NK1 also associates with the FcR γ chain in both NK cells and NK1+ T cells (40), providing an alternate mechanism for recruitment of signal transducing complexes. Further characterization of CD161-associated signal-transducing molecules should provide a molecular mechanism for the involvement of CD161 in positive regulation of human Vα24inv T cell responses to CD1d.

The blocking of Vα24inv T cell responses to CD1d+ target cells by CD161 antibodies indicates that these target cells and their physiological CD1d+ counterparts in vivo can express CD161 ligand(s). Although CD161 is a member of the C-type lectin superfamily, it is not clear that carbohydrate alone can be the ligand. As discussed above, one possible CD161 ligand is CD1d itself. In this model, CD161 contributes to CD1d recognition directly as a coreceptor (8), as CD4 and CD8 bind MHC class II and I molecules, respectively. An alternative suggested above is that human CD161 acts like CD28 and binds a true costimulatory ligand on physiological CD1d+ target cells. CD1d on CHO cells is insufficient to activate Vα24inv T cells without mild glutaraldehyde fixation (19), which has been found in other systems to artificially substitute for costimulatory signals (51, 53). Similarly, fixation markedly increases Vα24inv T cell response to CD1d+ Hela transfectants, but not to the B lymphoblastoid cells used in this study (M. Exley, unpublished observations). Therefore, such a CD161 costimulatory ligand may only be expressed by certain cell types.

In summary, we have found that human CD161 functions not as a direct stimulatory structure but as a costimulatory molecule for human Vα24inv T cell responses to their physiological ligand, CD1d. Activation of resting CD161+ Vα24inv T cells via the TCR in combination with signals mediated by CD161 ligation led to proliferation, and both Th1- and Th2-type cytokine secretion. However, the potent granule-mediated CD1d-restricted cytotoxic activity of preactivated Vα24inv T cells was CD161-independent. The costimulation of CD1d recognition through CD161 appears to reflect a different mechanism for activation of human Vα24inv T cells compared with rodent NK cells and NK1+ T cells, both of which NK-P1 is directly stimulatory and associated with p56ck (3, 4, 37, 38, 48).
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