Requirements for CD1d Recognition by Human Invariant Vα24+ CD4−CD8− T Cells

By Mark Exley,* Jorge Garcia,* Steven P. Balk,* and Steven Porcelli‡

*From the *Cancer Biology Program, Hematology/Oncology Division, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215; and ‡Lymphocyte Biology Section, Division of Rheumatology, Immunology, and Allergy, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115*

Summary

A subset of human CD4−CD8− T cells that expresses an invariant Vα24-JαQ T cell receptor (TCR)-α chain, paired predominantly with Vβ11, has been identified. A series of these Vα24 Vβ11 clones were shown to have TCR-β CDR3 diversity and express the natural killer (NK) locus–encoded C-type lectins NKR-P1A, CD94, and CD69. However, in contrast to NK cells, they did not express killer inhibitory receptors, CD16, CD56, or CD57. All invariant Vα24+ clones recognized the MHC class I–like CD16 molecule and discriminated between CD1d and other closely related human CD1 proteins, indicating that recognition was TCR-mediated. Recognition was not dependent upon an endosomal targeting motif in the cytoplasmic tail of CD1d. Upon activation by anti-CD3 or CD1d, the clones produced both Th1 and Th2 cytokines. These results demonstrate that human invariant Vα24+ CD4−CD8− T cells, and presumably the homologous murine NK1+ T cell population, are CD1d reactive and functionally distinct from NK cells. The conservation of this cell population and of the CD1d ligand across species indicates an important immunological function.

The CD1 locus encodes a family of conserved nonpolymorphic proteins structurally related to MHC class I and II proteins (1–5). Human and murine CD1-restricted T cell lines and clones that recognize lipid antigens (6–8) or hydrophobic peptide antigens (9) have been identified, indicating that CD1 proteins can function as specialized antigen-presenting molecules. A distinct function for murine CD1d appears to be as a ligand or antigen-presenting molecule recognized by a population of T cells that express the NKR-P1C (NK1) cell surface C-type lectin (10–14). NK1 is otherwise restricted to NK cells and these NK1+ T cells have also been referred to as NK T cells or natural T cells (15, 16). Phenotypically, these cells are either CD4+CD8− or CD4−CD8− (double negative, DN[1]), and forced expression of CD8 in transgenic mice results in the deletion of this population (12, 17). Most strikingly, the majority of these cells use an invariant TCR-α chain (Vα14-Jα281) that pairs preferentially with Vβ8, 7 or 2 (17–20). NK1+ T cells appear to play a role in regulating immune responses, based upon their ability to rapidly produce large amounts of IL-4 after stimulation with anti-CD3 in vivo (21–24). However, these cells also produce large amounts of IFN-γ,

and production of this cytokine can be specifically induced by stimulation through NK1 (25). The immunological functions of these cells and the physiologically relevant CD1d-presenting cells mediating their activation remain to be determined.

Analyses of the CD1 genes in humans and other species indicate that the proteins fall into two groups, CD1a-, b-, and c-like (group 1), and CD1d-like (group 2) (3, 26). The murine CD1 locus appears unique in that it has deleted the group 1 genes, and contains only a duplicated group 2 gene (27, 28). This observation suggests that there may be important functional differences between the CD1 proteins in mice and other species. Nonetheless, a human invariant TCR-α chain closely related to the murine invariant Vα14-Jα281 TCR has been identified as a predominant TCR used by TCR-α/β DN T cells from multiple normal donors (29). This human invariant TCR-α is generated by a rearrangement between Vα24 (TCRAV24) and JαQ with no N-region diversity. Subsequent studies have shown that the human invariant Vα24-JαQ TCR-α chain associates preferentially with Vβ11 (TCRBV11) (30–32), which is homologous to murine Vβ8. This invariant TCR may also be expressed by a small proportion of CD4+ T cells, but not CD8+ T cells (31). These observations suggest that human invariant Vα24+ T cells are homologous to murine NK1+ T cells.

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[1]*Abbreviations used in this paper:* CHO, Chinese hamster ovary; DN, double negative; KIR, killer cell inhibitory receptor; RT, reverse transcriptase.

To better understand the function of these cells and their requirements for specific activation, a series of human invariant Vα24+Vβ11+ DN T cell clones were established and characterized. TCR-β sequence analysis demonstrated that these cells were derived from a polyclonal population with no evidence of a shared β chain CDR3 motif. Phenotypically, the clones expressed high levels of NKR-P1A, the only known human homologue of rodent NK1 (33). They also expressed CD94 and CD69, two other C-type lectins closely linked to NKR-P1A in a chromosomal region referred to as the NK locus. However, these cells did not express the NK cell-associated p58 or p70 HLA class I killer cell inhibitory receptors (KIRs; 34, 35) or other markers of NK cells including CD16, CD56, or CD57. Upon stimulation, the clones secreted cytokines associated with both Th1 and Th2 cells, including IFN-γ and IL-4, respectively. Of the four characterized human CD1 proteins, each clone specifically recognized only CD1d expressed on human or hamster cell transfectants. CD1d recognition was not dependent upon the specific TCR-β chain CDR3 sequence. Moreover, deletion of an endosomal targeting sequence motif in the cytoplasmic tail of CD1d (4, 5, 36) did not effect recognition, suggesting that recognition by these cells was not dependent upon efficient targeting of CD1d to a specialized endosomal compartment involved in antigen processing. These results demonstrate that human invariant Vα24+Vβ11+ DN T cells are a specialized population of CD1d-specific T cells. The results also indicate that the similarities between this T cell population, and presumably the homologous murine NK1+ T cell population, to NK cells may be limited to the expression of certain closely linked NK locus–encoded C-type lectins.

Materials and Methods

Cell Lines and Clones. T cell lines and clones were derived and phenotypic analyses performed essentially as described (32). In brief, DN Vα24+Vβ11+ human peripheral blood T cell lines and clones were established by sequential negative (CD4CD8) and positive (Vα24Vβ11) magnetic bead and FACS® sorting, respectively, of human peripheral blood T cells followed by stimulation with PHA-P (reconstituted according to the supplier's instructions and used at a final dilution of 1:2,000; Difco, Detroit, MI) and IL-2 (1.5 nM; Ajinomoto, Yokohama, Japan) in the presence of irradiated (5,000 rads) peripheral blood mononuclear cell feeders. Clones were established by limiting dilution. Clones and lines were maintained by restimulation every 3–6 wk as above with phenotype monitored by FACS®. A human IL-2–dependent NK cell line, NKL, (37) was provided by Drs. M. Robertson (Indiana University Medical Center, Indianapolis, IN) and J. Ritz (Dana Farber Cancer Institute, Boston, MA).

Antibodies and Phenotypic Analyses of T Cells. The following antibodies were obtained from the fifth Leukocyte Workshop unless otherwise indicated: anti-Vα24 (C15B2) and anti-Vβ11 (C21D2) (provided by Dr. A. Lanzavecchia [Basel Institute for Immunology, Basel, Switzerland]); anti–TCR-α/β (BMA031; provided by Dr. R.G. Kurrle, Boehringwerke, Marburg, Germany); anti-CD3 (SPV-T3b and OKT3; provided by Dr. H. Spits [Netherlands Cancer Center, Amsterdam, Netherlands] and American Type Culture Collection [Rockville, MD], respectively); anti-CD4 (OKT4; American Type Culture Collection); anti-CD8α (OKT8; American Type Culture Collection); anti-CD8β (2ST8.5H7; from Dr. E. Reinherz [Dana Farber Cancer Institute]); anti-CD16 (ABA2B1); anti-CD28 (9.3); anti-CD56 (MEM188, MOC-1, 0218); anti-CD57 (TB01, TB02); anti-CD69 (PharMingen, San Diego, CA); anti-CD94 (HP-3D9; PharMingen); anti-NKR-P1A (DX1 and DX12, provided by Dr. L. Lanier (DNAX, Palo Alto, CA); HP-3G10 and 191B8); anti-p58 KIR (NK workshop mAbs GL183, EB6, CH-L, and HP-3E4); and anti-p70 KIR (DX9; provided by Dr. L. Lanier). Isotype control mAbs were P3 (IgG₁), 4A7.6 (IgG2a, provided by Dr. D. Olive [Institut National de la Sante et de la Recherche Medicale, Marseille, France]), and MPC11 (IgG2b).

CD1d-specific mAbs were raised from mice immunized with CD1d–IgG fusion proteins. Further characterization of these CD1d proteins and antibodies will be reported (Balk, S., and S. Porcelli, manuscript in preparation). CD1d mAbs were purified from low IgG serum (GIBCO BRL, Gaithersburg, MD) containing tissue culture medium by protein G (Pharmacia, Piscataway, NJ) chromatography. FACS® analysis was by indirect immunofluorescence as described (32). Positive controls included an NK cell line as above and other cell lines and clones derived from peripheral blood. TCR transcripts were amplified by reverse transcriptase (RT)-PCR using variable and constant region-specific primers as described previously (29, 38). Sequences were determined directly from the PCR products on an automated DNA sequencer (ABI 373A).

CD1 Transfectants. Chinese hamster ovary (CHO) and C1R cells were transfected with a CD1d cDNA (3) in the pSRα-neo expression vector (39), followed by G418 selection and FACS® to generate lines stably expressing CD1d. C1R cells stably transfected with CD1a, b, and c in the pSRα-neo vector were described previously (38). The CD1d-CD1a chimeric protein was generated using an Xba1 restriction site located at the 3′ end of the α3 domain in CD1a and CD1d. The C1R line established with this chimera construct in the pSRα-neo vector was uniformly CD1d positive after G418 selection and was used in these experiments without further enrichment for CD1d+ cells.

Functional Analysis of T Cells. T cell activation for cytokine analysis was done using 10⁵ T cells/well in 96-well flat-bottomed plates coated with anti-CD3 at 10 μg/ml for 48 h. Stimulation with CD1 was done using equal numbers of T cells and stimulator cells (10⁵/well) for 48 h, unless otherwise indicated. For CHO cells, a 30-s glutaraldehyde fixation (0.05% in PBS) was used to bypass the need for costimulatory molecules (40). For stimulation by CHO and C1R transfectants, PMA was included at 1 ng/ml, except where stated otherwise. PHA control stimulations were carried out using a 2,000-fold dilution as described above. For antibody blocking experiments, CD1d transfectants were mixed with mAb dilutions immediately before addition of T cells.

Released cytokine levels were determined by ELISA with matched antibody pairs in relation to cytokine standards. The antibodies for IFN-γ were from Endogen, Inc. (Boston, MA) and the others were from PharMingen. T cell proliferation was determined by incorporation of [³H]thymidine (0.5 mCi/well) using glutaraldehyde fixed (0.05%) or irradiated (5,000 rads) stimulator cells where indicated. Results shown are means of triplicate samples with error bars representing standard deviations.

Results

Isolation and TCR Analysis of Invariant Vα24-JαQ DN T Cells. Circulating DN T cells were isolated from the pe-

Table 1. *CDR3 Sequences of Vα24⁺Vβ11⁺ Clones*

T cell	Vα24	JαQ*	Vβ11	CDR3	Jβ
				TCR-β	
DN1.10B3	+	+	CAS	REGAMGTGELF	FGEG Jβ2.2
DN2.B9	+	+	CAS	SATRALTGSDTQY	FGPG Jβ2.3
DN2.C6	+	+	CAS	SFLDRDYSYNEQF	FGPG Jβ2.1
DN2.C7	+	+	CAS	SENRQGAGYEQY	FGPG Jβ2.7
DN2.C9	+	+	CAS	2 Vβ11 sequences‡	
DN2.D5	+	+	CAS	SERTTNTGELF	FGEG Jβ2.2
DN2.D6	+	+	CAS	SVRPGGNEQF	FGPG Jβ2.1
DN2.D7	+	+	CAS	SDGEQANTEAF	FGQG Jβ1.1
DN3.2	+	+	CAS	SATIRDRASGYT	FGSG Jβ1.2
SP3.1 (CD4)	+	−	CAS	SDTRVGGELF	FGEG Jβ2.2
SP3.4 (CD4)	+	−	CAS	SLGESNQPQH	FGDG Jβ1.5
SP3.7 (CD4)	+	−	CAS	SVPGPAYEQY	FGPG Jβ2.7
SP3.11 (CD4)	+	−	CAS	SDTRVGGELF	FGEG Jβ2.2
SP3.15 (CD4)	+	−	CAS	GTQGNTEAF	FGQG Jβ1.1
SP3.19 (CD4)	+	−	CAS	EYGGPSYGYT	FGSG Jβ1.2

*Sequencing confirmed that all JαQ⁺ TCRs expressed the invariant Vα24 TCR-α chain.
‡Sequencing indicated that two Vβ11 transcripts were expressed, presumably reflecting two clones.

ripheral blood of two healthy donors (DN1 and DN2) by anti-CD4 and -CD8 depletion (29, 32). DN Vα24⁺ T cells were then positively selected from these populations using the C15B12 mAb, specific for Vα24 (41), and clones were established by limiting dilution. Vα24⁺ T cell lines from these populations were also established by bulk stimulation with PHA, and the majority of the cells in these lines (75–95%) were found to be Vβ11⁺ using the C21D2 mAb (31). DN and single positive T cells from a third healthy donor (DN3 and SP3, respectively) were isolated by positive selection with the Vα24 and Vβ11 mAbs and cloned by limiting dilution. As reported previously, the majority of these latter clones were CD4⁺, but only the DN clones from this donor expressed JαQ (32).

The TCR structure of a series of eight DN Vα24⁺ Vβ11⁺ clones from donor 2 and single clones from donors 1 and 3 was analyzed. Sequence analysis demonstrated that the DN clones from donors 1 and 3 and all but one of the clones from donor 2 expressed the invariant Vα24 TCR-α chain (Table 1). Significantly, in one invariant Vα24⁺ clone (DN2.C7), the Vα24-encoded serine was apparently removed during recombination and the serine was regenerated through an N-region addition and recombination 2 bp further 5′ in JαQ (Fig. 1). These results confirmed the high frequency of the invariant Vα24 TCR among DN Vα24⁺ T cells, and suggested that this TCR is strongly selected based upon its protein structure, rather than being generated exclusively by a developmentally programmed precise joining of Vα24 and JαQ gene segments.

In contrast to the invariant TCR-α structure of the DN

Vα24⁺Vβ11⁺ clones, multiple distinct TCR-β sequences were identified (Table 1). The TCR-β sequences from a series of CD4⁺, Vα24⁺Vβ11⁺ clones that did not use the invariant Vα24 were also determined for comparison. The identification of multiple Vβ sequences in the clones from donor 2 demonstrated that the DN invariant Vα24⁺ population may be derived from a large number of independent clones in single donors. The TCR-β chains were also noteworthy for their markedly diverse CDR3 structures and Jβ usage. There was no suggestion of a common CDR3 sequence motif, and even CDR3 length was quite variable. This TCR-β diversity raised the possibility that these clones might recognize diverse antigens in spite of their invariant TCR-α chain. Alternatively, the lack of conserved TCR-β chain structure may indicate that the TCR-β CDR3 did not contribute significantly to recognition by the TCRs of these cells.

Expression of Cell Surface Proteins Associated with NK Cells. The relationship between invariant Vα24⁺ DN T cells and NK cells was explored using a panel of mAbs rec-

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                        V              N              J
                   cysvalvalser                 aspargglyser
invariant Vα24-JαQ Vα24-TGTGTGGTGAGC            GACAGAGGCTCA-JαQ

                   cysvalval       ser          aspargglyser
clone DN2.C7       Vα24-TGTGTGGTG  T             CCGACAGAGGCTCA-JαQ
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Figure 1. Invariant Vα24⁺ TCR-α nucleotide and derived amino acid sequences. DN invariant Vα24⁺ T cell clone cDNA was sequenced. One clone (DN2.C7) had a distinct nucleotide sequence, which resulted in an identical amino acid sequence as shown.
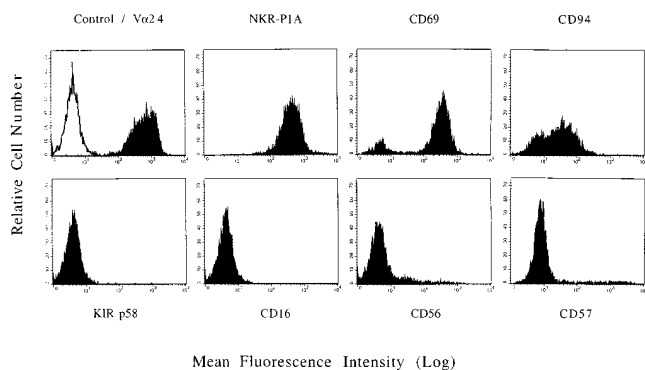
Figure 2. Expression of NK-associated proteins by invariant $V\alpha24^+$ T cells. FACS® profiles of a representative DN invariant $V\alpha24^+$ T cell clone (DN2.C9) 4 wk after PHA stimulation. T cells were stained with mAbs against the antigens shown or isotype-matched control mAb at 10 μg/ml and with anti-IgG FITC conjugate for 30 min each before FACS® analysis with propidium iodide gating on viable cells. (*Top, left to right*) P3 isotype control (*open histogram*) and $V\alpha24$ (*solid histogram*), NKR-P1A, CD69, CD94. (*Bottom, left to right*) p58 KIR (GL183 shown), CD16, CD56, CD57.

ognizing proteins expressed predominantly by NK cells. Humans appear to have only a single NK1-like gene, the homologue of murine NKR-P1A, that is expressed by NK cells and, at low levels, by a subpopulation of peripheral blood T cells (33). Nonetheless, NKR-P1A was expressed at high levels by each of the invariant $V\alpha24^+$ clones (Table 2 and Fig. 2), but not by any of the CD4+ $V\alpha24^+V\beta11^+$ clones that did not express the invariant $V\alpha24$-JαQ rearrangement (data not shown).

CD69, CD94, and the NKG2 family are additional C-type lectins linked to NKR-P1A in the human NK locus. CD69 is an early and transient T cell activation marker (42), but expression of CD69 by each of the invariant

$V\alpha24^+$ clones persisted at high levels for long periods (>3 wk) after in vitro stimulations (Table 2 and Fig. 2). CD94, a possible NK cell receptor for class I proteins (43–46), was also expressed at high levels by all of the invariant $V\alpha24^+$ clones. In contrast, prolonged high level expression of these NK locus–encoded proteins was not observed on any of a series of similarly derived CD4+ clones or on more than a very small fraction of PHA-stimulated PBLs (data not shown). Cell surface expression of the NKG2 family (47), members of which may associate with CD94 (48), could not be assessed as there are no NKG2-specific, surface-reactive NKG2 antibodies readily available (Bach, F., and J. Houchins, personal communications).

In contrast to the NK locus–encoded C-type lectins, other functionally important receptors expressed by NK cells, but encoded at other genetic loci, were not expressed at significant levels by the invariant $V\alpha24^+$ clones. These included the p58 and p70 HLA class I–specific KIRs, CD16, CD56, and CD57 (Table 2 and Fig. 2). Finally, each clone expressed low and variable levels of CD28 and CD8α, but not CD8β. CD8α was similarly detected at low levels on some cells from the CD4+ $V\alpha24^+$ invariant $V\alpha24^-$ clones (Table 2 and data not shown), consistent with its appearance as a late activation antigen. Taken together, these results further supported the conclusion that human invariant $V\alpha24^+$ T cells and murine invariant $V\alpha14$-Jα281 NK1+ T cells are homologous. However, the relationship of these cells to classical NK cells appeared limited to their expression of NK locus genes, but not of genes encoded elsewhere that are characteristically expressed by NK cells.

Effector Functions of Invariant $V\alpha24$ T Cells. Cytokine production by the series of invariant $V\alpha24V\beta11$ clones in response to stimulation by plate-bound anti-CD3 was assessed. For comparison, a series of CD4+ $V\alpha24^+$-JαQ$^-$ $V\beta11^+$ clones was also analyzed and IL-4/IFN-γ ratios compared

Table 2. *Expression of NK-associated Proteins by Invariant $V\alpha24^+$ T Cells*

| T Cell | CD4 | CD8α | NK locus molecules | | | NK markers | | | |
			NKR-P1A	CD69	CD94	CD16	CD56	CD57	KIR
DN2.B9	−	±	++	++	++	−	−	−	−
DN2.C6	−	±	++	ND	+	ND	ND	ND	−
DN2.C7	−	±	++	++	++	ND	ND	ND	−
DN2.C9	−	+	++	++	+	−	±	±	−
DN2.C11	−	±	++	ND	ND	−	−	±	−
DN2.D5	−	±	++	++	+	−	±	−	−
DN2.D6	−	±	++	++	+	−	±	−	−
DN2.D7	−	±	++	++	+	−	−	−	−
SP3.5B2	+++	±	−	ND	ND	−	−	−	−

Summary of DN invariant TCR+ clones and controls FACS® data. T cells 2–4 wk after PHA stimulation were stained with mAbs against the antigens shown or isotype-matched controls at 10 μg/ml and anti-IgG FITC conjugate before FACS® analysis. −, <5% gated positive, MFI <10; ±, <50% gated or MFI <100; +, >50% gated, MFI <100; ++, >50% gated, MFI >100; +++, >90% gated, MFI >1,000. α/β-TCR/CD3, $V\alpha24$, and $V\beta11$ mAb staining was in the ++ to +++ range. All cells shown were CD8β−. KIR expression was by staining with mAb against four p58 and the NKB1 p70 molecules (see Materials and Methods).
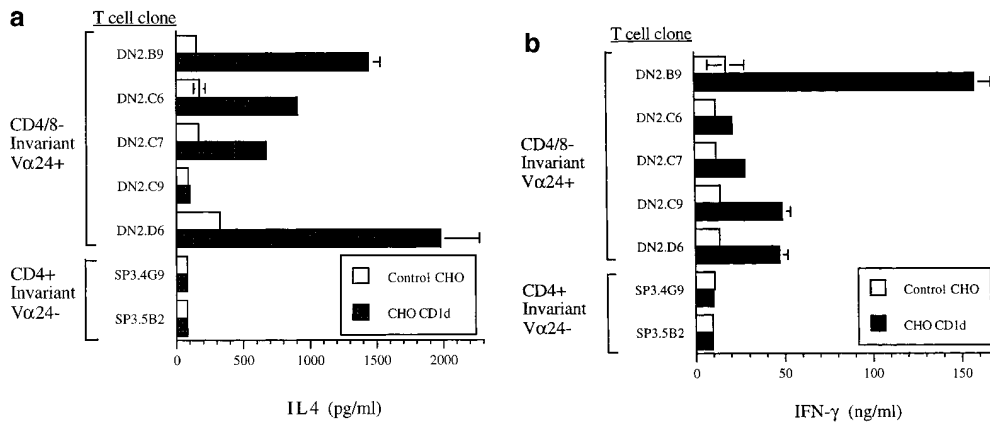
Figure 3. Invariant $V\alpha24^+$ T cells responded to CD1d CHO transfectants specifically. DN invariant $V\alpha24^+$ T cell clones (*DN2.B9*, *C6*, *C7*, *C9*, and *D6*) and control CD4$^+$ $V\alpha24^+$ invariant TCR-negative T cell clones (*SP3.4G9* and *5B2*), all at 2×10^5/well were stimulated with 0.05% glutaraldehyde-fixed CD1d$^+$ CHO transfectants or control CHO cells (2×10^5/well). PMA (1 ng/ml) and IL-2 (1 nM) were included, and secreted IL-4 and IFN-γ measured at 48 h by ELISA in triplicate (standard deviations shown). Similar results were obtained without IL-2. (*a*) IL-4; (*b*) IFN-γ.

(Table 3). With the exception of DN2.D7, the invariant $V\alpha24^+$ clones all produced substantial levels of IFN-γ and IL-4, associated with Th1 and Th2 T cells, respectively. Compared to the CD4$^+$ clones, there was a trend towards higher levels of IL-4 production by the invariant $V\alpha24^+$ clones, whether assessed based upon absolute IL-4 production or IL-4/IFN-γ ratios. This contrasts, to some extent, with an apparent bias in humans towards the generation of T cells that produce much higher levels of IFN-γ relative to IL-4 (Th1 type cells) in the absence of polarizing stimuli (49, 50). However, there was overlap between the invariant $V\alpha24^+$ DN clones and the CD4$^+$ clones with respect to IL-4 and IFN-γ production, and IL-4/IFN-γ ratio, indicating that the invariant $V\alpha24^+$ DN T cells had a phenotype that did not fall clearly into the Th1 or Th2 categories. The DN clones also produced IL-13, and some produced IL-10 at levels that were not clearly distinct from the

CD4$^+$ cells.

Recognition of CD1d-transfected CHO Cells by DN Invariant $V\alpha24^+$ Clones. CD1d recognition was assessed initially using CD1d-transfected CHO cells. Each of the five invariant $V\alpha24^+$ clones assayed responded specifically to the CD1d-transfected CHO cells based upon T cell proliferation (not shown) and cytokine release (Fig. 3). Recognition of CD1d required PMA and mild aldehyde fixation of the target cells, which have been shown in other systems to substitute for certain physiological costimulatory signals (40). Fig. 3 *a* shows that the CD1d transfectants stimulated IL-4 production from each of the clones except DN2.C9, with the highest levels produced by DN2.B9 and DN2.D6. The CD1d transfectants also strongly stimulated IFN-γ production from three of these clones (DN2.B9, DN2.C9, and DN2.D6) and modest, but specific, IFN-γ release by the other two clones (Fig. 3 *b*). In contrast, the invariant

Table 3. *Cytokine Responses of Invariant TCR$^+$ T Cells to Mitogenic Stimulus*

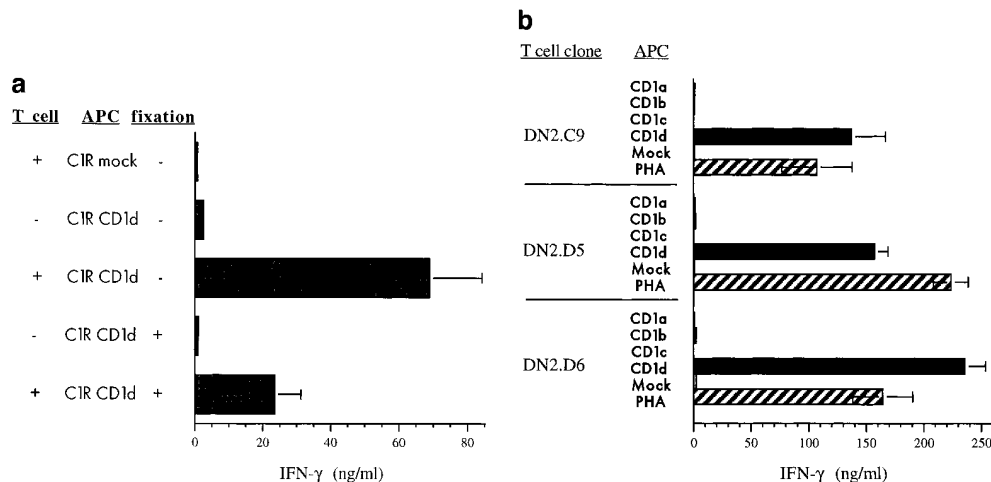| T cell	IFN-γ	IL-4	IL-10	IL-13	GM-CSF	IL-4/IFN-γ
DN2.B9	7,743	2,498	<100	7,853	6,410	0.323
DN2.C6	20,480	8,723	12,690	3,379	15,510	0.435
DN2.C7	31,300	10,780	<100	34,240	32,690	0.345
DN2.C9	13,390	1,658	<100	4,557	6,670	0.123
DN2.D5	9,536	5,304	5,660	11,960	12,940	0.556
DN2.D6	31,180	15,720	15,300	63,895	68,500	0.500
DN2.D7	5,919	414	6,220	1,520	9,880	0.070
SP3.3B2	8,491	1,681	34,910	26,770	23,190	0.196
SP3.3D10	35,390	1,942	88,980	19,690	35,330	0.055
SP3.4C6	39,710	1,903	218,200	24,590	28,050	0.048
SP3.4G9	21,060	767	<100	24,120	39,420	0.036
SP3.5B2	2,656	4,723	<100	17,270	6,700	1.667

T cells (10^5/well) were stimulated with plate-bound mAb (10 μg/ml) against CD3 or NKR-P1A, supernatants collected at 48 h, and ELISA was with cytokine mAb pairs and standards (PharMingen), results shown as pg/ml. Antibody-free and isotype control mAb wells had <610 pg/ml of all cytokines tested. Detection limits were <100 pg/ml for cytokine ELISA. Similar results, with slightly higher responses and backgrounds, were obtained in the presence of 1 nM IL-2. T cell proliferation was also determined at 96 h (not shown).
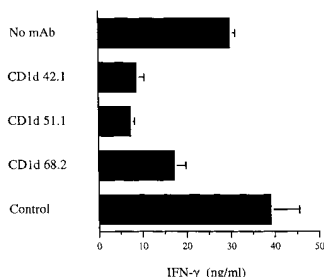
Figure 4. CD1d antibodies inhibited invariant Vα24+ T cell recognition of targets. DN invariant Vα24+ T cell clone DN2.D5 (10^5/well) was stimulated with fixed CD1d+ CHO cell transfectants (10^5/well) as in Fig. 3. Control and CD1d-specific mAb were included at 0.67 μg/ml, and secreted IFN-γ measured by ELISA. Higher concentrations of mAb gave similar results except for 68.2, where inhibition was more complete.

Vα24− clones did not respond specifically to the CD1d transfectants (Fig. 3, *a* and *b*, and data not shown). The failure of the DN2.C9 clone to produce significant IL-4 in response to the CD1d transfectant was consistent with the relatively low ratio of IL-4/IFN-γ produced by this clone in response to anti-CD3 stimulation (Table 3). Indeed, the relative levels of IL-4 and IFN-γ release in response to CD1d from each of the clones were comparable to those observed with anti-CD3 (Table 3).

Antibody inhibition studies were performed to confirm that CD1d was recognized and to rule out the possibility that peptide fragments of CD1d were the actual target of the invariant Vα24+ clones. Two mAbs that recognized native CD1d protein (42.1 and 51.1) could almost completely block activation of the DN2.D6 T cell clone at concentrations ⩾0.2 μg/ml (Fig. 4 and data not shown). A third anti-CD1d mAb (68.2) partially blocked, but only at higher concentrations. Several isotype-matched control antibodies used for blocking had no significant effect. Directly comparable results were seen with the second clone analyzed (DN2.D5; data not shown).
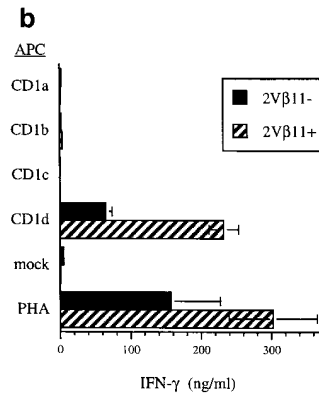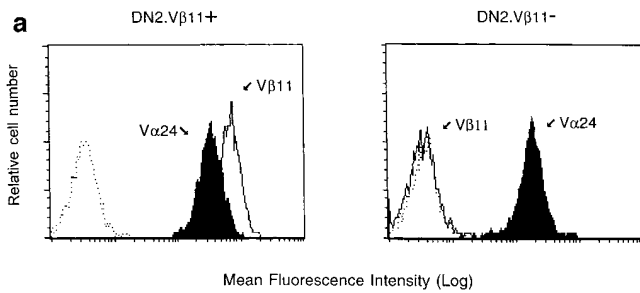
Recognition of CD1d-expressing B Cell Transfectants. Stimulation of invariant Vα24+ T cells by CD1d+ CHO cells required both PMA and aldehyde fixation, presumably due to the absence of necessary costimulatory ligands on the CHO cells. Although the target cells that mediate activation of invariant Vα24+ T cells in vivo are not known, normal B cells express CD1d (51) and may be a relevant CD1d-presenting cell. Therefore, the C1R HLA-A and -B negative B lymphoblastoid cell line (52), which does not express detectable CD1d (our unpublished data), was used to confirm the results in CHO cells and to determine whether the need for nonphysiological costimulation could be reduced or eliminated. CD1d-transfected C1R cells specifically stimulated each of the invariant Vα24+ DN T cell clones tested based upon IFN-γ and IL-4 production and T cell proliferation (Fig. 5 *a* and data not shown), confirming the results in CHO cells. Stimulation did not require aldehyde fixation, but phorbol ester was still necessary.

The fine specificity of CD1d recognition was further assessed using C1R cells stably transfected with CD1a, b, or c versus CD1d. The CD1a, b, and c transfectants were not significantly more active than the mock transfectant in stimulating IFN-γ (Fig. 5 *b*) or IL-4 production (not shown) from any of the clones examined, although they were able to stimulate CD1a–, b–, or c–reactive T cell clones, respectively (6, 53). In marked contrast, the CD1d C1R transfectants stimulated the production of IFN-γ (Fig. 5 *b*) and IL-4 (not shown) at levels directly comparable to those produced in response to PHA.

CD1d Recognition by Invariant Vα24+ T Cells Not Expressing Vβ11. Polyclonal DN T cell lines selected for expression of Vα24 were sorted into Vβ11+ or Vβ11− populations and examined to determine whether Vβ11 was necessary for CD1d recognition. FACS® analyses showed that virtually all of the cells in both lines were Vα24+ (Fig. 6 *a*), and previous

Figure 5. Invariant Vα24+ T cells responded to CD1d B cell transfectants. T cell clones (10^5/well) were incubated for 48 h with either fixed (0.025 or 0.05% glutaraldehyde, latter shown) or unfixed C1R human B cells (10^5/well) transfected with CD1a, b, c, d, or plasmid alone. Results with the two fixations were indistinguishable. PMA (1 ng/ml) was included and PHA as positive control is shown for comparison. Representative IFN-γ cytokine ELISA results of multiple experiments are shown. (*a*) Production of IFN-γ by DN2.C9 incubated for 48 h with C1R ± fixation. (*b*) IFN-γ cytokine responses of three representative DN2 T cell clones incubated for 48 h with unfixed C1R transfected with CD1a, b, c, d, mock, or mock with PHA.

Figure 6. Invariant $V\alpha24^+$ T cells that did not express $V\beta11$ responded to CD1d. (*a*) Representative FACS® profiles of two DN invariant TCR^+ T cell lines (*DN2.Vβ11+*, *left*; and *DN2.Vβ11−*, *right*). T cells were stained with 10 μg/ml of mAbs against $V\alpha24$, $V\beta11$, or isotype control mAb and with anti-IgG FITC conjugate for 30 min each before FACS® analysis of viable cells. (*b*) T cell lines (10^5/well) were incubated with unfixed C1R human B cells (10^5/well) transfected with CD1a, b, c, d, mock, or mock with PHA as in Fig. 5 and IFN-γ production results are shown.

RT-PCR analyses of these lines showed that both expressed primarily or exclusively the invariant $V\alpha24$ (32). The line designated as $DN2.V\beta11^-$ had no significant $V\beta11^+$ population, whereas the line designated as $DN2.V\beta11^+$ was virtually all $V\beta11^+$ (Fig. 6 *a*). It is of interest that cells in the $V\beta11^-$ line consistently expressed slightly lower TCR levels, based upon staining with the anti-$V\alpha24$ mAb (Fig. 6 *a*) and anti-TCR mAbs (not shown). The relationship of this observation to the preferential use of $V\beta11$ by invariant $V\alpha24^+$ T cells is not clear, but could reflect greater stability of the invariant $V\alpha24$ when paired with $V\beta11$.

CD1 recognition by cells in both lines was compared using the panel of CD1-transfected C1R cells. Both the $V\beta11^+$ and $V\beta11^-$ lines were activated specifically by the CD1d-transfected C1R cells (Fig. 6 *b*). Although the response by the $V\beta11^-$ line was quantitatively less, the responses by both lines were comparable to the corresponding PHA responses and most likely represented maximal activation for each line. These results demonstrated that T cells expressing the invariant $V\alpha24$ TCR-α paired with $V\beta$s other than $V\beta11$ can mediate CD1d recognition.

Contribution of the CD1d Endoplasmic Targeting Motif to Recognition. Human CD1b, c, d, and murine CD1d, but not human CD1a, have short cytoplasmic tails containing a sequence motif, Tyr-X-X-Z (where *X* is any amino acid and *Z* is a hydrophobic amino acid), shown to regulate the intracellular trafficking of many transmembrane proteins

(54). Previous studies demonstrated a critical role for this motif in the endosomal localization of human CD1b (36). To determine whether this sequence is necessary for the cell surface expression and function of CD1d, the transmembrane domain and cytoplasmic tail from CD1a was fused to the CD1d ectodomain. A C1R cell line transfected with this CD1d-CD1a chimera expressed moderate levels of CD1d at the cell surface (Fig. 7 *a*). The level of CD1d expression was lower than in the C1R line expressing wild-type CD1d, as this latter line was initially sorted for CD1d$^+$ cells.

Despite lower levels of CD1d expression, the C1R cells expressing the CD1d-CD1a chimeric protein were very effective at activating invariant $V\alpha24^+$ clones. The DN2.D5 clone produced both IFN-γ (Fig. 7 *b*) and IL-4 (not shown) in response to the chimeric protein at levels comparable to those induced by PHA stimulation. Identical results were seen with a second clone, DN2.D6 (not shown). Therefore, deletion of the targeting motif did not impair CD1d recognition by invariant $V\alpha24^+$ DN T cells.

Discussion

This report demonstrated the expression of invariant $V\alpha24^+$ TCRs by a distinct population of CD1d-reactive DN T cells that appear to be closely related, both phenotypically and functionally, to murine NK1$^+$ T cells. A se-
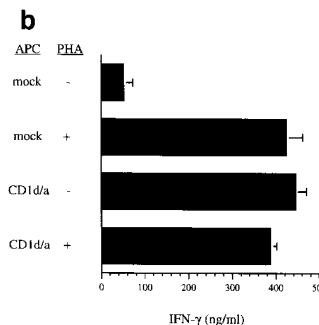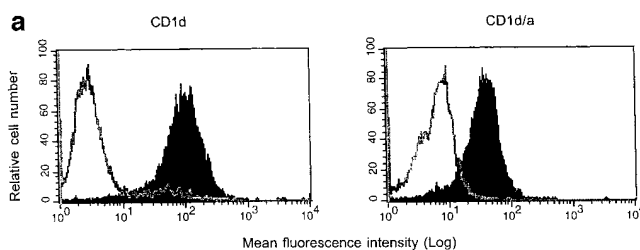
Figure 7. Invariant $V\alpha24^+$ T cells responded to chimeric CD1d with intracellular CD1a. (*a*) FACS® profiles of C1R CD1d (*left*) and CD1d/a chimera (*right*) transfectants stained with normal mouse serum (*open histogram*) or 42.1 CD1d-specific mAb (*solid histogram*). (*b*) DN2.D6 T cell clone (10^5/well) was incubated with unfixed C1R human B cell CD1d/a chimera or mock transfectants (10^5/well) and IFN-γ cytokine ELISA results obtained.

ries of invariant $V\alpha24^+V\beta11^+$ DN T cell clones were shown to recognize CD1d expressed either by a hamster (CHO) or human B cell (C1R) line. The fine specificity of this recognition was demonstrated by the failure of these clones to recognize CD1a, b, or c transfectants. Moreover, antibody blocking studies indicated that intact CD1d, rather than peptides derived from this protein, were recognized by these clones. These results provided strong evidence that CD1d recognition by these clones was mediated directly by their TCRs.

The $V\beta11$ chains from these clones were sequenced to determine whether this population was monoclonal or polyclonal and whether there were structural constraints on the CDR3 region. This analysis revealed a unique $V\beta11$ chain with extensive N-region diversity in each of the clones isolated from a single donor, demonstrating that multiple independently derived clones contributed to this population. Sequence analysis of the $V\alpha24$-$J\alpha Q$ chains also revealed a clone with distinct codon usage in the V-J junction, indicative of N-region addition. These observations suggest that the TCRs used by these clones are generated through V-(D)-J recombination mechanisms and subsequent positive selection as with conventional T cells.

The $V\beta11$ sequence analysis also revealed the lack of apparent structural constraints on the CDR3 region, since there was marked variability in CDR3 length, $J\beta$ usage, and sequence. There was no suggestion of a sequence motif that distinguished the $V\beta11$ CDR3 regions associated with the invariant $V\alpha24$ chain from those associated with other noninvariant $V\alpha24$ chains. This pattern of CDR3 independent recognition by one or several $V\beta$ chains could be consistent with selection or expansion by a superantigen (possibly CD1d associated). However, conventional superantigen recognition is not dependent upon a $V\alpha$ chain CDR3. It also appeared unlikely that these heterogeneous $V\beta11$ chains mediated recognition of distinct CD1d-presented antigens since each clone recognized CD1d expressed by both hamster and human cells without the deliberate addition of an antigen. Therefore, the current data suggest that $V\beta11$ may be structurally favored as an invariant $V\alpha24$ partner and/or mediate direct contacts with CD1d. However, pairing of the invariant $V\alpha24$ with $V\beta11$ was not an absolute requirement for CD1d recognition since analysis of an invariant $V\alpha24^+V\beta11^-$ DN T cell line demonstrated that the invariant $V\alpha24$ can pair with other $V\beta$s to generate CD1d-reactive TCRs.

Although CD1d recognition by multiple clones was demonstrated without the deliberate addition of an antigen, this did not rule out CD1d presentation of one or a small number of conserved ubiquitous endogenous or serum-derived antigens. CD1 has been shown to present lipid antigens (6–8, 53) and hydrophobic peptide antigens (9). Consistent with these observations, the crystal structure of murine CD1d reveals a potential deep hydrophobic antigen-binding cavity (55). The pathway(s) through which CD1 may acquire such hydrophobic or other antigens are not clear, but appear distinct from the MHC class I pathway (6, 56–58). Significantly, the cytoplasmic tails of human

CD1b, c, and d, and murine CD1d contain a short tyrosine-based signal that has been implicated in trafficking from the plasma membrane to endosomal compartments (54), and a recent immunogold electron microscopy study demonstrated that a large fraction of CD1b molecules were located intracellularly in an endosomal compartment (36). Based upon these observations, one hypothesis has been that CD1 proteins are targeted to an acidic endosomal compartment and are there loaded with antigen.

To address this hypothesis in the case of CD1d, the cytoplasmic tail of CD1d was replaced with the cytoplasmic tail of CD1a, which lacks recognizable targeting motifs. CD1a proteins appear to traffic to the cell surface through the default secretory pathway and do not enter an endosomal compartment (Sugita, M., M. Brenner, and S. Porcelli, unpublished data). The chimeric protein was recognized by invariant $V\alpha24^+$ DN T cell clones despite the loss of the endosomal targeting signal, indicating that endosomal trafficking mediated by this signal was not necessary for CD1d recognition by this cell population. Taken together, the data in this report indicate that T cell recognition of CD1d mediated by the invariant $V\alpha24$ TCR may not involve a specific antigen, although presentation of a conserved cellular antigen acquired through a pathway that is independent of the endosomal targeting motif cannot be excluded. Moreover, it remains possible that CD1d presents specific foreign antigens in vivo and that the in vitro responses reflect relatively low affinity interactions mediated by CD1d binding to diverse nonspecific antigens.

In addition to specific antigen recognition by the TCR, the activation of conventional T cells is dependent upon the recruitment of $p56^{lck}$ to the TCR complex by the CD4 or CD8 accessory proteins. The invariant $V\alpha24^+$ DN clones express $p56^{lck}$ (data not shown) and it is very likely that there are other accessory proteins that couple it to the TCR in these cells. The consistent high level expression of NKR-P1A by human invariant $V\alpha24^+$ DN T cell clones, the expression of NK1 (NKR-P1C) by the homologous murine cell population, and the presence of a $p56^{lck}$ binding motif in the cytoplasmic tails of the murine NKR-P1 proteins make these clear candidate accessory proteins (59, 60). However, the presence of invariant $V\alpha^+$ T cells in mouse strains that do not express NK1 or other NKR-P1 proteins argues against such a critical role for NKR-P1. It should also be noted that the human NKR-P1A protein does not contain the putative $p56^{lck}$ binding site (33) and, in preliminary biochemical studies, we have been unable to demonstrate an association between human NKR-P1A and $p56^{lck}$ (Exley, M., unpublished data).

The human invariant $V\alpha24^+$ T cell clones also expressed two other C-type lectins, CD69 and CD94, encoded in a chromosomal region that has been termed the NK locus. CD94 may heterodimerize with NKG2 proteins (48), another family of C-type lectins encoded in the NK locus (47), and this complex may be an MHC class I receptor (44, 48). CD69 is an early and transient T cell activation antigen (42), but its expression by invariant $V\alpha24^+$ DN T cell clones was persistent. This suggests that invariant

Vα24$^+$ DN T cells may remain in an activated state longer than conventional T cells. Alternatively, given the high level of expression of other NK locus–encoded proteins observed on invariant Vα24$^+$ T cells, CD69 expression may reflect constitutive transcriptional activity of the NK locus in these cells which is not directly related to T cell activation.

Although the expression of NKR-P1 and CD94 at high levels suggests some relationship to NK cells, the invariant Vα24$^+$ DN T cells did not express a number of other molecules that play roles in NK cell function, such as CD16, CD56, and CD57. In particular, the invariant Vα24$^+$ DN T cells did not express p58 or p70 KIRs, although expression of family members not recognized by the multiple antibodies used here remains possible. Consistent with the mAb data, preliminary RT-PCR amplification experiments with consensus KIR primers have similarly failed to detect KIR expression (data not shown). This is in contrast to recent data showing that KIRs may be expressed in other T cell subpopulations (61–64). These observations indicate that the link between invariant Vα24$^+$ DN T cells and NK cells may be transcriptional activation of the NK locus, with limited functional overlap between these cell populations.

Cytokine production by invariant Vα24$^+$ DN T cells was also analyzed and the results supported conclusions reached in the mouse that these cells can produce significant levels of IL-4 in response to activation (20–23). However, they also produced other cytokines, particularly IFN-γ, at substantial levels, and their regulatory functions in vivo are probably complex. Murine NK1$^+$ T cells are responsible for the acute production of IL-4 in response to anti-CD3 in vivo (23), but this type of stimulus is clearly nonphysiological. It is unlikely that the function of this cell population is to determine systemic levels of IL-4 or other cytokines, and more likely that the IL-4 produced in response to anti-CD3 stimulation reflects the primed activation state of these cells in vivo. A reasonable alternative hypothesis is that invariant Vα24$^+$ T cells function through cell–cell interactions to provide individual CD1d$^+$ target cells with IL-4, IFN-γ, or other cytokines that in turn direct the further proliferation and/or differentiation of these target cells.

B cells express CD1d (51) and represent one possible target cell for invariant Vα24$^+$ T cells. However, CD1d may also be widely expressed (51, 65) and invariant Vα24$^+$ T cells may, therefore, have functionally important interactions with a number of different cell types. In particular, the large fraction of T cells in murine bone marrow and liver that are NK1$^+$ (66, 67) presumably interact locally with CD1d$^+$ cells and may play roles in regulating B cell maturation, myeloid development, or hepatic immune function. Finally, recent reports indicate that loss of invariant Vα24$^+$ T cells in humans or of the homologous NK1$^+$ T cell population in mice is associated with disease progression in several autoimmune diseases (68–70). Although the precise functions of invariant Vα24$^+$ T cells and their murine homologues remain to be clarified, the conservation of this cell population and of the CD1d ligand across species suggests an important immunological function.

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