An Invariant T Cell Receptor α Chain Is Used by a Unique Subset of Major Histocompatibility Complex Class I–specific CD4$^+$ and CD4$^-$8$^-$ T Cells in Mice and Humans

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Summary

The mouse thymus contains a mature T cell subset that is distinguishable from the mainstream thymocytes by several characteristics. It is restricted in its usage of T cell receptor (TCR) Vβ genes to Vβ8, Vβ7, and Vβ2. Its surface phenotype is that of activated/memory cells. It carries the natural killer NK1.1 surface marker. Furthermore, though it consists entirely of CD4$^+$ and CD4$^-$8$^-$ cells, its selection in the thymus depends solely upon major histocompatibility complex (MHC) class I expression by cells of hematopoietic origin. Forced persistence of CD8, in fact, imparts negative selection. Here, we have studied the TCR repertoire of this subset and found that, whereas the β chain V-D-J junctions are quite variable, a single invariant α chain Vα14-J281 is used by a majority of the TCRs. This surprisingly restricted usage of the Vα14-J281 α chain is dependent on MHC class I expression, but independent of the MHC haplotype. In humans, a similar unusual population including CD4$^-$8$^-$ cells can also be found that uses a strikingly homologous, invariant α chain Vα24-JQ. Thus, this unique Vα-Jα combination has been conserved in both species, conferring specificity to some shared nonpolymorphic MHC class I/peptide self-ligand(s). This implies that the T cell subset that it defines has a specialized and important role, perhaps related to its unique ability to secrete a large set of lymphokines including interleukin 4, upon primary stimulation in vitro and in vivo.

The development and function of the minor subset of CD4$^-$8$^-$ double negative (DN)[1] TCR-α/β$^+$ T cells and their relationship to the mainstream CD4$^+$ or CD8$^+$ T cells have been elusive issues (1). In the mouse thymus, a set of mature CD4$^+$ (2–7), but not CD8$^+$ (4), cells has recently been described that is strikingly similar to the DN thymocytes because it shares the membrane expression of activated/memory and NK markers and the restricted use of Vβ8, Vβ7, and Vβ2 TCR-β chains. This population has the unique potential to secrete a large set of lymphokines, including IL-4, upon primary stimulation in vitro (2, 3, 7) and in vivo (8), thus its physiological role may be to influence the Th1/Th2 differentiation of an immune response (9). It is surprising that the selection element for both these CD4$^+$ (4) and DN (4, 10) cells has been shown to be an MHC class I molecule presented by cells of hematopoietic origin. In addition, and equally surprising, was the finding that such selection is largely CD8 independent and that the persistence of CD8 instead imparts negative selection on these cells (4). Based on these findings, we suggested that this subset of T cells might express a particular set of TCRs with a level of affinity for the MHC class I ligand that was sufficient for CD8-independent positive selection, the negative selection threshold for CD8$^+$ cells being reached as the TCR level increased later during the developmental process, after the CD4$^+$8$^+$ double positive (DP) precursor stage (4).

We have thus analyzed the TCR repertoire used by these cells. We show here that their Vα usage is even more restricted than their Vβ usage. In fact, they use a single invariant α chain, Vα14-J281, whereas their Vβ8, Vβ7, and Vβ2 TCR-β chains have diverse V-D-J junctions. Moreover, this α chain is virtually identical to a human α chain, Vα24-JQ, that is expressed as an invariant chain by a related subset of DN T cells. Thus, the restricted repertoire of these cells is not likely to be due to an interaction with some putative superantigens, but instead to the recognition of a restricted set of MHC class I– peptide ligands that is conserved in both mice and humans.

Materials and Methods

Mice. 7–9-wk-old, specific pathogen-free C57BL/6, MHC-I$^-$ (β$_2$-microglobulin$^-$ [β$_2$m$^-$]) (11), originally provided by Dr. R.

[1] *Abbreviations used in this paper:* β$_2$m, β$_2$-microglobulin; DN, double negative; DP, double positive; HSA, heat stable antigen.

Jaenisch (Massachusetts Institute of Technology, Cambridge, MA) and backcrossed eight times to C57BL/6, B10 (H-2^b), B10.D2 (H-2^d), B10.A (H-2^a), B10.BR (H-2^k), B10.M (H-2^f), B10.RIII (H-2^r), B10.S (H-2^s), B10.Q (H-2^q), B10.SM (H-2^v), B10.P (H-2^p), and B10.PL (H-2^u) mice were obtained from the National Institute of Allergy and Infectious Diseases barrier facility (Bioqual, Frederick, MD). MHC-II$^-$ ($A_\beta{}^b{}^-$ [12], backcrossed four times to C57BL/6) were obtained from GenPharm International (Mountain View, CA). CD8.1 transgenic mice (13) were obtained from B. J. Fowlkes (National Institutes of Health) after eight backcrosses to B10.BR.

Cell Preparation and FACS® Sorting. Mature mouse thymocyte subsets were purified after one-step killing at 37°C with J11d.2 (anti–heat stable antigen [HSA]) mAb and rabbit complement followed by centrifugation over a density gradient (Lympholyte; Cedarlane, Hornby, Canada) and by three-color staining (as indicated in figure legends, using antibodies obtained from Pharmingen, San Diego, CA) for cell sorting with a FACStar^{PLUS}® (Becton Dickinson & Co., Mountain View, CA) equipped with an argon and a dye laser. After sorting, cell samples were divided into duplicates and digested for genomic DNA analysis or extracted for mRNA analysis as described below.

Human PBL were isolated from healthy volunteers after centrifugation over Ficoll gradient (Ficoll-Paque; Pharmacia, Uppsala, Sweden), and treated with a cocktail of anti-CD19, CD14, CD4, and CD8 coated paramagnetic beads (Miltenyi Biotec, Bergish Gladbach, Germany) and passed through magnetic columns (magnetic cell separation [MACS] system; Miltenyi Biotech) to enrich for DN T cells. The cell preparation was subsequently stained with anti-CD8-PE (Amac, Inc., Westbrook, ME), anti-TCR-α/β-FITC (T Cell Sciences, Inc., Cambridge, MA) and biotinylated anti-CD4 (Caltag Laboratories, San Francisco, CA) followed by streptavidin-RED613 (Caltag Laboratories) and FACS® sorted to purify TCR-α/β^+ DN PBL.

T Hybridoma Generation. CD44^{hi} mature thymocytes were obtained from 9-wk-old C57BL/6 mice after depleting thymocytes with J11d.2 (anti-HSA) and 3.155 (anti-CD8) mAbs plus rabbit complement, removing 3G11$^+$ and leukocyte cell adhesion molecule 1–positive cells with biotinylated mAb 3G11 (7), MEL-14, and streptavidin-coated paramagnetic beads (Miltenyi Biotec) using the MACS system, staining with anti-CD5-APC, anti-CD44-FITC, and anti-CD4-PE, and sorting CD5^{hi}CD44^{hi}CD4$^+$ and CD5^{hi}CD44^{hi}CD4$^-$ (DN) cells. 10^6 cells of each subset were stimulated, in the presence of 4×10^6 γ-irradiated (30 Gy) low density (Percoll 55% fraction) spleen cells, with soluble anti-TCR-α/β TCR mAb (H57) at 1 μg/ml and recombinant mouse IL-7 (Genzyme, Cambridge, MA) at 20 ng/ml in culture medium containing 10% FCS as described elsewhere (3). Recombinant mouse IL-2 (25 U/ml) (Biosource, Camarillo, CA) was added at day 2 of the culture. At day 4, 10^6 blast cells were fused with 10^6 aminopterin-resistant TCR-α/β^- BW5147 thymoma cells using standard procedures (14), plated at 1,000 and 3,000 cells per well in 96-well microplates, and selected in HAT medium. 27 TCR-α/β^+ hybridomas were studied: 19 derived from CD4 and 8 from DN.

Oligonucleotides. All probes and primers were obtained from Bioserve Biotechnologies (Laurel, MD) and used without further purification. Probes were digoxigenin-labeled using the digoxigenin oligonucleotide 3′-end labeling kit as indicated by the manufacturer (Boehringer Mannheim, Indianapolis, IN). The following primers were used, modified from Casanova et al. (15): 3′C_α: GAAGCTTGTCTGGTTGCTCCAG, 5′ V_α14: CTAAGCACAGCACGCTGCACA, 5′ V_α11: CCCTGCACATCAGGGATGCC for the V_α amplification; and 5′ C_α: CCCTCTGCCTGTTCACCGACTT and 3′ C_α: CTCGGTCAACGTGGCATCACA for C_α amplification. V_α14-J281 rearrangements were quantified using the above V_α14 primer with the 3′ J281 primer: CAGGTATGACAATCAGCTGAGTCC. For V_β-specific amplifications, 5′ V_β8:GCATGGGCTGAGGCTGATCCA, 5′ V_β7: TACAGGGTCTCACGGAAGAAGCG, and 5′ V_β2: TCACTGATACGGAGCTGAGGC were used with a 3′ C_β primer: GACCCCACTGTGGACCTCCTT. For sequencing of the PCR products, nested primers on C_β: CCAAGCACACGAGGGTAGCCTT and C_α: TCGGTGAACAGGCAGAGGGTG were used. The probes used for quantitation of PCR products were TGTCATCCAGCAGGGTGG for V_α14-J281, TCCAAGAGCAATGGGGCC for V_α-C_α, and GAGACCAACGCCACCTAC for C_α-C_α.

For human PBL samples, the primer pair used for PCR amplification was V_α24: CACAAAGCAAAGCTCTCTGCACA and C_α: GCCACAGCACTGTTGCTCTTG; sequencing was performed using a nested C_α primer: TAGGCAGACAGACTTGTCACTGGAT.

Nucleic Acid Preparation. DNA was prepared by lysing 2×10^4 (unless specified otherwise) cells in 10 mM Tris-HCl, pH 9, 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.5% Tween 20, and 100 μg/ml Proteinase K at 56°C for 2 h and at 95°C for 20 min.

Total RNA was extracted with the RNAzole kit (TelTest, Austin, TX) and ethanol-precipitated with addition of 5 μg of glycogen (Boehringer, Mannheim), and resuspended in 20 μl of Di-ethyl-pyro-carbonate (DEPC) water. Reverse transcription was carried out as described (16): briefly, 5 μl of RNA was denatured for 5 min at 65°C, quickly chilled on ice, and incubated in 20 μl reverse transcription buffer (100 mM KCl, 20 mM Tris-HCl (pH 9.0 at 25°C), 7 mM $MgCl_2$, 1 mM dNTP, 2 mM dithiothreitol, 100 μM hexanucleotides, 5 U/reaction RNase inhibitor, and 4 U/reaction of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). Incubation was for 10 min at room temperature and 1 h at 42°C. Reverse transcription was stopped by incubation at 95°C for 5 min and at 99°C for 1 min.

Quantitative PCR. Quantitative PCR amplification was carried out as previously described (16). 1 μl of cDNA was added to 50 μl of amplification mixture (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.01% gelatin, and 0.1% Triton X-100) containing 0.25 μM 5′ and 3′ primers with 1.25 U/reaction Taq polymerase and overlaid with mineral oil (Sigma Chemical Co., St. Louis, MO). Temperature was initially at 94°C for 4 min, followed by cycles at 94° for 45 s, 60° for 1 min, and 72° for 1 min in a thermal cycler (model PTC-100; M-J Research Inc., Watertown, MA). At sequential cycle numbers, 5 μl of the reaction mixture was sampled through oil and transferred onto avidin-coated microtiter plates containing 95 μl of TE buffer for quantitation of the amplified products in a liquid-hybridization-ELISA assay with luminometry readings (16).

Single Cell Analysis. Mature thymocytes enriched as described above were stained with anti-CD4-RED613 (GIBCO BRL, Gaithersburg, MD), anti-CD44-FITC, and anti-TCR-α/β-biotin (H57) followed by Streptavidin-PE. TCR-α/β^+CD44^{lo}CD4$^+$, TCR-α/β^+CD44^{hi}CD4$^+$, and TCR-α/β^+CD44^{hi} DN cells were sorted and plated into 96-well plates, using an autoclone unit, at 1 or 10 cells per well filled with 100 μl of PBS. After centrifugation, PBS was replaced with 10 μl of lysing solution and individual wells amplified with a V_α14-J281 primer pair for 46 cycles before quantitation of the amount of PCR products with a V_α14-specific probe.

Sequencing. The following polyclonal sequencing of TCR-α chains was performed. After reverse transcription, cDNAs from fresh sorted populations were amplified with V_α14-C_α (mouse) or

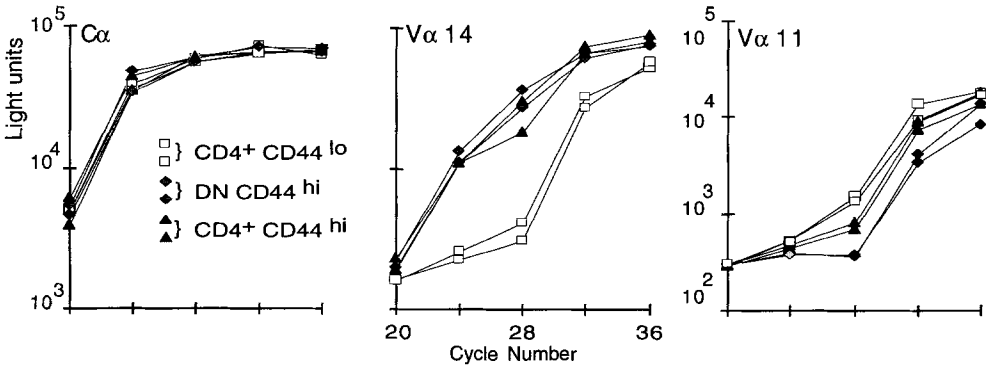

Figure 1. Increased expression of $V_\alpha14$ mRNA in mature $CD44^{hi}$ thymocytes. Mature thymocytes (obtained after anti-HSA plus C killing) were stained with anti-CD4-PE, anti-CD44-FITC, and anti-CD8-APC and sorted to obtain the $CD44^{lo}CD4^+$, $CD44^{hi}CD4^+$, and $CD44^{hi}DN$ subsets. 10^5 sorted cells were divided into duplicate samples of 0.5 × 10^5 cells and processed for quantitative RT-PCR of C_α, $V_\alpha14$, and $V_\alpha11$ mRNAs.

$V_\alpha24$-C_α (human) primers for 40 cycles, purified (Magiclean; Promega), and sequenced using a nested primer in the constant region and the Cyclist kit (Stratagene, La Jolla, CA) with [^{33}P]dATP. Data were digitalized with a phosphoimager (Molecular Dynamics, Inc., Sunnyvale, CA) and background was subtracted using the National Institutes of Health Image 1.52 software with the vertical 1D subtraction procedure.

The following hybridoma TCR-α and -β chain sequencing was performed. After characterization of V_β usage by flow cytometry using a panel of anti-V_β antibodies (Pharmingen), V_β-specific PCR amplification was carried out on cDNA using a C_β primer and $V_\beta8$-, $V_\beta7$-, or $V_\beta2$-specific primers. PCR products were cycle sequenced using a nested primer in the C_β region. For TCR-α chains, $V_\alpha14$-J281 rearrangements were scored by amplifying genomic DNA extracted from 10^5 hybridoma cells with the $V_\alpha14$-J281 primer pair and ethidium bromide staining of agarose gel electrophoresis. DNA from eight individual $V_\alpha14^+$ hybridomas was amplified with the $V_\alpha14$-J281 primer pair and sequenced with the same $V_\alpha14$ primer as used for amplification.

Results

A Single TCR-α Chain Is Used by $CD44^{hi}$ $NK1.1^+$ TCR-α/β^+ Mature Thymocytes. To analyze the TCR V_α repertoire of the $CD44^{hi}NK1.1^+$ TCR-α/β^+ thymocytes, we isolated them from the mature thymic population based on their expression of the activated, $CD44^{hi}$ phenotype. Using the few available anti-V_α antibodies, we found that although ~20% of the mainstream ($CD44^{lo}$) cells expressed one of the $V_\alpha2$-, $V_\alpha8$-, or $V_\alpha11$-TCR-α chains by FACS® analysis, virtually none of these V_α chains were expressed among $CD44^{hi}$ cells (data not shown). These results indicated that $CD44^{hi}$ cells might use a rather restricted set of V_α. To identify these V_α, we compared the expression of different TCR-α mRNAs in $CD44^{hi}$ and $CD44^{lo}$ mature thymocytes by quantitative reverse polymerase chain reaction (RT-PCR) using V_α-specific primers (15, 16). We found (Fig. 1) that $V_\alpha14$ cDNA was strikingly increased in FACS®-sorted $CD44^{hi}CD4^+$ as well as DN cells, as compared with $CD44^{lo}$ cells, as judged by the difference in the number of amplification cycles necessary to generate similar amounts of PCR products. This increase was estimated to be in the range of 24-fold, using an external standard scale (data not shown). Quantitation of a panel of other V_α mRNAs showed a moderate decrease, usually in the range of two to three fold, as seen in Fig. 1 for $V_\alpha11$. This relative conservation of $V_\alpha11$ mRNA in $CD44^{hi}$ cells most likely represents nonallelically excluded and/or out of frame mRNAs (17), since FACS® analysis of $V_\alpha11$ expression showed a 14-fold decrease (0.5% in $CD44^{hi}$ vs 7% in $CD44^{lo}$ cells; data not shown). When the $CD44^{hi}$ cells were sorted according to their V_β expression, the large increase in $V_\alpha14$ mRNA was seen in all of the subpopulations studied ($V_\beta8.2^+$, $V_\beta7^+$, or $V_\beta8.2^-$) (Fig. 2), suggesting that TCRs with different V_β nevertheless use $V_\alpha14$. In addition, direct polyclonal sequencing from the C_α end of the PCR products obtained with a C_α-$V_\alpha14$ primer pair, generated a readable sequence for $CD44^{hi}$ but not $CD44^{lo}$ cells (Fig. 3), showing that the $V_\alpha14^+CD44^{hi}$ cells use predominantly one J region, J281, and that they do not display significant heterogeneity in their V-J junction. Experiments where $CD44^{hi}$-derived products were diluted into $CD44^{lo}$-derived products before polyclonal sequencing indicated that at least 50% of the amplified products need to

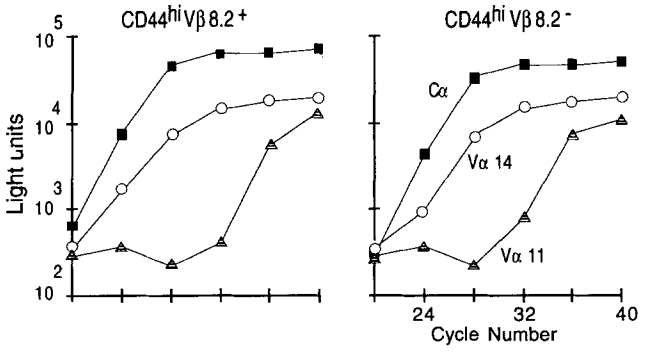

Figure 2. $V_\alpha14$ is paired with various V_β in mature $CD44^{hi}$ thymocytes. Mature $CD44^{hi}$ thymocytes were enriched as described in Materials and Methods and sorted as $CD5^{hi}$ (anti-CD5-APC), $CD44^{hi}$ (anti-CD44-FITC) cells, and $V_\beta8.2$ positive or negative (anti-$V_\beta8.2$-biotin plus avidin-PE) or $V_\beta7$ positive (anti-$V_\beta7$-biotin plus avidin-PE) cells. Quantitation of C_α, $V_\alpha14$, and $V_\alpha11$ mRNAs was carried out on duplicate samples of 3 × 10^5 ($V_\beta8.2^+$ and $V_\beta8.2^-$) and 7.5 × 10^4 ($V_\beta7^+$) cells and is represented as averaged values at sequential cycles of RT-PCR amplification.

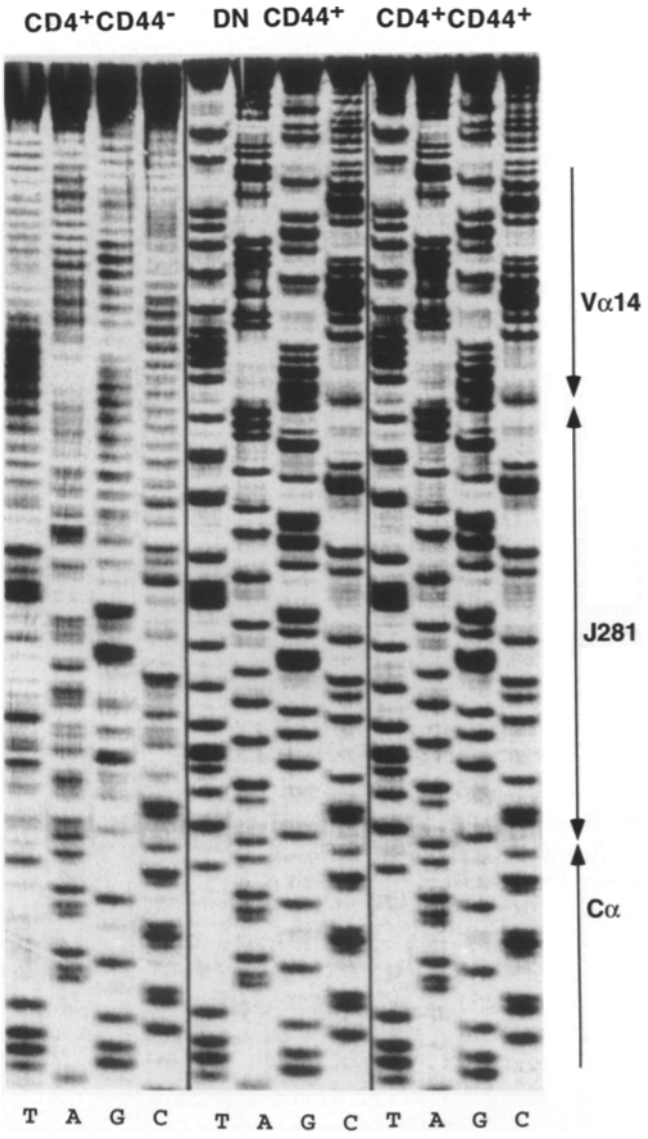

Figure 3. Polyclonal sequencing of $V_\alpha 14$ TCR-α chain in $CD4^+CD44^{lo}$, DN $CD44^{hi}$, and $CD4^+CD44^{hi}$ mature thymocytes. At the bottom of the sequencing gel, the constant region is readable for the $CD44^{lo}$ as well as the $CD44^{hi}$ samples, whereas above, the J (*J281*) and V (*$V_\alpha 14$*) regions are readable only for the $CD44^{hi}$ samples.

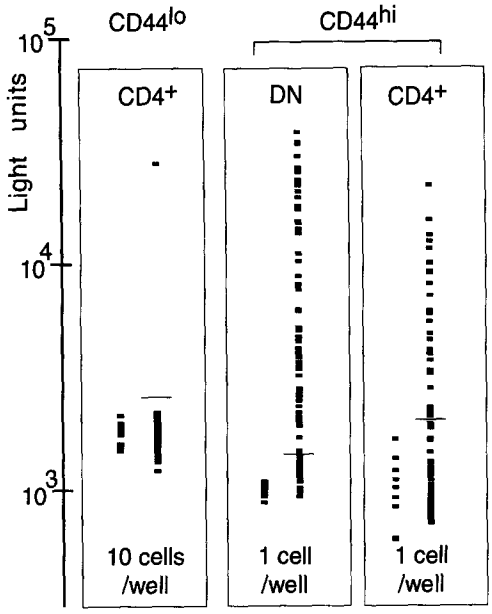

Figure 4. Presence of the $V_\alpha 14$-J281 rearrangement at the single cell level in $CD44^{hi}$ mature thymocytes. The left set of dots in each panel represents control wells without cells. Each dot represents an individual well where the presence of $V_\alpha 14$-J281 DNA rearrangement was assayed by PCR.

be identical to generate such a readable sequence reaction (data not shown).

To measure the frequency of $V_\alpha 14$-J281 TCR^+ cells in vivo, we sorted $CD44^{hi}$ cells into 96-well plates at 1 cell/well and amplified genomic DNA with a specific $V_\alpha 14$-J281 primer pair. 64 of 91 and 27 of 79 of the wells were positive in the $CD44^{hi}$DN and $CD44^{hi}CD4^+$ sets, respectively (Fig. 4). In contrast, only 1 of 84 wells was positive among the mainstream $CD44^{lo}CD4^+$ cells plated at 10 cells/well. Thus, at least 34–70% of the $CD44^{hi}$ cells had rearranged $V_\alpha 14$ to J281. Furthermore, because only 55% of the wells scored positive in parallel plates amplified with a C_β-specific primer pair (data not shown), the frequency of $V_\alpha 14$-J281 rearrangements is probably underestimated by this method because of the technical limitations in handling single cell plated wells.

To analyze the V_α and V_β junctional regions, we generated T cell hybridomas from either $CD4^+$ or DN FACS®-sorted mature $CD44^{hi}$ thymocytes. These hybridomas were representative of the fresh $CD44^{hi}$ cell population because 24 of 27 expressed either $V_\beta 8$, $V_\beta 7$, or $V_\beta 2$ TCR chains (Table 1). 23 of these 24 (96%) hybridomas had rearranged their $V_\alpha 14$ segment to J281 confirming that $CD44^{hi}$ cells use this $V_\alpha 14$-J281 TCR-α chain almost exclusively. The α chains from eight of these hybridomas were sequenced and their V-J junctions found to be nearly monomorphic, with a glycine in position 93 and an aspartate in position 94, with a single exception in which a valine replaced glycine 93 (Table 2). Glycine 93 was either coded by a GGC corresponding to the genomic sequence of the V region, or by GGG or GGT, indicating trimming of the 3′ end of the V region with at least one N addition. In one instance, aspartate 94 was coded by a GAC instead of a GAT implying that the 5′ end nucleotides (TAGAT) of the J region were trimmed and replaced by TGAC. The invariant size of the junctional region and the tendency to conserve the glycine 93–aspartate 94 sequence, despite nucleotide changes, unambiguously indicates that the α chain CDR3 region is selected at the amino acid level, most probably in order to maintain the specificity of the TCR. In contrast (Table 1), the V_β chains used many different J regions (10 of the 12 available J_β genes) and displayed a large variety of D/N regions of different sizes, sug-

Table 1. *TCR-β Chain V-D-J Junctional Amino Acid Sequences and Frequency of V_α14-J281 Rearrangements in T Cell Hybridomas Derived from DN and CD4⁺ Mature TCR-α/β⁺ CD44^{hi} Thymocytes*

Hybridoma	Origin	V_β	V_β	N/D	J_β	J_β	V_α 14-J281
DN13H1	DN	2	CTC	KAAGGD	TEVFFG	1.1	+
411D1	CD4	2	CTCSA	DWEG	SAETLYFG	2.3	+
414A2	CD4	7	CASS	SDRAD	TGQLYFG	2.2	+
431G11	CD4	8.1	CASS	RR	SAETLYFG	2.3	+
432F6	CD4	8.2	CASG	ATGAT	NTEVFFG	1.1	+
431D12	CD4	8.2	CASGD	AGQGPA	NTEVFFG	1.1	+
DN14F9	DN	8.2	CASG	GQG	NSDYTFG	1.2	+
DN32H6	DN	8.2	CAS	RE	SNERLFFG	1.4	+
432G7	CD4	8.2	CASGD	AGTGRVN	NPAPLFG	1.5	+
431A11	CD4	8.2	CASGD	KG	FRPLYFA	1.6	−
431G5	CD4	8.2	CASGD	AGG	TGQLYFG	2.2	+
DN32D3	DN	8.2	CASGD	PDI	QNTLYFG	2.4	+
DN31E12	DN	8.2	CASGD	AWTGSG	QNTLYFG	2.4	+
DN14C9	DN	8.2	CASG	PDWAG	NTLYFG	2.4	+
432B9	CD4	8.2	CASG	WGG	QDTQYFG	2.5	+
411B10	CD4	8.2	CASGD	YGERMGGR	QDTQYFG	2.5	+
431F10	CD4	8.2	CASGD	GLG	QDTQYFG	2.5	+
DN32F3	DN	8.2	CASG	PPGLGL	YEQYFG	2.6	+
431 E13	CD4	8.3	CAS	RDGRGH	TEVFFG	1.1	+
432B8	CD4	8.3	CAS	KHEGTAR	APLFG	1.5	+
DN13A1	DN	8.3	CASSD	GWGGA	AETLYFG	2.3	+
411G1	CD4	8.3	CASSD	AE	DTQYFG	2.5	+
432 E4	CD4	8.3	CASS	EEVDWG	YEQYFG	2.6	+
432F5	CD4	8.3	CASSD	PTVP	YEQYFG	2.6	+
Others	DN (3)	2⁻, 7⁻, 8⁻					− (3)

Table 2. *Nucleotide and Amino Acid Sequences of V_α14-J281 Junctions from Eight T Cell Hybridomas Derived from DN and CD4⁺ Mature TCR-α/β⁺ CD44^{hi} Thymocytes*

Germline V_α14	TGT	GTG	GTG ctgtgTA	GGC Gcac	GAT	AGA	GGT	TCA	GCC	Germline J281
V_α Sequences										
				93	94					
5/8	TGT	GTG	GTG	GGC	GAT	AGA	GGT	TCA	GCC	
	C	V	V	G	D	R	G	S	A	
1/8	. . .	. . .	. . .	GGG*	GAT	. . .	. . .	. . .	. . .	
	−	−	−	G	D	−	−	−	−	
1/8	. . .	. . .	. . .	GTA	GAT	. . .	. . .	. . .	. . .	
	−	−	−	V	D	−	−	−	−	
1/8	. . .	. . .	. . .	GGT	**GAC**	. . .	. . .	. . .	. . .	
	−	−	−	G	D	−	−	−	−	

* N additions are in bold characters, and are underlined.

gesting an absence of gross structural constraints in the CDR3 regions. Altogether, these results suggest that the CD44^{hi} NK1.1$^+$ TCR-α/β^+ thymocytes do not constitute a distinct lineage using a genetically programmed TCR gene rearrangement (such as, for example, the $V_\gamma3^+\gamma/\delta$ T cells; 18, 19) but rather that they are selected after recognition by their TCR of thymic MHC–peptide complexe(s). It is interesting to note that V_α14-J281 invariant α chains have previously been described in KLH-specific suppressor hybridomas and subsequently reported in various mouse tissues in vivo using RNase protection assays and quantitative PCR analysis (20–22). Although neither the cellular subset that expresses V_α14-J281, nor the associated β chains had been characterized, it is likely that the NK1.1$^+$ TCR-α/β^+ population studied by us is the main source of the V_α14-J281 rearrangements identified by these authors. Gut lymphocytes have also been reported to contain cells bearing a modified version of this invariant α chain with a deletion of aspartate 94 (23), emphasizing the importance of the CDR3 region in the selection process and suggesting that a slightly different MHC/peptide ligand is expressed in the gut.

The MHC Ligand. To characterize the MHC ligand required for selection of these cells, we performed a systematic analysis of their TCR repertoire in a panel of MHC congenic B10 mice. Surprisingly, we found the same frequencies of cells bearing $V_\beta8$, $V_\beta7$, and $V_\beta2$ positive TCRs, as well as a similar increase of V_α14-J281 rearrangements in all mice tested (Fig. 5). Using MHC knockout mice (11, 12), we confirmed that MHC class I but not II expression is required for the selection of these V_α14-J281$^+$ cells (Fig. 6). We also confirmed that CD4$^+$ but not CD8$^+$ thymocytes include V_α14-J281$^+$ cells and that forced (transgenic) expression of CD8 results in deletion of the V_α14-J281$^+$ cells from the CD44^{hi} thymocyte population (Fig. 6). This suggests that TCRs made of a V_α14-J281 α chain and of a $V_\beta8$, $V_\beta7$, or $V_\beta2$ β chain have a particular affinity for their MHC class I ligand and that they bind it in a classical way, allowing the interaction between CD8 and MHC class I (24, 25). Altogether, these data suggest that the putative selecting element for the CD44^{hi}NK1.1$^+$ TCR-α/β^+ thymocyte population may be a nonpolymorphic MHC class I molecule, located either inside or outside the MHC complex region, a hypothesis previously put forward (4, 10) because, like CD1 or Tla (26, 27), it is selectively expressed on bone marrow-derived but not epithelial thymic cells. Alternatively, though less likely, this ligand could be a nonpolymorphic peptide degenerately presented by polymorphic MHC molecules (28–31).

A Subset Similar to the Mouse NK1.1$^+$ TCR-α/β^+ Population Exists in Humans. There are some striking homologies between human and mouse TCR usage in DN T cell populations. For example, among the V_β chains found in human DN lymphocytes ($V_\beta2$, $V_\beta8$, $V_\beta11$, and $V_\beta13$ [32–34], $V_\beta11$ and $V_\beta13$ are closer in sequence to the mouse $V_\beta8$ and $V_\beta7$ chains expressed in CD44^{hi}NK1.1$^+$ mouse thymocytes than to other mouse V_β (34, and data not shown). Even more striking is the similarity that we found between the α chains.

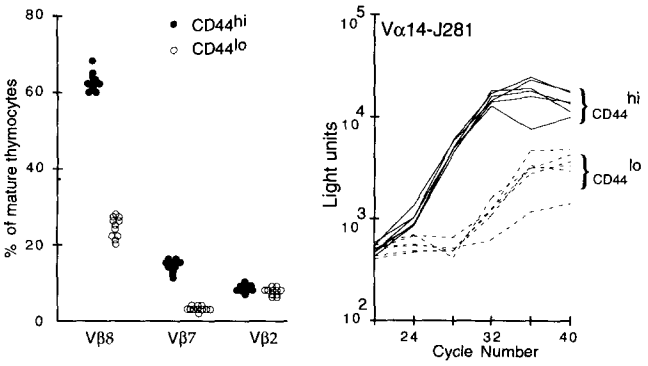

Figure 5. TCR-β and -α repertoire of mature CD44^{hi} thymocytes in MHC congenic mice. (*Left*) Percentage of $V_\beta8^+$, $V_\beta7^+$, and $V_\beta2^+$ cells determined by FACS® analysis among CD44^{hi} and CD44^{lo} subsets of B10 (H-2^b), B10.D2 (H-2^d), B10.A (H-2^a), B10.BR (H-2^k), B10.M (H-2^f), B10.RIII (H-2^r), B10.S (H-2^s), B10.Q (H-2^q), B10.SM (H-2^v), B10.P (H-2^p), and B10.PL (H-2^u) mice. (Right) Quantitation of V_α14-J281 rearrangements among FACS®-sorted CD44^{hi} and CD44^{lo} subsets of B10, B10.D2, B10.BR, B10.M, B10.RIII, and B10.S mice. Values are averaged from duplicate samples of 2 × 10^4 cells.

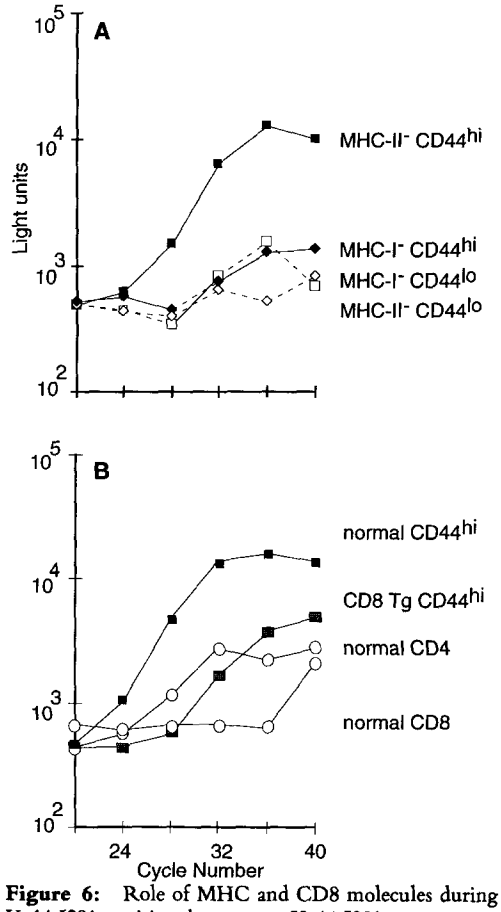

Figure 6: Role of MHC and CD8 molecules during the selection of V_α14-J281–positive thymocytes. V_α14-J281 rearrangements are measured as average values from duplicate samples of 2 × 10^4 sorted cells. (*A*) CD44^{hi} or CD44^{lo} mature TCR-α/β^+ thymocytes obtained from Aβ^{b-} (*MHC-II$^-$*) and β_2m$^-$ (*MHC-I$^-$*) mice. (*B*) CD4$^+$, CD8$^+$, or CD44^{hi} mature TCR-α/β^+ thymocytes from normal B10.BR mice (note that 24% of the CD4$^+$ and 1.5% of the CD8$^+$ population were CD44^{hi}) and CD44^{hi} mature TCR-α/β^+ thymocytes from CD8.1 transgenic B10.BR mice.

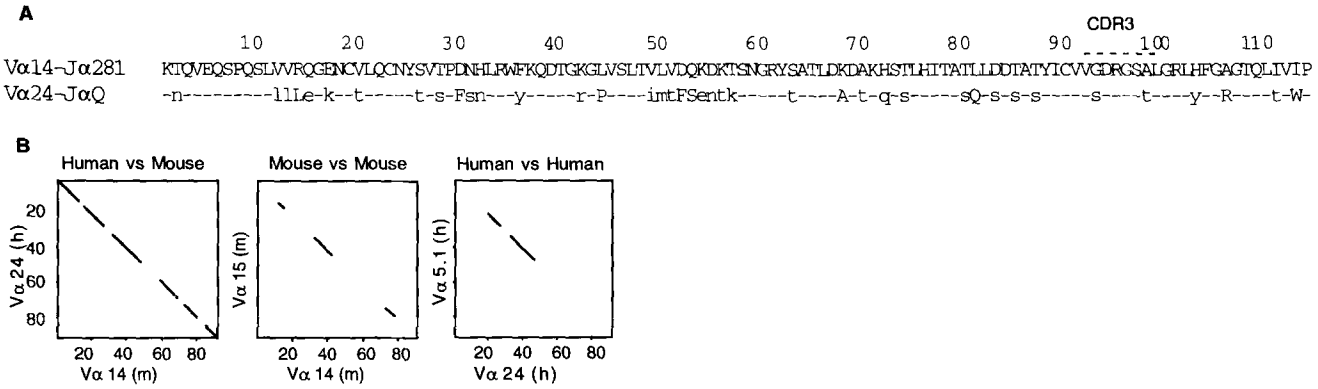

Figure 7. The human homologues of mouse $V_\alpha14$ and J281 gene products. (*A*) Alignments of mouse $V_\alpha14$-J281 and human $V_\alpha24$-JQ. (*B*) Pustell matrix comparison (43) of $V_\alpha14$ and $V_\alpha24$, $V_\alpha14$ and the closest mouse V_α ($V_\alpha15$), $V_\alpha24$ and the closest human V_α ($V_\alpha5.1$). In Pustell matrix, each dot represents a similarity above 60% in a window of eight amino acids centered on the *x* and *y* coordinates as calculated with the Mac Vector program, using a PAM250 scoring matrix (IBI, New Haven, CT).

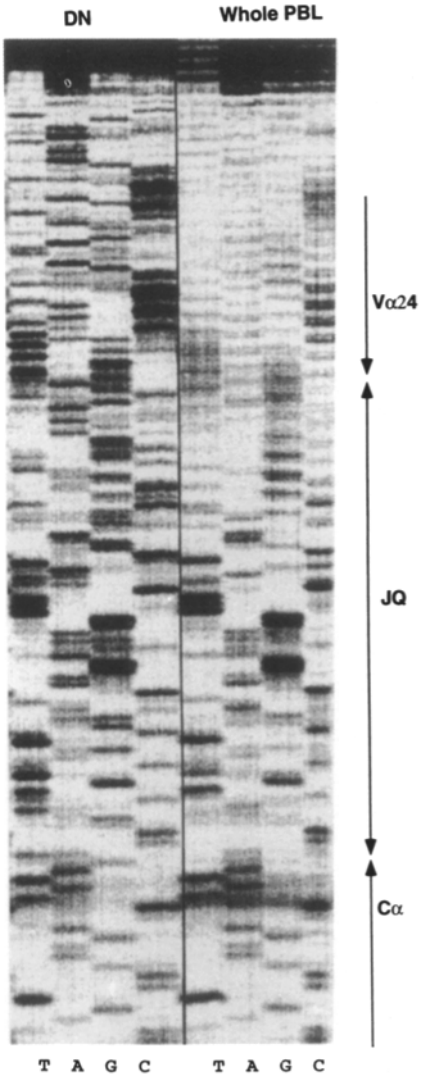

Figure 8. The human homologue of mouse NK1.1$^+$ TCR-α/β^+ cells. Polyclonal sequencing of $V_\alpha24^+$ TCR α chains from human DN and unseparated PBL. TCR-α/β^+ DN cells (0.6% of human PBL), were FACS®-sorted and compared with whole PBL (80% of which are TCR-α/β^+).

It has been reported that $V_\alpha24$-JQ is expressed as an invariant (without junctional variability) chain in some human DN PBL and clones (32, 33). According to GenBank, the human $V_\alpha24$ has more homology to mouse $V_\alpha14$ than any other mouse V_α has and the closest protein sequence to mouse J281 is that of human JQ (Fig. 7, *A* and *B*). Using the Blast analysis software (35), the homology score between $V_\alpha14$ and $V_\alpha24$ was 316 whereas the closest other mouse or human V_α was at 180 (mouse $V_\alpha15$) and 223 (human $V_\alpha5.1$), respectively; most of the other V_α are below 160 (identity would be 480 and homology scores are considered significant above 50). The closest protein sequence to the mouse J281 recorded in GenBank is that of human JQ (score = 80 and identity is at 107) whereas all other mouse or human J regions have a score below 61. In accordance with the mouse data, we found a readable JQ sequence after direct polyclonal sequencing (from the C_α end) of $V_\alpha24$-C_α PCR amplified products from freshly isolated human peripheral blood DN cells (Fig. 8), confirming their predominant use of JQ without junctional polymorphism. The importance of the junctional region for the selection of this cell population is indicated by the conservation of the unique VVG (or S) DRGS sequence in the CDR3 of both mice and humans.

Discussion

Do NK1.1$^+$ $V_\alpha14^+$ Thymocytes Originate from Mainstream Thymocyte Precursors or Do They Constitute a Distinct Lineage? Altogether, these results suggest that a unique recombination of one V_α and one J_α TCR gene, and a corresponding nonpolymorphic MHC class I molecule/peptide(s) for which this TCR-α chain has affinity, have been conserved in both mice and humans. The rare occurrence of such a V_α-J_α recombination and its selection at the amino acid rather than the nucleotide level, suggest that this population does not represent a separate lineage that is genetically programmed to rearrange this particular set of TCR genes. The possibility

that this population is composed of cells that have escaped the "classical" positive selection process and are expanded as mature thymocytes by the recognition of self or environmental antigens is also unlikely, because, if this were the case, one would not expect to see a TCR repertoire that is solely restricted by nonpolymorphic MHC class I/peptide ligand(s). Rather, we favor a model where mainstream thymocyte DP precursors expressing the appropriate V_α14-J281/V_β8, V_β7, or V_β2 TCRs recognize nonpolymorphic MHC class I/peptide ligand(s) selectively displayed on the surface of bone marrow–derived thymic cells. They undergo a stochastic downmodulation of CD4, CD8, or both CD4 and CD8. The resulting $CD4^+$ and DN cells, because of their intrinsic (CD8 independent) affinity for the selecting ligands, are rescued at the second stage of the positive selection process, following a model similar to the one proposed by Chan et al. (36), whereas the $CD8^+$ cells, after upregulation of their TCR level, reach the negative selection threshold and are eliminated. The particular $CD44^+NK1.1^+$ surface phenotype and the unique lymphokine secretion potential of this subset could be related to the particular affinity of the TCRs for the MHC class I molecule and/or the type of APC involved in presenting the selecting ligand.

The MHC Class I Ligand. Two findings suggest that a potential candidate for this conserved MHC molecule may lie in the Qa-1 family of genes. First, anti-Qa-1 $CD8^+$ mouse T cell clones predominantly use the same V_β chains (V_β8, V_β7, and V_β2) as the $NK1.1^+$ TCR-α/β^+ T cells (37). Second, although most MHC genes, whether polymorphic or not, have diverged widely between species, Qa-1 molecules are very homologous to the rat RTBM.1 molecule and share unique features of their peptide binding cleft with both the rat RTBM.1 and the human HLA-E molecules (38). As for the peptides involved in the selection of this cell subset, the evidence that these cells can be obtained in long-term fetal thymic organ culture (4) suggests that they must be self-antigen(s). In line with the observation that tryptic digests of heat shock proteins stabilize the surface expression of Qa-1 (39), one may speculate that the selecting peptides are members of the widely conserved family of stress proteins. Once in the periphery, these cells might respond to upregulated levels of these same peptides or, alternatively, to foreign antigens that are structurally related, in an analogous manner to the γ/δ T cells that respond to heat shock proteins (40).

What Is the Function of the $NK1.1^+$ $V_\alpha14^+$ Thymocytes? The conservation through speciation events of such an unusual T cell subset bearing an invariant TCR-α chain associated with the recognition of nonpolymorphic MHC class I/self peptide(s) strongly argues for an important, though yet uncharacterized, function for these cells. Since their particular TCRs are unlikely to recognize most foreign pathogens, they could be recruited after upregulation of their self-ligand(s) or recognition of variant, foreign peptides. Alternatively, their reactivation may result from downregulation of the NK-like molecules (or of their ligands), as it was recently suggested that some of these may bind MHC class I molecules and transduce inhibitory signals (41). One potential role for the $NK1.1^+V_\alpha14^+$ thymocytes is hinted at by their unique property of being able to produce large quantities of IL-4 upon primary stimulation in vitro (3, 7) and within minutes of TCR cross-linking in vivo (8). Because the early secretion of IL-4 appears to be critical for the generation of Th2-type humoral immune responses over Th1-type cell-mediated responses, the $NK1.1^+V_\alpha14^+$ T cells may play a role in determining the Th1/Th2 phenotype of some immune responses to particular pathogens or to self-antigens (9, 42).

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Note added in proof: In the same issue of the Journal, P. Dellabona et al. (44) report a detailed analysis of the human blood $V\alpha24\text{-}JQ^+$ T cells that further demonstrates the similarity to the mouse $NK1.1^+$ $V\alpha14\text{-}J281^+$ T cell subset.

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