An Invariant T Cell Receptor α Chain Defines a Novel TAP-independent Major Histocompatibility Complex Class Ib–restricted α/β T Cell Subpopulation in Mammals

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Summary

We describe here a new subset of T cells, found in humans, mice, and cattle. These cells bear a canonical T cell receptor (TCR) α chain containing hAV7S2 and AJ33 in humans and the homologous AV19-AJ33 in mice and cattle with a CDR3 of constant length. These T cells are $CD4^-CD8^-$ double-negative (DN) T cells in the three species and also CD8αα in humans. In humans, their frequency was ~1/10 in DN, 1/50 in $CD8\alpha^+$, and 1/6,000 in $CD4^+$ lymphocytes, and they display an activated/memory phenotype ($CD45RA^{lo}CD45RO^+$). They preferentially use hBV2S1 and hBV13 segments and have an oligoclonal Vβ repertoire suggesting peripheral expansions. These cells were present in major histocompatibility complex (MHC) class II– and transporter associated with antigen processing (TAP)-deficient humans and mice and also in classical MHC class I– and CD1-deficient mice but were absent from β2-microglobulin–deficient mice, indicating their probable selection by a nonclassical MHC class Ib molecule distinct from CD1. The conservation between mammalian species, the abundance, and the unique selection pattern suggest an important role for cells using this novel canonical TCR α chain.

Key words: invariant T cell receptor α chain • $CD4^-CD8^-$ T cells • humans • cattle • mice

B and T lymphocytes display a wide repertoire of antigen receptors, made by random recombination of V, (D), and J segments and trimming/addition of nucleotides at the junctions between these rearranged segments (1). Besides the mainstream lymphocytes, three different types of cell subpopulations have been described that have a limited repertoire diversity: the B1 B cell subset (2, 3), some γ/δ T cell subpopulations (4), and the TCR α/β^+ NK1 T cells (5). Restricted repertoires seem to define discrete lymphocyte subpopulations at the frontier between innate and adaptive immunity, as these selected repertoires may allow the presence of a high frequency of preexisting cells reactive against phylogenetically conserved antigens (6, 7). Alternatively, cells with such a restricted repertoire may play an immunoregulatory role or another physiologic function, such as the wound healing, that results from the secretion of keratinocyte growth factor (8) by γ/δ dendritic epithelial cells (DECs)[1] recognizing self-ligands (9).

B1 cells, which appear early during embryonic life, use recurrent VDJ combinations with few "N" additions (10), and seem to be selected by endogenous ligands (11). Similarly, some γ/δ T cell subsets appear early during ontogeny and use peculiar V-J combinations without N additions (12). These recurrent sequences seem to be favored by enzymatic constraints linked to the recombination process, such as homologous region–guided recombination (4).

F. Tilloy and E. Treiner contributed equally to this work.

[1]*Abbreviations used in this paper:* APC, allophycocyanin; β2m, β2-microglobulin; B6, C57BL/6; DEC, dendritic epithelial cell; DN, $CD4^-CD8^-$ double-negative; IEL, intraepithelial lymphocyte; TAP, transporter associated with antigen processing; TC, Tricolor.

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Cells bearing these invariant γ/δ TCRs seem to be also selected by endogenous ligands as, in mice deficient for these segments, they are replaced by cells expressing other VγVδ combinations but similar clonotypic motifs (13).

NK1 T cells use an "invariant" α chain (mAV14AJ18 in mice [14], hAV24AJ18 in humans [14, 15]) with a CDR3 of constant length paired with a limited number of Vβ segments (BV2S1, 7S1, and 8S2 in mice, BV11S1 in humans). NK1 T cells display peculiar phenotypic characteristics: they are $CD3^{int}$, $CD4^+$, or $CD4^-CD8^-$ (DN), and have both activation ($CD44^{hi}$ and $3G11^{lo}$) and NK markers (NKRP1, Ly49) (5). In vivo development of NK1 T cells requires the MHC class Ib CD1d molecule (16), whose expression is β2-microglobulin (β2m) dependent but transporter associated with antigen processing (TAP) independent (17). In both humans and rodents, mature NK1 T cells recognize some glycolipids (such as α-galactosylceramide) in a CD1d-restricted fashion (18, 19) as well as some exogenous protozoan glycosylphosphatidylinositol (GPI [20]), but the exact endogenous ligand(s) presented physiologically by CD1d have not yet been identified. The most salient feature of NK1 T cells is their ability to secrete very rapidly large amounts of IL-4 in a primary response (21). They can also secrete large amounts of IFN-γ, especially when triggered through their NKRP1 receptor (22). However, their true in vivo functions are still enigmatic, though there is evidence that they may be involved in Th1/Th2 class polarization (23), in IL-12–induced tumor rejection (24), and in autoimmunity (25–27).

Mainstream α/β T lymphocytes express either CD8 or CD4 accessory molecules and respond to peptidic antigens presented in low amounts by either MHC class I or class II molecules. The absence of accessory molecules in the DN T cell subpopulation suggests that these cells may recognize high-density ligands (such as α-galactosylceramide or glycosylphosphatidylinositol presented by CD1 for NK1 T cells) and/or display TCRs with high affinity. Indeed, most γ/δ T cells are DN or CD8αα, and the NK1 T cells comprise both DN (and $CD8\alpha\alpha^+$ in humans [28]) and $CD4^+$ cells, though the CD4 molecule in these latter does not seem to play a role in the binding affinity (14, 29). DN α/β^+ T cells represent between 0.5 and 2% of PBLs in normal individuals and comprise the aforementioned NK1 T cells and other cells of unclear specificity or functions. Some of these also recognize CD1d, while others are specific for glycolipids presented by CD1a, b, or c (30–32).

NK1 T cells thus far represent the only known T cell subset that has conserved its TCR, its specificity, and its functional features between rodents and primates, suggesting that they may play an ancient and important physiological role (18). Here, we report on the existence of another phylogenetically conserved population of T cells in mammals, defined by the expression of a novel canonical TCR α chain comprising the hAV7S2 and AJ33 elements in humans and the homologous AV19 and AJ33 elements in mice and cattle. $V\alpha7/19\text{-}J\alpha33^+$ T cells show an oligoclonal TCR β chain repertoire and are selected by a β2m-dependent but TAP-independent molecule that is neither a classical MHC class I molecule nor CD1d. The conservation between species of T cells displaying this limited repertoire and their unusual restriction pattern suggest that this population may serve a function complementary to that played by NK1 T cells.

Materials and Methods

Antibodies. In humans, the following antibodies, anti-CD28–PE, anti-CD45RA–PE, anti-CD45RO–PE, anti-CD57–FITC, anti-CD56–PE, anti-α/β TCR–allophycocyanin (APC), anti-α/β TCR–PE, anti-CD8β–PE, biotinylated anti-CD4, and anti-CD8α, were obtained from Immunotech; anti-CD27–FITC and anti-α/β TCR–FITC were purchased from PharMingen. Anti-CD8α–FITC and –Tricolor (TC), anti-CD4–FITC and –PE, and anti-CD3–TC were obtained from Caltag Laboratories. An anti-CD4–FITC (Diaclone) recognizing an epitope distinct from the anti-CD4 magnetic beads was also used. Streptavidin–Red 613 was purchased from GIBCO BRL. Streptavidin-PE and -TC were obtained from Caltag Laboratories.

In mice, anti-CD4 (RM4-5 or RM4-4)–PE, anti-CD8α– or anti-CD8β–FITC, biotinylated or Cychrome-conjugated anti-α/β TCR and streptavidin-APC were obtained from PharMingen. Anti-α/β TCR–TC was obtained from Caltag Laboratories. Bovine anti-CD4 and anti-CD8α antibodies were provided by J. Naessens, International Laboratory for Research on Animal Diseases (ILRAD, Nairobi, Kenya).

Human Cell Preparations. Heparinized blood was obtained from healthy donors or from MHC-deficient patients, and PBMCs were purified by Ficoll-Hypaque (Amersham Pharmacia Biotech) gradient centrifugation. Cells were frozen in liquid nitrogen until use.

Bovine Cell Separations. Bovine blood was provided by Mr. Noé from the GENA Laboratory, Institut National de la Recherche Agronomique (INRA, Jouy en Josas, France). After Ficoll-Hypaque separation, PBLs were labeled with anti-CD4 and/or anti-CD8 (both Ig2a), which were revealed with an FITC-conjugated goat anti-Ig2a serum. Cells were FACS® sorted into CD4 or CD4/CD8 positive or negative fractions as indicated, and the relative amounts of boCα- or boAV19-boAJ33 transcripts were estimated by kinetic PCR (see below).

Mice. C57BL/6 ($H\text{-}2^b$) (B6), BALB/c ($H\text{-}2^d$), 129 ($H\text{-}2^b$), DBA/2 ($H\text{-}2^d$), and CBA ($H\text{-}2^k$) mice were bred in our own specific pathogen–free animal facility. $\beta2m^{-/-}$ and $I\text{-}A^{b-/-}$ mice, backcrossed nine times to B6, were obtained from the Centre National de la Recherche Scientifique central animal facility (CNRS, Orleans, France). $TAP^{-/-}$ B6/129 and $CD8^{-/-}$ deficient mice were obtained from The Jackson Laboratory. $K^{b-/-}$ $D^{b-/-}$ mice have been described (33). $CD1^{-/-}$ mice were generated by S.-H. Park and A. Bendelac (unpublished results).

Immunofluorescence Studies. Surface phenotyping was carried out on a FACSCalibur™ cytometer (Becton Dickinson). Three-color sorting (CD4, CD8α, and α/β TCR staining) was carried out on a FACS Vantage™ (Becton Dickinson) to obtain α/β^+ DN, $\text{-}CD8\alpha^+$, or $\text{-}CD4^+$ cells. Reanalyzed fractions were >99% pure. In some experiments, DN cells were seeded into 96-well PCR microplates at the indicated cell concentrations using a single cell deposition unit (Becton Dickinson). $DN/CD8^+$ fractions, enriched or depleted for a given marker, were obtained by FACS® sorting negative and positive fractions for this marker after quadruple staining with anti-CD4, anti-CD8α, anti-α/β TCR, and the relevant marker (either CD8β, CD45RA, CD45R0, CD56, CD57, CD28, or CD27).

In some experiments, DN and $CD8\alpha^+$ enriched fractions were

obtained using paramagnetic beads and the VarioMacs system (Miltenyi Biotec). In brief, cells at 10^7/100 μl were resuspended in PBS, 0.5% BSA, 5 mM EDTA and incubated with anti-CD4 paramagnetic microbeads, washed once, resuspended in 500 μl of the same buffer, and passed through an RS column. Effluent was collected as the DN^+CD8^+ fraction. After thorough washes of the column, the eluate was collected as $CD4^+$ fraction. In these conditions, the $CD4^+$ fraction was >95% pure.

Oligonucleotides. All primers and probes were obtained from Genosys Biotechnologies and used without further purification. Probes were FITC conjugated. The following primers have been described previously (14): mAV14, mAJ18, mAC-5′, mACV, mAC-3′, hACV, hAC-5′, and hAC-3′. The following primers were used (m, bo, and h stand for murine, bovine, and human; p is for probe; in and out are for the inner and outer primer, respectively, in the case of nested PCR): mAV19, CACTTTCCTGAGCCGCTCGAA; mAJ33, biotin-TTAGCTTGGTCCCAGAGCCCC; pmAV19, GCTTCTGACAGAGCTCCAG-FITC. The mouse-specific Vβ and Vα primers were slightly modified from Casanova et al. (34). The human Vβ primers were derived from Puisieux et al. (35) as modified by Martinon et al. (36). The other human primers were as follows: hAV7S2, biotin-CCTTAGTCGGTCTAAAGGGTACAG; hAJ33, CCCAGCGCCCCAGATTAA; hBJ1S2, GTCTCCCTCAGTCTGGCTCCG; hBJ1S3in, GGTGGTAAGGTCAGAGGCTCTTTTATC; hBJ1S3out, TCTGTGTGAGGGAGAGAAACG; hBJ1S5, CAAGACACACCAAAGGGAACGC; hBJ2S2, CTCTCCCAGCACCCAGAACCA; hBJ2S4, CGCACAAAAACCCGAGCGCAG; hBJ1S6in, CCAGGCACCCCCGAGTCAAGA; hBJ1S6out, GGCAACACAGCAGAGCAACAA; hBJ2S7out, TCGTCCTCTCCAGTCTCGCCT; hBJ2S7in, CGCCCTCTGCTCAGCTTTCCG; and hAJ33in, CAAAAGCTGACTTGCAGGCATCG. In cattle, the following primers were used: boAV19, biotin-CATTCCTTAGACGCTCTGATGCACA; boAJ33, GCCCCAGATCCACTGATAGTTGC; pboAV19, FITC-GTGGAGTTCCTTCAGAAGGAG; bo5′CA, biotin-AGGACCCCAACCCCACTGTGT; bo3′CA, CTTCAGGAGGAGGATGCGGAAC; and pboCA, FITC-CACTGGATTGGGGGCTTC.

Nucleic Acid Preparation. Genomic DNA was obtained as crude cell lysate by adding to the cell pellets (1–10^6 cells) a solution containing 200 μg/ml proteinase K (Promega Corp.), 0.5% Tween 20 (Sigma-Aldrich), 10 mM Tris-HCl, pH 9, 50 mM KCl, 2.5 mM $MgCl_2$. After resuspension, this solution was incubated for 2 h at 56°C, and the proteinase K was denatured by incubation at 95°C for 20 min.

Total RNA was extracted from 1–5 × 10^5 cells with the RNAble solution (Eurobio), ethanol precipitated with addition of 2 μl Pellet Paint coprecipitant (Novagen), and resuspended in 20 μl sterile water. Reverse transcription was carried out as described with random hexamers and oligo-dT priming (37).

Quantitative Kinetic PCR. Quantitative kinetic ELISA PCR was carried out as described (37). Polyclonal samples were divided into two aliquots before RNA extraction except in a few instances in which the amount of sorted cells was too low (<2 × 10^4). Average values are shown.

In the most recent experiments, an ABI prism 7700 (Perkin-Elmer) apparatus was used instead of the ELISA assay to monitor the amount of amplicon during the PCRs. This apparatus is based on the 5′ to 3′ nuclease activity of Taq polymerase (38), which allows the release of a fluorescent reporter during the PCR. A probe labeled with both a reporter and a quencher dye is spiked into the PCR mix at the beginning of the reaction. The sequences of the Taqman probes used in this study are the following: hCA, mGCATGTGCAAACGCCTTCAACAACAqp; hAV7.2, mTGAAAGACTCTGCCTCTTACCTCTGTGCqp; mAC, mCTCCCAAATCAATGTGCCGAAAACCAqp; mAV19, mTCCAGATCAAAGACTCTGCCTCATACCTCTGqp; and mAV14, mCACCCTGCTGGATGACACTGCCACqp; where m preceding each sequence stands for FAM and qp following for TAMRA blocked with a phosphate. When using the ABI prism 7700 apparatus, the following primers were also used: hAV7S2, TCCTTAGTCGGTCTAAAGGGTACAG; hAJ33, CCAGCGCCCCAGATTAA; hAC-5′, ACCCTGACCCTGCCGTGT; hAC-3′, GGCTGGGGAAGAAGGTGTCTT; mAV14, TGGGAGATACTCAGCAACTCTGG; mAJ18, CCAGCTCCAAAATGCAGCC; mAV19, CTTTCCTGAGCCGCTCGAA; mAJ33, CTTGGTCCCAGAGCCCC; mAC-5′, CCTCTGCCTGTTCACCGACTT; and mAC-3′, CGGTCAACGTGGCATCACA. In these quantitative kinetic PCR methods, for two samples containing the same amount of Cα, a shift of *n* cycles along the x-axis of the two amplification curves represents an $\sim 1.8^n$-fold difference in the two samples studied.

Single Cell Repertoire Analysis. Single human DN α/β T cells were sorted into 84 wells of PCR plate. 11 of the remaining wells were used as negative controls and 1 as positive. After cell lysis, PCR amplification was carried out for 25 cycles using the following primers: 29 Vβ, 7 Jβ, AV7S2out, AJ33out, and as positive control AV24 and AJ18out. The resulting reaction mix was diluted fivefold, and aliquots were amplified in a second PCR reaction with an AV7S2/AJ33 primer pair for 40 cycles. The specific AV7S2-AJ33 amplicons were detected by ELISA or using the Taqman assay. Aliquots of the AV7S2-AJ33–positive wells from the first PCR reaction were then PCR amplified in 24 individual PCR reactions with 1 (or 2) specific Vβ primer(s) and a mixture of 7 Jβ primers for 45 cycles. The positive reactions were detected by agarose gel electrophoresis, and the amplicons were sequenced as described below.

Sequencing. Polyclonal sequencing was performed after amplification for 42 cycles with hAV7S2, boAV19, or mAV19 and either AJ33 or Cα primer pairs. The amplicons were purified using the Wizard PCR clean-up system (Promega Corp.) and sequenced using either a nested primer in the Cα constant region (in mouse) or the same AV19 or AV7S2 primer as used for amplification, as appropriate. Sequences were performed with the Thermosequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech) with ^{33}P-ddNTP.

T–T Hybridoma Generation. DN and $CD8^+$ T cells were obtained from $TAP^{-/-}$ mice after depleting $CD4^+$ T cells with anti-CD4 antibody (GK1.5 and 172.4) plus rabbit complement (Behring AG) followed by centrifugation over a density gradient (Lympholyte M; Cedarlane Laboratories). 2 × 10^6 cells were stimulated, in the presence of 4 × 10^6 γ-irradiated (2,500 cGy) splenic cells, for 2 d with soluble anti-α/β TCR (H57) at 7.5 μg/ml which had been precoated for 1 h at 37°C in flat-bottomed 96-well plates. Cells were cultured for an additional 2 d with IL-2 (100 U/ml) in complete culture medium: RPMI 1640, 15% FCS, 2 mM l-glutamine (GIBCO BRL), penicillin 100 U/ml, streptomycin 100 μg/ml, 50 μM β-ME. At day 4, 8 × 10^6 cells were fused with 2.5 × 10^7 TCR α/β^- BW5147 thymoma cells using standard procedures, plated at 4 × 10^4 cells per well in 96-well microplates, and selected in HAT medium. Resulting clones were screened for expression of AV19-AJ33 rearrangement on genomic DNA followed by sequencing. Vβ were characterized by 17 Vβ-specific PCR reactions, and the resulting amplicons were then sequenced. Results were verified by immunofluorescence with specific anti-Vβ antibodies.

Results

A previous analysis of TCR α chain repertoire of human DN T cells indicated that besides the invariant hAV24AJ18 TCR α chain, another TCR α chain was frequently expressed that also had a restricted AVAJ usage and recurrent junctional features (39). This α chain, which was encoded by rearranged hAV7S2AJ33 elements, had a CDR3 of constant length but some variability in the two junctional codons. A GenBank search showed that among the 20 TCR α chains sequenced in cattle, one chain (BOTCRA14) used the homologous Vα and Jα segments with a CDR3 of the same length (40). In mice, no homologous invariant α chain had been described, but the corresponding Vα (mAV19) and Jα (mAJ33) segments display high homology with their human counterparts, as there is only one amino acid difference between the murine AJ33 and its human counterpart (41, 42). Based on these observations, which suggested the existence of another conserved T cell population in mammals, we undertook an extensive analysis of the frequency and repertoire of the T cells bearing this particular combination of AVAJ elements in these three species.

Canonical hAV7S2AJ33 TCR α Chains, as well as Their Murine and Bovine Homologues, Are Abundantly Expressed in DN T Cells

Homologous hAV7S2AJ33 or bo/mAV19AJ33 Transcripts Are Enriched in Human, Bovine, and Murine DN T Cells. To determine the frequency and coreceptor phenotype of T cells bearing hAV7S2AJ33 TCR α chains, PBLs were separated into highly purified α/β DN, $CD4^+$, or $CD8\alpha^+$ (i.e., $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$) subsets, and the amount of hAV7S2AJ33-encoded transcripts in duplicate fractions was quantified by kinetic PCR. Although the amounts of Cα chain were similar in all samples (Fig. 1 A), there was a large shift to the left of the hAV7S2AJ33 amplification curves for the DN (8.5 cycles) and the $CD8\alpha^+$ fractions (5 cycles) (Fig. 1 B) compared with the $CD4^+$ fractions, indicating that DN and $CD8\alpha^+$ cells contained ~150–360- and 19–32-fold more hAV7S2AJ33 transcripts, respectively, than $CD4^+$ lymphocytes. hAV7S2AJ33 transcripts were enriched in DN and $CD8\alpha^+$ T cell preparations from all individuals tested, and on average the enrichment levels of hAV7S2AJ33 transcripts in DN T cells were much higher than those observed for the NK1 T cell–specific invariant α chain hAV24AJ18 (data not shown).

Because a similar invariant α chain had been sequenced once in cattle (40), we examined its distribution within $CD4^+$, $CD8^+$, DN, or ($CD4^+$ plus $CD8\alpha^+$) sorted cells obtained from bovine blood. As shown in Fig. 1, C and D, the levels of boAV19AJ33 transcripts in the DN fractions were as high as those observed in humans. No boAV19AJ33 amplification could be obtained with the $CD8\alpha^+$ fraction, and the comparison of $CD4^+$ and $CD4^+$ plus $CD8\alpha^+$ amplification curves suggested that bovine $CD8\alpha^+$ cells did not express high amounts of boAV19AJ33 transcripts.

In mice, no canonical TCR α chain rearrangement involving mAV19 and mAJ33, the murine elements that are homologous to hAV7S2 and hAJ33, respectively, had been described. However, quantitative PCR analysis of mAV19AJ33 transcripts within highly purified murine α/β DN, $CD4^+$, and $CD8\alpha^+$ lymphocytes indicated that, as in human and bovine blood, increased mAV19AJ33 expression was observed in the DN fraction from lymph nodes (Fig. 1, E and F). The shift of the amplification curves between DN and $CD4^+$ cell preparations was lower than in humans and cattle,

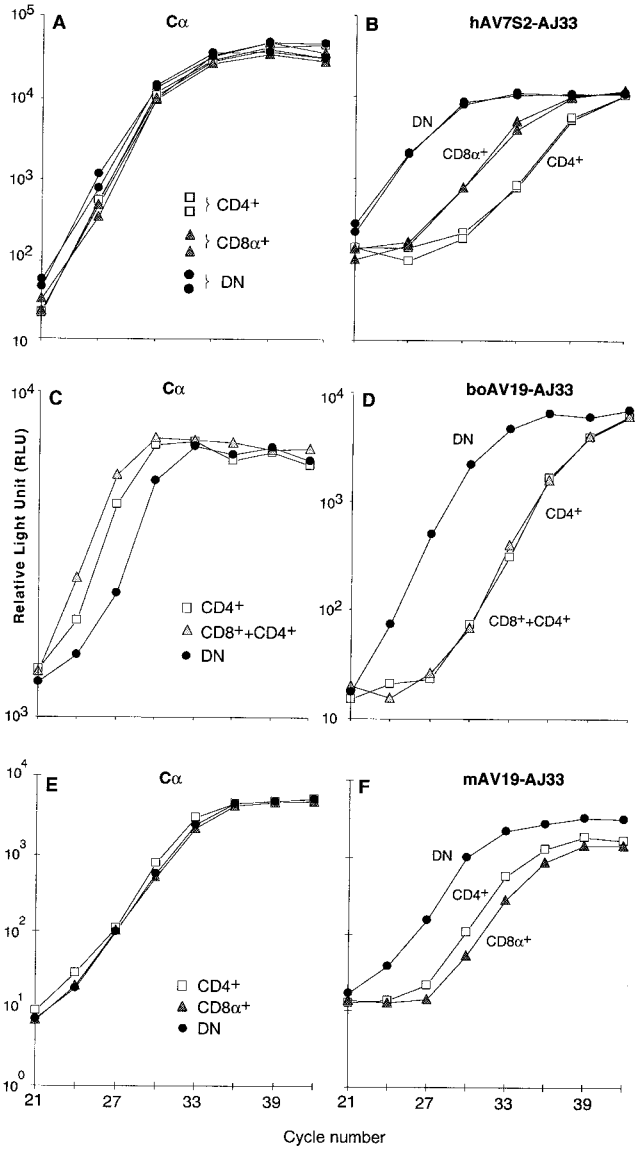

Figure 1. High levels of hAV7S2/AV19-AJ33 TCR α chain are present in DN T cells from three mammal species: the indicated cell subpopulations were purified by FACS® sorting from human (A and B) or bovine (C and D) PBLs and from mouse lymph nodes (E and F). Duplicate RNA preparations were obtained and, after reverse transcription, PCR was carried out for the indicated gene segments. The amount of amplicon was quantified by ELISA at the indicated cycle. Averaged values of duplicates are shown for C–F. $CD8\alpha^+$ fraction contains both $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ TCR α/β^+ T cells. In cattle, no amplification could be obtained with boAV19-AJ33 primers in the $CD8\alpha^+$ fraction, and thus the corresponding curves are not displayed.

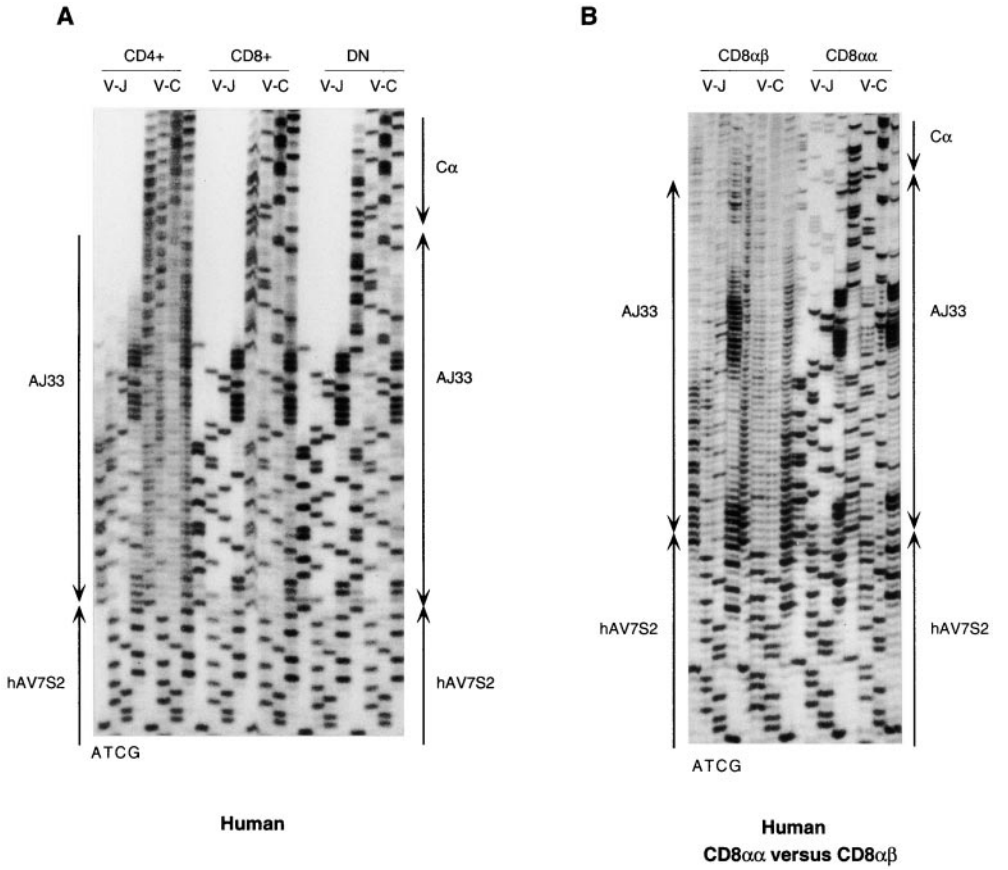

Figure 2. (A) The CDR3 of hAV7S2-AJ33 TCR α chain is of constant length in DN T cells and in human CD8αα$^+$ T cells. PCR amplifications from hAV7S2 to Cα (V-C) or to AJ33 (V-J) were carried out on cDNA from the T cell subpopulations indicated, and polyclonal sequencing was carried out using the same AV7S2/AV19 primer as used for amplification. Panel B displays polyclonal sequencing of FACS®-sorted CD8αβ$^+$ or CD8αα$^+$ TCR α/β$^+$ human PBLs.

suggesting that mAV19AJ33-bearing cells were less abundant in mice.

hAV7S2-AJ33, mAV19AJ33, and boAV19AJ33 Transcripts Display the Same CDR3 Length in the Three Species. To examine the length of the CDR3 regions of the hAV7S2AJ33 transcripts and their homologues in mice and cattle, we carried out polyclonal sequencing of Vα-Cα or Vα-Jα amplicons obtained from the various lymphocyte subpopulations in these species. As shown in Fig. 2 A, a readable hAV7S2AJ33 sequence was obtained from hAV7S2-Cα amplicons derived from DN and CD8α$^+$ samples but not from CD4$^+$ samples. Therefore, this indicated that in the former but not the latter cells, the hAV7S2 segment was predominantly rearranged to the hAJ33 segment and comprised a CDR3 of constant length. The polyclonal hAV7S2AJ33 sequence displayed some heterogeneity at the VJ junction, in agreement with previous results from Porcelli et al. showing some variability of the two N-encoded amino acids (39).

In cattle, the picture was slightly different, as polyclonal sequencing of boAV19-Cα amplicons demonstrated the predominant presence of the invariant boAV19AJ33 chain in DN cells, but not in CD4$^+$ or CD8$^+$ cells (data not shown). At the VJ junction, the polyclonal sequence derived from DN cells displayed some junctional heterogeneity, indicating also some variability of the two amino acids encoded by N additions. In mice, polyclonal sequencing of the mAV19-Cα amplicons showed a fully readable sequence for the DN but not for the CD8α$^+$ fraction (data not shown). Moreover, the VJ sequence displayed no heterogeneity and corresponded to a "germline" junction without nucleotide trimming or N addition (see also Table

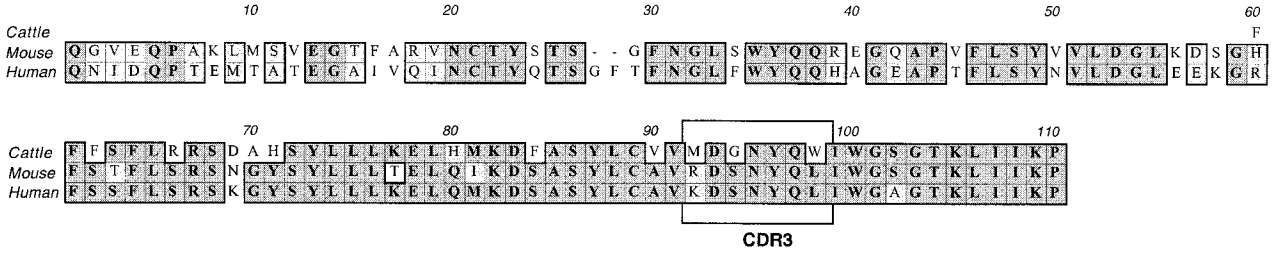

Figure 3. High homology of human, cattle, and mouse hAV7S2/AV19-AJ33 α chains. Homologies were scored using the default PAM250 of ClustalW as implemented in MacVector software (Oxford Technology). Identities are bold and shaded in gray; similarities are light gray. The CDR3 region is boxed.

II below). Altogether, these results demonstrated that in the three species, DN α/β^+ T cells were enriched for cells bearing TCR α chains with highly homologous AVAJ elements (hAV7S2AJ33, mAV19AJ33, and boAV19AJ33) and constant CDR3 length as shown in the multiple alignment displayed in Fig. 3. This novel canonical TCR α chain will be referred to hereafter as the "invariant" Vα7/19-Jα33 TCR α chain.

Direct Numbering of Cells Bearing Invariant Vα7/19-Jα33 Chains. A large amount of invariant α chain transcripts does not formally demonstrate the existence of a large number of invariant TCR α chain–bearing cells, as this transcript could have been expressed at very high levels in a few cells. To more directly estimate the number of cells using the invariant Vα7-Jα33 chain, single α/β^+ DN cells from three different subjects were sorted into PCR plates using a single cell deposition unit. FACS®-sorted α/β^+ TCR CD8α^+ or CD4$^+$ cell suspensions were also serially diluted into PCR plates to get 12 replicates of the indicated cell concentrations. After cell lysis, hAV7S2AJ33 PCR amplification was carried out on genomic DNA with luminometry detection of the amplicons. As shown in Fig. 4 A, the assay was sensitive enough to detect a single cell harboring the relevant rearrangement and yielded a frequency of Vα7-Jα33$^+$ DN cells of $\sim$1/7.4. Using limiting dilution analysis (LDA), the frequency observed for CD8α^+ cells was 1/36 (confidence interval: 1/25–1/70; Fig. 4 B). Sequence analysis of amplicons from single cell preparations obtained with DN and CD8α^+ cells demonstrated the presence of invariant Vα7-Jα33 transcripts in all cases. A similar analysis on CD4$^+$ cells yielded a frequency of Vα7-Jα33$^+$ cells of 1/1,093 (1/751–1/2,003; Fig. 4 C). Importantly, sequencing of the positive wells at concentrations where the Vα7-Jα33 amplicons were derived from one cell showed that out of six positive wells, two corresponded to a rearrangement of the hAV7S2 to hAJ34 (which were amplified because of the short genomic distance [$\sim$700 bp] separating hAJ34 from hAJ33), three corresponded to a rearranged Vα7-Jα33 chain with a different CDR3 length, and only one corresponded to the invariant Vα7-Jα33 chain. Thus, the actual frequency of CD4$^+$ cells harboring the invariant Vα7-Jα33 chain should be $\sim$1/6,000.

In mice, Vα19-Jα33$^+$ cells were less numerous than in humans. Their frequency within α/β DN lymph node cells was 1/56 (Fig. 5 A), compared with $\sim$1/7.5 in humans (Fig. 4 A).

Surface Phenotype and TCR β Repertoire of Vα7/19-Jα33–bearing Cells

Invariant Vα7-Jα33–bearing Cells Express Memory Markers and Show a Biased Usage of hBV2 and hBV13 TCR β Chains. In the absence of anti-hAV7S2 or anti-mAV19 antibody, we used the following strategy to characterize the surface phenotype of the population expressing the invariant Vα7-Jα33 chain. α/β DN and CD8α^+ cells were depleted or enriched for a given marker by FACS® sorting, and the amounts of Vα7-Jα33 transcripts in the positive and negative fractions were quantified and compared with the amounts of amplified Cα transcripts, in order to take into

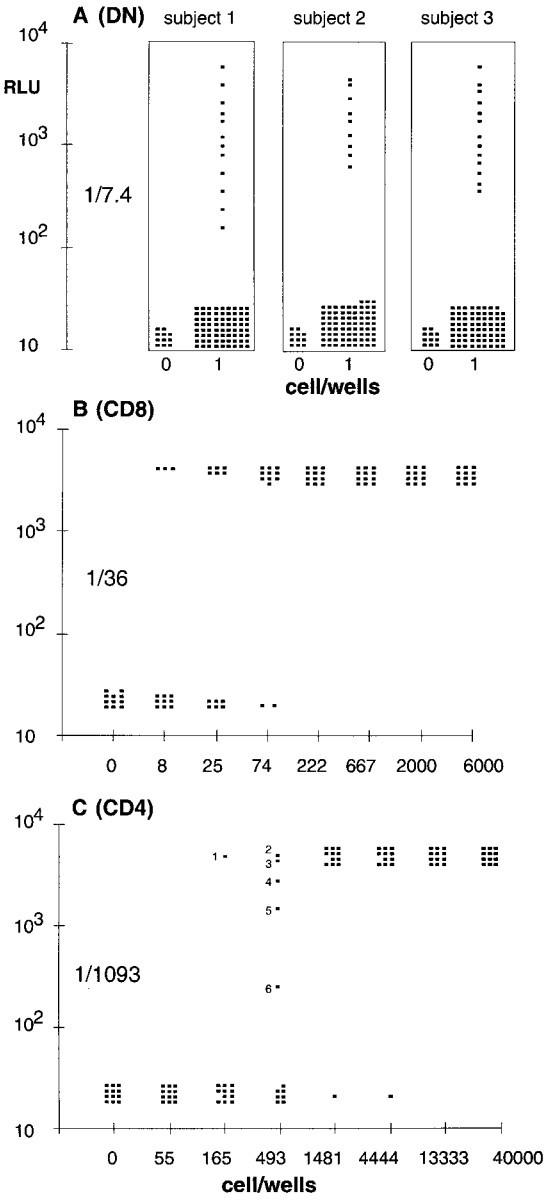

Figure 4. Direct enumeration of AV7S2/AV19-AJ33–bearing cells by PCR–limiting dilution analysis and single cell PCR. (A) DN cells obtained from three human subjects were seeded into microtiter plates using a FACS® single cell deposition unit. After cell lysis, PCR was carried out with AV7S2 and AJ33 primers, and the amount of amplicons was quantified by ELISA. (B and C) α/β CD8α^+ or CD4$^+$ cells were obtained by FACS® sorting, serially diluted, distributed in a microtiter plate at the indicated cell concentration, and processed as above. In C (CD4$^+$ cells), the amplicons of the six wells indicated (which had a high probability of clonality) were sequenced. The sequences were as follows:

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1-GCT GTG ATG TCG AT
2-GCT GTG AGA GAG ATC GGA
3-GCT GTG AGA GAT
4-GCT GNN ACT CGA
5-GCT GTG AGA GAG GAT AAC-AJ34-intron-AJ33
6-GCT GTG AGG GGG GGG GAA AAC-AJ34-intron-AJ33
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Only one (sequence 3) corresponds to the canonical AV7S2-AJ33 rearrangement (see text for details). In B and C, frequencies were calculated by maximum likelihood analysis.

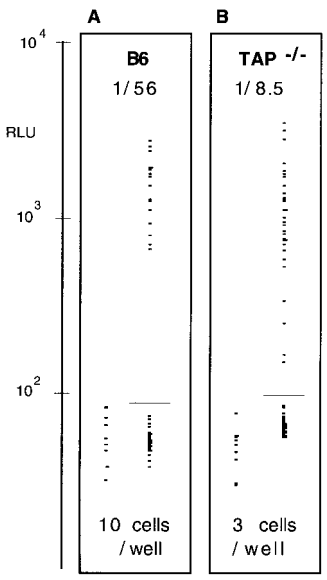

Figure 5. mAV19-AJ33$^+$ cells are more frequent in TAP$^{-/-}$ mice. The indicated number of DN T cells from B6 (A) or TAP$^{-/-}$ (B) mice were seeded in PCR microplates, and the presence of the mAV19-AJ33 rearrangement was detected by PCR-ELISA.

account variations in cell number. Fig. 6 displays an example of such an experiment, where we examined whether the CD8 molecules expressed by Vα7-Jα33$^+$ cells were either heterodimeric (αβ) or homodimeric (αα), since CD8αα$^+$ cells represent between 5 and 25% of CD8α$^+$ TCR α/β$^+$ lymphocytes in human PBLs (data not shown). There were ~45-fold more Vα7-Jα33 transcripts in the CD8αα$^+$ fraction than in the CD8αβ$^+$ fraction. This result was confirmed by polyclonal sequencing of the amplicons obtained after amplification with hAV7S2/Cα (V-C) or hAV7S2/hAJ33 (V-J) primers: no readable sequence was obtained from the CD8αβ$^+$ fraction, whereas the invariant α chain was present in the CD8αα$^+$ fraction (Fig. 2 B). The absence of a readable sequence in the CD8αβ$^+$ fraction using V-J amplification indicates the near absence of the invariant α chain in this fraction. Using this strategy, it could be established that Vα7-Jα33$^+$ cells had mainly a memory phenotype (i.e., CD45R0$^+$CD45RA$^-$) and were CD56$^-$CD57$^-$CD28$^+$CD27$^+$ (data not shown).

Despite many attempts using different lymphokine mixtures, we were unable to obtain a large enough number of T cell clones expressing the invariant Vα7-Jα33 chain. Therefore, the TCR β chain repertoire of DN cells bearing invariant Vα7-Jα33 chain was directly estimated by single cell PCR analysis. After single cell deposition of DN α/β cells into PCR plates, cells were lysed and a first PCR reaction was carried out using hAV7S2, hAJ33, and a mixture of 29 Vβ and 7 Jβ. This first reaction was then amplified in a second PCR with nested AV7S2/AJ33 primers allowing us to find the wells containing Vα7-Jα33$^+$ cells. Using the reaction mixture of the first PCR corresponding to the positive wells, 24 individual PCR reactions were set up using 1 (or 2) Vβ and a mixture of 7 Jβ primers. This allowed us to get a specific band for 0–2 Vβ which was then sequenced using the relevant Vβ primer. In 9 independent experiments carried out on DN cells obtained from 3 different subjects, the frequency of Vα7-Jα33$^+$ wells ranged between 6 and 16/84. Among the 92 Vα7-Jα33$^+$ wells studied, a β chain could be assigned and sequenced in 37 cases (40%), and we found that there was a heavy bias toward Vβ13 and Vβ2. Out of 13 Vβ sequenced in donor A, 7 were BV13$^+$ and 3 were BV2$^+$. In donor B, 8/12 were BV13$^+$ and 4/12 were BV2$^+$. In donor C, 7/12 were BV13$^+$ and 2/12 were BV2$^+$. However, despite this biased usage of BV13 or BV2 regions, the TCR β chains derived from Vα7-Jα33$^+$ cells did not show obvious restrictions in Jβ usage or in CDR3 length (Table I). Sequence analysis of VαJα junctions confirmed the presence of Vα7-Jα33 chains with the canonical CDR3 length and revealed significant variations in amino acid composition at the two N-encoded positions (Table I). Moreover, the presence of repeated TCR α and β chain junctional sequences in different cells from the same subject cloned in different PCR plates and the fact that these TCR chains were not found in other subjects indicated that this subset had undergone clonal expansion in vivo, an interpretation that is consistent with its memory phenotype (see above). Overall, Vα7-Jα33$^+$ cells appear to use a semirestricted repertoire that is expanded as needed.

TCR β Chain Repertoire of Vα19-Jα33$^+$ Cells. Because the frequency of Vα19-Jα33$^+$ cells is not as high in mice as in

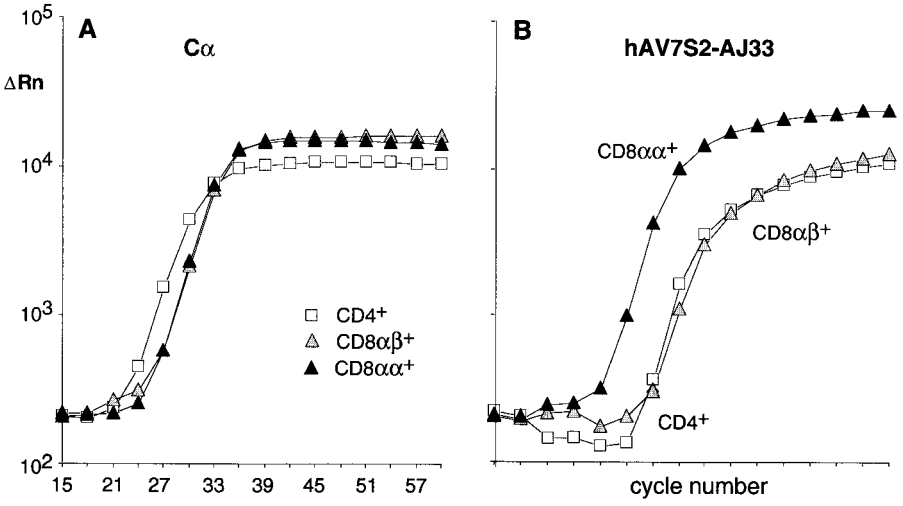

Figure 6. hAV7S2-AJ33–bearing cells are CD8αα$^+$ or DN. The indicated TCR α/β$^+$ fractions were FACS® sorted using a four-color staining with anti-CD4–TC, anti-CD8α–FITC, anti-CD8β–PE, and anti-α/β TCR–APC. The amounts of Cα (A) or hAV7S2-AJ33 (B) transcripts were quantified by kinetic PCR using the ABI Prism 7700 apparatus (Taqman). This experiment was repeated with cells from another subject with identical results. ΔRn, difference in fluorescence relative units.

Table I. *Vβ Repertoire of Human Invariant AV7S2-AJ33 TCR α Chain–bearing Cells*

Germline AV7S2 TGT GCT GTG AGAGAcacggtgact[a]
tttgtgtctgtgTG GAT AGC germline AJ33

subject	Cell	Vα-Jα CDR3	Vβ	CDR3	Jβ	PCR plate #
A	C5	GCC GTG AAG GAT AGC A V K D S	13.x	CAS SPSIGGGGEQ FFG	2.1	2
	E6	GCC CCC CTG GAT AGC A P L D S	13.2	CAS SYPPDQGNTI YFG	1.3	1
	D8-F7-G10[b]	GCT GTG AAG GAT AGC A V K D S	13.3	CAS SVWIGEGSGEL FFG	2.2	3
	F7[b]	GCT GTG AAG GAT AGC A V K D S	13.3	CAS SVWIGEESGEL FFG	2.2	1
7 Vβ13/13	G1	GCT GTG ATG GAT AGC A V M D S	13.5	CAS SDGTSIDTQ YFG	2.3	3
	G3	GCT GTG CTG GAT AGC A V L D S	2.1	CSA SKGENYEQ YFG	2.7	2
	E10	GCT GTC ATG GAT AGC A V M D S	2	CSA FVGGGNTEA FFG	1.1	3
3 Vβ2/13	C2	GCT GCC ATG GAT AGC A A M D S	2	CSA SEGEHSYEQ YFG	2.7	3
	E1[c]	GCT GTG ATG GAT AGC A V M D S	24	CAT SRDGTSGSGATVL TFG	2.6	2
2 Vβ24/13	D2[c]	GCT GTG ATG GAT AGC A V M D S	24	CAT SRDGTSGSGATVL TFG	2.6	1
1 Vβ15/13	F1	GCT GTG ATC GAT AGC A V I D S	15	CAT MIPADTGEL FFG	2.2	2
B	B10	GCT GGG ATG GAT AGC A G M D S	13.3	CAS TLGQEQQPQ HFG	1.5	1
	G2	GCT TCC ATG GAT AGC A S M D S	13.3	CAS SEIDPNTGEL FFG	2.2	1
	F3-C1[d]	GCT TCC ATA GAT AGC A S I D S	13.3	CAS SEIDPNTGEL FFG	2.2	2
	G8	GCT GCC ATG GAT AGC A A M D S	13.3	CAS SGGDSGEL FFG	2.2	1
	C10	GCT GCC GAG GAT AGC A A E D S	13.3	CAS SYEVSGANVL TFG	2.6	1
	A10	GCT GCA TTG GAT AGC A A L D S	13.5	CAS SVSGGTDTQ YFG	2.3	1
8 Vβ13/12	A12	GCT GGG TTG GAT AGC A G L D S	13.5	CAS SRSGGNPIDIQ YFG	2.3	1
	D5	GCT GCT TTG GAT AGC A A L D S	2	CSA RVGRGVTEA FFG	1.1	1
	D8	ND[f]	2	CSA RDGGRDSGEL FFG	2.2	1
	A6	GCT GTG ATG GAT GAC A V M D S	2.1	CSA SPPDRATSPL HFG	1.6	3
4 Vβ2/12	E12	GCT GCT ATA GAT AGC A A I D S	2.1	CSA RSLAEPYEQ YFG	2.7	3
C	B11	GCT GTG AGA GAT AGC A V R D S	13.2	CAS NQGDVDTGEL FFG	2.2	2
	B9	GCT GTG ATG GAT AGC A V M D S	13.2	CAS SPSSGGYNEQ FFG	2.1	1
	B8	GCT AGC ATG GAT AGC A S M D S	13.2	CAS SYLGSGANVL TFG	2.6	3
	C10	GCT GTG GTG GAT AGC A V V D S	13.3	CAS SPFGGTDTR YFG	2.3	2
	A11	GCT GTG AGA GAT AGC A V R D S	13.3	CAS SAAVEQGNTI YFG	1.3	3
	G3	GCT TTC ATG GAT AGC A F M D S	13.5	CAS SDKGGSTGE LFG	2.2	1
7 Vβ13/12	D3	GCT GTC CAG GAT AGC A V Q D S	13.5	CAS SAEGAPDTQ YFG	2.3	3
	F7[e]	GCT TTT ATG GAT AGC A F M D S	2	CSA RTSGDFGEQ FFG	2.1	2
2 Vβ2/12	E5[e]	GCT TTT ATG GAT AGC A F M D S	2.1	CSA RTSGDFGEQ FFG	2.1	1
1 Vβ3/12	E1	GCT GTC TTG GAT AGC A V L D S	3	CAS SRGILDAGEL FFG	2.2	1
1Vβ7/13	D6	ND[f]	7.2	CAS SQGLQQGNYEQ YFG	2.7	2
1 Vβ19/12	F1	GCT GTA TTG GAT AGC A V L D S	19	CAN LDRPSNIGEL FFG	2.2	1
1 Vβ20/12	A10	GCT GTG CTG GAT AGC A V L D S	20	CAW RSQGSYGH TFG	1.2	2

Single TCR α/β$^+$ DN T cells were FACS® sorted into 96-well PCR plates. After screening for the presence of the invariant AV7S2-AJ33 chain, individual TCR α and β chains of the positive wells were characterized and sequenced as described in Materials and Methods. The β chain could be assigned for 40% of the wells. Three independent PCR plates were studied for each subject, as indicated in the last column.

[a]Small caps code for the recombination sequence signal.

[b, c, d, e]Identical TCR α and β sequences probably representing a single expanded clone.

[f]Not done.

humans, the strategy followed to characterize the surface phenotype of human Vα7-Jα33$^+$ cells was not successful in mice. Therefore, to characterize the TCR β chain repertoire of this subset, we took advantage of the fact that Vα19-Jα33$^+$ cells are enriched in TAP$^{-/-}$ mice (see below). We generated T–T hybridomas from DN enriched lymph node cells from TAP$^{-/-}$ mice, and screened for expression of Vα19-Jα33 transcripts. In agreement with the single cell analysis (Fig. 5 B), 16/168 hybridomas studied were Vα19-Jα33$^+$. As shown in Table II, in all cases, these hybridomas carried Vα19-Jα33 chains with the canonical CDR3 length and in all but two cases, the Vα19-Jα33 junction was germline encoded. The Vβ segments used were predominantly BV8$^+$ (7/16) and BV6$^+$ (4/16). No restriction in the Jβ repertoire was apparent.

Development and Selection of Vα7/19-Jα33–bearing Cells

Ontogeny of Vα7/19-J33–bearing Cells. NK1 T cells are not found in neonates and accumulate after birth (5). Therefore, we examined cord blood lymphocytes for Vα7/19-Jα33 sequences by quantitative PCR and polyclonal sequencing of Vα7-Cα or Vα7-Jα33 amplicons on cDNA obtained from enriched DN/CD8$^+$ or CD4$^+$ fractions. We were unable to find significant amounts of invariant Vα7-Jα33 transcripts in cord blood in the six samples studied. However, significant amounts of this chain could be obtained in young children, albeit in lower amounts than in adults (data not shown).

Similarly, Vα19-Jα33–bearing cells were not found in spleen or thymus of mouse neonates (data not shown). They were present in lymph nodes and blood in large amounts and in smaller amounts in the spleen in the five mouse strains tested, as shown in Table III (top), which displays a summary of the studies examining the tissue distribution of

Table II. *Summary of the Mouse Vβ Repertoire of T–T mAV19-AJ33$^+$ Hybridomas*

Genomic mAV19
TGT GCT GTG AGG xxx
tgtgtG GAT AGC AAC genomic mAJ33

Clone	mAV19-AJ33	Vβ	CDR3	Jβ
26G12	GCT GTG AGG GAT AGC AAC A V R D S N	8.1	CAS SDVGEGSDY TFG	1.2
22A5	GCT GTG AGG GAT AGC AAC A V R D S N	8.1	CAS SDAQHYEQ YFG	1.4
23G6	GCT GTG AGG GAT AGC AAC A V R D S N	8.1	CAS RDNYAEQ FFG	2.1
16H2	GCT GTG AGG GAT AGC AAC A V R D S N	8.2	CAS GDGGAYAEQ FFG	2.1
6H2	GCT GTG AGG GAT AGC AAC A V R D S N	8.2	CAA GTGGALYAEQ FFG	2.1
8H12	GCT GTG AGG GAT AGC AAC A V R D S N	8.2	CAS RDCANTGQL YFG	2.2
25F11	GCT GTG AGG GAT AGC AAC A V R D S N	8.2	CAS GDGRTGGERQ YFG	2.7
23B1	GCT GTG AGG GAT AGC AAC A V R D S N	6	CAS SQPQQGYNSP YFA	1.6
7D11	GCT GTG AGG GAT AGC AAC A V R D S N	6	CAS STGGDNYAEQ FFG	2.1
11F1	GCT GTG **C**TG'GAT AGC AAC A V L D S N	6	CAS SIGGDYAEQ FFG	2.1
22B11	GCT GTG AGG GAT AGC AAC A V R D S N	6	CAS SRLGSNIGQL YFG	2.2
17E6	GCT GTG AGG GAT AGC AAC A V R D S N	2	CSA DPGQGCERL FFG	1.4
9C2	GCT GTG AGG GAT AGC AAC A V R D S N	5	CAS SLGTGSAETL YFG	2.3
4H1	GCT GTG AGG **GAC'G**GC AAC A V R D G N	7	CAS SSSGGYNSSPL YFA	1.6
24E11	GCT GTG AGG GAT AGC AAC A V R D S N	7	CAS SLFAGSP YFG	2.7
2G8	GCT GTG AGG GAT AGC AAC A V R D S N	14	CAW RGSGDAETL YFG	2.3

*In bold and underlined, N additions.

Table III. *Strain and Tissue Distribution, and MHC Selection of mAV19-AJ33$^+$ T Cells*

Strain	Haplotype	Lymph nodes	Spleen	PBLs	Thymus	Bone marrow	Liver	IELs	Lamina propria
BALB/c	H-2^d I-A$^+$/I-E$^+$	+++	+	+++	+	+	−	−	−
DBA/2	H-2^d I-A$^+$/I-E$^+$	+++							
CBA	H-2^k I-A$^+$/I-E$^+$	+++	+						
129	H-2^b I-A$^+$/I-E$^-$	+		+++					
C57BL/6	H-2^b I-A$^+$/I-E$^-$	+++		+++	+				
NUDE B6	H-2^b I-A$^+$/I-E$^-$	−	−						

	B6	I-A$^{b-/-}$	β2m$^{-/-}$	K$^{b-/-}$/D$^{b-/-}$	TAP$^{-/-}$	CD1$^{-/-}$	CD8$^{-/-}$
mAV19-AJ33	++	++	−	++	+++	++	++
(fold increase compared with B6)		(2)		(4)	(8)	(2)	(4)
mAV14-AJ18	++	++	−	++	++	−	++

DN α/β$^{+/+}$ T cells were purified from the indicated organs in different mouse strains. Expression of the invariant mAV19-AJ33 TCR α chain was estimated by quantitative kinetic PCR and confirmed by polyclonal sequencing (see Materials and Methods, and Figs. 1 and 2). Tissue distribution results are compared with the expression in sorted CD4$^+$ α/β$^+$ T cells: +++, high expression (>3-cycle shift to the left); +, no increase; −, absence as judged by polyclonal sequencing of mAV19-AJ33 amplicons. Selection results (bottom) in the lymph nodes of the different MHC-deficient mice are compared with the expression in DN cells from B6 lymph nodes using quantitative PCR. All of the results were also confirmed by polyclonal sequencing after mAV19-Cα, or -AJ33 amplification as appropriate.

Vα19-Jα33–bearing cells. Importantly, although more than half of the human invariant Vα7-Jα33$^+$ cells were CD8αα$^+$ cells, which might suggest an extrathymic development pathway (43), invariant Vα19-Jα33 rearrangements were not detected in nude mice either by quantitative PCR (Table III, top) or polyclonal sequencing (data not shown), indicating that the thymus is required for their development.

Development of Vα7-Jα33$^+$ Cells Is Not Affected in MHC Class II– and TAP-deficient Patients. The restriction element of Vα7-Jα33$^+$ cells could be either a class I or class II MHC molecule, as they do not express CD4 or CD8αβ accessory molecules. To address this issue, we quantified the expression of the Vα7-Jα33 invariant chain in PBLs obtained from MHC-deficient patients. MHC class II–deficient patients harbor low but significant numbers of CD4$^+$ α/β lymphocytes whose Vβ repertoire is grossly normal (44). CD4$^+$, CD8α$^+$, and DN enriched fractions were prepared and analyzed by quantitative PCR. Although the amount of Cα was much lower in the DN than in the CD4$^+$ samples (Fig. 7 A), the amounts of Vα7-Jα33 transcripts were comparable in both samples (Fig. 7 B), therefore indicating an enrichment of Vα7-Jα33 transcripts within DN cells and, presumably, selection of invariant Vα7-Jα33–bearing cells in this patient. Accordingly, the predominance of the invariant Vα7-Jα33 sequence within DN cells was confirmed by polyclonal sequencing (data not shown). Similar results were obtained in the two other patients studied. Taken together, these results strongly suggest that the restriction element of the invariant Vα7-Jα33$^+$ cells is not an MHC class II molecule.

There is no MHC class I–deficient patient described to date, but we could obtain PBLs from two TAP-deficient siblings (45). In these patients, α/β$^+$ TCR CD8α$^+$ cells are reduced in numbers at birth but may expand with age. α/β$^+$ TCR CD4$^+$ and CD8α$^+$ cells were separated in one of these patients, and the amount of Cα and Vα7-Jα33 transcripts was quantified in the two fractions. Despite lower amounts of Cα in the CD8α$^+$ fraction, the amount of Vα7-Jα33 transcripts was much higher in the CD8α$^+$ fraction (Fig. 7, C and D). Enrichment for Vα7-Jα33 transcripts was also demonstrated in a PHA-stimulated CD8α$^+$ line derived from the other TAP-deficient patient, when compared with either unsorted PBL-derived cell line from the same patient or to CD8α$^+$ cells from healthy donors (data not shown). Actually, the larger amounts of Vα7-Jα33 transcripts in the CD8α$^+$ α/β T cells of TAP-deficient patients is in agreement with the higher proportion of CD8αα$^+$ cells in these patients (data not shown). This indi-

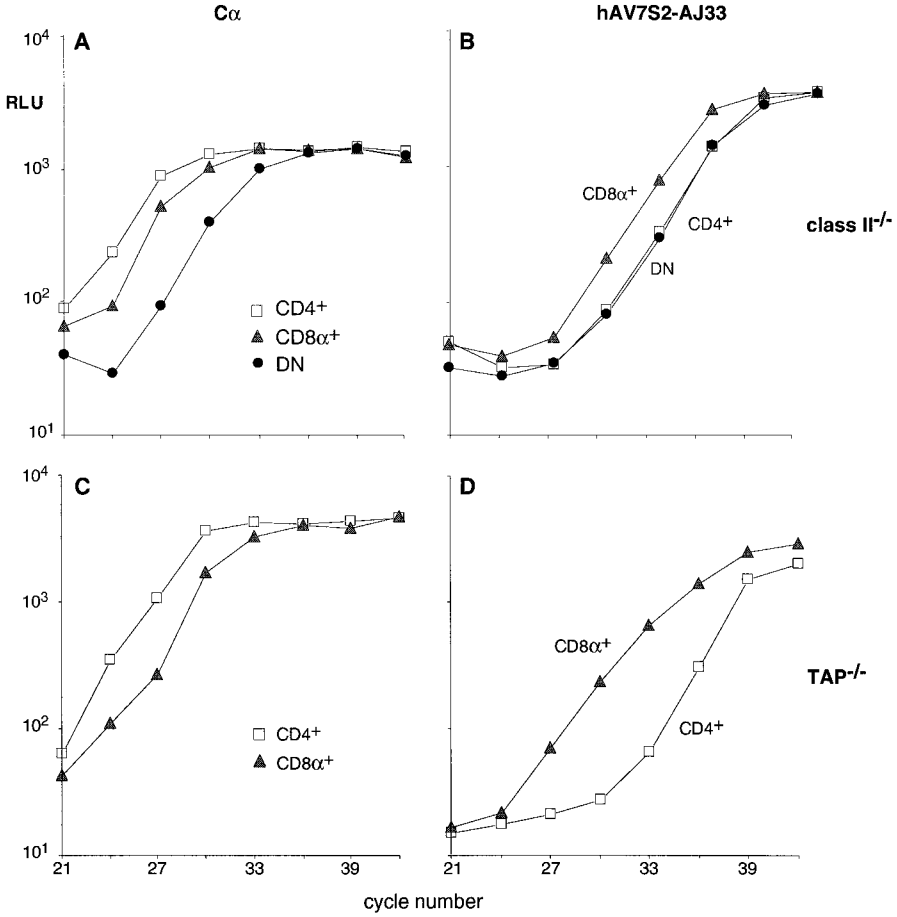

Figure 7. AV7S2-AJ33–bearing cells are present in MHC class II– and TAP-deficient patients. The indicated cell subpopulations were obtained from an MHC class II– (A and B) or a TAP-deficient (C and D) patient. The amounts of Cα or hAV7S2-AJ33 transcripts were quantified by kinetic PCR-ELISA. Two other MHC class II–deficient patients and another TAP-deficient patient were studied with similar results.

cates that T cells bearing this invariant α chain are probably not selected by a TAP-dependent MHC class I molecule.

Thus, the selecting molecule for the invariant Vα7-Jα33$^+$ cells appeared to be neither an MHC class II nor a classical MHC class I molecule. However, because the defect in MHC class II expression may not be complete and the level of MHC class I expression in TAP-deficient patients is still 1% of normal, no definitive conclusion could be drawn from these studies. Therefore, we turned to the mouse, where carefully controlled studies using well-characterized MHC-deficient mice can be performed.

The Selecting Ligand for Vα19-Jα33$^+$ Cells Is β2m-dependent, TAP-independent, and Distinct from CD1. The amount of Vα19-Jα33 transcripts was examined in α/β$^+$ DN lymph node cells from several MHC-deficient strains. Because the frequency of Vα19-Jα33$^+$ cells and the percentage of DN cells are lower in mice, the differences between positive and negative samples are smaller in mice than in humans. However, despite higher levels of Cα in the β2m$^{-/-}$ samples, there were fewer Vα19-Jα33 transcripts than in control or I-A$^{b-/-}$ mice (Fig. 8, A and B). Polyclonal sequencing of Vα19-Jα33 amplicons from β2m$^{-/-}$ samples showed the complete absence of invariant Vα19-Jα33 transcripts. This was consistent with an MHC class I or β2m-dependent class I–like selection of the invariant Vα19-Jα33$^+$ cells. However, the relevant molecule is not a classical MHC class I molecule because the amount of invariant Vα19-Jα33 transcripts was higher in the DN cells from K$^{b-/-}$D$^{b-/-}$ mice than in controls, though the Cα levels were lower. These results suggest that the MHC class I selecting molecule is not a classical one and that, in the absence of classical class I molecules, the invariant chain–bearing DN cells are less diluted by mainstream cells. In Fig. 8, C and D, Vα19-Jα33 transcripts were quantified in CD8-, CD1-, and TAP-deficient mice, B6 and β2m$^{-/-}$ mice being used as positive and negative controls, respectively. There was no decrease in the amounts of Vα19-Jα33 transcripts in CD1-deficient mice, indicating that Vα19-Jα33$^+$ cells were not selected by CD1 or a CD1-bound ligand. The curves for TAP$^{-/-}$ samples were actually shifted to the left, indicating an increased frequency of Vα19-Jα33$^+$ cells in TAP$^{-/-}$ mice, in agreement with the result shown in Fig. 5 B where the frequency of Vα19-Jα33$^+$ cells in DN TAP$^{-/-}$ mice was 1/8.5 (i.e., a sixfold increase compared with B6; Fig. 5 A). The Vα19-Jα33 curve corresponding to the CD8-deficient mice is similar to that of B6, indicating the presence of Vα19-Jα33 invariant chains in CD8$^{-/-}$ mice. This was confirmed by polyclonal sequencing (data not shown). Altogether, these results, summarized in Table III (bottom), indicate that Vα19-Jα33$^+$ cells do not require CD8 for their selection and that the selecting molecule is a β2m-dependent, TAP-independent molecule distinct from CD1.

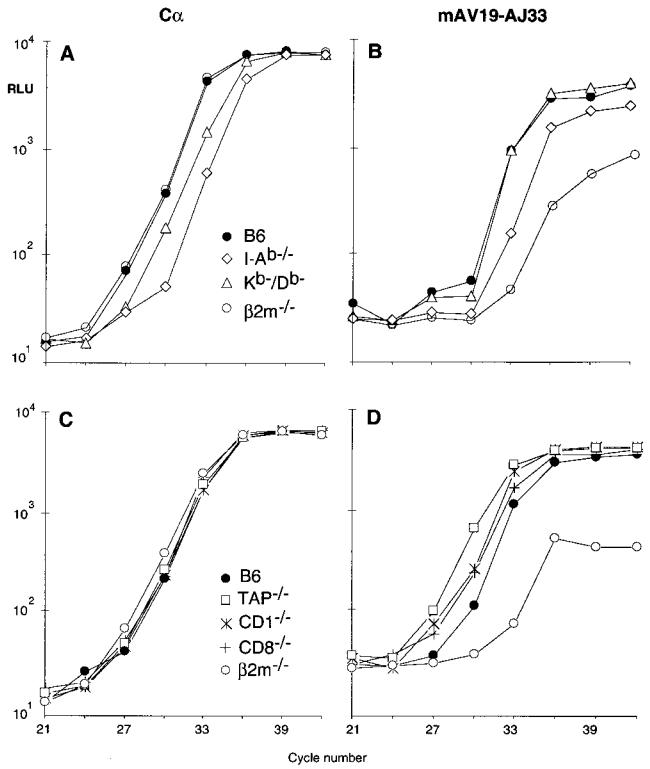

Figure 8. AV19-AJ33$^+$ cells are selected by a β2m-dependent, TAP-independent nonclassical MHC–like molecule distinct from CD1. DN lymph node cells were obtained from the indicated mice, and the amounts of Cα (A and C) and mAV19-AJ33 (B and D) transcripts were quantified by kinetic PCR-ELISA. All results were confirmed by polyclonal sequencing.

Discussion

Invariant Vα7.2-Jα33 TCR α chain and its murine and bovine homologues were found in three different mammalian species and, thus, define a new phylogenetically conserved T cell population using a canonical TCR α repertoire. In humans, DN and CD8αα$^+$ cells bearing invariant Vα7-Jα33 chain accumulate after birth and become quite abundant, as they represent ~0.1–0.2% of all human PBLs. Therefore, it is legitimate to wonder why such cells have not been previously reported. This may be due to the lack of a stringent Vβ repertoire restriction, or to the difficulties in growing clones expressing this invariant chain (our unpublished observations), together with methodological limitations linked to TCR α chain repertoire analysis (e.g., lack of allelic exclusion [46]). However, this invariant α chain had already been described in DN cells by Porcelli et al. (39) and, among the 317 random CDR3 sequences reported by Moss and Bell (47), 4 (1.2%) had a sequence corresponding to the Vα7-Jα33 invariant α chain. In the same study, this canonical sequence was not found in cord blood samples or in CD4$^+$ cells, but represented 3/40 (7.5%) CDR3 sequences derived from CD8α$^+$ cells. This age and cell distribution is in agreement with our own measurements (data not shown). In cattle, this CDR3 was found in 1/20 TCR α chains randomly sequenced (40). In mice, random cloning of TCR α chains revealed one example of this invariant Vα19-Jα33 α chain (48). It should be stressed that as shown in Fig. 3, the AJ33 segments are quasi-identical in the three species. The mouse and human Jα loci are entirely homologous, with the same genomic organization and an average similarity of 71% including the Jα coding sequences (41, 42, 49). The AJ33 is the most similar Jα segment between mouse and human sequences with only one amino acid difference; the two other most similar human/mouse pairs (AJ23 and AJ24) have four and three amino acid differences, respectively (41). This quasi identity between mouse and human AJ33 segments suggests a strong selective pressure for these segments.

In humans and cattle, the TCR α chain displayed some junctional diversity, whereas in mice a genomic sequence without trimming or N additions was found in most cases (Table II). However, a murine sequence made by trimming and reconstitution of the canonical sequence through N additions was found in two hybridomas (11F1 and 4H1 in Table II), indicating that the TCR in mice is selected at the protein level. In humans, the invariant TCR α chain was associated with a limited number of Vβ segments (mostly hBV2 and hBV13), and the same TCR with the same nucleotide sequence was present in different cells from two subjects, suggesting oligoclonal expansions. Despite this combinatorial restriction, TCR β chain diversity of invariant Vα7-Jα33$^+$ cells remained extensive, since most Jβ segments and seven Vβs were found associated with CDR3s of variable length. In mice, five different Vβs were observed with a high proportion of mBV6 and mBV8. Importantly, the murine orthologous segments of hBV13 are mBV8 and mBV6 (50–52).

This semi-invariant repertoire selected at the protein level is reminiscent of the repertoire observed in murine epithelial DECs and in murine and human NK1 T cells. In DECs, an invariant repertoire generated from genomic sequences is selected at the protein level, as the same epitope recognizable by an mAb can be reconstituted by different Vγ segments when the original GV1 and GV2 segments are inactivated by homologous recombination (13). For NK1 T cells, selection at the protein level was also apparent (14), and the transgenic overexpression of the invariant mAV14AJ18 was sufficient to induce a large increase in the number of NK1 T cells (53).

The restriction element of Vα7/19-Jα33$^+$ cells is probably an MHC class Ib molecule distinct from CD1d, though a β2m-derived peptide presented by a "nonclassical" class II molecule cannot be formally excluded. The selecting molecule should be present in both mice and humans. Murine Qa-1 and human HLA-E might be good candidates, given their structural homology and their ability to bind to homologous CD94/NKG2 receptors in both species (54–56). However, when using HLA-E tetramers complexed with HLA-G leader sequence–derived peptide (56), we were unable to find any difference in expression levels of the invariant Vα7-Jα33 α chain between FACS®-sorted HLA-E tetramer–positive and –negative (DN plus CD8α$^+$) α/β$^+$ TCR PBL fractions (data not shown). Moreover, the in-

variant Vα19-Jα33 chain was not expressed by three TAP-independent anti–Qa-1 clones provided by J. Forman (University of Texas, Dallas, TX [57]; data not shown). Nonetheless, no definitive conclusions as to the HLA-E/Qa-1 specificity of the invariant TCR can be drawn from these negative results because the invariant TCR may recognize HLA-E complexed with another peptide. Another candidate class I molecule that is widely expressed in both mice and humans is the recently described MR1 molecule (58, 59). Identification of the selecting element is underway by testing reactivity of mouse hybridomas against different cell types and MHC class I/Ib transfectants.

The expression of CD8αα on Vα7-Jα33$^+$ cells could argue for an extrathymic development pathway (43). However, these cells were not found in nude mice (Table III, top), in human intraepithelial lymphocytes (IELs) (Cerf-Bensussan, N., E. Treiner, and O. Lantz, unpublished results), or in murine IELs or lamina propria lymphocytes (Guy-Grand, D., F. Tilloy, and O. Lantz, unpublished results), and their frequency was not particularly high in mouse bone marrow (Table III, top). Thus, Vα7/19-Jα33$^+$ cells seem to be mainly thymus dependent. In this respect, the fact that we have been unable to find large numbers of these cells in human or mouse thymus (data not shown) does not preclude an intrathymic development. Indeed, if the antigen is not present in sufficient amounts in the thymus, these cells may not accumulate locally and may be diluted out by mainstream α/β T cells in the periphery. The absence of significant numbers of Vα19-J33$^+$ cells in mouse CD8α$^+$ cells (Fig. 1 F, and polyclonal sequencing after V-J amplification [not shown]) is in agreement with the extremely low numbers (<0.4%) of CD8αα$^+$ cells in murine blood or lymph nodes (data not shown). The CD8αα expression on some invariant Vα7-Jα33$^+$ cells and its complete absence on Vα19-Jα33$^+$ counterparts is reminiscent of the phenotype of NK1 T cells, which may express CD8αα$^+$ in humans (28) but never in mice. The reasons for such phenotypic differences between mice/cattle and humans as well as the role of CD8αα expression in human PBLs are not clear. There have been contradictory reports concerning the role of homo- versus heterodimeric CD8 (60–63). In addition, CD8αα expression in human PBLs might reflect an activated status rather than a particular differentiation pathway (64).

Concerning the development pathway of the Vα7/19-Jα33 cells, in the three species there seems to be no expression of the invariant α chain in the CD8αβ$^+$ T cells, strong enrichment for this sequence within DN cells (as well as within human CD8αα$^+$ cells), and persistence of the invariant sequence in the CD4$^+$ subset. Indeed, in humans, the frequency of invariant α chain–bearing cells was ~1/6,000 in the CD4$^+$ fraction. Moreover, polyclonal sequencing of Vα7-Jα33 amplicons from CD4$^+$ cells (Fig. 2 A) revealed the presence of canonical CDR3 at a low, though readable, frequency, whereas the canonical CDR3 was undetectable in CD8αβ$^+$ samples (Fig. 2 B). In mice and cattle, there was also a small but reproducible shift of the CD4$^+$ amplification curves to the left compared with the CD8α$^+$ curves, suggesting some expression of the invariant α chain in murine or bovine CD4$^+$ cells. Accordingly, a readable canonical sequence in Vα19-Jα33 amplicons was detected in the CD4$^+$ but not in the CD8α$^+$ fraction in both species (data not shown). The absence of any readable sequence after V-J amplification of CD8α/β$^+$ cells suggests that the CD8αβ$^+$ Vα7/19-Jα33–bearing cells are deleted by negative selection, as are NK1 T cells in CD8 transgenic mice (14). These results are compatible with the hypothesis that Vα7/19-Jα33 cells follow a normal T cell development pathway but that, in contrast with NK1 T cells where the numbers of CD4 and DN NK1 T cells are similar, CD4$^+$ T cells bearing the Vα7/19-Jα33 α chain are diluted out by mainstream lymphocytes.

A memory phenotype associated with an oligoclonal repertoire may be related to recognition of either an endogenous ligand or a ubiquitous pathogen. In favor of an endogenous ligand is the finding that invariant Vα7-Jα33$^+$ cells existed in an 18-mo-old child who had not been vaccinated with Calmette-Guérin bacillus (BCG) and in several subjects who had negative EBV and CMV serologies (data not shown). On the other hand, the abundance of Vα7/19-Jα33–bearing cells in humans and cattle contrasting with a lower number of these cells in mice could be related to the clean environment in which laboratory mice are housed. The two hypotheses are not mutually exclusive, as cells selected by an endogenous ligand could be ready to react against a highly prevalent pathogen.

Both NK1 T cells and Vα7/19-Jα33–bearing cells display semi-invariant repertoires (i.e., one monomorphic α chain and a biased Vβ chain repertoire), suggesting that their TCRs may have similar characteristics, probably a high affinity for a selecting ligand by the invariant α chains, as the transgenic overexpression of AV14AJ18 is sufficient to greatly increase the number of NK1 T cells (53). Together with the similarities in their restricting elements (a TAP-independent, β2m-dependent nonclassical MHC class I–like molecule), the absence of accessory molecule (CD4 or CD8) involvement suggests that they may recognize a high-density ligand that might therefore be a saccharide or a glycolipid.

Are there other invariant TCR α chains defining other T cell subpopulations? We did not find any after measuring the amounts of several other α chains which had been found either in DN T cells (39) or in a "regulatory" subpopulation (65–67; Tilloy, F., and O. Lantz, unpublished observations). However, the definite resolution of this issue awaits a systematic study of the expression of all AV-AJ combinations, and other populations may exist in other organs in the same way that γ/δ subpopulations are distributed.

Concerning the functions of Vα7/19-Jα33$^+$ lymphocytes, future analysis of the mAV19AJ33 transgenic mice we have made will certainly help address this point. The high frequency of Vα7/19-Jα33$^+$ T cells and their extensive phylogenetic conservation in mammals both argue for an important physiological function. Because Vα7/19-Jα33$^+$ and NK1 T cells are selected by distinct ligands, they may complement each other and act synergistically either in defense mechanisms against broadly distributed pathogens or in immune/nonimmune homeostatic processes.

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