

# An Invariant T Cell Receptor $\alpha$ Chain Defines a Novel TAP-independent Major Histocompatibility Complex Class Ib-restricted $\alpha/\beta$ 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)  $\alpha$  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<sup>-</sup>CD8<sup>-</sup> double-negative (DN) T cells in the three species and also CD8 $\alpha$ <sup>+</sup> in humans. In humans, their frequency was  $\sim$ 1/10 in DN, 1/50 in CD8 $\alpha$ <sup>+</sup>, and 1/6,000 in CD4<sup>+</sup> lymphocytes, and they display an activated/memory phenotype (CD45RA<sup>lo</sup>CD45RO<sup>+</sup>). They preferentially use hBV2S1 and hBV13 segments and have an oligoclonal V $\beta$  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  $\beta$ 2-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  $\alpha$  chain.

Key words: invariant T cell receptor  $\alpha$  chain • CD4<sup>-</sup>CD8<sup>-</sup> 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  $\gamma/\delta$  T cell subpopulations (4), and the TCR  $\alpha/\beta$ <sup>+</sup> 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 immuno-

regulatory role or another physiologic function, such as the wound healing, that results from the secretion of keratinocyte growth factor (8) by  $\gamma/\delta$  dendritic epithelial cells (DECs)<sup>1</sup> 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  $\gamma/\delta$  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).

<sup>1</sup>Abbreviations used in this paper: APC, allophycocyanin;  $\beta$ 2m,  $\beta$ 2-microglobulin; B6, C57BL/6; DEC, dendritic epithelial cell; DN, CD4<sup>-</sup>CD8<sup>-</sup> double-negative; IEL, intraepithelial lymphocyte; TAP, transporter associated with antigen processing; TC, Tricolor.

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

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

NK1 T cells use an “invariant”  $\alpha$  chain (mAV14AJ18 in mice [14], hAV24AJ18 in humans [14, 15]) with a CDR3 of constant length paired with a limited number of  $V\beta$  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 (NKR1, Ly49) (5). In vivo development of NK1 T cells requires the MHC class Ib CD1d molecule (16), whose expression is  $\beta 2$ -microglobulin ( $\beta 2m$ ) dependent but transporter associated with antigen processing (TAP) independent (17). In both humans and rodents, mature NK1 T cells recognize some glycolipids (such as  $\alpha$ -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- $\gamma$ , especially when triggered through their NKR1 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  $\alpha/\beta$  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  $\alpha$ -galactosylceramide or glycosylphosphatidylinositol presented by CD1 for NK1 T cells) and/or display TCRs with high affinity. Indeed, most  $\gamma/\delta$  T cells are DN or  $CD8\alpha\alpha$ , 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  $\alpha/\beta^+$  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  $\alpha$  chain comprising the hAV7S2 and AJ33 elements in humans and the homologous AV19 and AJ33 elements in mice and cattle.  $V\alpha 7/19$ - $J\alpha 33^+$  T cells show an oligoclonal TCR  $\beta$  chain repertoire and are selected by a  $\beta 2m$ -dependent but TAP-independent molecule that is neither a clas-

sical 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- $\alpha/\beta$  TCR-allophycocyanin (APC), anti- $\alpha/\beta$  TCR-PE, anti-CD8 $\beta$ -PE, biotinylated anti-CD4, and anti-CD8 $\alpha$ , were obtained from Immunotech; anti-CD27-FITC and anti- $\alpha/\beta$  TCR-FITC were purchased from PharMingen. Anti-CD8 $\alpha$ -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-CD8 $\alpha$ - or anti-CD8 $\beta$ -FITC, biotinylated or Cychrome-conjugated anti- $\alpha/\beta$  TCR and streptavidin-APC were obtained from PharMingen. Anti- $\alpha/\beta$  TCR-TC was obtained from Caltag Laboratories. Bovine anti-CD4 and anti-CD8 $\alpha$  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<sup>®</sup> sorted into CD4 or CD4/CD8 positive or negative fractions as indicated, and the relative amounts of boC $\alpha$ - or boAV19-boAJ33 transcripts were estimated by kinetic PCR (see below).

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

**Immunofluorescence Studies.** Surface phenotyping was carried out on a FACSCalibur<sup>™</sup> cytometer (Becton Dickinson). Three-color sorting (CD4, CD8 $\alpha$ , and  $\alpha/\beta$  TCR staining) was carried out on a FACS Vantage<sup>™</sup> (Becton Dickinson) to obtain  $\alpha/\beta^+$  DN, -CD8 $\alpha^+$ , or -CD4<sup>+</sup> 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<sup>+</sup> fractions, enriched or depleted for a given marker, were obtained by FACS<sup>®</sup> sorting negative and positive fractions for this marker after quadruple staining with anti-CD4, anti-CD8 $\alpha$ , anti- $\alpha/\beta$  TCR, and the relevant marker (either CD8 $\beta$ , CD45RA, CD45RO, 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  $10^7/100 \mu\text{l}$  were resuspended in PBS, 0.5% BSA, 5 mM EDTA and incubated with anti-CD4 paramagnetic microbeads, washed once, resuspended in 500  $\mu\text{l}$  of the same buffer, and passed through an RS column. Effluent was collected as the DN<sup>+</sup>CD8<sup>+</sup> fraction. After thorough washes of the column, the eluate was collected as CD4<sup>+</sup> fraction. In these conditions, the CD4<sup>+</sup> 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, mA5-5', mA5V, mA5-3', 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-TTAGCTTGGTCCCA-GAGCCCC; pmAV19, GCTTCTGACAGAGCTCCAG-FITC. The mouse-specific V $\beta$  and V $\alpha$  primers were slightly modified from Casanova et al. (34). The human V $\beta$  primers were derived from Puisieux et al. (35) as modified by Martinon et al. (36). The other human primers were as follows: hAV7S2, biotin-CCT-TAGTCGGTCTAAAGGGTACAG; hAJ33, CCCAGCGCC-CAGATTAA; hBJ1S2, GTCTCCCTCAGTCTGGCTCCG; hBJ1S3in, GGTGGTAAGTTCAGAGGCTCTTTTATC; hBJ1S3-out, TCTGTGTGAGGGAGAGAAACG; hBJ1S5, CAAGACAC-ACCAAAGGGAACGC; hBJ2S2, CTCTCCAGCACCCAGACCA; hBJ2S4, CGCACAAAACCCGAGCGCAG; hBJ1S6in, CCAGGCACCCCGAGTCAAGA; hBJ1S6out, GGCAACA-CAGCAGAGCAACAA; hBJ2S7out, TCGTCCTCTCCAGT-CTCGCCT; hBJ2S7in, CGCCCTCTGCTCAGCTTTCCG; and hAJ33in, CAAAAGCTGACTTGCAGGCATCG. In cattle, the following primers were used: boAV19, biotin-CATTCCTTA-GACGCTCTGATGCACA; boAJ33, GCCCCAGATCCACT-GATAGTTGC; pboAV19, FITC-GTGGAGTTCCTTCAGAAG-GAG; 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  $\mu\text{g}/\text{ml}$  proteinase K (Promega Corp.), 0.5% Tween 20 (Sigma-Aldrich), 10 mM Tris-HCl, pH 9, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>. 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  $1-5 \times 10^5$  cells with the RNable solution (Eurobio), ethanol precipitated with addition of 2  $\mu\text{l}$  Pellet Paint coprecipitant (Novagen), and resuspended in 20  $\mu\text{l}$  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 ( $<2 \times 10^4$ ). 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 fol-

lowing: hCA, mGCATGTGCAAACGCCTTCAACAACAq; hAV7.2, mTGAAAGACTCTGCCTCTTACCTCTGTGCq; mAC, mCTCCCAAATCAATGTGCCGAAAACCAq; mAV19, mTCCAGATCAAAGACTCTGCCTCATACCTCTGq; and mAV14, mCACCCTGCTGGATGACACTGCCACq; 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, TCCTTAGTTCGGTCTAAAGGGTACAG; hAJ33, CCAGCG-CCCCAGATTAA; hAC-5', ACCCTGACCCCTGCCGTGT; hAC-3', GGCTGGGGAAGAAGGTGTCTT; mAV14, TGG-GAGATACTCAGCAACTCTGG; mAJ18, CCAGCTCCAA-AATGCAGCC; mAV19, CTTTCCTGAGCCGCTCGAA; mAJ33, CTTGGTCCCAGAGCCCC; mA5-5', CCTCTGC-CTGTTCCACCGACTT; and mA5-3', CGGTCAACGTGGC-ATCACA. In these quantitative kinetic PCR methods, for two samples containing the same amount of C $\alpha$ , 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  $\alpha/\beta$  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 V $\beta$ , 7 J $\beta$ , 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 V $\beta$  primer(s) and a mixture of 7 J $\beta$  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 C $\alpha$  primer pairs. The amplicons were purified using the Wizard PCR clean-up system (Promega Corp.) and sequenced using either a nested primer in the C $\alpha$  constant region (in mouse) or the same AV19 or AV7S2 primer as used for amplification, as appropriate. Sequences were performed with the Thermosequense radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech) with <sup>32</sup>P-ddNTP.

**T-T Hybridoma Generation.** DN and CD8<sup>+</sup> T cells were obtained from TAP<sup>-/-</sup> mice after depleting CD4<sup>+</sup> 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).  $2 \times 10^6$  cells were stimulated, in the presence of  $4 \times 10^6$   $\gamma$ -irradiated (2,500 cGy) splenic cells, for 2 d with soluble anti- $\alpha/\beta$  TCR (H57) at 7.5  $\mu\text{g}/\text{ml}$  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  $\mu\text{g}/\text{ml}$ , 50  $\mu\text{M}$   $\beta$ -ME. At day 4,  $8 \times 10^6$  cells were fused with  $2.5 \times 10^7$  TCR  $\alpha/\beta$ -BW5147 thymoma cells using standard procedures, plated at  $4 \times 10^4$  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. V $\beta$  were characterized by 17 V $\beta$ -specific PCR reactions, and the resulting amplicons were then sequenced. Results were verified by immunofluorescence with specific anti-V $\beta$  antibodies.

## Results

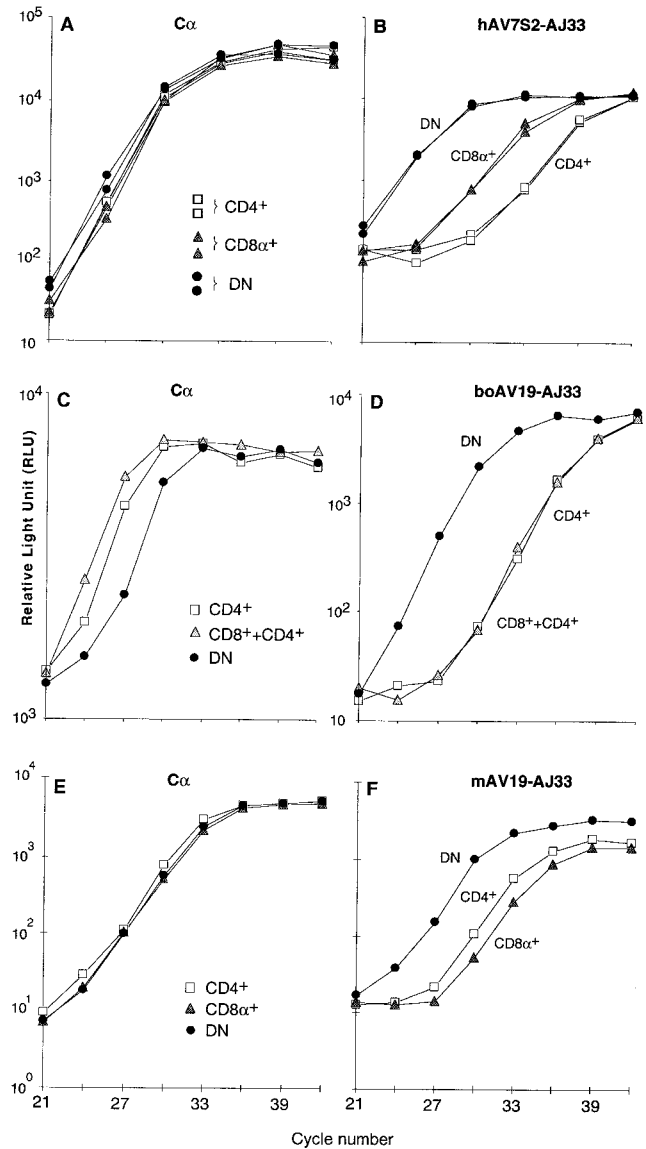
A previous analysis of TCR  $\alpha$  chain repertoire of human DN T cells indicated that besides the invariant hAV24AJ18 TCR  $\alpha$  chain, another TCR  $\alpha$  chain was frequently expressed that also had a restricted AVAJ usage and recurrent junctional features (39). This  $\alpha$  chain, which 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  $\alpha$  chains sequenced in cattle, one chain (BOTCRA14) used the homologous V $\alpha$  and J $\alpha$  segments with a CDR3 of the same length (40). In mice, no homologous invariant  $\alpha$  chain had been described, but the corresponding V $\alpha$  (mAV19) and J $\alpha$  (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 $\alpha$ 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  $\alpha$  chains, PBLs were separated into highly purified  $\alpha/\beta$  DN, CD4<sup>+</sup>, or CD8 $\alpha$ <sup>+</sup> (i.e., CD8 $\alpha\alpha$ <sup>+</sup> and CD8 $\alpha\beta$ <sup>+</sup>) subsets, and the amount of hAV7S2AJ33-encoded transcripts in duplicate fractions was quantified by kinetic PCR. Although the amounts of C $\alpha$  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 $\alpha$ <sup>+</sup> fractions (5 cycles) (Fig. 1 B) compared with the CD4<sup>+</sup> fractions, indicating that DN and CD8 $\alpha$ <sup>+</sup> cells contained ~150–360- and 19–32-fold more hAV7S2AJ33 transcripts, respectively, than CD4<sup>+</sup> lymphocytes. hAV7S2AJ33 transcripts were enriched in DN and CD8 $\alpha$ <sup>+</sup> 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  $\alpha$  chain hAV24AJ18 (data not shown).

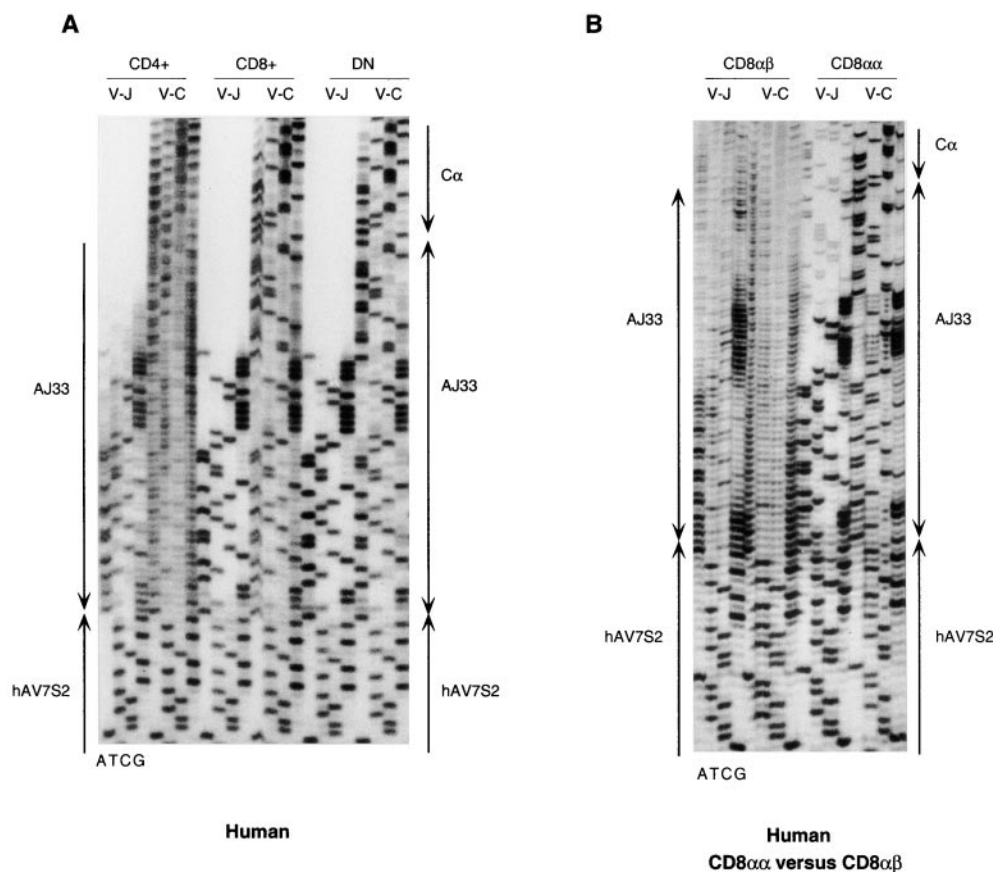
Because a similar invariant  $\alpha$  chain had been sequenced once in cattle (40), we examined its distribution within CD4<sup>+</sup>, CD8<sup>+</sup>, DN, or (CD4<sup>+</sup> plus CD8 $\alpha$ <sup>+</sup>) sorted cells obtained from bovine blood. As shown in Fig. 1, C and D, the levels of boAV19AJ33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

In mice, no canonical TCR  $\alpha$  chain rearrangement involving mAV19 and mAJ33, the murine elements that are homologous to hAV7S2 and hAJ33, respectively, had been de-



**Figure 1.** High levels of hAV7S2/AV19-AJ33 TCR  $\alpha$  chain are present in DN T cells from three mammal species: the indicated cell subpopulations were purified by FACS<sup>®</sup> 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 $\alpha$ <sup>+</sup> fraction contains both CD8 $\alpha\alpha$ <sup>+</sup> and CD8 $\alpha\beta$ <sup>+</sup> TCR  $\alpha/\beta$ <sup>+</sup> T cells. In cattle, no amplification could be obtained with boAV19-AJ33 primers in the CD8 $\alpha$ <sup>+</sup> fraction, and thus the corresponding curves are not displayed.

scribed. However, quantitative PCR analysis of mAV19AJ33 transcripts within highly purified murine  $\alpha/\beta$  DN, CD4<sup>+</sup>, and CD8 $\alpha$ <sup>+</sup> 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<sup>+</sup> cell preparations was lower than in humans and cattle,



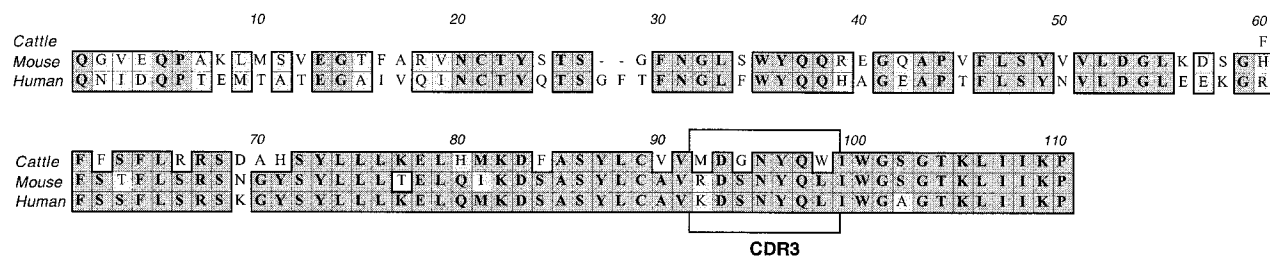
**Figure 2.** (A) The CDR3 of hAV7S2-AJ33 TCR  $\alpha$  chain is of constant length in DN T cells and in human CD8 $\alpha\alpha$ <sup>+</sup> T cells. PCR amplifications from hAV7S2 to C $\alpha$  (V-C) or to AJ33 (V-J) were carried out on cDNA from the T cell subpopulations indicated, and polyclonal sequencing was carried out using the same AV7S2/AV19 primer as used for amplification. Panel B displays polyclonal sequencing of FACS<sup>®</sup>-sorted CD8 $\alpha\beta$ <sup>+</sup> or CD8 $\alpha\alpha$ <sup>+</sup> TCR  $\alpha/\beta$ <sup>+</sup> 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 V $\alpha$ -C $\alpha$  or V $\alpha$ -J $\alpha$  amplicons obtained from the various lymphocyte subpopulations in these species. As shown in Fig. 2 A, a readable hAV7S2AJ33 sequence was obtained from hAV7S2-C $\alpha$  amplicons derived from DN and CD8 $\alpha$ <sup>+</sup> samples but not from CD4<sup>+</sup> 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-C $\alpha$  amplicons demonstrated the predominant presence of the invariant boAV19AJ33 chain in DN cells, but not in CD4<sup>+</sup> or CD8<sup>+</sup> 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-C $\alpha$  amplicons showed a fully readable sequence for the DN but not for the CD8 $\alpha$ <sup>+</sup> 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  $\alpha$  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.

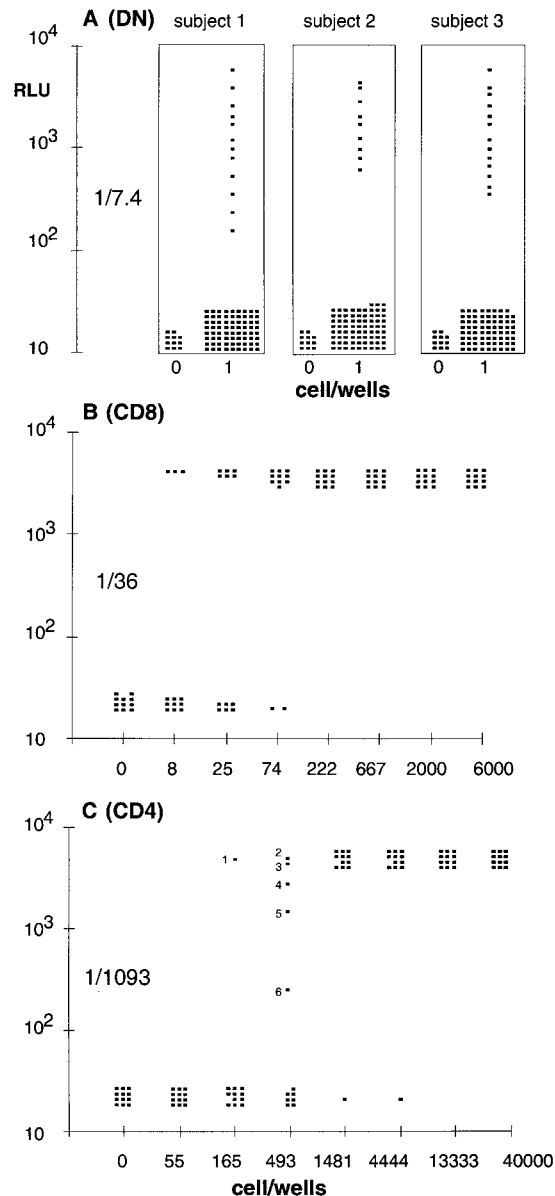
II below). Altogether, these results demonstrated that in the three species, DN  $\alpha/\beta^+$  T cells were enriched for cells bearing TCR  $\alpha$  chains with highly homologous AVAJ elements (hAV7S2AJ33, mAV19AJ33, and boAV19AJ33) and constant CDR3 length as shown in the multiple alignment displayed in Fig. 3. This novel canonical TCR  $\alpha$  chain will be referred to hereafter as the “invariant”  $V\alpha 7/19-J\alpha 33$  TCR  $\alpha$  chain.

**Direct Numbering of Cells Bearing Invariant  $V\alpha 7/19-J\alpha 33$  Chains.** A large amount of invariant  $\alpha$  chain transcripts does not formally demonstrate the existence of a large number of invariant TCR  $\alpha$  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  $V\alpha 7-J\alpha 33$  chain, single  $\alpha/\beta^+$  DN cells from three different subjects were sorted into PCR plates using a single cell deposition unit. FACS<sup>®</sup>-sorted  $\alpha/\beta^+$  TCR  $CD8\alpha^+$  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 lumimetry 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  $V\alpha 7-J\alpha 33^+$  DN cells of  $\sim 1/7.4$ . Using limiting dilution analysis (LDA), the frequency observed for  $CD8\alpha^+$  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  $CD8\alpha^+$  cells demonstrated the presence of invariant  $V\alpha 7-J\alpha 33$  transcripts in all cases. A similar analysis on  $CD4^+$  cells yielded a frequency of  $V\alpha 7-J\alpha 33^+$  cells of 1/1,093 (1/751–1/2,003; Fig. 4 C). Importantly, sequencing of the positive wells at concentrations where the  $V\alpha 7-J\alpha 33$  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  $V\alpha 7-J\alpha 33$  chain with a different CDR3 length, and only one corresponded to the invariant  $V\alpha 7-J\alpha 33$  chain. Thus, the actual frequency of  $CD4^+$  cells harboring the invariant  $V\alpha 7-J\alpha 33$  chain should be  $\sim 1/6,000$ .

In mice,  $V\alpha 19-J\alpha 33^+$  cells were less numerous than in humans. Their frequency within  $\alpha/\beta$  DN lymph node cells was 1/56 (Fig. 5 A), compared with  $\sim 1/7.5$  in humans (Fig. 4 A).

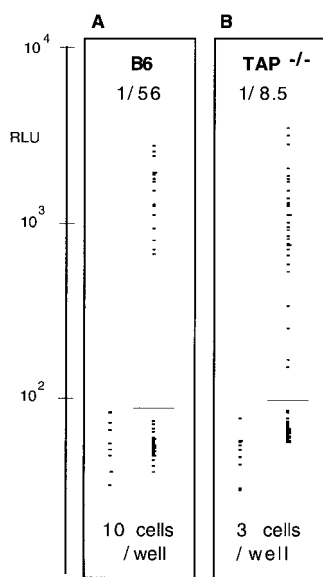
#### Surface Phenotype and TCR $\beta$ Repertoire of $V\alpha 7/19-J\alpha 33$ -bearing Cells

**Invariant  $V\alpha 7-J\alpha 33$ -bearing Cells Express Memory Markers and Show a Biased Usage of hBV2 and hBV13 TCR  $\beta$  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  $V\alpha 7-J\alpha 33$  chain.  $\alpha/\beta$  DN and  $CD8\alpha^+$  cells were depleted or enriched for a given marker by FACS<sup>®</sup> sorting, and the amounts of  $V\alpha 7-J\alpha 33$  transcripts in the positive and negative fractions were quantified and compared with the amounts of amplified C $\alpha$  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<sup>®</sup> 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)  $\alpha/\beta$   $CD8\alpha^+$  or  $CD4^+$  cells were obtained by FACS<sup>®</sup> 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:

- 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 GAA AAC-AJ34-intron-AJ33
- 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.



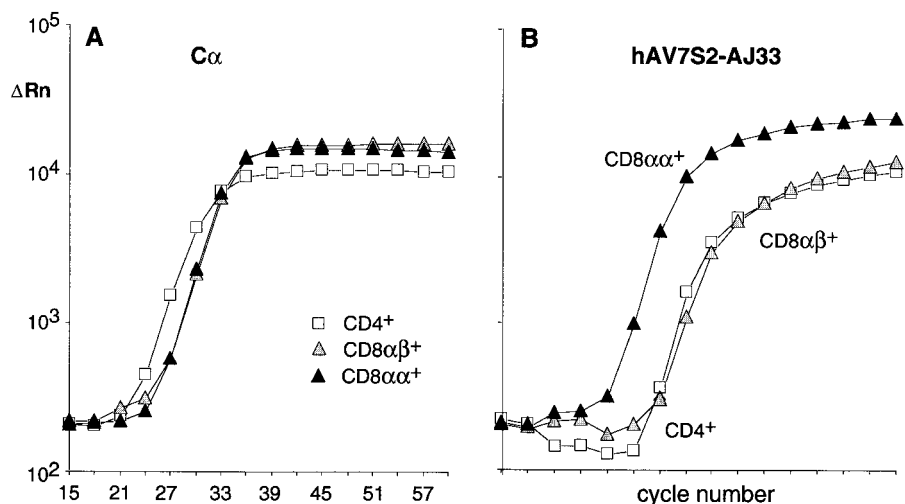
**Figure 5.** mAV19-AJ33<sup>+</sup> cells are more frequent in TAP<sup>-/-</sup> mice. The indicated number of DN T cells from B6 (A) or TAP<sup>-/-</sup> (B) mice were seeded in PCR microplates, and the presence of the mAV19-AJ33 rearrangement was detected by PCR-ELISA.

account variations in cell number. Fig. 6 displays an example of such an experiment, where we examined whether the CD8 molecules expressed by V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> cells were either heterodimeric ( $\alpha\beta$ ) or homodimeric ( $\alpha\alpha$ ), since CD8 $\alpha\alpha$ <sup>+</sup> cells represent between 5 and 25% of CD8 $\alpha$ <sup>+</sup> TCR  $\alpha/\beta$ <sup>+</sup> lymphocytes in human PBLs (data not shown). There were  $\sim$ 45-fold more V $\alpha$ 7-J $\alpha$ 33 transcripts in the CD8 $\alpha\alpha$ <sup>+</sup> fraction than in the CD8 $\alpha\beta$ <sup>+</sup> fraction. This result was confirmed by polyclonal sequencing of the amplicons obtained after amplification with hAV7S2/C $\alpha$  (V-C) or hAV7S2/hAJ33 (V-J) primers: no readable sequence was obtained from the CD8 $\alpha\beta$ <sup>+</sup> fraction, whereas the invariant  $\alpha$  chain was present in the CD8 $\alpha\alpha$ <sup>+</sup> fraction (Fig. 2 B). The absence of a readable sequence in the CD8 $\alpha\beta$ <sup>+</sup> fraction using V-J amplification indicates the near absence of the invariant  $\alpha$  chain in this fraction. Using this strategy, it could be established that V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> cells had mainly a memory phenotype (i.e., CD45R0<sup>+</sup>CD45RA<sup>-</sup>) and were CD56<sup>-</sup>CD57<sup>-</sup>CD28<sup>+</sup>CD27<sup>+</sup> (data not shown).

Despite many attempts using different lymphokine mix-

tures, we were unable to obtain a large enough number of T cell clones expressing the invariant V $\alpha$ 7-J $\alpha$ 33 chain. Therefore, the TCR  $\beta$  chain repertoire of DN cells bearing invariant V $\alpha$ 7-J $\alpha$ 33 chain was directly estimated by single cell PCR analysis. After single cell deposition of DN  $\alpha/\beta$  cells into PCR plates, cells were lysed and a first PCR reaction was carried out using hAV7S2, hAJ33, and a mixture of 29 V $\beta$  and 7 J $\beta$ . This first reaction was then amplified in a second PCR with nested AV7S2/AJ33 primers allowing us to find the wells containing V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> 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) V $\beta$  and a mixture of 7 J $\beta$  primers. This allowed us to get a specific band for 0–2 V $\beta$  which was then sequenced using the relevant V $\beta$  primer. In 9 independent experiments carried out on DN cells obtained from 3 different subjects, the frequency of V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> wells ranged between 6 and 16/84. Among the 92 V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> wells studied, a  $\beta$  chain could be assigned and sequenced in 37 cases (40%), and we found that there was a heavy bias toward V $\beta$ 13 and V $\beta$ 2. Out of 13 V $\beta$  sequenced in donor A, 7 were BV13<sup>+</sup> and 3 were BV2<sup>+</sup>. In donor B, 8/12 were BV13<sup>+</sup> and 4/12 were BV2<sup>+</sup>. In donor C, 7/12 were BV13<sup>+</sup> and 2/12 were BV2<sup>+</sup>. However, despite this biased usage of BV13 or BV2 regions, the TCR  $\beta$  chains derived from V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> cells did not show obvious restrictions in J $\beta$  usage or in CDR3 length (Table I). Sequence analysis of V $\alpha$ J $\alpha$  junctions confirmed the presence of V $\alpha$ 7-J $\alpha$ 33 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  $\alpha$  and  $\beta$  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, V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> cells appear to use a semirestricted repertoire that is expanded as needed.

**TCR  $\beta$  Chain Repertoire of V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> Cells.** Because the frequency of V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> cells is not as high in mice as in



**Figure 6.** hAV7S2-AJ33-bearing cells are CD8 $\alpha\alpha$ <sup>+</sup> or DN. The indicated TCR  $\alpha/\beta$ <sup>+</sup> fractions were FACS<sup>®</sup> sorted using a four-color staining with anti-CD4-TC, anti-CD8 $\alpha$ -FITC, anti-CD8 $\beta$ -PE, and anti- $\alpha/\beta$  TCR-APC. The amounts of C $\alpha$  (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.  $\Delta$ Rn, difference in fluorescence relative units.

**Table I.**  $V\beta$  Repertoire of Human Invariant AV7S2-AJ33 TCR  $\alpha$  Chain-bearing Cells

Germline AV7S2 TGT GCT GTG AGAGAcacaggtgact <sup>a</sup> ttgtgtctgtgtg GAT AGC germline AJ33							
subject	Cell	V $\alpha$ -J $\alpha$ CDR3	V $\beta$	CDR3	J $\beta$	PCR plate #	
A	G5	GCC GTG AAG GAT AGC A V K D S	13.x	CAS SPS1GGGGEQ	FRG	2.1 2	
	E6	GCC CCC CTG GAT AGC A P L D S	13.2	CAS SVYPPDGNIT	YFG	1.3 1	
	D8-F7-G10 <sup>b</sup>	GCT GTG AAG GAT AGC A V K D S	13.3	CAS SWITGEGSSSEL	FRG	2.2 3	
		GCT GTG AAG GAT AGC A V K D S	13.3	CAS SWITGEGSSSEL	FRG	2.2 1	
	7 V $\beta$ 13/13	G1	GCT GTG AAG GAT AGC A V M D S	13.5	CAS SGG1SIDITQ	YFG	2.3 3
		G3	GCT GTG CTG GAT AGC A V L D S	2.1	CSA SKGENMEQ	YFG	2.7 2
	3 V $\beta$ 2/13	E10	GCT GTG ATG GAT AGC A V M D S	2	CSA FVGGNITEA	FRG	1.1 3
		C2	GCT GCC ATG GAT AGC A A M D S	2	CSA SEBBSHYEQ	YFG	2.7 3
	2 V $\beta$ 24/13	E1 <sup>c</sup>	GCT GTG ATG GAT AGC A V M D S	24	CAT SRDGTSSGGATVNL	TRF	2.6 2
		D3 <sup>d</sup>	GCT GTG ATG GAT AGC A V M D S	24	CAT SRDGTSSGGATVNL	TRF	2.6 1
1 V $\beta$ 15/13	F1	GCT GTG ATG GAT AGC A V I D S	15	CAT MIPADTGEI	FRG	2.2 2	
B	B10	GCT GGG ATG GAT AGC A G M D S	13.3	CAS TLGQEQEQPQ	FRG	1.5 1	
	G2	GCT TCC ATG GAT AGC A S M D S	13.3	CAS SEIDPNIGEL	FRG	2.2 1	
	F3-C1 <sup>d</sup>	GCT TCC ATG GAT AGC A S I D S	13.3	CAS SEIDPNIGEL	FRG	2.2 2	
		G8	GCT GCC ATG GAT AGC A A M D S	13.3	CAS SGG1SSGSEL	FRG	2.2 1
	C10	GCT GCC GAG GAT AGC A A E D S	13.3	CAS SYEVSQANVL	TRF	2.6 1	
		A10	GCT GCA TTG GAT AGC A A L D S	13.5	CAS SVSGETDITQ	YFG	2.3 1
	8 V $\beta$ 13/12	A12	GCT GGG TTG GAT AGC A G L D S	13.5	CAS SESGGNFDITQ	YFG	2.3 1
		D5	GCT GCT TTG GAT AGC A A L D S	2	CSA RVRGNITEA	FRG	1.1 1
	4 V $\beta$ 2/12	LB	ND <sup>e</sup>	2	CSA RDGGKDSGSEL	FRG	2.2 1
		A6	GCT GTG ATG GAT AGC A V M D S	2.1	CSA SPEDRATSEL	FRG	1.6 3
B12		GCT GCT ATG GAT AGC A A I D S	2.1	CSA RELAEPEYEQ	YFG	2.7 3	
B11		GCT GTG AGA GAT AGC A V R D S	13.2	CAS NQSDVDITGEL	FRG	2.2 2	
C	B9	GCT GTG ATG GAT AGC A V M D S	13.2	CAS SPSGGYNEQ	FRG	2.1 1	
	E8	GCT AGC ATG GAT AGC A S M D S	13.2	CAS SVLGGGANVL	TRF	2.6 3	
	C10	GCT GTG CTG GAT AGC A V V D S	13.3	CAS SPPGGTDIR	YFG	2.3 2	
		A11	GCT GTG AGA GAT AGC A V R D S	13.3	CAS SAAVEGGNIT	YFG	1.3 3
	7 V $\beta$ 13/12	G3	GCT TTC ATG GAT AGC A F M D S	13.5	CAS SIKGSSITGE	LFG	2.2 1
		D8	GCT GTC CAG GAT AGC A V Q D S	13.5	CAS SAEGAFDITQ	YFG	2.3 3
	2 V $\beta$ 2/12	F7 <sup>f</sup>	GCT TTT ATG GAT AGC A F M D S	2	CSA RKSQDFGEQ	FRG	2.1 2
		E5 <sup>g</sup>	GCT TTT ATG GAT AGC A F M D S	2.1	CSA RKSQDFGEQ	FRG	2.1 1
	1 V $\beta$ 3/12	B1	GCT GTC TTG GAT AGC A V L D S	3	CAS SRGTDAGEI	FRG	2.2 1
	1 V $\beta$ 7/13	D5	ND <sup>e</sup>	7.2	CAS SQELQGNVEQ	YFG	2.7 2
1 V $\beta$ 19/12	F1	GCT GTA TTG GAT AGC A V L D S	19	CAN LDRESNIGEL	FRG	2.2 1	
1 V $\beta$ 20/12	A10	GCT GTG CTG GAT AGC A V L D S	20	CNW RSQGSVGH	TRF	1.2 2	

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

<sup>a</sup>Small caps code for the recombination sequence signal.

<sup>b, c, d, e</sup> Identical TCR  $\alpha$  and  $\beta$  sequences probably representing a single expanded clone.

<sup>f</sup>Not done.

humans, the strategy followed to characterize the surface phenotype of human V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> cells was not successful in mice. Therefore, to characterize the TCR  $\beta$  chain repertoire of this subset, we took advantage of the fact that

V $\alpha$ 19-J $\alpha$ 33<sup>+</sup> cells are enriched in TAP<sup>-/-</sup> mice (see below). We generated T-T hybridomas from DN enriched lymph node cells from TAP<sup>-/-</sup> mice, and screened for expression of V $\alpha$ 19-J $\alpha$ 33 transcripts. In agreement with the single cell analysis (Fig. 5 B), 16/168 hybridomas studied were V $\alpha$ 19-J $\alpha$ 33<sup>+</sup>. As shown in Table II, in all cases, these hybridomas carried V $\alpha$ 19-J $\alpha$ 33 chains with the canonical CDR3 length and in all but two cases, the V $\alpha$ 19-J $\alpha$ 33 junction was germline encoded. The V $\beta$  segments used were predominantly BV8<sup>+</sup> (7/16) and BV6<sup>+</sup> (4/16). No restriction in the J $\beta$  repertoire was apparent.

#### Development and Selection of V $\alpha$ 7/19-J $\alpha$ 33-bearing Cells

**Ontogeny of V $\alpha$ 7/19-J $\alpha$ 33-bearing Cells.** NK1 T cells are not found in neonates and accumulate after birth (5). Therefore, we examined cord blood lymphocytes for V $\alpha$ 7/19-J $\alpha$ 33 sequences by quantitative PCR and polyclonal sequencing of V $\alpha$ 7-C $\alpha$  or V $\alpha$ 7-J $\alpha$ 33 amplicons on cDNA obtained from enriched DN/CD8<sup>+</sup> or CD4<sup>+</sup> fractions. We were unable to find significant amounts of invariant V $\alpha$ 7-J $\alpha$ 33 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, V $\alpha$ 19-J $\alpha$ 33-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 V $\beta$  Repertoire of T-T mAV19-AJ33<sup>+</sup> Hybridomas

Germline mAV19 TGT GCT GTG AGG xxx ttgtgtg GAT AGC AAC germline mAJ33						
Clone	mAV19-AJ33	V $\beta$	CDR3	J $\beta$		
26G12	GCT GTG AGG GAT AGC AAC A V R D S N	8.1	CAS SDVGESSDY	TRF	1.2	
22A5	GCT GTG AGG GAT AGC AAC A V R D S N	8.1	CAS SDNQHVEQ	YFG	1.4	
23G6	GCT GTG AGG GAT AGC AAC A V R D S N	8.1	CAS RDNVABQ	FRG	2.1	
16H2	GCT GTG AGG GAT AGC AAC A V R D S N	8.2	CAS GEGGAYABQ	FRG	2.1	
6H2	GCT GTG AGG GAT AGC AAC A V R D S N	8.2	CAA GTGGALYABQ	FRG	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 GEGRTGGFERQ	YFG	2.7	
23B1	GCT GTG AGG GAT AGC AAC A V R D S N	6	CAS SQPQGGYNSP	YFA	1.6	
7D11	GCT GTG AGG GAT AGC AAC A V R D S N	6	CAS STGGDNVABQ	FRG	2.1	
11F1	GCT GTG <u>CTG</u> GAT AGC AAC A V L D S N	6	CAS SGGDYABQ	FRG	2.1	
22B11	GCT GTG AGG GAT AGC AAC A V R D S N	6	CAS SRLSGNITGQL	YFG	2.2	
17B6	GCT GTG AGG GAT AGC AAC A V R D S N	2	CSA DPGQCCERL	FRG	1.4	
9C2	GCT GTG AGG GAT AGC AAC A V R D S N	5	CAS SLGTGSABITL	YFG	2.3	
4H1	GCT GTG AGG <u>GAC</u> GAC AAC A V R D G N	7	CAS SSSGGYNSSEL	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	CNW RGGSDAETL	YFG	2.3	

\*In bold and underlined, N additions.



**Table III.** Strain and Tissue Distribution, and MHC Selection of mAV19-AJ33<sup>+</sup> T Cells

Strain	Haplotype	Lymph nodes	Spleen	PBLs	Thymus	Bone marrow	Liver	IELs	Lamina propria
BALB/c	H-2 <sup>d</sup> I-A <sup>+</sup> /I-E <sup>+</sup>	+++	+	+++	+	+	-	-	-
DBA/2	H-2 <sup>d</sup> I-A <sup>+</sup> /I-E <sup>+</sup>	+++							
CBA	H-2 <sup>k</sup> I-A <sup>+</sup> /I-E <sup>+</sup>	+++	+						
129	H-2 <sup>b</sup> I-A <sup>+</sup> /I-E <sup>-</sup>	+		+++					
C57BL/6	H-2 <sup>b</sup> I-A <sup>+</sup> /I-E <sup>-</sup>	+++		+++	+				
NUDE B6	H-2 <sup>b</sup> I-A <sup>+</sup> /I-E <sup>-</sup>	-	-						
	B6	I-A <sup>b-/-</sup>	β2m <sup>-/-</sup>	K <sup>b-/-</sup> /D <sup>b-/-</sup>	TAP <sup>-/-</sup>	CD1 <sup>-/-</sup>	CD8 <sup>-/-</sup>		
mAV19-AJ33 (fold increase compared with B6)	++	++ (2)	-	++ (4)	+++ (8)	++ (2)	++ (4)		
mAV14-AJ18	++	++	-	++	++	-	++		

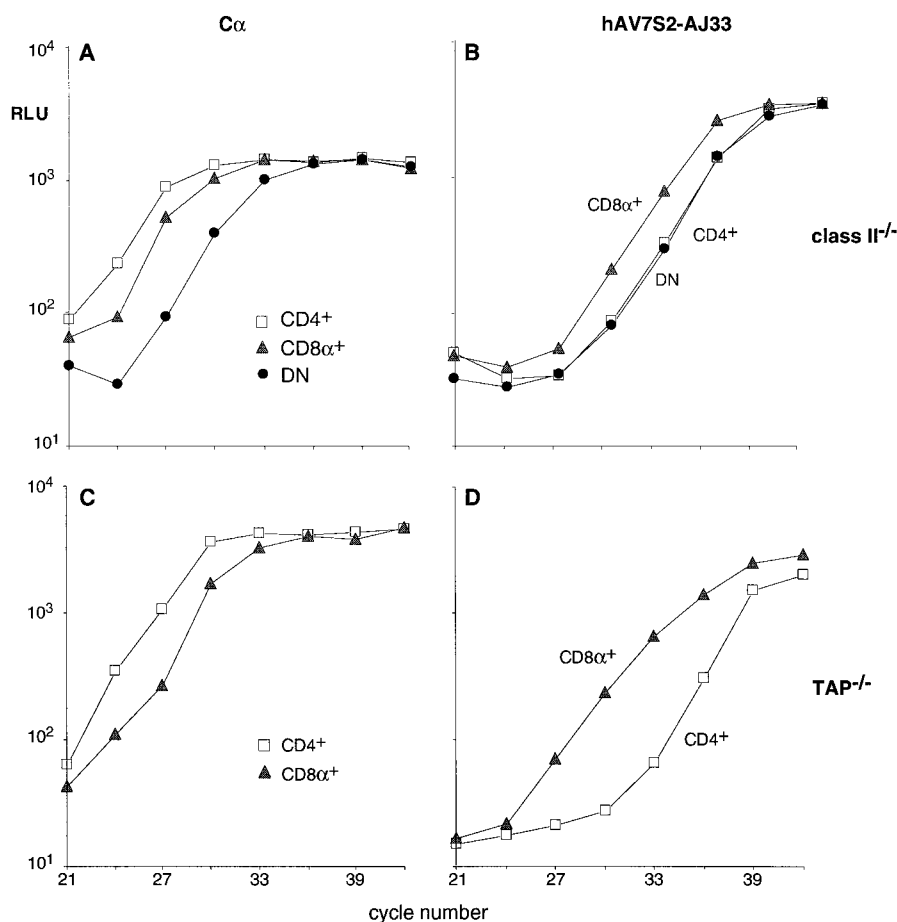
DN  $\alpha/\beta^{+/+}$  T cells were purified from the indicated organs in different mouse strains. Expression of the invariant mAV19-AJ33 TCR  $\alpha$  chain was estimated by quantitative kinetic PCR and confirmed by polyclonal sequencing (see Materials and Methods, and Figs. 1 and 2). Tissue distribution results are compared with the expression in sorted CD4<sup>+</sup>  $\alpha/\beta^{+}$  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-C $\alpha$ , or -AJ33 amplification as appropriate.

V $\alpha$ 19-J $\alpha$ 33-bearing cells. Importantly, although more than half of the human invariant V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> cells were CD8 $\alpha\alpha$ <sup>+</sup> cells, which might suggest an extrathymic development pathway (43), invariant V $\alpha$ 19-J $\alpha$ 33 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 V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> Cells Is Not Affected in MHC Class II- and TAP-deficient Patients.* The restriction element of V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> cells could be either a class I or class II MHC molecule, as they do not express CD4 or CD8 $\alpha\beta$  accessory molecules. To address this issue, we quantified the expression of the V $\alpha$ 7-J $\alpha$ 33 invariant chain in PBLs obtained from MHC-deficient patients. MHC class II-deficient patients harbor low but significant numbers of CD4<sup>+</sup>  $\alpha/\beta$  lymphocytes whose V $\beta$  repertoire is grossly normal (44). CD4<sup>+</sup>, CD8 $\alpha$ <sup>+</sup>, and DN enriched fractions were prepared and analyzed by quantitative PCR. Although the amount of C $\alpha$  was much lower in the DN than in the CD4<sup>+</sup> samples (Fig. 7 A), the amounts of V $\alpha$ 7-J $\alpha$ 33 transcripts were comparable in both samples (Fig. 7 B), therefore indicating an enrichment of V $\alpha$ 7-J $\alpha$ 33 transcripts within DN cells and, presumably, selection of invariant V $\alpha$ 7-J $\alpha$ 33-bearing cells in this patient. Accordingly, the

predominance of the invariant V $\alpha$ 7-J $\alpha$ 33 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 V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> 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,  $\alpha/\beta^{+}$  TCR CD8 $\alpha$ <sup>+</sup> cells are reduced in numbers at birth but may expand with age.  $\alpha/\beta^{+}$  TCR CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> cells were separated in one of these patients, and the amount of C $\alpha$  and V $\alpha$ 7-J $\alpha$ 33 transcripts was quantified in the two fractions. Despite lower amounts of C $\alpha$  in the CD8 $\alpha$ <sup>+</sup> fraction, the amount of V $\alpha$ 7-J $\alpha$ 33 transcripts was much higher in the CD8 $\alpha$ <sup>+</sup> fraction (Fig. 7, C and D). Enrichment for V $\alpha$ 7-J $\alpha$ 33 transcripts was also demonstrated in a PHA-stimulated CD8 $\alpha$ <sup>+</sup> line derived from the other TAP-deficient patient, when compared with either unsorted PBL-derived cell line from the same patient or to CD8 $\alpha$ <sup>+</sup> cells from healthy donors (data not shown). Actually, the larger amounts of V $\alpha$ 7-J $\alpha$ 33 transcripts in the CD8 $\alpha$ <sup>+</sup>  $\alpha/\beta$  T cells of TAP-deficient patients is in agreement with the higher proportion of CD8 $\alpha$ <sup>+</sup> cells in these patients (data not shown). This indi-



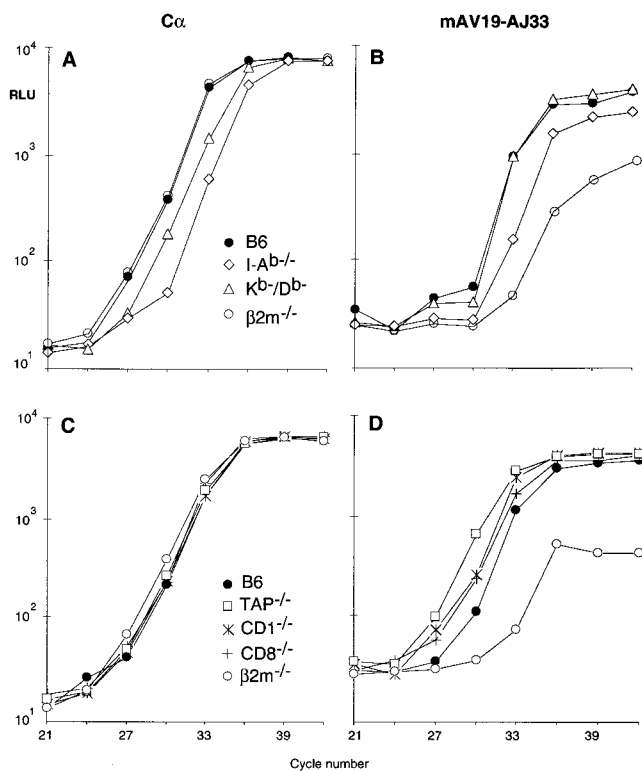
**Figure 7.** AV7S2-AJ33-bearing cells are present in MHC class II<sup>-/-</sup> and TAP-deficient patients. The indicated cell subpopulations were obtained from an MHC class II<sup>-/-</sup> (A and B) or a TAP-deficient (C and D) patient. The amounts of C $\alpha$  or hAV7S2-AJ33 transcripts were quantified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

icates that T cells bearing this invariant  $\alpha$  chain are probably not selected by a TAP-dependent MHC class I molecule.

Thus, the selecting molecule for the invariant V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> 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 V $\alpha$ 19-J $\alpha$ 33<sup>+</sup> Cells Is  $\beta$ 2m-dependent, TAP-independent, and Distinct from CD1.* The amount of V $\alpha$ 19-J $\alpha$ 33 transcripts was examined in  $\alpha/\beta^+$  DN lymph node cells from several MHC-deficient strains. Because the frequency of V $\alpha$ 19-J $\alpha$ 33<sup>+</sup> 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 C $\alpha$  in the  $\beta$ 2m<sup>-/-</sup> samples, there were fewer V $\alpha$ 19-J $\alpha$ 33 transcripts than in control or I-A<sup>b-/-</sup> mice (Fig. 8, A and B). Polyclonal sequencing of V $\alpha$ 19-J $\alpha$ 33 amplicons from  $\beta$ 2m<sup>-/-</sup> samples showed the complete absence of invariant V $\alpha$ 19-J $\alpha$ 33 transcripts. This was consistent with an MHC class I or  $\beta$ 2m-dependent class I-like selection of the invariant V $\alpha$ 19-J $\alpha$ 33<sup>+</sup> cells. However, the relevant molecule is not a classical MHC class I molecule

because the amount of invariant V $\alpha$ 19-J $\alpha$ 33 transcripts was higher in the DN cells from K<sup>b-/-</sup>D<sup>b-/-</sup> mice than in controls, though the C $\alpha$  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, V $\alpha$ 19-J $\alpha$ 33 transcripts were quantified in CD8<sup>-</sup>, CD1<sup>-</sup>, and TAP-deficient mice, B6 and  $\beta$ 2m<sup>-/-</sup> mice being used as positive and negative controls, respectively. There was no decrease in the amounts of V $\alpha$ 19-J $\alpha$ 33 transcripts in CD1-deficient mice, indicating that V $\alpha$ 19-J $\alpha$ 33<sup>+</sup> cells were not selected by CD1 or a CD1-bound ligand. The curves for TAP<sup>-/-</sup> samples were actually shifted to the left, indicating an increased frequency of V $\alpha$ 19-J $\alpha$ 33<sup>+</sup> cells in TAP<sup>-/-</sup> mice, in agreement with the result shown in Fig. 5 B where the frequency of V $\alpha$ 19-J $\alpha$ 33<sup>+</sup> cells in DN TAP<sup>-/-</sup> mice was 1/8.5 (i.e., a sixfold increase compared with B6; Fig. 5 A). The V $\alpha$ 19-J $\alpha$ 33 curve corresponding to the CD8-deficient mice is similar to that of B6, indicating the presence of V $\alpha$ 19-J $\alpha$ 33 invariant chains in CD8<sup>-/-</sup> mice. This was confirmed by polyclonal sequencing (data not shown). Altogether, these results, summarized in Table III (bottom), indicate that V $\alpha$ 19-J $\alpha$ 33<sup>+</sup> cells do not require CD8 for their selection and that the selecting molecule is a  $\beta$ 2m-dependent, TAP-independent molecule distinct from CD1.



**Figure 8.** AV19-AJ33<sup>+</sup> cells are selected by a  $\beta$ 2m-dependent, TAP-independent nonclassical MHC-like molecule distinct from CD1. DN lymph node cells were obtained from the indicated mice, and the amounts of C $\alpha$  (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 V $\alpha$ 7.2-J $\alpha$ 33 TCR  $\alpha$  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  $\alpha$  repertoire. In humans, DN and CD8 $\alpha$ <sup>+</sup> cells bearing invariant V $\alpha$ 7-J $\alpha$ 33 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 V $\beta$  repertoire restriction, or to the difficulties in growing clones expressing this invariant chain (our unpublished observations), together with methodological limitations linked to TCR  $\alpha$  chain repertoire analysis (e.g., lack of allelic exclusion [46]). However, this invariant  $\alpha$  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 V $\alpha$ 7-J $\alpha$ 33 invariant  $\alpha$  chain. In the same study, this canonical sequence was not found in cord blood samples or in CD4<sup>+</sup> cells, but represented 3/40 (7.5%) CDR3 sequences derived from CD8 $\alpha$ <sup>+</sup> 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  $\alpha$  chains randomly sequenced (40). In mice,

random cloning of TCR  $\alpha$  chains revealed one example of this invariant V $\alpha$ 19-J $\alpha$ 33  $\alpha$  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 J $\alpha$  loci are entirely homologous, with the same genomic organization and an average similarity of 71% including the J $\alpha$  coding sequences (41, 42, 49). The AJ33 is the most similar J $\alpha$  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  $\alpha$  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  $\alpha$  chain was associated with a limited number of V $\beta$  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  $\beta$  chain diversity of invariant V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> cells remained extensive, since most J $\beta$  segments and seven V $\beta$ s were found associated with CDR3s of variable length. In mice, five different V $\beta$ s 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 DEC<sub>s</sub> and in murine and human NK1 T cells. In DEC<sub>s</sub>, 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 V $\gamma$  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 V $\alpha$ 7/19-J $\alpha$ 33<sup>+</sup> cells is probably an MHC class Ib molecule distinct from CD1d, though a  $\beta$ 2m-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 V $\alpha$ 7-J $\alpha$ 33  $\alpha$  chain between FACS<sup>®</sup>-sorted HLA-E tetramer-positive and -negative (DN plus CD8 $\alpha$ <sup>+</sup>)  $\alpha$ / $\beta$ <sup>+</sup> TCR PBL fractions (data not shown). Moreover, the in-

variant V $\alpha$ 19-J $\alpha$ 33 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 CD8 $\alpha\alpha$  on V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> 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, V $\alpha$ 7/19-J $\alpha$ 33<sup>+</sup> 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  $\alpha/\beta$  T cells in the periphery. The absence of significant numbers of V $\alpha$ 19-J $\alpha$ 33<sup>+</sup> cells in mouse CD8 $\alpha\alpha$ <sup>+</sup> cells (Fig. 1 F, and polyclonal sequencing after V-J amplification [not shown]) is in agreement with the extremely low numbers (<0.4%) of CD8 $\alpha\alpha$ <sup>+</sup> cells in murine blood or lymph nodes (data not shown). The CD8 $\alpha\alpha$  expression on some invariant V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> cells and its complete absence on V $\alpha$ 19-J $\alpha$ 33<sup>+</sup> counterparts is reminiscent of the phenotype of NK1 T cells, which may express CD8 $\alpha\alpha$ <sup>+</sup> in humans (28) but never in mice. The reasons for such phenotypic differences between mice/cattle and humans as well as the role of CD8 $\alpha\alpha$  expression in human PBLs are not clear. There have been contradictory reports concerning the role of homo- versus heterodimeric CD8 (60–63). In addition, CD8 $\alpha\alpha$  expression in human PBLs might reflect an activated status rather than a particular differentiation pathway (64).

Concerning the development pathway of the V $\alpha$ 7/19-J $\alpha$ 33 cells, in the three species there seems to be no expression of the invariant  $\alpha$  chain in the CD8 $\alpha\beta$ <sup>+</sup> T cells, strong enrichment for this sequence within DN cells (as well as within human CD8 $\alpha\alpha$ <sup>+</sup> cells), and persistence of the invariant sequence in the CD4<sup>+</sup> subset. Indeed, in humans, the frequency of invariant  $\alpha$  chain-bearing cells was  $\sim$ 1/6,000 in the CD4<sup>+</sup> fraction. Moreover, polyclonal sequencing of V $\alpha$ 7-J $\alpha$ 33 amplicons from CD4<sup>+</sup> cells (Fig. 2 A) revealed the presence of canonical CDR3 at a low, though readable, frequency, whereas the canonical CDR3 was undetectable in CD8 $\alpha\beta$ <sup>+</sup> samples (Fig. 2 B). In mice and cattle, there was also a small but reproducible shift of the CD4<sup>+</sup> amplification curves to the left compared with the CD8 $\alpha$ <sup>+</sup> curves, suggesting some expression of the invariant  $\alpha$  chain in murine or bo-

vine CD4<sup>+</sup> cells. Accordingly, a readable canonical sequence in V $\alpha$ 19-J $\alpha$ 33 amplicons was detected in the CD4<sup>+</sup> but not in the CD8 $\alpha$ <sup>+</sup> fraction in both species (data not shown). The absence of any readable sequence after V-J amplification of CD8 $\alpha/\beta$ <sup>+</sup> cells suggests that the CD8 $\alpha\beta$ <sup>+</sup> V $\alpha$ 7/19-J $\alpha$ 33-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 V $\alpha$ 7/19-J $\alpha$ 33 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<sup>+</sup> T cells bearing the V $\alpha$ 7/19-J $\alpha$ 33  $\alpha$  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 V $\alpha$ 7-J $\alpha$ 33<sup>+</sup> 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 V $\alpha$ 7/19-J $\alpha$ 33-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 V $\alpha$ 7/19-J $\alpha$ 33-bearing cells display semi-invariant repertoires (i.e., one monomorphic  $\alpha$  chain and a biased V $\beta$  chain repertoire), suggesting that their TCRs may have similar characteristics, probably a high affinity for a selecting ligand by the invariant  $\alpha$  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,  $\beta$ 2m-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  $\alpha$  chains defining other T cell subpopulations? We did not find any after measuring the amounts of several other  $\alpha$  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  $\gamma/\delta$  subpopulations are distributed.

Concerning the functions of V $\alpha$ 7/19-J $\alpha$ 33<sup>+</sup> lymphocytes, future analysis of the mAV19AJ33 transgenic mice we have made will certainly help address this point. The high frequency of V $\alpha$ 7/19-J $\alpha$ 33<sup>+</sup> T cells and their extensive phylogenetic conservation in mammals both argue for an important physiological function. Because V $\alpha$ 7/19-J $\alpha$ 33<sup>+</sup> 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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