

## **An Invariant V $\alpha$ 24-J $\alpha$ Q/V $\beta$ 11 T Cell Receptor Is Expressed in All Individuals by Clonally Expanded CD4<sup>-</sup>8<sup>-</sup> T Cells**

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### **Summary**

The T cell receptor (TCR)- $\alpha/\beta$  CD4<sup>-</sup>8<sup>-</sup> (double negative, DN) T cell subset is characterized by an oligoclonal repertoire and a restricted V gene usage. By immunizing mice with a DN T cell clone we generated two monoclonal antibodies (mAbs) against V $\alpha$ 24 and V $\beta$ 11, which have been reported to be preferentially expressed in DN T cells. Using these antibodies, we could investigate the expression and pairing of these V $\alpha$  and V $\beta$  gene products among different T cell subsets. V $\alpha$ 24 is rarely expressed among CD4<sup>+</sup> and especially CD8<sup>+</sup> T cells. In these cases it is rearranged to different J $\alpha$  segments, carries N nucleotides, and pairs with different V $\beta$ . Remarkably, V $\alpha$ 24 is frequently expressed among DN T cells and is always present as an invariant rearrangement with J $\alpha$ Q, without N region diversity. This invariant V $\alpha$ 24 chain is always paired to V $\beta$ 11. This unique V $\alpha$ 24-J $\alpha$ Q/V $\beta$ 11 TCR was found in expanded DN clones from all the individuals tested. These findings suggest that the frequent occurrence of cells carrying this invariant TCR is due to peripheral expansion of rare clones after recognition of a nonpolymorphic ligand.

Growing evidence indicates that TCR- $\alpha/\beta$  CD4<sup>-</sup>8<sup>-</sup> (double negative, DN) T cells constitute a peculiar subset with respect to ontogeny, specificity, lifespan, and TCR V gene usage. These cells do not appear to undergo classical positive and negative selection in the thymus (1–4), and it has been suggested that they may develop extrathymically (5–7). With respect to specificity, it has been shown that in mice  $\alpha/\beta$  DN T cells recognize bacteria (8), and in humans, recognize monomorphic CD1 molecules as such or in association with bacterial products (9–11). A particularly striking feature is the presence within this population of expanded clones that persist for years with the same clonal size (11, 12). Furthermore  $\alpha/\beta$  DN T cells display a restricted TCR V gene usage (13, 14). Molecular analysis of random cDNAs from human  $\alpha/\beta$  DN T cells revealed a preferential expression of particular V $\alpha$  and V $\beta$  such as V $\alpha$ 24 and 7 and V $\beta$ 2, 8, 11, and 13 (13, 15) suggesting a biased TCR usage. However, this method could not address whether there could be a preferential pairing of the various V gene products and whether the frequent occurrence of a given sequence is due to a frequent rearrangement or to the clonal expansion of the corresponding T cells.

We have previously isolated from a healthy donor an expanded  $\alpha/\beta$  DN T cell clone (CO9) that expresses V $\alpha$ 24 paired to V $\beta$ 11. The CO9 V $\alpha$  sequence consists of a V $\alpha$ 24-J $\alpha$ Q rearrangement with no N region. This sequence was already reported in a leukemic patient (16), and can be

detected by PCR-oligotyping in peripheral blood lymphocytes of all donors tested (11). Furthermore Porcelli et al. (13) reported that this sequence could be detected in cDNA isolated from DN T cells.

To identify the possible existence of this clonotype in different subsets and individuals we immunized mice with the CO9 clone and produced two monoclonal antibodies to V $\alpha$ 24 and V $\beta$ 11. Using these reagents we could show that expanded clones carrying the invariant V $\alpha$ 24-J $\alpha$ Q paired to V $\beta$ 11 TCR are present in all individuals in the  $\alpha/\beta$  DN T cell subset. This remarkable TCR conservation and clonal expansion suggest a major role for nonpolymorphic highly conserved ligands in the selection of the  $\alpha/\beta$  DN T cell repertoire.

### **Materials and Methods**

**mAbs and Flow Cytometry.** Two mouse mAbs were produced from mice immunized with T cell clone CO9 (11). The anti-V $\alpha$ 24 mAb (C15, IgG1) recognizes V $\alpha$ 24 independently of V $\beta$  and J $\alpha$  and has been already described (17). From the same fusion we isolated an anti-V $\beta$ 11 antibody (C21, IgG2a). T cells stained by this antibody were sorted and cloned. All the clones analyzed express V $\beta$ 11 with different N regions, J $\beta$  and V $\alpha$  (this work and Dellabona, P., unpublished results). Anti-CD8 (OKT8, IgG2a) was obtained from American Type Culture Collection (Rockville, MD) and anti-CD4 (10A12, IgG2a) was a gift of Dr. Eddy Roosnek (Hôpital Cantonal Universitaire, Geneva, Switzerland). All the

antibodies were used in indirect immunofluorescence followed by subclass-specific FITC- or PE-conjugated goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL). The stained cells were analyzed on a FACScan® or sorted on a FACStar plus® (Becton Dickinson & Co., Mountain View, CA).

**PCR Reaction and Sequencing.** The methods for isolation and maintenance of T cell clones have been previously described (11). PCR reactions were carried out as described (11). Briefly, total RNA was extracted from T cell clones, reverse transcribed into cDNA, and amplified using the following oligonucleotides: V $\alpha$ 24 5'-GCACTGTGACGACGACACAAAGCAGAGC-3'; J $\alpha$ Q 5'-GGATAGAAATCCAGACGGTCAACTGAGTTCC-3'; V $\beta$ 11 5'-GACCCTGAATCTGCCAGGCCCTCACATACCTCTCA-3'; J $\beta$ 2.1 5'-GTTGAGGACGACTAGCACGGTGAGCCCTGTCCCTGG-3'; C $\alpha$  5'-TGCTCTTGAATCCATAGACCTCATGTC-3'; and C $\beta$  5'-TGC-TGACCCCACTGTCGACCTCCTTCCCATT-3'. PCR products were fractionated on low melt agarose gels, eluted, and directly sequenced (18) using the following primers: C $\alpha$  5'-CAGACAGACTTGTCACT-3'; and C $\beta$  5'-TGCTTCTGATGGCTCAA-3'.

**Oligotyping and Heteroduplex Analysis.** Oligotyping on PCR products was performed according to a published protocol (11), using the following <sup>32</sup>P-labeled oligonucleotides as probes: C $\alpha$  5'-CAGACAGACTTGTCACT-3'; J $\alpha$ Q 5'-GGATAGAAATCCAGACGGTCAACTGAGTTCC-3'; invariant V $\alpha$ 24-J $\alpha$ Q junction (N-CO9) 5'-TGGTGAGCGACAGAG-3'.

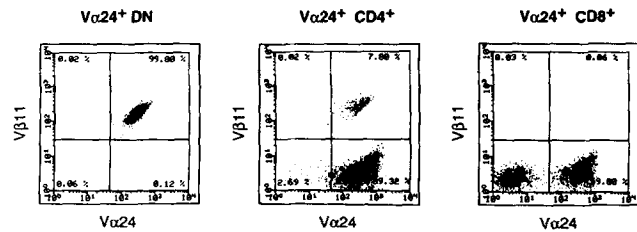
For heteroduplex analysis (19) the PCR products were heated at 94°C for 5 min, cooled down at 64°C for 1 h and run on a 12% native acrylamide gel in 0.5× Tris borate EDTA buffer at 15 mA for 12 h at 4°C. The gels were stained with ethidium bromide and photographed under UV light.

## Results and Discussion

**V $\alpha$ 24 Is Expressed in Association with V $\beta$ 11 on  $\alpha/\beta$  DN T Cells.** We have previously described an expanded  $\alpha/\beta$  DN clone (CO9) that expresses V $\alpha$ 24 and V $\beta$ 11 (11). Since these two V genes are frequently found among random cDNA clones generated from DN cells (13), we were interested to study whether this pairing would be selected in the DN subset of all individuals.

By immunizing mice with CO9 we obtained two mAbs recognizing V $\alpha$ 24 and V $\beta$ 11 (17 and our unpublished observations). By two-color staining, we found that cells expressing V $\alpha$ 24 are very rare (~0.1%) among CD8<sup>+</sup> T cells, but more frequent (~0.5%) in the CD4<sup>+</sup> and especially in the DN subset, where they may account for a substantial fraction (2–10%) of all cells (data not shown). To analyze the pairing of V $\alpha$ 24 with V $\beta$ , we first sorted V $\alpha$ 24<sup>+</sup> T cells from the CD4<sup>+</sup>, CD8<sup>+</sup>, and DN T cell subsets. The sorted cells were expanded in short-term polyclonal lines and analyzed by two-color fluorescence for the expression of V $\alpha$ 24 and V $\beta$ 11. Strikingly, in 4 out of 5 individuals, all V $\alpha$ 24<sup>+</sup> cells within the DN compartment express V $\beta$ 11, whereas in one, about half of the cells express this V $\alpha$ /V $\beta$  pair (Fig. 1 and Table 1). In the CD4<sup>+</sup> compartment, T cells expressing V $\alpha$ 24 and V $\beta$ 11 are present as a minor fraction of the total V $\alpha$ 24<sup>+</sup> cells whereas they are absent in the CD8 compartment.

**The V $\alpha$ 24 Paired to V $\beta$ 11 in DN T Cells Is Identical to the CO9 Chain.** To dissect the structure of the V $\alpha$ 24 TCR chains expressed in DN, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells, we prepared



**Figure 1.** V $\alpha$ 24 pairs selectively with V $\beta$ 11 within the  $\alpha/\beta$  DN T cell compartment. V $\alpha$ 24<sup>+</sup> T cells were sorted from the CD4<sup>+</sup>, CD8<sup>+</sup>, and DN compartment (donor ALA), expanded, and analyzed for expression of V $\alpha$ 24 and V $\beta$ 11 by two-color fluorescence.

cDNA from the short-term V $\alpha$ 24<sup>+</sup> polyclonal T cell lines. The cDNA was amplified with V $\alpha$ 24- and C $\alpha$ -specific oligonucleotides to obtain a fragment encompassing the V-J junction. The PCR product was fractionated on agarose gels, blotted, and hybridized with oligonucleotide probes specific for C $\alpha$ , J $\alpha$ Q, and the V-J junction of CO9 to detect the expression of total V $\alpha$ 24, J $\alpha$ Q, and of the CO9 V $\alpha$ 24-J $\alpha$ Q junction that lacks N region diversity. As shown in Fig. 2 A, in all three donors studied the CO9 invariant V $\alpha$ 24-J $\alpha$ Q junction was prominent among DN T cells, was expressed at a much lower level among CD4<sup>+</sup>, and was absent in CD8<sup>+</sup> cells. A similar pattern of expression was observed for J $\alpha$ Q indicating that this J $\alpha$  is used preferentially for this type of invariant  $\alpha$  chain. Note, however, that CD4<sup>+</sup> clones with V $\alpha$ 24-J $\alpha$ Q rearrangement but N region addition can be found, for example clone T6, which is shown as a control for the oligotyping (Fig. 2 A).

To get additional information on the heterogeneity of the V $\alpha$ 24 chains in the different T cell subsets, the same V $\alpha$ 24-C $\alpha$  PCR products were subjected to heteroduplex analysis. As shown in Fig. 2 B, PCR products from the DN cells are substantially homogeneous since they gave a prominent band of homoduplex. Direct sequencing of the homoduplex band from donors CDO and FOL gave the exact CO9 junctional sequence (data not shown and Table 2), indicating that the dominant V $\alpha$ 24 sequence present among the DN subset is identical to CO9. In contrast, the product amplified from CD4<sup>+</sup> or CD8<sup>+</sup> T cells gave a clear heteroduplex pattern indicating a substantial heterogeneity of the products, although in some cases clear bands of homo- and heteroduplexes were superimposed on the polyclonal pattern.

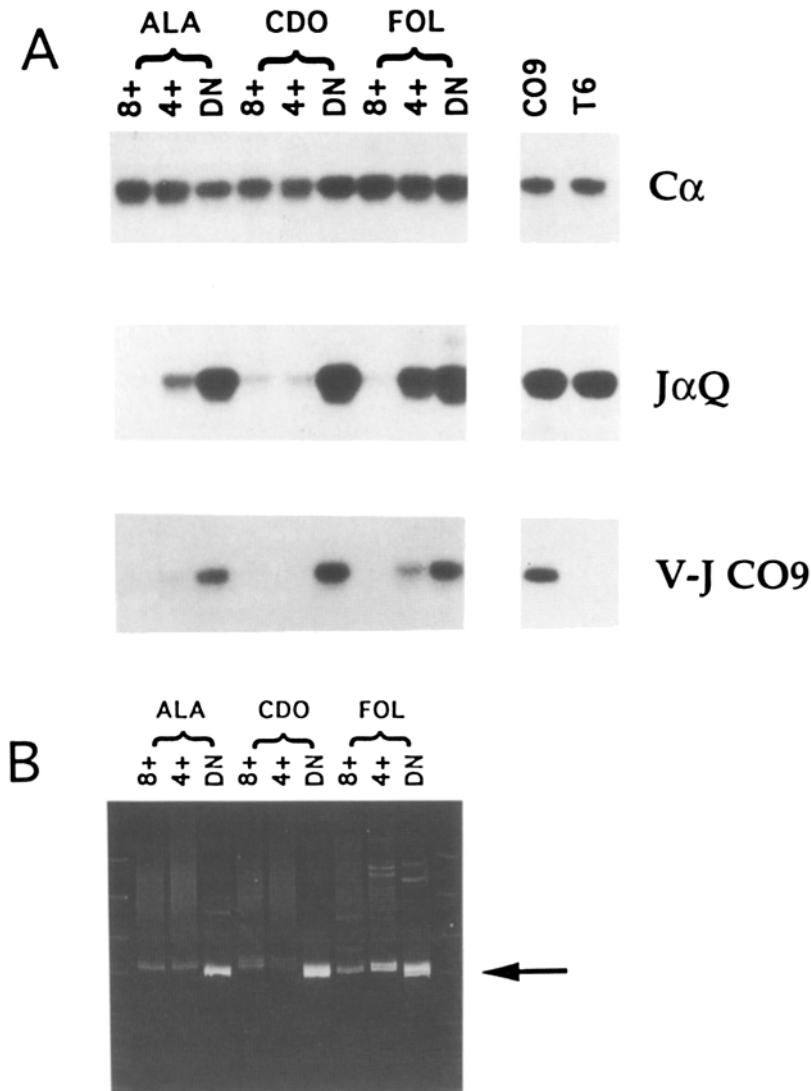
We conclude that the invariant V $\alpha$ 24-J $\alpha$ Q chain is found mainly among the DN cells of all individuals and, in some individuals, also in the CD4<sup>+</sup> compartment, although at a lower level.

**TCR Structure of V $\alpha$ 24<sup>+</sup>/V $\beta$ 11<sup>+</sup> DN T Cell Clones.** The previous results show that DN T cells express an invariant V $\alpha$ 24 paired with V $\beta$ 11. To determine the structure of the associated  $\beta$  chain we directly sorted peripheral T cells stained by both anti-V $\alpha$ 24 and anti-V $\beta$ 11 antibodies and isolated a panel of 48 independent T cell clones. The cDNA prepared from each clone was amplified with oligonucleotides specific for the relevant V, J, and C segments and the V-C products

**Table 1.** Pairing of V $\alpha$ 24 with V $\beta$ 11 in Single Positive and DN T Cells

Polyclonal lines:	V $\alpha$ 24 <sup>+</sup> CD4 <sup>+</sup>		V $\alpha$ 24 <sup>+</sup> CD8 <sup>+</sup>		V $\alpha$ 24 <sup>+</sup> DN	
	$\alpha$ 24	$\alpha$ 24/ $\beta$ 11	$\alpha$ 24	$\alpha$ 24/ $\beta$ 11	$\alpha$ 24	$\alpha$ 24/ $\beta$ 11
ALA	96	8	59	0	99	99
CDO	97	27	84	0	94	94
FOL	98	15	46	2	99	85
FSA	88	2	78	0	85	82
SDE	91	10	37	0	42	21

V $\alpha$ 24<sup>+</sup>CD4<sup>+</sup>, V $\alpha$ 24<sup>+</sup>CD8<sup>+</sup>, and V $\alpha$ CD4<sup>-</sup>8<sup>-</sup> cells were sorted from PBMC of five normal donors, expanded in culture for 6 d and analyzed by FACS<sup>®</sup> for the expression of the V $\alpha$ 24 and V $\beta$ 11 as in Fig. 1. In all donors  $\alpha/\beta$  DN cells did not exceed 2% of peripheral blood mononuclear cells.



**Figure 2.** The V $\alpha$ 24 chain that pairs with V $\beta$ 11 in the  $\alpha/\beta$  DN subset is homogeneous and identical to the invariant V $\alpha$ CO9. (A) RNA was extracted from short-term lines derived from three of the five donors described in Table 1 and reverse transcribed into cDNA. The cDNA was amplified with primers specific for V $\alpha$ 24 and C $\alpha$ . The PCR products were hybridized with labeled oligonucleotides specific for C $\alpha$ , J $\alpha$ Q, and the invariant V $\alpha$ 24–J $\alpha$ Q junctional sequence of clone CO9 (N-CO9). Controls include CO9 and T6, a CD4<sup>+</sup> clone that expresses V $\alpha$ 24 joined to J $\alpha$ Q by a short N region (17). (B) The same PCR products as in A were subjected to heteroduplex analysis on native acrylamide gels. The arrow indicates the migration of the homoduplex bands, which were eluted from the gel and directly sequenced. The sequence was identical to that of CO9 (see also Table 2).

**Table 2.** TCR Structure of  $V\alpha 4^+/V\beta 11^+$  T Cell Clones

Phenotype	Clonotype frequency	TCR structure	
<b>Donor 1</b>			
DN	16/22	valser <u>V<math>\alpha</math>24-GTGAGC</u>	asparggly GACAGAGGC-J $\alpha$ Q
		serser <del>glu</del> V $\beta$ 11-AGCAGTGAAT	serglygly <u>CGGGGGG</u> asnthrglu GAACACTGAA-J $\beta$ 1.1
CD4 <sup>+</sup>	1/22	valser V $\beta$ 24-GTGAGC	alagly GCGGGT glyglyser GGAGGAAGC-J $\alpha$ T
		V $\beta$ not 11	J $\beta$ not 2.1
CD4 <sup>+</sup>	1/22	valser V $\alpha$ 24-GTGAGC	glygly GGGGGA serglygly TCAGGAGGA-J $\alpha$ W
		V $\beta$ not 11	J $\beta$ not 2.1
<b>Donor 2</b>			
DN	7/26	valser V $\alpha$ 24-GTGAGC	asparggly GACAGAGGC-J $\alpha$ Q
DN	3/26	serser <del>glu</del> tyr V $\beta$ 11-AGCAGTGAATA	lysthrsergly <u>TAAGACTAGCGGGC</u> protyrasnglu CCTACAATGAG-J $\beta$ 2.1
		valser V $\alpha$ 24-GTGAGC	asparggly GACAGAGGC-J $\alpha$ Q
CD8 <sup>+</sup>	1/26	serser <del>glu</del> V $\beta$ 11-AGCAGTGA	pheglyglylyls GTTCGGTGGAAA asnthr GAACACC-J $\beta$ 2.2
		V $\alpha$ 24	J $\alpha$ not Q
CD8 <sup>+</sup>	2/26	serser <del>glu</del> V $\beta$ 11-AGCAGTGA	glyalaalaglytrp GGGGCGGCCGGGTGG gluthrgln GAGACCCAG-J $\beta$ 2.5
		val V $\alpha$ 24-GTG	alaarg GCCCGA asparggly GACAGAGGC-J $\alpha$ Q
CD4 <sup>+</sup>	12/26	serser <del>glu</del> V $\beta$ 11-AGCAGTGA	serglyglyglyargser TCTGGAGGGGGGAGGT aspthr CAGATACG-J $\beta$ 2.3
		V $\alpha$ 24 V $\beta$ 11	J $\alpha$ not Q J $\beta$ not 2.1
CD4 <sup>+</sup>	2/26	V $\alpha$ 24 serarg V $\beta$ 11 AGCAG	J $\alpha$ not Q glyglu CCGGGGAG-J $\beta$ 2.2

$V\alpha 24^+$  T cells from donor 1 (1.1%  $\alpha/\beta$  DN T cells) or  $V\alpha 24^+/V\beta 11^+$  T cells from donor 2 (1.5%  $\alpha/\beta$  DN T cells) were sorted and immediately cloned by limiting dilution. RNA from the clones was reverse transcribed into cDNA and amplified by PCR. The primers were chosen to recognize the TCR of the  $\alpha/\beta$  DN clone CO9. Where indicated, the PCR product was purified from the agarose gel and directly sequenced. Underlined are residues that may be encoded by D $\beta$ 1 or D $\beta$ 2. The V $\beta$ 11 germ line encoded nucleotides have been identified on the basis of the germ line sequence provided by Dr. Leroy Hood (University of Washington, Seattle, WA).

were sequenced. Table 2 summarizes the results of this analysis in two different healthy donors with normal levels of  $\alpha/\beta$  DN cells. In donor 1, all DN clones showed the same TCR- $\alpha$  and - $\beta$  sequence. The fact that the  $\beta$  chain carries the same N region indicates that all the clones derive from a single T cell that has expanded in vivo. In the second donor two expanded DN clonotypes were detected, each with a characteristic N region and J $\beta$ . Finally, all the three DN clonotypes carry the invariant CO9  $\alpha$  chain.

T cell clones carrying V $\alpha$ 24 and V $\beta$ 11 could also be found among CD4<sup>+</sup> and CD8<sup>+</sup> T cells. However, in this case the V $\alpha$ 24 carries N region or is rearranged to J $\alpha$  segments other than J $\alpha$ Q (Table 2).

In conclusion, the use of mAbs and T cell clones allowed to identify an almost invariant TCR expressed on expanded clones of  $\alpha/\beta$  DN T cells in all individuals tested. This receptor consists of an invariant V $\alpha$ 24-J $\alpha$ Q with no N region nucleotides, paired with V $\beta$ 11 bearing chains that carry N regions of different lengths and sequence and can use different J $\beta$  segments.

There is a striking similarity between the invariant human V $\alpha$ 24-J $\alpha$ Q chain and the mouse V $\alpha$ 14-J $\alpha$ 281 described by Taniguchi et al. (20). Both lack junctional diversity and are highly homologous (62% aminoacid homology in the V region and 9 out of 10 identical residues in CDR3; reference

21). It is tempting to speculate that the lack of N region may be related to a late V $\alpha$  rearrangement occurring after loss of terminal transferase and possibly extrathymically, as it has been shown for the mouse V $\alpha$ 14 (20).

There are two mechanisms that may account for the frequent occurrence of a particular TCR. The first is that the TCR is frequently generated by an homology-mediated recombination event (22–26) or that the  $\alpha/\beta$  pairing is forced by molecular constraints. The second mechanism is that rare clones carrying specific receptors are expanded by recognition of their specific ligand. Our data clearly point to the second possibility because there is no evidence of molecular constraints for V $\alpha$ 24 rearrangement and pairing and, most important, in every individual V $\alpha$ 24<sup>+</sup> DN T cells derive from a single or a few expanded T cell clones as demonstrated by  $\gamma$  rearrangements (11) and V $\beta$  N region diversity (this study).

The clonal expansion of these cells clearly carries the hallmark of antigen-driven selection and implies that the selective antigen must be very similar in all individuals and thus, most likely, not polymorphic (27). Although this ligand has not been characterized yet, it may be an antigen bound to a nonpolymorphic antigen-presenting molecule such as CD1, as described by Brenner et al. (9).

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Note added in proof: In this issue, Lantz and Bendelac (28) describe in the mouse a CD4<sup>-</sup>CD8<sup>-</sup> T cell population that uses an invariant TCR V $\alpha$ 14-J $\alpha$ 281 chain strikingly homologous to the human invariant V $\alpha$ 24-J $\alpha$ Q.

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