Dual T Cell Receptor β Chain Expression on Human T Lymphocytes

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Summary

Allelic exclusion of lymphocyte antigen receptor chains has been hypothesized as a mechanism developed by the immune system to ensure efficient lymphocyte repertoire selection and tight control of lymphocyte specificity. It was effectively shown to be operative for both the immunoglobulin (Ig) and the T cell receptor (TCR) β chain genes. Our present observations suggest that close to 1% of human T lymphocytes escape this allelic control, and express two surface TCR β chains with distinct superantigenic reactivities. Since this high frequency of dual β chain expressors did not result in any dramatic immune dysregulations, these results question the need for a mechanism ensuring clonal monospecificity through allelic exclusion.

Lymphocyte antigen receptors are composed of two variable glycoprotein subunits, the Ig heavy and light chains on B cells and the TCR α and β or γ and δ chains on T cells. The genes coding for Ig and TCR chains are formed through somatic rearrangement of V, D, and J elements (for reviews see references 1 and 2). Given its diploid nature, any lymphocyte clone could theoretically express up to four distinct combinations of antigen receptor chains on its surface. Since clonal plurispecificity, possibly resulting from expression of multiple antigen receptors on the same cell, is expected to lead to immune dysregulations (that is, autoimmunity), control processes ensuring allelic exclusion of antigen receptor chains were postulated more than a decade ago, and their existence demonstrated through elegant Ig and TCR transgenic mouse models (3–5).

More recently it has become clear that the stringency of allelic exclusion greatly differs from one antigen receptor chain gene to another. In the case of TCR β chain genes, expression of any productively rearranged gene prevents further rearrangement in the β locus, a process referred to as "genotypic" exclusion (6). Such a genotypic control is illustrated by the arrest of endogenous TCR β gene rearrangements in mice carrying a functional or truncated β transgene (5, 7). However, similar transgenic studies have failed to demonstrate a dramatic influence of functional α transgenes on the occurrence of rearrangements within endogenous TCR α loci (8–12). Moreover, studies performed on normal T cells have revealed the expression of distinct productive α transcripts (13) or even distinct surface α chains (14) on a fraction of murine and human T cell clones.

Although lack of allelic exclusion of α chains can result in a violation of the "one cell, one receptor" rule, current ontogenic models suggest that dual α chain expression would have limited physiological consequences. Experiments with normal and transgenic T cells suggest that a developing lymphocyte will keep on rearranging its α loci until it produces a TCR α chain able to pair with the available TCR β chain and form a TCR showing sufficient affinity for self MHC–peptide complexes (12, 13, 15). The corollary to this hypothesis, which proposes a close coupling between arrest of TCR α gene rearrangements within the thymocyte and its positive selection, is that any lymphocyte carrying two distinct α chains should systematically express one α chain unable to form a selectable TCR, that is, with a physiologically relevant specificity. Therefore lack of genotypic exclusion of TCR α chain genes, which would merely be the consequence of a low frequency of selectable α chains within the immature repertoire, should remain compatible with maintainance of clonal monospecificity. This might not necessarily be true in the case of dual TCR β chain expression. First, since rearrangements within TCR β loci are completed before production of TCR α chains and subsequent α/β TCR selection (for reviews see references 16 and 17), the two α/β TCR present in dual β expressors could not in theory be sequentially tested for affinity to a given MHC–peptide complex. Second, because β chains, unlike α chains, display superantigenic reactivity (for a review see reference 18), dual TCR β chain expression should systematically lead to bispecificity, possibly hampering regulation of immune responses directed against exogenous superantigens. Hence, this may justify the need for a tight control of TCR β gene rearrangement, whose primary aim would be to ensure clonal monospecificity through maintainance of

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TCR β chain clonal distribution. This interpretation is put into question by the present data, which suggest that a normal immune function can accommodate itself to a relatively high frequency of dual β chain expressors.

Materials and Methods

Antibodies. The following mAbs were used for flow cytometry and sorting experiments: BMA031 (pan β), 360 (anti-Vγ9), LE89 (anti-Vβ3), IMMU157 (anti-Vβ5.1), 36213 (anti-Vβ5.2), 3D11 (anti-Vβ5.3), OT145 (anti-Vβ6.7), 56C5.2 (anti-Vβ8), S511 (anti-Vβ12.2), JU74 (anti-Vβ13.3), 417.5F3 (anti-Vβ19), IG125 (anti-Vβ21.3), and IMMU546 (anti-Vβ22) (19–25). The Vβ nomenclature used is from reference 26.

Generation of T Cell Lines and Clones. PBL from healthy donors were sorted, cultured, and cloned as previously described (27, 28). In brief, cells were incubated with TCR V-specific mAbs for 45 min, washed once, and rotated for 4 h at 4°C with magnetic beads coated with sheep anti-mouse Ig (Dynal, Oslo, Norway). After eight washes, bead-adherent cells were cultured in medium (RPMI 1640, 10% human serum, 1 mM L-glutamine, rIL-2, and leukoagglutinin) and irradiated PBL and B lymphoblastoid cells. Beads were removed between day 5 and 8, cells were cultured further, and at day 14, they were subjected to a second immunomagnetic separation. After two more weeks of culture, cells were cloned by limiting dilution in culture medium and irradiated feeder cells. Growing colonies with a probability of monoclonality above 95% were kept for further analysis.

Flow Cytometry Analysis. Cells were phenotyped by indirect immunofluorescence (IF)[1] as follows. Cells were incubated first with the unconjugated mAbs (single or pooled Vβ-specific mAbs, 1:1,000 ascites) at 4°C for 30 min; second with FITC-conjugated rabbit anti-mouse Ig for 30 min at 4°C; third with normal mouse serum (1:10 dilution) for 10 min at room temperature; fourth with biotinylated Vβ-specific mAbs for 30 min at 4°C; and fifth with PE-conjugated streptavidin for 30 min at 4°C. Between each incubation, cells were washed twice with PBS, 1% BSA. Cells were analyzed by flow cytometry on a FACScan® (Becton Dickinson & Co., Mountain View, CA) using LYSYS II software. To have an accurate estimate of infrequent subsets (representing <0.2% of the total population), 0.4×10^{-6} events were accumulated during the analysis. Staining background was reduced by gating out dead cells using propidium iodide (read on the FL3 channel). To reduce the proportion of doublets, which could represent up to 2% of the total population under "standard" conditions, two-color immunofluorescence (2C-IF) using a Vβ8-specific mAb (red fluorescence) vs pooled, unconjugated mAbs directed against other Vβ regions (green fluorescence) was performed on cells sorted with the Vβ8-specific mAb. Under these conditions, because of the scarcity of "green-positive" events (<0.5%), the proportion of doublets remained negligible (as demonstrated by control 2C-IF using a Vβ8-specific mAb (red) vs an irrelevant Vγ9-specific mAb (green) (see Fig. 1 *C*).

Functional Assays. Reactivity of T cell clones towards recombinant staphylococcal enterotoxins B and E (SEB and SEE) was assessed by proliferation and lymphokine production assays. To this end, resting T cells (taken more than 3 wk after the last mitogenic stimulation) (10^4 cells/well) were cocultured with irradiated MHC class II–positive Burkitt's lymphoma cells (RAJI) in the absence or presence of superantigen (SAg) (10 ng/ml). Culture supernatants, harvested at day 1, were tested for leukemia inhibiting factor (LIF) activity using DA.1a cells as described (29). T cell clone proliferation was estimated at day 2 according to standard protocols (30). The specificity and purity of the SAgs used were controlled by staining analysis of PBL cultured for 10 d in the presence of either culture medium alone or supplemented with recombinant SEB or SEE. In all instances, coculture with SEB lead to a dramatic amplification of Vβ3, Vβ12, Vβ14, and Vβ19 positive cells and disappearance of Vβ5.1, Vβ8, Vβ16, Vβ17, and Vβ21 positive cells within the culture, whereas reciprocal results were observed after coculture with SEE (our unpublished results), which is in accordance with previous reports (31, 32).

Amplification and Sequencing of TCR Transcripts. Molecular analysis of TCR β transcripts was performed as previously described (33) on several T cell clones stained by two distinct Vβ-specific mAbs (in all cases, 100% of the cells were recognized by the two antibodies). Reverse transcription of T cell clone RNA, amplification, and sequencing were performed using the following primers: Vβ3 (5'TAG AAT TCA TGG GAA TCA GGC TCC TC), Vβ5 (5'TAG AAT TCA TGG GCT CCA GGC TGC TCT GTT), Vβ6 (5'TCG AAT TCA CCA TGG GCA CCA GGC TCC TCT G), Vβ8 (5'ATG ATG CGG GGA CTG GAG TTG CTC), Vβ12 (5'GAC AAA GGA GAA GTC TCA GAT GCC), Vβ19 (5'TAG AAT TCA TGA GCA ACC AGG TGC TCT GCT), Vβ21 (5'AAA GGA GTA GAC TCC ACT CTC), Vβ22 (5'AAT TCT CAG TTG AAA GGC CTG), and Cβ (5'GGG AGA TCT CTG CTT CTG ATG GCT C). V, NDN, and J assignments were deduced from a comparative analysis of cDNA sequences with those of germline elements. The Vβ and Jβ nomenclatures used were from Wilson et al. (26) and from Toyonaga et al. (34).

Results

Detection by Flow Cytometry of Peripheral T Lymphocytes Recognized by Distinct Vβ-specific mAbs. We recently described T cell clones expressing two distinct TCR γ chains on their surface, and demonstrated by 2C-IF that 1–7% of PBL were recognized by mAbs directed against distinct Vγ regions (33). In the course of this study, PBL-derived α/β T cell lines were analyzed in parallel by 2C-IF using mAbs directed against distinct Vβ regions, to compare the stringency of TCR γ vs. TCR β chain exclusion. In light of previous studies suggesting a tight genotypic exclusion of TCR β chain genes (5), the proportion of cells recognized by two distinct Vβ-specific mAbs was expected to be low, if not negligible. Therefore we set up technical conditions that allowed detection and accurate quantification of cell subsets representing <0.2% of the total population (see Materials and Methods). Under these conditions, double positive cells recognized by both a Vβ8-specific mAb and a mAb specific for another Vβ-region were reproducibly detected in all the cell lines studied (Fig. 1 *B* and Table 1).

Three possible artifacts could explain the above results: (*a*) a binding of the FITC-conjugated antiserum to the PE-conjugated mAb; (*b*) a doublet discrimination problem (that is, due to lack of discrimination by the flow cytometer soft-

[1] *Abbreviations used in this paper:* IF, immunofluorescence; rMFI, relative mean fluorescence intensity; SEB, staphylococcal enterotoxin B; SEE, staphylococcal enterotoxin E; SAg, superantigen; 2C-IF, two-color immunofluorescence.

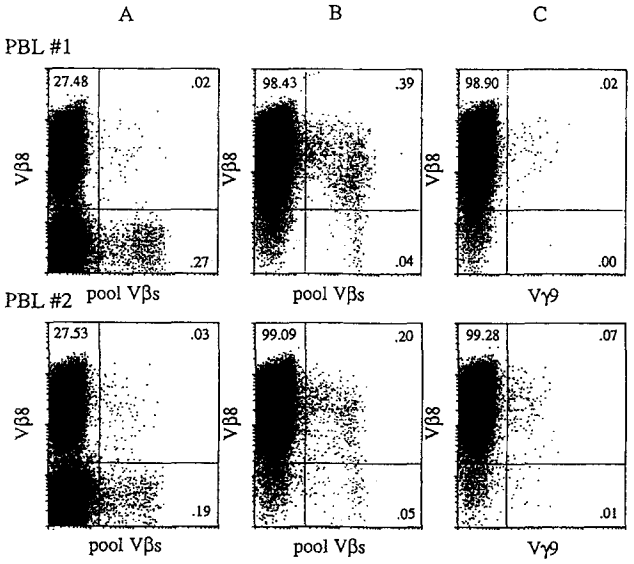

Figure 1. Flow cytometry analysis of cultured PBL lines using Vβ-specific mAbs. Shown are the dot-plot fluorescence histograms (4×10^5 events accumulated) of Vβ8+ T cell lines derived from PBL of two healthy donors (*#1: upper plots* and *#2: lower plots*). Cells were stained by 2C-IF using the Vβ8-specific mAb (red fluorescence, *ordinate*) and either a pool of Vβ-specific mAb (green fluorescence, *abscissa*) (*B*) or an irrelevant Vγ9-specific mAb (green fluorescence) (*C*). In *A*, cells were stained by single-color IF with either the Vβ8-specific mAb (red) or with the pool of Vβ-specific mAb (green). The two samples were then mixed at a 1:2 ratio before flow cytometry analysis (in this case, although >98% of the cells were Vβ8+, only one third of them were stained with the Vβ8 mAb because of the mixing procedure). mAb included in the pool were specific to the following Vβ regions: Vβ3, Vβ5.1, Vβ5.2, Vβ5.3, Vβ6.7, Vβ12, Vβ19, Vβ21, and Vβ22. Note the presence of double positive cells in B (*upper right quadrant*) but not in *A* and *C*, and the persistence of FITC-labeled cells in *A* (*lower right quadrant*), which indicated that DP cells were not due to a doublet discrimination problem or to an artifactual binding of the FITC-conjugated antiserum to the Vβ8 mAb.

ware between a doublet of single positive events and a double positive event); and (*c*) a cross-reactivity of some Vβ-specific mAbs to other variable regions. The first possibility was ruled out by 2C-IF experiments using irrelevant isotype-matched mAbs (Fig. 1 *C* and data not shown). Moreover, the fact that no double positive events were detected when analyzing mixed samples stained by 1C-IF using either the PE- or FITC-conjugated mAbs allowed us to rule out a doublet discrimination problem (Fig. 1 *A*). Finally, the staining profiles of the double positive population was not compatible with cross-reactivity of some of the Vβ-specific mAbs tested. Instead, the inverse correlation noted between staining intensities obtained with the Vβ8-specific mAbs vs the pool of Vβ-specific mAbs was strongly indicative of the presence of two distinct TCR β chains on the same cell, competing for pairing with limiting amounts of other TCR or CD3 components (Fig. 1 *B*).

All T Cell Clones Recognized by Distinct Vβ-specific mAbs Express Two Distinct Productively Rearranged TCR β Genes. To formally prove the existence of dual β expressors, we generated T cell lines after sequential sorting of PBL using one

Table 1. *Frequency of Dual β Expressors within Cultured Vβ8+ PBLs Estimated by 2C-IF*

PBL	Frequency of dual β expressors	
	Observed	Theoretical
476	0.37	1.23
765	0.13	0.43
477	0.15	0.50
751	0.22	0.73
767	0.18	0.59
770	0.17	0.57
26	0.49	1.63
27	0.23	0.77
Mean	0.25	0.83

Proportions of cells recognized by two distinct Vβ-specific mAbs among eight Vβ8+ PBL-derived cell lines were calculated by substracting the percent double positive cells estimated by 2C-IF using a Vβ-specific mAb (red) vs an irrelevant mAb (Vγ9 specific, green) (see Fig. 1 *C*) to the percent double positive cells estimated by 2C-IF using the Vβ8 mAb (red) vs a pool of mAbs directed against the Vβ3, 5, 6.7, 12, 13.3, 19, 21, and 22 regions (green) (see Fig. 1 *B*). Considering that the pool of Vβ-specific mAb used here recognized ~30% of PBL (data not shown), the theoretical frequency of dual β expressors was deduced by multiplying the observed frequency by 3.33.

Vβ-specific mAb first and then a pool of mAbs specific for other distinct Vβ regions (Fig. 2), and cloned them by limiting dilution. More than 50 clones recognized by two distinct Vβ-specific mAbs were generated this way (see for example, Fig. 2 *D*). It is noteworthy that the relative mean fluorescence intensities (rMFI) obtained with each Vβ-specific mAb, which were calculated by dividing the MFI obtained with the Vβ mAb by the MFI obtained with a pan β mAb, greatly varied form one dual β-expressing clone to another (Fig. 3 and data not shown). Moreover, within a population of clones recognized by the same combination of Vβ mAbs (e.g., Vβ8 and Vβ19), the rMFI obtained with each Vβ-specific mAb were inversely correlated (Fig. 3). Again these results, which were reminiscent of those obtained with dual TCR γ- and dual TCR α chain–expressing clones (14, 33), strongly suggested the presence of distinct β chains on the above cells.

To formally prove this point, we performed a molecular analysis of TCR β transcripts derived from T cell clone RNA, after reverse transcription and polymerase chain amplification using Cβ- and Vβ-specific primers followed by sequencing of the VDJβ junctions. In all cases, surface phenotype of clones recognized by two distinct Vβ-specific mAbs matched the expression of the corresponding productive TCR β chain gene transcripts (Fig. 4).

Superantigenic Reactivity of Dual TCR β Chain Expressors. A salient feature of TCR β chains is their ability to interact via germline residues located in their V region with so-

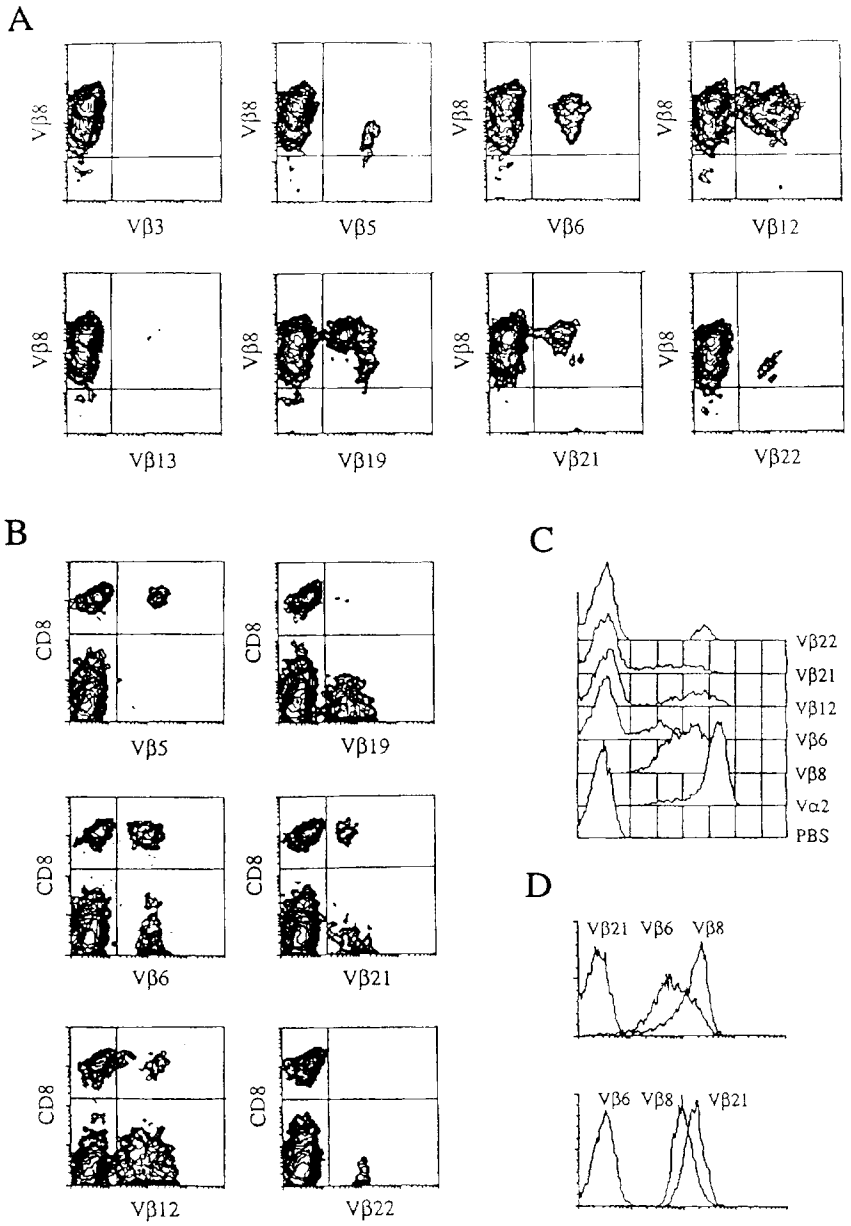

Figure 2. Flow cytometry analysis of dual TCR β chain–expressing cells. (*A*) 2C-IF analysis of a T cell line (β8βX-1) generated after sequential sorting of PBL using a Vβ8-specific mAb and a pool of mAbs specific to other Vβ regions. The pool of Vβ-specific mAbs used for sorting is the same as that in Fig. 1. Note the presence of almost all the possible Vβ8VβX combinations in this cell line (see also Table 3). (*B*) CD8 coreceptor expression by dual β chain expressors. Cells from line β8βX-1 were stained with PE-conjugated CD8 mAb (*ordinate*) and various Vβ-specific mAbs (*abscissa*). All cells were recognized by the Vβ8-specific mAb (see *A*). Reciprocal results were obtained with a PE-conjugated CD4 mAb (data not shown). Note that the majority of the subsets recognized by a given pair of Vβ-specific mAbs comprised $CD4^+$ and $CD8^+$ cells. (*C*) 1C-IF analysis of $V\alpha2^+$ cells derived from line β8βX-1. Cells were sorted using a Vα2.1-specific mAb, cultured and analyzed by IF. All cells were recognized by the Vα2- and Vβ8-specific mAb, some were also recognized by the Vβ22-, Vβ21-, Vβ12-, and Vβ6-specific mAb, which indicated that $V\alpha2^+$ cells from line β8βX-1 were still highly polyclonal. (*D*) Flow cytometry analysis of two T cell clones derived from line β8βX-1. Shown are the overlayed fluorescence histograms obtained after IF staining of two T cell clones (#10, *upper histograms* and #5, *lower histograms*) using Vβ21-, Vβ6- and Vβ8-specific mAbs. The presence of distinct β chains in clones #10 and #5 was also confirmed by molecular analysis (see Fig. 4).

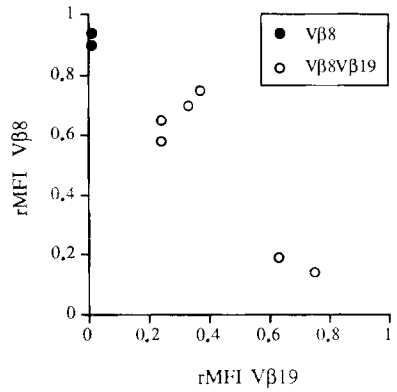

Figure 3. Surface expression levels of $V\beta8^+$ and $V\beta19^+$ chains on dual $V\beta8V\beta19$ expressors. The relative mean fluorescence intensities (*rMFI*) obtained with a Vβ8 (*ordinate*) and a Vβ19-specific mAb (*abscissa*), calculated by dividing the MFI obtained with the Vβ mAb by the MFI obtained with a pan β mAb BMA031 (19), were estimated on two $V\beta8^+$ T cell clones (*filled circles*) and 6 $V\beta8^+V\beta19^+$ T cell clones (*unfilled circles*). Note the inverse correlation between the Vβ8 and Vβ19 rMFI within the dual β-expressing cell population.

called bacterial or viral SAgs (that is, staphylococcal enterotoxins or products of the mouse mammary tumor virus [MMTV]-LTR). We therefore tested whether dual TCR β chain expressors could be activated by SAgs interacting with each of their surface β chains. In humans, SEB activates $V\beta3^+$ and $V\beta19^+$ cells whereas SEE activates $V\beta8^+$ cells (31, 32, and our own unpublished results). Accordingly, $V\beta8^+$ clones proliferated and produced cytokines when cultured in the presence of recombinant SEE but not recom-

Clone	Phenotype	Vβ		NDN	Jβ		Vβ		NDN	Jβ	
3β3	Vβ19 Vβ8	19	CASS	FWTGG	SNQPQHF	1.5	8	CAS	STGAGGF	SYEQYF	2.7
5β1	Vβ19 Vβ8	19	CASS	TRGEEV	YNSPLHF	1.6	8	CASS	LARS	YGYTF	1.2
5β2	Vβ19 Vβ6	19	CAS	RQGI	YGYTF	1.2	6	CASS	PTVSP	TKTGELFF	2.2
5β3	Vβ19 Vβ3	19	CA	GL	NTEAFF	1.1	3	CAS	ETSGGA	NYGYTF	1.2
5β4	Vβ19 Vβ8	19	CASS	IGDIP	YEQYF	2.7	8	CASS	TRAAR	QFF	2.1
5β7	Vβ19 Vβ3	19	CAS	GGAYE	QYF	2.7	3	CASS	LPGRS	SYEQYF	2.7
5β10	Vβ19 Vβ8	19	CASS	IVPDEHGG	YEQYF	2.7	8	CASS	LQGLV	NTEAFF	1.1
1	Vβ8 Vβ12	8	CASS	LSGSG	EKLFF	1.4	12	CASS	GGLAVLSYG	QYF	2.7
5	Vβ8 Vβ21	8	CASS	FSRGE	SPLHF	1.6	21	CASS	PTDFPGTTA	DTQYF	2.9
10	Vβ8 Vβ6	8	CASS	LWETGVY	EQFF	2.1	6	CASS	YYTPGG	EKLFF	1.4
11	Vβ8 Vβ22	8	CAS	PRRGGGRD	TEAFF	1.1	22	CASS	RRGGVNL	PQHF	1.5

Figure 4. Deduced amino acid sequences of TCR β chain gene transcripts expressed in PBL-derived T cell clones. The T cell clone phenotype was deduced from IF analysis (100% of the cells were recognized by the Vβ-specific mAb mentioned in the figure). These sequence data are available from EMBJ/EBI data bank under accession numbers X84265 to X84283.

binant SEB, whereas reciprocal results were obtained with Vβ3$^+$Vβ19$^+$ clones (Table 2 and data not shown). It is noteworthy that Vβ8$^+$Vβ19$^+$ cells responded to both SEE and SEB, which indicated that their two TCR β chains were functional (Table 2).

Frequency of Dual TCR β Chain Expressors within Human PBL. The frequency of dual β chain expressors was calculated from a 2C-IF analysis of Vβ8$^+$ lines derived from eight individuals, under experimental conditions that allowed estimation of frequencies with an accuracy of <0.05%. On average, .25% of Vβ8$^+$ cells were recognized by a pool of mAbs directed against 10 other Vβ regions (Vβ3, Vβ5.1, Vβ5.2, Vβ5.3, Vβ6.7, Vβ12, Vβ13.3, Vβ19, Vβ21, and Vβ22) (Table 1). Considering that these mAbs recognized altogether about 30% of PBL, the mean theoretical frequency of dual TCR β chain expressors should be close to 0.8–1.0%. Such a high frequency is in accordance with the extensive polyclonality of the T cell lines that were generated after sequential sorting using Vβ8- and the pool of Vβ-specific mAbs. Indeed, most of the expected Vβ8/Vβx combinations were detected in the majority of the cell lines (Fig. 2 *A* and Table 3). Moreover, within a given line, most subsets recognized by a pair of Vβ-specific mAbs comprised CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ cells (Fig. 2 *B* and data not shown). Finally, IF analysis of Vα2$^+$ cells derived from the above lines still demonstrated the presence of subsets expressing distinct Vβ combinations (Fig. 2 *C*).

Discussion

Despite genotypic exclusion of TCR β chains, which has been clearly demonstrated in murine T lymphocytes (5–7), and which is likely to occur in human T cells as well, our present observations indicate that a significant fraction of peripheral lymphocytes in adults carries two distinct functional TCR β chains. As for any biological process, an escape from TCR β allelic exclusion was somewhat expected. However

Table 2. *Reactivity of Dual TCR β Chain-expressing T Cell Clones towards Staphylococcal SAgs*

T cell clone	Phenotype	[^{3}H]TdR uptake		LIF production	
		SEB	SEE	SEB	SEE
		cpm × 10^{-3}		*a.u.*	
3β4	Vβ8$^+$	0.6	13.8	0.0	6.5
5β3	Vβ3$^+$Vβ19$^+$	11.1	0.4	5.0	0.0
5β7	Vβ3$^+$Vβ19$^+$	ND	ND	19.5	0.0
5β10	Vβ8$^+$Vβ19$^+$	21.2	22.0	37.0	31.0
3β3	Vβ8$^+$Vβ19$^+$	13.3	17.8	7.0	8.0

T cell clones were cultured with irradiated MHC class II–positive Raji Burkitt's lymphoma cells in the absence or presence of either recombinant SEE or SEB (SAg). LIF activity in the culture supernatant was estimated by a biological assay (29) and expressed as arbitrary units (a.u) per ml. None of the clones tested produced LIF when cultured with Raji cells alone (data not shown). T cell clone proliferative response to SAg was calculated by substracting the [^{3}H]TdR uptake estimated after coculture with Raji cells alone to the [^{3}H]Tdr uptake estimated after coculture with Raji and SAg. Note the response of Vβ8$^+$Vβ19$^+$ T cell clones to both the SEE and SEB.

Table 3. *TCR Vβ Surface Expression of PBL-derived Lines Generated after Sequential Sorting Using a Vβ8-specific mAb First and then a Pool of mAbs Specific to Vβ3, 5, 6, 8, 12, 13, 19, 21, and 22*

PBL line	Vβ3	Vβ5	Vβ6	Vβ8	Vβ12	Vβ13	Vβ19	Vβ21	Vβ22
β8βX-1	0.2	3.8	14.4	98.0	34.7	0.5	31.3	7.2	2.5
β8βX-2	4.5	0.2	7.6	99.1	58.1	0.2	7.5	9.4	4.4
β8βX-3	2.4	1.1	39.9	99.9	1.9	0.3	11.0	14.0	23.8
β8βX-4	7.9	2.6	10.5	99.9	14.5	0.1	29.4	8.5	20.6

Note that subsets expressing most possible Vβ8VβX combinations are detected in most lines, attesting to their extensive polyclonality. In each of these cell lines, we could demonstrate the presence of at least 10–15 distinct clones differing by the Vβ combination expressed and CD4/CD8 coreceptor expression. Since all these lines were generated from 10^5 Vβ8$^+$ cells, the minimal observed frequency is $\geq 1\text{–}1.5 \times 10^{-4}$. The 10–20-fold difference between this minimal estimate and the frequency deduced from flow cytometry analyses (Fig. 1) is easily explained by the fact that (*a*) clones were defined on the basis of two criteria only (Vβ combination and coreceptor expression); (*b*) our sorting efficiency was much below 100% (under standard conditions it was at most 20%, our unpublished observations); and (*c*) the starting population was probably not composed of distinct clones.

the relatively high frequency of dual β expressors estimated here (that is, close to 1%) raises questions regarding its physiological consequences and the way cells with such an unusual phenotype were generated.

Rearrangements of TCR β genes are activated within early $CD3^-CD4^-CD8^-$ thymocytes. At that stage, expression of any productively rearranged β chain gene will lead to a transient arrest of the thymocyte recombination machinery, presumably upon pairing between the β chain gene product and a surrogate α chain (35). TCR α gene rearrangements are activated at a later stage of development (that is, within $CD4^+CD8^+$ thymocytes), when the β loci are no longer accessible to the recombinases. According to this scheme, three major hypotheses might be put forward to account for the generation of dual TCR β chain expressors. First, escape from β genotypic exclusion might be due to an intrinsic inability of the first β chain produced to generate a recombinase modulating signal. However, this possibility seems rather unlikely because such a signal can even be provided by truncated β chains (7) and because both the β chains on dual expressors were shown to be functional (Table 2). Second, escape from β chain exclusion might happen if for some unknown reasons, rearrangements occur synchronously on the two β alleles or if signaling through the first β chain produced is delayed. Third, an accessibility of the β loci to the recombinases at an abnormally late stage of development (that is, at the $CD4^+CD8^+$ stage) might result in secondary TCR β rearrangements.

As mentioned, one might expect the frequency of cells with two receptors with distinct physiologically relevant specificities to be higher among dual β than dual α expressors. However, it is likely that for several reasons exposed below, a relatively high incidence of dual β expressors (even in the order of 1%) will have limited consequences on a normal immune function. First, as mentioned previously, current models suggest that a thymocyte will stop its recombination machinery once it has produced a selectable α chain, able to form a receptor with sufficient affinity for self MHC–peptides. Consequently, the fact that T cells have almost systematically rearranged their two α alleles (6) would indicate that in most cases, the first α chain produced was unable to generate a recombination modulating signal, which would mean that the proportion of selectable α/β TCR within the immature (unselected) repertoire is extremely low. Therefore, the probability that both TCR on dual β expressors will be self MHC restricted (that is, physiologically relevant) should be almost negligible. Second, even if a dual expressor turned out to be plurispecific, it would likely be controlled by the powerful negative selection processes operating in the thymus and in the periphery. Third, although the in vivo significance of superantigenic responses is yet unclear, the fact that the immune system tolerates a relatively high frequency of clones truly bispecific towards distinct SAgs might indicate that clonal plurispecificity has limited consequences in terms of immune response specificity. Fourth and more importantly, demonstration of the functionality of the two TCRs present on dual α, β, or γ expressors in in vitro assays may not necessarily mean that these cells can be efficiently activated in vivo via each of their surface receptors. In this respect, several indirect observations suggest that cells expressing a single surface receptor have a strong selective advantage over dual TCR expressors. For instance, in transgenic mice carrying several copies of an Ig light chain transgene, because each copy is independently hypermutated in the course of the Ig affinity maturation process, any B cell from these animals should express different transgenic light chains on its surface. In fact only those B cells expressing a single transgenic chain (the one conferring a high affinity for the selecting antigen), but having phenotypically excluded the improperly mutated ones, are amplified during secondary B cell responses (36). Similarly a preliminary analysis of human peripheral γ/δ T cells suggests the occurrence of a γ chain phenotypic exclusion within subsets subjected to an in vivo peripheral selection (our own unpublished observations). The hypothesis of a phenotypic exclusion occurring during selection of mature T cells might well explain the apparent discrepancies between observations made on human vs murine T cells. Indeed, whereas a relatively high incidence of dual α (14) or dual β expressors (this study) has been observed within human PBL, murine T cell clones were generally shown to express a single TCR α chain on their surface, despite the presence of two productive TCR α rearrangements in a large fraction of them (6). Similarly, dual β expression in the mouse has been thus far formally proven in one case only (37). Since in most murine studies, unlike human ones, clones have been selected in vivo or in vitro by specific antigenic stimuli before analysis, an antigen-driven phenotypic exclusion might have occurred in the former, but not the latter case. A recent study, which demonstrates that within T cells expressing identical TCRs partial modulation of CD8 coreceptors on the responding cells converts an agonist Ag into an antagonist one (38), may provide some clues on the way dual TCR expressors (which express intermediate levels of each TCR) would be eliminated or diluted out in the course of an antigen response.

All the above considerations suggest that to an extreme point of view, allelic exclusion is not meant to ensure clonal monospecificity. However, they also suggest that expression of multiple receptors on the same cell may constitute a disadvantage in the course of antigen-driven selection of peripheral T cells. Therefore, the occurrence of a control mechanism limiting the heterogeneity of the TCR β chains expressed on the T cell clone surface should greatly improve the efficiency of central and peripheral T cell selection.

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