Surface Expression of Two Distinct Functional Antigen Receptors on Human γδ T Cells

François Davodeau, Marie-Alix Peyrat, Isabelle Houde, Marie-Martine Hallet, Gennaro De Libero, Henri Vié, Marc Bonneville*

Lymphocytes recognize antigens with highly variable heterodimeric surface receptors. Although four distinct antigen receptors could in principle be produced by any lymphocyte, only one functional combination of receptor chains has thus far been found expressed on their surface. Examination of human γδ T cells revealed a population that violated this rule by expressing on their surface two distinct functional γδ T cell receptors (TCRs) that used different TCRγ gene alleles. Thus, current models for T cell clonal selection may need modification, and a possible escape mechanism for autoreactive TCRs is suggested.

Numerous studies provide evidence for mechanisms of antigen (Ag) receptor allelic exclusion that operate at various levels in both B and αβ T cells. In the case of TCRβ or immunoglobulin heavy chain genes, exclusion is achieved at the genomic level, that is, a productive rearrangement on one Ag receptor locus prevents complete rearrangement at the locus carried on the other chromosome, a process referred to as "genotypic" allelic exclusion (*1, 2*). However, such a stringent mechanism does not apply to TCRα chain genes, because several αβ T cell clones carry two productive TCRα rearrangements (*2, 3*). Despite this qualification, no examples of αβ T cells that express two TCRα chains on the cell surface have been found in physiological situations, which suggests the existence of "phenotypic" exclusion processes operating posttranscriptionally (*3*). Regulation of TCR γδ gene rearrangements remains unclear. Two productively rearranged TCRγ genes were found in murine γδ T cell clones (*4, 5*), but in all cases the two γ genes were not allelic, so that these observations could merely reflect a lack of intrachromosomal exclusion. We show that in humans, regulation of TCRγ gene allelic exclusion is very inefficient or absent and frequently results in γδ T cell clones that express two distinct functional γ chains on their surface.

In the course of flow cytometry analysis of several human γδ T cell lines, we noticed that a significant fraction of cells were recognized by two monoclonal antibodies (mAbs), 7B6 and 4A11, specific to distinct variable (V) γ regions ($V_\gamma9$ and $V_\gamma4$, respectively) (Fig. 1A). Out of 11 $4A11^+$ polyclonal cell lines derived from peripheral blood lymphocytes (PBLs) of healthy donors, the proportion of $V_\gamma9^+,V_\gamma4^+$ cells ranged from 1.1 to 6.9% (mean ± SD, 2.7 ± 2.0%), indicating that the occurrence of γδ T cells with this phenotype was a general phenomenon (*6*). Moreover, analysis of several $4A11^+,7B6^+$ cloned cells indicated that ratios of mean fluorescence intensities (MFIs) obtained with $V_\gamma4$- and $V_\gamma9$-specific mAbs, although stable for a given clone (*6*), varied greatly from one clone to another (Fig. 1B) and were inversely correlated (Fig. 1C) (*7*). Thus, 7B6 and 4A11 epitopes were probably carried on distinct γ chains, which competed for pairing with limiting amounts of δ chains.

Nonetheless, to formally rule out a possible cross-reactivity of the 7B6 mAb to a set of $V_\gamma4$ chains or, reciprocally, of the 4A11 mAb to a set of $V_\gamma9$ chains, we analyzed TCRγ gene transcripts in several $7B6^+,4A11^+$ T cell clones and confirmed the presence of productive $V_\gamma4$ and $V_\gamma9$ transcripts in all the clones studied (Fig. 2). Several T cell clones expressed $V_\gamma4$ and $V_\gamma9$ genes rearranged to constant $C_\gamma1$- and $C_\gamma2$-associated joining (J) elements, respectively (for example, clones B16 and C7), or reciprocally (for example, clones B4 and B22). Because the γδ interchain disulfide bridge involves a cysteine present in the $C_\gamma1$ region but absent in the $C_\gamma2$ region (*8*), the above clones should express on their surface both covalently and noncovalently linked γδ TCR.

This could be confirmed directly by immunoprecipitation experiments using mAb to $V_\gamma9$ (anti-$V_\gamma9$) (7B6), mAb to $V_\gamma4$ (anti-$V_\gamma4$) (4A11), and mAb to pan-δ (510) (Fig. 3). The $V_\gamma4$-specific mAb precipitated from lysate of B16 T cell clone a 70- to 80-kD species under nonreducing (NR) conditions, which resolved under reduction (R) into 43- and 40-kD bands corresponding, respectively, to TCRδ and TCRγ chains (*9*). In contrast, material precipitated from B16 cells by anti-$V_\gamma9$ consisted of two species of 43 and 53 kD under R and NR conditions, which should correspond to TCRδ chain and $VJC2_\gamma$

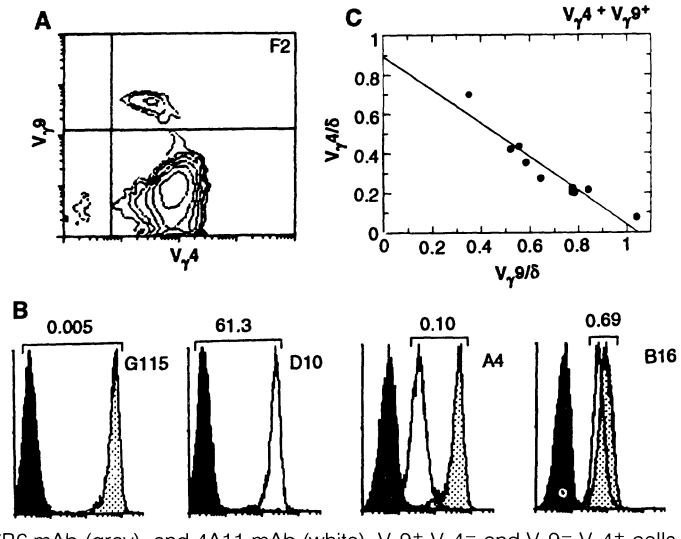

Fig. 1. Flow cytometry analysis of human γδ PBL lines and clones. (**A**) Double-color immunofluorescence analysis of a human γδ T cell line (F2), with $V_\gamma4$-specific 4A11 and $V_\gamma9$-specific 7B6 mAbs. Note the presence of γδ T cells recognized by both mAbs (upper right quadrant). (**B**) Fluorescence-activated cell sorter (FACS) analysis of two $4A11^+,7B6^+$ T cell clones (A4 and B16) derived from line F2. Shown are the fluorescence histograms obtained with an irrelevant control mAb (black), 7B6 mAb (gray), and 4A11 mAb (white). $V_\gamma9^+,V_\gamma4^-$ and $V_\gamma9^-,V_\gamma4^+$ cells (G115 and D10, respectively) were included as controls. $V_\gamma4/V_\gamma9$ MFI ratios are indicated at the top of each histogram. (**C**) Inverse correlation between the relative staining intensities obtained with 4A11 and 7B6 mAbs among $4A11^+,7B6^+$ T cell clones ($r = -0.95$, $P < 0.001$). Linear MFI values were obtained from flow cytometry analysis of ten $4A11^+,7B6^+$ T cell clones. Linear regression analysis was performed on Vγ4/δ MFI ratios (abscissa) versus Vγ9/δ MFI ratios (ordinate) calculated from cell staining analysis using 4A11, 7B6, and 510 (pan-δ) mAbs. γδ T cell lines were isolated after immunomagnetic sorting with pan-δ (510) mAb (*14*), cultured with irradiated PBLs and B lymphoblastoid cells in recombinant interleukin-2–supplemented culture medium, and cloned by limiting dilution as described (*15*). Cells were stained by indirect immunofluorescence with saturating amounts of mAb according to standard procedures (*15*) and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, California).

F. Davodeau, M.-A. Peyrat, I. Houde, M.-M. Hallet, H. Vié, M. Bonneville, INSERM U211, Institut de Biologie, 44035 Nantes, France.
G. De Libero, Department of Research, Institute of Experimental Immunology, 4031 Basel, Switzerland.
*To whom correspondence should be addressed.

Clone	Expressed γ genes										Expressed δ gene				
	V_γ				J_γ	C_γ	V_γ			J_γ	C_γ	V_δ			J_δ
B1	4	DG	LE	YYK	2	2	9	WEV	ASK	YYK	2	2	1	LG	DLPAELVLGAMIMMG	DK	1
B4	4	DG	AR	K	2	2	9	WE	ESW	FKI	P1	1	1	LGE	LHSYVLGDRKN	TDK	1
2B55	4	DG	SGPH	YK	2	2	9	W	V	YYK	2	2	1	LGE	PALGGSPPGY	TDK	1
A4	4	DG	PATT	K	2	2	9	WEV	AP	YYK	2	2	2	CDT	RDAVRRGIHPF	K	1
B22	4	DG	TAIH	YYK	2	2	9	WEV	I	ELG	P	1	2	C	AEVRSGGYLH	SWD	3
D5	4	DG	PR	YK	1	1	9	WEV	LCRQS	LG	P	1	2	CDT	GN	TDK	1
C7	4	DG	W	YK	1	1	9	WEV	FRFD	YK	2	2	3	CA	FSRLLGD	TDK	1
B16	4	DG	HKL	YK	1	1	9	WE	ANH	K	2	2	5	CAA	SLPSFLKR	K	1
D7	4	DG	PWL	FKI	P1	1	9	WE	PPWD	YK	1	1			ND		

Fig. 2. Deduced V(D)J junctional amino acid sequences (*16*) of TCRγ and TCRδ transcripts from 4A11$^+$,7B6$^+$ γδ T cell clones. Clones derived from line F2 (see Fig. 1, A and B) are listed on the vertical axis. All clones expressed two productively rearranged VJC_γ genes and a $VDJC_\delta$ gene comprising various V_δ ($V_\delta1$, $V_\delta2$, $V_\delta3$, or $V_\delta5$). $J_\gamma P1$, $J_\gamma P$, and $J_\gamma1$ are located upstream of $C_\gamma1$, and $J_\gamma2$ is located upstream of $C_\gamma2$ [nomenclature of Lefranc and Rabbitts (*17*)]. ND, not determined. All clones were recognized by $V_\gamma4$- and $V_\gamma2,3,4$-specific mAbs [4A11 and 23D12 (*18*), respectively] and $V_\gamma9$-specific mAbs [7B6, Y102, 360 (*14*), and Tiγa (*19*)]. RNA derived from T cell clones was reverse-transcribed with 3′ primers [C_γ, GGGTTACTGCAGCAGTAGTGTATC (*20*); or C_δ, GGGTTACTTCTCGGTATGAACTATGGC (*21*)] and then amplified by PCR after addition of 5′ primers [$V_\gamma9$, ATCTGCAGGCACTGTCAGAAAGGAATC (*22*); $V_\gamma4$, ATTGATACTGGAAATCT (*22*); $V_\delta1$, GCCTTAACCATTTCAGCC (*23*); $V_\delta2$, TTGCAAAGAACCTGGCTG (*23*); $V_\delta3$, TCACTTGGTGATCTCTCC (*23*); and $V_\delta5$, TCTCTCTGCACATTGTGC (*23*)] as described (*24*). After purification on low melting point agarose, amplified material was sequenced with double-strand template according to USB Sequenase kit procedure.

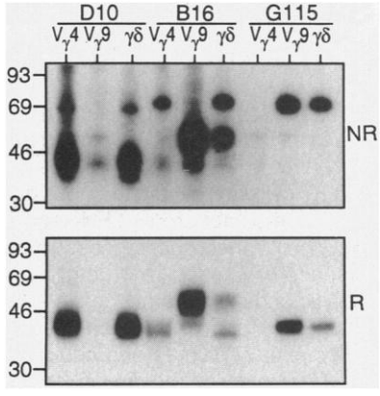

Fig. 3. Immunoprecipitation of disulfide-linked $V_\gamma4$ and nondisulfide-linked $V_\gamma9$ chains from B16 T cell clone. The γδ T cells were labeled with ^{125}I, and lysates were precipitated with 4A11 (anti-$V_\gamma4$), 7B6 (anti-$V_\gamma9$), and 510 (pan-δ) mAbs and resolved on SDS–polyacrylamide gel electrophoresis (PAGE) minigel under nonreducing (NR) and reducing (R) conditions. Molecular size markers are shown on the left (in kilodaltons). B16 is a 4A11$^+$,7B6$^+$ T cell clone, G115 is a 4A11$^-$,7B6$^+$ T cell clone, and D10 is a polyclonal 4A11$^+$ cell line. The 4A11 mAb precipitated from B16 cell lysate disulfide-linked material [apparent molecular mass (M_r) ~80 kD under NR conditions and 40 to 43 kD under R conditions], whereas 7B6 mAb precipitated from the same cells two nondisulfide-linked species (M_r of 53 and 43 kD). The G115 clone expressed a disulfide-linked TCR (M_r of 80 kD under NR conditions and 40 to 43 kD under R conditions) precipitated by 7B6 and 510 mAbs but not by 4A11 mAb. The D10 cell line expressed both disulfide-linked and nondisulfide-linked TCR precipitated by 4A11 mAb. The presence of a minor population of 7B6$^+$ cells (about 1%) among D10 cells probably accounts for the faint signal obtained with the 7B6 mAb. The γδ T cells were isolated from adult PBLs as described in Fig. 1, and 2×10^7 cells were labeled with ^{125}I and lysed in NP40 buffer (*25*). Lysates were precipitated with 4A11, 7B6, and 510 mAbs and material resolved on SDS-PAGE in the presence or absence of 2-mercaptoethanol as described (*25*).

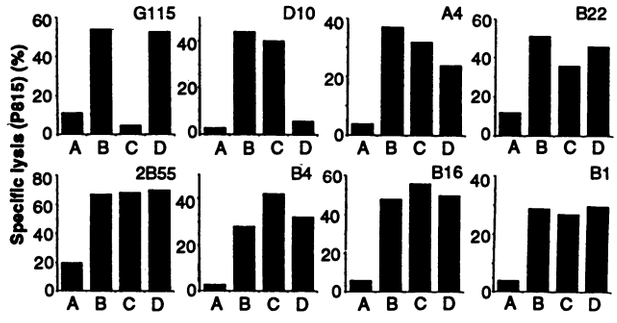

Fig. 4. Both $V_\gamma4$- and $V_\gamma9$-specific mAbs induce redirected lysis of P815 target cells by $V_\gamma9^+$,$V_\gamma4^+$ T cell clones. Cytolytic activity of γδ T cell clones against ^{51}Cr-labeled P815 target cells was estimated in the absence or presence of mAb [lane A, W6/32 (pan–major histocompatibility complex class I); lane B, 510 (pan-δ); lane C, 4A11 (anti-$V_\gamma4$); lane D, 7B6 (anti-$V_\gamma9$)]. The $V_\gamma9^+$,$V_\gamma4^-$ T cell clone G115 and $V_\gamma9^-$,$V_\gamma4^+$ D10 cells (see Fig. 3) were included as control effectors. None of the clones killed P815 cells in the absence of mAb (*26*). Both 4A11 and 7B6 mAbs induced efficient P815 lysis by all $V_\gamma4^+$,$V_\gamma9^+$ cytotoxic T cell clones studied (A4, B22, 2B55, 2B24, B16, and B1). Similar results were obtained with other $V_\gamma4$- and $V_\gamma9$-specific mAbs (*26*). We carried out cell-mediated lysis assay as described (*15*), using ^{51}Cr-labeled P815 (Fc receptor–positive) target cells. The cytotoxic assay was done in the absence or presence of various mAbs (hybridoma supernatant at 1:4 final dilution).

chain with triplicated exon II, respectively (9). All the above species were also precipitated with the mAb to pan-δ (Fig. 3). Thus, in agreement with the sequence analysis, B16 clone expressed on its surface a δ chain covalently linked to a $V_\gamma4J_\gamma C_\gamma1$ chain and noncovalently linked to a $V_\gamma9J_\gamma C_\gamma2$ chain.

These $V_\gamma4^+$,$V_\gamma9^+$ clones expressed TCRδ chain genes that comprised various V_δ gene elements ($V_\delta1$, $V_\delta2$, $V_\delta3$, or $V_\delta5$) (Fig. 2). Moreover, combined flow cytometry and molecular analysis of the $V_\delta1^+$ clones demonstrated the presence of a single δ chain, paired with either the $V_\gamma9$ or the $V_\gamma4$ chains on their surface (*10*).

The lytic machinery of cytotoxic T lymphocytes is classically activated after cross-linking the TCR by mAbs or Ags. Therefore, because most $V_\gamma4^+$,$V_\gamma9^+$ T cell clones described above were cytotoxic, we could test the functionality of their surface TCR by redirected lysis of Fc receptor–positive target cells (P815) with mAbs to the TCR (Fig. 4). Lysis of P815 cells by a $V_\gamma9^+$,$V_\gamma4^-$ clone (G115) was triggered by mAb to $V_\gamma9$ but not to $V_\gamma4$, whereas reciprocal results were obtained with $V_\gamma9^-$,$V_\gamma4^+$ cells (D10). In contrast, both mAbs induced P815 lysis by all cytotoxic $V_\gamma4^+$,$V_\gamma9^+$ clones studied (for example, clones B1 and B16) (Fig. 4), which indicates that both of their TCRs were able to transduce an activating signal.

Our data show that in humans, TCRγ genes, like TCRα genes, are not strictly allelically excluded at the genomic level, an assumption also supported by analysis of γ alleles in a random panel of γδ T cell clones (*11*). Moreover, unlike TCRα genes (*2*), the frequent occurrence of two productive VJCγ gene rearrangements in the same cells resulted in surface expression of two distinct γ chains on a significant fraction of cultured γδ PBLs [Fig. 1 and (6)]. These results are reminiscent of data demonstrating the presence of γ proteins encoded by distinct TCRγ isotypes in some murine T cell clones (*5*), although in those studies surface expression and functionality of the expressed γ proteins were not addressed (*5*).

The explanation for the distinct behavior of TCRα and TCRγ chains is unclear. Perhaps TCRβ chains are structurally less permissive for pairing with different TCRα chains than are TCRδ chains for pairing with TCRγ chains, so that in αβ T cells, one TCRα polypeptide would systematically compete out the other. Alternatively, some αβ T cells may express two surface α chains, but such cells may not be detected, given the extensive combinatorial diversity of the TCRα chain gene and the limited number of available V_α-specific mAbs.

According to current models of TCR

clonal selection, the presence of two distinct TCRs on the same cell should have limited consequences. Because the signal to switch off the thymocyte recombination machinery seems to be closely dependent on an interaction of the surface TCR with a specific thymic ligand during development (*12*), lack of genotypic exclusion of TCRα and TCRγ chain genes should allow a given T cell to try several TCR chains until it expresses a functionally relevant (that is, selectable) TCR. Thus, if two surface α or γ chains are expressed on the lymphocyte surface, one of these receptors, the one that was not selected for, should have little chance to encounter its nominal ligand in the periphery. However, many TCRs also have affinity for superantigens, which bind germline V-specific residues (*13*) and hence should be efficiently recognized by both selected and nonselected TCRs. In this context, the probability that a lymphocyte expressing two surface TCRs would encounter ligands for each of its expressed Ag receptors becomes more significant. If one of these ligands is an autoantigen, one could imagine that such cells might initiate an autoimmune reaction.

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7. Among ten clones expressing $V_\gamma9$ chains only, linear MFI values obtained with 7B6 mAb were strictly correlated with MFI values obtained with 510 (pan-δ) mAb ($r = 0.96$, $P < 0.001$). We obtained similar results with cells expressing $V_\gamma4$ chains only, using 4A11 mAb instead of 7B6 mAb ($r = 0.94$, $P < 0.001$). In contrast, analysis of $4A11^+,7B6^+$ T cell clones demonstrated a lack of correlation between MFI values obtained with either $V_\gamma4$- or $V_\gamma9$-specific mAb versus pan-δ mAb. However, a strong correlation was observed between cumulated linear MFI values obtained with each V_γ-specific mAb versus pan-δ mAb ($r = 0.96$, $P < 0.001$). Accordingly, staining intensities obtained with V-specific mAb relative to pan-δ were inversely correlated ($r = -0.95$, $P < 0.001$) (Fig. 1C). Taken together, this analysis strongly suggests that 7B6 and 4A11 mAbs recognized distinct γ chains on the surface of $7B6^+,4A11^+$ clones.
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10. $4A11^+,7B6^+$ T cell clones were analyzed by indirect immunofluorescence with the $V_\delta1$-specific mAb A13 [S. Ferrini *et al.*, *Eur. J. Immunol.* **19**, 57 (1989)], the $V_\delta2$-specific mAb 389 (*14*), and mAb 515, which recognizes all δ chains except those comprising the $V_\delta1$ region (I. Houde *et al.*, in preparation). Clones B1, B4, and 2B55 were $A13^+,389^-,515^-$, clones A4, B22, and D5 were $A13^-,389^+,515^+$, and clones C7 and B16 were $A13^-,389^-,515^+$ (M.-A. Peyrat and M. Bonneville, unpublished results). Because none of the $V_\delta1^+$ T cell clones (B1, B4, or 2B55) bound 515 mAb, they expressed δ chains comprising the $V_\delta1$ region exclusively. Because direct sequencing of polymerase chain reaction (PCR) products amplified from cDNA of clones with $V_\delta1$ and C_δ primers yielded a single in-frame sequence (Fig. 2), a single δ chain paired with either the $V_\gamma9$ or the $V_\gamma4$ chains was expressed on their surface.
11. Expressed TCRγ genes were sequenced by PCR in a panel of 28 randomly chosen T cell clones. Among the clones, three expressed two distinct productive γ transcripts ($V_\gamma8J_\gamma2C_\gamma2$ and $V_\gamma8J_\gamma2C_\gamma2$; $V_\gamma4J_\gamma1C_\gamma1$ and $V_\gamma4J_\gamma P1C_\gamma1$; and $V_\gamma4J_\gamma2C_\gamma2$ and $V_\gamma9J_\gamma PC_\gamma1$) (F. Davodeau, M.-M. Hallet, M. Bonneville, unpublished results).
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16. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Switch of CD8 T Cells to Noncytolytic $CD8^-CD4^-$ Cells That Make T_H2 Cytokines and Help B Cells

François Erard,* Marie-Thérèse Wild, Jose A. Garcia-Sanz, Graham Le Gros

$CD8^+$ T cells are a major defense against viral infections and intracellular parasites. Their production of interferon-γ (IFN-γ) and their cytolytic activity are key elements in the immune response to these pathogens. Mature mouse $CD8^+$ T cells that were activated in the presence of interleukin-4 (IL-4) developed into a $CD8^-CD4^-$ population that was not cytolytic and did not produce IFN-γ. However, these $CD8^-$ cells produced large amounts of IL-4, IL-5, and IL-10 and helped activate resting B cells. Thus, CD8 effector functions are potentially diverse and could be exploited by infectious agents that switch off host protective cytolytic responses.

The activation of resting $CD4^+$ T cells by antigens in the presence of IL-4 directs their development toward the production of IL-4, IL-5, and IL-10 cytokines (T_H2 phenotype) (*1*). We sought to establish whether the phenotype of CD8 cells could also be influenced by IL-4. The basic experimental outline consisted of activating $CD8^+$ cells (sorted with a fluorescence-activated cell sorter) for 6 days in in vitro primary cultures in the presence and absence of IL-4 and then analyzing the resulting phenotype with respect to surface markers and expression of functional activities. The low frequency of antigen-specific CD8 cells made the use of antigen-stimulated primary culture systems impractical. Therefore, we used a primary culture system activated by a mitogen [phorbol 12-myristate 13-acetate (PMA) + ionomycin + IL-2] to ensure a high efficiency of CD8 cell activation, confirmed by limiting dilution analysis (*2*). Small numbers of $CD8^+$ cells (800 cells per well) were seeded in the mitogen-activated primary cultures because this ensured that the activation and proliferation of the CD8 cells were independent of accessory cells (*2*).

Fluorescence flow cytometry–sorted $CD8^+$ cells (>99% purity) were stimulated to grow in primary cultures that contained different combinations of PMA, ionomycin, IL-2, and IL-4 (*3*). After 6 days of culture, the cells were stained for the expression of the αβ T cell receptor (TCR), Thy-1.2, CD2, CD4, CD8, CD44 (Pgp1), and CD45 (T200) surface markers. All cells (99%) from each primary culture condition expressed TCRαβ and Thy-1.2, and expression was not affected either by activation or by the addition of IL-4 (Fig. 1). After activation, CD44 expression was increased to a similar degree in each culture condition. However, CD8 completely disappeared from cells cultured with PMA,

F. Erard, M.-T. Wild, G. Le Gros, Department of Allergy/Immunology, Pharmaceuticals Research Division, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland.
J. A. Garcia-Sanz, Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel, Switzerland.

*To whom correspondence should be addressed.