In Vivo Persistence of Expanded Clones Specific for Bacterial Antigens within the Human T Cell Receptor α/β CD4$^-$8$^-$ Subset

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Summary

We analyzed the T cell receptor (TCR) rearrangements of 100 TCR-α/β CD4$^-$CD8$^-$ (double negative [DN]) T cell clones from normal individuals. We found that in four out of six donors this subset contains expanded clones that often account for 0.5% and, in one individual, even 7% of all peripheral blood lymphocytes. By combining limiting dilution analysis and N region oligotyping of polymerase chain reaction amplified TCR cDNA, we could measure the clonal size and show that two of these expanded clones remain stable in size for up to 4 yr in peripheral blood. The expanded clones analyzed ex vivo are not cycling and CD45 RA^{hi} RO^{lo}, but express high levels of $\alpha 4/\beta 1$ integrins, suggesting that they may have reverted to resting cells after activation. One of these expanded DN clones proliferates in vitro in response to *Escherichia coli* presented by monocytes cultured in GM-CSF plus IL-4 and kills CD1a$^+$ Molt-4 cells. In contrast to what was found in the α/β DN subset, α/β CD4$^+$ T cell clones specific for a tetanus toxin epitope showed a very small clonal size (<1 in 10^7) and could not be reisolated after 2 yr. Taken together, these results indicate that large clonal size and persistence are distinctive features of α/β DN cells specific for bacterial antigens. These cells may use antigen-presenting cells, restriction molecules, and selection routes different from those used by antigen-specific CD4$^+$ T cells.

The majority of mature TCR-α/β T cells express either the CD4 or CD8 coreceptor which participates in both recognition of the MHC–peptide complex and T cell activation (for a review see reference 1). A minor subset of α/β T cells that is double negative (DN)[1] for CD4 and CD8 has been described in the thymus and in the periphery of mice and humans (2, 3). This subset differs in many properties from the α/β CD4$^+$ or CD8$^+$ single positive (SP) T cells.

First, it is not clear whether α/β DN cells are thymus derived. Indeed, in the thymus, this subset appears late in ontogeny after α/β SP cells (2, 4). In addition, it has been reported that they can differentiate in bone marrow or liver of euthymic, athymic, or thymectomized mice (5–7). Second, mouse α/β DN cells do not follow the rules of negative and positive selection that apply to SP cells. Indeed, they express a restricted repertoire, which contains potentially autoreactive Vβ segments (7, 8) or autoreactive TCR that are deleted in SP cells of the same animal (9, 10). In addition, α/β DN cells do not contain increased levels of Vβ that are positively selected in SP cells (8). Although the potential autoreactivity of the α/β DN subset has never been directly proven, they are greatly expanded in the *lpr* and *gld* autoimmune mouse strains (11), where they accumulate as resting cells in LNs, after proliferation in the liver (12). In humans, α/β DN cells have been found expanded up to 50% of peripheral blood in patients with autoimmune diseases (13, 14). Third, α/β DN have unique growth requirements, since they proliferate in response to IL-3 (3, 15).

There is no consensus on the specificity and restriction of α/β DN T cells. It has been shown in the mouse that these cells increase in number after intraperitoneal injection of bacteria (16). Porcelli et al. (17) have reported that some human α/β DN clones recognize CD1a molecules. In addition, the same group has recently reported that some α/β DN clones respond in vitro to mycobacterial antigens presented by CD1b molecules, indicating that this cell subset may have unique restriction specificities (18).

All these results suggest that the repertoire and lifestyle of α/β DN T cells may differ from SP T cells. Thus, we were interested to establish the clonal make-up of this subset

[1] *Abbreviations used in this paper:* DN, double negative; LDA, limiting dilution assay; SP, single positive; TT, tetanus toxoid.

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in humans, since we had preliminary unpublished results that independent α/β DN clones isolated from the same donor carried the same patterns of TCR-β and -γ rearrangements, suggesting that they may have derived from the same clone that was expanded in vivo.

We have developed a method based on PCR oligotyping which allows us to quantitate and monitor a clonotype and show that α/β DN T cells comprise expanded clones that can account for 0.5–7% of all peripheral blood mononuclear cells. One of these expanded clones specific for *Escherichia coli* persists for at least 4 yr and displays a phenotype in vivo that is noncycling, $\alpha 4/\beta 1$ integrin high, CD45RA^{hi} RO^{lo}.

Materials and Methods

Cell Cultures. T cells were cloned and maintained according to established methods (19). Stimulation of T cells in limiting dilution with antigen was performed according to reference 20. For antigen presentation experiments, peripheral blood adherent cells were expanded in vitro as described by Porcelli et al. (18) in RPMI 10% FCS supplemented with 50 ng/ml human recombinant GM-CSF and 500 U/ml human recombinant IL-4. When collected after 6–8 d, ~60% of these cells were large, nonadherent with a typical veiled appearance, and expressed high levels of CD1a, CD1b, and CD1c and low levels of CD14. These cells were mitomycin C treated and 2 × 10^4 were cultured with 3 × 10^4 cells from α/β DN clones in 200 μl RPMI 10% FCS in the presence or absence of heat-killed *E. coli* (JM109 strain) and anti-CD1 antibodies (1:200 dilution of ascites). T cell proliferation was measured after 40 h by thymidine incorporation. Cytotoxicity was performed according to standard methods (21).

Cell Staining. PBMC were stained with mouse mAb 10A12 (γ2a anti-CD4), OKT8 (γ2a anti-CD8), and WT31 (γ1 anti-CD3/TCR-α/β), followed by goat anti-mouse γ2a FITC plus anti γ1 PE antisera (Southern Biotechnology Associates, Birmingham, AL). The WT31$^+$, CD4$^-$ CD8$^-$ cells were sorted on a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA), immediately cloned by limiting dilution at 0.2 cells per well (50–80% efficiency), and expanded using irradiated allogeneic PBMC, PHA, and human rIL2. For three-color FACS® analysis, purified PBMC were stained with biotin-WT31, FITC-anti Leu2a (CD8), and FITC-anti Leu3a (CD4), plus one of the following antibodies: PE–anti-DR, PE–anti-CD25, PE–anti-Leu17 (CD38), PE–anti-Leu8 (Lam-1) (Becton Dickinson & Co.), PE-UCHL1 (anti-CD45R0) (Dako Corp., Carpinteria, CA), PE-2H4.RD1 (anti-CD45RA) (Coulter Corp., Hialeah, FL), K20 (anti-CD29, mouse γ2a), HP2/1 (anti–very late antigen [VLA]4, mouse 1) (Immunotech, Marseille, France), anti-CD69 (mouse μ, a gift of Dr. C. Cosulich, IST, Genoa, Italy), and anti-CD31 (mouse γ1) (a gift of Dr. C. Mackay, Basel Institute for Immunology). Biotin-labeled mAbs were revealed with APC–streptavidin (Molecular Probes Inc., Eugene, OR), and unlabeled mAbs were revealed with PE–goat anti-mouse Ig subclass specific antisera (Southern Biotechnology Associates). Samples were analyzed on a FACStar Plus®. Cytospin preparations of sorted α/β CD4$^-$8$^-$ and CD4$^+$/CD8$^+$ T cells were fixed with acetone and stained with the anti-BCL-2 mAb 100 (22) followed by APAAP (Dako Corp.). Cell cycle analysis was performed using 7-AAD (Sigma Chemical Co., St. Louis, MO) in combination with WT31, anti-CD4, and -CD8 mAbs as described (23). Samples were analyzed on a FACScan® (Becton Dickinson & Co.), using an electronic gate to exclude cell doublets. The DNA histograms were analyzed using a CELLFIT program (Becton Dickinson & Co.). Ascitic fluid of antibodies to CD1a (NA1/34), CD1b (WM25), and CD1c (10C3) was donated by F. Calabi (Medical Research Council, Cambridge, UK).

DNA Extraction and Blotting. High molecular weight DNA was digested with EcoRI (New England Biolabs Inc., Beverly, MA), fractionated on 0.8% agarose gel and blotted to N-Hybond membrane (Amersham Corp., Arlington Heights, IL) according to standard procedures (24). The probes were labeled (10^9 cpm/μg) using a random priming kit (Boehringer Mannheim, Mannheim, Germany). Filters were hybridized overnight at 68°C in 5× SSPE, 0.1% SDS, 1% BLOTTO, washed at 65°C in 0.1× SSC, 0.1% SDS, and exposed. The TCR β locus probe was a 0.4-kb BglI fragment derived from the Cβ2 cDNA (25). The TCR γ locus probe was a 0.7-kb HindIII–EcoRI Jγ1 fragment (26).

Anchor-primed PCR Cloning and Sequencing. Total RNA was extracted from >5 × 10^6 cells as described (27). Single-strand cDNA was synthesized from 5 μg total RNA using oligo $(dT)_{12-18}$ primer and AMV reverse transcriptase (Boehringer Mannheim). The anchor-primed PCR amplification was performed by a modification of a published method (28). The cDNA was ethanol precipitated three times and poly (dG) tailed in 40 μl containing 0.1 mM dGTP and 15 U terminal deoxitransferase in the buffer supplied by the manufacturer (BRL, Gaithersburg, MD) for 30 min at 37°C. The cDNA was phenol extracted, ethanol precipitated, and resuspended in 200 μl of water. 10 μl of each cDNA sample was amplified using 100 ng (about 15 pmol) of the following primers: a poly (dG) complementary primer 5′-GCATGCGCGCGCGGCCGCGGAGG$(C)_{14}$-3′ containing a SacII site, together with either a TCR Cβ antisense primer 5′TGCTGACCCCACTGTCGACCTCCTTCCCATT-3′ containing a SalI site, or with a TCR Cα antisense primer 5′-CGTATCTGTTTCAAAGCTTTTCTCGACCAG-3′, containing a HindIII site. The amplifications were performed in 30 μl using a PCR kit (Perkin Elmer Corp., Norwalk, CT), with the following profile: 94°C for 15 s, 56°C for 60 s, 60°C for 15 s, and 72°C for 45 s. The PCR products were digested either with SacII plus SalI (TCR-β) or with SacII plus HindIII (TCR-α), cloned into pBluescript (Stratagene, La Jolla, CA) and multiple isolates were sequenced. The nomenclature of the TCR α and β genes is according to references 29–31.

Limiting Dilution and PCR Oligotyping. Total PBMC or sorted α/β DN cells were stimulated in a limiting dilution assay (LDA) as described above. Replicate cultures were grown to >5 × 10^6 cells. Total RNA was extracted and 0.5 μg of it was reverse transcribed. One tenth of the cDNA was amplified in 30 μl, using 80 ng of each primer. For the TCR β chain amplification, the following primers were used: Cβ 5′TGCTGACCCCACTGTCGACCTCCTTCCCATT-3′, containing a SalI site, with either F20 Vβ 5′-AGTACAGGATCCTATGGGAGCCT-3′, containing a BamHI site, or CO9 Vβ 5′-GTGCGTCGACTATCAGGCTCCTCTGCTACAT-3′, containing a SalI site. For the TCR α chain amplification, the primers were: Cα 5′-TGCTCTTGAATTCCATAGACCTCATGTC-3′, which has an EcoRI site, with either F20 Vα 5′-CTTTTCAGTCGACACTCTGGTTCAAGGC-3′, or CO9 Vα 5′-GAATACACAAGTCGACTCTGGGAGC-3′, both containing a SalI site. The PCR reactions were heated once for 2 min at 94°C, followed by 20 s at 60°C, 30 s at 72°C, and 10 s at 94°C for 35 cycles, followed by a 10-min extension at 72°C. 10 μl of each PCR reaction were fractionated on 2% agarose gel, alkaly blotted onto N-Hybond$^+$ nylon membrane, and hybridized on with ^{32}P-labeled N region–specific oligonucleotides in 6× SSPE, 1% BLOTTO (dry skim milk), 1 mM EDTA, and 0.1% SDS. The filters were washed twice at room temperature in 6× SSPE, 0.1% SDS for 10 min, once at the hybridization temperature for 10 min, and once at the

indicated temperature for 10 min. Optimal hybridization and washing temperatures were determined in cell mixing experiments, in which the PCR oligotyping was performed on scaled numbers of the relevant clones mixed with constant numbers of PHA blasts from unrelated donors. The oligonucleotides, hybridization, and washing temperatures were: F20 β 5'-CATAGTCCCAAGCCCCTGATAGA-3' (50°C and 60°C); F20 α 5'-GCTGCAGGATTTTCTGGTGCCTA-3' (50°C and 60°C); CO9 β 5'-TGAACTTGGGACTGAAGGAA-3' (40°C and 45°C); and CO9 α 5'-GTGGTGAGCGACAGAGGC-3' (40°C and 48°C). For tetanus toxoid (TT) specific CD4$^+$ clones, replicated cultures were set in the presence of 10 μg/ml of TT (Connough, Ontario, Canada) and expanded to ~2–3 × 10^6 cells. The RNA was extracted, the cDNA synthesized and amplified as described above, using the same Cα and Cβ oligonucleotides, plus one of the following V region oligonucleotides: Vβ2 5'-GGACAGAATTCTCATCAACCATGCAAGCCTGACCT-3', containing an internal EcoRI site; Vα1 5'-ACCCTGGTCGACGGCATTAACGGTTTTGAGGCTGGA-3'; Vα8 5'-GGCATTGTCGACCATTCGTTCAAATGTGGGCAAAAG-3'; Vα17 5'-CCAACAGTCGACTGGGAAAGGCCGTGCATTATTGAT-3'; Vα215'-CCAGCGTCGACAGCAAAATTCACCATCCCTGAGCAG-3', all containing an internal SalI site. For N region oligotyping, hybridization was carried out at 37°C as described above, using the following oligonucleotides (20): clonotype AL15.1 α 5'GCAGGGTCCTACAATGCCAGA-3' and β 5'CTCCCCCGGGGATACTATGGC-3'; ALp2III6.1 α 5'-GCAGCAAGCGGCGGCCAGTTC-3' and 5'-AGATCGGACCCGGCTACAAGT-3'; ALp2III4.3 α 5'-GCAGCGACCTTGTATGGTCAG-3' and β 5'-AGAGATCCGGGGGGGCAGGCGGGGTTT-3'; AL17.3 α 5'-GCAGCAAGCAGCCGGAAACACA-3' and β 5'-CGAGCAAGCCCTACCTACGAG-3'; AL4.1 α 5'-GCAGCTCGTCAGGGCGGATCT-3' and β 5'-AGGGGAGGGGGAAGGCCCCAG-3'; AL8.1 α 5'-GCAGCAGAGAATTATGGAGGA-3' and β 5'AAGACCGGGACTAGCAGATAC; and AL9.2 α 5'-GTTGTGAGTGCGGATACCGGC-3' and β 5'-AGAGGTCTCCCTGGGACTAGC-3'. Filters were washed twice for 15 min in 3 M Tetra methyl ammonium chloride (Sigma Chemical Co.), 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.1% SDS at the stringent temperatures determined according to published protocol (32): 63°C for all the 21-nucleotide-long N region oligonucleotides, and 68°C for the 24-nucleotide-long ALp2III4.3 β. The limiting dilution data were evaluated as described (33).

PCR Cloning an Sequencing. α/β DN T cell clones isolated in 1991 were PCR oligotyped and five positive clones from each donor were selected. The TCR-α and -β cDNAs were amplified, digested with the appropriate restriction enzymes, gel purified using Prep-A-Gene matrix (Bio-Rad Laboratories, Richmond, CA), and ligated to pBluescript. The ligated product was electroporated into JM105 bacteria and recombinant colonies were sequenced using Sequenase 2.0 (US Biochemical, Cleveland, OH).

Results

The Human α/β DN Subset Contains Expanded Clones. Four years ago, α/β DN T cells were sorted from the peripheral blood of two healthy individuals and immediately cloned by limiting dilution with high efficiency. The clones were characterized by Southern blot analysis using TCR Cβ1-2 (25) and Jγ1-2 probes (26), and the results are summarized in Table 1. In donor FO, who had 10% α/β DN T cells in peripheral blood, 8/11 clones showed the same pattern of β plus γ rearrangement, suggesting that these cells might be clonally related. In donor CO, who had 1% α/β DN T cells, two predominant clonotypes were found, each accounting for ~30% of the DN clones. Four additional donors revealed a somewhat higher clonal heterogeneity: in two of them, GC and PD, identical patterns of rearrangements were found in four and two pairs of independent clones, whereas the remaining two donors showed distinct patterns in all the clones analyzed. Since the cells were not expanded in vitro before cloning, these findings suggest that the α/β DN population of normal individuals often contains expanded clones of large size comprising 0.3–0.4% and, in one individual, even 7% of PBLs.

Table 1. *Clonal Relationship among Independently Isolated α/β DN T Cell Clones in Normal Individuals*

Donor (age)	Year	Percent α/β DN T cells	Frequency of clones sharing a unique TCR β plus γ rearrangement pattern
			%
FO (29)	1988	10	8/11 (73)
CO (40)	1988	1	6/20 (30)
			6/20 (30)
GC (30)	1991	2	2/11 (18)
			2/11 (18)
			2/11 (18)
			2/11 (18)
PD (33)	1991	2	2/20 (10)
			2/20 (10)
GS (39)	1991	2	0/20
AL (42)	1991	1	0/20

DN T cells were sorted from peripheral blood and cloned by limiting dilution (19). DNA was extracted from independent clones, digested with EcoRI, and analyzed by Southern blot using Cβ1-2 and Jγ1-2 probes (24, 25). The frequency of clones sharing the same β plus γ rearrangement is shown.

The TCR α and β genes from the two most frequent clonotypes of donors FO and CO were cloned by anchor-primed PCR (28) and sequenced (Fig. 1). Clones F20 and CO9 express different Vα and Vβ and do not share any particular pattern in the N region. Interestingly, the α chain of CO9 has a very short N region (GAC) and is identical in the V, N, and J regions to an already published sequence (34).

Clonotype Identification by PCR Oligotyping. Having the TCR-α and -β sequence, it was possible, using LDA and PCR olgiotyping, to determine the size and persistence of these two clonotypes with time. Different numbers of PBMC, or sorted α/β DN cells, were stimulated in replicate cultures with PHA, feeder cells, and IL-2 and expanded to ~5 × 10^6 cells. To detect the presence of the clonotypic TCR transcripts, RNA was extracted from each culture, reverse transcribed and amplified using Vα plus Cα or Vβ plus Cβ oligonucleotides. The amplified products were fractionated on

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                                                                        Clonotype F20

       Vβ               N/D1                      Jβ
GCC.AGC.AGC   TCT.ATC.AGG.GGC.TTG.GA           C.TAT.GGC.TAC           Vβw23.2-Jβ1.2
 A   S   S     S   I   R   G   L   D              Y   G   Y

       Vα                N                        Jα
TTC.TGT.GCT        GCA.GGA.TTT                 TCT.GGT.GCC.TAC         Vα1.1-JαIGR08
 F   C   A          A   G   P                   S   G   A   Y


                                                                        Clonotype CO9

       Vβ               N/D2                      Jβ
GCC.AGC.AG    T.GAA.CTT.GGG.ACT.GAA.GGA.A      AC.AAT.GAG.CAG          Vβ11-Jβ2.1
 A   S   S        E   L   G   T   E   G         N   N   E   Q

       Vα                N                        Jα
GTG.GTG.AGC.             GAC.                  AGA.GGC.TCA.ACC         VαLINV-JαQ
 V   V   S                D                     R   G   S   T
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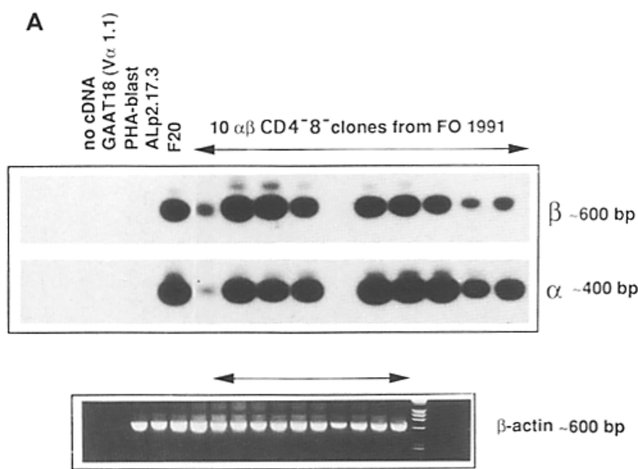
Figure 1. Sequences of the TCR α and β genes from clonotypes F20 and CO9. The complete sequence was obtained by anchor-primed PCR (27). The V and J segments were already described (28, 30). VαLINV corresponds to Vα24 (28). Shown is the junctional sequence. Oligonucleotides used for N region typing are underlined.



an agarose gel, blotted, and hybridized with ^{32}P-labeled N region–specific oligonucleotides under high stringency conditions. The system is very sensitive, allowing the detection of about 10 cells among 10^6 irrelevant lymphocytes (data not shown), and is highly specific. An example (Fig. 2 *A*) shows that the F20 clonotypic oligonucleotides do not hybridize to amplified products of unrelated T cell clones or allogeneic PHA blasts. The only exception is the CO9 α N region probe, which gives a signal in polyclonally activated blasts from all individuals tested. This "invariant" α chain is present in the peripheral blood of six out of six donors tested and is preferentially expressed in the α/β DN subset (Dellabona, P., manuscript in preparation).

Detection of In Vivo Expanded, Persistent α/β DN Clones. When 10 α/β DN clones isolated in 1991 from donor FO

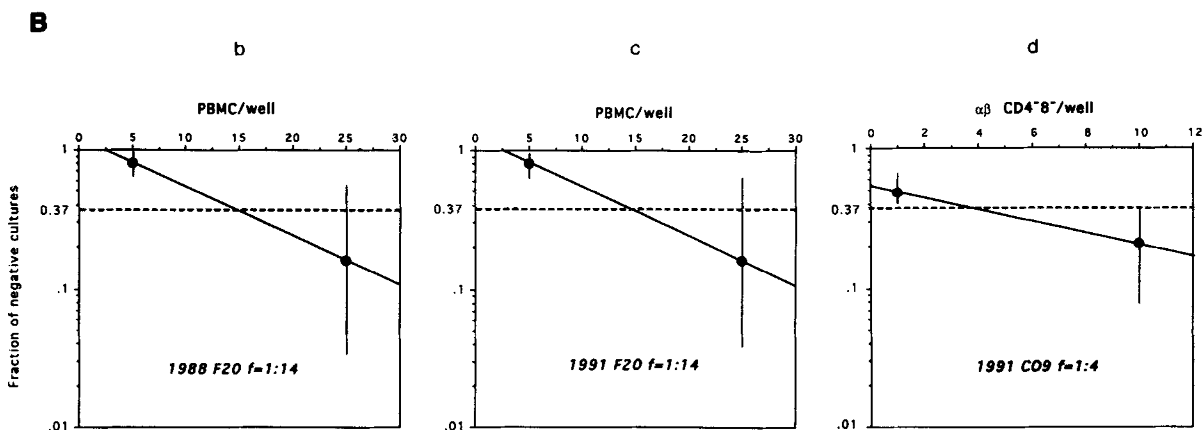


Figure 2. Clonotypic frequency determined by LDA-PCR. (*A*) Example of oligotyping for the F20 clonotype performed on PCR-amplified α and β gene products of 10 α/β DN clones from donor FO isolated in 1991, irrelevant control clones (*GAAT18*, which shares the Vα1.1 gene with *F20*, and *ALp2.17*), and allogeneic PHA blasts. β-actin is used as control. (*Right*) Size of the amplified products. (*B*) Frequency of the F20 and CO9 clonotypes estimated by LDA-PCR oligotyping. The PCR oligotyping was performed three times. Each point corresponds to 15–30 replicates. For *b*, the fraction of negative wells (FO) at 5 cells/well = 0.83, at 25 cells/well = 0.16; in *c* FO at 5 cells/well = 0.87, at 25 cells/well = 0.16; in *d* Fo at 1 cell/well = 0.48, at 10 cells/well = 0.21.

were analyzed, nine were positive by oligotyping, proving that the frequency of the F20 clonotype after 4 yr was similarly high. As shown in Fig. 2 *B*, the frequency of clonotype F20, as measured by LDA, was one in 14 PBMC in samples frozen in 1988, and one in 14 PBMC in samples obtained in 1991, indicating that this expanded clonotype was also strikingly stable in size, composing ∼7% of clonable PBL over a 4-yr period. For clonotype CO9, the frequency determined in 1991 was one in four α/β DN cells, a frequency comparable to the one estimated in 1988 by Southern blot analysis of random α/β DN clones (Table 1). To confirm the specificity of the oligotyping, we cloned sorted α/β DN T cells from these donors in 1991, identified several new F20 and CO9 clonotypes and sequenced the TCR α and β chains of five such T cell clones from each donor. All the sequences were identical to those of the original clones isolated in 1988, thus proving the persistence of the nonmutated, expanded T cell clones (data not shown).

Phenotype of α/β DN Clones In Vivo. In view of the long persistence of F20 and CO9, it was of interest to investigate their functional status and phenotype. Three-color FACS® analysis revealed that the α/β DN population in these donors did not express high levels of early-intermediate activation markers such as CD69, CD25, CD38, and HLA-DR (data not shown). Interestingly, fresh ex vivo cells expressed a CD45RA high, R0 low phenotype (Fig. 3), whereas the corresponding clones in culture acquired high levels of CD45R0 (data not shown). Furthermore, in donor FO, the expanded α/β DN population expressed high levels of VLA-4 and CD29 and low-intermediate levels of CD31 and leukocyte adhesion molecule (LAM) 1 (Fig. 4), a phenotype indicative of previous stimulation (35–38).

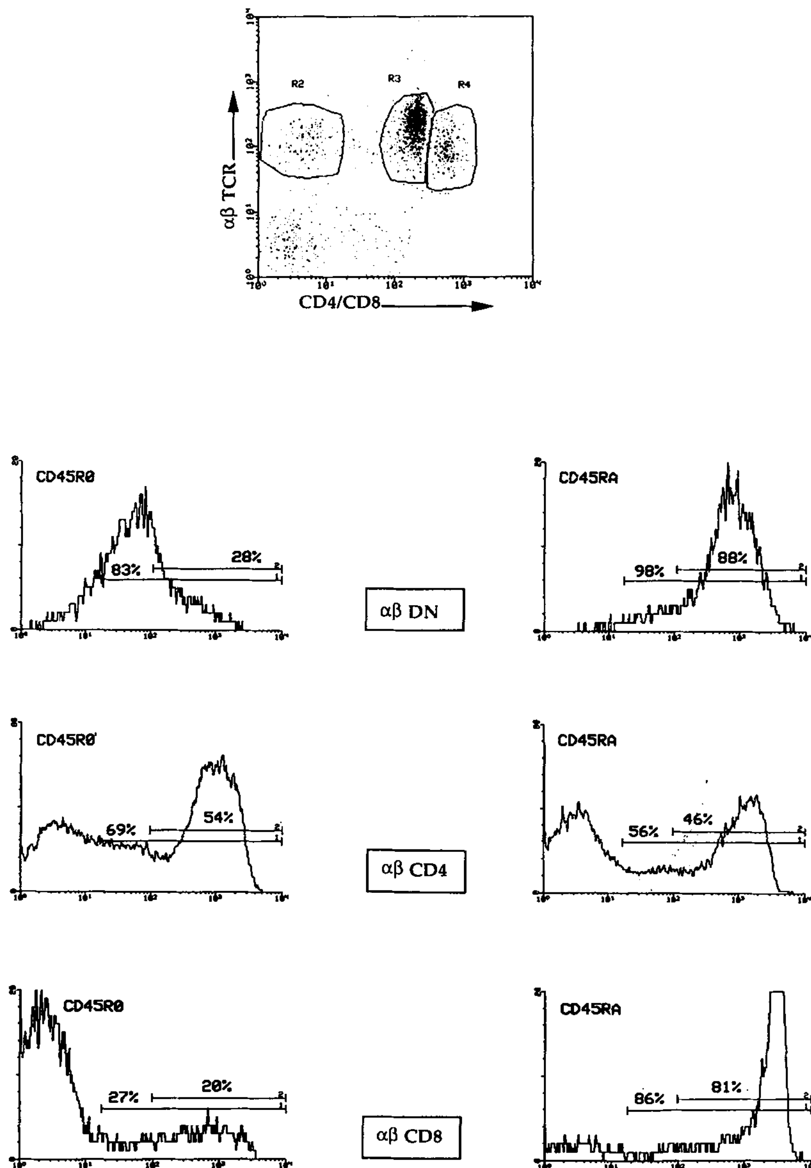


Figure 3. CD45 expression in freshly isolated α/β T cells of donor FO. (*Top*) Gates used for the three-color analysis: (*R2*) α/β DN, (*R3*) $CD4^+$, and (*R4*) $CD8^+$. In the histograms, the lower gate shows the percentage of positive cells in comparison to an isotype-matched irrelevant antibody, whereas the upper gate shows the percentage of cells expressing high levels of the marker.

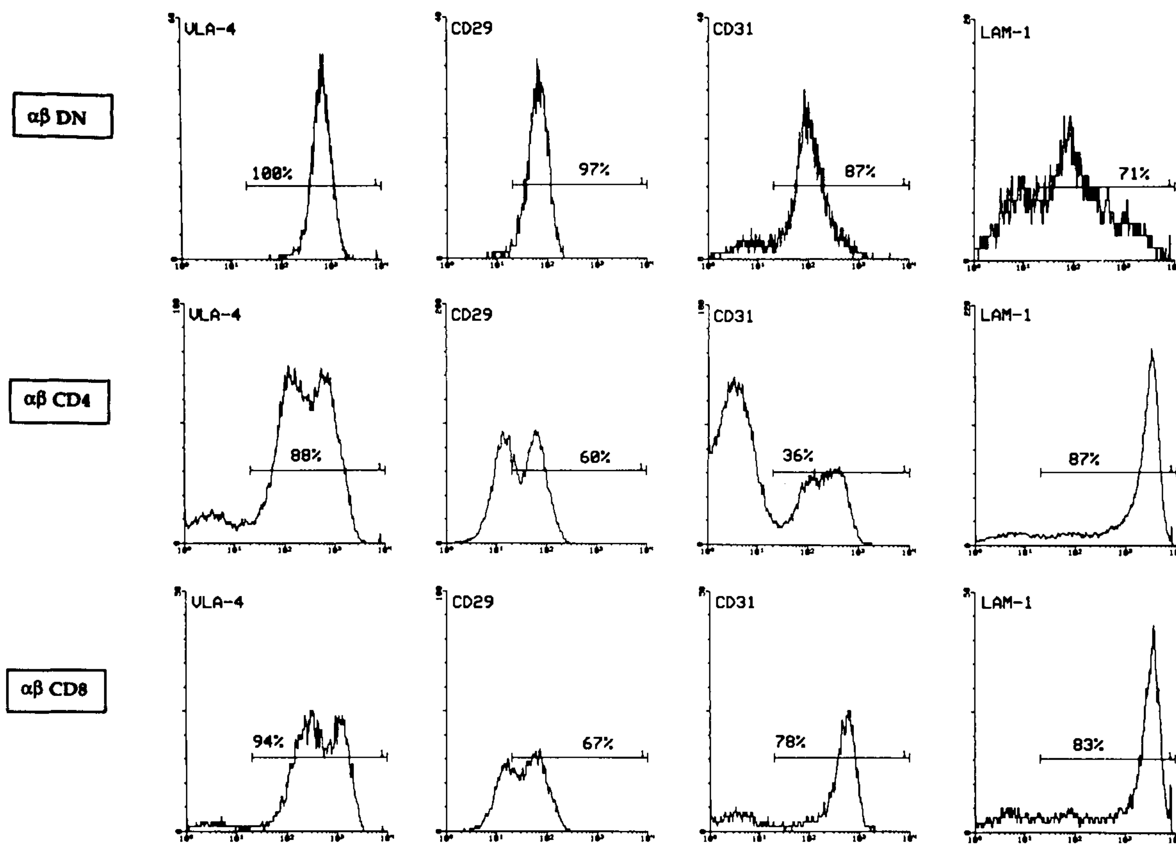


Figure 4. Expression of adhesion molecules VLA-4, CD29, CD31, and LAM-1 in freshly isolated α/β T cells of donor FO. The different populations were gated as in the upper panel of Fig. 3. The percentage of positive cells is indicated.

Cell cycle analysis of fresh PBMC revealed that <1% of α/β DN cells were in the S-G2M phase (data not shown). The level of Bcl-2 expression in freshly sorted DN cells was comparable to that of the $CD4^+$ and $CD8^+$ peripheral blood T cells (data not shown). In conclusion, in peripheral blood, the cells of the large and persisting α/β DN clones display a resting phenotype, with surface markers typical of Ag-experienced cells, but with $CD45RA^{hi}$, $R0^{lo}$.

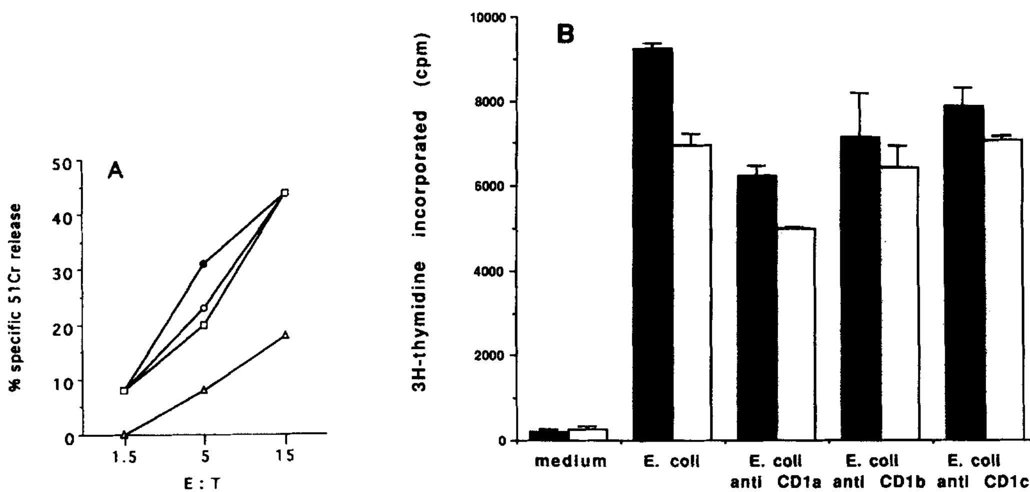


Figure 5. Specificity of clone F20. (*A*) Killing of Molt-4 cells by clone F20 in the absence (●) or presence of anti-CD1A (△), -CD1b (○), and -CD1c (□) mAbs. (*B*) Proliferative response of triplicate cultures (plus 1 SD) of clone F20 cultured with autologous (□) or allogeneic (■) $CD1^+$ monocytes in the presence or absence of heat-killed *E. coli* and anti-CD1 mAbs.

An Expanded α/β DN Clone Recognizes E. coli and CD1a. Since the α/β DN clones were selected with PHA, it is difficult to identify the antigen that might have driven in vivo the clonal expansion. When tested against a panel of target tumor cell lines, we noticed that clone F20 killed Molt-4 and the killing could be inhibited by anti-CD1a mAb as already described (Fig. 5 *A*, 17). After the recent report by Porcelli et al. (18), we asked whether clone F20 might also recognize bacterial proteins. We therefore expanded monocytes with GM-CSF and IL-4 (18) to obtain cells that express a high level of CD1. These cells were mitomycin C treated and cultured with F20 T cells in the presence or absence of bacterial antigens. As is evident from Fig. 5 *B*, both autologous and allogeneic CD1$^+$ monocytes could stimulate proliferation of clone F20 in the presence of heat-killed *E. coli*. Addition of anti CD1 mAbs caused only a modest inhibition of proliferation, which was, however, more clear with anti-CD1a. These findings raise the possibility that CD1a alone or associated with bacterial antigens may be the stimulus for the clonal expansion and persistence of F20 in vivo.

Clonal Size and Persistence of Antigen-specific CD4$^+$ T Cells. To ask whether large clonal size and persistence are unique features of α/β DN cells, we analyzed the frequencies and persistence of clones specific for a TT epitope in a donor who was repeatedly boosted with this vaccine.

As previously reported (20), several α/β CD4$^+$ T cell clones specific for the TT 830-844 were isolated from independent cultures in 1989. The frequency of TT-specific cells in peripheral blood was ~one in 3,000 and all the clones, except two, were unrelated as determined by TCR-α and -β sequences. After 2 yr, 200 replicate cultures were stimulated with TT, each containing 10^5 PBMC, and the responding cells from the individual cultures were expanded and analyzed by PCR oligotyping to detect the presence of seven clonotypes that had been isolated in 1989. Although in control cell mixing experiments we were able to detect the signal of 10 cells of a given clonotype in a million of allogeneic PBMC, none of the seven clonotypes could be identified among the TT-specific cells expanded 2 yr later (data not shown). This experiment shows that the class II–restricted response to a single epitope is highly polyclonal, and that each of the clones has a very small clonal size. Thus, large clonal size and persistence are not the key features of an antigen-specific response, at least in the CD4$^+$ compartment.

Discussion

Our analysis represents, to our knowledge, the first systematic attempt to measure in vivo the clonal expansion and persistence of T cells. We have used PCR oligotyping and limiting dilution analysis to measure the frequency of clones within the α/β DN and α/β CD4$^+$ subset. Two major findings emerge from this study. First, the α/β DN subset contains expanded clones that can account for up 0.5% and, in one donor, even 7% of PBL. Second, the expanded clones can persist for up to 4 yr with comparable size and a phenotype that is noncycling, CD45RA^{hi} RO^{lo} and VLA-4^{hi}.

The size of these α/β DN clones is remarkable. Since PBL are ~5×10^9, the size of these clones varies from 5×10^6 to 4×10^8, but is certainly underestimated, since most (~98%) of all T lymphocytes are outside the blood (39). It is possible that α/β DN T cells are compartmentalized in peripheral lymphoid or nonlymphoid organs such as liver, bone marrow, and LN in mice (5, 6) and skin in humans (40), and it is therefore possible that in peripheral blood we are measuring only a selected fraction of the clones.

The α/β DN expanded clones in the blood are noncycling and express a CD45RA^{hi} RO^{lo} phenotype. Since the same cells switch to RO^{hi} when activated in vitro, it is conceivable that they may have reverted to RA^{hi} sometimes after being activated (41–44). Indeed, when isolated from the blood, the expanded clones express high levels of $\alpha 4/\beta 1$ integrins and low levels of L-selectin and CD31, a phenotype that has been attributed to memory T cells (37, 38). This phenotype resembles that of α/β DN cells in autoimmune MRL-Mp-*lpr/lpr* mice (45) or in transgenic mice, where α/β DN cells expand and persist in the presence of a self-antigen (9).

How can such a large clonal size be maintained and what is the driving force for clonal expansion? To explain the persistence of expanded clones, we can consider two mechanisms. First, the clones may have expanded once by antigenic stimulation and subsequently persist in a resting state for 4 yr as long-lived memory cells that have reverted their CD45R0 phenotype to RA. Alternatively, these clones may undergo continuous expansion in peripheral organs (for instance in the intestine, where they are exposed to *E. coli* antigens), and the cells that revert to the resting state may reenter the recirculating pool, while others may die.

The lack of CD4 and CD8 coreceptors would suggest that these cells may not recognize classical class I or II molecules. Indeed, it has been shown that some human α/β DN T cell clones recognize CD1a molecules as such or CD1b in association with bacterial antigens (17, 18; clone F20). It is possible that chronic stimulation with a common bacterial antigen such as *E. coli* may drive a sustained proliferation resulting in a large clonal expansion as is the case for clone F20.

An alternative and testable possibility is that the clones may recognize self-MHC molecules and the affinity of recognition may be tuned by fluctuating levels of CD4 or CD8 molecules, as has been shown in a transgenic model (46).

The oligoclonal expansion and persistence of α/β DN T cells is in striking contrast with our finding that α/β CD4$^+$ clones specific for a TT peptide epitope are present at low frequency (<1 in 10^7) and cannot be detected in a sample of 2×10^7 lymphocytes after 2 yr. Although the characterization of T cell clonal size and persistence in the α/β SP subset is quite preliminary and outside the scope of this work, it seems to fit the current paradigms of immune response and memory, i.e., polyclonality (20) and requirement for continuous antigenic stimulation to maintain a memory cell pool (47, 48).

Contrary to our results with α/β CD4$^+$ clones, which conform to current models of T cell response, the observation with DN clones is unexpected. The oligoclonal expan-

sions in this subset could be explained if the T cell repertoire for a putative selecting antigen is small. Alternatively, these cells could be expanded and maintained by different mechanisms.

These results suggest that α/β DN T cells may represent a separate lineage with rules of selection and dynamics in periphery different from the SP. In some aspects, the α/β $CD4^-8^-$ subset resembles B1 (Ly-1) B cells, which are known to be created once in ontogeny, persist because of self-renewal capacity, and recognize self as well as common environmental antigens (49–51).

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