

Identification of a Committed T Cell Precursor Population in Adult Human Peripheral Blood

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Summary

Here, we report data concerning the discovery in adult human peripheral blood of a precursor cell population able to differentiate into CD4⁺CD3⁺αβ⁺ mature T cells. These cells, which represent 0.1–0.5% of total peripheral blood mononuclear cells (PBMC), express substantial levels of CD4, but lack CD3 surface expression. At a molecular level, they express the pre-T cell receptor α (pTα) gene, CD3-γ, CD-δ and CD-ε, and *RAG-1* recombination enzyme and have initiated rearrangements in the T cell receptor (TCR)-β locus (D–J). Moreover, low levels of CD3ε protein, but not of TCR-β chain, can be detected in their cytoplasm. Our results suggest that CD4⁺CD3⁻ cells identified in peripheral blood are different from CD3⁻CD4⁺CD8⁻ thymocytes and may contain precursors of an extrathymic T cell differentiation pathway.

In murine T cell development, early thymocytes that productively rearrange the TCR-β locus are selected to continue maturation. This happens because of the coupling of the rearranged β chain with the invariant pre-TCR-α (pTα)¹ protein (1, 2). This pre-TCR regulates early T cell development; later stages are under the control of the mature TCR, composed of the α and β chains (3). The TCR-β-pTα heterodimer is associated with CD3 molecules (4), and signals triggered by the pre-TCR induce expansion and differentiation of immature precursor cells (5).

Analysis of pTα gene-deficient mice provided formal proof that expression of the pre-TCR is required in the transition of CD25⁺ double-negative (DN) T cell precursors into small CD4⁺CD8⁺ thymocytes (5). This transition normally occurs through a stage in which maturing thymocytes proliferate vigorously and lose the expression of CD25. Nevertheless, low levels of mature αβ T cells can be detected in the periphery of pTα knockout mice, suggesting that the expression of pTα is necessary for quantitative expansion of maturing thymocytes, but not crucial for differentiation.

Using murine pTα cDNA as a probe, we were able to show that a comparable gene was expressed in human thymocytes (6). Amino acid sequence comparison between

human and mouse pTα cDNAs revealed high sequence homology in the extracellular as well as the transmembrane region, but complete divergence in the cytoplasmic region. Recently, another group cloned human pTα cDNA and performed a comparison of the developmental regulation of pTα, TCR-β, TCR-α, and *RAG-1* gene expression, providing a picture of the maturational progression of early human intrathymic stages (7).

We have previously shown that murine pTα expression is exquisitely T lineage specific and occurs in pro-T cells outside the thymus, in the earliest T cell precursors identified in the thymus, and in sites that support extrathymic T cell development (gut and liver), (8). Consistent with these observations, pTα RNA could not be detected in human non-T cells including B, NK, myeloid, and dendritic cells (7). Here, we have used the expression of the pTα gene as a tool to identify a human T cell precursor in the peripheral blood of adult donors.

Material and Methods

Antibodies. Cell staining was performed using the following mouse anti-human mAbs: phycoerythrin-conjugated (R-PE)-CD4, fluorescein-conjugated FITC-CD3, FITC-CD14, R-PE-CD5, biotin-CD10, R-PE-CD33, biotinylated HLA-DR, and RPE-CD34 (Becton Dickinson, San Jose, CA); tricolor CD4, biotinylated CD2 and RPE-CD7 (Caltag Laboratories, San Francisco, CA), CD45RA (IgG2b) and B7-2 (IgG2b) (PharMingen, San Diego, CA), FITC-pan-TCR-αβ (T Cell Diagnostic, Inc., Boston, MA). To deplete samples from CD3⁺ cells, we used TR66 mAb (9) that was FITC conjugated according to standard procedures. For TCR-β cytoplasmic staining we used HB-9283 supernatant (Amer-

¹Abbreviations used in this paper: ACDU, automatic cell deposit unit technique; CB, cord blood; DN, double negative; DP, double positive; FTOC, fetal thymic organ culture; GL, germline; HS, human serum; ISP, immature single positive; MMLV, murine maloney leukemia virus; pTα, pre-T cell receptor α gene; SP, single positive.

ican Type Culture Collection). Second-step antibodies used were goat anti-mouse IgG1 R-PE or FITC conjugated (Southern Biotechnology Associates, Inc.)

Surface Staining and Sorting of Lymphocytes. PBMC were separated from 50–60 ml blood from 25–33-yr-old male volunteers by standard Ficoll gradient centrifugation, resuspended in PBS containing 2% FCS at 10^7 cells per ml, and stained following standard procedures. Stained samples were analyzed and sorted on either FACScan[®], FACStar Plus[®], or FACS Vantage[®]. Sorted populations were reanalyzed by the same machines to test purity.

Cell Depletion. A single-cell suspension was first stained, as described previously, with FITC-conjugated TR66 mAb and, subsequently, PBMC were depleted of CD3⁺ cells using Dynabeads (Milan, Switzerland).

Cytoplasmic Staining. Sorted cells ($0.5\text{--}1 \times 10^5$) were washed twice in PBS and fixed in 500 μ l of 2% paraformaldehyde for 20 min at room temperature. Samples were washed again twice with PBS and, subsequently, lysed in 1 ml Saponin buffer (0.5% Saponin, 10 mM Hepes, 5% FCS) for 10 min at room temperature. Samples were then stained for 30 min at room temperature with HB-9283 supernatant specific for TCR- β cytoplasmic chain or CD3-FITC. After washing twice with Saponin buffer, goat anti-mouse IgG1 R-PE antibody was added in the case of TCR- β cytoplasmic staining. As background controls, for the TCR- β cytoplasmic staining we used the second-step antibody alone (goat anti-mouse IgG1 R-PE) and for CD3 cytoplasmic staining an irrelevant FITC-conjugated antibody (CD20; Becton Dickinson).

Cell Cycle Analysis. Sorted cells ($0.5\text{--}1 \times 10^5$) were fixed in 2 ml 70% ethanol at 4°C overnight. Samples were then centrifuged at 2,000 rpm for 5 min and resuspended in 250 μ l RNase A (Sigma, Buchs, Switzerland) (0.5 mg/ml in 0.1 M Tris, pH 7.5, 0.1 M NaCl) and incubated for 30 min at 37°C. Without centrifuging, 250 μ l pepsin (Sigma, Buchs, Switzerland) (1 mg/ml in 0.4% HCl) were added and samples were incubated for 15 min at 37°C. Without centrifuging, 500 μ l ethidium bromide (0.02 mg/ml, 0.2 M Tris, pH 8–8.5, 0.5% BSA) were added and samples were incubated for another 15 min at room temperature protected from light with aluminum foil. At the end of incubation, samples were kept on ice until analysis. Analysis was performed on a FACScan[®], equipped with the doublets discriminating module (DDM).

Reverse Transcription-PCR. In the analysis of pT α and CD3 expression in the total population, cells (0.5×10^5) were directly sorted into 500 μ l of RNazol (Cinna Scientific, Houston, TX) in Eppendorf tubes. Total RNA was extracted according to the protocol of the manufacturer. cDNA was prepared with random hexamer primers and reverse transcribed with a Superscript kit (GIBCO BRL, Gaithersburg, MD). In the analysis of pT α expression in a limited amount of cells, we used the automatic cell deposit unit technique (ACDU): cells (50, 20, 10, 5, and 1) were directly sorted in 5 μ l PBS in 96-well PC plates type H (Costar, Cambridge, MA). After sorting, plates were immediately put on dry ice and left for 30 min. cDNA was made directly in the plates after heating for 2 min at 95°C using MMLV reverse transcriptase and random hexamer primers at 37°C. Primers used were oligonucleotides specific for pT α (5'-GGCACACCCTTTCCTTCTCTG-3' and 5'-GCAGGTCTGGCTGTAGAAGC-3'), for CD3 ϵ (5'-GTCTCCATCTCTGGAACCACAG-3' and 5'-GGCCTTTCTATTCTTGCTCCAG-3'), for CD3 γ (5'-GGAGGAATTCATGACATGGAACAGGGGAAGG-3' and 5'-ACTCGAATTCTGAGTTCAATTCCTCCTCAAC-3'), for CD3 δ (5'-GTGAATTGCAATACCAGCATC-3' and 5'-GCTGTA-CTGAGCATCATCTC-3') and for RAG-1 (5'-CCAAATTG-

CAGACATCTCAAC-3' and 5'-CAACATCTGCCTTCACATCGATCC-3') gene transcripts. PCRs were done in a 30 μ l reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 10 mM mixed dNTP, 10 pM of each oligonucleotide primer, and 1 U AmpliTaq DNA polymerase (Roche Diagnostic System). DNA was amplified for 35 cycles at an annealing temperature of 55°C for pT α and RAG-1 and 63°C for CD3 with a thermal cycling machine (Perkin-Elmer Cetus). A 9- μ l portion of each amplified product was examined by 1.3% agarose gel electrophoresis and stained with ethidium bromide. In the case of ACDU procedure, a first PCR was done directly in the plates on total cDNA samples in 85 μ l reaction at 55°C annealing temperature for 30 cycles using an Hybaid Omnigen PCR machine. Subsequently, 2 μ l of each sample was used for a second PCR following the procedure described above. Primers used were the same for both PCRs. Primers to detect DJ β rearrangements at a transcriptional level were the following: 5'-TGGGAGGGGCTGTTTTTGT-3' and 5'-GATCTCATAGAGGATGGTGGC-3'.

DNA Extraction and PCR. DNA was prepared from cells by directly sorting them in PBS. Subsequently, 5 μ l of proteinase K (20 mg/ml; Merck, Darmstadt, Germany) was added and sample were left at 55°C for 2 h. Phenol/chloroform extraction and DNA precipitation followed, according to standard procedures. PCR conditions were as described elsewhere (10) using TBF1 (upstream, the D β 1 segment) and TBR1 (downstream, the J β 1–6 element) primers (10) for genomic DJ β amplification.

Southern Blot Analysis. Some of the gels containing PCR products were blotted for 2 h with 0.4N NaOH and subsequently hybridized, according to standard procedures, with the following different oligonucleotides: for RAG-1 gene product with 5'-ACC-ATCCACAGGACCATTGGACTGG-3', for DJ β rearrangements of the TCR- β locus with TBR3 (10), for immature D-J-C transcripts from the β locus with a C β -specific oligo 5'-GAGCCAT-CAGAAGCAGAGATC-3'.

Hybrid Human/Mouse Fetal Thymic Organ Cultures. The in vitro development of human T cells was studied using the hybrid human/mouse fetal thymic organ culture (FTOC) (11). Fetal thymi from embryos of RAG-1-deficient mice were dissected on day 15–16 of gestation and precultured for 5 d in the presence of 1.35 mM 2-deoxyguanosine (Sigma, St. Louis, MO) to remove endogenous thymocytes. Next, the thymic lobes were cocultured for 2 d in hanging drops in wells of a Terasaki plate with human progenitor cells and transferred to nucleopore filters, which were layered over gelfoam rafts in 6-well plates (Costar). The lobes populated with human cells were cultured for the indicated number of days in Yssel's medium (12) supplemented with 2% normal human serum and 5% FCS. To analyze differentiation of human cells, the mouse thymi were dispersed into single cell suspensions and stained with mAbs specific for human cell surface antigens.

Liquid Cultures in the Presence of Cytokines. To test T cell differentiation potential, 1×10^4 sorted cells were cocultured with irradiated (3,000 rads) allogeneic PBMC as feeder cells in U-bottomed 96-well plates in complete RPMI medium with 5% human serum (HS), 0.1 μ g/ml PHA, 1,000 U/ml IL-2, and 500 U/ml IL-7. After 10 d, growing cells were stained and analyzed by FACS[®].

Limiting Dilution Analysis. Flow cytometry-sorted progenitor cells were carefully spun down and diluted in the same medium described above for T cell differentiation, containing 1×10^6 /ml irradiated allogeneic PBMC as feeder cells. 1 cell/well was distributed in Terasaki plates. After 10–15 d, growing wells were transferred in U-bottomed 96-well plates and cells stained and analyzed by FACS[®].

Results

Identification of a $pT\alpha^+$ Population in Peripheral Blood. By PCR analysis of retrotranscribed mRNA of PBMC of adult donors we were able to detect low levels of $pT\alpha$ message in all of the samples tested (data not shown; Fig. 1 B, lane 1). Depletion of $CD3^+$ T cells from PBMC did not abrogate the detection of $pT\alpha$ expression by PCR. This result was expected, because mature T cells have completely lost expression of $pT\alpha$ (6–8). In view of our previously demonstration (6) that the thymic subset expressing the highest levels of $pT\alpha$ is $CD3^-CD4^+CD8^-$ (immature single positive, ISP), we investigated whether expression of CD4 was shared by $pT\alpha^+$ cells in PBMC. Fig. 1 demonstrates that this is indeed the case, because sorted $CD4^+$, $CD3^-$ cells (Fig. 1 A, shown in the box, R2) expressed $pT\alpha$ (Fig. 1 B, lane 2). No $pT\alpha$ message could be detected in total PBMC depleted of $CD4^+CD3^-CD14^-$ cells (Fig. 1 B, lane 3). Monocytes, which also express low levels of CD4 but are $CD3^-$, were excluded from the selection by staining with a specific mAb (CD14) and on the basis of their size (Fig. 1 A). Interestingly, the physical parameters (FSC versus SSC) of the $pT\alpha^+$ subset differ substantially from those of mature

lymphocytes and monocytes, in that $pT\alpha^+$ cells have a size and a cell complexity intermediate between lymphocytes and monocytes (Fig. 1 A, bottom). This cell population represents, in different healthy adult individuals analyzed (25–35 yr old), 3–9% of gated cells (R1) and 0.1–0.5% of the total PBMC, obtained by Ficoll preparation.

Partial DJ β Rearrangement and β Gene Transcription in $pT\alpha^+$ Cells in Peripheral Blood. Rearrangement of B cell (13) and T cell (14) antigen receptor gene loci to form functional VDJ gene segments is a key molecular event in lymphocyte ontogeny. In the thymus, DJ β rearrangements precede V(D)J β rearrangements (15). Therefore, the pattern of TCR β gene rearrangements represents a helpful marker to assess the maturational stage of a given population and its possible commitment to the T cell lineage. To analyze the rearrangement status of the TCR- β locus (DJ β versus germline configuration) in the $pT\alpha^+$ population observed in human peripheral blood, genomic DNA was isolated from $CD4^+CD3^-CD14^-$ $pT\alpha^+$ cells, and a recently described (10) PCR-based method was used, to amplify the DJ $\beta 1$ gene region specifically. Relevant controls in which

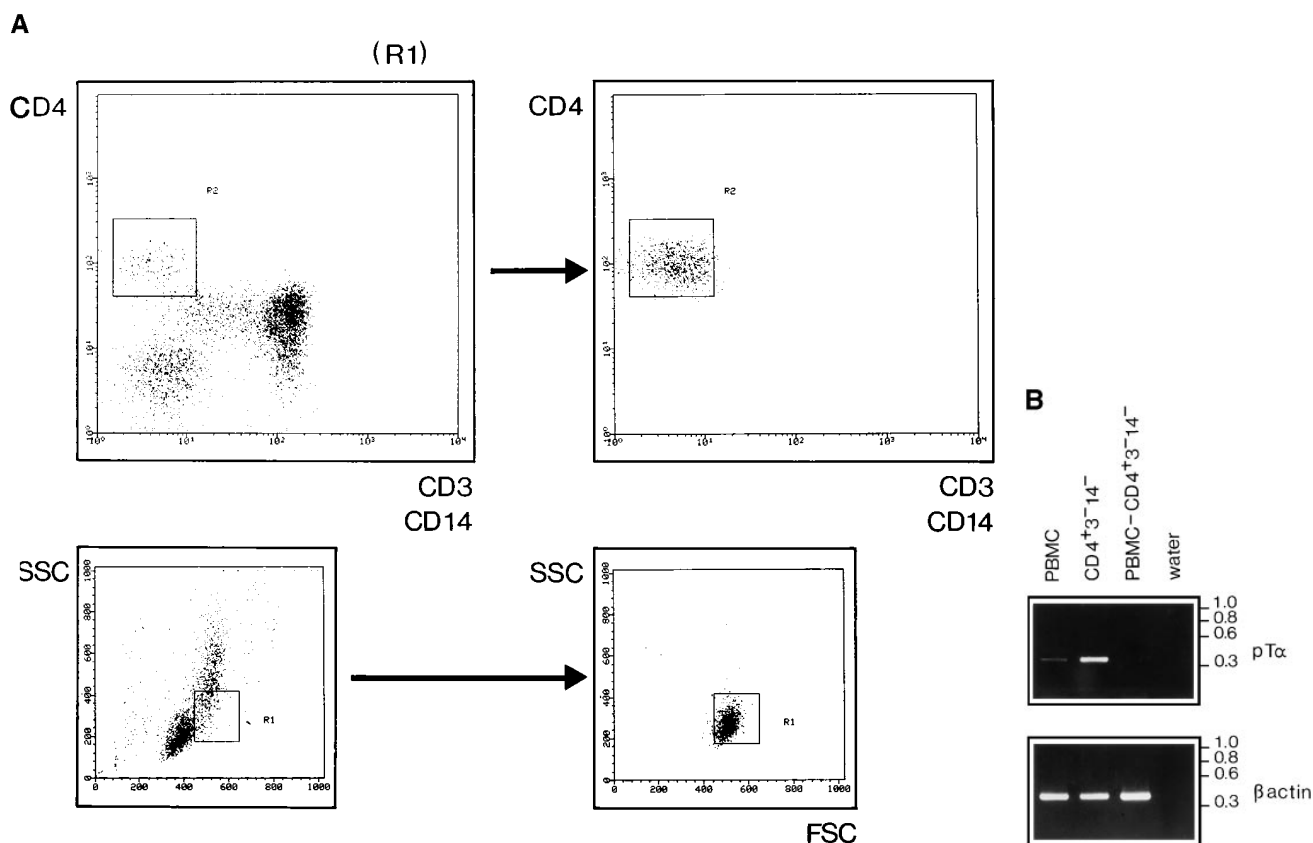


Figure 1. (A) FACS[®] analysis of presorted and sorted cells from PBMC. Mononuclear cells were separated from total PB by standard Ficoll gradient centrifugation, CD3 depleted using FITC-TR66 mAb and stained with CD4-PE- and CD14-FITC-specific mAbs. Top left, CD3/CD14 versus CD4 staining pattern of cells gated through R1 (FSC versus SSC, bottom left). R1 determined by backgating R2 into FSC versus SSC (see also size pattern, bottom right). Top and bottom right, cells after sorting (purity close to 100%). (B) $CD4^+CD3^-CD14^-$ sorted cells express $pT\alpha$. RT-PCR analysis for the expression of $pT\alpha$ (top) and β -actin (bottom) was performed on total PBMC, $CD4^+CD3^-CD14^-$ sorted cells according to gates shown in A and total sorted PBMC gating out $CD4^+CD3^-CD14^-$ (PBMC, $CD4^+CD3^-CD14^-$). To ascertain that PCR products shown were derived from $pT\alpha$ cDNA, gels were blotted and probed with a $pT\alpha$ -specific oligonucleotide (data not shown).

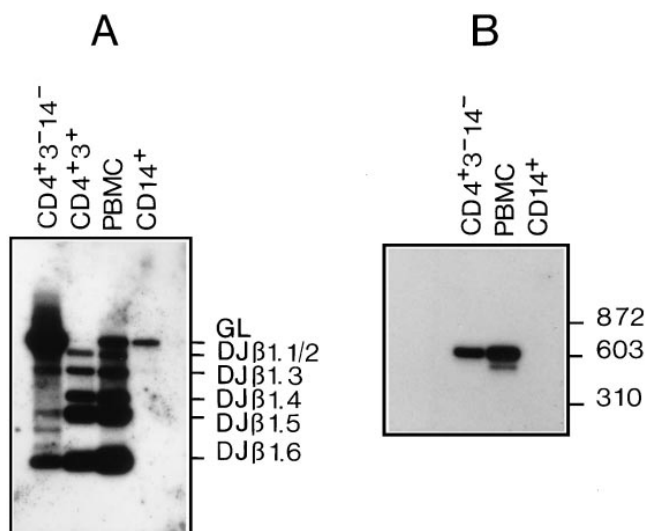


Figure 2. Detection of partial (D-J) rearrangements of the TCR- β locus. (A) At DNA level. Genomic DNA was isolated from sorted CD4⁺CD3⁻CD14⁻, CD4⁺CD3⁺, CD14⁺, and total PBMC and amplified by PCR using TBF1 and TBR1 primers to detect DJ β 1-J β 1 rearrangements. Normal PBMC and monocytes (CD14⁺) were used as positive and negative controls, respectively. PCR products were blotted and hybridized with TBR3 probe. PCR products, ranging from 200 to 3,000 bp, and the J β segment used are indicated. GL, germline. (B) At RNA level. Total RNA was first isolated from sorted CD4⁺CD3⁻CD14⁻, CD14⁺, and from total PBMC and, subsequently, RT-PCR using TBF1 and C β primer set was performed. PCR products were blotted and probed with a C β -specific primer.

DNA was obtained either from CD4⁺CD3⁺ mature T lymphocytes, CD14⁺ monocytes (obtained from FACS[®] sorting), or from unseparated PBMC were run in parallel. The primer combination used in this method allows to amplify, in a single PCR reaction, a germline-derived DNA fragment of 3 kb and/or several shorter DNA products derived from DJ β rearranged loci. PCR products are then blotted and hybridized with an internal oligonucleotide probe.

Several DJ β rearrangements (DJ β 1.3, DJ β 1.5, and DJ β 1.6) could be demonstrated in genomic DNA from CD4⁺CD3⁻CD14⁻ progenitors, in addition to a dominant germline band (GL) (Fig. 2 A). As expected, monocytes (CD14⁺) were characterized by the presence of an amplification product only in germline configuration, while in mature T cells (CD4⁺CD3⁺) and total PBMC all the different DJ β rearrangements were present. As expected, a band corresponding to germline gene configuration also could be detected in PBMC because of the presence of non-T populations. This result suggests the presence of T cell lineage committed cells within the pT α ⁺ CD4⁺CD3⁻ population.

To ascertain whether the presence of DJ β rearrangements was paralleled by transcription of the rearranged genes, we tested for the presence of transcripts corresponding to the partial DJ β rearrangements by RT-PCR. Total RNA was isolated from sorted peripheral populations (CD4⁺CD3⁻CD14⁻ and CD14⁺) or total PBMC and reverse transcribed into cDNA before amplification with a primer specific for the upstream D β 1 sequence together with a C β -

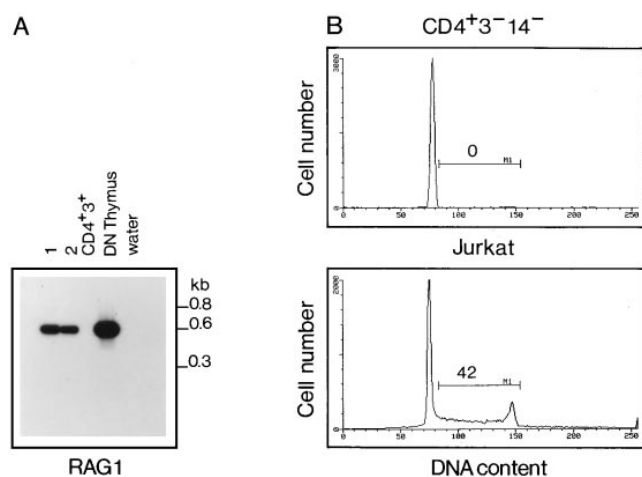


Figure 3. CD4⁺CD3⁻CD14⁻ cell population expresses *RAG-1* and is not cycling. (A) RT-PCR analysis on two different samples of sorted CD4⁺CD3⁻CD14⁻ PBMC (1, 2), mature T cells (CD4⁺CD3⁺) and DN thymocytes (DN Thymus). PCR products were blotted and probed with a *RAG-1*-specific oligonucleotide. (B) Cell cycle analysis on sorted CD4⁺CD3⁻CD14⁻ (top) and on Jurkat T cell line (bottom).

specific primer. PCR products were analyzed by Southern blotting with an internal oligo specific for the C β region. All cDNA were controlled for integrity with β -actin primers before D-J-C amplification (data not shown). Results are shown in Fig. 2 B, in which the presence of a transcript of 600 bp, corresponding to partial DJ β rearrangement, is detected both in unfractionated PBMC and sorted CD4⁺CD3⁻CD14⁻ cells, but not in CD14⁺ monocytes. Taken together, these results show that both rearrangement of, and transcription from TCR- β loci are present in a fraction of the pT α ⁺ cell population present in peripheral blood.

The CD4⁺CD3⁻CD14⁻ Cell Population Expresses *RAG-1* Gene and Is Not Cycling. Previous studies have shown that analysis of the expression of *RAG-1* and *RAG-2* genes and of TCR transcription are useful to trace early events during T cell development both in murine and in human T cell development (7, 16). As independent confirmation of DJ β rearrangement of the TCR- β locus of CD4⁺CD3⁻CD14⁻ cells, we tested for *RAG-1* gene expression in two different preparations of CD4⁺CD3⁻CD14⁻ cells from human peripheral blood. *RAG-1* message could indeed be detected in both preparations (Fig. 3 A, lanes 1 and 2), as well as in DN cells from thymus, but not in mature CD4⁺CD3⁺ T cells.

Because CD3⁻CD4⁺CD8⁻ immature thymocytes are actively cycling, we then asked whether peripheral pT α ⁺, CD4⁺CD3⁻CD14⁻ cells were also characterized by active proliferation. Analysis of DNA content in the potential T cell precursor population revealed that all of the cells were in G₀-G₁ phase of the cell cycle, while TCR- $\alpha\beta$ ⁺ Jurkat T cell line showed also G₂-S phases (Fig. 3 B). Thus, CD4⁺CD3⁻CD14⁻ cells are in a resting state, express *RAG-1* and pT α , and carry DJ rearrangements at the TCR- β locus.

Cell Surface Phenotype Analysis. CD4⁺CD3⁻CD14⁻ cells were isolated by flow cytometry as described above (see

CD4⁺CD3⁻CD14⁻

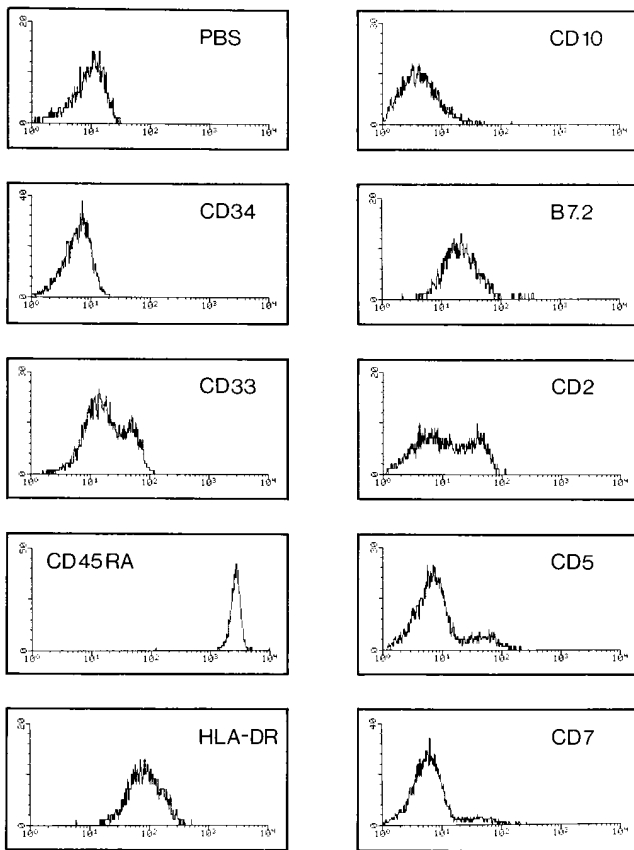


Figure 4. Cell surface analysis of pT α ⁺ cells. Mononuclear cells were separated with standard Ficoll gradient and depleted of CD3⁺ cells with FITC-TR66 mAb. Three-color analysis was then performed using CD4- and CD14-specific mAbs in combination with a third antibody of interest (histograms of which are shown in the figure): in the case of biotinylated antibodies (CD2, CD10, and HLA-DR), CD4-R-PE and CD14-FITC and, subsequently, APC were used; in the case of directly PE-labeled antibodies (CD5, CD7, CD33, and CD34), CD4-tricolor and CD14-FITC were used. When staining with CD33 or B7-2 mAbs we had to sort CD4⁺CD3⁻CD14⁻ cells, as shown in Fig. 1, and restrain them with one of the two antibodies that were then detected with mouse anti-human IgG2.

Fig. 1) and subsequently stained with a set of monoclonal antibodies specific for cell surface markers. Fig. 4 shows that sorted cells homogeneously expressed high levels of CD45RA, intermediate levels of MHC class II HLA-DR, low levels of the costimulatory molecule B7-2, but did not express the hematopoietic progenitor cell surface antigen CD34 or the neutral endopeptidase CD10, both present on a T, B, NK, and DC cell precursor population recently described in human bone marrow (17). Only a very small fraction, around 2–5% of sorted cells, expressed CD5 and CD7, found on mature and immature T cells. A higher fraction, 15–40% depending on the different individuals analyzed, expressed the T- and NK-specific molecule CD2. CD33, expressed on myeloid progenitors and monocytes, also presented a bimodal distribution with most of

the cells expressing low levels of the marker (CD33^{dim}). Both CD33⁺ and CD33⁻ cells expressed pT α (data not shown). CD4⁺CD3⁻CD14⁻ cells did not exhibit detectable levels of the lineage-specific cell markers CD1a, CD8, CD16, and CD56, CD19 and CD20, $\gamma\delta$ TCR and CD83 (data not shown).

Expression of CD3 Subunits but Not TCR- β Chain in CD4⁺CD3⁻CD14⁻ PBMC. Although the pT α ⁺ peripheral blood cell population does not express CD3 on the cell surface, as assessed by FACS[®] analysis, we investigated whether transcripts for the different components of this TCR-associated signalling molecule could be detected. For this purpose, cDNA from CD4⁺CD3⁻CD14⁻ sorted cells was amplified with three different sets of primers specific for CD3- γ , CD3- ϵ , and CD3- δ transcripts, respectively. Analysis of the PCR products showed that CD4⁺CD3⁻CD14⁻ cells expressed all three CD3 components, as can be observed in the T cell line MOLT 4 (Fig. 5 A); CD3- γ was found to be expressed in low amount, because it appeared barely detectable on agarose gels after staining with ethidium bromide; nonetheless, the presence of the message was readily detectable after hybridization with a specific internal probe of the blotted PCR products (data not shown).

The concomitant expression of transcripts of pT α and CD3 components in the CD4⁺CD3⁻CD14⁻ cell population, together with the described rearrangement of TCR- β genes, raised the possibility that, at this stage, such cells might assemble in their cytoplasm a putative human pre-TCR complex consisting of a TCR- β chain associated with the pT α chain. To test this possibility we performed cytoplasmic staining to detect the presence of TCR- β and CD3- ϵ proteins in CD4⁺CD3⁻CD14⁻ cells. Flow cytometric analysis shown in Fig. 5 B revealed that while pT α ⁺ cells do not express TCR- β chain in the cytoplasm, all cells within this population express CD3- ϵ protein, but at lower levels than in a mature human T cell clone. Specificity of the CD3- ϵ cytoplasmic staining was confirmed by relevant controls, and in particular by the lack of any FACS[®] profile shift in similarly stained B lymphocytes and dendritic cells (data not shown). The absence of TCR- β chain in the cytoplasm of CD4⁺CD3⁻CD14⁻ cells is consistent with lack of VDJ β rearrangements at DNA level (data not shown). These data indicate that while some components of such putative human pre-TCR, and in particular CD3- ϵ , are not only transcribed, but also translated into proteins, others, like the TCR- β chain are only present in the form of D-J transcript, while no translation can be detected.

Only a Fraction of CD4⁺CD3⁻CD14⁻ Expresses pT α . To define whether all CD4⁺CD3⁻CD14⁻ cells or, on the other hand, only a fraction of them expressed pT α , we performed PCR-based analysis of pT α expression on discrete numbers of sorted CD4⁺CD3⁻CD14⁻ PBMC obtained with the ACDU technique. In brief, 50, 20, 10, or 5 CD4⁺CD3⁻CD14⁻ cells were directly sorted in 96-well plates and cDNA was synthesized directly in the plates. Two rounds of PCR followed, a first one on the total cDNA obtained and a second one on a fraction of the first

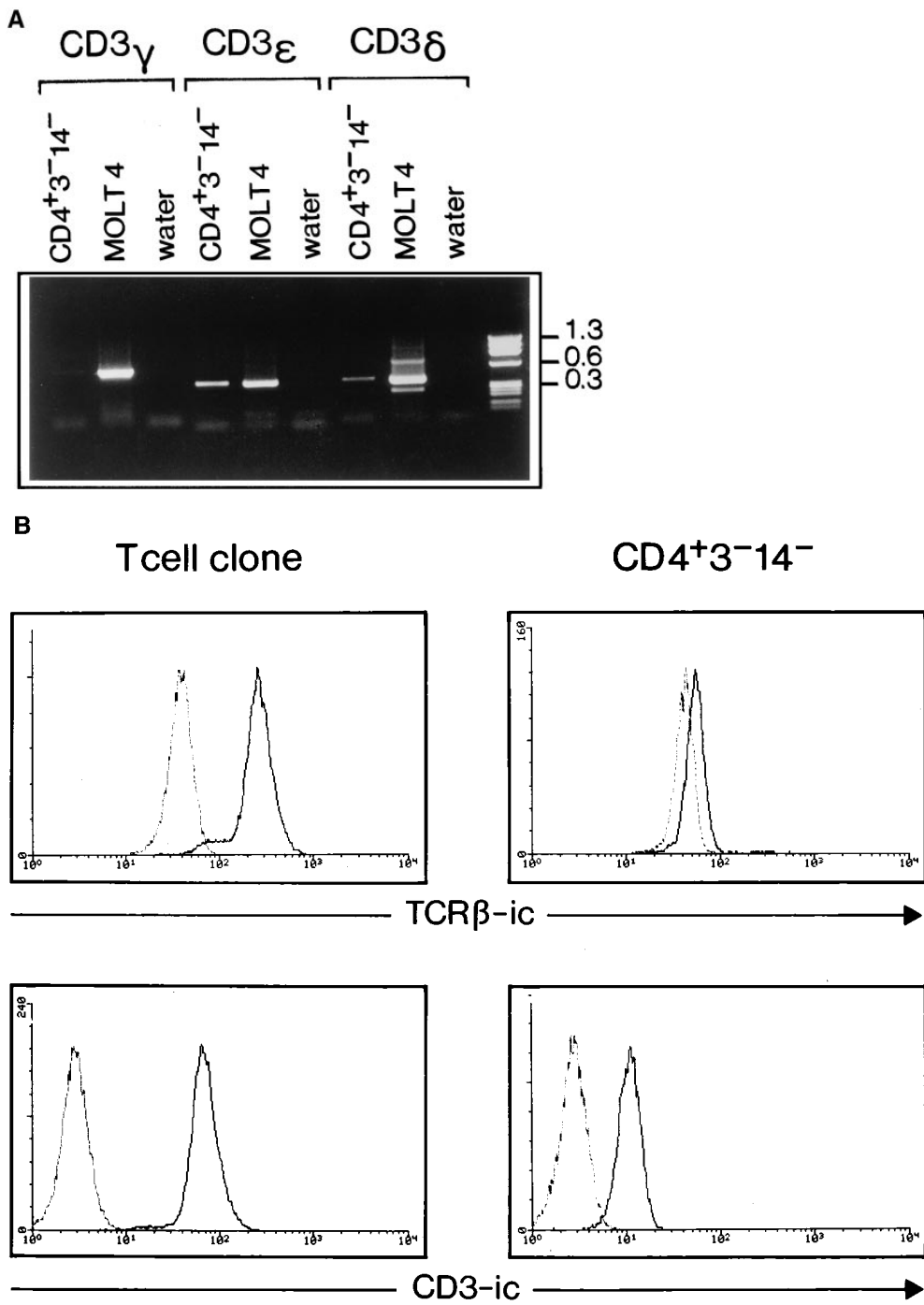


Figure 5. (A) CD3- γ , CD3- ϵ , and CD3- δ transcripts are expressed in pT α ⁺ cells. RT-PCR analysis with primers specific for three components (γ , ϵ , and δ) of the CD3 signaling molecule was performed on CD4⁺CD3⁻CD14⁻ cell population. The T cell line MOLT4 and water were used as positive and negative controls, respectively. (B) CD3- ϵ molecule, but not TCR β chain, is present in the cytoplasm of pT α ⁺ cells. Cytoplasmic staining was performed with a TCR- β -specific (top right and left) or a CD3- ϵ -specific (bottom right and left) mAbs on a T cell clone (right top and bottom) and on CD4⁺CD3⁻CD14⁻ sorted PBMC (left top and bottom). In the case of TCR- β cytoplasmic staining, mouse anti-human IgG1 was subsequently used. Dotted histogram lines represent negative controls that were the following: for β -cytoplasmic, mouse anti-human IgG1 alone and for CD3-cytoplasmic, CD20-FITC.

PCR product (2 μ l). For both PCRs, the same pT α -specific primers were used.

When we initially restricted our analysis to duplicates of each sorted cell aliquots (50, 20, 10, 5, cells; Fig. 6, top left), a positive reaction could be detected in all the wells, down to the ones that contained 5 cells/well. To try and get a more accurate and reliable estimate of the frequency, ten separate PCR reactions were run, each on a well containing 5 cells (Fig. 6, top right). The analysis of the results showed a positive signal in 50% of the samples. Assuming

that our PCR was 100% efficient on these 5-cell samples, a frequency of pT α ⁺ cells of 1/2 can be calculated using Poisson's equation.

Next, we asked whether differences existed in the frequency of pT α ⁺ cells in the two subsets that could be identified by differential expression of CD2. Therefore, we separated our starting population into CD2⁺ and CD2⁻ (see Fig. 4) subsets and performed the same PCR-based analysis. This showed an enriched pT α ⁺ cell frequency in the CD2⁻ subset where all samples containing 5 cells/well

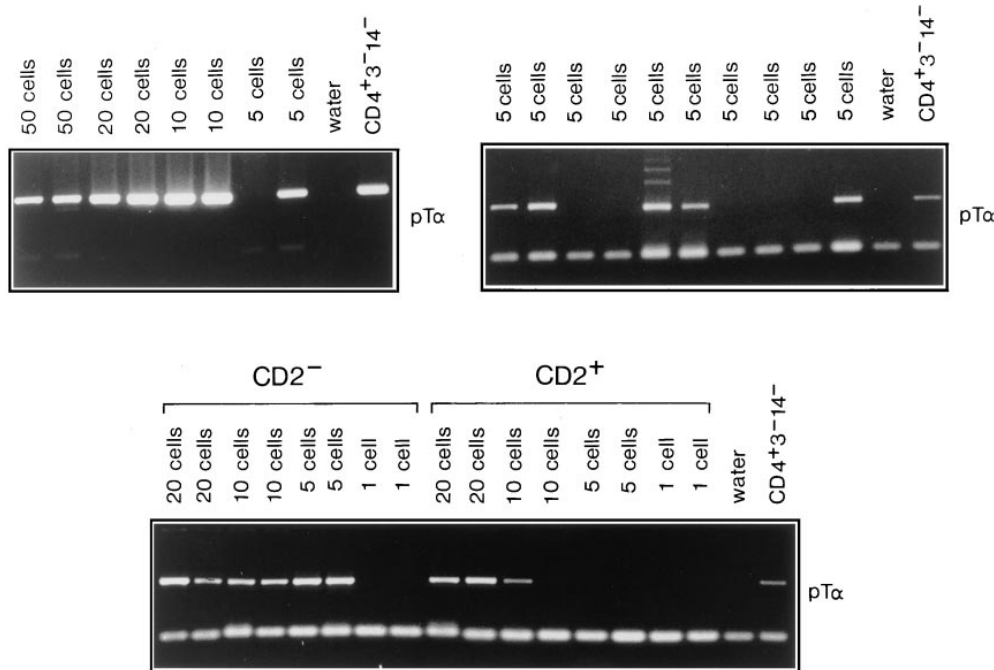


Figure 6. pT α expression in the CD2⁻ and CD2⁺ of CD4⁺CD3⁻CD14⁻ cells. Limited amounts (50, 20, 10, 5) of CD4⁺CD3⁻CD14⁻ cells were directly sorted in 96-well plates and analyzed for the expression of pT α gene by two rounds of PCR using the same primers (*top right and left*). The analysis was first performed on different cell numbers in duplicate (*top left*) and then on the same cell number 10 times (*top right*). In the bottom panel, the same analysis on different cell numbers (20, 10, 5, 1) in duplicate of CD2⁻ and CD2⁺ fractions of CD4⁺CD3⁻CD14⁻ cells is shown. CD4⁺CD3⁻CD14⁻ total sorted cell population and water were the positive and negative controls, respectively.

scored positive, whereas only 50% of the samples containing 10 cells/well showed a positive PCR reaction in the CD2⁺ subset (Fig. 6, *bottom*).

T Cell Differentiation Potential. The fact that the CD4⁺CD3⁻CD14⁻ PB cell population was found to express CD3- γ , CD3- δ , and CD3- ϵ , pT α , and *RAG-1* transcripts, and has partially rearranged the TCR- β locus strongly suggested that committed T cell precursors were present. Moreover, the phenotype of these cells resembles that of the CD3⁻CD4⁺CD8⁻ ISP thymocytes. These latter cells differentiate into DP and CD4⁺TCR- $\alpha\beta$ ⁺ cells in mouse FTOC. To analyze the T cell developmental potential, CD4⁺CD3⁻CD14⁻ peripheral blood cells were introduced into FTOC. After 3 wk of incubation only few human cells were recovered from the FTOC with CD4⁺CD3⁻CD14⁻ peripheral blood cells. To determine accurately the phenotype of these cells, we stained with anti-CD45, anti-CD4, and anti-CD3 or anti-CD8. Fig. 7 shows that almost all human cells expressed high levels of CD3 and CD4. The CD4⁺ cells completely lack CD8. This pattern of differentiation is distinct from what we observe with CD3⁻CD4⁺CD8⁻ ISP thymocytes, which give rise to a majority of DP and SP cells (Fig. 7).

As the CD2⁻ and CD2⁺ fractions were differentially enriched in pT α -expressing cells, we asked whether either population could give rise to mature T cells when cultured in the presence of irradiated feeder cells, IL-2, IL-7, and PHA. CD3⁻CD4⁺CD8⁻ thymocytes are completely unable to grow under these conditions (Spits, H., unpublished data). However, when 1×10^5 sorted potential precursor cells were cultured under these conditions, we observed that, while CD2⁺ cells developed into either CD4⁺CD3⁺ resembling mature T cells or CD4⁺CD3⁻ cells (Fig. 8 A), CD2⁻ cells were not able to expand. CD4⁺CD3⁻ cells de-

veloping from the CD2⁺ fraction were found to have CD3 and TCR- β proteins in the cytoplasm and, after restimulation, gradually acquired CD3 surface expression (data not shown). pT α expression was not present in both CD4⁺CD3⁺ and CD4⁺CD3⁻ phenotypes obtained (data not shown).

To characterize further the progeny obtained from CD4⁺CD3⁻CD14⁻ starting population, we cloned it by limiting dilution. Cloning frequencies ranging from 5 to 10% were observed in five different experiments performed; in addition, according to what was previously shown in bulk culture experiments, all clones were CD4⁺, but expressed different levels of CD3 molecule on the cell surface, vary-

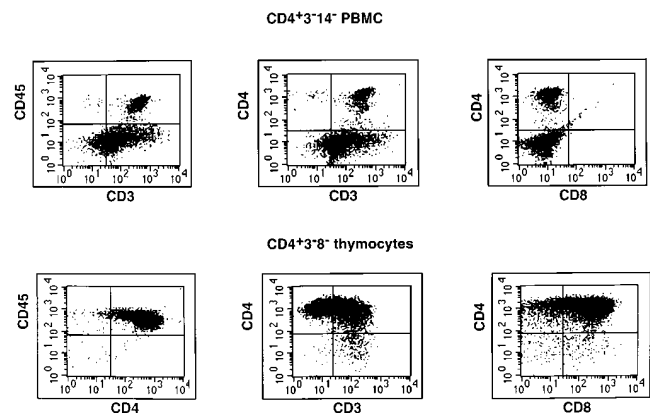


Figure 7. The capacities of CD4⁺CD3⁻CD14⁻ PBMC and CD4⁺CD3⁻CD8⁻ thymocytes of developing into T cells in a FTOC. PBMC and thymocytes were cultured for 3 wk with mouse *RAG-1*^{-/-} thymic lobes as indicated in Materials and Methods and analyzed with PE, FITC, and tricolor-labeled mAbs against CD45 (to discriminate between mouse and human cells) CD3, CD4, and CD8.

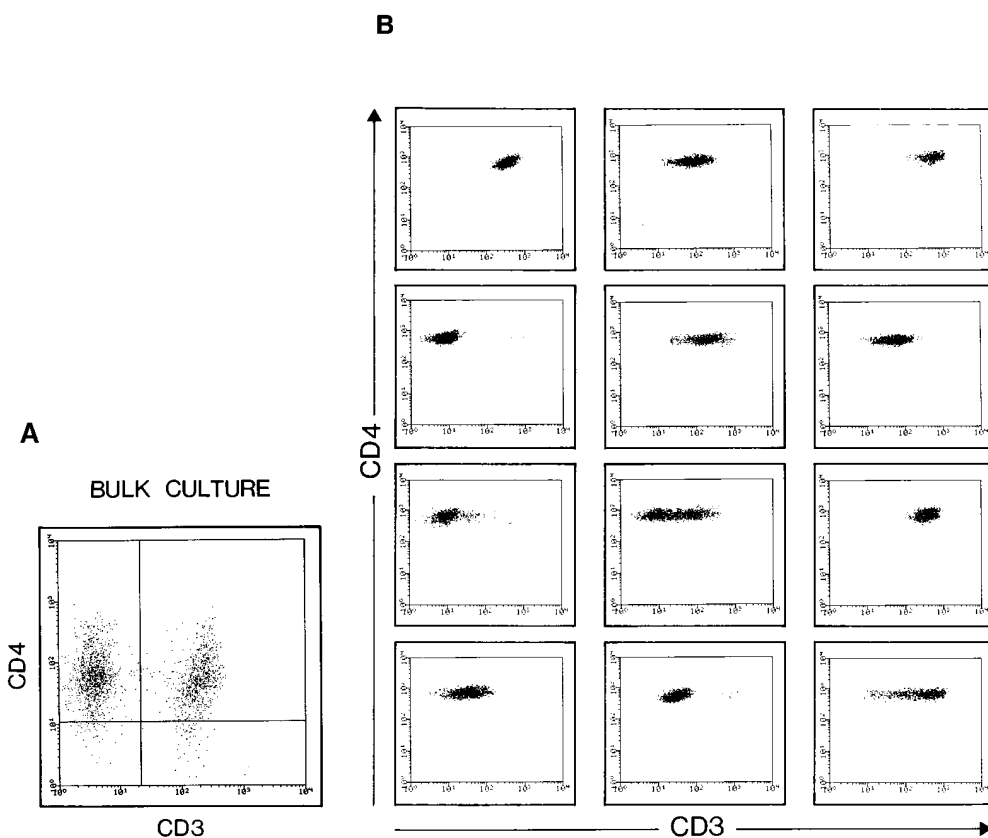


Figure 8. CD3^{low} to CD3^{high} T cells can be generated from the CD2⁺ fraction of CD4⁺CD3⁻CD14⁻ PB cell population. (A) FACS[®] profile of cultured CD2⁺, CD4⁺CD3⁻CD14⁻ cells after 10 d of growth in IL-2, IL-7, PHA in the presence of allogeneic irradiated feeder cells. Double staining with CD4-PE and CD3-FITC is shown. (B) FACS[®] profiles of some representative clones obtained by limiting dilution of CD4⁺CD3⁻CD14⁻ sorted cells. Culture conditions used were the same as described above for bulk cultures. Double staining with CD4-PE and CD3-FITC is shown.

ing from CD3^{high} to CD3⁻. In Fig. 8 B, some representative clones are shown; interestingly, some of them were characterized by a smear in CD3 expression. All the different clones obtained, independently from their level of CD3 expression, became, after repeated stimulations, CD3^{high} and TCR- $\alpha\beta$ ⁺, suggesting that stimulation favored the assembling of a functional TCR on the cell surface.

Discussion

In the present work, we describe a novel population of T cell progenitors present in human peripheral blood. This cell population lacks CD3 on the cell membrane, expresses CD4, but not the early hematopoietic progenitor cell marker CD34. The latter molecule has been shown to be expressed on all of the early T cell progenitor populations described until now, both inside (19) and outside the thymus (17, 20–22). Despite the absence of CD34, these cells have characteristics of committed T cell progenitors. Specifically, these cells show partial TCR- β locus rearrangements (DJ but not VDJ) and have transcripts of genes of pT α and *RAG-1*. Moreover, these cells express CD3- δ , CD3- γ , and CD3- ϵ genes. The fact that these CD4⁺CD3⁻ cells do not express CD34 and that they were found to be the only fraction in total PBMC positive for pT α , is in contrast with some recently published data (7) in which CD34⁺ cells isolated from both adult and cord blood (CB) were found to be pT α ⁺. We failed to detect pT α message in CD34⁺ cells present in either PB (data not shown) and CB (Blom, B.,

and H. Spits, manuscript in preparation). This discrepancy may be due to differences in the purification of CD34⁺ cells; moreover, CD34⁺ cells were obtained with G-CSF treatment (7); it is possible that in this way pT α ⁺ cells are mobilized from bone marrow, where we can detect pT α RNA in CD34⁺ cells (data not shown).

Cells within this population can develop into CD4⁺TCR- $\alpha\beta$ ⁺ cells in both FTOC as well as with IL-2, IL-7, and PHA. This latter development is not due to selective outgrowth of mature T cells, because the cloning efficiency (5–10%) is much higher than the theoretical contamination of cell surface CD3⁺ cells (0.1–0.5%). In addition, the possibility that CD4⁺CD3⁻CD14⁻ cells are derived from activated mature CD4⁺ T cells that have downregulated the TCR seems to be highly unlikely because, in contrast with mature T cells, these cells do not express the TCR- β protein in the cytoplasm. Moreover, also the relatively low levels of CD3- ϵ protein in the cytoplasm, size and cell complexity (FSC versus SSC) distinguish the CD4⁺CD3⁻CD14⁻ cells from mature T cells.

The development of CD4⁺CD3⁻CD14⁻ peripheral blood cells into CD4⁺TCR- $\alpha\beta$ ⁺ cells in both a human/mouse hybrid FTOC as well as with IL-2, IL-7, and PHA clearly demonstrates the presence of T cell precursors in adult PBMC and could suggest that they are prothymocytes on their way to the thymus. Cells, similar to the CD4⁺ peripheral blood cells, with partial DJ β rearrangements and pT α gene expression have been identified in day 15.5 murine fetal blood and were shown to be able to home in the

Table 1. Phenotypic and Functional Comparison between CD3⁻CD4⁺CD8⁻ Immature Single-positive Cells (CD4⁺CD3⁻, ISP) Present in Thymus and CD4⁺CD3⁻ Cells Identified in Peripheral Blood

	Thymus CD4 ⁺ CD3 ⁻ , ISP	Peripheral blood CD4 ⁺ CD3 ⁻
pTα	+	+
cCD3	+	+
cTCR-β	-	-
CD1a	+	-
CD2	100%	15-40%
CD7	100%	2-5%
CD5	100%	2-5%
CD10	nt	-
CD45Rα	±	+
CD33	-	±
B7-2	nt	+
HLA-DR	+	+
Cell cycle	+++	-
TCR-β locus		
DJβ rearrangement	+	+
FTOC	DP, SP	SP
PHA, IL-2, IL-7	-	+

nt, not tested. + + +, a lot.

thymus and to give rise to DP and SP thymocytes (23). A fraction (~65%) of these fetal blood murine cells also expressed CD4 (Rodewald, H.R., personal communication). It is possible that the CD4⁺CD3⁻CD14⁻ PBMC are the direct precursors of the CD3⁻CD4⁺CD8⁻ ISP cells in the thymus. These latter cells are actively dividing, express CD1, CD5, and CD7, and develop into DP and SP cells in an FTOC and in human thymic fragments transplanted SCID mice. However, although CD4⁺CD3⁻CD14⁻ cells and CD3⁻CD4⁺CD8⁻ ISP thymocytes share expression of CD4 and of pTα, the two populations are distinct both with respect to phenotypic and differentiation characteristics (Table 1). The most conspicuous phenotypic difference concerns the expression of CD1a, which is present on all ISP thymocytes but not on the CD4⁺CD3⁻CD14⁻ PBMC and CD45RA, which has an opposite expression pattern.

More importantly, CD4⁺CD3⁻CD14⁻ PBMC are not able to develop in the hybrid human/mouse FTOC in double positive (DP) cells, but only into single-positive (SP) CD4⁺CD3⁺ cells, whereas DP and few CD3⁺ SP are obtained from ISP thymocytes in the same experimental conditions. Moreover, it is impossible to expand ISP thymocytes with IL-2, IL-7, PHA, and feeder cells as observed for CD4⁺CD3⁻CD14⁻ PBMC both in bulk culture and in limiting dilution (results not shown). If these CD3⁻CD4⁺ PBMC are prothymocytes, one should assume that cells of different developmental stages can enter the thymus, because the thymus also contains CD34⁺CD1⁻ progenitor cells with the TCR in germline configuration and the capacity to develop into T cells, DC, and NK cells (18). One should also expect to find a population that is intermediate between CD3⁻CD4⁺ PBMC and ISP thymocytes. Such a population has not yet been described in the human thymus. Based on the fact that the thymus in adults is severely involuted, it may be reasoned that there is not an influx of new progenitors into the thymus in adults. This notion, together with the observations pointing to substantial differences with ISP thymic precursors, in particular with respect to the capacity to develop into DP cells in a FTOC, could raise the alternative possibility that CD4⁺CD3⁻CD14⁻ cells represent an extrathymic pathway of T cell development. Studies with nude mice have shown that mature T cells can develop in aged mice in the absence of a thymus (24), indicating that this organ is not required for development of some T cells. CD3⁻Thy-1^{low} cells have been found in the bone marrow of nude mice that have partial DJ rearrangements of the TCR-β locus (25). A transcript containing TCR-β constant region sequences but not variable region sequences was amplified, suggesting that an unrearranged TCR-β gene locus is transcriptionally active in this bone marrow population. These characteristics of Thy-1^{low}CD3⁻ cells in nude bone marrow cells are very similar to those of the CD3⁻CD4⁺ PBMC population described here. T cells that develop in nude mice have a different TCR-Vβ repertoire than euthymic controls (26) and some TCR-Vβ can be expressed on extrathymically developed cells that are deleted in euthymic controls (27). The TCR-Vβ repertoire of the CD3⁺ cells that develop from the CD4⁺CD3⁻CD14⁻ has not yet been investigated in detail. However, we can exclude that these circulating PBMC are the precursors of those T cells that express a monomorphic TCR (Vα24, Vβ11) (28, 29), because none of the CD4⁺CD3⁺ cells developing from CD4⁺CD3⁻CD14⁻ PBMC cells were found to be Vα24⁺Vβ11⁺ (data not shown).

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