2005

Human peripheral CD2-/lo T cells: an extrathymic population of early differentiated, developing T cells

Matthew Loza

Patrizia Luppi

Kerstin Kiefer

Eric Martin

Jennifer L. Szczytkowski-Thomson
Messiah University, jthomson@messiah.edu

See next page for additional authors

Human peripheral CD2-/lo T cells: an extrathymic population of early differentiated, developing T cells.

Authors:

Loza MJ

Luppi P

Kiefer K

Martin ES

Szczytkowski JL

Perussia B

Source:

International immunology [Int Immunol] 2005 Sep; Vol. 17 (9), pp. 1213-25.

We previously reported that a subset of human peripheral blood CD3+ T cells expresses low-to-null CD2 levels (CD2-/lo), produces type 2 cytokines and is inducible to differentiate to functionally mature IFN-gamma+ cells. Multiple-color immunofluorescence analysis indicated that this population, representing <0.1% of the T cells in fresh lymphocytes, contains subsets that are phenotypically immature, including CD4-CD8- and CD3+TCR- cells. Ex vivo, the CD2-/lo cells can proliferate (carboxyfluorescein diacetate succinimidyl ester analysis) independently from exogenous stimulation, respond to CD3-mediated stimulation with significantly greater proliferation than the autologous mature cells and their subsets are inducible to undergo in vitro a developmental sequence similar to that reported for the phenotypically similar thymic populations. This is especially evident for the CD4+CD8+ subset. CD2-/lo T-cell populations exhibit a TCR repertoire (Vbeta chain distribution) that is complete but different (complementarity determining region R3 analysis) from that of the autologous CD2+ T cells. These characteristics distinguish peripheral CD2-/lo T cells as possible early differentiated T cells that may undergo extrathymic maturation, and potentially contribute to maintain the peripheral naive T-cell pool. These findings define the existence of phenotypically immature T

cells in the periphery. Also, given the high numbers of CD2-/lo T cells generated, upon ex vivo culture, from peripheral lymphocytes of all adult and neonatal individuals tested, they have relevance to clinical applications for immune reconstitution of T cells, as well as myeloid cells, via myeloid colony-stimulating factors and type 2 cytokines.

Introduction

Several lines of evidence support the possible existence of self-peptide-reactive T cells in the periphery that may variably participate in autoimmune reactions (1), including responses to self-antigens on tumor cells (2), or in homeostatic proliferation, especially in lymphopenic conditions (3, 4). Whether these cells are mature, 'tolerized' cells, or also include T cells that possibly escaped the thymus, or bypassed it, at an early differentiation stage is a matter of debate.

Phenotypically or functionally immature cells of most hematopoietic lineages, including CD34+ immature progenitor cells, are present in small proportions in human peripheral blood. Among lymphocytes, most B cells exiting the bone marrow undergo terminal differentiation of their B cell receptor in the periphery (5). We also reported that functionally immature NK cells, expressing only CD161, are present in small proportions in the peripheral blood of all individuals tested (6, 7). These cells have high proliferative potential and are solely capable of type 2 cytokine production, but are inducible to undergo terminal phenotypic and functional differentiation (8). Unlike mature NK cells producing also or exclusively type 1 cytokines, they do not express other known markers of mature NK cells, including CD2, a differentiation antigen shared with the majority of mature peripheral T cells.

CD2 is absent on CD3− thymocytes at their earliest developmental stages, but becomes expressed early in T-cell development, approximately simultaneously with CD3, the signaling moiety of the TCR. We have reported that low proportions of CD3+ T cells in human peripheral blood express low-to-null CD2 levels (CD2−/lo). Like the immature peripheral NK cells, they produce type 2 cytokines but no IFN-γ, and can be induced to undergo functional maturation to IFN-γ-producing cells ex vivo (9). Like immature cells of most lineages, and the peripheral CD2−/lo NK cells, these T cells, which are mostly CD3+ and thus distinct from T-cell precursors, have high proliferative potential (8, 10). Based on these considerations, and the importance of identifying relatively immature peripheral T cells for their potential use for immune reconstitution in clinical settings, we tested the hypothesis that the peripheral CD3+CD2−/lo cells contain cells that share functional and phenotypic characteristics with immature T lymphocytes.

Methods

CD2−/lo and CD2+ lymphocytes and clones

Lymphocytes were separated from healthy adult and neonatal (umbilical cord) peripheral blood (approved for procurement and use according to institutional guidelines) (11). Rosetting with sheep erythrocytes treated with 2-aminoethylisothiouronium bromide (EAET; Sigma Chemical Co., St Louis, MO, USA) was used to deplete most mature CD2+ T and NK cells (12). Most leukocytes expressing receptors for the Fc portion of IgG (FcγR) (residual mature NK, B and myeloid cells) were depleted from the cells in the

E−AET

cell fraction after adherence to immune complexes [rabbit IgG-sensitized erythrocyte monolayers (EA)] (12). Based on the percentage of cells depleted, the proportion of CD2−/lo CD3+ T cells in the leukocyte population remaining after this procedure represents a >50-fold enrichment compared with that (<0.1%) in the original population, and an ∼500-fold enrichment within the CD3+ T-cell population. These cells were used in the analyses described for freshly isolated E−AET
lymphocytes. Although this population may contain other immature cell types [e.g. immature myeloid and NK cell progenitors (6) and IFN-α-producing cells (13)], CD2−/lo T cells were unambiguously distinguished from them by gating based on CD3 and CD2 staining. CD3+ lymphocytes in the E+AET
cell fraction (>99.9% CD2+ on reanalysis) are referred to as CD2+ T cells. Similar results were obtained with E−AET
lymphocytes regardless of the lymphocyte source (adult or neonatal).

Freshly separated
E−AETEA−
and E+AETEA−
cells were cloned (one cell per well), in the initial presence of PHA-P (0.5 μg ml−1; Sigma Chemical Co.), recombinant IL (rIL)-2 (50 U ml−1), anti-IL-12 mAb and 50-Gy-irradiated PBMCs, in medium containing 5% autologous plasma, and maintained after the first week in medium containing rIL-2 only.

Cultured CD2−/lo T cells

Homogeneous CD2−/lo T-cell populations were obtained from the

E−AETEA−

cell fractions following depletion of residual myeloid, B, and mature NK and CD2+ T cells by indirect anti-Ig rosetting (14) or sorting (flow cytometry) with mAb to CD2 (B67.1 + OKT11), CD56 (B159.5), NKp46 (9E2, provided by M. Colonna) and HLA-DR (B33.1). The extremely low numbers of cells recovered do not allow reanalysis of the purified cells. Even if reanalysis were possible, undetectable CD2 may reflect levels of expression below the limit of sensitivity of the detection system. Thus, the CD3+ cells obtained after either procedure are referred to as CD2−/lo T cells. These cells were cultured in RPMI-1640 medium supplemented with 5% autologous plasma, human rIL-2- (50 U ml−1), rIL-4- (10 ng ml−1), IL-12-neutralizing mAb C8.6 (5 μg ml−1, cells provided by G. Trinchieri), plate-bound CD3 (OKT3, 5 μg ml−1) and soluble CD28 mAb (9.3, 2 μg ml−1). The cells were transferred to a new plate after 3 days, and subcultured as needed with IL-2 only, without CD3-mediated stimuli. Cells from the

E−AETEA−

fractions, rather than the more rigorously purified CD2−/lo T cells, were used in cultures performed in the absence of CD3-mediated stimulation. When indicated, PHA-P, 0.5 μg ml−1, was used in combination with CD28 mAb for CD3-mediated stimulation in secondary cultures of T cell populations sorted after culture (see below). Similar results were obtained with cultured CD2−/lo T cells regardless of the lymphocyte source (adult or neonatal).

Immunofluorescence and cell sorting

The mAbs used for surface phenotyping and cell sorting from cultured cells (immunofluorescence, flow cytometry) were CD2 (B67.1, biotin), CD3 [4.1, PE and PE-Texas Red (PETR)], CD4 (S3.5, PETR), CD5 (B36.1, biotin and FITC), CD8 (B116.1, biotin and FITC), CD45RA (EM56, TriColor) CD45RO (UCHL1, FITC), TCRαβ (BMA031, FITC) and TCRγδ (B1, FITC). mAbs produced in our laboratory were directly labeled in-house, the others were from Caltag Laboratories (Burlingame, CA, USA) or PharMingen (San Diego, CA, USA). Biotin-labeled mAbs were detected with CyChrome– or PE–streptavidin. Thresholds for positivity were set in each population based on isotype-matched Ig as control (ref. 8, 9, 15, data not shown). Samples were analyzed on an XL-MCL automated analytical flow cytometer and sorted on an EPICS Elite flow cytometer (Beckman Coulter, Miami, FL, USA). Listmode data were analyzed with the WinMDI Flow Cytometry Application (J. Trotter, The Scripps Research Institute, La Jolla, CA, USA). When sorting antigen (Ag)−/lo and Ag+ populations, ~10% of the cells with, respectively, the highest and the lowest fluorescence intensities were excluded. Stringency of purification was confirmed on restaining and analysis of cell aliquots immediately after sorting (data not shown). Percentages of positive cells are reported after background subtraction.

For carboxyfluorescein diacetate succinimidyl ester (CFSE) analysis of proliferation by flow cytometry, freshly obtained

E+AET

and E−AET

or cultured CD2−/lo and CD2+ T cells, as indicated, were labeled with CFSE (0.25–0.75 μM, 107 cells ml−1, 8 min, 37°C; Molecular Probes, Eugene, OR, USA). Identical cell numbers were

then cultured in a 3-day culture. This time was specifically chosen based on: (i) the question asked (i.e. do the freshly obtained cells proliferate under minimal stimulation?) and (ii) the need to limit the culture time, since CD2 becomes expressed on cells originally CD2−/lo early during culture (9), thus limiting identification of cells corresponding to the original peripheral CD2−/lo cells. Cells from identical cultures of CFSE-labeled cells with mimosine (300 μM, Sigma Chemical Co.), to inhibit G1–S transition, and of non-labeled cells were used to establish the 0-division and the maximal limit of resolution of division peaks, respectively (8, 10). The numbers of divisions the cells have undergone during culture were defined based on distinct peaks with sequentially halving CFSE fluorescence intensity, starting with that of mimosine-treated cells as the 0-division peak (8). The reported results have been obtained analyzing identical numbers of the cells of interest (i.e. ∼2 × 104 CD3+CD2−/lo or CD3+CD2+ cells, as indicated).

Reverse transcriptase–PCR analysis for pre-TCRα and RAG-2

RNA was extracted from freshly isolated
E−AETEA−
and E+AET,
or the indicated CD2−/lo and CD2+ cultured cell populations from adult and neonatal lymphocytes using TRIzol (GIBCO BRL, Gaithersburg, MD, USA); control total human thymus RNA was from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). mRNA was reverse transcribed using 0.5 μg oligo(dT) and the SuperScript First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA, USA), each according to the manufacturer's instructions. PCRs for pre-TCRα detection were performed in 25-μl reaction volumes using 1 μl cDNA, 10 pmol of each

primer and AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Reaction conditions were a 10-min denaturing step, 95°C, followed by 40 cycles of 1 min, 94°C; 1 min, 65°C, and 2 min, 72°C (pre-TCRα); or 30 cycles of 30 s, 94°C; 30 s, 55°C, and 30 s, 72°C (recombination-activating gene 2 [RAG-2] and β-actin control). PCR products were resolved in 2% agarose gels, stained with ethidium bromide and visualized using the Quantity One gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA). The primers used were pTα forward 5′-GTCCAGCCCTACCCACAGGTGT-3′, pTα reverse 5′-CTGGGGGCAGGTCCTGGCTGTAGAAGCCTCTC-3′ [adapted from (16)]; RAG-2 forward 5′-GAAGCCAGATATGGTCATTCCA-3′, RAG-2 reverse 5′-AGTCAGGATTGCACTGGAGACA-3′; β-actin forward 5′-ACACTGTGCCCATCTACGAGGG-3′, β-actin reverse 5′-ATCATGGAGTTGAAGGTAGTTTCG-3′.

TCR Vβ repertoire

Each of the 26 individual TCR Vβ cDNA families and subfamilies were amplified, from cDNA prepared from freshly isolated and cultured CD2−/lo and CD2+ cells from adult and neonatal lymphocytes as above, using a panel of 5′-Vβ primers (17) together with a 6-carboxyfluorescein (Applied Biosystems) and 3′-Cβ oligonucleotide (18, 19). Internal positive controls for each Vβ family segment were a pair of constant region oligonucleotides 5′-Cα and 3′-Cα, tagged with 4,7,2′,7′-tetrachloro-6-carboxyfluorescein (Applied Biosystems), 0.2 μmol (18, 19). The Vβ and Cβ oligonucleotide primers were used each at a 0.5-μmol final concentration in a 50-μl reaction mixture. PCR conditions [28 cycles, in order to remain in the linear portion of the reaction (20)]

were denaturation, 94°C, 30 s; annealing, 60°C, 30 s; extension, 70°C, 45 s. The PCR products were resolved in 5% polyacrylamide non-denaturing gels (ABI 377 DNA Sequencer, Applied Biosystems). Analysis used GeneScan software (Applied Biosystems) to quantify fluorescent peak heights and areas corresponding to each band. The relative abundance of each Vβ chain transcript was estimated based on the ratio between the Vβ (experimental) and Cα (internal positive control) fluorescent areas for each Vβ gene family. Percent expression of each Vβ gene family was calculated relative to the sum (100%) of the ratios of all 26 Vβ RNA (percent corrected ratios) as described (21). The average values of two independent PCR performed on each cDNA sample for each Vβ gene family were calculated and are reported as mean percent corrected ratios.

Complementarity diversity region 3 length analysis

TCR Vβ cDNAs were amplified as above, with the exception that 40 PCR cycles were performed, to maximize the likelihood of detecting all Vβ present in the populations. After amplification, each PCR fluorescent product was purified using QIAquick PCR purification Kit (Qiagen Inc., Valencia, CA, USA). Each purified sample (2 μl) was mixed with size standard (1μl) in 24 μl formamide buffer, heated (95°C, 5 min) and resolved on an ABI Prism 3100 Genetic Analyzer (PE Biosystem, Wellesley, MA, USA) for 45 min. The Vβ-specific amplification resulted in a number of discrete bands (8–10 for each Vβ family, spaced by three nucleotides), each corresponding to the abundance of T cells expressing TCR with the defined complementarity diversity region (CDR) 3 length. Data analysis used GeneScan 3.7 software (PE Biosystem). The proportion of CDR3 of individual lengths within each TCR Vβ family or

subfamily was calculated as the ratio between the area of each fluorescent peak and the sum of the areas of all peaks.

Cell-mediated cytotoxicity

Cytotoxicity was tested in 4-h 51Cr release assays, using autologous and allogeneic PHA-blasts or EBV-transformed B cell lines as target cells.

Results

Phenotype of freshly isolated peripheral CD2−/lo T cells

CD3+CD2−/lo T cells have been identified in all adult and neonatal blood samples tested (9). They represent <0.1% of the CD3+ T cells but were enriched ~50-fold in freshly isolated peripheral blood lymphocytes (PBLs) partially depleted of

E+AET

cells (9). Although CD3− pre-T cells may also be contained within the CD2−/lo cell population, no attempt was made to define their existence in the periphery, and the CD3+ cells discussed in this report (defined as CD2−/lo or early differentiated T cells) do not correspond to them. However, brief consideration was given to CD3+TCR− cells, presumably CD3+ pre-T cells. Phenotypic analysis indicated that this CD3+ cell population contained mostly CD4−/loCD8− [double negative (DN)] and CD4+hiCD8− [CD4 single positive (SP)] cells, with few CD8+hiCD4− (CD8 SP) and CD4+CD8+ [double positive, (DP)] cells (Fig. 1A). The CD2−/lo DN T cells contained on an average ~50% TCRαβ+ cells, with most remaining cells expressing TCRγδ (Fig. 1B), except for a small proportion of CD3+TCR− cells which could be reproducibly

detected only using the two TCR mAbs in combination (Fig. 1A and C). Instead, the CD2+ (E+AET)

T cells contained CD4 SP and CD8 SP cells at the expected 2 : 1 ratio characteristic of peripheral T cells, and minimal proportions of DN, DP and TCRγδ+ cells.

Phenotype of freshly isolated peripheral CD2−/lo T cells. Surface phenotype was analyzed (immunofluorescence, flow cytometry) in the

E−AET

EA− (E−AET)

and E+AET

cells from freshly isolated lymphocytes. (A) CD4 (PETR), CD8 (FITC), CD5 (FITC) and TCR (αβ and γδ, FITC) expression within gated CD3+ (PE) CD2− or CD2+ (biotin, detected with SA-CyChrome) populations (y-axis). Here, and in all pertinent Figures, isotype-matched Ig were used as control to set threshold of positivity (data not shown). Proportion of cells (mean ± SD, background subtracted) with the phenotype indicated on the x-axis within E−AETCD2− (open square) or E+AETCD2+

(filled square) T-cell populations. DN (CD4−CD8−), DP (CD4+CD8+), CD4 SP (CD4+CD8−), CD8 SP (CD4−CD8+), n = 5 for each; CD5−, n = 4, and TCR−, n = 3; *P < 0.05. (B) TCR and CD2 expression in gated CD3+ or CD3+ DN E−AET

lymphocytes. Representative of two experiments (one neonatal, one adult lymphocyte samples). (C) Expression of the indicated differentiation antigen in total (top) and gated CD3+ (middle and bottom), CD2−/lo (left, E−AET

) or CD2+ (right, E+AET

) lymphocytes (gated populations highlighted in the top panels). Representative of three experiments (two neonatal, one adult lymphocyte samples). (D) Pre-TCRα, mRNA expression (RT–PCR) in E−AET

and E+AET

adult lymphocytes; total thymus as positive control.

Phenotype of freshly isolated peripheral CD2−/lo T cells. Surface phenotype was analyzed (immunofluorescence, flow cytometry) in the \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{-}}\) \end{document} EA−\batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \((\mathrm{E}_{\mathrm{AET}}^{{-}})\) \end{document} and \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document} cells from freshly isolated lymphocytes. (A) CD4 (PETR), CD8 (FITC), CD5 (FITC) and TCR (αβ and γδ, FITC) expression within gated CD3+ (PE) CD2− or CD2+ (biotin, detected with SA-CyChrome) populations (y-axis). Here, and in all pertinent Figures, isotype-matched Ig were used as control to set threshold of positivity (data not shown). Proportion of cells (mean ± SD, background subtracted) with the phenotype indicated on the x-axis within \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts}

\usepackage{amsmath} \pagestyle{empty} \begin{document}
\(\mathrm{E}_{\mathrm{AET}}^{{-}}\mathrm{CD}2^{{-}}\) \end{document} (open square) or \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document}
\(\mathrm{E}_{\mathrm{AET}}^{{+}}\mathrm{CD}2^{{+}}\) \end{document} (filled square) T-cell populations. DN (CD4−CD8−), DP (CD4+CD8+), CD4 SP (CD4+CD8−), CD8 SP (CD4−CD8+), n = 5 for each; CD5−, n = 4, and TCR−, n = 3; *P < 0.05. (B) TCR and CD2 expression in gated CD3+ or CD3+ DN \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document}
\(\mathrm{E}_{\mathrm{AET}}^{{-}}\) \end{document} lymphocytes. Representative of two experiments (one neonatal, one adult lymphocyte samples). (C) Expression of the indicated differentiation antigen in total (top) and gated CD3+ (middle and bottom), CD2−/lo (left, \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document}
\(\mathrm{E}_{\mathrm{AET}}^{{-}}\) \end{document}) or CD2+ (right, \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document}
\(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document}) lymphocytes (gated populations highlighted in the top panels). Representative of three experiments (two neonatal, one adult lymphocyte samples). (D) Pre-TCRα, mRNA expression (RT–PCR) in \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document}

\(\mathrm{E}_{\mathrm{AET}}^{{-}}\) \end{document} and \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document} adult lymphocytes; total thymus as positive control.

The CD3+CD2−/lo lymphocytes population contained a CD5−/lo population, mostly DN (Fig.1A, right, and C), that was rare, as expected, among the CD2+ T cells. They also contained a significant proportion of cells lacking mature TCR (Fig. 1A right). The possibility was tested that in the peripheral CD2−/lo T cells, CD3 may also be found associated with a pre-TCR complex not recognized by the available anti-TCR mAb and composed of TCRβ and pre-TCRα subunits (or the equivalents for γδ T cells). In thymic T cells (22), CD3 is first expressed associated with a pre-TCR complex composed of the pre-TCRα and the antigen-binding TCRβ chains (23), whereas expression of high CD5 levels, and of the CD4 and CD8 co-receptors, occurs only upon successful TCRα chain DNA rearrangement (24). Reverse transcriptase (RT)–PCR analysis indicated expression of pre-TCRα mRNA in CD2−/lo but not CD2+ lymphocytes (Fig. 1D). Further molecular characterization indicated that, unlike pre-Tα and in agreement with, and possibly predicted by, previous reports (25, 26), RAG-2 expression was not distinctive of the CD2−/lo or CD2+ populations (data not shown). Since IFN-α-producing cells, which may express pT-α mRNA (27), may be present as contaminants in the

E−AET

(EA−) fraction, we can not formally exclude the possibility that also these cells, and not only, possibly, pre-T cells, contribute, in part, to the pTα mRNA detected by RT–PCR in this population.

CD45 isoforms expressed in freshly isolated CD2−/lo T cells

The proportions of both CD45RA+CD45RO− and CD45RA+CD45RO+ cells in CD2−/lo T cells from freshly purified
E−AET
CD3+ T-cell fractions were similar in adult and neonatal PBLs (Fig. 2A). E−AET
DN T cells, which are predominantly CD2−/lo, were mostly CD45RA+CD45RO− (Fig. 2B). Instead, the DP and SP populations contained both CD45RA+CD45RO−lo and CD45RA−loCD45RO+ cells (data not shown). As expected, the neonatal E+AET
CD2+ T cells exhibited naive CD45RA+CD45RO− phenotype (Fig. 2A), while the same population in adults included both CD45RA+CD45RO− (naive) and CD45RA−loCD45RO+ (effector–memory or antigen experienced) cells (Fig. 2B), but no CD45RA+CD45RO+ cells. This phenotype, which, in the thymus, is found almost exclusively in medullary thymocytes (28–30), was exclusively detectable in the SP peripheral CD2−/lo population. Cells with similar phenotype (CD45RA+loCD45RO+/lo) have also been reported early after bone marrow reconstitution (31). Altogether, the results establish that freshly isolated peripheral CD2−/lo T cells contain subsets with phenotypes reminiscent of those found in developing thymic T cells. The CD4 SP cells, especially those expressing CD4 at low density, may represent immature cells, or classical CD4 SP mature cells recently developed, in vivo, from the DP cells.

Fig. 2.

CD45 isoforms expressed in freshly isolated CD2−/lo T-cells. (A) Expression of CD45RA and CD45RO (bottom panel) in gated CD3+CD2−/lo (left, \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{-}}\) \end{document}) or CD3+CD2+ (right, \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document}) freshly isolated neonatal lymphocytes (gated populations highlighted in the top panels). (B) CD45RA and CD45RO expression in gated CD3+ DN \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \((\mathrm{E}_{\mathrm{AET}}^{{-}})\) \end{document} or CD3+\batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \((\mathrm{E}_{\mathrm{AET}}^{{+}})\) \end{document} freshly isolated adult lymphocytes. (A and B) are representative, respectively, of four and two experiments, each with both neonatal and adult lymphocyte samples.

Open in new tabDownload slide

CD45 isoforms expressed in freshly isolated CD2−/lo T-cells. (A) Expression of CD45RA and CD45RO (bottom panel) in gated CD3+CD2−/lo (left,

E−AET

) or CD3+CD2+ (right, E+AET

) freshly isolated neonatal lymphocytes (gated populations highlighted in the top panels). (B) CD45RA and CD45RO expression in gated CD3+ DN (E−AET)

or CD3+ (E+AET)

freshly isolated adult lymphocytes. (A and B) are representative, respectively, of four and two experiments, each with both neonatal and adult lymphocyte samples.

CD45 isoforms expressed in freshly isolated CD2−/lo T-cells. (A) Expression of CD45RA and CD45RO (bottom panel) in gated CD3+CD2−/lo (left, \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{-}}\) \end{document}) or CD3+CD2+ (right, \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document}) freshly isolated neonatal lymphocytes (gated populations highlighted in the top panels). (B) CD45RA and CD45RO expression in gated CD3+ DN \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \((\mathrm{E}_{\mathrm{AET}}^{{-}})\) \end{document} or CD3+\batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \((\mathrm{E}_{\mathrm{AET}}^{{+}})\) \end{document} freshly isolated adult lymphocytes.

(A and B) are representative, respectively, of four and two experiments, each with both neonatal and adult lymphocyte samples.

Proliferative potential of freshly isolated CD2−/lo T cells

We tested the possibility that, consistent with the proliferative capability of immature cells, and of the type 2 cytokine+ T cells in peripheral blood (10), freshly obtained CD2−/lo T cells might have a greater proliferative potential than the corresponding mature CD2+ T cells, being possibly capable of proliferation in the absence of overt stimulation. Up to ~5% of the freshly isolated CD2−/lo T cells, but no detectable CD2+ T cells, proliferated in a 3-day culture in the absence of exogenously added cytokines, even when mAbs neutralizing IL-2 and IL-15 were added (Fig. 3). Moreover, the CD2−/lo T cells proliferated significantly in response to IL-2. Unpublished data evaluating proliferation after a 6-day culture in the presence of IL-2 alone indicated that the CD2−/lo T cells derived from the originally CD2−/lo T cell population had undergone an average number of divisions smaller than those undergone by the T cells in the CD2+ T cell cultures. However, the proportion of CD2−/lo T cells that had undergone divisions on days 3 and 6 of culture with IL-2 alone remained constant, whereas that of the CD2+ T cells was lower on day 6 than on day 3. These data indicate that some of the CD2+ T cells that had proliferated died (both in culture with IL-2 and more so in the presence of TCR-mediated stimulation), whereas the CD2−/lo T cells did not appear to have a net loss of proliferating cells (32). Indeed, in cultures with IL-2 alone, their cell numbers increased ~103-fold over a 2-week period. As previously reported (ref. 9, data not shown), these cells maintained phenotypic and functional characteristics identical to those of the cells freshly isolated from peripheral blood (e.g. type 2

cytokine production) with the exception that CD2 became detectable on a proportion of them (ref. 9, data not shown).

Fig. 3.

Proliferative potential of freshly isolated CD2−/lo T cells. Identical numbers of CFSE-labeled freshly isolated \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{-}}\) \end{document} (left) and \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document} (right) PBLs from the same adult donor were cultured for 3 days with the stimuli and mAb indicated at right. The anti-IL-15 mAb (10 μg ml−1) was provided by Immunex. Histograms of relative cell numbers versus CFSE content within gated CD3+CD2−/lo (left) and CD2+ lymphocytes (right) are shown; the proportions of cells undergoing the indicated number of divisions (DIV, x-axis; M in the histograms) are reported. Dotted lines: mimosine-treated (right peak) or non-labeled (left peak) controls. Identical numbers of events (20 000) were analyzed for CD3+CD2−/lo cells within the \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{-}}\) \end{document} population and for CD3+CD2+ cells in the \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document} population. Representative of three experiments (one neonatal, two adult lymphocyte samples).

Open in new tabDownload slide

Proliferative potential of freshly isolated CD2−/lo T cells. Identical numbers of CFSE-labeled freshly isolated
E−AET
(left) and E+AET
(right) PBLs from the same adult donor were cultured for 3 days with the stimuli and mAb indicated at right. The anti-IL-15 mAb (10 μg ml−1) was provided by Immunex. Histograms of relative cell numbers versus CFSE content within gated CD3+CD2−/lo (left) and CD2+ lymphocytes (right) are shown; the proportions of cells undergoing the indicated number of divisions (DIV, x-axis; M in the histograms) are reported. Dotted lines: mimosine-treated (right peak) or non-labeled (left peak) controls. Identical numbers of events (20 000) were analyzed for CD3+CD2−/lo cells within the E−AET
population and for CD3+CD2+ cells in the E+AET
population. Representative of three experiments (one neonatal, two adult lymphocyte samples).

Proliferative potential of freshly isolated CD2−/lo T cells. Identical numbers of CFSE-labeled freshly isolated \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{-}}\) \end{document} (left) and \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document} (right) PBLs from the same adult

donor were cultured for 3 days with the stimuli and mAb indicated at right. The anti-IL-15 mAb (10 μg ml−1) was provided by Immunex. Histograms of relative cell numbers versus CFSE content within gated CD3+CD2−/lo (left) and CD2+ lymphocytes (right) are shown; the proportions of cells undergoing the indicated number of divisions (DIV, x-axis; M in the histograms) are reported. Dotted lines: mimosine-treated (right peak) or non-labeled (left peak) controls. Identical numbers of events (20 000) were analyzed for CD3+CD2−/lo cells within the \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{-}\) \end{document} population and for CD3+CD2+ cells in the \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{+}\) \end{document} population. Representative of three experiments (one neonatal, two adult lymphocyte samples).

Phenotype of cultured CD2−/lo T cells

In culture with IL-2 for up to 1 month after an initial 3-day ‘priming’ in the presence of IL-4, and CD3- and CD28-mediated stimulation, the CD3+ cells in the CD2−/lo population maintained their original, functionally immature (ref. 9, data not shown) phenotype. Under the same conditions during a 2-week culture of 104 freshly purified CD2−/lo T cells, their numbers increased ∼104-fold and, consistent with the absolute number of cells capable of proliferating under these conditions, we could obtain routinely ∼108 cells. Consistent with our previous findings (9), 60 ± 40% of these cells (n = 10) up-regulated CD2 expression during culture.

However, they remained predominantly DN or CD4 SP (Fig. 4A). Like in freshly isolated T cells, CD8 SP cells were found almost exclusively in the CD2+ fraction from these cultures (Fig. 4A). CD2 is expressed on thymocytes before positive and negative selection, thus before the DP stage. Similarly, the DP T cells detected after culture were CD2+. CD3+CD5−/lo (Fig. 4A and B) and CD3+TCR− cells (Fig. 4A and C), predominantly CD2− and DN, were also detected in proportions similar to those in freshly isolated CD2−/lo T cells. These populations contained both αβ and γδ TCR+ cells, with high inter-donor variability (50 ± 40% of TCR+ cells for both, n = 5).

Fig. 4.

Phenotype of cultured CD2−/lo T-cells. (A) Analyses were performed as in Fig.1(A) on gated CD2− (open square) or CD2+ (filled square) CD3+ cells from cultures of homogeneous CD2−/lo T-cell populations with rIL-2, rIL-4, IL-12-neutralizing and CD28+CD3 mAb. CD4 versus CD8, n = 6; CD5−, n = 10; TCR−, n = 5; *P < 0.05. (B and C) Density plots of the indicated antigens within total (B, top); gated CD3+ (B, middle and bottom), CD2− (B, left) or CD2+ (B, right) cells; gated CD3+CD4− (C, left) or CD3+CD4+ cells (C, right) in cultured CD2−/lo lymphocytes. Representative of three experiments each (two neonatal, one adult lymphocyte samples).

Open in new tabDownload slide

Phenotype of cultured CD2−/lo T-cells. (A) Analyses were performed as in Fig.1(A) on gated CD2− (open square) or CD2+ (filled square) CD3+ cells from cultures of homogeneous CD2−/lo T-cell populations with rIL-2, rIL-4, IL-12-neutralizing and CD28+CD3 mAb. CD4 versus CD8, n = 6; CD5−, n = 10; TCR−, n = 5; *P < 0.05. (B and C) Density plots of the indicated antigens

within total (B, top); gated CD3+ (B, middle and bottom), CD2− (B, left) or CD2+ (B, right) cells; gated CD3+CD4− (C, left) or CD3+CD4+ cells (C, right) in cultured CD2−/lo lymphocytes. Representative of three experiments each (two neonatal, one adult lymphocyte samples).

Phenotype of cultured CD2−/lo T-cells. (A) Analyses were performed as in Fig.1(A) on gated CD2− (open square) or CD2+ (filled square) CD3+ cells from cultures of homogeneous CD2−/lo T-cell populations with rIL-2, rIL-4, IL-12-neutralizing and CD28+CD3 mAb. CD4 versus CD8, n = 6; CD5−, n = 10; TCR−, n = 5; *P < 0.05. (B and C) Density plots of the indicated antigens within total (B, top); gated CD3+ (B, middle and bottom), CD2− (B, left) or CD2+ (B, right) cells; gated CD3+CD4− (C, left) or CD3+CD4+ cells (C, right) in cultured CD2−/lo lymphocytes. Representative of three experiments each (two neonatal, one adult lymphocyte samples).

CD3+ T cells from cultures of CD2−/lo cells from several individuals were predominantly CD45RA−loCD45RO+ (data not shown). Like thymocytes with this phenotype, these cells did not survive TCR-mediated stimulation, whereas the few CD45RA+CD45RO+ cells present survived re-stimulation with low doses CD3 mAb or PHA, which resulted in decreased CD45RO expression (data not shown). Altogether, these data support the contention that the phenotype and functions of fresh and cultured peripheral blood CD2−/lo T cells are identical.

Developmental capacity of CD2−/lo peripheral T cell subsets

Given the limited numbers of CD2−/lo T cells obtainable from freshly separated PBL, the ability of peripheral CD2−/lo, TCR−, CD5−/lo and DN and DP (Fig. 5A–D, respectively) T cells to

develop into phenotypically mature T cells was analyzed using subsets purified to homogeneity after sorting from the cultured CD2−/lo cells (Fig. 5). In agreement with previous results (9), CD2 was detectable in a proportion (20–25%) of the originally CD2−/lo T cells regardless of exogenous CD3-mediated stimulation. Consistent with recently induced and submaximal expression, the surface CD2 levels were lower than those on the autologous CD2+ T cells concomitantly sorted and cultured under identical conditions. After culture, most DP T cells became SP (CD4 or CD8), while CD4 SP and CD8 SP T cells maintained their original phenotypes. Identical results were obtained regardless of CD3-mediated stimulation in the initial culture of the CD2−/lo T cells (data not shown). Also, within the CD3+ population, >48% of the TCR− cells became TCR+ regardless of exogenous CD3-mediated stimulation. Only ∼25% of the T cells from these cultures expressed CD2 at low levels, whereas ∼40% of the CD3+TCR+ cells sorted and cultured in parallel expressed it, mostly at high levels. In other experiments, ∼40% of the CD3+ TCR− population remained CD5−/lo after a 3-day culture, whereas only ∼10% of the cells from the TCR+ population exhibited this phenotype (data not shown). A small proportion of the CD5−/lo T cells became CD5+ after culture. After a 3-day culture including CD3-mediated stimulation, a proportion of the DN T cells became CD4 SP, with minor proportions of DP and CD8 SP cells also reproducibly detected (Fig. 5D, bottom). Lower proportions of CD4 SP and DP, but higher proportions of CD8 SP cells, were also detected in the absence of exogenous CD3-mediated stimulation (Fig. 5D, top).

Fig. 5.

Phenotype of developing CD2−/lo T cells. CD2−/lo and CD2+ (A), TCR (αβ and γδ)− and TCR+ (B), CD5−/lo (C) and DN, DP, CD4 SP and CD8 SP populations (D, original populations highlighted in the top panel) were purified to homogeneity (sorting) from cultured CD2−/lo T

cells. After a 3-day culture of identical cell numbers with rIL-2 alone (IL-2, A and B, as indicated; and D, middle panel) or with rIL-2, PHA-P and CD28 mAb (IL-2 + PHA, A and B, as indicated; and in: C; D, bottom panel), surface phenotype was analyzed in gated CD3+ (A; B, bottom panel; C, right panel; D) and total lymphocytes (B, top panel; C, left panel). Representative of three experiments each (two neonatal, one adult lymphocyte samples). The relatively high proportion of CD8 SP T cells, restricted to this blood sample, allows a convincing phenotypic definition of these cells, when present.

Open in new tabDownload slide

Phenotype of developing CD2−/lo T cells. CD2−/lo and CD2+ (A), TCR (αβ and γδ)− and TCR+ (B), CD5−/lo (C) and DN, DP, CD4 SP and CD8 SP populations (D, original populations highlighted in the top panel) were purified to homogeneity (sorting) from cultured CD2−/lo T cells. After a 3-day culture of identical cell numbers with rIL-2 alone (IL-2, A and B, as indicated; and D, middle panel) or with rIL-2, PHA-P and CD28 mAb (IL-2 + PHA, A and B, as indicated; and in: C; D, bottom panel), surface phenotype was analyzed in gated CD3+ (A; B, bottom panel; C, right panel; D) and total lymphocytes (B, top panel; C, left panel). Representative of three experiments each (two neonatal, one adult lymphocyte samples). The relatively high proportion of CD8 SP T cells, restricted to this blood sample, allows a convincing phenotypic definition of these cells, when present.

Phenotype of developing CD2−/lo T cells. CD2−/lo and CD2+ (A), TCR (αβ and γδ)− and TCR+ (B), CD5−/lo (C) and DN, DP, CD4 SP and CD8 SP populations (D, original populations highlighted in the top panel) were purified to homogeneity (sorting) from cultured CD2−/lo T cells. After a 3-day culture of identical cell numbers with rIL-2 alone (IL-2, A and B, as

indicated; and D, middle panel) or with rIL-2, PHA-P and CD28 mAb (IL-2 + PHA, A and B, as indicated; and in: C; D, bottom panel), surface phenotype was analyzed in gated CD3+ (A; B, bottom panel; C, right panel; D) and total lymphocytes (B, top panel; C, left panel). Representative of three experiments each (two neonatal, one adult lymphocyte samples). The relatively high proportion of CD8 SP T cells, restricted to this blood sample, allows a convincing phenotypic definition of these cells, when present.

Proliferative potential of CD2−/lo T cell subsets

The above data indicate that the early differentiated peripheral T cells at the distinct developmental stages can undergo in vitro a developmental progression similar to that of phenotypically similar thymocytes. This is more evident for the DP population. CFSE (Fig. 6) and [3H]thymidine incorporation analyses (data not shown) indicated that the difference in the average number of divisions in culture with CD3-mediated stimulation versus that in cultures without it (IL-2 included in both only to allow cell survival) was greater for the SP than the DP and DN cells. SP cells were also more sensitive than DP and DN cells to CD3-mediated cell death, as indicated by their lower cell number in cultures despite the higher degree of proliferation [see (32) for a thorough explanation]. Cell survival was minimal for any subset in culture with CD3-mediated stimulation in the absence of added cytokines. Culture with IL-15 gave identical results (data not shown). As indicated by increased cell numbers in the absence of increased proliferation compared with the no-cytokine condition, IL-7 allowed survival of the immature T cells in the absence of IL-2, rather than directly inducing their proliferation, without preventing CD3-mediated activation-induced cell death in any subset. The behavior of the

peripheral SP compared with DN and DP cells appears similar to that of the phenotypically homologous lymphocyte subsets in the thymus (i.e. greater proliferative responsiveness to CD3-mediated stimulation and greater susceptibility to negative selection of the SP thymocytes).

Fig. 6.

Proliferative potential of CD2−/lo T cell subsets. Identical cell numbers of CFSE-labeled cultured CD2−/lo T cells were cultured for 3 additional days without or with PHA-P and CD28 mAb, without (diamond) or with added rIL-2 (square) or rIL-7 (triangle). CFSE analysis was performed on the gated CD3+ T cell subsets indicated at the left (identical cell numbers analyzed). Reported is the proportion of cells (y-axis) that had undergone the respective number of divisions (x-axis); in parentheses are the numbers of cells (×104) in each subset after culture under each condition. The original cell numbers (×104) were 12.7 (DN), 0.45 (DP), 15.7 (CD4 SP) and 40.5 (CD8 SP). Representative of three experiments (two neonatal, one adult lymphocyte samples).

Open in new tabDownload slide

Proliferative potential of CD2−/lo T cell subsets. Identical cell numbers of CFSE-labeled cultured CD2−/lo T cells were cultured for 3 additional days without or with PHA-P and CD28 mAb, without (diamond) or with added rIL-2 (square) or rIL-7 (triangle). CFSE analysis was performed on the gated CD3+ T cell subsets indicated at the left (identical cell numbers analyzed). Reported is the proportion of cells (y-axis) that had undergone the respective number of divisions (x-axis); in parentheses are the numbers of cells (×104) in each subset after culture under each condition. The original cell numbers (×104) were 12.7 (DN), 0.45 (DP), 15.7 (CD4

SP) and 40.5 (CD8 SP). Representative of three experiments (two neonatal, one adult lymphocyte samples).

Proliferative potential of CD2−/lo T cell subsets. Identical cell numbers of CFSE-labeled cultured CD2−/lo T cells were cultured for 3 additional days without or with PHA-P and CD28 mAb, without (diamond) or with added rIL-2 (square) or rIL-7 (triangle). CFSE analysis was performed on the gated CD3+ T cell subsets indicated at the left (identical cell numbers analyzed). Reported is the proportion of cells (y-axis) that had undergone the respective number of divisions (x-axis); in parentheses are the numbers of cells (×104) in each subset after culture under each condition. The original cell numbers (×104) were 12.7 (DN), 0.45 (DP), 15.7 (CD4 SP) and 40.5 (CD8 SP). Representative of three experiments (two neonatal, one adult lymphocyte samples).

TCR Vβ repertoire of CD2−/lo T cells

All TCR Vβ families (analyzed by semi-quantitative RT–PCR) were represented (complete repertoire) in the

E−AET

and the E+AET

populations in freshly isolated adult and neonatal lymphocytes, though in different proportions for ∼25% of Vβ families (Fig. 7A). Differences in frequency of Vβ families could potentially be muted by contaminating CD2+ T cells, which are ∼25–75% of the E−AET

population. These differences in a minority of the Vβ families were maintained in the cells from cultures of each T cell population purified to homogeneity (CD2−/lo or CD2+) from the same

donor and cultured under identical conditions that allow proliferation without phenotypic changes (Fig. 7B). Based on the very minor fraction of Vβ11+ cells in this analysis, the CD3+CD2−/lo cell population mostly contained ‘classical’ T cells, rather than being predominantly represented by NKT cells. Because the amount of T cell RNA that can be isolated from fresh, purified CD2−/lo T cells is insufficient for CDR3 length analysis, only cultured CD2−/lo cells were compared with CD2+ T cells for CDR3 length analysis. Freshly isolated and cultured CD2+ T cells had nearly identical distributions of CDR3 lengths in all Vβ families, verifying that expansion stimulated with CD3+CD28 mAb minimally affects the distribution of CDR3 lengths in Vβ families. CDR3 length analysis of TCR Vβ families in CD2+ and CD2−/lo T cells from the same individuals (Fig. 7C) indicated that the differences in TCR Vβ usage identified above corresponded to complete, but qualitatively distinct, repertoires in the two cell populations. In both neonatal (data not shown) and adult cultured CD2−/lo cells (Fig. 7C), the distribution of the relative proportions of TCR with different CDR3 lengths within most Vβ families was distinct from that in the autologous CD2+ T cells, with many non-Gaussian. In the CD2+ T cells, both cultured and freshly separated, the distribution was instead predominantly Gaussian, with a few exceptions in adults.

Fig. 7.

TCR Vβ repertoire and CDR3 length analysis. (A) TCR Vβ RNA transcript expression was analyzed in freshly isolated EAET (white) and autologous \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document} (black) adult (top) and neonate

(bottom) lymphocytes, or (B) adult CD2−/lo T cells (white) and autologous CD2+ T cells (black) after cultures under identical conditions. For each TCR Vβ gene family (x-axis), the average value from two independent PCRs was calculated and expressed as corrected ratio (y-axis). (C) CDR3 length of all the TCR Vβ families and subfamilies was analyzed in cultured CD2−/lo, cultured \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document} and freshly separated \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document} lymphocytes (as indicated) from two individual adults. The proportions (y-axis) of CDR3 of individual lengths (x-axis, relative nucleotide number) are reported for eight randomly chosen TCR Vβ gene families.
Open in new tabDownload slide

TCR Vβ repertoire and CDR3 length analysis. (A) TCR Vβ RNA transcript expression was analyzed in freshly isolated EAET (white) and autologous
E+AET
(black) adult (top) and neonate (bottom) lymphocytes, or (B) adult CD2−/lo T cells (white) and autologous CD2+ T cells (black) after cultures under identical conditions. For each TCR Vβ gene family (x-axis), the average value from two independent PCRs was calculated and expressed as corrected ratio (y-axis). (C) CDR3 length of all the TCR Vβ families and subfamilies was analyzed in cultured CD2−/lo, cultured E+AET
and freshly separated E+AET

lymphocytes (as indicated) from two individual adults. The proportions (y-axis) of CDR3 of individual lengths (x-axis, relative nucleotide number) are reported for eight randomly chosen TCR Vβ gene families.

TCR Vβ repertoire and CDR3 length analysis. (A) TCR Vβ RNA transcript expression was analyzed in freshly isolated EAET (white) and autologous \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document} (black) adult (top) and neonate (bottom) lymphocytes, or (B) adult CD2−/lo T cells (white) and autologous CD2+ T cells (black) after cultures under identical conditions. For each TCR Vβ gene family (x-axis), the average value from two independent PCRs was calculated and expressed as corrected ratio (y-axis). (C) CDR3 length of all the TCR Vβ families and subfamilies was analyzed in cultured CD2−/lo, cultured \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document} and freshly separated \batchmode \documentclass[fleqn,10pt,legalpaper]{article} \usepackage{amssymb} \usepackage{amsfonts} \usepackage{amsmath} \pagestyle{empty} \begin{document} \(\mathrm{E}_{\mathrm{AET}}^{{+}}\) \end{document} lymphocytes (as indicated) from two individual adults. The proportions (y-axis) of CDR3 of individual lengths (x-axis, relative nucleotide number) are reported for eight randomly chosen TCR Vβ gene families.

In spite of the skewed repertoire, overt reactivity of the CD2−/lo T cells to hematopoietic self-antigen was excluded at the population level based on the lack of cytotoxicity of bulk populations of these cells against mitogen-activated or EBV-transformed autologous lymphoblasts even after CD3-mediated priming (data not shown). However, preliminary evidence indicates the presence of clones with anti-self-reactivity in the CD2−/lo but not the CD2+ T-cell population (Table 1). Further analysis is needed to exclude potential autoreactivity at a clonal level to other non-hematopoietic tissue antigens.

Table 1.

Autoreactivity of clonal $CD2^{-/lo}$ versus $CD2^{+}$ T cell populations

$CD3^{+}$ clones generated from the $CD2^{-/lo}$ and the $CD2^{+}$ T cell populations from two individual adult donors were tested for cytotoxicity against autologous and allogeneic EBV-transformed B cells lines (donor 1) or PHA-blasts (donor 2). Reported are the numbers of cytotoxic clones over the total number of clones tested. By microscopic examination, all clones were similar in size. Specific lysis was <10% for all clones considered negative for cytotoxicity. For all clones considered positive, specific lysis was >30%. CD3-redirected cytotoxicity by the total $CD2^{-/lo}$ and $CD2^{+}$ T cell populations (both after culture under conditions maintaining their original composition and functions) was similar (~40% cytotoxicity at the 25:1 effector:target ratio tested).

Donor	Population	Target cells	
		Autologous	**Allogeneic**
1	$CD2^{-/lo}$	9/72	3/72
	$CD2^{+}$	0/16	0/16
2	$CD2^{-/lo}$	4/101	3/101
	$CD2^{+}$	0/101	6/101

Discussion

We show that human peripheral CD2−/lo T cells, representing <0.1% of the CD3+ T cells in PBLs from all adult and neonatal individuals tested, contain subsets phenotypically and functionally similar to those found in developing early differentiated thymocytes, including CD3+TCR−, presumably pre-TCRα+, cells. However, formal evidence for pre-Tα expression by the CD3+TCR− cells is required to exclude the scenario that all of the pre-Tα mRNA detected may have derived from minor contaminant IFN-α-producing cells. The CD2−/lo T cells can proliferate ex vivo independently from exogenous stimulation, and respond to CD3-mediated stimulation with significantly greater proliferation than the autologous mature CD2+ cells. This is, to our knowledge, the first report identifying extrathymic T-cell populations that,

phenotypically reminiscent of early lymphocytes developing in the thymus, can be induced to differentiate to mature T cells (CD4 or CD8 SP cells) in vitro in the absence of a thymus-equivalent environment. Other leukocytes with defined biological role are present in peripheral blood in undetectable proportions [e.g. CD34+ cells, undetectable, <0.1% (33); IFN-α-producing cells, ~0.01% (34)]. The fact that the CD2−/lo T cells also represent <0.1% of the peripheral T cells can not be taken to exclude a possible in vivo role for these cells in physiologic or pathologic conditions. In this respect, it is noteworthy that, together with CD34+ progenitors (35), T cells expressing very early developmental markers (e.g. TdT) are characteristically detectable in atopic dermatitis skin infiltrates (36). Also, the present data confirm and extend our previous reports indicating that at least a proportion of the T cells capable solely of type 2 cytokine production are early differentiated highly proliferative T lymphocytes (9, 10), and support a possible manipulation of this human peripheral T-cell population for therapeutic interventions.

The phenotypic and developmental similarities between peripheral CD2−/lo T cells and thymocytes make them possible progenitors to T cells undergoing extrathymic maturation, and potential contributors to maintain the peripheral naive T cell pool. It is possible that their contribution predominates later in life, when the thymic output is mostly lost. Earlier in life, the thymus may serve as a central site to efficiently organize the rapid generation of new T cells, providing a self-contained, macrophage-rich environment to accommodate the massive cell death occurring during thymic development. The origin (thymus dependent or independent) of the peripheral CD2−/lo T cells, and their relationship to other ‘extrathymic’ subsets [e.g. the gut-associated TCRγδ+ (37) and CD8αα+ T cells (38), or the T cells with immature phenotype in

atopic skin infiltrates (36)] need to be established. The possibility that at least part of the CD2−/lo T cells may migrate to peripheral lymph nodes is suggested by preliminary evidence indicating that a variable fraction of these cells are CD62L+ (our unpublished results).

The TCR repertoires of the early differentiated and the mature TCRαβ+ peripheral T cells are different. Although the limited numbers of freshly separated CD2−/lo T cells prevented repertoire (CDR3) analysis immediately after separation, it seems probable that major changes did not occur under the culture conditions used, because the repertoire of the CD2+ T cells was very similar to that of the freshly separated cells cultured under identical conditions. The distribution of CDR3 with different lengths in the neonatal CD2−/lo T cells, mostly naive and foreign antigen inexperienced, was skewed compared with that in the CD2+ T cells. In the latter it was for most part Gaussian, as expected of a mature, naive T cell population. This may reflect differences in 'education' of the two T-cell pools, depending on positive and negative selection. Most mature neonatal blood CD2+ T cells have likely undergone thymic education. Instead, the source(s) of the CD2−/lo T cells remains unknown, leaving it unclear whether they have undergone selection (positive or negative) in the thymus, or in alternative location(s) (e.g. bone marrow or blood itself). However, their presence in relatively higher proportions among the T cells in granulocyte colony-stimulating factor-mobilized peripheral blood (M.J.L. and B.P., unpublished results) suggests that at least a proportion of them may derive directly from the bone marrow. 'Education' of the peripheral early differentiated cells, if it occurs at some point, may differ from that occurring in the thymus. For example, the former may have undergone some degree of positive but not negative selection, or the efficiency of selection and self-antigen

expression may differ between the thymic and the possible extrathymic environments responsible for education.

Our observations have implications to future avenues of research, like definition of whether the observed differences in the TCR Vβ repertoire correspond, in part, to expression of 'unique' TCR specificities, and the role that differences in selection may play to shape the repertoire of the early differentiated versus the mature peripheral T cells. Potential autoreactivity of the early differentiated cells to specific tissue antigen, for which preliminary evidence has been presented, may be expected if negative selection is less efficient in the periphery than in the thymus, and may provide insight into the origin of T cell contributions to autoimmune disorders. Study of the same cells may help define how positive and negative selections occur outside the human thymic microenvironment. It may also help understanding the origin of acute T-cell leukemias with phenotype similar to that of developing thymocytes, or of the peripheral CD4+CD8+ DP T cells described in rare pathological conditions [reviewed in (39)].

The peripheral blood CD2−/lo T cells are low in numbers, but can rapidly undergo extensive proliferation ex vivo in response to CD3-mediated stimulation, routinely resulting in large numbers of these cells after ex vivo cultures started from ~100 ml of blood. Like the freshly separated cells, those derived from their cultures produce predominantly type 2 cytokines (ref. 9, data not shown), and can be readily induced to mature, losing the ability to produce these cytokines as they gain that to produce IFN-γ and finally IL-10 (9). Both proliferation and functional maturation can occur, in the absence of exogenous CD3-mediated stimulation, in response to IL-2 (or IL-15) or monokines (IL-12+IL-15+IL-18+IFN-α), respectively (9). Thus,

the CD2−/lo T cells appear to constitute a useful cell population for novel immune therapeutic approaches independently from their likely but yet-to-be-defined physiological role. Their collection and culture before myeloablation for stem cell or bone marrow engraftment for treatment of tumors or genetic diseases would allow returning them (genetically manipulated or not) to patients, providing them with an autologous T-cell population that can readily expand and mature, thus possibly shortening the time for immune reconstitution. Given their predominant type 2 cytokine profile (9), they may facilitate the development of other immune cell components or increase efficiency of hematopoiesis. Accordingly, IL-4, IL-5 and IL-13, granulocyte monocyte colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α, all produced by these cells, contribute to the generation, mobilization and survival of various hematopoietic cell types, including granulocytes and monocytes, and thus aid reconstituting hematopoiesis and host defenses. Early differentiated T cells may also contribute to protect against pathogens directly, via cells expressing TCR either 'unique' to this population or over-represented compared with the mature cells. Depending on differences in the selection process, they may contain cells with anti-tumor reactivity absent in the mature population. This is a likely possibility when tumor-associated antigens corresponding to differentially expressed self-antigens probably have induced deletion of the tumor-reactive mature T cells.

T cells with 'unique' specificities may also be harmful. T cells potentially reacting to self-antigens may accumulate and, once adoptively transferred to autologous donors after ex vivo culture, their increased numbers might contribute to autoimmunity, especially if they do not receive further peripheral 'education' leading to their deletion. Likewise, dysregulation of the peripheral early differentiated cells may contribute to the onset of autoimmunity. This would not

be the expected case in healthy individuals because, given the extremely low numbers of peripheral CD2−/lo T cells, the potentially self-reactive cells may be present at too low frequency to be effective, or may be deleted by yet-to-be characterized peripheral negative selection processes before being able to elicit clinically apparent anti-self-responses. However, if self-reactive T cells are detected among the CD2−/lo T-cell population, these can be manipulated to delete them or replaced, likely with similar therapeutic efficacy, with immature CD161+CD56− NK cells, which also produce IL-5, IL-13, GM-CSF and TNF-α (8). Thus, in the same endeavor, ex vivo expanded autologous immature NK cells may also be beneficial, and especially indicated in cases of T cell leukemias, where reconstitution with autologous early differentiated normal T cells is likely impossible, unless they can be distinguished from the leukemic ones.

Supported, in part, by USPHS grants AI055842, CA56036 (B.P.) and T32-CA09683 (M.J.L.).

We thank V. Berghella and the Staff of the Obstetrics and Gynecology Division, Thomas Jefferson Hospital, for providing the umbilical cord blood samples, and the personnel in the KCC Flow Cytometry Facility for assistance.

References

1. Theophilopoulos, A. N., Dummer, W. and Kono, D. H. 2001. T cell homeostasis and systemic autoimmunity. J. Clin. Invest.108:335.

2 Dummer, W., Niethammer, A. G., Baccala, R. et al. 2002. T cell homeostatic proliferation elicits effective antitumor autoimmunity. J. Clin. Invest.110:185.

3 Cho, B. K., Rao, V. P., Ge, Q., Eisen, H. N. and Chen, J. 2000. Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. J. Exp. Med.192:549.

4 Ge, Q., Hu, H., Eisen, H. N. and Chen, J. 2002. Different contribution of thymopoiesis and homeostasis-driven proliferation to the reconstitution of naive and memory T cell compartments. Proc. Natl Acad. Sci. USA99:2989.

5 Jelinek, D. F. 2000. Regulation of B lymphocyte differentiation. Ann. Allergy Asthma Immunol.

84:375.

6 Bennett, I. M., Zatsepina, O., Zamai, L., Azzoni, L., Mikheeva, T. and Perussia, B. 1996 . Definition of a natural killer NKR-P1A+/CD56−/CD16− functionally immature human NK cell subset that differentiates in vitro in the presence of interleukin 12. J. Exp. Med.184:1845.

7 Loza, M. J., Peters, S. P., Zangrilli, J. G. and Perussia, B. 2002. Distinction between IL-13+ and IFN-γ+ NK cells and regulation of their pool size by IL-4. Eur. J. Immunol.32:413.

8 Loza, M. J. and Perussia, B. 2001. Final steps of natural killer cells maturation: a general model for type 1-type 2 differentiation? Nat. Immunol.2:917.

9 Loza, M. J. and Perussia, B. 2002. Peripheral immature CD2−/lo T cell development from type 2 to type 1 cytokine production. J. Immunol.169:3061.

10 Loza, M. J. and Perussia, B. 2003. Accumulation of type 2 cytokine+ T cells depends on differentiation-independent proliferation of pre-existing type 2 T cells. Eur. J. Immunol.33:939.

11 Bennett, I. M. and Perussia, B. 1996. Purification of peripheral blood natural killer cells. In Jones, G. E., ed., Methods in Molecular Medicine: Human Cell Culture Protocols, p. 161. Humana Press Inc., Fotoma, NJ, USA.

12 Perussia, B., Trinchieri, G. and Cerottini, J. C. 1979. Functional studies of Fc receptor-bearing human lymphocytes: effect of treatment with proteolytic enzymes. J. Immunol.123:681.

13 Perussia, B., Fanning, V. and Trinchieri, G. 1985. A leukocyte subset bearing HLA-DR antigens is responsible for in vitro alpha interferon production in response to viruses. Nat. Immun. Cell Growth Regul.4:120.

14 Perussia, B., Ramoni, C., Anegon, I., Cuturi, M. C., Faust, J. and Trinchieri, G. 1987. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells co-cultured with B lymphoblastoid cell lines. Nat. Immun. Cell Growth Regul.6:171.

15 Loza, M., Faust, J. S. and Perussia, B. 2003. Multiple color immunofluorescence for cytokine detection at the single cell level. Mol. Biotechnol.

23:245.

16 Res, P. C., Couwenberg, F., Vyth-Dreese, F. A. and Spits, H. 1999. Expression of pTalpha mRNA in a committed dendritic cell precursor in the human thymus. Blood94:2647.

17 Choi, Y. W., Kotzin, B., Berron, L., Callahan, J., Marrack, P. and Kappler, J. 1989. Interaction of Staphylococcus aureus toxin “superantigens” with human T cells. Proc. Natl Acad. Sci. USA86:8941.

18 Luppi, P. and Trucco, M. 1996. Superantigens in insulin-dependent diabetes mellitus. Springer Semin. Immunopathol.17:333.

19 Luppi, P., Rudert, W. A., Zanone, M. M. et al. 1998. Idiopathic dilated cardiomyopathy: a superantigen-deriven autoimmune disease. Circulation98:777.

20 Weidmann, E., Whiteside, T. L., Giorda, R., Herbermann, R. B. and Trucco, M. 1992. The T-cell receptor V beta gene usage in tumor-infiltrating lymphocytes and blood of patients with hepatocellular carcinoma. Cancer Res.52:5913.

21 Luppi, P., Zanone, M. M., Hyoty, H. et al. 2000. Restricted TCR V beta gene expression and enterovirus infection in type 1 diabetes: a pilot study. Diabetologia43:1484.

22 Weiss, A., Dazin, P. F., Shields, R., Fu, S. M., and Lanier, L. L. 1987. Functional competency of T cell antigen receptors in human thymus. J. Immunol.139:3245.

23 Groettrup, M., Ungewiss, K., Azogui, O. et al. 1993. A novel disulfide-linked heterodimer consists of the T cell receptor beta chain and a 33 kd glycoprotein. Cell75:283.

24 von Boehmer, H. and Fehling, H. J. 1997. Structure and function of the pre T-cell receptor. Annu. Rev. Immunol.15:443.

25 Cooper, C. J., Orr, M. T., McMahan, C. J. and Fink, P. J. 2003. T cell receptor revision does not solely target recent thymic emigrants. J. Immunol.171:226.

26 Ma, A., Pena, J. C., Chang, B. et al. 1995. Bclx regulates the survival of double-positive thymocytes. Proc. Natl Acad. Sci. USA.92:4763.

27 Colonna, M., Trinchieri, G. and Liu, Y. 2004. Plasmacytoid dendritic cells in immunity. Nat. Immunol.5:1219.

28 Janossi, G., Bofill, M., Rowe, D., Muir, J. and Beverly, P. C. 1989. The tissue distribution of T lymphocytes expressing different CD45 polypeptides. Immunology66:517.

29 Serra, H. M., Krowka, J. F., Ledbetetr, L. A. and Pilarski, L. M. 1998. Loss of CD45R (Lp220) represents a post-thymic differentiation event. J. Immunol.140:1435.

30 Deans, J. P., Wilkins, J. A., Coixia, S., Pruski, E. and Pilarski, L. M. 1991. Prolonged expression of high molecular mass CD45RA isoform during the differentiation of human progenitor thymocytes to CD3+ cells in vitro. J. Immunol.147:4060.

31 Heitger, A., Neu, N., Kern, N. et al. 1997. Essential role of the thymus to reconstitute naive (CD45RA+) T-helper cells after human allogeneic bone marrow transplantation. Blood90:850.

32 Loza, M. J. and Perussia, B. 2004. Differential regulation of NK cell proliferation by type I and type II IFN. Int. Immunol.16:23.

33 Thomas, T., Abraham, S. and Lansdorp, P. 2002. Flow Cytometry and Immunoselection of Human Stem Cells. Humana Press, Totowa, NJ, USA.

34 Cella, M., Jarrossay, D., Facchetti, F. et al. 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce high amounts of type I interferon. Nature Med.5:868.

35 Mastrandrea, F., Cadario, G., Nicotra, M. R. and Natali, P. G. 1999. Hematopoietic progenitor cells in atopic dermatitis skin lesions. J. Investig. Allergol. Clin. Immunol.9:386.

36 Mastrandrea, F., Cadario, G., Bedello, P. G., Nicotra, M. R. and Natali, P. G. 1998. Expression of T-lineage early developmental markers by cells establishing atopic derma infiltrates. J. Investig. Allergol. Clin. Immunol.8:359.

37 Lees, R. K., Ferrero, I. and MacDonald, H. R. 2001. Tissue-specific segregation of TCR gamma delta+ NKT cells according to phenotype TCR repertoire and activation status: parallel with TCR alpha beta+ NKT cells. Eur. J. Immunol.31:2901.

38 Guy-Grand, D., Pardigon, N., Darche, S., Lantz, O., Kourilsky, P. and Vassalli, P. 2001. Contribution of double-negative thymic precursors to CD8α−α+ intraepithelial lymphocytes of the gut in mice bearing TCR transgenes. Eur. J. Immunol.31:2593.

39 Parel, Y. and Chizzolini, C. 2004. CD4+ CD8+ double positive (DP) T cells in health and disease. Autoimmun. Rev.3:215.