A NOVEL SUBSET OF CD2⁻, CD3/T CELL RECEPTOR α/β⁺ HUMAN PERIPHERAL BLOOD T CELLS

Phenotypic and Functional Characterization of Interleukin 2-dependent CD2⁻CD3⁺ T Cell Clones

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During fetal ontogeny, T cell progenitors entering the thymus undergo a coordinate pathway of differentiation. Discrete steps of intrathymic maturation can be monitored by the sequential appearance of cell surface glycoproteins, of which CD2 (T11, sheep erythrocyte receptor) is thought to be the first T lineage-specific differentiation antigen (1). It is only after expression of CD2 that differentiating thymocytes proceed to acquire CD4 plus CD8 antigens, and finally segregate into two mutually exclusive subsets of CD2⁺CD3⁺CD4⁺CD8⁻ and CD2⁺CD3⁺CD4⁻CD8⁺ mature thymocytes (1, 2). With the appearance of the CD3 molecular complex, thymocytes first express γ/δ and later α/β TCR heterodimers (3–5). This prevailing view of T cell ontogeny predicts that CD2 is expressed on all CD3⁺ T cells (1). Recently, however, CD2⁻CD3⁺ T cells have been identified both in fetal human spleen and thymus (6), and in a subset of CD3⁺TCR γ/δ⁺ peripheral blood T cells from one individual (7). In addition, CD2⁻ stable variants have been selected from the CD3/TCR⁺ leukemic Jurkat line (8). Together, these recently published data suggest that the expression of a functional CD3/TCR complex is not invariably linked to the simultaneous expression of the CD2 differentiation antigen.

In this article we show that a minor fraction of CD2⁻ cells can be regularly identified among CD3⁺TCR-α/β⁺ peripheral blood T cells isolated from healthy donors. In addition, we have established IL-2-dependent long-term clones with a stable CD2⁻CD3⁺TCR-α/β⁺ phenotype that do not express detectable levels of CD2 mRNA. Functional studies indicated that CD2⁻, CD3/TCR⁺ T cell clones could be induced to proliferate, produce IL-2, and display cytotoxic effector function by mAbs directed against CD3 or TCR. These data identify a previously unrecognized minor subset of CD2⁻ CD3⁺TCRα/β⁺ human peripheral blood T cells and demonstrate that expression of CD2 is not a prerequisite for the surface expression of a functional CD3/TCR-α/β⁻ molecular complex on mature human T cells.

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Materials and Methods

*Enrichment of CD2⁻CD3⁺ T Cells from Peripheral Blood.* Ficoll-Hypaque-separated PBMC were depleted of plastic-adherent cells by incubation for 90 min at 37°C in RPMI 1640/10% FCS. Nonadherent cells were separated into E-rosetting ("T cells") and nonrosetting ("non-T") cells as described (9). E⁻ (i.e., non-T) cells were further depleted of surface Ig⁻ B cells by panning on sheep anti-human Ig-coated petri dishes (10). The resulting cell population is referred to as E⁻sIg.

*Establishment of CD2⁻CD3⁺ T Cell Clones.* E' and E⁻sIg cells were incubated for 20 min on ice with FITC-Leu 5b mAb (anti-CD2; Becton Dickinson & Co., Mountain View, CA) plus phycoerythrin (PE)-conjugated Leu 4 mAb (anti-CD3; Becton Dickinson & Co.). After washing in ice-cold PBS/1% FCS, the cells were analyzed on a EPICS V cell sorter (Coulter Electronics, Hialeah, FL) on the basis of forward angle light scatter and fluorescence intensity. Single cells of CD2⁺CD3⁺ and CD2⁻CD3⁺ phenotype were sorted and delivered into wells of 96-well, round-bottomed microtiter plates (Nunc, Roskilde, Denmark) with the aid of the EPICS V autcloning device. The culture medium was RPMI 1640 (Biochrom KG, Berlin, FRG) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, antibiotics, and 10 mM Hepes buffer (complete medium). The cultures were supplemented with 10⁵ irradiated MNC feeder cells, PHA (1 µg/ml) and 50 U/ml recombinant human IL-2 (11), and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Growing clones were expanded in complete medium supplemented with 50 U/ml rIL-2. Established clones were re-stimulated every 2-3 wk with PHA (0.5 µg/ml) plus irradiated feeder cells (10⁵ PBMC/ml plus 10⁵ EBV-transformed B cell line/ml).

*Phenotypic Characterization of Clones.* 8-12 d after feeding, the clones were stained in a direct or indirect staining procedure with the following panel of mAbs: Leu 4-PE (anti-CD3), Leu 2a-PE (anti-CD8), OKT 4-FITC (anti-CD4; Ortho Pharmaceuticals, Raritan, NJ), BMA 031 (anti-TCR-α/β; reference 12), and TCRδ1 (anti-TCR-γ/δ; T Cell Sciences, Cambridge, MA). In addition, the following set of anti-CD2 mAbs directed against the Tll1, Tll2, or Tll3 epitope of CD2 (13) were used: Leu 5b, OKT 11, MT 1100, 6F10.3, Tll2, MT 910, Tll3 (13-15). For indirect staining, FITC-conjugated F(ab)₂ goat anti-mouse IgG (Tago Inc., Burlingame, CA) was used as a second-step reagent.

*Northern Blot Analysis.* RNA was prepared from E-rosetting T cells or established clones by homogenization in guanidium thiocyanate followed by centrifugation through a cushion of CsCl. Total RNA (20 µg/lane) was electrophoresed on 1% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with nick-translated DNA probes. The filters were processed as described (9) except that washing was carried out in 0.2 x SSC with 0.1% SDS at 37°C. The CD2 cDNA probe (16) was kindly provided by Dr. W.A. Sewell (Imperial Cancer Research Fund, London, U.K.). The TCR-β gene probe was a purified Bgl II/Eco RV fragment from the TCR-β cDNA (17) and was obtained from Dr. T. Mak (Ontario Cancer Research Institute, Toronto, Canada).

*Cell Proliferation and IL-2 Production.* Cloned T cells were cultured at 5 x 10⁴ cells per well in round-bottomed microtiter plates (Nunc) previously coated for 12 h with 10 µg/ml goat anti-mouse Ig (Tago Inc.). Anti-CD3/TCR mAbs or phorbolester TPA (Sigma Chemical Co., Deisenhofen, FRG) plus ionomycin (Calbiochem-Behring Corp., San Diego, CA) were added to the cultures as indicated in Results. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 2 d, 75 µl of culture supernatant was removed from each well for determination of IL-2 content. The remaining cell pellet was pulsed for 6 h with 1 µCi [³²P]dR (specific activity 5 Ci/mmol) per well, and [³²P]dR uptake was measured in a Packard liquid scintillation counter. IL-2 present in cell-free culture supernatants was detected in a bioassay on IL-2-dependent murine CTLL cells. IL-2-induced proliferation of CTLL cells was visualized by a colorimetric method (cleavage of tetrazolium salt MTT) as described (18). The results are expressed as the mean optical density (OD) ± SD of triplicate cultures measured at 570 nm.

*Cytotoxicity Assay.* Cloned T cells were incubated at various E/T ratios with 2,000 ³¹Cr-labeled P815 target cells in V-shaped microtiter plates in the absence or presence of OKT3 (20 ng/ml) or PHA (1 µg/ml). After 4 h at 37°C, 100 µl of supernatant was removed for counting.
in a Packard gamma-counter. Spontaneous $^{51}$Cr-release was determined in wells that had received no responder cells, and maximal $^{51}$Cr-release was measured in wells that had been vigorously resuspended. Specific lysis was calculated as follows: \[ \text{Percent specific lysis} = 100 \times \left( \frac{\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}}}{\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontaneous}}} \right) \].

Results

Presence of CD2+CD3+ T Cells in Peripheral Blood. We used simultaneous two-color cytofluorometry to study the cell surface expression of CD2 and CD3 antigens on subsets of macrophage-depleted PBMC obtained from normal blood donors. We took advantage of the fact that CD2 is the receptor for sheep E (19) and separated MNC into E+ (CD2+) and E− subpopulations, arguing that CD2− T cells should not form E-rosettes. After additional removal of surface slg+ B cells from E− cells by panning on anti-Ig-coated dishes, we analyzed CD2 and CD3 expression on E+ and E slg− cell fractions. As shown in a representative isometric display of two-color flow cytometric analysis in Fig. 1 A, E+ cells contained, as expected, a major population of CD2+CD3+ (mature T cells) and a minor population of CD2+CD3− cells (mostly NK cells; reference 20). In contrast, staining of E−slg− cells with anti-CD2 plus anti-CD3 mAbs clearly revealed a distinct peak of CD2−CD3+ cells (Fig. 1 B). We determined in six independent experiments that CD2−CD3+ accounted for 0.1−0.8% of PBMC.

Characterization of CD2−CD3+ Long-term Clones. The above results indicated that CD2−CD3+ T cells constitute a minor fraction of normal PBMC. To characterize these cells in more detail, we established IL-2-dependent long-term clones of CD2−CD3+ (and conventional CD2+CD3+) T cells with the aid of an EPICS V cell sorter and autoclone device. A detailed phenotypic analysis of four representative clones is shown in Fig. 2. Clones 2 and 3 were derived from one individual, while clones 5 and 10 were each derived from a second and third individual, respectively. In addition to mAbs against CD3, CD4, CD8, and TCR-α/β, we used a panel of anti-CD2 mAbs directed against the three known epitopes of CD2 (13). As seen in Fig. 2, clones 3, 5, and 10 did not express any of the well-characterized CD2 epitopes, despite the fact that they all had a CD3+TCR-α/β+CD4+CD8− phenotype and thus were mature T cells. Control clone 2 was clearly CD2+ and reacted with all anti-CD2 mAbs tested except anti-T11/3. It is known that the T113 epitope is expressed on activated but not on resting T cells (13). Since cloned cells were analyzed 8−10 d after feeding with IL-2-supplemented medium, it is not surprising that T113 was no longer detectable on clone 2. The T113 epitope was, however, readily induced on clone 2 shortly after restimulation with PHA (not shown). In agreement with

![Fig. 1. Two-color cytofluorographic analysis of CD2 and CD3 expression on peripheral blood lymphocytes subsets. E-rosette-purified T cells (A) and E-rosette- plus B cell-depleted (E−slg−) cells (B) were stained with FITC-conjugated Leu 5b (anti-CD2) plus PE-conjugated Leu 4 (anti-CD3) mAbs. Log red fluorescence (CD3) is depicted on the y-axis, log green fluorescence (CD2) on the x-axis, and relative cell numbers on the z-axis.](image-url)
The phenotypic analysis, clones 3, 5, and 10 did not form rosettes with sheep erythrocytes, while clone 2 did (not shown). These results thus clearly demonstrate that long-term clones with a stable CD2−CD3+TCR-α/β+ phenotype can be established from human peripheral blood. The clones described here have been in continuous culture for >6 mo.

Northern Blot Analysis of CD2 mRNA Expression. To investigate whether the CD2− or CD2+ phenotype corresponded to the level of CD2 mRNA, we performed Northern blot analysis of total RNA from 11 different clones and E-rosetted polyclonal T cells (Fig. 3 A). As expected from the phenotypic analysis, CD2+CD3+ clone 2 (lane 2) and several other CD2+CD3+ clones (lanes 4, 8, 9, 12) expressed
FIGURE 3. Northern blot analysis of CD2 and TCR Cβ mRNA expression. Total RNA from E-rosetting T cells (lane 1) or from IL-2-dependent long-term clones (lanes 2-12) was electrophoresed on 1% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with nick-translated DNA probes. The same filter was used for hybridization with the CD2 (A) and Cβ probes (B). Exposure times were 2 d (CD2) and 3 d (TCR Cβ), respectively. The approximate sizes of hybridizing RNA species are indicated. E* cells (lane 1) and clones 2, 3, 4, 12 (corresponding to lanes 2, 3, 4, 12) were derived from donor I, clones 5 and 6 (lanes 5 and 6) from donor II, clones 7-9 (lanes 7-9) from fetal thymus (reference 6), and clones 10 and 11 (lanes 10 and 11) from donor III.

both the 1.7- and 1.3-kb CD2 transcripts (16). In further agreement with the surface marker analysis, CD2-CD3+ clones 3, 5, 6, 7, and 10 (lanes 3, 5, 6, 7, 10) did not express detectable levels of either 1.7- or 1.3-kb CD2 mRNA. Although CD2-CD3+ clones 3, 5, 6, and 10 lacked CD2 transcripts, they did express full-length 1.3-kb TCR Cβ transcripts (Fig. 3 B), thus lending further support to the notion that these clones are mature CD3+ TCR-α/β+ T cells. Also shown in Fig. 3 A is CD2 mRNA expression in freshly isolated (polyclonal) T cells (lane 1) and in two previously characterized clones (reference 6) established from fetal human thymus (lanes 7 and 8). The fetal clone displayed in lane 7 was CD2-CD3+, while the clone in lane 8 was CD2+CD3+. Accordingly, CD2 mRNA was absent in clone 7, while clone 8 expressed both 1.7- and 1.3-kb transcripts. Furthermore, clone 7 lacked TCR Cβ transcripts and clone 8 expressed only a truncated TCR Cβ mRNA (Fig. 3 B), in agreement with the TCR-γ/δ+ phenotype as revealed by reactivity with mAb TCRδ-1 and nonreactivity with mAb BMA 031 (6). Together, these data unequivocally demonstrate that the failure to react with anti-CD2 mAbs in our series of CD2-CD3+ T cell clones was due to the specific absence of CD2 gene expression.

Triggering of CD2-CD3+ T Cell Clones via CD3/TCR. T cell clones were cultured in the presence of anti-CD3 or anti-TCR mAbs in microculture plates previously coated with goat anti-mouse Ig, and IL-2 secretion and [3H]Tdr uptake were determined after 2 d. As shown in Table I, a proliferative response of CD2- clones 3 and 7 was efficiently triggered by OKT3 (anti-CD3) mAb. In addition, both clones responded vigorously to stimulation with anti-TCR mAbs; while clone 3 (TCR-α/β+, see Fig. 2) was activated by anti-TCR-α/β mAb BMA 031 but not by anti-TCR-γ/δ mAb TCRδ-1, the TCR-γ/δ+ (see above) fetal clone 7 was vigorously
activated by mAb TCR6-1. Where tested (e.g., clone 3), CD2− clones were also stimulated by a combination of phorbolester TPA plus ionomycin. The proliferative response of CD2− clones to anti-CD3/TCR stimulation was followed by secretion of significant amounts of IL-2 as visualized by a colorimetric assay (cleavage of MTT) on IL-2-dependent CTLL cells (Table I).

CD2− T cells lack the “alternative” CD2-dependent activation pathway (13). Therefore, we were interested to investigate whether the absence of CD2 expression would possibly alter the threshold of the amount of anti-CD3/TCR mAb required for activation via CD3/TCR. To address this question, CD2−CD3+ and CD2+CD3+ clones were cultured in the presence of anti-CD3 or anti-TCR mAbs titrated over a wide range of concentrations. As illustrated in Fig. 4, proliferation of the CD2− clone 3 could be efficiently stimulated by as little as 0.5 ng/ml OKT3 mAb, while the CD2+ control clone 4 required 5 ng/ml. Comparable results (not shown) were obtained when T cell clones were stimulated with titrated concentrations of anti-TCR mAb BMA 031. From these results we concluded that the absence of CD2 antigen expression did not reduce the sensitivity of T cell triggering via the CD3/TCR molecular complex.

Finally, triggering of cytotoxic effector function was tested as a third functional parameter to analyze the impact of lack of CD2 expression on CD3/TCR-mediated T cell signaling. To this end, T cell clones were incubated at various effector/target ratios with 51Cr-labeled Fc receptor-positive P815 target cells in the absence or presence of OKT3 (20 ng/ml) or PHA (1 μg/ml). As shown in Fig. 5, neither CD2− (clone 5 and 7) nor CD2+ (clone 4) T cells lysed P815 in the absence of OKT3 mAb.
or PHA. On the other hand, cytotoxic effector activity could be triggered by anti-CD3 mAb OKT3 (and by PHA) independent of whether T cell clones did (clone 4) or did not (clone 5 and 7) express CD2.

Discussion

Our results demonstrate that a small subset of CD3/TCR⁺ peripheral blood T cells do not express the CD2 antigen. Although CD2⁻ cells have been previously identified among CD3/TCR-γ/δ⁺ T cells from one particular individual (7), as well as among CD3⁺ T cells in fetal spleen and thymus (6), the present study is the first to identify CD2⁻ cells within nontransformed CD3/TCR-α/β⁺ human T cells. CD2⁻/CD3⁺ T cells were enriched by depletion of macrophages, E-rosetting T cells and B cells, and accounted for 0.1-0.8% of all PBMC. IL-2-dependent long-term clones of CD2⁻/CD3⁺ T cells did not react with a panel of anti-CD2 mAbs directed against the three known epitopes of CD2 and failed to express detectable levels of CD2 mRNA; only one of six cell surface CD2⁻ clones (clone 11, Fig. 3 A) showed a faint band of 1.7-kb CD2 mRNA. With respect to its maturational stage, this clone appears to correspond to previously described CD2⁻ mutants of the CD3⁺ leukemic
Jurkat line (8). As reported by Moingeon et al. (8), cell surface CD2− Jurkat variants expressed strongly reduced yet detectable levels of either 1.7- or 1.3-kb CD2 mRNA. It should be stressed that the CD2− Jurkat mutants described by Moingeon et al. (8) were derived by mutagenesis and immunoselection from CD2+ parental cells. In contrast, our present series of CD2−CD3+ clones was not derived by mutagenesis or any other selection procedure except initial cell sorting. The CD2− clones established here thus represent a minor subset of normal PBMC. Five of six cell surface CD2−CD3+ T cell clones completely lacked detectable CD2 transcripts.

A widely accepted model of T cell ontogeny proposes that CD2 is one of the earliest if not the first T cell–specific cell surface glycoprotein (1). Recent evidence indicates, however, that CD3 mRNA expression precedes CD2 mRNA expression during fetal murine development (21). In addition, a major population of CD4+CD8− “double-negative” sheep peripheral blood T cells is CD2− (22). Taken together with the identification of CD2−CD3+ T cells in fetal human tissue (6) and adult peripheral blood (this article), the hypothesis that CD2 is necessarily expressed on all CD3/TCR+ T cells may not be of general validity.

Human T cells can be activated through two major pathways: one of these involves antigen recognition via T cell receptor, whereas the other is antigen independent. Antigen-specific T cell activation can be mimicked by mAbs directed against the CD3/TCR molecular complex (23), while the antigen-independent pathway is initiated by a combination of mAbs directed at two distinct epitopes of the CD2 antigen (13, 24). From previous studies there is strong evidence, however, for an interdependence of both pathways. Thus, it has been found that activation of T cells via CD2 requires the simultaneous expression of a functional CD3/TCR complex (25–27). In addition, activation of T cells via CD2 can be modulated by anti-CD3 mAbs (28) and, vice versa, TCR-dependent, antigen-specific T cell stimulation is influenced by anti-CD2 mAbs (29, 30). It has been less clear, however, whether activation of T cells via CD3/TCR is absolutely dependent on the simultaneous coexpression of CD2. Recently, a CD2−, CD3/TCRγ/δ+ cell population has been described in one particular individual; these cells showed a normal response to stimulation with anti-CD3 and anti-TCR-γ mAbs (7). Similarly, cell surface CD2− variants of the CD3/TCR+ leukemic Jurkat T cell line were found to respond to anti-CD3 or anti-TCR signaling by Ca2+ influx and IL-2 production (8). These studies have not established, however, whether normal (i.e., nontransformed) T cells with a conventional TCR-α/β molecule can be activated via CD3/TCR in the absence of CD2 expression. Our present results now clearly demonstrate that CD2−, CD3+ T cell clones can be induced to proliferate, secrete IL-2, and display cytotoxic effector function by mAbs directed against CD3 or TCR. Thus, at least with respect to the three functional parameters tested, signaling via CD3/TCR appeared to be fully maintained in CD2− T cell clones. This was true for both α/β and γ/δ TCR-expressing T cells; while anti-CD3 mAb OKT3 triggered both types of clones, mAb BMA 031 activated TCR-α/β+ clones and mAb TCRδ1 activated TCR-γ/δ+ clones, respectively.

In conclusion, our studies have identified and characterized a novel subset of CD2−CD3/TCR-α/β+ human peripheral blood T cells. In addition, we have shown that T cell signaling via TCR-γ/δ or -α/β can proceed in the absence of CD2 antigen
expression. Further studies will be required, however, to address the question how the lack of CD2 expression affects the antigen-specific activation of cloned T cells.

Summary

It is generally believed that CD2 (T11, sheep erythrocyte receptor) is expressed on all human T cells. In the present study we have identified and characterized a minor subset of CD2⁻/CD3⁺/TCRα/β⁺ T cells in the peripheral blood of healthy individuals. CD2⁻/CD3⁺ T cells were enriched in PBMC depleted of plastic-adherent macrophages, E-rosetting (i.e., CD2⁺) T cells and surface Ig⁺ B cells. CD2⁻/CD3⁺ T cells accounted for 0.1-0.8% of PBMC in six individuals. IL-2-dependent long-term clones of CD2⁻/CD3⁺ T cells neither reacted with a panel of anti-CD2 mAbs nor expressed detectable levels of CD2 mRNA by Northern blot analysis. These clones, however, expressed a full-length TCR Cγ mRNA and reacted with mAbs against TCR-α/β, CD3, and CD4, and thus were mature T cells. CD2⁻/CD3⁺/TCR⁺ T cell clones could be triggered into proliferation, IL-2 production, and cytotoxic effector activity by anti-CD3 and anti-TCR mAbs. We conclude that (a) a minor subset of CD2⁻/CD3⁻/TCR⁺ T cells is present in normal peripheral blood; and (b) expression of CD2 at the level of protein and/or mRNA is not required for T cell signaling via the CD3/TCR molecular complex.

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