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Title

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Permalink

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Journal

The Journal of experimental medicine, 164(1)

ISSN

0022-1007

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Publication Date

1986-07-01

DOI

10.1084/jem.164.1.339

Peer reviewed

HUMAN CD3⁺ T LYMPHOCYTES THAT EXPRESS NEITHER CD4 NOR CD8 ANTIGENS

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Differential expression of the CD4 and CD8 antigens on T lymphocytes has been used as a basis to construct models of thymic differentiation, and to identify functionally distinct subsets of T cells (1, 2). In the thymus, most cells coexpress both CD4 and CD8, while minor subpopulations express the phenotypes $CD4^-,8^-$; $CD4^+,8^-$; and $CD4^-,8^+$ (1, 2). It has been proposed that during thymic ontogeny $CD4^-,8^-$ precursor cells differentiate into $CD4^+,8^+$ thymocytes. Upon maturation, $CD4^+,8^+$ thymocytes acquire the CD3/Ti complex and presumably lose either CD4 or CD8, then $CD3^+,4^+,8^-$ and $CD3^+,4^-,8^+$ T lymphocytes migrate into peripheral tissues (1, 2). Mature $CD3^+,4^+,8^-$ T cells augment Ig secretion by B lymphocytes and facilitate the generation of cytotoxic T cells, whereas $CD3^+,4^-,8^+$ T cells predominately mediate cytotoxicity and immune suppression (3, 4). In general, antigenic recognition by $CD4^+$ T cells is restricted by class II MHC gene products, whereas antigenic recognition by $CD8^+$ cells is restricted by class I MHC antigens (5, 6). It has been generally accepted that all peripheral T cells express either CD4 or CD8, with a minor population expressing both antigens. However, we have recently observed that a small subset of human peripheral blood $CD3^+$ T lymphocytes express neither CD4 nor CD8. Herein, we describe the characteristics of this unique subset of T cells.

Materials and Methods

Isolation of Peripheral Blood $CD3^+,4^-,8^-$ T Lymphocytes. Mononuclear cells from normal peripheral blood (Stanford Blood Center, Palo Alto, CA) were isolated using Ficoll/Hypaque. Monocytes were depleted by plastic adherence. Nonadherent cells were stained with a mixture of FITC-conjugated anti-Leu-2 (CD8), FITC-conjugated anti-Leu-3 (CD4), and phycoerythrin (PE)-conjugated anti-Leu-4 (CD3) (7). After extensive washing in ice cold PBS, the cells were resuspended in PBS containing 1% FCS. Lymphocytes expressing CD3, but lacking both CD4 and CD8, would therefore show specific red (PE) fluorescence, but not green (FITC) fluorescence, $CD3^+,4^-,8^-$ cells were isolated to >95% purity using a FACS 440 (Becton Dickinson Immunocytometry Systems, Mountain View, CA) (7). Fluorochrome-conjugated, isotype-matched control antibodies were used to establish the specificity of antibody binding (7).

Antibodies. Anti-Leu-1, -Leu-2, -Leu-3, -Leu-4, -Leu-5, -Leu-6, -Leu-11, and -Leu-12 react with the CD5, CD8, CD4, CD3, CD2, CD1, CD16, and CD19 differentiation antigens, respectively. Anti-Leu-19 reacts with the Leu-19 (NKH-1) antigen, which is present on NK cells and a subset of T lymphocytes (8).

Immunofluorescence and Flow Cytometry. Methods of immunofluorescence, flow cytometry, and data analysis have been presented elsewhere (7, 9).

Cytotoxicity Assays. ⁵¹Cr-labeled tumor cells were used as targets in a 4-h radioisotope release assay (7).

Cell Culture. Cells were cultured in RPMI 1640 supplemented with 10% FCS (KC

A   Freshly Isolated Peripheral Blood Lymphocytes

B   r IL-2 Cultured CD3⁺ 4⁻, 8⁻ Peripheral Blood Lymphocytes
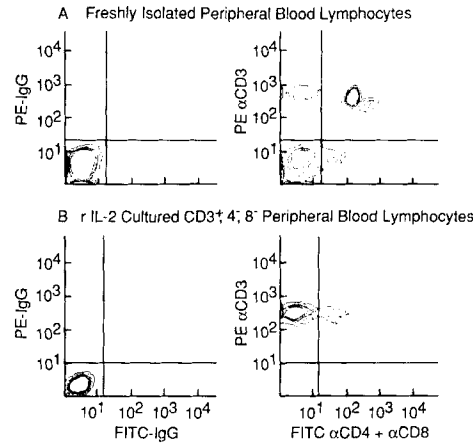
FITC-IgG   FITC αCD4 + αCD8

FIGURE 1.   CD3⁺ T cells expressing neither CD4 nor CD8. (*A*) Lymphocytes were stained with FITC-IgG and PE-IgG control antibodies (*left*) or PE anti-Leu-4 (CD3) and a mixture of FITC anti-Leu-3 (CD4) and FITC anti-Leu-2 (CD8) (*right*). Based on the control sample, the contour plot was divided into quadrants representing unstained cells (*lower left*), cells with only red fluorescence (*upper left*), cells with red and green fluorescence (*upper right*), and cells with only green fluorescence (*lower right*). Lymphocytes expressing CD3, but lacking both CD4 and CD8 would therefore be present in the upper left quadrant (i.e., red is positive, green is negative). In this individual, 79.8% of lymphocytes expressed the CD3 antigen, and 8.5% were CD3⁺,4⁻,8⁻. In the Ig isotype–matched control sample, <0.01% of lymphocytes were present in the *upper left quadrant* and >98% were unstained (*lower left quadrant*). α, anti. (*B*) In vitro cultured CD3⁺,4⁻,8⁻ lymphocytes were stained as described in (*A*). 90% of the lymphocytes were CD3⁺4⁻,8⁻. Note that 10% of the lymphocytes were stained both red (CD3⁺) and dim green (*upper left*). Immunofluorescent staining with only FITC anti-Leu-2 and PE anti-Leu-4 gave an identical profile, indicating that these cells were CD3⁺,CD8^{dim+} (not shown). No CD4⁺ lymphocytes were detected in these cultures. α, anti.

Biologicals, Lexena, KS), 1 mM glutamine, 100 µg/ml gentamicin, and 1000 U/ml rIL-2 (Cetus Corp., Emeryville, CA).

Results and Discussion

Peripheral blood lymphocytes from normal donors were stained with PE anti-Leu-4 (CD3) and a mxiture of FITC anti-Leu-2 (CD8) and FITC anti-Leu-3 (CD4) and were analyzed by flow cytometry. A small subset of cells expressed CD3 antigen (a component of the T cell antigen receptor), but lacked both CD4 and CD8 (Fig. 1). Based on analysis of >20 normal blood donors, ~3% of blood lymphocytes comprised CD3⁺,4⁻,8⁻ T lymphocytes (range, <0.5–23%) (~78% of lymphocytes were CD3⁺). The specificity of the staining was confirmed by showing that an identical percentage of lymphocytes was observed using a reciprocal set of reagents (i.e., FITC anti-Leu-4 and a mixture of PE anti-Leu-2 and PE anti-Leu-3), and by direct three-color immunofluorescence analysis using FITC anti-Leu-2, PE anti-Leu-3, and APC anti-Leu-4 (not shown).[1] By three-color immunofluorescence,[2] we also determined that >90% of the CD3⁺,4⁻,8⁻

[1] Identical results have also been obtained using the T3, T4 and T8 mAbs (Coulter Immunology, Hialeah, FL).

[2] This was determined by three-color immunofluorescence by staining lymphocytes with APC streptavidin/biotin anti-Leu-4 (CD3), a mixture of PE anti-Leu-2 and PE anti-Leu-3, and a third FITC-conjugated antibody (e.g., FITC anti-Leu-1 [CD5], FITC anti-Leu-5 [CD2], etc.). By gating on CD3⁺,4⁻,8⁻ (APC⁺,PE⁻-stained) lymphocytes, it was possible to determine the proportion of CD3⁺,4⁻,8⁻ cells that expressed the antigen detected by the FITC-conjugated antibody.

cells coexpressed the CD2 and CD5 pan T cell antigens, but did not express antigens associated with B cells (CD19), NK cells (CD16), thymocytes (CD1), or monocytes (Leu-M3).

$CD3^+,4^-,8^-$ lymphocytes were isolated to >90% purity using a FACS. 10^5 cells were placed into culture in medium containing rIL-2. After 2 wk, ~5 × 10^6 cells were recovered and reanalyzed for expression of CD4 and CD8. >99% of the rIL-2-cultured lymphocytes expressed CD3, and 90% were $CD3^+,4^-,8^-$ (Fig. 1B). We failed to detect any cells (<0.5%) that expressed either CD4 or CD16. These results clearly show that $CD3^+,4^-,8^-$ T lymphocytes can proliferate in response to rIL-2 without deliberate antigenic stimulation and that the $CD3^+,4^-,8^-$ phenotype is stably maintained. It should be noted however that 10% of the rIL-2-cultured $CD3^+$ T cells did express CD8 antigen, but in approximately one-tenth of the amount present on typical blood $CD3^+,8^+$ T lymphocytes. Lymphocytes expressing "dim" CD8 have been described; however, in these prior reports most $CD8^{dim+}$ cells lacked CD3 and were $CD3^-$, $CD16^+$ NK cells (7). Whether $CD3^+,CD4^-,CD8^{dim+}$ lymphocytes were generated from the $CD3^+,4^-,8^-$ population or were a contaminant in the isolation procedure is unknown.

Morphological examination revealed that >80% of $CD3^+,4^-,8^-$ cells cultured only in rIL-2 were large lymphoblasts with abundant cytoplasm containing predominant azurophilic granules (Fig. 2A). In contrast, >90% of freshly isolated $CD3^+,4^-,8^-$ cells were agranular lymphocytes, although a small proportion of granular cells was observed in some donors (Fig. 2B). Since both NK cells and some CTL possess this characteristic granular morphology, these cells were tested for cytotoxic activity. $CD3^+,4^-,8^-$ T cells cultured only in rIL-2 lysed NK-sensitive K562 and NK-insensitive JY targets (Fig. 3A). Since K562 lacks cell surface expression of both class I and II MHC antigens, these results show that rIL-2-cultured $CD3^+,4^-,8^-$ T lymphocytes recognize and lyse tumor cell targets without MHC restriction. Freshly isolated $CD3^+,4^-,8^-$ T cells did not lyse K562 (Fig. 3B). The rIL-2-activated $CD3^+,4^-,8^-$ cells could be distinguished from freshly isolated $CD3^+,4^-,8^-$ cells based on expression of Leu-19 antigen. >70% of rIL-2-stimulated $CD3^+,4^-,8^-$ cells expressed Leu-19, whereas generally <10% of freshly isolated $CD3^+,4^-,8^-$ cells expressed Leu-19. Recently, we have shown that the Leu-19 antigen is expressed on essentially all peripheral blood $CD3^-$ NK cells, but is also present on a small subset of freshly isolated peripheral blood $CD3^+$ T lymphocytes (most of which express CD8) that mediate non-MHC-restricted cytotoxicity (8).

The cytotoxicity mediated by rIL-2-cultured $CD3^+,4^-,8^-$ lymphocytes against K562 was significantly augmented by the presence of soluble anti-Leu-4 antibody in the cytotoxicity assay (Fig. 4). Although anti-CD3 antibodies generally inhibit the cytotoxic function of antigen-specific CTL activity against the specific target antigen, it has been observed that anti-CD3 antibodies actually induce non-specific lyse of inappropriate targets (10). The finding that anti-CD3 induces, rather than inhibits, the cytotoxicity of the rIL-2-activated $CD3^+,4^-,8^-$ cells suggests that this population may be polyclonal, containing cytotoxic cells of several specificities which are triggered in the presence of anti-CD3.

These results are compatible with the hypothesis that the peripheral blood $CD3^+,4^-,8^-$ population is polyclonal, and that a $Leu-19^+$ cytotoxic subset within
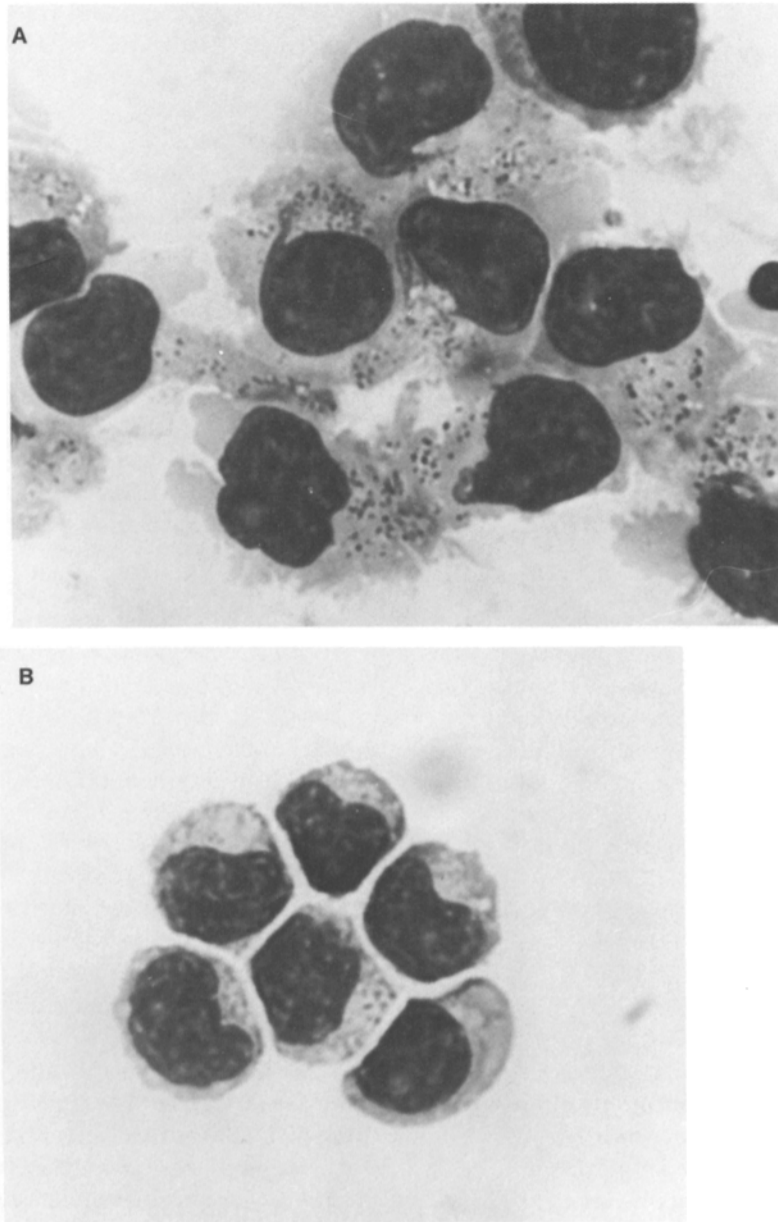
FIGURE 2. Morphology of $CD3^+,4^-,8^-$ T lymphocytes. Cells were cytocentrifuged onto glass slides and stained with Giemsa. (A) rIL-2-cultured $CD3^+,4^-,8^-$ lymphocytes; (B) freshly isolated peripheral blood $CD3^+,4^-,8^-$ lymphocytes. Magnification, × 6,000.

the $CD3^+,4^-,8^-$ population can proliferate directly in response to rIL-2, without deliberate antigenic stimulation. Further studies on the precursors of the cyto-toxic cells within the $CD3^+,4^-,8^-$ subset will be necessary to identify the frequency and phenotype of these cells, and investigate their relationship to the lymphokine-activated killer phenomenon.

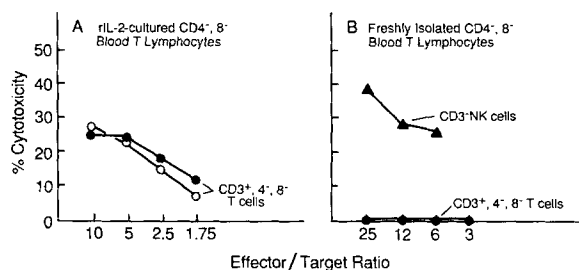The existence of $CD3^+,4^-,8^-$ T lymphocytes shows that a minor subset of

FIGURE 3. Cytotoxicity mediated by $CD4^-,8^-$ T lymphocytes (*A*) An rIL-2-cultured $CD3^+,4^-,8^-$ cell line shown in Fig. 1B, was assayed for cytotoxicity against K562 (*closed symbols*) and JY (*open symbols*). (*B*) Lymphocytes were stained with PE anti-Leu-1 and a mixture of FITC anti-Leu-2, FITC anti-Leu-3, and FITC anti-Leu-12. Lymphocytes that showed exclusively red immunofluorescence (i.e., $CD5^+,4^-,8^-,12^-$) were isolated with a FACS to >95% purity. These freshly isolated $CD5^+,4^-,8^-$ T lymphocytes (●) were assayed for cytotoxicity against K562. In these experiments, T cells were identified with an antibody against CD5 rather than CD3, since antibodies against CD3 have been shown to either induce or inhibit cytotoxic function (10). >95% of the $CD5^+,4^-,8^-,12^-$ lymphocytes coexpressed CD3, as determined by three-color immunofluorescence analysis. FITC anti-Leu-12 (CD19, pan B cell antigen) was used to exclude any $CD5^+$ B lymphocytes from the T lymphocyte population. As a positive control, NK cells (▲), i.e., the $CD5^-,4^-,8^-,19^-$ population, were isolated from the same individual's blood and assayed for cytotoxicity.
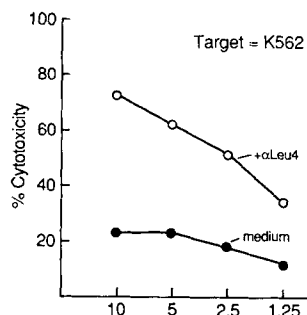

FIGURE 4. Effect of anti-CD3 on cytotoxicity. rIL-2-cultured $CD3^+,4^-,8^-$ cells were assayed for cytotoxicity against K562 in the presence (○) or absence (●) of soluble anti-Leu-4 antibody (final concentration 2 μg/ml) in the 4 h radioisotope release assay. α, anti; abscissa, E/T ratio.

functionally competent peripheral T cells apparently does not require either CD4 or CD8 expression. Furthermore, these studies raise questions regarding the origin of these unique T cells. Recently, De La Hera et al. (11) have reported that in vitro culture of $CD3^+,4^-,8^-$ thymocytes in IL-2 results in the generation of non-MHC-restricted cytotoxic cells. It seems likely that these thymocytes are the immediate precursor for the $CD3^+,4^-,8^-$ T lymphocytes that we have detected in peripheral blood.

Summary

$CD3^+$ T lymphocytes expressing neither CD4 nor CD8 antigens exist in normal human peripheral blood in low frequency (~3% of lymphocytes). The $CD3^+,4^-,8^-$ phenotype was stably maintained after in vitro culture in IL-2. Culture of $CD3^+,4^-,8^-$ cells in only rIL-2 generated cytotoxic T cells that lysed NK-sensitive and NK-insensitive tumor cell targets without MHC restriction. These experiments clearly show that phenotypically and functionally competent T cells expressing neither CD4 nor CD8 are present in normal peripheral blood.

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