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Human double-negative ($CD4^-CD8^-$) T cells bearing $\alpha\beta$ T cell receptor possess both helper and cytotoxic activities

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SUMMARY

Expression of CD4 or CD8 on the cell surface is an important guide for discriminating the immunological functions of T cells. However, a minor T cell subset, which lacks both CD4 and CD8 molecules but bears the usual form of T cell receptor (TCR) $\alpha\beta$ ($CD4^-CD8^-TCR\alpha\beta^+$ T cells), has recently been found not only in mice but also in humans, and its role in immune response is now of considerable interest. In order to clarify the characteristics of this newly defined T cell subpopulation, we established five IL-2-dependent $CD4^-CD8^-TCR\alpha\beta^+$ T cell clones from the peripheral blood of a healthy individual, and examined their various biological functions. It was found that all clones not only helped B cells in immunoglobulin production, but also exerted major histocompatibility complex-unrestricted cytotoxicity. Although their CD3/TCR complexes were functionally competent, the cytotoxicity seemed to be mediated via unknown molecules other than the CD3/TCR complex, as evidenced by the failure of CD3 MoAb to inhibit the cytotoxic activity. Our present findings showed that $CD4^-CD8^-TCR\alpha\beta^+$ T cells possess potential bifunction, i.e. helper and cytotoxic activities. Their roles in the pathogenesis of immunodeficiency are discussed.

Keywords double-negative T cells cytotoxicity helper function $\alpha\beta$ T cell receptor

INTRODUCTION

The diverse functions of T cells in the immune system are mediated by multiple subpopulations that are often discriminated by their surface structures. Most $CD3^+$ T cells bear the $\alpha\beta$ form of the T cell receptor (TCR) which recognizes complexes of foreign antigen with products of the major histocompatibility complex (MHC) (Zinkernagel & Doherty, 1979; Buus *et al.*, 1986; Dembić *et al.*, 1986). However, recent studies have revealed that a small subset of T cells bears another form of TCR, the heterodimer of γ and δ. It is unique in that most of the $TCR\gamma\delta^+$ T cells lack both CD4 and CD8 molecules (Brenner *et al.*, 1986; Lanier & Weiss, 1986). In addition to $TCR\gamma\delta^+$ T cells, another T cell subset lacking both CD4 and CD8, and expressing the $\alpha\beta$ form of TCR ($CD4^-CD8^-TCR\alpha\beta^+$ T cells) has been recently identified in the murine thymus (Fowlkes *et al.*, 1987). Since $CD4^-CD8^-TCR\alpha\beta^+$ T cells have been found to accumulate in lymphoid organs of MRL-*lpr/lpr* and C3H/HeJ-*gld/gld* autoimmune-prone mice (Hashimoto *et al.*, 1987; Miescher *et al.*, 1987), their biological functions are of considerable interest. Although an analogous subset has been recently found in human thymus (Toribio *et al.*, 1988), skin (Groh *et al.*, 1989), and peripheral blood (Lanier, Ruitenberg & Phillips, 1986; Londei *et al.*, 1989; Shivakumar, Tsokos & Datta, 1989), the detailed characteristics of this newly defined T cell subpopulation remain obscure.

In the present study we established IL-2-dependent $CD4^-CD8^-TCR\alpha\beta^+$ T cell clones from the peripheral blood lymphocytes (PBL) of a healthy individual and examined their biological functions. $CD4^-CD8^-TCR\alpha\beta^+$ T cells were found to possess simultaneously both helper and cytotoxic activities.

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MATERIALS AND METHODS

Cell separation

Peripheral blood mononuclear cells (PBMC) were isolated from a healthy individual by Ficoll-Conray gradient centrifugation. T cells were enriched by passage through a nylon wool column. Sheep erythrocyte rosette-forming (E^+) and non-rosette-forming (E^-) cells were separated using the sheep-erythrocyte-rosetting method, and E^- cells were further treated with CD3 MoAb OKT3 (Ortho Pharmaceutical Corp., Raritan, NJ) and complement (C) as described below to deplete T cells.

Monoclonal antibodies

OKT3 (anti-CD3), OKT4 (anti-CD4), and OKT8 (anti-CD8) were purchased from Ortho Pharmaceutical Corp. Leu11b (anti-CD16) and NKH1A (anti-CD56), which react with most

natural killer (NK) cells, were purchased from Becton Dickinson (Mountain View, CA) and Coulter (Hialea, FL), respectively. Phenotype analysis was performed by direct immunofluorescence using an Epics profile flow cytometer (Coulter) as described previously (Yasukawa, Inatsuki & Kobayashi, 1988) with the following MoAbs: Leu5b (CD2), Leu4 (CD3), Leu3a (CD4), Leu2a (CD8), Leu11 (CD16), Leu8, WT31 (anti-TCR$\alpha\beta$) (Becton Dickinson), MO1 (CD11b), 4B4 (CD29), 2H4 (CD45RA) (Coulter), KOLT-2 (CD28) (Nichirei, Tokyo, Japan), and TCRδ1 (anti-TCR$\gamma\delta$) (T Cell Sciences, Cambridge, MA). Although WT31 MoAb may also react with TCR$\gamma\delta^+$ T cells when used at very high concentrations (Van de Griend *et al.*, 1988), it did not react with TCR$\gamma\delta^+$ T cell clones at the concentration we used (data not shown).

Generation of IL-2-dependent CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cell clones

Cells were suspended at a concentration of $1-2 \times 10^7$ cells/0·1 ml in phosphate-buffered saline (PBS), pH 7·2, containing 0·02 ml of each of the OKT4, OKT8, Leu11b, and NKH1A MoAbs. After 30 min of incubation on ice, the cells were washed, and then non-toxic rabbit C (Cedarlane, Ontario, Canada) was added at a final dilution of 1/3. After an incubation for 1 h at 37°C, the cells were washed twice and suspended in RPMI 1640 medium supplemented with 10 mmol/*l* HEPES buffer and 10% heat-inactivated fetal calf serum (GIBCO, Grand Island, NY) (this medium is referred to as the culture medium). CD4$^+$ and CD8$^+$ cells did not exceed 1% of this MoAb-treated population. The cells were then seeded at a concentration of one per well in round-bottomed microtitre wells containing 0·2 ml of culture medium with 0·05 U/ml recombinant IL-2 (rIL-2) (Takeda Chemical Industries, Osaka, Japan), phytohaemaglutinin (PHA)-P (DIFCO Laboratories, Detroit, MI) at a final dilution of 1:1000, and 1×10^5 X-irradiated mixed allogeneic PBMC. After 10 days of culture at 37°C in a 5% CO_2 incubator, the growing cells were transferred to 16-mm wells and analysed for phenotype with a flow cytometer. CD4$^-$CD8$^-$TCR$\alpha\beta^+$ clones were selected and expanded in culture medium containing rIL-2. X-irradiated PBMC and PHA were added to the wells every alternate week.

Helper activity for immunoglobulin production

One hundred thousand clone cells and 1×10^6 autologous E$^-$ cells suspended in 2 ml of culture medium were cultured in a 16-mm well. For the control, 3×10^6 PBMC, 1×10^6 E$^-$ cells, 2×10^6 E$^+$ cells, and 1×10^6 E$^-$ cells plus 2×10^6 E$^+$ cells were cultured under the same conditions. After 6 days, culture supernatants were collected and then assayed for IgG and IgM production. The amounts of IgG and IgM were assessed by an ELISA using microtitre plates coated with goat anti-human IgG and IgM (Cappel, Malvern, PA), respectively (Matsumoto, Mochizuki & Kobayashi, 1989). Purified human IgG and IgM (Tago, Burlingame, CA) were used as reference standard. Before these experiments clone cells were cultured for more than 1 week after the last addition of feeder cells and PHA and were extensively washed.

Cytotoxic assays

^{51}Cr-release assays were carried out as described previously (Yasukawa & Zarling, 1984a). Briefly, 1×10^4 target cells labelled with ^{51}Cr ($Na_2{}^{51}CrO_4$) (New England Nuclear, Boston, MA) and 1×10^5 effector cells were incubated together in 0·2 ml of culture medium in a round-bottomed microtitre well. After a 5-h incubation, 0·1 ml of supernatant was collected from each well. The percentage of specific ^{51}Cr release was calculated using the following formula:

$$\frac{(\text{ct/min experimental release} - \text{ct/min spontaneous release})}{(\text{ct/min maximal release} - \text{ct/min spontaneous release})} \times 100.$$

The spontaneous release from the target cells never exceeded 20% of the maximal release. In some experiments, CD3 MoAb OKT3 was added to the culture at a final dilution of 1:200 and ^{51}Cr-release assays were performed as described above. Clone cells were cultured for more than 1 week after the last feeding and were extensively washed before cytotoxic assays in order to remove PHA from the culture medium.

Proliferative response of clone cells to the stimulation with CD3 monoclonal antibody

Proliferative assays were performed as described previously with a slight modification (Geppert & Lipsky, 1987). Briefly, 2×10^4 clone cells in 0·2 ml of culture medium were seeded in a flat-bottomed microtitre well which had been previously coated with CD3 MoAb by incubation with MoAb OKT3 diluted in PBS. The plates were incubated at 37°C in a CO_2 incubator for 72 h. For the final 16 h of incubation, 1 μCi of ^{3}H-thymidine (New England Nuclear) was added to each well, and the cells were then harvested on glass fibre filter paper using a semiautomatic multiple cell harvester. The incorporation of ^{3}H-thymidine was determined by liquid scintillation counting.

Determination of free cytoplasmic Ca^{2+} concentration

Analysis of the free cytoplasmic Ca^{2+} concentration ($(Ca^{2+})_i$) in clone cells in response to CD3 MoAb was performed as described previously (Inatsuki, Yasukawa & Kobayashi, 1989). Briefly, clone cells were suspended in the labelling solution at a concentration of 5×10^6/ml, and acetoxymethyl ester of Fura-2 (Dojin Chemical, Tokyo, Japan) was added at a final concentration of 2 μmol/*l*. After incubation for 30 min at 37°C in the dark, the cells were washed twice with labelling solution and resuspended at $2{\cdot}5 \times 10^6$ cells/ml for the measurement of $(Ca^{2+})_i$. The fluorescence of the cell suspension was monitored with a CAF-100 Ca^{2+} analyser (Japan Spectroscopic, Tokyo, Japan). The cell suspension was subjected to excitation at 340 nm or 380 nm and fluorescence was measured at 510 nm. CD3 MoAb OKT3 was added at a final concentration of 1/400. Anti-mouse IgG goat serum (Cappel) for cross-linking of CD3 molecules was added at final dilution of 1/400.

RESULTS

Generation of CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cell clones

PBMC of the healthy individual, M.Y., which were depleted of CD4-, CD8-, CD16- and CD56-positive cells, were seeded at 1 cell per well in the presence of rIL-2, PHA and feeder cells in 576 wells. Thirty clones were generated and screened by phenotypic analysis with a flow cytometer. Five clones positive for CD3 and WT31, but negative for CD4, CD8, CD16 and TCRδ1, were selected. These clones were designated MY-3, MY-13, MY-20, MY-24 and MY-27 and expanded for further experiments. As shown in Table 1, surface phenotype of these clones was almost identical except for the expression of Leu8.

Table 1. Surface phenotype of clones

Clone	CD2	CD3	CD4+CD8	CD11b	CD16	CD28	CD29	CD45RA	Leu8	WT31	TCRδ1
MY-3	99	99	3	69	0	75	84	17	9	95	0
MY-13	99	99	0	83	2	87	96	56	77	99	0
MY-20	99	99	0	64	1	78	80	33	11	98	1
MY-24	99	99	2	83	0	82	90	25	15	97	0
MY-27	99	99	3	70	0	80	83	28	65	97	1

Phenotype of clones was determined by direct immunofluorescence assay using a flow cytometer, as detailed in Materials and Methods. Results are expressed as percentages of positive cells.

Table 2. Helper activity of clones in immunoglobulin production by B cells

Cells cultured	IgG (μg/ml)	IgM (μg/ml)
PBMC	2·5	0·2
E^+	<0·1	<0·1
E^-	<0·1	<0·1
$E^+ + E^-$	0·1	0·1
MY-3+E^-	3·3	>1·0
MY-13+E^-	0·7	>1·0
MY-20+E^-	2·9	>1·0
MY-24+E^-	0·8	>1·0
MY-27+E^-	0·6	>1·0

PBMC (3×10^6), E^+ cells (2×10^6), E^- cells (1×10^6), E^+ cells+E^- cells ($2 \times 10^6 + 1 \times 10^6$) and clone cells+$E^-$ cells ($1 \times 10^5 + 1 \times 10^6$) were cultured in 16-mm wells. The culture supernatants were collected after 6 days, and the productions of IgG and IgM were determined by ELISA, as detailed in Materials and Methods.

Table 3. Cytotoxic activity of clones

	Per cent specific ^{51}Cr release from K562 cells	
Clone	No antibody	OKT3
MY-3	42·0	56·8
MY-13	45·4	55·9
MY-20	58·1	70·5
MY-24	29·9	45·4
MY-27	43·4	47·3

^{51}Cr-release assays were performed at an effector-to-target cell (E:T) ratio of 10:1 with and without pretreatment of clone cells with CD3 MoAb.

Table 4. Proliferative response of clones stimulated by immobilized CD3 MoAb

	(^3H)TdR incorporated (ct/min) (S.E.M.)	
Clone	No antibody	Immobilized OKT3
MY-3	1417 (48)	8797 (1016)
MY-13	7988 (858)	28 291 (1323)
MY-20	2791 (669)	63 764 (1421)
MY-24	5712 (818)	14 234 (2346)
MY-27	2028 (173)	13 187 (511)

Incorporation of ^3H-thymidine into 2×10^4 clone cells cultured with and without immobilized CD3 MoAb OKT3 was determined during the final 16 h of a 72-h incubation. The values represent the mean counts per minute (s.e.m.) of triplicate wells.

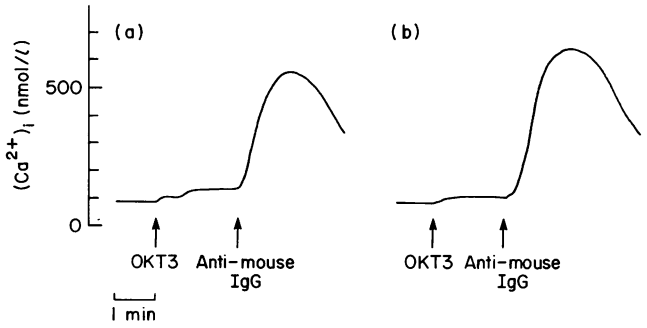

Fig. 1. Analysis of $(Ca^{2+})_i$ mobilization in clone cells in response to CD3 MoAb. (a) MY-3 and (b) MY-20 were loaded with Fura-2, and the fluorescence of each cell suspension was monitored as described in Materials and Methods. OKT3 and anti-mouse IgG antibody were added at the times indicated. $(Ca^{2+})_i$ (nmol/l) is displayed on the ordinate.

Helper activity of clones in immunoglobulin production

We first addressed the question of whether these $CD4^-CD8^-TCR\alpha\beta^+$ T cell clones might have the ability to help B cells in immunoglobulin production. Although a little spontaneous immunoglobulin production from whole PBMC was detected, either E^- cells (B cells and macrophages) alone or E^+

cells (T cells) alone were capable of producing little or no IgG and IgM. However, the immunoglobulin production was partially restored by co-culturing E^+ cells with E^- cells. Based on these data, we examined the helper activity of $CD4^-CD8^-TCR\alpha\beta^+$ T cell clones by co-culturing clone cells with autologous E^- cells. We found that, as shown in Table 2, both IgG and IgM production by B cells was markedly augmented by co-culturing with the clone cells without any exogenous mitogen.

Cytotoxic activity of clones

We next examined the cytotoxicity of $CD4^-CD8^-TCR\alpha\beta^+$ T cell clones. As shown in Table 3, all clones lysed NK-sensitive K562 cells, indicating that $CD4^-CD8^-TCR\alpha\beta^+$ T cells have MHC-unrestricted cytotoxic activity in addition to helper activity for immunoglobulin production by B cells. It has been shown that CD3/TCR complex is essential for the mediation of antigen-specific cytotoxicity in both $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes (CTL), based on the findings that CD3 or anti-TCR MoAb inhibited their cytotoxicity (Landegren *et al.*, 1982; Meuer *et al.*, 1982; Meuer *et al.*, 1983). Moreover, MHC-unrestricted cytotoxicity mediated by both $CD4^+$ and $CD8^+$ CTL was found to be inhibited also by CD3 MoAb in most cases (Thiele & Lipsky, 1989). In contrast to these previous findings of conventional CTL, cytotoxicity of our $CD4^-CD8^-TCR\alpha\beta^+$ T cell clones was not inhibited, but rather augmented somewhat by CD3 MoAb. These data might suggest that the cytotoxic mechanisms of double-negative $TCR\alpha\beta^+$ CTL are different from those of MHC-restricted $CD4^+$ and $CD8^+$ CTL.

Proliferation of clones stimulated by immobilized CD3 monoclonal antibody

The evidence that CD3 MoAb did not inhibit the cytotoxic activity of $CD4^-CD8^-TCR\alpha\beta^+$ T cell clones led us to the question whether CD3 molecules on double-negative $TCR\alpha\beta^+$ T cells are functional molecules triggering T cell activation. In order to clarify this point, we first examined whether $CD4^-CD8^-TCR\alpha\beta^+$ T cell clones proliferate in response to the stimulation with immobilized MoAb. The results, presented in Table 4, clearly demonstrated that double-negative $TCR\alpha\beta^+$ T cell clones proliferated in response to CD3/$TCR\alpha\beta$ triggering, comparable to that described in the usual single-positive T cells. These data indicated that human $CD4^-CD8^-TCR\alpha\beta^+$ T cells express functionally competent CD3/TCR complexes.

$(Ca^{2+})_i$ mobilization in clone cells in response to CD3 monoclonal antibody

In order to confirm further the functional competence of CD3/$TCR\alpha\beta$ complex on the clones, the elevation of $(Ca^{2+})_i$ by stimulation with CD3 MoAb was examined. As shown in Fig. 1, $CD4^-CD8^-TCR\alpha\beta^+$ T cell clones MY-3 and MY-20 showed as significant an increase of $(Ca^{2+})_i$ as that of single-positive $TCR\alpha\beta^+$ T cells, reported previously by us (Inatsuki *et al.*, 1989). Similar results were obtained when three other clones were tested for the elevation of $(Ca^{2+})_i$ induced by CD3 MoAb (data not shown).

DISCUSSION

In the present study we demonstrated for the first time that $CD4^-CD8^-TCR\alpha\beta^+$ human T cells can simultaneously exert both cytotoxic and helper functions without any exogenous stimulation. In a previous study we revealed that herpes simplex virus (HSV)-specific human $CD4^+$ T cells could simultaneously induce anti-HSV antibody production as well as lyse HSV-infected cells in a human leukocyte antigen (HLA) class II-restricted manner (Yasukawa & Zarling, 1984a, 1984b; Yasukawa *et al.*, 1988). Moreover, alloantigen-specific and virus-specific multifunctional T cell clones have been reported by other investigators (Widmer & Bach, 1981; Kaplan *et al.*, 1984; Yarchoan *et al.*, 1986). These findings suggest that some T cells can simultaneously exert dual functions which were previously thought to be mediated by distinct T cell subpopulations, $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. Our present study provided evidence that this multifunctionality is not restricted to some single-positive T cells but is also mediated by $TCR\alpha\beta^+$ T cells lacking both CD4 and CD8.

Since the discovery of double-negative T cells bearing the $\alpha\beta$ form of TCR, their functional properties have been studied by several workers. In spite of these extensive investigations, the functions of $CD4^-CD8^-TCR\alpha\beta^+$ T cells which have been reported so far are controversial. Borst *et al.* (1987) derived a double-negative $TCR\alpha\beta^+$ T cell clone from the peripheral blood of a healthy adult, but this clone had little cytolytic activity. In contrast, Groh *et al.* (1989) first reported the spontaneous MHC-unrestricted cytotoxic activity of human double-negative $TCR\alpha\beta^+$ T cells derived from the skin. Londei *et al.* (1989) found that double-negative $TCR\alpha\beta^+$ T cells derived from peripheral blood lacked spontaneous cytotoxic activity but could be induced to lyse target cells after activation of their TCR by CD3 MoAb. Moreover, Brooks *et al.* (1990) have recently reported the establishment of two double-negative $TCR\alpha\beta^+$ T cell lines with distinct functions, derived from the peripheral blood of a patient with combined immunodeficiency. One of these lines showed spontaneous MHC-unrestricted cytolytic activity and the other line became cytotoxic only in the presence of lectin or CD3 MoAb. In contrast to these previous reports describing varied results, five clones generated in the present study uniformly showed strong MHC-unrestricted cytotoxicity without any exogenous stimulation. Our present study thus indicates that further investigation is needed to clarify whether the functional heterogeneity in cytotoxicity reported previously is mediated by distinct $CD4^-CD8^-TCR\alpha\beta^+$ T cell subpopulations or by a single T cell subset at different phases of the cell cycle.

Although both spontaneous cytotoxicity and cytotoxicity induced by CD3 MoAb or lectin have been reported for $CD4^-CD8^-TCR\alpha\beta^+$ T cells as described above, their target antigens and the cell surface structures recognizing target cells have remained obscure. Porcelli *et al.* (1989) described a $CD4^-CD8^-TCR\alpha\beta^+$ T cell line that lysed target cells in an MHC-unrestricted manner through specific recognition of the CD1a molecules. Rivas *et al.* (1990) have recently generated $CD4^-CD8^-TCR\alpha\beta^+$ cytotoxic T cell clones which were restricted by HLA class I in their cytotoxicity, following repeated stimulation of PBL with an allogeneic lymphoblastoid cell line. MHC class I-restricted cytotoxic T cells lacking both CD4 and CD8 were also derived from murine lymphocytes (Wadsworth, Yui & Greene, 1989). These CD1a-specific and MHC class I-specific double-negative $TCR\alpha\beta^+$ T cells were found to use their CD3/TCR complexes for recognition of target structures based on the findings that MoAbs against CD3 and TCR inhibited

their cytotoxic activity. In contrast to these 'antigen-specific' double-negative TCRαβ⁺ T cells, cytotoxicity of all clones generated in the present study was not inhibited by CD3 MoAb. This suggests that the cytotoxicity of our clones was mediated via molecules other than CD3/TCR complexes. The evidence that our clones proliferated in response to immobilized CD3 MoAb and that $(Ca^{2+})_i$ rose rapidly following stimulation of the clone cells with CD3 MoAb indicated that the CD3/TCR complexes of our CD4⁻CD8⁻TCRαβ⁺ T cell clones were indeed competent. Therefore, it can be considered that CD4⁻CD8⁻TCRαβ⁺ T cells with MHC-unrestricted cytotoxicity possess at least two distinct antigen-recognition receptors; one is the CD3/TCR complex and the other is an unknown receptor which mediates NK-like non-specific cytotoxicity. Such a dual receptor model was previously proposed in the usual single-positive TCRαβ⁺ T cells and also in TCRγδ⁺ T cells. By blocking experiments using MoAbs, Moretta *et al.* (1984) revealed that alloantigen-specific human T cell clones could mediate both antigen-specific and NK-like cytotoxicity, and that two independent recognition structures were involved in this dual activity. Koide, Rivas & Engleman (1989) also reported similar dual cytotoxic activity via distinct mechanisms in human TCRγδ⁺ T cell clones. In order to clarify the immunological properties of double-negative TCRαβ⁺ T cells, it is necessary to further identify the ligands of their CD3/TCR complexes and the structures mediating MHC-unrestricted cytotoxicity.

In addition to cytotoxic activity, the CD4⁻CD8⁻TCRαβ⁺ T cell clones generated in the present study all exhibited helper function for polyclonal immunoglobulin production by autologous B cells. Recently, it has been reported that a double-negative TCRαβ⁺ T cell line derived from the peripheral blood of a patient with active systemic lupus erythematosus induced the production of anti-DNA autoantibodies by autologous B cells (Shivakumar *et al.*, 1989). Moreover, a murine CD4⁻CD8⁻TCRαβ⁺ T cell line with helper function for B cells has been isolated from the spleen of an MRL-*lpr*/*lpr* mouse which is known to develop autoimmune manifestations (Pelkonen & Palacios, 1990). Although these recent reports describing the helper function of CD4⁻CD8⁻TCRαβ⁺ T cells were based on data obtained from individuals with pathological autoimmune status, the present study has demonstrated that double-negative TCRαβ⁺ T cells of healthy individuals also possess the physiological potential to induce polyclonal immunoglobulin production by B cells. We examined whether anti-DNA autoantibodies are produced by co-culturing CD4⁻CD8⁻TCRαβ⁺ T cell clones derived from a healthy adult with autologous B cells, and found that no autoantibody was detected in the supernatant of this co-culture (data not shown). This result suggested that CD4⁻CD8⁻TCRαβ⁺ T cells do not themselves have the potential to induce autoantibody production, but that the primary cause of autoantibody production in patients with autoimmune diseases might be their abnormal B cell repertoire.

In conclusion, we have for the first time demonstrated multifunctionality of a newly defined T cell subpopulation, CD4⁻CD8⁻TCRαβ⁺ T cells. Although association between autoimmune diseases and excessive activation of double-negative TCRαβ⁺ T cells has been considered, their physiological roles *in vivo* remain obscure. Further characterization of CD4⁻CD8⁻TCRαβ⁺ T cells would lead to greater understanding of the precise mechanisms of immune responses and would shed light on the pathogenesis of various immunological diseases of unknown etiology.

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