Biochemical Differences in the αβ T Cell Receptor–CD3 Surface Complex between CD8⁺ and CD4⁺ Human Mature T Lymphocytes*

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We have reported the existence of biochemical and conformational differences in the αβ T cell receptor (TCR) complex between CD4⁺ and CD8⁺ CD3γ-deficient (γ⁻) mature T cells. In the present study, we have furthered our understanding and extended the observations to primary T lymphocytes from normal (γ⁺) individuals. Surface TCR-CD3 components from CD4⁺ γ⁺ T cells, other than CD3γ, were detectable and similar in size to CD4⁺ γ⁺ controls. Their native TCR-CD3 complex was also similar to CD4⁺ γ⁺ controls, except for an αβ(δε)δζ₂ instead of an αβγεδζζ₂ stoichiometry. In contrast, the surface TCRα, TCRβ, and CD3δβ chains of CD8⁺ γ⁺ T cells did not possess their usual sizes. Using confocal immunofluorescence, TCRα was hardly detectable in CD8⁺ γ⁻ T cells. Blue native gels (BN-PAGE) demonstrated the existence of a heterogeneous population of TCR-CD3 in these cells. Using primary peripheral blood T lymphocytes from normal (γ⁺) donors, we performed a broad epistopic scan. In contrast to all other TCR-CD3-specific monoclonal antibodies, RW2-8C8 stained CD8⁺ better than it did CD4⁺ T cells, and the difference was dependent on glycosylation of the TCR-CD3 complex but independent of T cell activation or differentiation. RW2-8C8 staining of CD8⁺ T cells was shown to be more dependent on lipid raft integrity than that of CD4⁺ T cells. Finally, immunoprecipitation studies on purified primary CD4⁺ and CD8⁺ T cells revealed the existence of TCR glycosylation differences between the two. Collectively, these results are consistent with the existence of conformational or topological lineage-specific differences in the TCR-CD3 from CD4⁺ and CD8⁺ wild type T cells. The differences may be relevant for cis interactions during antigen recognition and signal transduction.

αβ T lymphocytes recognize peptide-major histocompatibility complex ligands by means of a multimeric protein complex termed the αβ T cell receptor (TCR) CD3 complex (TCR-CD3). This structure is composed of a variable αβ TCR dimer that binds antigens and three invariant dimers (CD3γ, δε, and ζ) that are in charge of TCR-CD3 complex transport, stabilization, and signal transduction (1). The minimum stoichiometry, therefore, is believed to be αβγεδζζ₂.

Mature CD4⁺ and CD8⁺ αβ T cells differ sharply in their major histocompatibility complex ligands, but their TCR-CD3 complex is believed to be qualitatively identical. The reduced αβ TCR-CD3 staining levels observed in CD8⁺ T cells, relative to CD4⁺ T cells, were therefore reported as quantitative under this assumption (2). Unexpectedly, peripheral blood αβ TCR-CD3 expression was shown to be more impaired in CD8⁺ than in CD4⁺ cells when CD3γ (3, 4) or CD3δ (5) was absent. These observations were followed by the description of conformational and biochemical differences in the TCR-CD3 complex between CD8⁺ and CD4⁺ CD3γ-deficient (γ⁻) T lymphocytes (6). Biosynthetic studies showed that CD8⁺ but not CD4⁺ γ⁻ T cells lacked normal TCRα. Instead, the CD4⁺ γ⁻ T cells contained a small αβ heterodimer composed of abnormally glycosylated TCRβ and an abnormally small CD3-associated chain that was not recognized by TCRα-specific antibodies.

In the present study, we have extended these biochemical studies to the cell surface and provide further evidence for the existence of qualitative differences in the αβ TCR-CD3 complex between CD8⁺ and CD4⁺ T lymphocytes, particularly when CD3γ is lacking, but also in normal T cells.

EXPERIMENTAL PROCEDURES

Cells—Herpesvirus saimiri-transformed T cell lines were derived with informed consent from the peripheral blood lymphocytes (PBLs) of a healthy congenital CD3γ-deficient (γ⁻) individual or the PBLs of normal donors (γ⁺) as described previously (7, 8). The T cell lines used in the experiments (DSF4, CD4⁺ γ⁻; CTRDC4, CD4⁺ γ⁺; DSF8, CD8⁺ γ⁺; and CTO, CD8⁺ γ⁻) had been cultured for 6–7 years when the experiments were performed. Cells had always been grown in parallel in 1:1 RPMI 1640 and 25 mM Heps/Panserin medium (from Invitrogen and Pan Biotech, Aidenbach, Germany, respectively) supplemented with 40 IU/ml human rIL-2 (Frederick Cancer Research and Development Center, NC1, National Institutes of Health, Frederick, MD, 10% FCS (Flow Laboratories), and 1% glutamine (BioWhitaker, Berkshire, UK). The medium was replaced every 3–4 days. The pre-TCR-expressing cell line SupT1 was grown in RPMI 1640 and 25 mM Heps supplemented with 10% FCS and 1% glutamine.

Human PBLs were obtained with informed consent from normal donors. Purified CD4⁺ and CD8⁺ T cells were isolated immunomagnetically using standard procedures according to manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Purities >90% were the rule. Human postnatal thymocytes were isolated from thymus fragments removed during corrective cardiac surgery of patients aged 1 month to 4 years with informed parental consent.

TCR Labeling and Immunoprecipitation—Surface labeling (125I and cycloheximide; MFI, mean fluorescence intensity; NANase, N-acetyl-neuraminidase; PBL, peripheral blood lymphocyte.)
biotin) and precipitation experiments were performed as described in Refs. 6 and 7. Cells were 125I-labeled using the lactoperoxidase method. Samples were lysed in Nonidet P-40 (Sigma)-containing lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 8, 10 mM iodoacetamide, and 1 mM phenylmethylsulfonyl fluoride) or in diithionite (or BrMg)-containing lysis buffer (1% diithionite (or BrMg), 50 mM Tris- HCl, 150 mM NaCl, 1 mM MgCl2, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 8 mM iodoacetamide). Supernatants, hereafter referred to as lysates, were collected and transferred to new tubes. The lysates were precleared twice with Sepharose beads (Amersham Biosciences) containing 1% Nonidet P-40 or 1% diithionite (or BrMg), depending on the detergent used for the lysis), followed by a 2-min centrifugation at 12,000 × g in an Eppendorf centrifuge at 4 °C. The precleared lysates were subsequently immunoprecipitated with normal mouse immunoglobulins (and data not shown) internally by the decreased binding of a CD43-specific antibody, as described (13). Blue Native (BN)-PAGE and Western Blotting—Published methods were used for the two-step affinity purification of the TCR (14). Briefly, cells were stimulated with pervanadate and lysed with 1% diithionite lysis buffer containing sodium orthovanadate. Phosphorylated proteins were purified with 1 μg of 4G10 and 3 μl of protein G-Sepharose, washed three times, and subsequently eluted in BN buffer (500 mM e-aminoacrylic acid, 20 mM NaCl, 10% glycerol, 2 mM EDTA, and 20 mM bis-Tris, pH 7) including 50 mM phenylphosphate, 1 unit of alkaline phosphatase, and 1% diithionite. Dephosphorylated TCRs were separated by BN-PAGE as described (15). Western blots were developed with the anti-Ly-2 line termed PerCP-C5.5 (data not shown) and internally by the decreased binding of a CD43-specific antibody, as described (13).

RESULTS

CD4+ and CD8+ CD3γ-deficient T Lymphocytes Express Biochemically Different Membrane αβ TCR-CD3 Complexes—Natural CD3γ-deficient (γ−) T cells were biotinylated or radiolabeled, lysed, and immunoprecipitated with CD3-specific as well as with TCR-specific mAb and compared with normal controls (γ+). The results showed that, in contrast to biosynthetic experiments, the amount of surface CD3-associated TCR (and γ) proteins was very low in γ− cells (Fig. 1A). This precluded a meaningful analysis of the TCR composition of the complex by this method. We have previously reported by immunoprecipitation that surface CD3δ from CD8+ γ− cells showed an impaired electrophoretic mobility in comparison with controls because of its abnormal glycosylation (Fig. 1A, right). This finding correlates with complete endo-β-N-acetylglucosaminidase H resistance (6). Therefore, we next analyzed the CD3δ from CD4+ γ− cells. The results demonstrated that, in contrast to CD8+ γ− cells, the CD3δ from CD4+ γ− cells was similar to that from controls, both in terms of relative mobility and endo-β-N-acetylglucosaminidase H sensitivity (Fig. 1B). The direct immunoprecipitation of TCR chains showed that CD4+ γ− cells were comparable with CD4+ γ+ cells in terms of TCR chain composition, whereas CD8+ γ− cells clearly lacked normal surface TCRα and TCRβ chains (Fig. 2). These differences were TCR-specific, because control immunoprecipitates of major histocompatibility complex class I molecules were equivalent in all cells.

To further characterize the surface TCR chains of the mutant cells, immunofluorescence confocal analyses were performed using the same mAb. Expression of the CD4 or CD8 coreceptors was used as a positive or negative control in each cell type. The results indicated that both TCRα and TCRβ chains showed normal (mostly surface) distribution and fluorescence intensities in CD4+ γ− lymphocytes (Fig. 3).

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cells also showed normal expression of TCRβ by this assay, but TCRα was hardly detectable and, when present (10–20% of cells), mostly intracellular. The discrepancy in TCRβ expression by CD4 γ− cells between these data and the immunoprecipitation results (above) may be due to technical differences, such as the use of acetone for confocal analysis.

In a last set of experiments, the native surface TCR-CD3 complexes from CD4+ and CD8+ mutant cells were compared (14, 15). The results indicated that the TCR-CD3 complex from CD4+ γ− T lymphocytes was similar to normal complexes from γ+ T cells (CD4+, CD8+, or Jurkat), although with delayed electrophoretic mobility (Fig. 4A). This observation was compatible with either a different stoichiometry or a different structure (such as glycosylation or conformation changes) of the mutant complex (see “Experimental Procedures”). To distinguish between these two possibilities, antigenic shift experiments were performed. One shift indicates that there is a single chain per complex, and two shifts denote two binding sites per complex. The results indicated that the mutant TCR-CD3 complex had a normal stoichiometry when compared with that of γ+ T cells (Jurkat, Fig. 4B, or PBL, not shown). It contained one TCRβ chain (hence a single αβ heterodimer) and two CD3ε chains (probably representing two CD3ε dimers, because γ was absent). In summary, these experiments support an αβ(βε)εε model for the mutant TCR-CD3 complex in CD4− γ− cells, where the γε dimer is replaced by the analogous βε dimer. Therefore, other types of modifications, such as glycosylation or conformation, may explain the delayed migration of the mutant complex. In sharp contrast, CD8− γ− cells expressed a very different TCR-CD3 complex as compared with their CD4+ γ− counterparts. Native TCR-CD3 complexes isolated from CD8− γ− cells migrated along a broad smear, suggesting the existence of a heterogeneous population of complexes in these cells (Fig. 4A). The stoichiometry of these heterogeneous TCR-CD3 complexes could not be determined by antigenic shift due to the basal broad smear.
From these studies we conclude that CD4⁺γ⁻ and CD8⁺γ⁻ cells shared a poor surface association of CD3 to TCR chains. We also conclude, by using several experimental approaches, that their surface αβ TCR-CD3 complexes differed biochemically in the CD3 and the TCR components, as well as in the complete native complex.

CD4⁺ and CD8⁺ Primary T Lymphocytes Express Biochemically Different Membrane αβ TCR-CD3 Complexes—Normal primary CD4⁺ T cells show higher TCR-CD3 staining levels than do CD8⁺ cells (−1.5-fold with OKT3; Ref. 2). This difference was interpreted as quantitative under the assumption that the αβ TCR-CD3 complex is identical in both cell types. However, as shown above and in Ref. 6, the analysis of γ TCR-CD3 complexes suggested the existence of hitherto unrecognized structural differences between both cell lineages. These results prompted us to search for qualitative αβ TCR-CD3 complex differences in normal CD4⁺ and CD8⁺ primary T lymphocytes. To test this hypothesis, a broad epoctic scan of the TCR-CD3 complex was performed by three-color flow cytometry using several CD3- or TCR-specific mAbs within gated CD4⁺ and CD8⁺ subsets. To exclude CD8⁺ NK cells (mostly CD8α⁺, which stain dull for CD8 mAb), only CD8bright cells (CD8αβ⁺ T cells) were analyzed. As a control, CD18-specific mAbs were used, which stained primary CD4⁺ and CD8⁺ T cells similarly. The data are depicted in Fig. 5A as MFI ratios of each mAb in CD4⁺ relative to CD8⁺ T cells, with representative histograms in Fig. 5B. The results showed that, for most of the mAbs assayed, CD4⁺ lymphocytes stained better (MFI ratio >1.5, Fig. 5A) than did CD8⁺ lymphocytes, as described (2). However this was not the case with two mAbs that stained CD8⁺ T cells similarly (MFI ratio = 1.2, WT31; Ref. 16) or even better (MFI ratio <1, RW2-8C8; Ref. 17) than did CD4⁺ T cells. These observations are not consistent with the quantitative interpretation but, rather, with the existence of conformational (i.e. qualitative) differences in the αβ TCR-CD3 complex between CD4⁺ and CD8⁺ T cells.

To determine whether these conformational differences were developmentally regulated or regulated by activation (as described for activated γδ T cells, 18), mature CD4⁺ and CD8⁺ T lymphocytes were analyzed and compared for CD3 (and CD18) expression in four consecutive differentiation stages as follows: 1) intrathymic single positive (CD4⁺CD8⁻ or CD4⁺CD8⁺) early mature T cells (gated as described under “Experimental Procedures”); 2) peripheral blood naive T cells (defined by CD45RA⁺ expression, which identifies recent thymic emigrants, Ref. 19); 3) peripheral blood memory T cells (CD45RO⁺); and 4) recently activated peripheral blood T cells (gated as CD69⁺ after phytohemagglutinin incubation) or constitutively activated H. saimiri (HVS)-transformed T cells (20). The results indicated that the increased RW2-8C8 binding of CD8⁺ as compared with that of CD4⁺ T cells was essentially independent of their activation or differentiation status, although it was more apparent in peripheral T cells (naive, memory, or activated) than in intrathymic single positive T cells (Fig. 5C). In contrast, increased UCHT1 binding of CD4⁺ T cells as compared with that of CD8⁺ T cells was clearly regulated by activation and differentiation, as it occurred only in intrathymic and naive T cells but not in memory or activated T cells. These changes were specific for the TCR-CD3 complex, as CD18 was regulated in an opposite fashion. Interestingly, equivalent UCHT1 binding of CD4⁺ and CD8⁺ memory T cells was acquired through both a decreased binding of CD4⁺ T cells and an increased binding of CD8⁺ T cells relative to their naive counterparts, whereas RW2-8C8 binding differences were unaltered by the naive/memory transition (not shown). Therefore, CD4⁺ and CD8⁺ T cells indeed regulated their αβ TCR-CD3
It has been proposed that lipid raft integrity affects the topological arrangement of the TCR-CD3 complex on the cell surface (10). Also, it is believed that a fraction of TCR-CD3 is raft-associated because of its association with the cytoplasmic portion of the CD8 (or CD4) coreceptor (21). We therefore reasoned that the conformational TCR-CD3 differences observed between CD4+ and CD8+ T cells could be due, at least in part, to the differential arrangement of lipid-raft-associated surface TCR-CD3 clusters or arrays. To test this hypothesis, primary T lymphocytes were treated with MβCD and analyzed for CD3 expression within the CD4+ and CD8bright T cell subsets using several TCR-CD3-specific mAbs (Fig. 5, D and E). MβCD disrupts lipid microdomains by extracting cholesterol from plasma membranes (22). WT31 and OKT3 were used as negative and positive controls for the TCR-CD3 complex, respectively, as described (11). Two different CD18-specific mAbs were used as additional negative controls (10). The results showed that the TCR-CD3 complex was more dependent on lipid raft integrity in CD8+ as compared with CD4+ T cells when probed with several antibodies (RW2-8C8, OKT3, Leu4, Cris7, UCHT1, and BMA031) but not with WT31 (as expected; Ref. 11). These results are consistent with the existence of more lipid raft-associated TCR-CD3 domains (or isoforms) in CD8+ than in CD4+ T cells. Interestingly, RW2-8C8 seemed to bind a relatively lipid raft-independent TCR-CD3 epitope (or isoform) in CD4+, which was lipid raft-dependent in CD8+ T cells. It was surprising to find that the lipid raft-dependent TCR-CD3-specific mAbs were those that bound CD4+ better than CD8+ T cells (compare Fig. 5, A and D).

Because the TCR-CD3 protein components are equivalent in CD8+ and CD4+ T cells, it was also possible that the conformational differences were due, in part, to glycosylation variability of the TCR-CD3 complex, as shown for γδ T cells (12, 18). To test this hypothesis, primary T cells were digested with NANAse and analyzed for CD3 expression within the CD4+ and CD8bright T cell subsets using RW2-8C8 and Leu4 (Fig. 6A). Decreased binding of CD43 was used as an internal control of NANAase treatment, as described (13). The results showed that NANAse-treated CD4+ T cells became indistinguishable from CD8+ T cells using RW2-8C8 but not Leu4, suggesting that NANA-associated glycosylation of the TCR-CD3 components in CD4+ T cells partially hides (or builds) the RW2-8C8 epitope.

To further characterize the RW2-8C8 epitope, competition experiments were performed on several mature and immature T lymphocyte types using phycoerythrin-labeled Leu4 (CD3-specific) or BMA031 (framework TCRαβ-specific). The results...
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Fig. 6. Characterization of the RW2-8C8 antibody. A, purified PBLs from normal donors were NANAse-treated (+) to remove sialic acid residues or left untreated (−) and analyzed for surface binding of RW2-8C8, Leu4, and CD43 (positive control) within CD4+ and CD8+ T cell subsets. B, mature or immature T cells were stained with Leu4-phycoerythrin (or UCHT1, not shown) or BMA031-phycoerythrin cell subsets. B24490 FIG. 6. Characterization of the RW2-8C8 antibody. A], mature or immature T cells were stained with Leu4-phycoerythrin or UCHT1, not shown) or BMA031-phycoerythrin cell subsets. B, mature or immature T cells were stained with Leu4-phycoerythrin or UCHT1, not shown) or BMA031-phycoerythrin cell subsets. B, mature or immature T cells were stained with Leu4-phycoerythrin or UCHT1, not shown) or BMA031-phycoerythrin cell subsets. B, mature or immature T cells were stained with Leu4-phycoerythrin or UCHT1, not shown) or BMA031-phycoerythrin cell subsets.

DISCUSSION

Lineage-associated αβ TCR-CD3 Variation—In the present report, we showed by several experimental approaches that CD4+ and CD8+ mature T lymphocytes do not express identical surface αβ TCR-CD3 complexes. First, CD3γ-deficient CD4+ and CD8+ T cells were shown to differ by the following three criteria: 1) CD3γ chain glycosylation (Fig. 1 and Ref. 6); before (thin histograms) or after (thick histograms) preincubation with unlabeled RW2-8C8, CD18, or UCHT1. Gated TCRγο− PBL reactivity patterns with BMA031 and RW2-8C8 are shown to illustrate that only the latter is CD3-specific (bottom right). C, representative reactivity patterns of RW2-8C8 (right) and Leu4 (left) in γCD4+ (open histograms) and CD8γο−CD4− (filled histograms) peripheral blood lymphocytes. D, comparative reactivity patterns of several mAbs with SupT1 (pTαβCD3γ), Jurkat (αβTCRγ), and CD4+CD8+ (DP) thymocytes (immature αβTCRγ).
2) TCRα and TCRβ chain content (Fig. 2 and 3; and 3) native αβTCR/CD3 migration (Fig. 4). Second, primary normal CD4+ and CD8+ T cells were shown to express conformationally and biochemically different surface αβTCR/CD3 complexes (Figs. 5 and 7, respectively), which were regulated by activation and differentiation for certain epitopes, likely due to lipid raft-dependent arrangements or glycosylation. These results confirm our previous biosynthetic and phenotypic studies in CD3γ-deficient T cells (6) and extend the observations to primary T lymphocytes from normal individuals. Biosynthetic studies showed that CD8+ but not CD4+ CD3γ-deficient T cells contained a small αβ heterodimer composed of abnormally glycosylated TCRβ and an abnormally small CD3-associatd chain that was not recognized by TCR-specific antibodies. However, the TCRα gene was normal, and the atypical 32-kDa TCRα observed intracellularly was indeed shown to be TCRα by protein sequencing using matrix-assisted laser desorption ionization time-of-flight (data not shown).

The TCR/CD3 complex is a very flexible structure. For instance, the γδ TCR (and γδ T cell development) can be quite CD3δ-independent in mice (5, 25). There are descriptions of CD3δ-specific mAb that do not bind the γδ TCR/CD3 complex unless it was deglycosylated previously (WT31, 12), and even T cell activation can modify CD3γ glycosylation (18) with clear biochemical consequences. Similarly, αβTCR/CD3γ expression is possible without CD3γ (4, 8) or δ (5), but not without both (26). Therefore, it is not surprising that further flexibility is present in CD4+ versus CD8+ T cells, particularly when CD3δ is absent, but also in normal donors. The mAbs used for surface TCR/CD3 detection were obtained using different antigens (bulk PBL or thyocytes, purified primary T cells or T cell lines, solubilized cell membranes, purified TCR/CD3 proteins, etc.) and different screening criteria (fresh, fixed, or tumor T cell binding, cytosisis blocking, PBL stimulation, etc.) (27). Therefore, a possible explanation for the observed lineage-specific binding differences could stem from the immunization or screening criteria used in each case; a situation that would favor naïve CD4+ and lipid raft-dependent over naïve CD8+ and lipid-raft-independent TCR/CD3 binding with certain mAbs. These differences could be exploited for therapeutic purposes, such as lineage-specific immunosuppression during graft rejection.

What could be the mechanism responsible for the observed lineage-dependent biochemical TCR/CD3 differences? Because all of the subunits are present in both cases (even with the same stoichiometry, if isolated with digitonin), we believe that there must be biochemical differences in the way the complex is assembled, glycosylated, trimmed, or topologically arranged in the cell surface in each T cell subset after the lineage decision is reached and apparently also upon antigen recognition (28).

The reported physical association of CD3δ with the raft-resident coreceptor molecules CD4 and CD8 on resting T lymphocytes could contribute to the observed differences (21, 29). In addition, the individual TCR/CD3 subunits could possess slightly distinct structures. These differences could be caused by developmentally acquired fundamental changes that distinguish the two cell types, including the glycosylation machinery (30, 31) or chaperones. For instance, the inactivation of a sialyltransferase (ST3Gal-I) strongly reduced peripheral CD8+ but not CD4+ T cell numbers (32). Further work is required to address this issue, perhaps through a comparative genomic approach that includes the chaperones and enzymes involved in glycosylation pathways. Interestingly, TCR/CD3 signaling capacity and dynamics are not affected by the observed biochemical differences, either with or without CD3δ (6–8), further suggesting that the changes are required for optimal receptor-coreceptor cis interactions during antigen recognition and signal transduction in each cell type (33). An alternative interpretation is that two different TCR populations exist on the cell membrane and that their relative proportions change in different T cell subsets. Antibodies such as RW2-8C8 may detect those changes.

αβTCR/CD3 Structure and Function—The results are consistent with a recent report on the structure and stoichiometry of the TCR/CD3 complex in vitro (34). The authors demonstrated that CD3γ is important for incorporation of ζ but not ε to the complex, as shown in Fig. 1A (CD3-associated ζ is very scarce in γγ cells) and in Ref. 6. They also proved that, whereas TCRα is strictly associated to ε dimers, TCRβ can interact with γε as well as with δε dimers. This apparent biochemical promiscuity may explain why surface TCR/CD3 adopts a αβεδεζεζ stoichiometry (Fig. 4) and why its expression is so notable despite the lack of CD3γ (7). Although the
same authors showed that isolated αβ and δε dimers do not normally assemble into αβ(δε)2 complexes in vitro, intrathymic selection mechanisms may expand, in vivo, the otherwise rare T cell precursors that manage to assemble a viable surface TCR-CD3 complex with such stoichiometry. In vivo observations further support this contention, because human CD3δy-deficient individuals showed significant T cell development with only mild lymphopenia and immunodeficiency, whereas CD3δ-deficient patients had no T cells and very severe clinical abnormalities (35).

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