

J Immunol 2016; 196:3509-3513; ;
http://www.jimmunol.org/content/196/9/3509.citation

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Identification of a putative second T-cell receptor

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Framework monoclonal antibodies have identified a population of human lymphocytes that express the T3 glycoprotein but not the T-cell receptor (TCR) α- and β-subunits. Chemical crosslinking experiments reveal that these lymphocytes express novel T3-associated polypeptides, one of which appears to be the product of the Ty gene. The other polypeptide may represent a fourth TCR subunit, designated Tδ.

Understanding T-cell recognition of antigen and the restriction of this process by major histocompatibility complex (MHC)-encoded antigens has been an important goal in immunology. A major step forward occurred with the immunological identification of clone-specific disulfide-linked heterodimers on T cells, composed of subunits termed TCR α and β (relative molecular mass (Mr) 50,000 (50K) and 40K, respectively)1,2. Genes that rearrange during thymic ontogeny and encode the TCR β5 and α6 subunits were isolated either by subtractive hybridization or by probing with oligonucleotides. A unique feature of the human TCR αβ was the observed co-modulation7, co-immunoprecipitation8, and required co-expression9 of the TCR αβ molecules with the T3 glycoprotein, which suggested that these two structures were related. Subsequently, the direct physical association of the two protein components was demonstrated by chemically crosslinking the TCR αβ molecules to the T3 glycoprotein and identifying the components of the crosslinked complex as the TCR β-subunit and the T3 glycoprotein 28K subunit11. A T3 counterpart is similarly associated with murine TCR αβ12,14. A third gene that rearranges in T cells, designated Ty, has been identified in mouse15 and man16,17. Ty gene rearrangements occur in lymphocytes with suppressor-cytotoxic as well as helper phenotypes and may produce a large number of Ty chains18-20. However, the function of the Ty gene is unknown. Furthermore, neither the protein encoded by the Ty gene nor its possible association with other structures (as occurs with TCR αβ and T3 glycoproteins) has been defined.

It appears increasingly likely that the TCR αβ molecule alone determines both antigen recognition and MHC restriction on at least some T cells21,22. However, it is unclear whether this receptor accounts for the process of T-cell selection during thymic ontogeny or for all antigen-specific recognition by mature T cells. For example, suppressor T lymphocytes remain an enigma; in some cases they delete or fail to rearrange TCR β gene23,24. Thus, it is of great importance to determine whether a second T-cell receptor exists, to define its structure (particularly with regard to the possible use of the Ty gene product) and ultimately to understand its function(s).

Here, framework monoclonal antibodies that react with shared determinants on human TCR αβ molecules were used to identify peripheral blood lymphocytes (PBL) that were T3 but did not react with these monoclonal antibodies. We reasoned that such lymphocytes might express non-α, non-β T3-associated structures and that each molecule might represent a second T-cell receptor complex. This strategy resulted in the identification of a T3-associated molecular complex on the surface of human lymphocytes that do not express detectable TCR α and β transcripts or polypeptides. We suggest that this complex is a putative second T-cell receptor.

Monoclonal antibodies

A murine framework antiserum that recognizes most human TCR αβ molecules has been reported previously25. Subsequently, a murine monoclonal antibody, called Β Framework 1 (BF1), that is reactive with shared determinants on the human TCR β-chain was obtained (M.B.B. et al, in preparation). The BF1 antibody reacts with most T3 human PBL and is capable of immunoprecipitating the TCR αβ heterodimer from all human T-cell lines examined that have αβ T-cell receptors and express the T3 glycoprotein. Immunoprecipitations from a panel of T-cell lines using BF1 demonstrate this reactivity as well as the heterogeneity of the TCR α- and β-subunits from different receptors (Fig. 1a). Like the framework antiserum26, this monoclonal antibody does not stain the surface of living T cells, but will react specifically with both membrane and cytoplasmic T-cell receptors after partial solubilization of the lymphocyte plasma membrane with 70% ethanol. Double staining of human PBL with fluorescein-conjugated anti-T3 monoclonal antibody and biotinyl-BF1 monoclonal antibody, followed by phycoerythrin-conjugated avidin reveals that BF1 recognizes 95-97% of peripheral blood T3 lymphocytes. However, it clearly defines a small population of T lymphocytes that is BF1+, yet T3 (Fig. 1c).

A second framework monoclonal antibody, WT31, initially thought to recognize the T3 antigen27, has recently been shown to react with a common epitope of human TCR αβ28. While double staining with anti-T3 monoclonal antibody (OKT3) and WT31 revealed that each antibody blocks binding of the other, one-colour fluorescence indicated that WT31 typically recognizes 1-3% fewer cells in peripheral blood than does anti-T3 (data not shown). WT31 efficiently binds to the surface of T cells (for example, in fluorescence-activated cell sorter (FACS) analyses) and is capable of immunoprecipitating the TCR αβ molecules, albeit inefficiently, from radiolabelled detergent lysates29 (see also Fig. 1a, lane 3). Thus, BF1 and WT31 appear to recognize all but a small fraction of human peripheral blood T3 cells, and define a subpopulation that is T3 but unreactive.

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Fig. 1 Reactivity of framework monoclonal antibodies recognizing TCR αβ α, Immunoprecipitates from 125I-labelled lymphocyte lysates were analysed by SDS-PAGE. The radiiodinated T-cell leukaemia lines HPB-MLT and Jurkat, the human T-lymphotropic virus type I-transformed cell line ANITA and resting PBL were solubilized in 1% Triton X-100 and immunoprecipitated with a control antibody, normal mouse serum (NMS) (lane 1) for each cell line) or framework antibody to TCR αβ antibody 81F1 (lane 2) for each cell line. β, 125I-labelled lymphocytes were solubilized in 0.1% Triton X-100 and immunoprecipitated with NMS (lane 1), anti-β3 antibody, UCHT-1 (ref. 40) (lane 2) or framework antibody to TCR αβ, WT31 (lane 3). The efficiency of immunoprecipitation with WT31 was improved at the lower concentration of Triton X-100 used here. α, Two-colour FACs analysis of normal adult PBL using anti-TCR αβ and anti-β3 monoclonal antibodies. PBL were stained first with FITC-conjugated anti-β3 antibody (OKT3) and then with biotinylated anti-TCR αβ antibody (81F1) followed by phycoerythrin-conjugated avidin (PE-avidin, Becton Dickinson). Red and green fluorescence were measured in comparison with nonspecific control FITC- and biotin-conjugated monoclonal antibodies (data not shown). Cells unreactive with either antibody are non-T cells (lower left corner); cells that are doubly positive, reacting with both OKT3 and 81F1, make up the large population of lymphocytes in the centre of the grid; cells that are βF1+ but OKT3- comprise the narrow group of lymphocytes (4% of the T3+ cells) observed along the x-axis.

Methods. Viable lymphocytes were isolated by Ficoll-Hypaque density gradient centrifugation for both SDS-PAGE and FACS analyses. For SDS-PAGE, lymphocytes were radioiodinated by the lactoperoxidase technique, solubilized in 1% Triton X-100 and immunoprecipitated using 1 μg of specific antibody (81F1 or UCHT-1) or 1 μl of NMS. Monoclonal antibody 187-1 (3 μg) was used as a second antibody for 81F1 and UCHT-1 (ref. 47). The immunoprecipitates were then analysed by SDS (10.5%) PAGE under reducing conditions. The 125I-labelled molecules were visualized by autoradiography as described previously. Two-colour cytofluorographic analysis was performed by first staining PBL with FITC-OKT3 for 45 min at 4°C. After washing, the lymphocytes were fixed in 1% paraformaldehyde for 15 min at 23°C and then incubated in 70% ethanol in phosphate-buffered saline (PBS) for 5 min at −20°C. After further washing, the cells were stained with biotinyl-βF1 followed by PE-avidin. Analysis was performed on an Ortho cytofluorograph.

<p>| Table 1 Phenotypes of cell lines derived from PBL of immunodeficiency patients |
|---------------------------------|------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source</th>
<th>Description</th>
<th>% Positive for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT31</td>
<td></td>
<td></td>
<td>T3</td>
</tr>
<tr>
<td>1 IDP1</td>
<td>Alto</td>
<td>A</td>
<td>50</td>
</tr>
<tr>
<td>2 IDP1</td>
<td>WT31* sort</td>
<td>A</td>
<td>100</td>
</tr>
<tr>
<td>3 IDP1</td>
<td>WT31* sort</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>4 IDP2</td>
<td>Fresh PBL</td>
<td>A</td>
<td>61</td>
</tr>
<tr>
<td>5 IDP2</td>
<td>PHA</td>
<td>A</td>
<td>100</td>
</tr>
<tr>
<td>6 IDP2</td>
<td>Alto</td>
<td>A</td>
<td>2</td>
</tr>
<tr>
<td>7 IDP2</td>
<td>PHA</td>
<td>A</td>
<td>12</td>
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The description of each cell line indicates either the conditions for activation of the lymphocytes, their source. Allantigen specific (allo) activated cultures were stimulated with irradiated allogeneic PBL at weekly intervals. Mitogen (phytohaemagglutinin, PHA)-activated lines were stimulated with a 1:1,000 dilution of PHA (Difco) at culture initiation. WT31* and WT31* sorted cell lines 2 and 3 (sort) were obtained from IDP1 cell line 1 by cell sorting analysis. All cell lines were propagated in vitro in media composed of RPMI 1640, 10% human serum and conditioned media containing 2-5 units of IL-2 as described previously. Visible lymphocytes were isolated by Ficoll-Hypaque density gradient centrifugation and stained with 0.5 μg of WT31, OKT3, OKT4 or OKT8 (Ortho, Raritan, New Jersey) for 30 min at 4°C. After washing, the cell pellets were stained again with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse F(ab')2 fragments. FACS analyses were performed on an Ortho cytofluorograph or a Coulter Epics as described previously. Specifically stained positive cells were determined relative to a negative control profile for each cell line (stained with nonspecific control monoclonal F.J.X6.Ag8). Cells having fluorescence intensity channel numbers greater than the intercept of the negative control profile with the baseline were counted as positive, and the per cent positive was calculated relative to the total number of cells counted.

with both of these framework monoclonal antibodies against the TCR αβ molecules. Evidence that the T3+ lymphocytes that are unreactive with βF1 are also unreactive with WT31 is presented below. We have used WT31 primarily for FACS analyses and βF1 primarily for immunoprecipitation studies.

**Isolation of WT31** T3+ lymphocytes

Efforts at growing the WT31 T3+ population from normal adult PBL proved difficult, as the WT31 T3+ lymphocytes usually overgrew the WT31 T3+ cells after mitogenic stimulation. However, growth of the WT31 T3+ population from the PBL of immunodeficiency patients was successful. Immunodeficiency patient 1 (IDP1) suffered from the bare lymphocyte syndrome, and lacked class II MHC antigen expression on lymphoid cells11,12, while immunodeficiency patient 2 (IDP2) suffered from an ectodermal dysplasia syndrome13 and displayed poor in vitro T-cell proliferative responses to mitogens.

After activation of PBL from IDP1 with alloantigen and propagation in conditioned media containing interleukin-2 (IL-2), the resultant cell line was observed to be ~50% WT31 T3+ and 50% WT31 T3+ (Table 1, cell line 1). Subsequent sorting of this cell line yielded homogeneous populations of WT31 T3+ cells and WT31 T3+ cells (Table 1, cell lines 2 and 3, respectively).

Cell lines were also obtained from IDP2; 63% of the PBL from IDP2 were T3+ and 1-3% fewer cells (61%) were WT31*, which is typical of normal PBL (Table 1, cell line 4). Activation of these IDP2 PBL with either phytohaemagglutinin (PHA) or alloantigen and propagation in vitro with conditioned media resulted in several cell lines, including a homogeneous WT31 T3+ cell line (Table 1, cell line 5), a homogeneous WT31 T3+ cell line (cell line 6) and, on a third occasion, a cell line that was 88% WT31 T3+ (with 12% contaminating WT31 T3+ cells) (cell line 7). Note that the WT31 T3+ popula-
tion contained both T4+T8+ and T4−T8− cells (cell lines 3, 6 and 7). Further phenotypic analyses revealed that this population was T11+ but negative for natural killer cell markers such as Leu 7, Leu 11 and OKM1 and for the immature thymocyte marker T6 (data not shown).

WT31+ T3+ cells are negative for TCR αβ

Having identified WT31+ T3+ and WT31− T3− populations, we sought to determine whether these lymphocytes expressed TCR αβ molecules that could be immunoprecipitated by βF1. βF1 immunochromically defined a heterodimeric structure on the surface of 125I-labelled WT31+ T3+ IDP1 lymphocytes (Fig. 2a, lane 5), it failed to recognize a similar protein on the WT31− T3− population from the same individual (Fig. 2a, lane 11). Similar analysis of IDP2 cell lines revealed a trace of TCR αβ on the 88% WT31+ T3+ cell line 7 (Fig. 2b, lane 2) consistent with the 12% contamination with WT31+ T3− cells. Thus, the WT31+ T3+ cell lines, identified by their cell surface reactivity with antibody WT31 on FACScan analysis, were also βF1−, as determined by lack of TCR αβ immunoprecipitation. Note that all WT31+ T3+ and WT31− T3− cell lines expressed similar amounts of T3 as determined by FACScan analysis (data not shown) and by immunoprecipitation with anti-T3 antibody (Fig. 2a, lanes 3 and 9, and c, lanes 2 and 4). However, the T3 molecule on WT31+ T3+ and WT31+ T3− lymphocytes was not identical to that on WT31+ T3+ cell lines, as revealed SDS-polyacrylamide gel electrophoresis (SDS-PAGE). One- (Fig. 2c) and two-dimensional (data not shown) gel analysis indicated that the difference in T3 was restricted to the light T3 subunits, which reproducibly exhibited different SDS-PAGE mobilities (Fig. 2c, arrowhead).

to determine whether the WT31+ βF1+ T3+ population lacked TCR αβ molecules, or alternatively expressed TCR αβ molecules that failed to react with the monoclonal antibodies used, we analysed the cells for messenger RNAs encoding the TCR α and β proteins. 2P-labelled complementary DNA (cDNA) encoding TCR α, TCR β and Ty were used to probe Northern blots containing whole-cell RNA from WT31+ βF1+ T3+ and WT31− βF1− T3− IDP2 cell lines and from HPB-MLT, which is known to contain mRNAs for TCR α, TCR β and Ty. No TCR α or β mRNA transcripts were detected in the RNA from the WT31+ βF1+ T3+ IDP2 cell line 6 (Fig. 3a, α-probe, lane 1; β-probe, lane 1), whereas expression of both was clearly detectable in RNA from HPB-MLT (Fig. 3a, α-probe, lane 2; β-probe, lane 2). Notably, Ty mRNA was present in the WT31+ T3+ cells at a level comparable to that in HPB-MLT (Fig. 3a, γ-probe, lanes 1 and 2). Thus, the WT31+ βF1+ T3+ lymphocytes lacked TCR α and β mRNA. Subsequent experiments on cell lines that were mostly WT31+ T3+ corroborated this result. For example, Northern blot analysis of IDP2 cell line 7 (88% WT31+ T3+) in comparison with IDP2 cell line 5 (WT31+ T3+), as well as with HPB-MLT cells, revealed only a trace of TCR α or β mRNA in the 88% WT31+ T3+ cells (con-
consistent with the 12% contamination with WT31 T3 cells) (Fig. 3a, lane 2 for each probe). Further, most of the β transcripts that could be detected were 1.0 and not 1.3 kilobases (kb) long and were probably nonfunctional. In contrast, the IDP2 cell line 5 (WT31 T3) expressed levels of both RNA species which were comparable to those in HPB-MLT (Fig. 3b, lane 1 for each probe). However, like the WT31 T3 lines shown in Fig. 3a, β and the WT31 T3 and the WT31 T3 lines showed levels of Ty RNA comparable to that in HPB-MLT (Fig. 3b, γ-probe).

Thus, the WT31 T3 cells lacked α and β T-cell receptor mRNAs and β T-cell receptor proteins (by immunoprecipitation and FACS analysis). The presence of Ty mRNA in WT31 T3 cells, while consistent with Ty protein expression, could not be taken as strong evidence for this, as many humoral cell lines that express Ty mRNA of normal size may express full-length transcripts that are out of frame because of defective joining of the V (variable) and J (joining) regions.

Non-α, non-β T3-associated molecules

We used chemical crosslinking to determine whether proteins analogous to the TCR αβ molecules existed on the WT31 T3 T3 cells. This technique has demonstrated the physical association of the TCR αβ molecules with the T3 glycoprotein. The bifunctional, cleavable reagent diethylenetriaminepentaacetic acid (DTPA), was used to crosslink labeled surface proteins of viable T lymphocytes. After crosslinking, the lymphocytes were solubilized in non-ionic detergent and immunoprecipitated with anti-α antibody. As expected, in the WT31 T3 T3 lymphocytes the TCR α and β-chains were found to be crosslinked to T3. For example, TCR αβ molecules (Fig. 2a, lane 4 and 6). However, despite the lack of reactivity with βF1 and the lack of TCR α or β mRNA, IDP1 cell line 3 (WT31 T3) and IDP2 cell line 7 (88% T31 T3), both also expressed two protein subunits (55K and 40K) that specifically crosslinked to T3 (Fig. 2a, lane 10 and b, lane 6). Note that the mobilities of these T3-associated molecules were clearly different from those of the TCR α and β-chains from WT31 T3 cell lines (compare Fig. 2a, lanes 4 and 10, or b, lanes 5 and 6).

As IDP2 cell line 7 (88% WT31 T3) contained 12% WT31 T3 cells (accounting for the lack βF1 immunoprecipitated, Fig. 2a, lane 2), the lysate from these cells was crosslinked, the TCR αβ protein by βF1. After crosslinking, no residual βF1-reactive material could be detected (Fig. 2b, lanes 8 and 11). When this βF1-reacted lysate from crosslinked cells was immunoprecipitated with anti-β, 55K and 40K subunits were still detected (Fig. 3a, lane 12).

Because these WT31 T3 T3 cell lines do not apparently express TCR α and β mRNAs, the molecules found specifically crosslinked to T3 on their cell surfaces cannot represent proteins encoded by the known TCR α or β genes. We therefore considered the possibility that one of these polypeptides represents the product of the rearranging Ty gene.

Isolation of T3-associated 55K subunit

cDNA clones representing the rearranging human Ty gene would encode a polypeptide with a predicted Mr of 40,000 (ref. 36). However, unlike the murine Ty gene, which does not reveal any N-linked glycosylation sites, the human Ty gene has five potential sites for N-linked glycosylation, four of which are located in the constant region. As a Ty protein that has not been isolated previously, it is unknown how many of these potential sites are used. However, a fully glycosylated human Ty protein may have a Mr of ~55,000. The heavy chain of the non-α, non-β T3-associated subunits identified on the WT31 T3 T3, IDP1 and IDP2 cell lines described in the present report had a relative mobility of 55K on SDS-PAGE (Fig. 2a, b). To determine whether this T3-associated heavy chain was serologically cross-reactive with or identical to the Ty protein, antisera were raised against synthetic peptides representing, respectively, a stretch of 17 amino acids (residues 5–21) from the variable region (αV, antisera) and a stretch of 24 amino acids (residues 117–136) from the constant region (anti-α, antisera) of the Ty amino-acid sequence deduced from a human cDNA clone. Both the anti-α, antisera and the anti-V, antisera immunoprecipitated a molecule with a Mr of 55,000 from the denatured lysate of WT31 T3 T3 cells (Fig. 4a, lanes 2 and 4). Such molecules could not be immunoprecipitated from lysates of WT31 T3 T3 cells, which express only nonfunctional Ty mRNA (data not shown).
To demonstrate that the 55K molecule immunoprecipitated by anti-\( \alpha \) and anti-\( \nu \) was, in fact, the heavy subunit that crosslinked to T3, an additional experiment was performed (Fig. 4b). A sample of DSP-crosslinked lysate from the WT31 \( \beta^{+} \) T3 cells was first immunoprecipitated with anti-T3, again demonstrating the association of 55K and 40K subunits with T3 (Fig. 4b, lane 2). In parallel, another aliquot of the crosslinked lysate was immunoprecipitated with an anti-T3 monoclonal antibody, and the immunoprecipitated T3-crosslinked polypeptides were eluted from the immunoabsorbent, under denaturing and reducing conditions in order to break the DSP crosslink. This eluate was then re-precipitated with anti-C, antisera; the 55K subunit that crosslinked to T3 was re-precipitated by the antisera (Fig. 4b, lane 5), indicating that the 55K subunits defined by these two approaches were identical.

**Conclusions**

Framework mononuclear antibodies (\( \beta^{+} \) and WT31) against the TCR \( \alpha \beta \) molecules were used to identify and isolate a WT31 \( \beta^{+} \) T3 lymphocyte population from the PBL of two immunodeficiency patients. By the criteria of both immunoprecipitation analysis with framework mononuclear antibody and Northern blot analysis using TCR \( \alpha \) and \( \beta \)-specific cDNA probes, polyclonal human T-cell line of this phenotype were shown to express neither TCR \( \alpha \beta \) mRNA transcripts nor polypeptides. Nevertheless, chemical crosslinking studies using the cleavable DSP reagent revealed the existence of a protein complex associated with the T3 glycoprotein on the surface of these cells. The heavier of the two subunits that crosslinked to T3 (55K) was also immunoprecipitated by two different antisera, one generated against a 17-amino-acid synthetic peptide corresponding to a part of the variable region and another generated against a 20-amino-acid synthetic peptide corresponding to a part of the constant region of the deduced amino-acid sequence of a rearranged Tc gene.\(^{2,3} \) Thus, it seems that the 55K protein is the Tc protein encoded by the rearranged Tc gene\(^{2,3} \) (or, less likely, a protein highly cross-reactive with it). Final proof of this, however, must await protein sequence determination and comparison with the deduced amino-acid sequences of rearranged Tc cDNA clones. The 40K polypeptide appears to be a novel fourth T3-associated protein that we term T3\(^{2,3} \). The T3 and T6 polypeptides may form a T3-associated heterodimeric structure (T6\( \times \)T3) on these cells that is analogous to the previously described Tc-cell receptor complex (TCR-\( \alpha \beta \)). Alternatively, since the cell lines examined are polyclonal, the 55K and 40K polypeptides may occur as monomeric T3-associated subunits on individual T-cell clones.

The complex described here has several important characteristics that might be expected of a second T-cell receptor, that is, it is physically associated with T3 and one of the T3-associated chains is recognized by several anti-T3 antisera. We do not know at present whether the T6 and T3 components are distinct gene products, whether they are covalently linked via an interchain disulfide bond, whether they display clonal heterogeneity on T cells, or if ligands that react specifically with this protein complex are capable of triggering cell proliferation or factor production as occurs with the TCR \( \alpha \beta \). However, in another study, human thymus-derived clones of the same phenotype as the IDP1 and IDP2 cell lines described here (\( \beta^{+} \) T3\(^{+} \)) were stimulated to proliferate and to secrete IL-2 in response to anti-T3 antibodies (see page 179 of this issue\(^{28} \)). Thus, pending the outcome of the peptide mapping and additional functional experiments, the T3-associated complex demonstrated here represents a likely candidate for a second T-cell receptor.

The function of the WT31 \( \beta^{+} \) T3 cells is unknown. Tc mRNA expression is highest early in murine thymic ontogeny and declines thereafter, suggesting that the Tc polypeptide may be functionally important only early in thymic ontogeny.\(^{27,28} \) Since the putative second T-cell receptor complex reported here has been identified in immunodeficiency patients, it may be expressed on cells arrested at an early stage of thymic ontogeny in these patients. For example, about 50% of the WT31 \( \beta^{+} \) T3 cells were T4\( \times \)T8 T3, which is similar in phenotype to an identified subpopulation of human thymocytes (see ref. 39 and J. Allison and J. Lanier, personal communication). However, it is clear that the \( \beta^{+} \) T3 phenotype occurs on a subpopulation of normal human PBL (Fig. 1c). These PBL may similarly express the non-\( \alpha \), non-T3 associated complex described here. If so, this putative second T-cell receptor may occur on mature cells of a lineage which is separate, although related, to that of TCR \( \alpha \beta \)-expressing T cells. Additional characterization of these novel T3-associated molecules and the cells that express them further our understanding of T-cell ontogeny as well as mature T-cell function.

We thank Drs W. T. Trowbridge and P. Beverley for providing monoclonal antibodies, Drs S. Burakoff, T. Springer and J. Fraser for critical comments on the manuscript, and B. Hoffman for assistance with two-colour cytofluorographic analysis. This work was supported by grant ACS-NY-MR-425, NIH grant SRO-115669, grant 2P50AI21163 and by budgets from the Leukemia Research Foundation and from Hoechst Akiziegellschaft.

**Note added in proof:** The 55K and 40K associated polypeptides on IDP2 cell lines are not disulphide-linked.