Low Activation Threshold As a Mechanism for Ligand-Independent Signaling in Pre-T Cells

Mariëlle C. Haks, Stanley M. Belkowski, Maria Ciofani, Michele Rhodes, Juliette M. Lefebvre, Sebastián Trop, Patrice Hugo, Juan Carlos Zúñiga-Pflücker and David L. Wiest

*J Immunol* 2003; 170:2853-2861; doi: 10.4049/jimmunol.170.6.2853

http://www.jimmunol.org/content/170/6/2853

---

**References**
This article cites 44 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/170/6/2853.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Low Activation Threshold As a Mechanism for Ligand-Independent Signaling in Pre-T Cells

Mariëllé C. Haks,* Stanley M. Belkowski,* Maria Ciofani,† Michele Rhodes,* Juliette M. Lefebvre,* Sebastián Trop,† Patrice Hugo,‡ Juan Carlos Zúñiga-Pflücker,† and David L. Wiest²*

Pre-TCR complexes are thought to signal in a ligand-independent manner because they are constitutively targeted to lipid rafts. We report that ligand-independent signaling is not a unique capability of the pre-TCR complex. Indeed, the TCRα subunit restores development of pTα-deficient thymocytes to the CD4⁺CD8⁺ stage even in the absence of conventional MHC class I and II ligands. Moreover, we found that pre-TCR and αβTCR complexes exhibit no appreciable difference in their association with lipid rafts, suggesting that ligand-independence is a function of the CD4⁺CD8⁻ (DN) thymocytes in which pre-TCR signaling occurs. In agreement, we found that only CD4⁺CD25⁺ DN thymocytes (DN3) enabled activation of extracellular signal-regulated kinases by the pre-TCR complex. DN thymocytes also exhibited a lower signaling threshold relative to CD4⁺CD8⁺ thymocytes, which was associated with both the markedly elevated lipid raft content of their plasma membranes and more robust capacitative Ca²⁺ entry. Taken together these data suggest that cell-autonomous, ligand-independent signaling is primarily a property of the thymocytes in which pre-TCR signaling occurs. The Journal of Immunology, 2003, 170: 2853–2861.

Development of immature CD4⁺CD8⁻ double negative (DN) thymocytes to the CD4⁺CD8⁺ double positive (DP) stage is linked to productive rearrangement of the TCRβ locus by signals transduced through a developmental fore-runner of the TCR termed the pre-TCR complex. Although it is clear that the transition from the DN to DP stage requires pre-TCR signaling, the way in which those pre-TCR signals are initiated remains unclear.

Whereas signaling through most lymphoid surface receptors, including the mature TCRαβ complex, is triggered by ligand-engagement, this does not appear to be the case for the pre-TCR complex, which is thought to signal in a cell-autonomous, ligand-independent manner. In support of this view, the extracellular Ig domains of both pTα and TCRβ are dispensable for pre-TCR function (1). In the absence of the exodomain Ig loops of pTα and TCRβ, the only potential ligand-binding sites remaining appear to be the exodomains of the CD3 subunits. CD3 subunits are expressed on the surface of thymocytes in the form of clonotype-independent CD3 complexes even in the absence of pTα or TCRβ, yet these CD3 complexes fail to transduce β-selection signals unless engaged by mAbs (2). Moreover, mAb engagement of pre-TCR complexes on pre-T cells arrests thymocyte development before their arrival at the DP stage (3). Taken together, these data suggest that engagement by a specific ligand is not responsible for initiation of pre-TCR signaling in vivo.

The ability of the pre-TCR to signal in a ligand-independent manner can be explained in two ways. First, it is possible that the ligand independence of the pre-TCR complex is an intrinsic property of the thymocyte subpopulation. Immature thymocytes in which pre-TCR signaling is initiated may have signaling thresholds that are sufficiently low to obviate the need for ligand engagement. Alternatively, the ability to transduce β-selection signals when not engaged by a ligand may be an innate property of the pre-TCR complex conferred upon it by pTα because pTα is the only receptor subunit unique to the pre-TCR complex (4). A recent report suggested that the ligand independence of the pre-TCR results from its constitutive targeting to specialized membrane domains termed rafts, which are highly enriched in sphingolipid, cholesterol, and signaling protein components and are important in activation of immunoreceptors (5–7). Because signaling molecules have been shown to be targeted to rafts via posttranslational modification with lipids, it was proposed that pre-TCR complexes are likewise targeted to rafts via palmitoylation of a juxtamembrane cysteine residue of pTα (6–9). However, mutagenesis of pTα-C176 did not impair the ability of the pre-TCR complex to signal development (10). Nevertheless, it was possible that another pTα motif might enable pre-TCR complexes to signal in a ligand-independent manner. pTα mutagenesis intended to address this possibility produced discrepant results. Two reports collectively indicated that the ectodomain as well as a large proportion of the intracellular tail of pTα were dispensable for proper pre-TCR function (11, 12); however, another report suggested that deletion

*Division of Basic Sciences, Immunobiology Working Group, Fox Chase Cancer Center, Philadelphia, PA 19111; †Department of Immunology, University of Toronto, Sunnybrook & Women’s College Health Sciences Center, Toronto, Ontario, Canada; ‡PROCREA Biosciences Inc., Montréal, Québec, Canada

Received for publication October 29, 2002. Accepted for publication January 8, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by American Cancer Society Grant RSG-01-084-01-LIB, National Institutes of Health Grants CA73656 and CA087047, National Institutes of Health Core Grant P01CA06927, and an appropriation from the Commonwealth of Pennsylvania. M.C.H. was supported by TALENT-Stipendium S 92-210 from the Netherlands Organization for Scientific Research (NWO-MW) and by the Fox Chase Cancer Center Board of Directors’ Postdoctoral Fellowship.

2 Address correspondence and reprint requests to Dr. David L. Wiest, Division of Basic Sciences, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111. E-mail address: DL_Wiest@fccc.edu

3 Abbreviations used in this paper: DN, CD4⁻CD8⁻; DP, CD4⁺CD8⁺; CP, connecting peptide; CRAC, Ca²⁺ release-activated Ca²⁺-; CTAB, cholera toxin B subunit; Tg, transgenic; DN3, CD4⁻CD25⁻CD4⁺CD8⁺; DN4, CD4⁻CD25⁻CD4⁺CD8⁺; eGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; FTOC, fetal thymic organ culture; GM1, GM1 ganglioside; LZR5, LZR-SpBMN-linker-IREs-eGFP; pTα, pre-Tα; pTαC₁₇₆, cysteine 176 of pTα; SA, streptavidin; TF-R, transferrin receptor.

Copyright © 2003 by The American Association of Immunologists, Inc.
of the cytoplasmic tail moderately impaired thymocyte development (10). Even in the lone report suggesting that the cytoplasmic tail of pTα plays a role in pre-TCR signaling, deletion of the tail only partially blocked thymocyte development suggesting that this is not the entire explanation. Consistent with this view, there is evidence suggesting that the pre-TCR is not unique in its ability to promote traversal of the β-selection checkpoint. Indeed, despite the fact that TCRα contains neither a juxtamembrane cysteine nor an extensive cytoplasmic tail and requires ligand engagement for raft targeting, the αβTCR complex was able to restore development of pTα-deficient thymocytes beyond the β-selection checkpoint (6, 13, 14). However, because the H-Y transgenic (Tg) TCR complexes used in these experiments were expressed in thymocytes containing selecting ligands, it is possible that MHC-ligand engagement of the αβTCR was responsible for the ability of TCRα to substitute for pTα during β-selection. Consequently, it remains unclear whether the ability to signal in a ligand-independent manner is a unique property of the pre-TCR conferred upon it by pTα or alternatively is an intrinsic property of the DN thymocytes in which β-selection occurs.

To distinguish between these possibilities, we asked whether the αβTCR complex could substitute for the pre-TCR complex in the absence of conventional ligand. We demonstrate that TCRα is able to complement pTα deficiency even in the absence of both MHC class I and class II. Moreover, we found only limited association of the pre-TCR complex with lipid rafts and this did not differ appreciably from that observed for αβTCR complexes. Importantly, we did observe that thymocytes exhibited developmental differences in their ability to support pre-TCR signaling. Only CD44+ CD25+ DN thymocytes (DN3) were able to support cell-autonomous activation of extracellular signal-regulated kinases (ERK) by the pre-TCR complex. DN thymocytes also appear to have a lower signaling threshold than DP thymocytes, as indicated by their ability to mobilize Ca²⁺ in response to ligand engagement. This may result from the high lipid raft content of their plasma membranes, which is far greater than that of DP thymocytes. Alternatively, it may reflect differences in expression or sensitivity of calcium channels, as DN thymocytes also exhibit more robust capacitative calcium (Ca²⁺) entry than do DP thymocytes. Taken together, these data support the view that the ability to signal in a ligand-independent manner is primarily a property of DN thymocytes, rather than a unique feature of the pre-TCR complex.

Materials and Methods

Mice

All mice were maintained under specific pathogen-free conditions in the American Association for the Accreditation of Laboratory Animal Care-accredited animal colony of the Fox Chase Cancer Center (Philadelphia, PA). Mice double deficient for MHC class I/class II were purchased from Taconic Farms (Germantown, NY); mice deficient for pTα were kindly provided by Dr. H. von Boehmer (Harvard University, Cambridge, MA); TCRα-deficient mice were purchased from the Jackson Laboratory (Bar Harbor, ME); and TCRβ-Tg SCID mice were generated by backcrossing the 2B4 TCRβ transgene onto the SCID background in our facility (15). The background strains of mice deficient for pTα, TCRα, and MHC class I/class II were all a combination of 129 and C57Bl/6. The background of the TCRβ-Tg SCID mice was C.B-17.

Flow cytometry

Thymic lobes were gently ground with a syringe plunger in PBA (PBS, 1% BSA, 0.02% NaN₃) to produce a single cell suspension that was filtered through mesh, dispensed into a round bottom microtiter plate (1 × 10⁵–1 × 10⁶ cells per well), and stained and analyzed as previously described (16).

Biotinylated, FITC-, PE- or APC-conjugated mAb specific for murine CD4, CD8α, CD8β, CD25, CD44, TCRβ, TCRγδ, and purified anti-mouse MHC class I H-2Kβ/H-2Dβ and anti-mouse MHC class II I-A/E mAb were obtained from BD Pharmingen (San Diego, CA). Where appropriate, avidin-Texas Red or streptavidin (SA)-APC (both from BD Pharmingen) were used as second-stage reagents.

Recombinant retrovirus production

Murine Flag-tagged pTα, Flag-tagged pTα-C1, pTα, TCRα, and TCRα-connecting peptide (CP)-pTα (17) constructs were generated by standard PCR methodologies and subcloned into the retroviral vector LZRSpBMN-linker-ires-eGFP (LZRS), which encompasses an internal ribosomal entry site (IRES) that allows cap independent translation of the enhanced green fluorescence protein (eGFP) marker. Retroviral vectors were transiently transfected into Phoenix-E packaging cells (Dr. G. Nolan, Stanford University, Stanford, CA) using the calcium phosphate transfection system according to the manufacturer’s protocol (Life Technologies, Paisley, Scotland).

Retroviral transduction of fetal thymocytes and fetal thymic organ culture (FTOC)

Single cell suspensions were prepared from day 14 fetal thymic lobes and transduced as previously described (18). Subsequently, equal numbers (n = 30,000) of thymocytes were transferred together with deoxyguanosine-treated fetal thymic lobes to a hanging drop in an inverted Terasaki well. After 48 h, thymocytes were examined by FACS analysis (2-day hanging drop), or alternatively, seeded lobes were placed on filter discs on gelfoam in a conventional FTOC system previously described (16) and cultured for another 2 days before FACS analysis (2-day hanging drop plus 2-day FTOC).

Analysis of lipid rafts by subcellular fractionation

A total of 20 × 10⁶ cells were surface-labeled with sulfo-NHS-LC-biotin as described (19) and lysed in 500 μl buffer A (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, and protease inhibitors) containing 0.4% Brij 58 for 1 h at 4°C. Cell lysates were adjusted to 40% sucrose (1 ml) and overlaid with 1 ml each of 30%, 20%, and 10% sucrose in buffer A. Six 400-μl fractions encompassing the low-density lipid rafts were collected from the top of the tube leaving 1600 μl as the high-density detergent soluble fraction. Fractions 2–6 (100 μl) were solubilized in 10 vol of buffer B containing 1% octylglucoside. Biotin-labeled proteins were isolated using SA-Sepharose (Pierce, Rockford, IL). The beads were washed with buffer B containing 0.2% octylglucoside and eluted by boiling in SDS. pTα-TCRβ and TCRαβ dimers were specifically isolated from the fractions with rabbit anti-pTα serum (4) and anti-TCRα mAb (clone H28-710), respectively, resolved on non-reducing SDS-PAGE gels, and visualized by blotting with HRP-SA as described (19). In addition, 100 μl of each gradient fraction were TCA precipitated, resolved by SDS-PAGE, and transferred to Immobilon-P (Millipore, Bedford, MA) membranes. For protein detection, membranes were immunoblotted with the indicated primary Abs. Bound Abs were detected by incubation with either protein A-HRP or protein G-HRP followed by ECL (Renaissance, NEN, Boston MA). For GM1 ganglioside (GM1) detection, blots were incubated with HRP-labeled cholera toxin B (CtxB) followed by ECL.

Immunofluorescence

Cells were incubated for 30 min at 37°C to allow attachment to poly-L-lysine-coated chamber slides, then fixed with 1% paraformaldehyde/PBS pH 7.0 for 10 min at room temperature (RT). Fixed cells were quenched in 50 mM NH₄Cl for 10 min at RT and subsequently blocked for 30 min with 1% BSA/BBSS containing 5% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were stained for 30 min at RT with the following reagents: hamster anti-CD3ε (clone 500A2), rat anti-TCR-α/β (clone 500A2), rat anti-transferrin receptor (TF-R) (clone 171.1.3), or biotinylated CtxB (Sigma-Aldrich, St. Louis, MO). After washing twice in 1% BSA/BBSS, bound mAb or CtxB was visualized by incubation for 30 min at RT with the appropriate fluorescent visualizing agent: goat anti-hamster Rhodamine Red, goat anti-rat-Rhodamine Red, goat anti-anti hamster Cy5, or streptavidin-Cy5 (all from Jackson ImmunoResearch Laboratories). After washing three times with 1% BSA/BBSS, stained samples were examined using a laser scanning confocal microscope (Bio-Rad, Hercules, CA).

Measurement of ERK activity

DN3, DN4, and DP thymocytes were sorted from TCRα-deficient mice with a FACSDiVa cell sorter. Sorted populations were ≥99.8% pure, as determined by post-sort analysis. Before cell sorting for DN3 and DN4 cells, DN cells were enriched by anti-CD8 complement-mediated lysis, as
previously described (20). γδTCR⁺ cells were not specifically excluded and in TCRα-deficient thymocytes comprise −0.3–0.7%, 8–13%, and 0.04–0.1% of the DN3, DN4, and DP subsets, respectively. Thymocytes were transfected with PathDetect reporter plasmids (Strategene, La Jolla, CA) by electroporation using a BTX ElectroCell Manipulator 600 (San Diego, CA), as previously described (21). Briefly, for each sample, 6.5 × 10⁶ cells in 250 μl RPMI 1640 supplemented with 20% FCS were incubated on ice for 10 min with either 20 μg of pFR-luc alone or with 20 μg of pFA-Elk. pCMV-βgal (10 μg; Stratagene) was included to determine transfection efficiency. DN3 and DN4 thymocytes were pulsed at 260 V, 1700 μF, 186 Ω with a time constant of ~85 ms. DP thymocytes were pulsed at 300 V, 800 μF, 186 Ω with a time constant of ~45 ms. After electroporation, cells were incubated on ice for 10 min and cultured in medium for 22 h. At 16 h posttransfection, cells were stimulated with 10 ng/ml PMA and 1 ng/ml ionomycin (Sigma-Aldrich), as indicated, and incubated for a further 6 h before analysis. Transfected thymocytes were assayed for luciferase and β-galactosidase activities using the Dual-Light reporter gene assay system (Tropix, Bedford MA), as described (21). Results represent the average luciferase activity of assays conducted in triplicate and indexed for β-galactosidase activity.

**Cytosolic Ca²⁺ mobilization**

Thymocytes (5 × 10⁶/ml) were loaded with the acetoxy-methyl ester of Indo-1 (5 μM; Sigma-Aldrich) by incubation in the dark for 45 min at 37°C in modified Dulbecco’s medium containing 10 mM HEPES buffer and 2% FCS (Ca-buffer) and supplemented with Indo-1 and 0.01% pluronic acid (Molecular Probes, Eugene, OR). Cells were washed twice and incubated with purified anti-Vβ3 (clone KJ25), anti-CD4, anti-CD8, anti-CD44, and anti-CD25 mAb for 30 min at 4°C. After two more washes, the cells were resuspended at 2 × 10⁶/ml in Ca-buffer and immediately analyzed on a FACSVantage SE flow cytometer at 37°C. After 30 s, cells were stimulated by addition of 30 μg/ml goat anti-hamster IgG (ICN Cap- pel, Costa Mesa, CA). Successful loading with Indo-1 was confirmed by the addition of 1 μM A23187 at the end of the experiment. For intracellular Ca²⁺ store depletion-activated Ca²⁺ influx, cells were loaded with Indo-1 and subsequently cell surface stained with CD4, CD8, CD44, and CD25 as previously described. Cells were washed twice and resuspended in Ca-buffer containing 2% BSA (instead of FCS) and 2 mM EGTA. Analysis followed immediately on a FACSVantage SE flow cytometer at 37°C. After 30 s, cells were treated with 100 nM thapsigargin (Molecular Probes), and after 6 min, 2 mM Ca²⁺ was added to the cells.

**Results**

**TCRα can overcome the arrest in pre-T cell development caused by pTα deficiency**

One of the most intriguing aspects of pre-TCR complex function is its ability to transduce signals in an apparently cell-autonomous, ligand-independent manner. To determine whether this capability is unique to the pre-TCR complex, we assessed whether TCRα was able to overcome the developmental arrest resulting from pTα deficiency (22). It is of interest to assess the ability of TCRα to do so because TCRα can be rearranged and expressed in the CD44⁺CD25⁺ DN3 thymocyte subset in which β-selection occurs (23, 24), and because TCRα lacks the structural features of pTα thought to underlie its constitutive targeting to lipid rafts and ligand-independent signaling (10). d14 pTα-deficient fetal thymocytes were retrovirally transduced with TCRα or pTα before reconstituting T cell-depleted MHC class I/class II double-deficient lobes (Fig. 1). Strikingly, expression of the TCRα subunit can effectively reverse the phenotype associated with pTα deficiency even in the absence of its MHC class I and class II ligands (Fig. 2A). It is important to note that the d14 pTα-deficient fetal precursors used to reconstitute MHC class I/class II-deficient lobes contain a small subpopulation of cells (~0.1%) with the potential of developing into MHC class I/class II-expressing thymic dendritic cells. To ensure that initiation of signaling by the mature αβTCR at the pre-T cell stage is indeed ligand-independent, blocking mAb to MHC class I H-2Kb/ H-2Db and MHC class II I-A/I-E were added immediately after transduction. The concentration of blocking anti-MHC class I/class II mAb used was sufficient to abolish positive selection in C57BL/6 lobes dissected at day 15 of gestation and cultured in FTOC for 11 days (our unpublished observation). Interestingly, even in the presence of blocking anti-MHC class I/class II mAb, expression of TCRα in pTα-deficient thymocytes resulted in the efficient generation of DN4 cells (Fig. 2B), strongly suggesting that the αβTCR complex does not require conventional ligand to signal.

We have recently demonstrated that the CP of TCRα confers more efficient assembly with CD3ζ than does the CP of pTα (17). To determine whether TCRα could substitute for pTα even when assembly of the αβTCR complex is rendered inefficient, we replaced the CP of TCRα with that of pTα. As shown in Fig. 2A,
expression of a TCRα protein encompassing the CP of pTα restores development of pTα-deficient thymocytes as efficiently as wild-type TCRα, indicating that the capacity of the αβTCR to signal in a ligand-independent manner does not depend on the CP. Taken together, these findings suggest that initiation of signaling without ligand engagement is not a unique property of the pre-TCR conferred upon it by pTα.

Only a small fraction of surface pre-TCR complexes localize to lipid rafts

Previous analysis suggested that pre-TCR complexes signal in a cell-autonomous, ligand-independent manner because most pre-TCR complexes (77%) are constitutively targeted to lipid rafts (5). αβTCR complexes, in contrast, are thought to require ligand engagement to be targeted to lipid rafts (6, 13). Because our analysis indicated that the pre-TCR was not unique in its ability to signal in a ligand-independent manner, we assessed whether pre-TCR and αβTCR complexes differed in their targeting to rafts using both immunofluorescence microscopy and subcellular fractionation (Fig. 3). SCID thymic lymphoma cell lines expressing either pre-TCR complexes (SL-343β1) or αβTCR complexes (SL-343αβ1) (19) were stained with GM1-binding CTxB subunit as a marker for lipid rafts and either anti-CD3 mAb to identify pre-TCR complexes (SL-343αβ1) (Fig. 3, A and B). Evaluation of the extent of overlap of the resultant staining patterns revealed that only a minority (an estimated 10%) of pre-TCR complexes colocalized with rafts, and this was not appreciably different for αβTCR complexes. Consistent with this observation, the staining pattern for the pre-TCR complex overlapped more extensively with that of the Tf-R, which is excluded from rafts (13, 26) (Fig. 3C). Confocal microscopy on primary thymocytes from TCRβ-Tg SCID mice also suggested that only a minor fraction of the pre-TCR complexes colocalize with lipid rafts (Fig. 3D). Finally, sucrose gradient fractionation revealed that >80% of surface pre-TCR complexes were excluded from rafts and this fraction was equivalent to that of the αβTCR complex (Fig. 3E). As previously described, the raft components, LAT and GM1, were detected in low-density raft-containing fractions while the cytosolic protein tubulin and
membrane receptor Tf-R were primarily present in the soluble high-density fraction. Taken together, our immunofluorescence and biochemical analyses suggest that pre-TCR complexes associate with rafts in approximately the same low stoichiometry exhibited by αβTCR complexes.

**DN and DP thymocytes differ in their ability to support ERK activation by the pre-TCR complex**

The observation that pre-TCR complexes are found in rafts in approximately the same low stoichiometry as αβTCR complexes is found supports the viewpoint that ligand-independent signaling is not unique to the pre-TCR complex but rather is an intrinsic property of the DN thymocytes in which pre-TCR signaling is initiated. To address this possibility, we asked whether DN and DP thymocytes differed in their ability to support ERK activation by the pre-TCR complex because several reports indicate that the Ras/Raf/MEK/ERK signaling cascade is important for development of thymocytes beyond the β-selection checkpoint (27-29). DN3, DN4, and DP thymocytes were isolated from TCRα-deficient mice and the endogenous levels of cellular ERK activity were measured using a reporter plasmid-based system (29). TCRα-deficient thymocytes were used because their inability to express the TCR would enable us to evaluate pre-TCR function in both DN and DP thymocytes. Importantly, ERK activity is not detected in pre-TCR complexes (30). The lack of any detectable ERK activity within DN4 and DP cells does not reflect an inability to induce this signaling cascade, as stimulation with PMA and ionomycin results in a strong induction of ERK activity (Fig. 4A). These data suggest that ligand-independent pre-TCR signaling is able to activate ERK in DN3 cells, but not in later developmental stages.

*Thymocytes at the DN stage display a lower signaling threshold compared with more mature thymocytes*

We hypothesized that one explanation for the ability of DN thymocytes to support ligand-independent signaling is that their signaling thresholds might be sufficiently low to obviate the need for ligand engagement. To investigate this possibility, we assessed the relative abilities of pre-TCR complexes expressed on DN and DP thymocytes to mobilize cytosolic Ca²⁺ in response to stimulation by a defined ligand. We used thymocytes from TCRβ-Tg SCID mice, which like the TCRα-deficient mice express the pre-TCR complex (but not αβTCR complexes) on both DN and DP thymocytes (31, 32). TCRβ-Tg SCID thymocytes were used instead of TCRα-deficient thymocytes, because we were only able to evoke Ca²⁺ responses after stimulation with the potent anti-Vβ3 mAb specific for the Vβ3 Tg (data not shown). Strikingly, Vβ3 cross-linking of pre-TCRs expressed at the surface of DN thymocytes, and in particular the DN4 subset, evoked a marked increase in cytosolic Ca²⁺ (Fig. 4B). In this particular assay, DN4 thymocytes represent the most appropriate population to compare with DP thymocytes because DN4 cells are the earliest population in which most cells express the pre-TCR complex. The increase in intracellular free Ca²⁺ was observed over a wide range of anti-Vβ3 mAb concentrations used and displayed a biphasic pattern with an initial rapid rise followed by decline to a sustained Ca²⁺ plateau. In sharp contrast, stimulation of pre-TCR complexes expressed at the DP stage resulted in only a slight increase in intracellular Ca²⁺. Anti-Vβ3 stimulation of DN3 thymocytes did not evoke a detectable increase in intracellular free Ca²⁺, presumably because only few cells in this subpopulation express the pre-TCR complex. These data, together with the finding that pre-TCR expression may be marginally higher in DP than in DN thymocytes from TCRβ-Tg SCID mice (Fig. 4C), suggest that DN thymocytes have a lower signaling threshold compared with DP thymocytes.

We reasoned that the greater increase in cytosolic Ca²⁺ observed following mAb stimulation of DN4 thymocytes might result in part from a greater capacity to facilitate Ca²⁺ influx through Ca²⁺ release-activated Ca²⁺ (CRAC) channels, which are characterized by receptor-independent opening in response to depletion of intracellular Ca²⁺ stores (33). Consequently we asked whether DN and DP thymocytes (from TCRβ-Tg SCID and TCRα-deficient mice) differed in their capacity to facilitate influx of extracellular Ca²⁺ following depletion of intracellular Ca²⁺ stores with the SERCA Ca²⁺-pump inhibitor, thapsigargin. Indeed, thapsigargin treatment resulted in a greater influx of extracellular Ca²⁺ in DN4 thymocytes than in DP thymocytes (Fig. 4D). Interestingly, thapsigargin treatment of DN3 thymocytes also resulted in greater Ca²⁺ influx, presumably because thapsigargin-mediated depletion of intracellular Ca²⁺ stores bypasses the requirement for a receptor signal. The increased Ca²⁺ influx in DN3 and DN4 thymocyte populations may result from expression of more CRAC channels or theoretically through their more efficient opening in response to store depletion.

Another parameter that might influence the signaling threshold of thymocytes is the raft content of their plasma membranes. In support, CD4⁺ memory T cells that are thought to have a lower signaling threshold than their naive counterparts also reportedly have a higher density of lipid rafts in their plasma membranes (34). Moreover, rafts have been shown to be required for effective Ca²⁺ mobilization induced by ligand engagement of the pre-TCR (35).
Consequently, we assessed raft content of the plasma membranes of thymocyte subpopulations (Fig. 5). Staining of wild-type fetal thymocytes with CTxB suggests that the plasma membranes of DN thymocytes, and in particular DN3 and DN4 cells, exhibit far higher lipid raft content than DP thymocytes (Fig. 5).

Taken together, this analysis suggests that the thymocyte subpopulations in which pre-TCR signaling occurs have a lower signaling threshold than do the subpopulations in which ligand-dependent signaling through the αβTCR complex occurs. The decreased signaling threshold is likely to be influenced by both the elevated lipid raft content of the plasma membrane of these cells and by their greater ability to support capacitative Ca\textsuperscript{2+} entry.

**Discussion**

Previous reports suggested that pre-TCR complexes signal in a cell-autonomous, ligand-independent manner because an undefined structural motif unique to pTα constitutively targets them to lipid rafts (5, 10). In this study we present data indicating that the pre-TCR is not unique in its ability to signal in a ligand-independent manner. Indeed, the TCRα subunit restores development of pTα-deficient thymocytes even in the absence of its MHC class I and class II ligands (Fig. 2), suggesting that when expressed at the DN stage, the αβTCR complex does not require conventional ligand to initiate signaling. This is significant because TCRα lacks motifs equivalent to those of pTα thought to underlie ligand independence of the pre-TCR complex. Moreover using both microscopic and biochemical approaches, we found only limited association of the pre-TCR complex with lipid rafts, and the fraction of surface pre-TCRs associated with lipid rafts did not differ appreciably from that observed for αβTCR complexes (Fig. 3), suggesting that ligand-independent signaling is primarily a function of the DN thymocytes in which β-selection signaling occurs.

Lipid microdomains act as repositories of signaling molecules that serve as essential platforms for signaling reactions in many cells (36). Specifically in T cells, the αβTCR complex is coupled to downstream signaling pathways through its ligand-induced association with rafts (6, 13). Although there has been no definitive demonstration that rafts are required for ligand-independent pre-TCR signaling, there is evidence consistent with that possibility (5), which raises the question of how the pre-TCR interaction with rafts is initiated in the absence of ligand. Previous analyses suggested that a motif particular to pTα mediates constitutive targeting of a large fraction of pre-TCR complexes (77%) to lipid rafts (5, 10). However, we find a much lower fraction of pre-TCR complexes associated with lipid rafts, which is estimated to be ~10% (Fig. 3). It is unclear why we find a substantially lower fractional association of pre-TCR complexes with rafts, but this may result from the use of an anti-TCRα Ab to assess the raft association of αβTCR complexes. Nevertheless, when SL-343αβ.1 cells were co-stained with anti-Vα11 and anti-CD3 Abs, we could not detect any significant difference in the staining patterns (data not shown).

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Only a small proportion of pre-TCR complexes localize in lipid rafts. Subcellular localization of pre-TCR and αβTCR complexes was assessed by laser scanning confocal microscopy (A–D) and sucrose gradient fractionation (E). Colocalization of pre-TCR complexes with GM1-containing lipid rafts was analyzed using a pre-TCR-expressing (SL-343β.1) SCID thymic lymphoma cell line (A) and primary thymocytes from 2B4-TCRβ-Tg SCID mice (D). Cells were stained with anti-CD3ε mAb to identify the pre-TCR complex and with CTxB to identify lipid rafts. We have verified that the anti-CD3 staining detects pre-TCR complexes (not clonotype-independent CD3 complexes) in SL-343β.1 cells because the staining is dependent upon transduction with TCRβ (data not shown). Colocalization of αβTCR complexes with lipid rafts was assessed by staining αβTCR-expressing SCID thymic lymphoma cells (SL-343αβ.1) with anti-TCR-Vα11 mAb and CTxB as previously described (B). It should be noted that because SL-343 lymphoma cells express pTα, cells transduced with TCRα and TCRβ (SL-343αβ.1) express both pre-TCR and αβTCR complexes. This required the use of an anti-TCRα Ab to assess the raft association of αβTCR complexes. Nevertheless, when SL-343αβ.1 cells were co-stained with anti-Vα11 and anti-CD3 Abs, we could not detect any significant difference in the staining patterns (data not shown). Colocalization of the pre-TCR with Tf-R was assessed by staining for the pre-TCR as in A and with anti-Tf-R mAb (C). Confocal slices of 0.5-μM thickness are depicted and the extent of overlap is revealed by yellow fluorescence in the electronically merged slices (A–D, right panels). E, Association of pre-TCR and αβTCR complexes with lipid rafts was assessed by sucrose gradient fractionation of surface biotin-labeled SL-343β.1 and SL-343αβ.1 cells. Surface-labeled pTα-TCRβ and TCRαβ heterodimers were isolated from the gradient fractions, resolved by non-reducing SDS-PAGE, and visualized by blotting with HRP-SA and chemiluminescence. Western blot analysis was performed on TCA-precipitated sucrose gradient fractions with Ab reactive to the indicated marker proteins. H, high-density detergent soluble fraction.
from differences in experimental procedures. For example, limited fixation conditions are required to maintain plasma membrane integrity of thymocytes and thymic lymphoma lines (data not shown). Consequently, the extensive colocalization of the pre-TCR with lipid rafts reported previously may result in part from staining of internal structures because lipid rafts form in the Golgi complex (36). Our finding that the pre-TCR and α/H9251/H9252 TCR do not differ significantly regarding the extent of raft localization has two important implications for ligand-independent signaling. First, because the α/H9251/H9252 TCR complex can promote traversal of the α/H9252-selection checkpoint in the absence of conventional ligand, the structural motif underlying this capability need not be particular to pT/α/H9251. Second, if constitutive raft association is important to signal without ligand engagement, association in relatively low stoichiometry is sufficient to initiate signaling. How then might raft localization be accomplished in the absence of ligand? Lipid modification of cysteine residues has been shown to be required for raft localization of a number of signaling molecules such as LAT, Ras, and Src kinases (7, 8, 37, 38). Nevertheless, the single juxtamembrane cysteine (C176) residue of pT/α/H9251, which can be palmitoylated in vitro, appears to be dispensable for pre-TCR function (Fig. 1) (5, 10). Recent analysis suggests that the cytoplasmic tail of pT/α/H9251 might be important because its deletion or mutagenesis partially impairs pre-TCR function (10); however, TCR/α/H9251 is able to signal without conventional ligand and lacks an extended cytoplasmic tail. Consequently, while the cytoplasmic tail of pT/α may be important for

FIGURE 4. DN thymocytes display a lower signaling threshold compared with DP thymocytes. A, DN3, DN4, and DP thymocytes were isolated from TCRβ-deficient mice and transfected with reporter plasmids (pFR-Luc + pFA-ELK) for the detection of ERK activity. ERK function was measured as induced luciferase activity in the transfected thymocyte subsets. Following transfection, thymocytes were cultured for 22 h; for stimulated DP thymocytes, PMA and ionomycin were added 6 h before analysis. The broken line shown represents the background luciferase activity from thymocytes transfected with the luciferase reporter construct (pFR-Luc) alone. Transfection efficiency was monitored by cotransfection with a control β-galactosidase expression plasmid. B, Mobilization of cytosolic Ca2+ was analyzed in electronically gated DN4 thymocytes (thick black line), total DN thymocytes (thick gray line), or DP thymocytes (thin black line) derived from adult 2B4-TCRβ-Tg SCID mice. Cells were treated with indicated concentrations of anti-Vβ3 mAb (clone KJ25) followed 30 s later by cross-linking with goat anti-hamster (GaH) Ab. Successful loading with Indo-1 was confirmed by the addition of 1 μM A23187 at the end of the experiment. Data are shown as the ratio of fluorescence of Ca2+-bound (violet)-Ca2+-unbound (blue) Indo-1. C, Cell surface expression of TCRβ was assessed by flow cytometry on electronically gated DN (open areas) and DP (shaded areas) thymocytes derived from 2B4-TCRβ-Tg SCID mice. D, Intracellular Ca2+ store depletion-activated Ca2+ influx was analyzed in electronically gated DN4 thymocytes (thick black line), DN3 thymocytes (thin gray line), total DN thymocytes (thick gray line), or DP thymocytes (thin black line) derived from 2B4-TCRβ-Tg SCID mice (top panel) and from TCRβ-deficient mice (bottom panel). Cells were treated with 100 nM thapsigargin (TG) 30 s after the analysis was initiated, and after 6 min 2 mM Ca2+ was added. Data are shown as the ratio of fluorescence of Ca2+-bound (violet)-Ca2+-unbound (blue) Indo-1.
expression of CRAC channels and/or (theoretically) more efficient coupling of their activation to modest depletion of intracellular signaling by ligand-independent manner only in DN3 thymocytes. Although the pre-TCR complex was able to activate ERK signaling in a ligand-independent manner (as indicated by ERK activity) appears to be lost at later stage, suggests that ligand-independence is a function of the cells in which β-selection signals are transduced. In support, we found that the pre-TCR complex was able to activate ERK signaling in a ligand-independent manner only in DN3 thymocytes. Although the mechanistic basis for the ability of DN thymocytes to support ligand-independent signaling by αβTCR and pre-TCR complexes remains unclear, we provide evidence that early DN thymocytes exhibit a lower signaling threshold than shown by DP thymocytes. Among the many adaptations (including differential expression of signaling molecules) that might contribute to setting cellular signaling thresholds, we have found that DN thymocytes exhibit both markedly elevated lipid raft content in their plasma membranes and a greater capacity to support capacitative Ca\(^{2+}\) entry (Figs. 4B and 5). The elevated lipid raft content could facilitate ligand-independent signaling through increasing the frequency or duration of undirected interactions of the pre-TCR complex with lipid rafts, such that the activation threshold can be exceeded even in the absence of ligand engagement. Alternatively, the frequency/duration of pre-TCR/raft association may not differ appreciably, and the increased raft content may instead serve to amplify comparatively weak signals. It is also possible that relatively weak signals may be amplified due to Ca\(^{2+}\) oscillations resulting from increased expression of CRAC channels and/or (theoretically) more efficient coupling of their activation to modest depletion of intracellular Ca\(^{2+}\) stores (33). The ability to signal in a ligand-independent manner (as indicated by ERK activity) appears to be lost at later stages of development perhaps due to induction of negative regulators of signaling such as CD5 or the Src-like adaptor protein, SLAP, which are both thought to be induced in response to pre-TCR signaling (39, 40). Interestingly, the signaling threshold can still be overcome in DN4 thymocytes by increasing pre-TCR signal strength through ligand engagement, although this ability is also lost upon arrival at the DP stage (Fig. 4B). Ligand engagement of the αβTCR complex is able to overcome the signaling threshold of DP thymocytes, presumably because its expression level is far higher than that of the pre-TCR complex.

Although we have found that the αβTCR complex can promote traversal of the β-selection checkpoint even in the absence of MHC class I/class II ligands, previous attempts to address this question have produced contradictory results. In one TCR-Tg model, pre-T cell development appeared normal both on wild type and pTα-deficient backgrounds (14). However, in other Tg models, premature expression of αβTCR complexes impaired progression beyond the β-selection checkpoint, reduced thymic cellularity, and caused accumulation of αβTCR-expressing DN thymocytes (41, 42). This may have resulted from ligand engagement of a Tg TCR complex that was overexpressed at an extremely early stage of development, and which induced either negative selection at the DP stage or developmental arrest of DN thymocytes. Therefore, one possible explanation for the contradictory results is that in model systems in which the αβTCR is expressed at relatively low physiologic levels, such as in our retrovirally transduced thymocytes (Fig. 1C), pre-TCR function is closely mimicked and differentiation toward the αβ T cell lineage is promoted. In contrast, premature overexpression of the αβTCR may result in an excessively intense signal that retards development toward the αβ T cell lineage. Therefore, it may be the strength of the signal transduced, rather than any intrinsic difference in pre-TCR and αβTCR structure, that is the primary determinant of a receptor’s ability to promote αβ T cell development. This view is corroborated by studies showing that increasing pre-TCR signal strength, either by ligation with anti-TCRβ mAb or by overexpressing signaling molecules (p38 mitogen-activated protein kinase and Lck), impairs development of thymocytes (3, 43, 44). Taken together, our findings support the view that the ability to signal in a ligand-independent manner is primarily a property of DN thymocytes rather than a unique feature of the pre-TCR complex. The modulation of cellular signaling thresholds during lymphoid development may be an important general mechanism for controlling receptor function.

Acknowledgments

We thank Drs. D. Kappes and A. Singer for critically reviewing the manuscript, Drs. S. Shinton and J. Boyd for excellent technical assistance, Dr. H. von Boehm for providing pTα-deficient mice, and Dr. B. Freedman for helpful discussions. We are also grateful for the assistance of the following core facilities at Fox Chase Cancer Center: Cell Culture, Cell Imaging, DNA Sequencing, DNA Synthesis, Flow Cytometry, Laboratory Animal, and Special Services.

References


