Signaling for NKT cell development: the SAP–FynT connection

Christine Borowski and Albert Bendelac

New studies demonstrate a critical role for the adaptor protein SAP (SLAM-associated protein) during NKT cell development. By connecting homotypic SLAM family receptor interactions with the FynT Src kinase, SAP may integrate a set of long-standing yet seemingly disparate observations characterizing NKT cell development. In fact, SAP-dependent signaling may underlie the development of multiple unconventional T cell lineages whose thymic selection relies on homotypic interactions between hematopoietic cells.

A hybrid lineage

With respect to their phenotype and function, NKT cells resemble a composite of two familiar hematopoietic lineages. Like conventional T cells, NKT cells bear a surface T cell receptor (TCR) that is coupled with CD3 subunits. However, like NK cells, NKT cells express NK1.1 as well as members of the Ly49 and NKG2 receptor families. In their reliance on TCR stimuli for activation, NKT cells bear semblance to naive conventional T cells. Yet, in the instantaneous nature of the cytokine secretion and lytic activity triggered by such TCR stimulation, they resemble NK cells. Considering their phenotypic and functional peculiarities, it is perhaps not surprising that the NKT cell developmental process seems as unusual as the cells it produces, and that the cellular and molecular mechanisms involved in this process remain poorly understood.

Unconventional selection

NKT cells appear to arise from the same thymocyte precursors as conventional T cells, likely passing through a $CD4^+CD8^+$ stage. However, unlike conventional T cells, NKT cells express a limited repertoire of TCRs, each of which contain a single segment of Vα and Jα DNA (Vα14-Jα18 in mice [Vα14i], Vα24-Jα18 in humans) combined with one of three Vβ segments (Vβ8, Vβ2, or Vβ7 in mice, Vβ11 in humans). These canonical TCRs recognize the self-glycosphingolipid iGb3 (1), which fully activates mature NKT cells both in vitro and in vivo during some microbial infections (2). Given the strong self-agonist activity of iGb3, it is paradoxical that Vα14i TCR^+ NKT cell precursors are not eliminated, as conventional T cells capable of self-agonist recognition usually undergo negative selection in the thymus.

Recently identified differences among signaling pathways and cell types used during conventional T and NKT cell development may play a major role in selection. Although essential for positive selection of conventional T cells, some components of the Ras–MAP kinase signaling pathway (Ras and Mek-1) appear dispensable for NKT cell selection (3). Conventional T cell development appears largely normal in the absence of the Src kinase FynT, but is ablated in mice lacking Lck (for review see reference 4). In contrast, NKT cells are absent in FynT-deficient mice (5, 6). The essential functions of Lck prior to the $CD4^+CD8^+$ developmental stage currently preclude assessment of its potential contribution to NKT cell selection. Lck enters the conventional TCR-driven selection signaling pathway by associating with the cytoplasmic regions of CD4 and CD8, whose extracellular domains bind MHC class II and Ia, respectively, on selector cells. Definitive demonstration of an interaction between CD4 or CD8 and CD1d is lacking, raising the possibility that Lck is dispensable for CD1d-driven NKT cell selection. However, considering the subtle but significant alterations of NKT cell TCR Vβ usage in CD8-deficient mice (7), a role for Lck during NKT cell selection cannot be excluded.

Adding to the novelty of their developmental pathway, NKT cells are selected exclusively by CD1d–glycolipid complexes expressed by other cortical $CD4^+CD8^+$ thymocytes, whereas conventional T cell precursors are selected by MHC–peptide complexes expressed on thymic epithelial cells. Expression of CD1d exclusively under the control of an MHC class II promoter (inactive in cortical $CD4^+CD8^+$ thymocytes) failed to support NKT cell development, raising the possibility that a feature other than CD1d expression that is unique to cortical $CD4^+CD8^+$ thymocytes is vital to NKT cell selection (8).

The FynT–SAP connection

The lack of understanding of FynT signaling has long frustrated the hope that FynT's involvement would illuminate the mechanisms guiding NKT cell development. Although some association between FynT and TCR subunit immunoreceptor tyrosine-based activation motifs has been demonstrated (9), a recent convergence of reports illustrating a direct interaction between FynT and SAP (also known as Sh2d1a, DSHP) provided an intriguing alternative explanation for the requirement of FynT in NKT cell development. The groups of Terhorst, Eck, and Veillette elegantly dissected the trimolecular interaction between the membrane-

C.B. and A.B. are at the University of Chicago, Chicago, IL 60637.

CORRESPONDENCE
A.B.: abendela@bsd.uchicago.edu

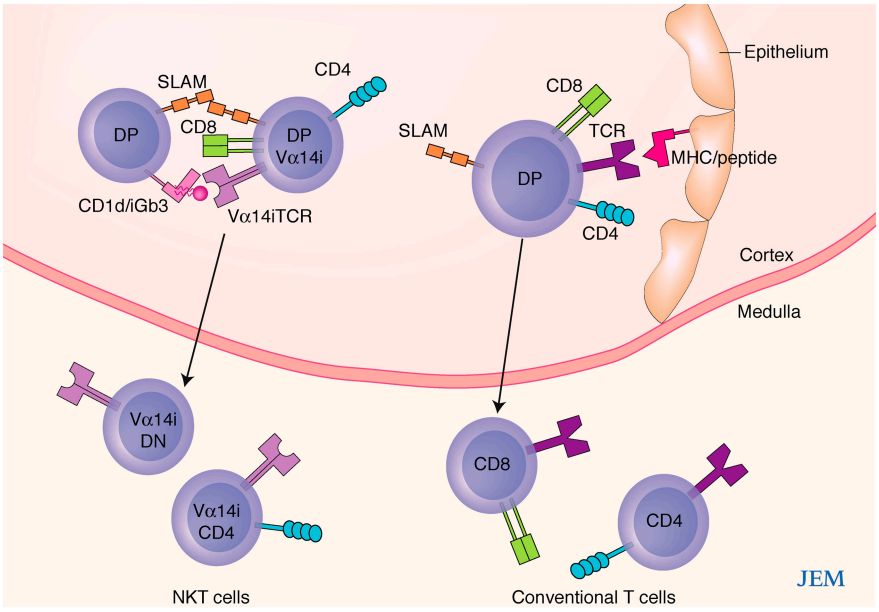

Figure 1. Potential scheme of SAP-dependent NKT cell selection. Homotypic interactions between Vα14i TCR$^+$ and CD1d/iGb3$^+$ cortical CD4$^+$CD8$^+$ double positive (DP) thymocyes allow simultaneous SAP-dependent homotypic interactions between unidentified SLAM family members. Interactions between CD8 and CD1d might also occur. Heterotypic interactions between conventional (non-Vα14i) TCR$^+$ DP thymocytes and MHC–peptide$^+$ thymic epithelial cells allow CD8–MHC class I interactions.

proximal SLAM tyrosine residue, the SAP SH2 domain, and the FynT SH3 domain (10–12). It is through the latter interaction that the auto-inhibitory loop structure of FynT is relieved, unleashing its tyrosine kinase activity. In humans, SAP mutations result in X-linked proliferative syndrome (XLP; reference 10). SAP-deficient mice exhibit defective T helper cell differentiation and altered responses to pathogens (13). Three new papers (14–16) now document a lack of NKT cells in the thymus, spleen, and liver of SAP-deficient mice, and confirm that the requirement for SAP is autonomous to developing NKT cells. Most strikingly, the authors extended their studies into humans, observing parallel NKT cell defects in peripheral blood samples from a cohort of XLP patients harboring a defined set of SAP mutations. These findings effectively insert SAP into the scheme of NKT cell selection.

Signaling upstream of SAP–FynT

The membrane-proximal mediator(s) that recruits SAP during NKT cell development remains unidentified. The SAP-associated SLAM family surface receptors are obvious candidates, particularly when considering the unique homotypic nature of the cellular interaction required for selection of Vα14i TCR$^+$ cortical CD4$^+$CD8$^+$ thymocytes into the NKT lineage. The SLAM family of immunoreceptors contains six members—SLAM (CD105), CD84, Ly108, Ly9, 2B4, and CRACC—all of which contain at least one extracellular immunoglobulin-like motif that is capable of binding ligands in either a heterotypic (2B4-CD48) or homotypic (CD150, CD84, Ly9, Ly108, and CRACC) manner. The cytoplasmic domains of all SLAM family members possess at least one copy of an immunoreceptor tyrosine-based switch motif (TIYxxV/I). It is through these motifs that SLAM receptors associate with SAP (with the exception of CRACC, which does not bind SAP; for review see reference 17). Only one homotypic SAP-binding SLAM family receptor, CD150 (SLAM), has been directly reported so far on the surface of murine cortical CD4$^+$CD8$^+$ thymocytes (18). Theoretically, interaction between the Vα14i TCRs of a cortical CD4$^+$CD8$^+$ thymocyte "selectee" with CD1d/iGb3 complexes of a cortical CD4$^+$CD8$^+$ thymocyte "selector" would allow simultaneous SAP-activating CD150–CD150 interactions (Fig. 1). Whether or not SLAM receptors are also expressed on CD1d$^+$ thymic dendritic cells or on macrophages is not known. However, expression on thymic epithelial cells is unlikely, as thus far SLAM receptors have been localized exclusively to hematopoietic lineages. The potential inability of thymic epithelial cells to provide partners for SAP-activating SLAM receptors may render these cell types unfit for selecting Vα14i TCR$^+$ cortical CD4$^+$CD8$^+$ thymocytes into the NKT lineage, thereby explaining the absence of NKT cells in mice expressing CD1d exclusively under the control of a MHC class II promoter (8).

Signaling downstream of SAP–FynT

The precise mechanism by which SAP–FynT activation ensures that a CD4$^+$CD8$^+$ cortical thymocyte enters the NKT lineage rather than the conventional T lineage is not known, but several downstream mediators recently linked to SAP–FynT signaling cascades may play a major role (Fig. 2). After recruitment by SAP, FynT phosphorylates and recruits the SH2 domain–containing inositol phosphatase (SHIP), Dok1/2 adaptor proteins, and the Ras GTPase-activating protein (RasGAP). By binding to RasGAP, Dok1/2 inhibits Ras-MAPK activation induced by a variety of stimuli (antigen, growth factor, and cytokine) in a variety of hematopoietic cell types. Dok2, which is expressed most prominently in T cells and macrophages (the two cell types that exhibit an overt phenotype in SLAM-deficient mice), is the only Dok bound to RasGAP after SLAM stimulation (19, 20). Indeed, the agonist nature of the NKT cell–selecting ligand iGb3, coupled with the inherent autoreactivity of peripheral NKT cells (held in check by inhibitory NK receptors), sug-

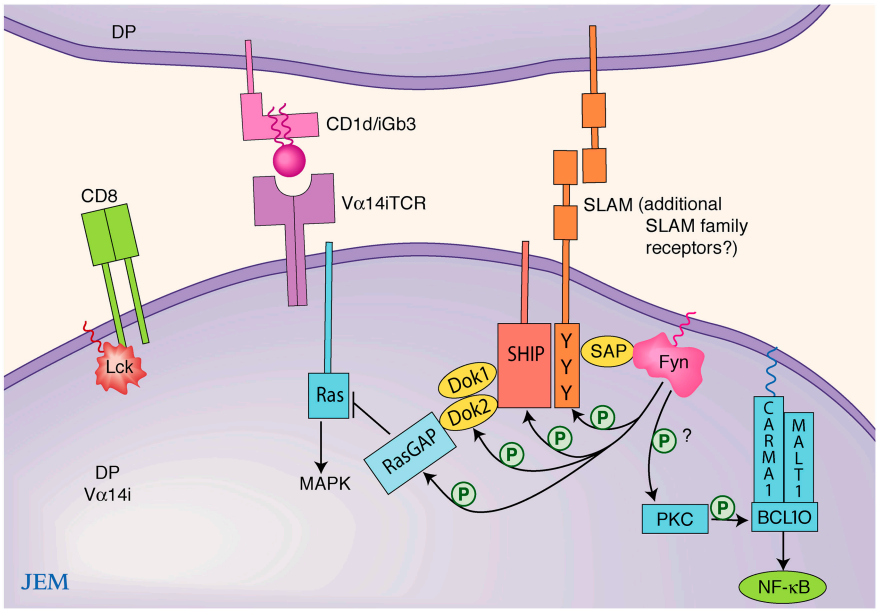

Figure 2. Potential signaling pathways triggered by ligation of SLAM family members. P, phosphorylation.

gest that SAP and its ability to activate the RasGAP signal dampener might be useful in avoiding deletion and in maintaining immune tolerance (21).

A separate signaling cascade links the SLAM-SAP–FynT complex to NF-κB via protein kinase θ (PKCθ) and the Bcl10 adaptor protein. This SAP–FynT-PKCθ–Bcl10 pathway may interact directly with TCR signaling cascades, as SAP- or FynT-deficient T cells exhibit defective recruitment of PKCθ and Bcl10 to the immunological synapse as well as decreased IκBα degradation and NF-κBp50 nuclear translocation in response to TCR stimulation (22). Interestingly, several recent reports implicate PKCθ, Bcl-10, and NF-κBp50 in NKT cell development and/or homeostasis. Mice deficient in PKCθ or Bcl10 exhibit a severe dearth of NKT cells in the thymus and spleen, respectively (23, 24). Mice expressing a dominant negative IκBα transgene and those lacking NF-κBp50 also contain severely diminished NKT cell populations, although the precise stage at which these molecules affect NKT cell development and/or homeostasis remains unclear (25, 26). Interestingly, similar to mice lacking PKCθ or Bcl10, mice deficient in CARMA1 or Malt1 adaptor proteins exhibit defective TCR-induced NF-κB activation (for review see reference 27). However, unlike PKCθ and Bcl10, CARMA1 and Malt1 appear dispensable for NKT cell development and survival, prompting Schmidt-Supprian et al. (23) to astutely suggest that the NKT cell defects in PKCθ- and Bcl10-deficient mice may therefore result from their involvement in NF-κB activation induced by TCR-independent receptors. In light of the new observations made in SAP-deficient mice, such receptors now appear likely to belong to the SLAM family.

A role for SAP and FynT in other unconventional T lineages?

The reason for the involvement of SAP in TCR-driven cellular selection in NKT cells but not conventional T cells is also open for discussion. The disparate expression patterns of molecules responsible for conventional T cell (MHC class Ia and II) and NKT cell (CD1d) selection must be considered. Cortical $CD4^+CD8^+$ thymocytes express both CD1d and SLAM receptors but lack MHC class Ia and II, rendering them capable of selecting NKT cells but not conventional T cells. Thymic epithelial cells, dendritic cells, and macrophages express MHC class Ia and II, but evidence localizing SLAM receptors to these cell types is currently lacking. Thus, although able to select conventional T cells, these cell types may not be suitable for NKT cell selection.

The SAP–FynT connection may provide a model for the selection of other unconventional T cell lineages. Similar to NKT cells, $CD8^+$ MHC class Ib–restricted T cells express an activated phenotype ($CD44^+$ β7 integrin$^-$ $CD11a^+$ $CD122^+$ $Ly6C^+$) before thymic export in uninfected mice, secrete cytokines with an innate-like response time after TCR stimulation, and recognize antigen-presenting molecules of limited diversity (H2-M3, Qa-1, and Qa-2). In addition, like NKT cells, $CD8^+$ MHC class Ib–restricted T cells are selected most efficiently by agonist ligands expressed on thymic hematopoietic cells (28, 29). When given no other option, small numbers of $CD8^+$ MHC class Ib–restricted T cells can be selected on thymic epithelial cells. However, this alternatively selected population lacks the full spectrum of activated surface integrins and cytokine receptors expressed by their normal counterparts. Similarly, $CD4^-$ $CD8^-$ αβTCR$^+$ cells in αβ TCR transgenic mice express some markers of activated and/or innate lymphocytes (CD44 and/or NK1.1), are selected by intrathymic hematopoietic cells presenting agonist MHC class Ia–peptide complexes, and were described by von Boehmer et al. (30) as harboring "benign autoreactivity" in vivo.

Overall, the selector cell type is the likely feature segregating the development of conventional and innate T cell lineages. Selector cell attributes responsible for enforcing this division of labor remain largely unidentified, but the new studies from the groups of Latour, Stein, and Rusung make a strong case for inclusion of SAP-activating surface receptors on this short list. That these receptors belong to the SLAM family is likely, but remains to be proven. However, insertion of SAP into the scheme of NKT cell selection highlights new

and exciting avenues of investigation, with the potential of unlocking selective secrets shared by NKT cells and other unconventional T cells.

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REFERENCES

1. Zhou, D., J. Mattner, C. Cantu III, N. Schrantz, N. Yin, Y. Gao, Y. Sagiv, K. Hudspeth, Y.P. Wu, T. Yamashita, et al. 2004. Lysosomal glycosphingolipid recognition by NKT cells. *Science*. 306:1786–1789.
2. Mattner, J., K.L. DeBord, N. Ismail, R.D. Goff, C. Cantu III, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, K. Hoebe, O. Schneewind, D. Walker, et al. 2005. Both exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature*. In press.
3. Alberola-Ila, J., K.A. Hogquist, K.A. Swan, M.J. Bevan, and R.M. Perlmutter. 1996. Positive and negative selection invoke distinct signaling pathways. *J. Exp. Med*. 184: 9–18.
4. Palacios, E.H., and A. Weiss. 2004. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene*. 23:7990–8000.
5. Gadue, P., N. Morton, and P.L. Stein. 1999. The Src family tyrosine kinase Fyn regulates natural killer T cell development. *J. Exp. Med*. 190:1189–1196.
6. Eberl, G., B. Lowin-Kropf, and H.R. MacDonald. 1999. Cutting edge: NKT cell development is selectively impaired in Fyn-deficient mice. *J. Immunol*. 163:4091–4094.
7. Bendelac, A., N. Killeen, D.R. Littman, and R.H. Schwartz. 1994. A subset of CD4+ thymocytes selected by MHC class I molecules. *Science*. 263:1774–1778.
8. Forestier, C., S.H. Park, D. Wei, K. Benlagha, L. Teyton, and A. Bendelac. 2003. T cell development in mice expressing CD1d directed by a classical MHC class II promoter. *J. Immunol*. 171:4096–4104.
9. Gauen, L.K., Y. Zhu, F. Letourneur, Q. Hu, J.B. Bolen, L.A. Matis, R.D. Klausner, and A.S. Shaw. 1994. Interactions of p59fyn and ZAP-70 with T-cell receptor activation motifs: defining the nature of a signalling motif. *Mol. Cell. Biol*. 14:3729–3741.
10. Sayos, J., C. Wu, M. Morra, N. Wang, X. Zhang, D. Allen, S. van Schaik, L. Notarangelo, R. Geha, M.G. Roncarolo, et al. 1998. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature*. 395:462–469.
11. Chan, B., A. Lanyi, H.K. Song, J. Griesbach, M. Simarro-Grande, F. Poy, D. Howie, J. Sumegi, C. Terhorst, and M.J. Eck. 2003. SAP couples Fyn to SLAM immune receptors. *Nat. Cell Biol*. 5:155–160.
12. Latour, S., R. Roncagalli, R. Chen, M. Bakinowski, X. Shi, P.L. Schwartzberg, D. Davidson, and A. Veillette. 2003. Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. *Nat. Cell Biol*. 5:149–154.
13. Wu, C., K.B. Nguyen, G.C. Pien, N. Wang, C. Gullo, D. Howie, M.R. Sosa, M.J. Edwards, P. Borrow, A.R. Satoskar, et al. 2001. SAP controls T cell responses to virus and terminal differentiation of TH2 cells. *Nat. Immunol*. 2:410–414.
14. Pasquier, B., L. Yin, M. Fondaneche, F. Relouzat, C. Bloch-Queyrat, N. Lambert, A. Fischer, G. de Saint-Basile, and S. Latour. 2005. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. *J. Exp. Med*. 201:797–803.
15. Nichols, K.E., J. Hom, S.Y. Gong, A. Ganguly, C.S. Ma, J.L. Cannons, S.G. Tangye, P.L. Schwartzberg, G.A. Koretzky, and P.L. Stein. 2005. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med*. 11:340–345.
16. Chung, B., A. Aoukaty, J. Dutz, C. Terhorst, and R. Tan. SLAM-associated protein controls natural killer T cell functions. 2005. *J. Immunol*. In press.
17. Veillette, A., and S. Latour. 2003. The SLAM family of immune-cell receptors. *Curr. Opin. Immunol*. 15:277–285.
18. Castro, A.G., T.M. Hauser, B.G. Cocks, J. Abrams, S. Zurawski, T. Churakova, F. Zonin, D. Robinson, S.G. Tangye, G. Aversa, et al. 1999. Molecular and functional characterization of mouse signaling lymphocytic activation molecule (SLAM): differential expression and responsiveness in Th1 and Th2 cells. *J. Immunol*. 163:5860–5870.
19. Latour, S., G. Gish, C.D. Helgason, R.K. Humphries, T. Pawson, and A. Veillette. 2001. Regulation of SLAM-mediated signal transduction by SAP, the X-linked lymphoproliferative gene product. *Nat. Immunol*. 2:681–690.
20. Wang, N., A. Satoskar, W. Faubion, D. Howie, S. Okamoto, S. Feske, C. Gullo, K. Clarke, M.R. Sosa, A.H. Sharpe, and C. Terhorst. 2004. The cell surface receptor SLAM controls T cell and macrophage functions. *J. Exp. Med*. 199:1255–1264.
21. Ikarashi, Y., R. Mikami, A. Bendelac, M. Terme, N. Chaput, M. Terada, T. Tursz, E. Angevin, F.A. Lemonnier, H. Wakasugi, and L. Zitvogel. 2001. Dendritic cell maturation overrules H-2D–mediated natural killer T (NKT) cell inhibition: critical role for B7 in CD1d-dependent NKT cell interferon γ production. *J. Exp. Med*. 194:1179–1186.
22. Cannons, J.L., L.J. Yu, B. Hill, L.A. Mijares, D. Dombroski, K.E. Nichols, A. Antonellis, G.A. Koretzky, K. Gardner, and P.L. Schwartzberg. 2004. SAP regulates T(H)2 differentiation and PKC-theta-mediated activation of NF-kappaB1. *Immunity*. 21:693–706.
23. Schmidt-Supprian, M., J. Tian, E.P. Grant, M. Pasparakis, R. Maehr, H. Ovaa, H.L. Ploegh, A.J. Coyle, and K. Rajewsky. 2004. Differential dependence of CD4+CD25+ regulatory and natural killer-like T cells on signals leading to NF-kappaB activation. *Proc. Natl. Acad. Sci. USA*. 101:4566–4571.
24. Stanic, A.K., J.S. Bezbradica, J.J. Park, L. Van Kaer, M.R. Boothby, and S. Joyce. 2004. Cutting edge: the ontogeny and function of Va14Ja18 natural T lymphocytes require signal processing by protein kinase C theta and NF-kappa B. *J. Immunol*. 172: 4667–4671.
25. Sivakumar, V., K.J. Hammond, N. Howells, K. Pfeffer, and F. Weih. 2003. Differential requirement for Rel/nuclear factor κB family members in natural killer T cell development. *J. Exp. Med*. 197:1613–1621.
26. Stanic, A.K., J.S. Bezbradica, J.J. Park, N. Matsuki, A.L. Mora, L. Van Kaer, M.R. Boothby, and S. Joyce. 2004. NF-kappa B controls cell fate specification, survival, and molecular differentiation of immunoregulatory natural T lymphocytes. *J. Immunol*. 172:2265–2273.
27. Lin, X., and D. Wang. 2004. The roles of CARMA1, Bcl10, and MALT1 in antigen receptor signaling. *Semin. Immunol*. 16:429–435.
28. Urdahl, K.B., J.C. Sun, and M.J. Bevan. 2002. Positive selection of MHC class Ib-restricted CD8(+) T cells on hematopoietic cells. *Nat. Immunol*. 3:772–779.
29. Bix, M., and D. Raulet. 1992. Inefficient positive selection of T cells directed by haematopoietic cells. *Nature*. 359:330–333.
30. von Boehmer, H., J. Kirberg, and B. Rocha. 1991. An unusual lineage of alpha/beta T cells that contains autoreactive cells. *J. Exp. Med*. 174:1001–1008.