A voltage-gated sodium channel is essential for the positive selection of CD4\(^+\) T cells

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The sustained entry of Ca\(^{2+}\) into CD4\(^+\)CD8\(^+\) double-positive thymocytes is required for positive selection. Here we identified a voltage-gated Na\(^+\) channel (VGSC) that was essential for positive selection of CD4\(^+\) T cells. Pharmacological inhibition of VGSC activity inhibited the sustained Ca\(^{2+}\) influx induced by positively selecting ligands and the \textit{in vitro} positive selection of CD4\(^+\) but not CD8\(^+\) T cells. \textit{In vivo} short hairpin RNA (shRNA)-mediated knockdown of the gene encoding a regulatory \(\beta\)-subunit of a VGSC specifically inhibited the positive selection of CD4\(^+\) T cells. Ectopic expression of VGSC in peripheral AND CD4\(^+\) T cells bestowed the ability to respond to a positively selecting ligand, which directly demonstrated that VGSC expression was responsible for the enhanced sensitivity. Thus, active VGSCs in thymocytes provide a mechanism by which a weak positive selection signal can induce the sustained Ca\(^{2+}\) signals required for CD4\(^+\) T cell development.

The generation of the adaptive immune response requires the production of a diverse T cell antigen receptor (TCR) repertoire that recognizes a wide range of foreign pathogens with the concurrent elimination of self-reactive T cells\(^{1–3}\). The stringent processes of positive and negative selection establish this anticipatory TCR repertoire during thymocyte development. In positive selection, a weak yet productive interaction of the TCR, self peptide and major histocompatibility complex (MHC) rescues CD4\(^+\)CD8\(^+\) double-positive (DP) thymocytes that express TCRs able to recognize self peptide–MHC from death by neglect. In negative selection, a strong TCR–self peptide–MHC interaction results in thymocyte death and thus deletion of potentially self-reactive DP thymocytes\(^{1–3}\). Discrimination between ‘weak’ and ‘strong’ TCR-peptide-MHC interactions is thought to depend on the duration of activation of the critical signaling molecules Ca\(^{2+}\) (refs. 4–8) and Erk\(^{9,10}\). However, the mechanism by which a weak TCR-self peptide–MHC interaction induces and maintains sustained Ca\(^{2+}\) and Erk signaling during successful positive selection has remained unknown. Whereas the Ca\(^{2+}\) release–activated Ca\(^{2+}\) (CRAC) pathway is crucial for peripheral T cell function\(^{8,11,12}\), genetic deletion of its two key components (ORAI and STIM) has no effect on the positive selection of CD4\(^+\) and CD8\(^+\) T cells\(^{8,13–15}\), which suggests that Ca\(^{2+}\) signaling by DP thymocytes during selection is complicated and may involve undefined ion channels in regulating a sustained Ca\(^{2+}\) signal during positive selection.

Here we report a previously unknown role for a VGSC in the sustained entry of Ca\(^{2+}\) into nonexcitable DP thymocytes that was necessary for positive selection of CD4\(^+\) T cells. The peptide gp250 has been identified as a naturally occurring self peptide that positively selects thymocytes from AND mice, which have transgenic expression of the AND TCR for gp250–I-E\(k\) in solution, gp250–I-E\(k\) induced a sustained Ca\(^{2+}\) signal and \textit{in vitro} positive selection of AND thymocytes, whereas the MCC agonist induced a transient Ca\(^{2+}\) signal and negative selection. Comparison of transcripts from AND DP thymocytes stimulated with gp250–I-E\(k\) with those stimulated with MCC–I-E\(k\) identified several candidate genes that might function specifically in positive selection. Through analysis of these candidates, we observed that VGSC (composed of a pore-forming SCN5A subunit and a regulatory SCN4B subunit) induced sustained Ca\(^{2+}\) influx during the positive selection of AND T cells. Specific pharmacological blockade of the VGSC with tetrodotoxin inhibited gp250-stimulated sustained Ca\(^{2+}\) signal and, notably, abolished the positive selection of AND thymocytes in reaggregate thymic cultures. A fusion protein of the SCN4B extracellular immunoglobulin domain and human immunoglobulin constant regions inhibited the gp250-stimulated sustained Ca\(^{2+}\) signal and the positive selection of AND DP thymocytes, which highlighted a critical role for this region of the SCN4B subunit during thymocyte selection. In nontransgenic, chimeric C57BL/6 mice reconstituted with bone marrow hematopoietic stem cells expressing shRNA that silenced the expression of SCN5A, less positive selection of CD4\(^+\) single-positive (CD4\(^{+}\)SP) thymocytes confirmed a requirement for VGSC during positive selection \textit{in vivo}. To substantiate our model, we explored the role of VGSC in antigen sensitivity of peripheral T cells through a gain-of-function assay. We reconstituted peripheral AND CD4\(^+\) T cells, which normally do not express VGSC, with human SCN5A and SCN4B. The human VGSC–reconstituted AND CD4\(^+\) T cells acquired the ability to respond to the positively selecting ligand gp250, to which they normally do not respond. Thus, the expression of a VGSC in DP thymocytes provides a mechanism through which a weak positive selection signal is amplified and sustained, resulting in the developmental process of positive selection.
RESULTS

Induction of a sustained Ca\(^{2+}\) flux by gp250

The endogenous positively selecting peptide gp250 for the AND TCR has no homology with the agonist MHC ligand but is recognized with a high degree of specificity. Surface plasmon resonance analysis showed that the binding affinity of the AND TCR with the gp250–I\(^{E_k}\) was very weak (>500 \(\mu\)M) relative to that of MCC–I\(^{E_k}\) (~13 \(\mu\)M; Fig. 1a). Staining AND DP thymocytes with gp250–I\(^{E_k}\) tetramers confirmed that weak affinity (Fig. 1b).

To examine early signaling events, we investigated Ca\(^{2+}\) influx in preselected AND H-2\(^{d}\) DP thymocytes deficient in recombination-activating gene 1 (Rag1\(^{-/-}\)) stimulated with plate-bound gp250–I\(^{E_k}\) immunoglobulin dimers. AND thymocytes were not selected on an H-2\(^{d}\) background and therefore arrested as preselection DP thymocytes, whereas transgenic expression of the TCR on the Rag1\(^{-/-}\) background prevented expression of TCRs of other specificities. Thus, essentially all thymocytes from AND Rag1\(^{-/-}\) H-2\(^{d}\) mice were resting, preselected DP thymocytes. By ratiometric imaging with the calcium indicator Fura-2 AM, we compared the pattern of Ca\(^{2+}\) influx in preselected AND DP thymocytes stimulated with the positively selecting peptide gp250–I\(^{E_k}\), the negatively selecting agonist peptide MCC–I\(^{E_k}\) or the nonselecting control peptide hemoglobin–I\(^{E_k}\). We found that gp250–I\(^{E_k}\) stimulated a strong and sustained Ca\(^{2+}\) influx (Fig. 1c and Supplementary Fig. 1a), which was maintained at its peak for more than 15 min, consistent with Ca\(^{2+}\) responses observed during positive selection. In contrast, MCC–I\(^{E_k}\) stimulated a transient Ca\(^{2+}\) influx, whereas the agonist MCC induced a strong yet transient signal.

Expression of VGSC components by preselected DP thymocytes

To identify genes encoding molecules critical for the gp250-induced sustained Ca\(^{2+}\) influx, we did transcriptional profiling of DP thymocytes. We stimulated preselected AND DP thymocytes for 7 h with I\(^{E_k}\) immunoglobulin dimers loaded with the positively selecting peptide gp250, the negatively selecting agonist peptide MCC or the nonselecting control peptide hemoglobin. We identified 28 genes upregulated by positive selection and downregulated in response to negative selection (Fig. 2a). Only one of these 28 genes, Scn4b, encoded an ion channel–related protein\(^{17-19}\). Scn4b encodes...
a regulatory β-subunit of VGSCs. We first confirmed by quantitative RT-PCR that the stimulation of AND DP thymocytes with gp250 maintained the expression of Scn4b, whereas stimulation with MCC downregulated Scn4b expression (Fig. 2b). Next we confirmed high expression of Scn4b in normal C57BL/6 DP thymocytes but not in mature single-positive thymocytes or peripheral T cells (Fig. 2c). Thus, Scn4b expression precisely correlated with positive selection both in vivo and in vitro, which indicated that SCN4B may participate in signaling during positive selection.

The functions of VGSCs have been extensively characterized in excitable cells, such as neurons and muscle cells, but to our knowledge have not been studied before in T cell development. VGSCs are composed of a pore-forming α-subunit along with one or two auxiliary regulatory β-subunits. Scn4B, a VGSC α-subunit, could potentially interact with ten different VGSC α-subunits. To identify the pore-forming α-subunit in DP thymocytes that pairs with SCN4B, we assessed which of ten potential VGSC α-subunits were expressed in preselected DP T cells. SCN5A was the VGSC α-subunit with the highest expression in preselected DP thymocytes, as assessed by quantitative RT-PCR, and was thus the strongest candidate for forming the pore of VGSCs in preselected DP thymocytes (Supplementary Fig. 2). Expression of Scn5a, like that of Scn4b, correlated with the positive selection (DP) and β-selection (DN3) checkpoints in thymocyte development (Fig. 2d). The expression of Scn5a mRNA was maintained during stimulation with gp250–I-Ek but decreased during stimulation with MCC–I-Ek (Fig. 2e). Thus, DP thymocytes expressed both a pore-forming subunit (SCN5A) and a regulatory subunit (SCN4B) of a VGSC, and expression of both subunits was specifically maintained by a positive selection signal.

**Blocking VGSC pore activity impairs positive selection**

To ascertain whether a VGSC was required for positive selection, we treated preselected AND DP thymocytes with tetrodotoxin, a specific inhibitor of VGSCs, in gp250-induced reaggregate cultures. Tetrodotoxin blocks the entry of Na+ into cells through VGSC α-subunits, with SCN5A being in the subset that requires higher tetrodotoxin concentrations to inhibit it. Blockade of VGSCs abolished the gp250-mediated positive selection of AND CD4+ T cells in reaggregate cultures (Fig. 3a). Furthermore, treatment with tetrodotoxin inhibited upregulation of the positive selection markers TCR in CD69 on DP thymocytes stimulated with gp250 (Supplementary Fig. 3). In the reaggregate cultures, we used the unstimulated ANV41.2 cortical thymic epithelial cell line transfected with cDNA encoding I-Ek and I-Ek in the expression vector pcEXV-3 (ref. 16) to minimize the number of endogenous positive-selecting ligands; this allowed us to add known self peptides, such as gp250. ANV41.2 cells, after treatment with interferon-γ (IFN-γ), upregulated the expression of MHC class I and II molecules and antigen-processing components and thus presented many endogenous peptides able to induce efficient positive selection, including AND and polyclonal T cells. In the presence of many positively selecting self peptides induced by IFN-γ, tetrodotoxin treatment also repressed the positive selection of AND CD4+ T cells in reaggregate cultures (Fig. 3b). When we added polyclonal DP thymocytes from C57BL/6 H-2k (B6.K) mice to the reaggregate cultures, a significant CD4+ population developed that was inhibited by treatment with tetrodotoxin, whereas the CD8+ SP (CD88P) T cell population that developed was not inhibited by tetrodotoxin (Fig. 3c). This finding suggested that VGSC was more critical for the positive selection of CD4+ T cells than for that of CD8+ T cells. Finally, tetrodotoxin-mediated inhibition of VGSC pore activity greatly diminished the sustained Ca2+ flux induced by gp250 stimulation from 131 nM to 52 nM (Fig. 3d,e) but did not alter the Ca2+ flux induced by stimulation with MCC agonist, a negative selection condition (Supplementary Fig. 4a). In reaggregate cultures containing a high dose of MCC, there was a disappearance of DP thymocytes regardless of the presence or absence of

![Figure 3](image-url)

**Figure 3** The pore-forming SCN5A subunit is critical in positive selection. (a) Flow cytometry (left) of thymocyte differentiation in reaggregate cultures of ANV41.2 cells transfected to express I-Ek and cultured for 96 h with preselected AND Rag1−/− H-2d DP thymocytes, along with 30 μM gp250, in the presence of vehicle (Ctrl) or 1 μM tetrodotoxin (TTX), and quantification of the frequency of the CD4SP subpopulation (right; each symbol represents a reaggregate culture, and small horizontal lines indicate the mean). Reaggregate cultures pulsed with 30 μM Hb served as negative controls. Numbers adjacent to outlined areas indicate percent CD4SP cells. *P = 0.007 (two-tailed Mann-Whitney test). (b) Flow cytometry of preselected AND Rag1−/− H-2d DP thymocytes cultured with 96 h with ANV41.2 cells that had been treated for 24 h with IFN-γ (100 units/ml). Numbers adjacent to outlined areas indicate percent CD4SP cells. *P = 0.0068 (two-tailed Mann-Whitney test). (c) Flow cytometry of preselected CD53− B6.K DP thymocytes cultured with IFN-γ–treated ANV41.2 cells for 96 h. Numbers adjacent to outlined areas indicate percent CD4SP cells (top left) or CD8SP cells (bottom right). NS, not significant; *P = 0.0027 (two-tailed Mann-Whitney test). (d) Intracellular Ca2+ concentration ([Ca2+]i) in AND Rag1−/− H-2d DP thymocytes stimulated with gp250–I-Ek in the presence of vehicle or TTX. (e) Peak (left) and mean (right) intracellular Ca2+ concentration from 7.5 min to 15 min. *P = 0.0099 and **P < 0.0001 (two-tailed Mann-Whitney test). Data are representative of three experiments with nine (a,c) or ten (b) cultures (a–c). Data are representative (n = 50 cells) of two experiments (d; error bars, s.e.m.) or two experiments with 50 cells each (e; error bars, s.d; n = 100 cells).
Regulation of pore activity by the SCN4B extracellular domain

SCN4B contains one extracellular immunoglobulin domain, a single transmembrane segment and a short intracellular tail. In excitable cells, the regulatory β-subunits of VGSCs have been shown to facilitate cell-cell interactions through the extracellular immunoglobulin domain and to modulate VGSC channel gating through either the extracellular immunoglobulin domain or the intracellular tail, which may favor an open VGSC at depolarized potentials.  

Thus, the extracellular immunoglobulin domain of SCN4B may interact with the immunoglobulin domains of other adhesion molecules on cortical thymic epithelial cells to facilitate cell-cell interactions or may act in cis with the extracellular portion of the SCN5A pore subunit regulating pore activity. To study the importance of the extracellular immunoglobulin domain of SCN4B, we generated an SCN4B-immunoglobulin fusion protein that would saturate potential ligands of SCN4B and thus disrupt cis or trans interactions mediated by SCN4B. The SCN4B-immunoglobulin fusion protein inhibited in vitro positive selection of AND thymocytes in gp250-mediated reaggregate cultures, whereas an unrelated immunoglobulin fusion protein did not. The SCN4B-immunoglobulin fusion protein also inhibited the positive selection of CD4+ polyclonal B6.K thymocytes but not the selection of CD8+ T cells. These findings demonstrated that SCN4B was essential for positive selection of CD4+ T cells in vitro.

To determine whether the SCN4B immunoglobulin domain was acting in cis or in trans, we assessed the effect of SCN4B-immunoglobulin fusion protein on the Ca\(^{2+}\) response, in which the DP thymocytes were the only cells present in the assay. In vitro, the SCN4B-immunoglobulin fusion protein inhibited the gp250-induced Ca\(^{2+}\) responses of preselected AND DP thymocytes in a way similar to that with tetrodotoxin treatment (Fig. 4d,e). This finding supported the proposal that the extracellular domain of SCN4B was essential for positive selection by its regulation of the SCN5A pore in cis.

**Scn5a** knockdown impairs CD4SP positive selection

We obtained in vivo genetic evidence of the requirement for VGSCs in positive selection through the use of bone marrow chimeras that expressed shRNA that silenced Scn5a expression in hematopoietic stem cells. We used green fluorescent protein (GFP) as a marker of transduction. Because of the lability of nonproliferating DP thymocytes, we could not transduce DP thymocytes by lentivirus in sufficient numbers to allow immunohistological analyses. Through in vitro single cell–based Ca\(^{2+}\) flux experiments, we identified a pool of two shRNA-GFP lentiviruses that targeted Scn5a (Scn5a-shRNA–GFP) and diminished the sustained Ca\(^{2+}\) flux in gp250–I-E\(^{a}\)-stimulated GFP+ AND DP thymocytes (Supplementary Fig. 5a,b). We transduced control cells with lentivirus expressing a nontargeting ‘scrambled’ shRNA–GFP that did not target any mouse transcripts, with which we compared all on-target effects. GFP+ cells transduced with lentivirus expressing scrambled shRNA–GFP showed no observed decrease in the gp250-induced sustained Ca\(^{2+}\) flux. Next we infected Ly5.1+ Sca-1+c-Kit+ C57BL/6 (B6) hematopoietic stem cells with lentivirus expressing Scn5a-shRNA–GFP or scrambled shRNA–GFP and intravenously injected these cells into lethally irradiated B6 donors. After 12 weeks, Scn5a-shRNA–mediated knockdown resulted in a significant decrease in CD4SP thymocyte populations from 18.5% to 13.2%, in contrast to the control groups of scrambled shRNA–GFP (Fig. 5a,b and Supplementary Fig. 5c,d). We observed a similar loss of mature CD4+ T cells in the periphery (Fig. 5c,d and Supplementary Fig. 5d). In contrast, there was no decrease in the double-negative (DN) or DP populations and there was a slight increase
Figure 5 Knockdown of Scn5a by shRNA impairs the positive selection of CD4SP cells in vivo. (a–h) Flow cytometry of thymocytes and splenocytes from lethally irradiated bone marrow B6 chimeras reconstituted with Ly5.1– B6 hematopoietic stem cells transduced with lentivirus expressing scrambled shRNA–GFP (Ctrl; n = 4 recipients) or Scn5a shRNA–GFP (Scn5A; n = 6 recipients), following analysis of recipient mice 12 weeks later. (a) Flow cytometry of thymocytes, gated on Ly5.1–GFP+ cells. Numbers adjacent to outlined areas indicate percent cells in each. (b) Frequency of DN, DP, CD4SP and CD8SP thymocytes among Ly5.1–GFP+ cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean. *P = 0.0095 (two-tailed Mann-Whitney test). (c) Flow cytometry of CD4+ and CD8+ splenocytes, gated on Ly5.1–GFP+ cells (numbers in plot as in a). (d) Frequency of splenocytes (presented as in b). (e) Flow cytometry of CD8SP cells, gated on Ly5.1–GFP+ CD8SP thymocytes. Numbers adjacent to outlined areas indicate percent immature (CD24–TCRβ+) CD8SP cells (top left) and mature (CD24–TCRβ+) CD8SP cells (bottom right). (f) Frequency of the immature and mature CD8SP cells in e (presented as in b). (g) TCRβ expression on DN cells in the presence of scrambled shRNA–GFP or Scn5a shRNA–GFP, gated on Ly5.1–GFP+ DN thymocytes. Numbers above bracketed lines indicate percent TCRβ+ cells (left) or TCRβ+ cells (right). (h) Frequency of TCRβ+ DN cells and TCRβ+ cells recovered from recipient mice, gated on Ly5.1–GFP+ DN cells (presented as in b). NS, not significant. Data are representative of two experiments (a–h).

in the CD8SP thymocyte population (9.58% to 10.02%; Fig. 5b). Separation of the CD8SP population into mature CD8SP and immature CD8SP cells showed that the slight increase (Fig. 5b) resulted from the accumulation of immature CD8SP T cells (Fig. 5c, f), whereas the TCRβ+CD24+ immature CD8SP population was not significantly larger (Fig. 5c, f). We observed no apparent difference in the upregulation of TCRβ expression of Scn5a-knockdown DN cells compared with that of scrambled shRNA control DN cells (Fig. 5g, h), which suggested a dispensable or less critical role for SCN5A in β selection. The inhibition of CD4SP selection but not of CD8SP selection was consistent with the results we obtained by tetradotoxin inhibition of in vitro positive selection of B6.K thymocytes (Fig. 3c). This difference in the effect on CD4SP cells was consistent with the present lineage-commitment models in which CD4SP

Figure 6 Peripheral AND CD4+ T cells acquire the ability to respond to positively selecting ligands by expression of human VGSCs. (a) Flow cytometry of AND hybridomas left untransfected (huVGSC+) or transfected (huVGSC−) with plasmids encoding human SCN5A and GFP (huSCN5A-GFP) and GFP or human SCN4B and DsRed (huSCN4B-DsRed). Numbers in quadrants indicate percent cells in each. (b) Expression of CD69 on untransfected and transfected AND hybridomas (as in a) stimulated overnight with plate-bound gp250–I-Ek or MCC–I-Ek. Black lines, unstimulated cells; blue line and blue fill, untransfected cells (huVGSC+); red line and red fill, transfected huVGSC+ cells. (c) Expression of CD69 on peripheral AND Rag1−/− H-2b CD4+ T cells transfected by electroporation with plasmids encoding human SCN5A-GFP or human SCN4B-DsRed, then allowed to ‘rest’ for 12 h and then stimulated for 10 h with plate-bound gp250–I-Ek or MCC–I-Ek. Cells in individual cultures were gated on GFP+DsRed+ (huVGSC+) or GFP–DsRed+ (huVGSC−). (d) Frequency of CD69+ cells among cells stimulated with gp250–I-Ek. P = 0.0139 (paired t test). Data are representative of two experiments (a, b) or three cultures in two independent experiments (c, d). error bars, s.d. for three cultures.)
cells require a sustained stronger signal than do CD8SP T cells. Thus, shRNA-mediated knockdown of Scn5a resulted in significantly less development of CD4+ T cells in B6 mice, which established the importance of VGSC in positive selection in vivo.

Reconstitution with VGSC enhances sensitivity to gp250
Preselected thymocytes are known to be more sensitive to TCR stimulation than are mature T cells. VGSCs were specifically expressed in preselected thymocytes and their expression was immediately down-regulated after thymocytes completed positive selection (Fig. 2c,e), which correlated with the change in sensitivity. To examine the potential contribution of VGSCs to the enhanced sensitivity of preselected thymocytes, we generated a cell line from AND T cell hybridomas that expressed human SCN5A and SCN4B proteins. We transfected AND T cell hybridomas by electroporation with both of these plasmids and sorted for AND hybridomas positive for expression of human VGSC to obtain a stable cell line and assess gain of function (Fig. 6a). AND T cell hybridomas that expressed human VGSC upregulated their expression of CD69 when stimulated with gp250–I-Ek, whereas VGSC− AND hybridomas did not (Fig. 6b). The expression of human VGSC allowed AND hybridomas to respond to positively selecting ligand stimulation, which suggested that the presence of human VGSC may make T cells more sensitive. Given that only preselected DP thymocytes express VGSC, whereas peripheral T cells do not, we then expressed human SCN5A and SCN4B in mouse AND peripheral CD4+ T cells to determine whether the expression of human VGSC makes peripheral CD4+ T cells as sensitive as preselected DP thymocytes. We transfected cDNA encoding human SCN5A and SCN4B by electroporation into peripheral AND CD4+ T cells. The human VGSC–positive population of AND peripheral CD4+ T cells was stimulated by positively selecting ligand gp250, as assessed by CD69 upregulation (Fig. 6c,d). Both populations responded to stimulation with the agonist peptide MCC (Fig. 6c). This gain-of-function study supported our hypothesis that the VGSC was essential for the positive selection of CD4+ T cells by increasing the sensitivity of DP thymocytes to a weak positively selecting ligand.

DISCUSSION
Positive selection is an exacting process during which DP thymocytes ‘translate’ a spectrum of TCR–self peptide–MHC interactions into the physiological outcome of commitment to the CD4SP or CD8SP T cell lineage or death. The molecules exclusively required for positive selection or specific lineage commitment are still poorly understood. Here we have identified a previously unknown role for a VGSC in the positive selection of CD4+ T cells. The specific expression of a SCN5A-SCN4B VGSC in DP thymocytes endowed these cells at a crucial developmental stage the ability to respond to positive selection signals, thereby ‘translating’ weak signals into a sustained one. After maturing into CD4+ or CD8+ T cells and emigrating to the periphery, T cells are poised to recognize foreign antigens. Furthermore, SCN5A is not expressed in mature CD4+ T cells. Thus, stage-specific expression of a VGSC empowers a DP thymocyte to respond to a positively selecting ligand, whereas no expression in peripheral T cells avoids autoreactivity. In the B cell lineage, Sncnb is expressed only at the pre-B cell and pro-B cell stages, during which weak receptor signals are critically involved in B cell development.

How do VGSCs increase the sensitivity of DP thymocytes to weak positively selecting ligand, how are they activated by downstream of the TCR signal, how are they regulated and how are they involved in activating sustained Ca2+ signal? In neurons and muscle cells, VGSC-mediated Na+ fluxes may lead to an increase in Ca2+ through the activation of voltage-gated Ca2+ channels; the regulatory subunit SCN4B may mediate high-frequency firing; the activity of pore SCN5A may be modulated by phosphorylation through several kinases, including Fyn, PKC, PKA, CaMKII and calmodulin. Essentially nothing is known about how VGSCs function in lymphocytes, but it is possible that activation of a kinase downstream of the TCR is important for the activation of VGSCs. In peripheral T cells, store-operated Ca2+ entry through CRAC channels is the main pathway for increasing intracellular Ca2+ concentrations, but T cell development and positive selection has shown to be normal in various pathways of separate deficiency in either of the two key CRAC components STIM or ORAI. One possibility is that there is an undefined channel(s) involved, which might function independently of STIM and ORAI and might control the influx of Ca2+ into DP thymocytes during positive selection. Alternatively, CRAC channels have an important but redundant role. An intriguing possibility is that DP thymocytes may dominantly use non–store-operated Ca2+ channels during positive selection. In a published study, a Ca2+ current mediated by L-type voltage-gated Ca2+ channels has been recorded in T cells and has been shown to be involved in peripheral T cell maintenance and commitment to the CD4+ T cell lineage. It would be informative to determine whether VGSCs serve as a linkage, followed by the receipt of a positive-selection TCR signal, to activate L-type voltage-gated Ca2+ channels or other channels that may carry Ca2+ to allow sustained influx of Ca2+ into DP thymocytes.

In our studies, negative selection induced a stronger Ca2+ flux than positive selection did, and such a higher Ca2+ peak might have had a key role in inhibiting channel activity and decreasing gene expression. In contrast, a weaker yet more sustained Ca2+ flux may activate calcineurin- and Erk-dependent pathways, leading to survival and maturation. We believe that the function of sustained kinetics of Ca2+ flux is key in supporting positive selection, as has been suggested, rather than the function of the magnitude of Ca2+ flux. Along with our observation, a published study has examined Ca2+ responses in MHC class II–restricted positive selection in thymic slices and has found that the naturally occurring positive selection on H2-k thymic slices induced a positive selection signal–specific sustained oscillation. Notably, in this model of thymic slices, preselected thymocytes may freely move around and sample multiple positively selecting ligands to result in the sustained Ca2+ oscillation. However, in our system, the preselected thymocytes in vitro had been forced to receive repetitive stimulation from the same positively selecting ligands, which led to sustained signaling. The Ca2+ kinetics in MHC class I–restricted positive selection have been described before. Preselected OT-I thymocytes (which have transgenic expression of an ovalbumin–specific TCR) induce Ca2+ influx at a slower rate after being stimulated by positively selecting tetramers of altered peptide ligand than after being stimulated by tetramers of the agonist ovalbumin. Also, altered peptide ligand p33 stimulates a quantitatively different Ca2+ flux than a strong agonist peptide, and intracellular Ca2+ concentration stimulated by p33 peptide reaches a steady state that remains high for at least 1 h (ref. 5). The use of an altered peptide ligand of agonist peptides and the thymocytes expressing MHC class I–restricted TCRs might account for some of the differences between those studies and our study here.

Another question raised by our study is whether VGSCs control the selection of only CD4+ T cells or the selection of both CD4+ T cells and CD8+ T cells. We found that inhibition of VGSCs by tetrodotoxin did
not affect the positive selection of polyclonal CD8+ T cells. Given that the starting point for MHC class I–mediated selection was the same DP population that expressed VGSC components, we wonder if a low-affinity antigen stimulating through an MHC class I–restricted TCR might fail to sustain VGSC expression. There is no direct evidence for this; however, it has been suggested that naturally occurring positively selecting MHC class I ligands can sustain Erk activation for the positive selection of CD8+ T cells, which might suggest that an MHC class I ligand can sustain the VGSC surface expression and function for a certain period of time to allow Ca2+ entry and activation of Erk. Thus, the surface expression of VGSC induced by MHC class I and MHC class II ligands remains to be determined.

In the Scn5a-knockdown experiments, we observed inhibition of the positive selection of CD4+ T cells but not of CD8+ T cells, and we noticed variability in the frequency of CD8+ T cells in bone marrow chimeras. There are two possible explanations for this result. The first is that there is a difference in the sensitivity of CD4 and CD8 positive selection for SCN5A and we only achieved a partial knockdown of Scn5a mRNA. As the asymmetric model suggests, commitment to the CD4+ T cell lineage requires stronger and more sustained positive selection signals than does commitment to the CD8+ T cell lineage, and our data support this model. The second hypothesis is that SCN5A controls only the selection of CD4+ T cells. The variability in the frequency of the CD8+ population might also have been caused by both of these mechanisms. If positive selection of CD8+ T cells requires less involvement of SCN5A, variable efficiency of VGSC depletion would present a variable possibility for DP thymocytes to complete CD8+ T cell development. In contrast, if CD4SP selection critically requires absolute participation of SCN5A, there would be a substantial development defect in CD4SP selection with less variability from mouse to mouse, consistent with our observations. The knockout of Scn5a is lethal to embryos and, therefore, conditional knockout of Scn5a–SCN4B VGSC controls the selection of only CD4+ T cells (such as themis and Thpok, do), or the selection of both CD4+ T cells and CD8+ T cells (such as calcineurin and Bcl11b do).

A similar rationale may be applied to our other observation that knockdown of Scn5a only resulted in defects in CD4SP positive selection but not in the β-selection of DN3 cells. A first possibility is the concern of incomplete knockdown of Scn5a mRNA. The second possibility is that only positive selection requires the participation of SCN5A because pre-TCR selection does not involve the recognition of peptide-MHC ligands but dimerization of pre-TCRs. In the future, it would worthwhile to define the precise role of SCN5A, if any, in early T cell development. Studies of the offspring of a cross of mice with a lckP-flanked Scn5a allele and mice expressing Cre recombinase under control of the promoter of the gene encoding the kinase Lck, in which Scn5a would be deleted at the DN stage, would be the most definitive way of assessing the role of VGSC in TCRβ selection.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession code. GEO: microarray data, GSE38909.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

W.-L.L. and P.M.A. designed the study and wrote the manuscript; W.-L.L. did the experimental work; and D.L.D. generated the AND hybridoma cell lines.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice and cells. MCC–I-Eβ-specific AND TCR-transgenic mice, B6.D (H-2b) mice, B6.K (H-2k) and B6 mice were from The Jackson Laboratory. All mice were bred and housed in specific pathogen-free conditions of the animal facility at the Washington University Medical Center. All use of laboratory animals was approved and done in accordance with the Washington University Division of Comparative Medicine guidelines. The ANV41.2 cortical epithelial cell line transfected with I-Eβ (provided by A.G. Farr) was cultured in Iscove’s DMEM containing 10% FCS, 1x nonessential amino acids, 2 mM GlutaMax, 1 mM sodium pyruvate, 0.05% gentamicin, 50 µM 2-mercaptoethanol and 0.5 µg/ml of the aminoglycoside G418.

Flow cytometry. Fluorophore-conjugated antibodies from commercial sources were as follows: fluorescein isothiocyanate–, allophycocyanin- or allophycocyanin-indocarbocyanine–conjugated anti-CD4 (GK1.5; BioLegend); phycoerythrin-indocarbocyanine–conjugated anti-CD8 (53-6.7; BioLegend); phycoerythrin-indocarbocyanine–conjugated anti-Ily5.1 (BioLegend); phycoerythrin–, allophycocyanin- or phycoerythrin-indocarbocyanine–conjugated anti-CD69 (1-11.2F3; BioLegend); phycoerythrin-indocarbocyanine–conjugated–anti-B220 (RA3-6B2; BioLegend) LIVE/DEAD Fixable Blue Dead Cell Stain (Invitrogen); or fluorescein isothiocyanate–conjugated or biotinylated anti-V,5 (RB-1; BD Pharmingen). All samples were analyzed on a FACSCalibur, FACSLISRII or FACSaria (BD), and data were analyzed with Flowjo software (TreeStar).

SPR analysis. Soluble AND TCR was generated in Escherichia coli using the chimeric expression system. The variable regions of mouse AND TCR were fused with the constant regions of human T cell clone LC13, to create plC13-ANDot and plC13-ANDβ constructs as ANC TCR α and β chains. AND α and β chains were expressed in inclusion bodies, refolded in an oxidized/reduced glutathione redox buffer and purified by fast protein liquid chromatography. The ligands gp250-I-Eβ–biotin, MCC–I-Eβ–biotin and hemoglobin–I-Eβ–biotin were refolded from inclusion bodies. The peptide-MHC ligands were attached to the surface plasmon resonance chip via streptavidin, and soluble AND TCRs were injected over the chip surface at a flow rate of 30 µl/min at 25 °C. The injected soluble AND TCR will be tested over various concentration ranges (by using twofold dilution) up to 500 µM. All measurements were baseline corrected by subtracting results of TCR injected over hemoglobin–I-Eβ–biotin ligands.

Tetramer-binding decay. Phycoerythrin-labeled I-Eβ tetramers in complex with MCC, gp250 or hemoglobin were generated. Irrelevant MHC-I tetramers (human CLIP–I-β2 from the US National Institutes of Health) were used as negative control. For tetramer staining, 1 × 10^6 T cells were stained on ice for 3 h with phycoerythrin-conjugated tetramer (50 µg/ml), fluorescein isothiocyanate–anti-V,5 (RR8-1; BD Pharmingen) and phycoerythrin-indocarbocyanine–anti-B220 (RA3-6B2). Cells positive for propidium iodide and B220 were gated out of the analysis.

Gene-expression analysis. Preselected AND Rag1−/− H-2d DP thymocytes were positively selected through the use of magnetic-activated cell sorting beads and were stimulated for 7 h with plate-bound gp250–I-Eβ, MCC–I-Eβ or hemoglobin–I-Eβ immunoglobulin dimers. For microarray analysis, as published, RNA was isolated with an RNeasy kit (Qiagen), and gene expression analyzed with a MouseRef-8 mouse Expression BeadChip (Illumina). A DNA-Chip Analyzer was used for normalization of the data and model expression values. Baseline expression was established with stimulation by the control hemoglobin–I-Eβ dimers.

Quantitative RT-PCR. RNA was obtained from preselected AND Rag1−/− H-2d sorted through the use of magnetic-activated cell sorting beads or B6 thymocytes purified by flow cytometry (all cells at various developmental stages) and was treated with 25 ng DNase. Then, cDNA was synthesized from the RNA with random hexamer primers (SuperScript II kit; Invitrogen). SYBR Green PCR master mix and an ABI7000 machine (Applied Biosystems) were used for quantitative RT-PCR. Cycling conditions were 50 °C for 2 min, followed by 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and a dissociation stage (95 °C for 15 s, 60 °C for 20 s and 95 °C for 15 s); β-actin was included for internal control for all samples. Changes were quantified by the change in cycling threshold (ΔCt; Fig. 2c.e) with standard curves or were calculated by the ΔΔCt method with nonselection hemoglobin–I-Eβ samples as the baseline sample and Actb as the reference gene (Fig. 2b.d). The following primers (5′ to 3′, forward and then reverse) were used: Scn5a, GGGCTTTTGGGTCTCTTC and GAGGTTCCTCAAAAGGCAATAACA; Scn5a, ATGGACAATCTCTGTTATCCCT and CCACCGGCTTTGTCTTACG; β-actin, CTAGGGCAACCGTGAAAG and ACCAGAGGACATAGGGGAC.

Analysis of intracellular Ca2+. Fura-2 AM Ca2+ imaging was done as published. Preselected AND Rag1−/− H-2d thymocytes were isolated through the use of CD8a MicroBeads (130-049-401; Miltenyi Biotech) and were loaded for 20 min at 37 °C with 5 µM Fura-2 AM (F-1221; Invitrogen). Cells (1 × 10^6) were loaded onto eight-chamber coverglass slides (177–402; Lab-Tek) that had been coated overnight at 4 °C with peptide-loaded I-Eβ immunoglobulin dimers (0.3 µg/ml). Ca2+ was imaged at 37 °C on a Zeiss Axiosvert 200 microscope equipped with a xenon arc lamp, and fluorescence was monitored in ratio mode. Cells being activated were quantified, and the reactive-cell frequency of gp250 stimulation was similar to that of MCC stimulation (both about 10%). Collected data were analyzed with MetaMorph software on randomly selected Ca2+–reactive cells. The curve for each cell was adjusted so that time 0 was when the Ca2+ started. The values of Rmin and Rmax were determined by exposure of cells to Ca2+–free solution containing 1 µM ionomycin to obtain Rmin and 10 M Ca2+ solution with 1 µM ionomycin to obtain Rmax (ref. 43). The intracellular Ca2+ concentration was calculated with an effective K value for Fura-2 AM of 260 nM (ref. 16). For treatment with pharmacological inhibitors, cells were ‘acutely’ treated with 1 µM tetrodotoxin or vehicle (1 µM citric acid pH 4.8) before the start of imaging. For treatment with immunoglobulin fusion proteins, cells were treated with 1 µg SCN4B–immunoglobulin fusion protein or 1 µg unrelated Cac3am4–immunoglobulin fusion protein just before the start of imaging.

Reaggregate cultures. In vitro positive selection reaggregates were done as described. Preselected and Rag1−/− H-2d or B6.K (H-2k) CD53+ preselection thymocytes were used. AND thymocytes are not selected on an H-2k background and are essentially all DP thymocytes. Preselection AND thymocytes were selected by CD8a MicroBeads (130-049-401; Miltenyi Biotech). Preselection B6K thymocytes were obtained by depleting CD53+ cell populations. ANV41.2 cortical thymic epithelia cells were pulsed with gp250 ligand (30 µg/ml) or control peptide hemoglobin (30 µg/ml) in peptide-mediated positive selection. In IFN-γ–induced positive selection, ANV41.2 cells were treated overnight with IFN-γ (100 units/ml), washed twice and used for reaggregate culture the next day. For treatment with pharmacological inhibitors, cells were treated with 1 µM tetrodotoxin or vehicle (1 µM citric acid, pH 4.8). For immunoglobulin fusion protein treatment, cells were treated with 1 µg SCN4B–immunoglobulin fusion protein or 1 µg unrelated Cac3am4–immunoglobulin fusion protein.

Production of lentivirus expressing shRNA. Five clones of shRNA targeting Scn5a (TRCN0000069003–TRCN0000069007; Sigma) and one nontargeting control shRNA (SHC002; Sigma) were cloned into plKO-puro-CMV-tGFP vector (Sigma). Scn5a shRNA–GFP or scrambled shRNA–GFP plasmid, and each packaging vector (delta 8.2, VSV-g) were cotransfected into HEK293 T cells. Supernatants containing virus particles were collected 48 h after transfection, filtered and concentrated by PEG 8000 precipitation. Two pairs of shRNAs (TRCN000069004 and TRCN000069007) resulted in a much lower sustained phase of Ca2+ influx (Supplementary Fig. 5a,b) and thus were selected for use in the bone marrow–chimera experiments.

Bone marrow chimeras. Lys5.1. B6 hematopoietic stem cells were enriched by c-Kit MicroBeads (130-091-224; Miltenyi Biotech), stained for c-Kit and Sca-1, and sorted as c-Kit+Sca-1+ population on a FACSariaII. Hematopoietic stem cells were resuspended at a density of 1 × 10^6 cells per 200 µl DMEM-F12, containing 10% FCS, 1x nonessential amino acids, 2 mM GlutaMax, 1 mM sodium pyruvate, 0.05% gentamicin, 50 M 2-mercaptoethanol, 50 ng/ml stem cell factor and 50 ng/ml thrombopoietin per well of a 96-well round-bottom plate. After 18–24 h of culture, 5 µl lentivirus stock was added to each well.

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Cells were adoptively transferred 24 h later into lethally irradiated B6 mice by retro-orbital injection on day 0 (at least 1 × 10^4 hematopoietic stem cells per mouse). After 12 weeks of reconstitution, cells were recovered from thymus and spleen of each recipient mouse and analyzed by flow cytometry.

**Expression of human VGSC in peripheral CD4+ T cells and AND hybridomas.** The human SCN5A-GFP construct was a gift from C. Nichols. The cDNA encoding human SCN4B was from Origene (RC223951) and was amplified by PCR and cloned into pcDNA3.1. A cassette encoding an internal ribosomal entry site and DsRed (6921-1; Clontech) was ligated into the pcDNA3.1-huSCN4B construct through the BamH1 and Not1 restriction sites. CD4+ peripheral T cells were isolated with CD4 magnetic-activated cell sorting beads, or AND hybridomas were separated by electrophoresis with human SCN5A-GFP and SCN4B-DsRed constructs (Amaxa Nucleofector kit for primary mouse T cells). After electroporation, primary peripheral AND CD4+ cells were allowed to ‘rest’ for 12 h, then were stimulated for 10 h with plate bound gp250–I-E^k (10 µg) or MCC–I-E^k (1 µg) along with 1 µg monoclonal antibody to CD28 (37.51; BioLegend), and analyzed by flow cytometry for CD69 expression. For AND hybridomas, after electrophoration, cells were stimulated for 24 h with plate-bound gp250–I-E^k (3 µg) or MCC/I-E^k (1 µg) along with CD28 (1 µg). Cells were stained with Live/Dead Blue Dead Cell Stain (L23105; Invitrogen) anti-CD4 and anti-CD69 antibodies.

**Generation of AND hybridomas.** CD4+ T cells from AND.Rag1<sup>−/−</sup> H-2<sup>k</sup> mice were stimulated for 3 d in vitro with MCC, and T cell hybridomas were generated with an established protocol<sup>44</sup> and the BW5147 αβ<sup>−</sup> fusion partner.

**Statistics.** All data were analyzed nonparametrically by the Mann-Whitney U-test or paired t test with Prism 4 or Prism 5 software (GraphPad). P values of less than 0.05 were considered significant.

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