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DLGH1 Is a Negative Regulator of T-Lymphocyte Proliferation▽†

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Discs large homolog 1 (DLGH1), a founding member of the membrane-associated guanylate kinase family of proteins containing PostSynaptic Density-95/Discs large/Zona Occludens-1 domains, is an ortholog of the *Drosophila* tumor suppressor gene Discs large. In the mammalian embryo, DLGH1 is essential for normal urogenital morphogenesis and the development of skeletal and epithelial structures. Recent reports also indicate that DLGH1 may be a critical mediator of signals triggered by the antigen receptor complex in T lymphocytes by functioning as a scaffold coordinating the activities of T-cell receptor (TCR) signaling proteins at the immune synapse. However, it remains unclear if DLGH1 functions to enhance or attenuate signals emanating from the TCR. Here, we used *Dlgh1* gene-targeted mice to determine the requirement for DLGH1 in T-cell development and activation. Strikingly, while all major subsets of T cells appear to undergo normal thymic development in the absence of DLGH1, peripheral lymph node *Dlgh1*$^{-/-}$ T cells show a hyperproliferative response to TCR-induced stimulation. These data indicate that, consistent with the known function of Discs large proteins as tumor suppressors and attenuators of cell division, in T lymphocytes, DLGH1 functions as a negative regulator of TCR-induced proliferative responses.

Engagement of the T-cell receptor (TCR) complex initiates signal transduction pathways involving protein and lipid kinases, phosphatases, and adapter proteins. Signals emanating from the TCR are thought to be modulated by the recruitment of transmembrane and cytosolic adaptor proteins, including PostSynaptic Density-95/Discs large/Zona Occludens 1 (PDZ) domain-containing scaffolds of the membrane-associated guanylate kinase (MAGUK) family. PDZ domains can bind to proteins via several mechanisms, the most common of which is through binding of conserved carboxyl-terminal sequences of proteins (8, 19, 27). Several studies suggest that PDZ domain proteins play critical functions in the compartmentalization of protein complexes and the asymmetric distribution of proteins into synaptic interfaces (3–5, 7, 15). In T cells, the evidence to date indicates that MAGUK proteins may interact with several proteins harboring PDZ ligands implicated in the regulation of TCR signaling and the coordination of T-cell polarity networks (16, 24, 25, 32). In addition to functioning as plasma membrane-proximal scaffolds, some PDZ proteins translocate to the nucleus and participate in transcriptional complexes, potentially allowing integration of structural functions with gene expression patterns, as has been shown for CASK (14).

While the majority of the literature concerning PDZ proteins has focused on their functions in neuronal and epithelial cells (3–5, 7, 15), recent studies have implied broader roles for these proteins in polarity determination in nonclassical synaptic systems, including in the immune synapse (10, 11, 32). For example, a founding member of the MAGUK family, Discs large homolog 1 (DLGH1), has recently been proposed to play a crucial role in regulating TCR signaling and T-cell polarity, along with the LAP family protein Scribble and the CARD family protein CARMA1, as well as several PAR family proteins (2, 12, 16, 23–25, 32, 33). However, the physiological relevance of these interactions is not completely understood. For example, it is unclear if DLGH1 plays an activating or inhibitory function in regulating TCR signaling (25, 32).

DLGH1 contains multiple protein interaction domains characteristic of proteins involved in signal transduction, including three N-terminal PDZ domains, a Src homology 3 domain, an i3 domain, a 4.1-binding domain, and a C-terminal guanylate kinase domain that lacks catalytic activity. Previous reports have shown that DLGH1 is expressed at high levels in developing and mature T cells and, following TCR activation, is recruited to lipid rafts and translocated to the immune synapse, where it can couple the TCR with Lck, Zap70, Vav, WASP, and p38 kinase and may influence signaling specificity (16, 24, 25, 32). However, the exact role of DLGH1 in T cells is unclear, as its downregulation by means of small interfering RNA (siRNA) in T-cell lines has been shown to alternatively attenuate and augment T-cell activation (24, 25, 32).

To gain further insights into the physiological role of DLGH1 in TCR signaling, we generated *Dlgh1*-deficient mice by gene targeting. Since mice harboring homozygous *Dlgh1*-

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null mutations show several developmental abnormalities and die perinatally (17), we used recombination-activating gene 2 (*Rag-2*)-deficient complementation to generate *Dlgh1*-deficient T cells. Here, we present biochemical and functional analyses of *Dlgh1*-deficient T cells which indicate that DLGH1 is dispensable for TCR signaling and for normal thymic development. However, DLGH1 is critical for regulation of T-cell proliferative responses. These results represent the first direct demonstration of a role for a MAGUK protein in lymphocytes using gene-targeted mice and indicate that DLGH1 functions in the negative regulation of T-lymphocyte proliferation, consistent with the role of the fly ortholog of the mammalian DLGH1, Discs large (DLG), which functions as an essential tumor suppressor and an attenuator of cell proliferation in imaginal discs during pupal development.

MATERIALS AND METHODS

Mice. The generation of the null allele of *Dlgh1* has been previously described (17). Mice were maintained as *Dlgh1* heterozygous in the specific-pathogen-free facility of Washington University School of Medicine in accordance with institutional policies for animal care and usage. C57BL/6 *Rag-2*$^{-/-}$ mice were sublethally irradiated (600 rads) and reconstituted with fetal liver cells from *Dlgh1*$^{+/+}$, *Dlgh1*$^{+/-}$, or *Dlgh1*$^{-/-}$ embryonic day 15.5 (E15.5) to E18 embryos. Survival rates were similar in lethally irradiated mice reconstituted with *Dlgh1*-sufficient and *Dlgh1*-deficient donor cells. The mice were analyzed 6 to 10 weeks following reconstitution. In all assays, no differences were found between *Dlgh1*$^{+/+}$ and *Dlgh1*$^{+/-}$ T lymphocytes, and mice reconstituted with *Dlgh1*$^{+/+}$ and *Dlgh1*$^{+/-}$ fetal liver cells were used interchangeably and designated wild type (WT). The generation of mice with a conditionally deleted *Dlgh1* allele is described in Fig. S2 and Materials and Methods in the supplemental material.

Flow cytometry. Single-cell suspensions were prepared from the spleens, thymus, and lymph nodes and stained with antibodies according to standard protocols. Antibody conjugates against the following markers were used: TCRβ, Foxp3, CD2, CD4, CD5, CD8, immunoglobulin D, immunoglobulin M, CD21/35, CD23, CD25, CD44, CD62L, and CD69 (BD Biosciences).

Stimulation and proliferation assays. Lymph node T cells were plated at 1×10^6/ml in complete Dulbecco's modified Eagle's medium-10% fetal bovine serum (FBS). The cells were stimulated with the indicated concentrations of soluble anti-CD3 and, when indicated, anti-CD28 (BD Biosciences) was used at 1 μg/ml. Proliferation assays using [^{3}H]thymidine were performed as previously described (6). Labeling with 1 μM carboxyfluorescein succinimidyl ester (CFSE Invitrogen) was performed on lymph node T cells. Briefly, cells were incubated with CFSE for 30 min at 37°C and then washed in complete medium. Labeled cells were cultured with the indicated stimuli for 72 h. The cells were labeled with 1 μM bromodeoxyuridine (BrdU) (Sigma Aldrich) for 1 h at 37°C. After being surface stained for CD4 and CD8, the cells were fixed with 4% paraformaldehyde, washed with staining buffer, and then frozen at −80°C in 10% FBS-90% dimethyl sulfoxide for at least 1 h. After being thawed, the cells were refixed and then permeabilized in 0.5% saponin-1% FBS/phosphate-buffered saline (PBS). The permeabilized cells were treated with 30 μg DNase I (Sigma Aldrich) for 1 h, washed, and then stained with antibodies against BrdU (Invitrogen), followed by 7-amino-actinomycin D (BD Biosciences).

Ca^{2+} fluxes. Cells were loaded with Fluo-4-AM (Invitrogen) (3 to 5 μg/ml) for 30 min at 37°C with occasional vortexing. The cells were stained with antibodies against CD4 or CD8, washed, resuspended in complete medium, and then analyzed by flow cytometry. Unstimulated cells were run through the cytometer for 20 seconds to establish a baseline and then stimulated with the indicated concentration of anti-CD3, immediately followed by cross-linking with sheep anti-hamster antibodies (Jackson ImmunoResearch Laboratories). Ca^{2+} fluxes were measured for an additional 5 min, followed by the addition of ionomycin (0.5 μg/ml) for an additional 1 min.

Cytokine assays. Purified lymph node T lymphocytes (10^5) were incubated with irradiated splenic cells from C57BL/6 *Rag-2*$^{-/-}$ mice and anti-CD3 antibodies for 24 h. Supernatant cytokine concentrations were analyzed using Cytometric Bead Array Assays (BD Biosciences) according to the manufacturer's instructions.

Immunoblotting and immunochemistry. Purified T cells were stimulated with anti-CD3, followed by cross-linking with anti-hamster antibodies. The cells were washed with cold PBS and then lysed in cold NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, supplemented with Complete protease inhibitors [Roche] according to the manufacturer's specifications) for 10 min at 4°C. The lysates were cleared by centrifugation at 14,000 × *g* for 10 min at 4°C. Samples were analyzed by Western blotting according to standard procedures using antibodies against phosphotyrosine (4G10; Upstate Biotechnology), phospho-Akt, phospho-p38, phospho-Erk1/2 (Cell Signaling Technology), and Erk2 (Santa Cruz). For T-cell-conjugate assays, white sulfate latex beads (Interfacial Dynamics) coated with anti-CD3 and anti-CD28 antibodies (10 μg/ml) were incubated with purified T cells at a ratio of 1 bead/cell for 30 min at 37°C. The conjugates were fixed with 4% paraformaldehyde for 10 min and then plated onto poly-L-lysine-coated coverslips. The cells were stained with fluorescently labeled phalloidin and the indicated fluorescent conjugated antibodies and analyzed by confocal microscopy.

Semiquantitative and real-time PCRs. CD8$^+$ T cells were purified from lymph node suspensions using magnetic sorting with anti-CD8 beads (Miltenyi Biotech; (purity, >90%). The cells were stimulated on plates coated with anti-CD3 (1 μg/ml) plus soluble anti-CD28 (1 μg/ml) for 18 h and harvested in Trizol (Invitrogen), and RNA was extracted according to the manufacturer's instructions. The extracted RNA was treated with 10 U RNase-free DNase for 15 min at 37°C. cDNA was generated using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. Semiquantitative PCR was performed on fourfold dilutions of the cDNAs. Real-time PCR was performed using an MX300P cycler (Stratagene).

RESULTS

DLGH1 is a negative regulator of T-cell proliferation. TCR ligation initiates multiple signaling cascades, culminating in the induction of cell division and proliferation. In this context, DLGH1 has been hypothesized to act as a scaffolding protein in T lymphocytes, the function of which is to integrate and coordinate multiple signaling cascades. To conclusively address the requirement for DLGH1 in the induction of proliferative responses in primary (naïve) T cells, we utilized mice harboring a gene-targeted *Dlgh1*-null allele (17). Since mice homozygous for the null allele of *Dlgh1* exhibit perinatal lethality (17), we used *Rag-2*-deficient complementation to generate *Dlgh1*-deficient T cells. To this end, irradiated *Rag-2*-deficient mice were reconstituted with E15.5 to E18 fetal liver cells from *Dlgh1*$^{-/-}$, *Dlgh1*$^{+/-}$, or *Dlgh1*$^{+/+}$ embryos. Of note, since no differences were observed in our analyses of *Dlgh1*$^{+/-}$ versus *Dlgh1*$^{+/+}$ cells (data not shown), we did not further distinguish between these genotypes in any subsequent designations. Accordingly, *Dlgh1*$^{+/-}$ and *Dlgh1*$^{+/+}$ cells were designated WT and *Dlgh1*$^{-/-}$ cells were designated knockout (KO). Strikingly, our analyses of TCR-induced proliferative responses of KO and WT T lymphocytes using [^{3}H]thymidine incorporation showed increased proliferation of KO T cells (Fig. 1A and B). Thus, compared with WT T lymphocytes, [^{3}H]thymidine incorporation was enhanced in both CD8$^+$ and CD4$^+$ KO T cells, although the effects were more pronounced with CD8$^+$ cells. This enhanced response was seen at all doses of anti-CD3 antibodies tested but was most pronounced when lower concentrations of stimulatory antibodies were used (Fig. 1A and B). Moreover, CFSE dye dilution assays demonstrated that KO T lymphocytes underwent several more divisions in response to stimulation with anti-CD3 or anti-CD3 and anti-CD28 antibodies than WT cells. Again, this effect was most pronounced at lower concentrations of activating stimuli (Fig. 1C and D). Of note, despite a clear reduction of the DLGH1 protein expression level in hemizygous cells (by approximately twofold) (17), the proliferative responses of *Dlgh1*$^{+/-}$ and *Dlgh1*$^{+/+}$ T cells were identical (data not shown), indicating that downregulating DLGH1 to about half of its normal level

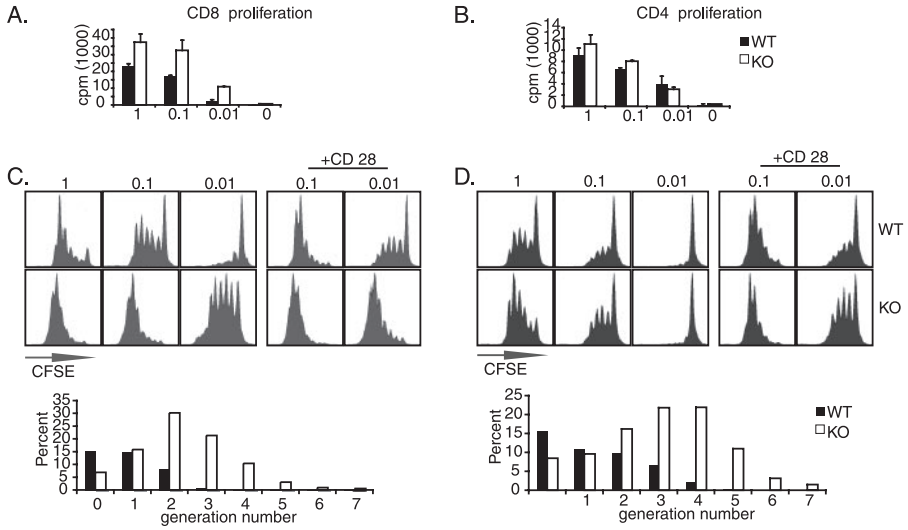

FIG. 1. Proliferation of *Dlgh1*$^{-/-}$ T lymphocytes. (A and B) Purifed CD8$^+$ and CD4$^+$ T cells from *Rag-2*-null mice reconstituted with WT or KO fetal liver cells were stimulated with the indicated concentrations of stimulatory anti-CD3 (μg/ml) with or without the addition of anti-CD28 (1 μg/ml) and analyzed for [^{3}H]thymidine incorporation. Shown are raw cpm values from one experiment, representative of three, all with similar results. The error bars indicate standard deviations. (C and D) Lymph node cells from mice reconstituted with WT or KO fetal liver cells were loaded with CFSE and stimulated with the indicated concentrations of anti-CD3 or anti-CD3 plus anti-CD28 (1 μg/ml). The cells were analyzed 72 h poststimulation. Shown is one representative sample from the indicated CD4$^+$ or CD8$^+$ gate ($n > 5$), all with similar results. Shown in bar graphs below the histograms is one representative experiment (in which the lymphocytes were stimulated with 0.1 μg/ml anti-CD3) showing the percentage of cells in each generation peak as calculated from the cell counts per peak.

did not affect T-cell proliferation. Taking these data together, we conclude from these experiments that in resting (naïve) T cells, the loss of DLGH1 leads to an exaggerated TCR-induced proliferative response.

Development of *Dlgh1*-deficient T lymphocytes. Exaggerated TCR-induced proliferative responses of KO T lymphocytes could potentially result from an abnormal developmental process, such as those observed in mice lacking negative regulatory proteins, like Cbl, Csk, or SHP-1/2 (1, 18, 26, 28), all of which show hyperresponsiveness to TCR stimuli. To determine if DLGH1 is required for normal T-cell development, we analyzed thymic and peripheral T-cell subsets that developed in *Rag-2*-deficient mice reconstituted with KO and WT fetal liver cells. We found that both the total numbers and percentages of thymocytes in double-negative (DN), double-positive, and single-positive CD4$^+$ and CD8$^+$ subsets were indistinguishable between mice reconstituted with KO and WT cells (Fig. 2 and Table 1). Also, the expression levels of TCR and several other surface markers, including CD24, CD5, and CD2, were similar in the two types of cells in all thymic subsets analyzed (Fig. 2). In addition, our analyses of DN subsets of thymocytes, which can be subdivided based on the expression of CD44 and CD25 into four populations, CD44$^+$ CD25$^-$ (DN1), CD44$^+$ CD25$^+$ (DN2), CD44$^-$ CD25$^+$ (DN3), and CD44$^-$ CD25$^-$ (DN4), showed no significant differences between KO and WT chimeras (data not shown). We also analyzed the populations of peripheral T lymphocytes in spleens and lymph nodes of mice reconstituted with WT and KO fetal liver cells, which showed normal numbers and percentages of mature CD4$^+$ and CD8$^+$ subsets (Fig. 2 and Table 1). Moreover, WT and KO peripheral T cells expressed similar levels of TCR and other surface markers, including CD62L, CD44, CD5, CD25, and CD69 (Fig. 2), and showed similar populations of T-regulatory cells, as indicated by staining for intracellular Foxp3 (Fig. 2). Taking these data together, we conclude from these experiments that DLGH1 is not required for normal thymocyte or peripheral-T-lymphocyte development in adult mice.

During thymic development, DLGH1 could be involved in the regulation of either thymocyte-intrinsic or thymocyte-extrinsic (or thymic stroma cell-intrinsic) signals, or both. For example, genes involved in Wnt signaling pathways, which have previously been implicated in DLGH1 function (13), can exert their developmental cues via thymic epithelial cell-intrinsic mechanisms (21, 29). Given that in *Rag2*-complemented *Dlgh1* chimeras thymic stroma is derived from the *Rag2*-deficient host tissue (which is *Dlgh1* sufficient), we analyzed the development of thymocytes in germ line *Dlgh1*-deficient embryos, in which both stroma and thymocyte progenitors lack DLGH1. Our analyses of *Dlgh1*-deficient fetal thymic development, starting immediately after thymic colonization with hematopoietic progenitors at E14 throughout the late stages of fetal development at E18, showed no substantial differences between KO and WT embryos (see Fig. S2 in the supplemental material). Thus, development of embryonic DN1 to -4, immature CD8$^+$ single-positive, and double-positive subsets is similar in *Dlgh1*-deficient and *Dlgh1*-sufficient embryos. We conclude from these experiments that DLGH1 is dispensable for normal thymic differentiation during fetal development.

As an alternative approach, we also generated and analyzed mice harboring a conditional ("floxed") *Dlgh1* allele, in which DLGH1 expression can be ablated in T-lineage cells in the presence of *lck*-Cre transgenes (see Fig. S2 in the supplemental material). Consistent with the results of analyses of *Rag-2*/*Dlgh1* chimeric mice, mice with conditionally deleted *Dlgh1* showed normal development of thymocytes and peripheral T

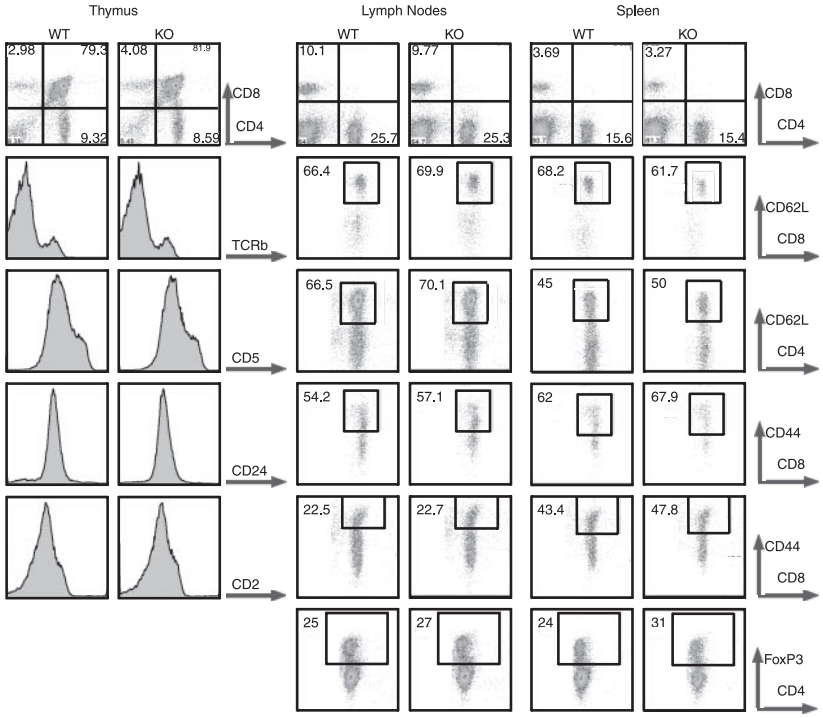

FIG. 2. Lymphocyte development in *Rag-2*-null mice reconstituted with KO fetal liver cells. Single-cell suspensions of thymocytes, splenocytes, or lymph node cells were counted, stained with the indicated fluorescent-antibody conjugates, and analyzed by flow cytometry as described in Materials and Methods. For analyses of CD44, CD62L, and Foxp3, the populations shown are from the indicated CD4 and CD8 gates. The data shown are from a representative experiment ($n > 5$).

lymphocytes (data not shown; see Fig. S2E and F in the supplemental material).

We also addressed the possibility that DLGH1 may be required for T-cell responses to chemokine gradients, such as stromal cell-derived factor 1α (SDF-1α), which have been implicated in the regulation of normal thymic egress (30). In this context, previous studies implicated Scribble, a DLGH1-related protein, in SDF-1α-mediated migration of T cells (16). To address this issue, we analyzed the migration of T lymphocytes from KO or WT chimeras in response to increasing concentrations of SDF-1α using a transwell assay. We found that SDF-1α-induced migration was indistinguishable between KO and WT T lymphocytes (see Fig. S3 in the supplemental material). These data indicate that in primary T lymphocytes, DLGH1 is not required for chemokine-induced migration. Collectively, our results support the hypothesis that *Dlgh1* deficiency results in a distortion of TCR signals that leads to an exaggerated proliferative response of T cells in vitro in the absence of major alterations in their development in either primary or secondary lymphoid organs.

TABLE 1. Lymphocyte populations in *Rag-2*-null mice reconstituted with *Dlgh1*$^{+/-}$ or *Dlgh1*$^{-/-}$ fetal liver cells[a]

T cells	No. of cells[b]	
	Dlgh1$^{+/-}$	*Dlgh1*$^{-/-}$
Thymus		
Total	140.5 ± 37.1	168.0 ± 44.2
CD4$^+$ CD8$^+$	107.9 ± 31.8	127.7 ± 38.9
CD4$^+$ CD8$^-$	15.8 ± 4.7	17.6 ± 6.0
CD4$^-$ CD8$^+$	5.7 ± 2.5	6.5 ± 2.9
CD4$^-$ CD8$^-$	8.1 ± 2.9	9.5 ± 4.2
Spleen		
Total	77.9 ± 38.3	88.2 ± 25.0
Total T cells	13.9 ± 8.2	13.9 ± 5.0
CD4$^+$	11.3 ± 6.9	11.1 ± 4.2
CD8$^+$	2.6 ± 1.3	2.8 ± 1.0
Lymph node		
Total	103.3 ± 33.3	108.2 ± 44.9
Total T cells	37.7 ± 11.9	39.0 ± 17.8
CD4$^+$	28.5 ± 9.3	28.5 ± 13.7
CD8$^+$	9.8 ± 3.7	11.1 ± 4.5

[a] Data are means ± standard deviations and represent multiples of 10^6 cells.
[b] *Dlgh1*$^{+/-}$ consisted of 12 mice, and *Dlgh1*$^{-/-}$ consisted of 13 mice.

Polarization of the actin cytoskeleton and recruitment of polarity and signaling proteins to the immune synapse in the absence of DLGH1. Actin cytoskeletal remodeling is critically important for sustained TCR signaling and activation. The i3 domain of DLGH1 has been shown to interact with proteins of the ezrin-radixin-moesin family, which couple proteins to the actin cytoskeleton. While DLGH1 is found in the immunological synapse following TCR cross-linking, a previous report indicated that cells expressing decreased levels of DLGH1 due to siRNA knockdown have diminished polymerized actin content and disrupted clustering and raft formation following TCR stimulation (25). To determine if DLGH1 is required for actin polymerization in primary *Dlgh1*-deficient T cells, we analyzed cytoskeletal remodeling and polarization toward a TCR-stimulatory target. Freshly isolated T lymphocytes from

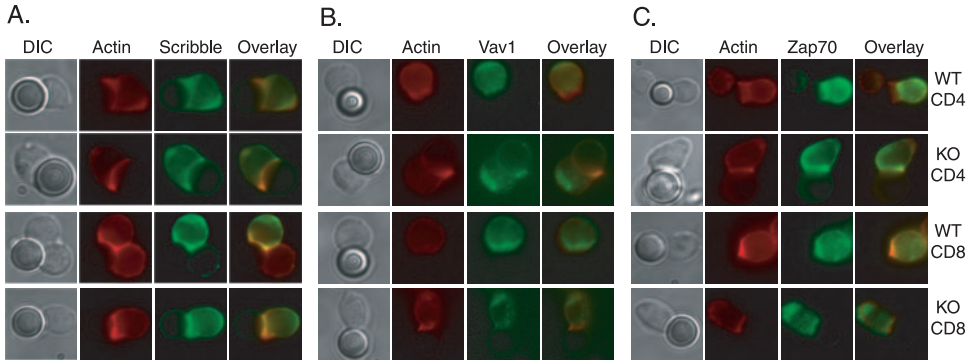

FIG. 3. Actin accumulation and recruitment of polarity and signaling proteins to the immune synapse in KO lymphocytes. (A to C) Purifed T cells from chimeric mice were incubated with anti-CD3-coated latex beads for 30 min. The cells were stained with phalloidin and antibodies against Scribble, Vav1, and Zap-70, followed by analysis by confocal microscopy. Shown is one experiment, representative of three. In each experiment, >60 conjugates were analyzed. DIC, differential interference contrast.

KO and WT chimeric mice were incubated with anti-CD3 and anti-CD28 antibody-coated latex beads and then analyzed for actin polymerization (as measured by accumulation of F-actin using phalloidin staining). F-actin accumulations were similar in WT and KO lymphocytes incubated with stimulatory beads (Fig. 3A to C). As expected, few bead-cell conjugates were found in cells incubated with control beads (coated with PBS/bovine serum albumin), nor did these conjugates show F-actin accumulation at the site of conjugation (data not shown).

Following TCR ligation and immune synapse formation, DLGH1 has been shown to colocalize with the polarity protein Scribble (16) and to associate with the signaling proteins Vav1 and ZAP-70, all of which are relocalized to the immune synapse following TCR cross-linking (16, 25, 32). To determine if the TCR-induced relocalization of these proteins is altered in the absence of DLGH1, we analyzed Scribble, Vav1, and ZAP-70 localization using conjugate assays. In both $CD4^+$ and $CD8^+$ cells, Scribble is localized to the immune synapse in a manner indistinguishable between KO and WT T cells (Fig. 3A). Moreover, Vav1 and ZAP-70 were found in the synapses of both KO and WT $CD4^+$ and $CD8^+$ T cells following TCR ligation (Fig. 3B and C). Similarly, assays analyzing the polarization of microtubule organizing centers toward the TCR-stimulatory target also showed no perturbations in the absence of DLGH1 (data not shown). Together, these data indicate that DLGH1 is not required for cytoskeletal remodeling and the relocalization of Scribble, Vav1, and ZAP-70 following TCR ligation in freshly isolated lymph node T cells.

TCR-induced signaling in *Dlgh1*-deficient T lymphocytes. DLGH1 contains multiple domains involved in signal transduction and associates with several proteins downstream of TCR activation (10, 11, 16, 24, 25, 32). However, it is unclear whether DLGH1 is required for the regulation of TCR-proximal signaling pathways in primary T cells. Given that DLGH1 is constitutively associated with the Shaker-related potassium channel Kv1.3 in T lymphocytes, which are implicated in the regulation of Ca^{2+} entry in T lymphocytes (10, 22), we first analyzed Ca^{2+} mobilization in freshly isolated T cells from KO and WT chimeric mice. Following TCR cross-linking with various doses of anti-TCR antibodies, both KO and WT $CD4^+$ and $CD8^+$ lymph node T cells showed Ca^{2+} fluxes with indistinguishable kinetics and magnitudes (Fig. 4A and data not shown). These data indicate that in T lymphocytes, DLGH1 is not required for Ca^{2+} mobilization following TCR cross-linking.

We next analyzed TCR-induced protein tyrosine phosphorylation patterns and activation of downstream signaling pathways in WT and KO T lymphocytes. Consistent with normal Ca^{2+} mobilization, the patterns of total protein tyrosine phosphorylation were similar in WT and KO T lymphocytes (Fig. 4B), and there were no significant differences in either the kinetics or the magnitudes of activation of Erk1/2, Akt, and p38 kinases (Fig. 4B; see Fig. S4 in the supplemental material). Moreover, the upregulations of CD25 and CD69, early markers of T-cell activation, were similar in TCR-stimulated WT and KO lymphocytes (see Fig. S5 in the supplemental material). To determine if DLGH1 is required for activation of nuclear factor of activated T cells (NFAT), we analyzed WT and *Dlgh1*-deficient T lymphocytes for their abilities to trans-

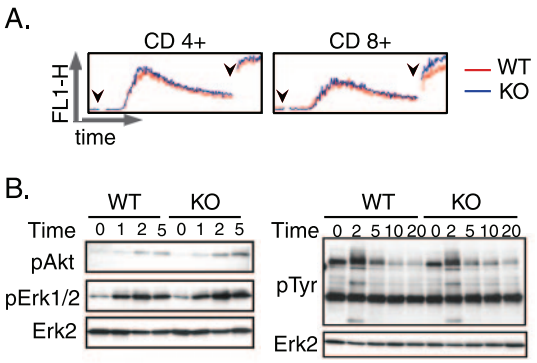

FIG. 4. Proximal signaling in KO T lymphocytes. (A) Lymph node cells from RAG chimeric mice were loaded with Fluo-4-AM and then stained with antibodies against CD4 (left) or CD8 (right). Cell suspensions were analyzed by flow cytometry for 20 seconds to determine the background and then for an additional 5 min after anti-CD3 (1 μg/ml) stimulation. Ionomycin was added at 5 min to achieve maximal Ca^{2+} signaling. In each plot, the first arrow indicates the addition of anti-CD3 and the second arrow indicates the addition of ionomycin. (B) Purified T cells from mice reconstituted with WT or KO donor cells were stimulated with 1 μg/ml anti-CD3 for the indicated times. Proteins were detected by Western blotting. Shown is one experiment, representative of three, all with similar results.

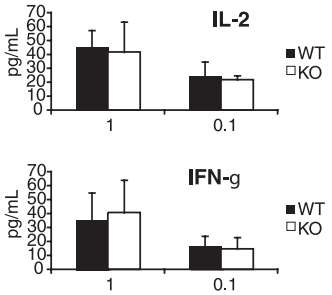

FIG. 5. IL-2 and IFN-γ production from *Dlgh1*-deficient T lymphocytes. The supernatants from purified T cells stimulated with the indicated concentrations of anti-CD3 antibodies for 24 h were assayed for IL-2 and IFN-γ production. Shown are the mean concentrations plus standard deviations from five experiments.

locate NFAT into the nucleus, which is mediated in the context of calcineurin-dependent dephosphorylation. In these experiments, we found that TCR stimulation led to a rapid translocation of NFAT to the nucleus in both WT and *Dlgh1*-deficient T cells, indicating that DLGH1 is not required for NFAT activation (data not shown; see Fig. S4 in the supplemental material). Together, these data suggest that DLGH1 is not essential for activation of major components of TCR-induced proximal signaling cascades leading to transcriptional activation of cytokine gene expression, including interleukin 2 (IL-2).

Cytokine production by *Dlgh1*-deficient T lymphocytes. The production of IL-2 is critically dependent on the TCR-induced activation and nuclear translocation of NFAT. Considering that IL-2 production is critical for the proliferation of naïve T lymphocytes and that NFAT activation has been linked to DLGH1 signaling (24, 25, 32), we hypothesized that the hyperproliferation of KO lymphocytes could result from enhanced IL-2 production. However, we found that TCR-induced IL-2 production was indistinguishable between WT and KO $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 5; see Fig. S6 in the supplemental material). Similarly, there were no significant differences in gamma interferon (IFN-γ) production between WT and KO T lymphocytes (Fig. 5; see Fig. S6 in the supplemental material). These data show that DLGH1 is not required for the induction of IL-2 or IFN-γ in primary T cells and suggest that the perturbations in proliferation observed in KO T cells do not result from deregulated cytokine production.

Increased S-phase entry and enhanced induction of c-*myc* in *Dlgh1*-deficient T lymphocytes. To further characterize the hyperproliferative response of *Dlgh1*-deficient T lymphocytes, we assayed the kinetics of cell cycle entry using BrdU incorporation and DNA content analyses in naïve resting T cells (which are predominantly in the G_0/G_1 phase of the cell cycle) upon TCR stimulation. Strikingly, these assays showed that compared to *Dlgh1*-sufficient T lymphocytes, an increased percentage of KO T lymphocytes entered S phase at 18 h poststimulation (Fig. 6). Consistent with the results from the [^{3}H]thymidine incorporation and CFSE dye dilution experiments, this increased S-phase entry was more pronounced in KO $CD8^+$ T cells and only modestly increased in KO $CD4^+$ T cells. Next, we analyzed the levels of transcripts of c-*myc*, a critical transcriptional regulator induced at the onset of proliferative responses of T lymphocytes. In these assays, we focused our attention on $CD8^+$ T cells, which exhibit a more profound increase in TCR-induced proliferation. Indeed, we observed that compared to the WT, c-*myc* transcript levels in unstimulated KO $CD8^+$ T cells were modestly increased, but that following TCR induction, KO $CD8^+$ T cells showed significantly greater induction of c-*myc* transcripts (Fig. 7A and B). These data indicate that *Dlgh1*-deficient T cells exhibit increased basal and induced expression of c-*myc*. Together, these results indicate that KO T cells show enhanced proliferation following TCR stimulation and that cell cycle entry is accelerated in *Dlgh1*-deficient lymphocytes, implicating

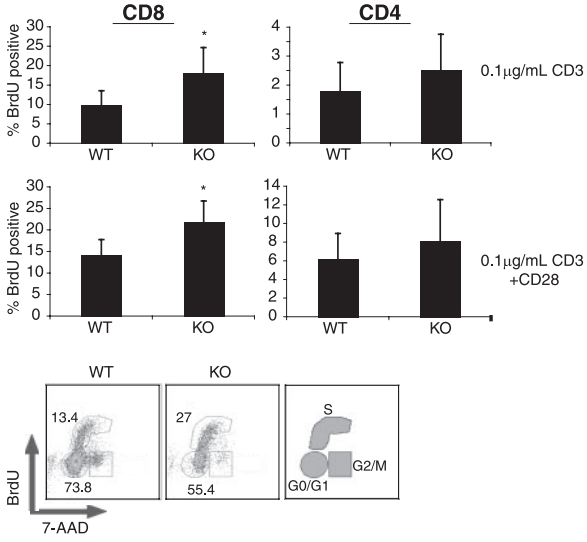

FIG. 6. KO $CD8^+$ T-cell entry into S phase following anti-CD3 stimulation. Lymph node T cells from mice reconstituted with WT or KO fetal liver cells were stimulated with the indicated concentrations of stimulatory anti-CD3 and anti-CD28 antibodies (1 μg/ml). The cells were stimulated for 18 h, pulsed with BrdU, and stained with antibodies against BrdU. Shown are the mean values plus standard deviations from five experiments.

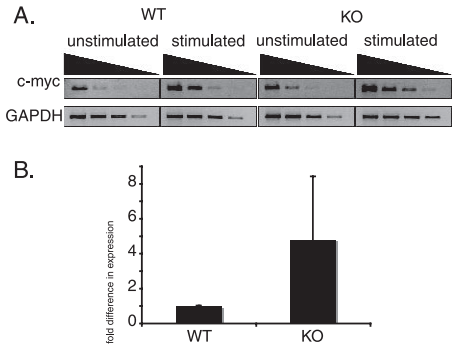

FIG. 7. c-*myc* transcripts in KO $CD8^+$ T cells. (A) Semiquantitative reverse transcription-PCR analysis of c-*myc* induction in purified $CD8^+$ T cells from WT- or KO-reconstituted mice stimulated for 16 h with 1 μg/ml anti-CD3 and anti-CD28. Fourfold serial dilutions of cDNA from one representative experiment of three is shown. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were used for normalization of cDNAs. (B) Quantitative RT-PCR analysis of purified $CD8^+$ T cells from WT- or KO-reconstituted mice stimulated for 16 h with 1 μg/ml anti-CD3 and anti-CD28. The data shown are mean values ($n = 3$) normalized to GAPDH.

DLGH1 as an essential regulator of the TCR-induced proliferative response in naïve T lymphocytes.

DISCUSSION

Although the majority of previous studies of PDZ domain-containing MAGUK proteins in mammalian systems have focused on their functions in neuronal and epithelial cells, more recent studies indicate that PDZ proteins are also involved in the regulation of T-lymphocyte activation and the establishment of synaptic polarity in the context of the immune synapse (10, 11, 16, 32). Additionally, several reports implicate DLGH1, a founding member of the MAGUK family, in regulating TCR signaling output. For example, our recent studies demonstrated that DLGH1 participates in the assembly of TCR-induced signaling complexes (32). Consistent with these findings, studies published by other groups indicated DLGH1 involvement in the recruitment of several key components of the TCR-induced proximal signaling complex, including Lck, ZAP-70, and WASP (15, 24, 25). However, important differences remain in the interpretation of experimental data in support of DLGH1 function downstream of the TCR, as our studies (32) and those of others (25) reached opposite conclusions regarding DLGH1 function either as a positive or a negative regulatory factor in TCR-induced responses.

Thus, in contrast to our report in which we concluded that overexpression of DLGH1 attenuates while downregulation of DLGH1 by siRNA enhances TCR signaling (32), Round et al. found that DLGH1 overexpression potentiates NFAT-dependent transcriptional activation while DLGH1 downregulation abrogates TCR-induced F-actin polymerization, lipid raft clustering, and TCR/CD3 capping and leads to defective cytokine production, including IL-2 and IFN-γ, due to a block in NFAT-dependent transcriptional activation (24, 25). The latter results are surprising, particularly given our previously published findings, but also in the context of a well-documented propensity of mammalian DLGH proteins, and their *Drosophila* ortholog, DLG, to function as tumor suppressors and attenuators of cell division, as revealed in various experimental systems to date (reviewed in reference 15). These differences could result from the different experimental models utilized by our group and in previous studies. Our initial studies used siRNA in immortalized T-cell lines (32), while Round et al. used siRNA knockdown of DLGH1 in OT-1 T cells that had been activated and expanded in culture (24, 25). Thus, it is conceivable that DLGH1 functions differently in regard to protein polarity and signaling in antigen-experienced T cells.

In this study, by utilizing *Dlgh1*-null lymphocytes, we were able to directly analyze the consequences of DLGH1 deficiency in development and activation of primary T cells. Since mice homozygous for the null allele of *Dlgh1* exhibit perinatal lethality (17), we utilized *Rag-2*-deficient complementation with *Dlgh1*-null fetal liver progenitors and mice with a conditionally targeted *Dlgh1* allele to generate T cells lacking DLGH1. Our experiments with these *Dlgh1*-deficient T cells conclusively establish three major findings. First, we show that DLGH1 is not essential for the differentiation of major thymic or peripheral subsets of αβ lineage T lymphocytes during both fetal and adult development. An important caveat to this conclusion is that we cannot rule out potential effects of TCR repertoire "shifts," which could conceivably compensate for gain or loss of signaling potency by individual TCR heterodimer complexes expressed by developing T cells. To conclusively address this issue, development and selection of *Dlgh1*-deficient thymocytes will need to be addressed in the context of "fixed" TCR repertoires, for example, by the introduction of conditional/inducible *Dlgh1*-null alleles into developing T cells that also harbor αβ TCR-transgenes in a positively selecting major histocompatibility complex background in the absence of expression of any endogenous α and β TCR chain genes. Our second major finding is that DLGH1 is not essential for TCR-induced F-actin accumulation and the recruitment of polarity proteins to the immune synapse, activation of the T-cell cytokines IL-2 and IFN-γ, or directional migration toward chemokine gradients. Thirdly, we show that in primary (naïve) T cells, DLGH1 functions as a negative regulator of TCR-induced proliferative responses in vitro. Thus, in the absence of any gross alterations in TCR proximal signal transduction events detectable in T cells lacking DLGH1 protein, DLGH1 appears to be required for normal regulation of TCR signaling outcome and proliferative responses. Alternatively, DLGH1 may function as an adaptor protein regulating signal intensity in other transduction pathways contributing to T-cell proliferation, such as those downstream of cytokine receptors like IL-2 or IL-4. Of note, in our experiments, even substantial downregulation of DLGH1 expression, such as that observed in T cells with a hemizygous *Dlgh1*-null mutation ($Dlgh1^{+/-}$) (which equaled or exceeded 50% of total DLGH1 expression), did not lead to any detectable changes in T-cell responses to TCR stimulation. Thus, our experiments show no evidence of DLGH1 haploinsufficiency in developing and mature T cells. We note however, that *Dlgh1* gene-deficient T cells may conceivably undergo an in vivo selection process and/or acquire some changes in their TCR signaling apparatus that may compensate for the gain or loss of TCR signaling potency during the course of their development. Also, a compensatory mechanism(s) must exist in vivo for the regulation of T-cell proliferation, as *Dlgh1*-deficient chimeras do not develop lymphoadenopathy, do not show any lymphoproliferative disorder, and can produce T-dependent humoral responses that appear similar to normal ones (data not shown).

Our studies suggest that DLGH1 is required for proper regulation of c-*myc* expression in primary T cells. Numerous previous studies have identified c-*myc* as a master regulator of a transcriptional program linking extracellular signals to cell cycle progression and cell division in a variety of cell types, including T cells (9, 31). These results are intriguing, as DLGH1 could potentially regulate c-*myc* via a pathway involving GSK3β and β-catenin (5, 20). However, at present we do not understand the exact mechanism that may underlie any such potential roles for DLGH1 in these signaling pathways in T cells, as our experiments to directly address the requirement for DLGH1 in β-catenin signaling in T cells were inconclusive (data not shown). Nevertheless, whatever the mechanism, enhanced c-*myc* may underlie the accelerated cell cycle entry and hyperproliferation in $Dlgh1^{-/-}$ T cells. Importantly, while loss of DLGH1 leads to considerable effects on c-*myc* expression, we note that the expression of other DLGH family members, notably DLGH3, may contribute redundant functions in the absence of DLGH1 in T cells. In this regard, although we did

not observe any substantial differences in the expression levels of other DLGH family members in the absence of DLGH1 (data not shown), it is also possible that the expression of other MAGUK family proteins, such as Scribble and Lgl, may provide a basis for partial functional redundancy with DLGH1 in the regulation of TCR-induced events. Genetic deletion of other DLGH family members and members of the related families of other MAGUK proteins, in conjunction with deletion of *Dlgh1*, will be required to define the limits of any such potential functional redundancies.

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We declare no conflict of interest.

REFERENCES

1. **Bachmaier, K., C. Krawczyk, I. Kozieradzki, Y. Y. Kong, T. Sasaki, A. Oliveira-dos Santos, S. Mariathasan, D. Bouchard, A. Wakeham, A. Itie, J. Le, P. S. Ohashi, I. Sarosi, H. Nishina, S. Lipkowitz, and J. M. Penninger.** 2000. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. Nature **403:**211–216.
2. **Betschinger, J., K. Mechtler, and J. A. Knoblich.** 2003. The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. Nature **422:**326–330.
3. **Caruana, G.** 2002. Genetic studies define MAGUK proteins as regulators of epithelial cell polarity. Int. J. Dev. Biol. **46:**511–518.
4. **Dimitratos, S. D., D. F. Woods, D. G. Stathakis, and P. J. Bryant.** 1999. Signaling pathways are focused at specialized regions of the plasma membrane by scaffolding proteins of the MAGUK family. Bioessays **21:**912–921.
5. **Etienne-Manneville, S., and A. Hall.** 2003. Cdc42 regulates GSK-3β and adenomatous polyposis coli to control cell polarity. Nature **421:**753–756.
6. **Fujikawa, K., A. V. Miletic, F. W. Alt, R. Faccio, T. Brown, J. Hoog, J. Fredericks, S. Nishi, S. Mildiner, S. L. Moores, J. Brugge, F. S. Rosen, and W. Swat.** 2003. Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cells. J. Exp. Med. **198:**1595–1608.
7. **Funke, L., S. Dakoji, and D. S. Bredt.** 2005. Membrane-associated guanylate kinases regulate adhesion and plasticity at cell junctions. Annu. Rev. Biochem. **74:**219–245.
8. **Giallourakis, C., Z. Cao, T. Green, H. Wachtel, X. Xie, M. Lopez-Illasaca, M. Daly, J. Rioux, and R. Xavier.** 2006. A molecular-properties-based approach to understanding PDZ domain proteins and PDZ ligands. Genome Res. **16:**1056–1072.
9. **Guo, Z., M. Dose, D. Kovalovsky, R. Chang, J. O'Neil, A. T. Look, H. von Boehmer, K. Khazaie, and F. Gounari.** 2007. β-Catenin stabilization stalls the transition from double-positive to single positive stage and predisposes thymocytes to malignant transformation. Blood **109:**5463–5472.
10. **Hanada, T., L. Lin, K. G. Chandy, S. S. Oh, and A. H. Chishti.** 1997. Human homologue of the *Drosophila* discs large tumor suppressor binds to p56lck tyrosine kinase and Shaker type Kv1.3 potassium channel in T lymphocytes. J. Biol. Chem. **272:**26899–26904.
11. **Hanada, T., L. Lin, E. V. Tibaldi, E. L. Reinherz, and A. H. Chishti.** 2000. GAKIN, a novel kinesin-like protein associates with the human homologue of the *Drosophila* discs large tumor suppressor in T lymphocytes. J. Biol. Chem. **275:**28774–28784.
12. **Hara, H., C. Bakal, T. Wada, D. Bouchard, R. Rottapel, T. Saito, and J. M. Penninger.** 2004. The molecular adapter Carma1 controls entry of IκB kinase into the central immune synapse. J. Exp. Med. **200:**1167–1177.
13. **Hering, H., and M. Sheng.** 2002. Direct interaction of Frizzled-1, -2, -4, and -7 with PDZ domains of PSD-95. FEBS Lett. **521:**185–189.
14. **Hsueh, Y. P., T. F. Wang, F. C. Yang, and M. Sheng.** 2000. Nuclear translocation and transcription regulation by the membrane-associated guanylate kinase CASK/LIN-2. Nature **404:**298–302.
15. **Humbert, P., S. Russell, and H. Richardson.** 2003. Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. Bioessays **25:**542–553.
16. **Ludford-Menting, M. J., J. Oliaro, F. Sacirbegovic, E. T. Cheah, N. Pedersen, S. J. Thomas, A. Pasam, R. Iazzolino, L. E. Dow, N. J. Waterhouse, A. Murphy, S. Ellis, M. J. Smyth, M. H. Kershaw, P. K. Darcy, P. O. Humbert, and S. M. Russell.** 2005. A network of PDZ-containing proteins regulates T cell polarity and morphology during migration and immunological synapse formation. Immunity **22:**737–748.
17. **Mahoney, Z. X., B. Sammut, R. J. Xavier, J. Cunningham, G. Go, K. L. Brim, T. S. Stappenbeck, J. H. Miner, and W. Swat.** 2006. Discs-large homolog 1 regulates smooth muscle orientation in the mouse ureter. Proc. Natl. Acad. Sci. USA **103:**19872–19877.
18. **Naramura, M., H. K. Kole, R. J. Hu, and H. Gu.** 1998. Altered thymic positive selection and intracellular signals in Cbl-deficient mice. Proc. Natl. Acad. Sci. USA **95:**15547–15552.
19. **Nourry, C., S. G. Grant, and J. P. Borg.** 2003. PDZ domain proteins: plug and play! Sci. STKE **2003:**RE7.
20. **Ohteki, T., M. Parsons, A. Zakarian, R. G. Jones, L. T. Nguyen, J. R. Woodgett, and P. S. Ohashi.** 2000. Negative regulation of T cell proliferation and interleukin 2 production by the serine threonine kinase GSK-3. J. Exp. Med. **192:**99–104.
21. **Outram, S. V., A. Varas, C. V. Pepicelli, and T. Crompton.** 2000. Hedgehog signaling regulates differentiation from double-negative to double-positive thymocyte. Immunity **13:**187–197.
22. **Panyi, G.** 2005. Biophysical and pharmacological aspects of K+ channels in T lymphocytes. Eur. Biophys. J. **34:**515–529.
23. **Plant, P. J., J. P. Fawcett, D. C. Lin, A. D. Holdorf, K. Binns, S. Kulkarni, and T. Pawson.** 2003. A polarity complex of mPar-6 and atypical PKC binds, phosphorylates and regulates mammalian Lgl. Nat. Cell Biol. **5:**301–308.
24. **Round, J. L., L. A. Humphries, T. Tomassian, P. Mittelstadt, M. Zhang, and M. C. Miceli.** 2007. Scaffold protein Dlgh1 coordinates alternative p38 kinase activation, directing T cell receptor signals toward NFAT but not NF-κB transcription factors. Nat. Immunol. **8:**154–161.
25. **Round, J. L., T. Tomassian, M. Zhang, V. Patel, S. P. Schoenberger, and M. C. Miceli.** 2005. Dlgh1 coordinates actin polymerization, synaptic T cell receptor and lipid raft aggregation, and effector function in T cells. J. Exp. Med. **201:**419–430.
26. **Schmedt, C., K. Saijo, T. Niidome, R. Kuhn, S. Aizawa, and A. Tarakhovsky.** 1998. Csk controls antigen receptor-mediated development and selection of T-lineage cells. Nature **394:**901–904.
27. **Sheng, M., and C. Sala.** 2001. PDZ domains and the organization of supramolecular complexes. Annu. Rev. Neurosci. **24:**1–29.
28. **Shultz, L. D., D. R. Coman, C. L. Bailey, W. G. Beamer, and C. L. Sidman.** 1984. "Viable motheaten," a new allele at the motheaten locus. I. Pathology. Am. J. Pathol. **116:**179–192.
29. **Terra, R., I. Louis, R. Le Blanc, S. Ouellet, J. C. Zuniga-Pflucker, and C. Perreault.** 2005. T-cell generation by lymph node resident progenitor cells. Blood **106:**193–200.
30. **Vianello, F., P. Kraft, Y. T. Mok, W. K. Hart, N. White, and M. C. Poznansky.** 2005. A CXCR4-dependent chemorepellent signal contributes to the emigration of mature single-positive CD4 cells from the fetal thymus. J. Immunol. **175:**5115–5125.
31. **Willert, K., and K. A. Jones.** 2006. Wnt signaling: is the party in the nucleus? Genes Dev. **20:**1394–1404.
32. **Xavier, R., S. Rabizadeh, K. Ishiguro, N. Andre, J. B. Ortiz, H. Wachtel, D. G. Morris, M. Lopez-Ilasaca, A. C. Shaw, W. Swat, and B. Seed.** 2004. Discs large (Dlg1) complexes in lymphocyte activation. J. Cell Biol. **166:**173–178.
33. **Yamanaka, T., Y. Horikoshi, Y. Sugiyama, C. Ishiyama, A. Suzuki, T. Hirose, A. Iwamatsu, A. Shinohara, and S. Ohno.** 2003. Mammalian Lgl forms a protein complex with PAR-6 and aPKC independently of PAR-3 to regulate epithelial cell polarity. Curr. Biol. **13:**734–743.