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Th2-Specific Immunity and Function of Peripheral T Cells Is Regulated by the p56Lck Src Homology 3 Domain

Margaret E. McCoy,* Fred D. Finkelman,† and David B. Straus*

T cell activation and effector function is essential for robust immunity. Ag TCR signals are known to regulate T lymphocyte differentiation, but the mechanisms involved in this regulation remain unclear. Recent work has demonstrated that the Src family protein tyrosine kinase p56Lck specifically links TCR signaling to activation of the MAPK pathway through the function of its Src homology 3 (SH3) domain. The MAPK pathway is involved in T cell activation and has previously been implicated in Th2 immunity. We have used Lck SH3 mutant knockin mice (LckW97A) to investigate the potential role of this regulatory mechanism in T lymphocyte activation and effector function. Our results demonstrate that Lck SH3 domain function regulates activation of T lymphocytes as indicated by reduced IL-2 production, CD69 induction, and proliferation of LckW97A T cells following TCR stimulation. Biochemical studies confirm that activation of the MAPK pathway is selectively altered following TCR ligation in LckW97A T lymphocytes. Phospho-ERK induction is reduced, but phospho-phospholipase Cγ is induced and calcium mobilization are largely unaffected. Immunization with DNP-keyhole limpet hemocyanin, heat-killed Brucella abortus, or infection with Nippostrongylus brasiliensis demonstrates selectively impaired Th2 immunity with reduced serum levels of IgG1, IgE, and IL-4. In vitro studies show that LckW97A T cells can differentiate into Th2-type cells, but they form IFN-γ-producing cells under conditions that normally favor Th2 development. These data indicate that the Lck SH3 domain controls T lymphocyte activation by regulating MAPK pathway induction and demonstrate a novel role for Lck in the regulation of Th2-type immunity. The Journal of Immunology, 2010, 185: 3285–3294.

The T lymphocyte subset of immune effector cells is essential for the formation of a functional adaptive immune response. The development of thymocytes and activation of mature T cells relies on signals received via the Ag TCR (1–3). Thymocyte development is known to be regulated by the nature of signaling through the TCR. However, much less is known about how TCR signals influence the differentiation and effector function of mature T lymphocytes.

Proper differentiation and effector function of T lymphocytes, particularly the Th subset, are critical for host survival and regulation of immune responses to pathogens (4, 5). Thus, understanding how components of the TCR signaling pathways regulate this process is of clinical interest for the control of immune responses to pathogen infections. It is known that altering the strength of TCR signaling can regulate differentiation of T lymphocytes (6). Initial studies showed that differentiation was influenced by altering Ag concentration (7, 8). More recent studies have shown that mutations in early TCR signaling components also modify differentiation (9–14). Independent work has implicated the MAPK pathway as a critical regulatory pathway downstream and independent of initial phosphorylation of the TCR and ZAP-70 and that this role in MAPK pathway activity is critical for normal activation and effector function of mature T cells. Furthermore, this work demonstrates that loss of function of the Lck SH3 domain specifically inhibits the ability of mature T cells to provide Th2 immunity in vivo following infection with the gastrointestinal helminth, Nippostrongylus brasiliensis.

Materials and Methods

Mice

C57BL/6 were obtained from The Jackson Laboratory (Bar Harbor, ME). Lck SH3 mutant knockin (LckW97A) mice (21) were backcrossed to C57BL/6 for either three or eight generations and maintained in aseptic housing until use. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University, Richmond, VA.

Analysis of cell surface phenotype

Single-cell suspensions were made from lymph nodes (LNs; inguinal, brachial, cervical, axillary) and spleen. Splenic preparations were used...
following RBC lysis with ammonium chloride treatment. The surface Ags of primary murine lymphocytes were evaluated by flow cytometry following cell surface staining by fluorochrome-conjugated Abs recognizing CD4, CD8, TCRβ, CD90.2, CD62L, CD44, and CD69. All Abs were obtained from BD Biosciences (San Jose, CA).

In vitro cell stimulations for proliferation and cytokine production

Single-cell suspensions of primary murine lymphocytes were labeled with 2.5 μM CFSE and plated at various cell concentrations. In some experiments, T cells were purified by negative selection using magnetic beads (Miltenyi Biotec, Auburn, CA). Cells were stimulated with 0.1–10 μg/ml bound or soluble anti-CD3 (2C11) and/or 1 μg/ml anti-CD28 Abs (with or without 10 ng/ml PMA) in 24-well tissue-culture plates. Cells were cultured in DMEM supplemented with 10% FBS, 1-glutamine, nonessential amino acids, penicillin, streptomycin, and 2-ME at 37˚C, 7.5% CO2 for 48 h. For CFSE analysis, cells were stained with anti-CD90.2 for 2 h, and cytometry, and intensity of CFSE staining was analyzed by FACS. For IL-2 analysis, supernatants were harvested after 24 h and analyzed using the OptEIA mouse IL-2 ELISA kit (BD Pharmingen, San Diego, CA) per manufacturer’s recommendations.

Activation marker induction

Single-cell suspensions of primary murine lymphocytes were plated at 0.5 × 106 cells/ml in 24-well tissue-culture plates and incubated in DMEM complete with various doses of soluble anti-CD3 (2C11) for 4 h at 37˚C, 7.5% CO2. Cells were then stained with anti-CD90.2 and anti-CD69–conjugated fluorochromes and analyzed by flow cytometry.

TCR signaling analysis

Single-cell suspensions of primary murine T lymphocytes were purified by negative selection using magnetic beads (Miltenyi Biotec) or by cell sorting using an FACSaria flow cytometer (BD Biosciences). Purified T cells were used at a concentration of 2–4 × 106 cells/ml in HEPEs-buffered saline (25 mM HEPEs, 125 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 1 mM CaCl2, 0.5 mM MgCl2; pH 7.4). Cells were warmed at 37˚C and stimulated with 3–5 μg/ml anti-CD3 Ab (2C11) for 3 min. Cells were lysed in 1% Nonidet P-40 with phosphatase and protease inhibitors, and insoluble material was removed by centrifugation. Lysates were denatured by the addition of SDS sample buffer and incubation at 100˚C. Samples were analyzed by Western blotting following SDS gel electrophoresis and transfer to polyvinylidene difluoride membrane. Primary Abs recognizing Lck (BD Biosciences), phospholipase C (PLC) γ1 (Biolegend, Carlsbad, CA), Erk, phospho-ERK, phospho-PLCγ1, p38, phospho-p38 (Cell Signaling Technology, Danvers, MA), and phosphotyrosine (PY; Upstate Group, Charlottesville, VA) were detected by incubation with primary Abs and HRP-conjugated secondary Abs (Southern Biotechnology Associates, Birmingham, AL) and detected using chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific, Rockford, IL). Calcium flux was measured by fluorimetry using a NOVO Star plate reader (BMG Labtech, Cary, NC). Lymphocytes were loaded with fura 2 and stimulated with biotinylated anti-CD3 followed by anti-biotin Ab (Vector Laboratories, Burlingame, CA), or were treated with ionomycin. Ratiometric analysis was performed using the ratio of 510–520 nm fluorescence at 340 and 380 nm excitation wavelengths.

Ag immunization

LckW97A mice 4–6 wk of age, sex- and age-matched with The Jackson Laboratory C57BL/6 wild-type (WT) mice, were injected s.c. with 100 μg DNP-keyhole limpet hemocyanin (DNP–KLH; Calbiochem, San Diego, CA) reconstituted in sterile deionized H2O in a 50:50 solution with Titer Max Gold adjuvant (Sigma-Aldrich, St. Louis, MO) on day 0. A boost was performed on day 14 by injecting 50 μg DNP–KLH s.c. without adjuvant. Blood was drawn via tail vein nick on days 0 (preboost), 14 (boost), and 21 at time of sacrifice. Alternatively, mice were injected i.p. with 0.2 ml 1:25 dilution of packed, heat-killed Brucella abortus and blood collected on days 7 and 10 postimmunization (24).

ELISA

Serum Ab levels were determined following DNP–KLH immunization or N. brasiliensis infection by ELISA. Ag-specific anti-DNP Abs were detected using IgG1, IgG2b, and IgG3 isotype-specific goat anti-mouse alkaline phosphatase-tagged secondary Abs (all from Southern Biotechnology Associates). For total serum IgE measurement following N. brasiliensis infection, plates were coated with B1E3 Ab and bound IgE detected with biotinylated R1E4 (primary Ab) and streptavidin–alkaline phosphatase (secondary reagent). Color change was initiated by reaction with phospho-nitrophenyl phosphate disodium salt substrate tablets (Sigma-Aldrich) and analyzed by absorbance OD at 405/650 on an ELISA reader. Reagents to detect IgE were a generous gift of Dr. Dan Conrad (Virginia Commonwealth University, Richmond, VA). IFN-γ serum levels were measured using BD Biosciences OptEIA Mouse IFN-γ reagents following the manufacturer’s recommendations (BD Biosciences).

Analysis of IFN-γ expression by intracellular staining

Mice were immunized with heat-killed B. abortus as described above and spleens removed after 7 d. Splenocytes were stimulated postlysing of RBCs with plate-bound anti-CD3 (2C11; 5 μg/ml) or 25 μl 1:25 dilution of packed heat-killed B. abortus cells in sterile PBS with 1 μg/ml anti-CD28. Following an initial 2-h incubation at 37˚C, monensin was added to block secretion and incubation continued for an additional 6 h. Cells were fixed for 5 min at room temperature in 3.2% paraformaldehyde and then permeabilized prior to staining with fluorescent anti–IFN-γ and anti-CD4 Abs. IFN-γ expression in the CD4+ lymphocyte population was determined by flow cytometry using electronic gating.

N. brasiliensis infection

LckW97A mice 4–8 wk of age, sex- and age-matched with The Jackson Laboratory C57BL/6 WT mice, were injected with 600–700 N. brasiliensis L3 larvae s.c. on day 0. Blood was drawn via tail vein nick on days 0, 8, 11, 14, 18, and 21 at time of sacrifice. N. brasiliensis L3 helminths were generously provided by Drs. Dan Conrad and Joe Urban (U.S. Department of Agriculture, Beltsville, MD). In vivo cytokine capture assay (25) was performed on days 5, 7, 10, and 12 of N. brasiliensis infection. To capture IL-4 for analysis, 10 μg biotin–BVD4–1D11 anti–IL-4 monoclonal Ab was injected i.p. Mice were bled 3 to 4 h postinjection, and IL-4–biotin–anti–IL-4 complexes were detected by coating microtiter ELISA plates with BVD6-24G2.3 and analyzed by luminescent ELISA (26).

In vitro T cell differentiation

CD4+ T lymphocytes were isolated from secondary lymphoid organs by negative selection from single-cell suspensions using magnetic beads and Abs against CD8, B220, IgM, IgD, CD44, CD62L, CD69, and culture continued with 5 ng/ml human IL-2 for 2 to 3 d before being restimulated with PMA and ionomycin, fixed, and levels of intracellular IFN-γ and IL-4 determined by staining and flow cytometry.

Results

Reduced numbers of CD8+ T cells and reduced CD4+ coreceptor expression in LckW97A peripheral T cells

p56Lck is essential for the activation and differentiation of T lymphocytes (19, 27, 28); however, the mechanisms through which Lck regulates these processes have not been established. To examine the potential mechanisms of regulation involving Lck in T cell activation and effector function, we have generated mice with a Lck allele carrying a tryptophan to alanine change (W97A) in the SH3 domain, which ablates ligand binding to this domain (21). Analysis of the LN and splenic peripheral T lymphocyte compartments in LckW97A mice showed a significantly reduced percentage of TCRβ cells compared with WT mice (Fig. 1A). Effects on T cell numbers were primarily due to reduction of the TCRβ+CD8+ cell population (29). Analysis of TCRβ+CD8+ cell population numbers in LckW97A mice, with CD4 expression modestly reduced. Expression of the CD8+ T lymphocyte population in LckW97A mice showed a significantly reduced percentage of TCRβ cells compared with WT mice (Fig. 1A).

For T cells from LckW97A mice, with CD8 expression modestly reduced. Expression of the CD8+ T lymphocyte population was significantly reduced compared with WT mice (Fig. 1A). For T cells from LckW97A mice, with CD8 expression modestly reduced. Expression of the CD8+ T lymphocyte population was significantly reduced compared with WT mice (Fig. 1A).
Activation of LckW97A T lymphocytes is significantly reduced in vitro

As Lck is known to be important for T cell activation, potential defects due to loss of function of the Lck SH3 domain in LckW97A mice were examined by observing outcomes of T lymphocyte activation in vitro. We assessed proliferation, cytokine production, and early activation marker expression in T cells from LckW97A mice. Proliferation studies were performed by staining LN cells.
with CFSE and stimulating with the anti-CD3 mAb 2C11. LckW97AT lymphocytes show a consistent 3- to 4.5-fold reduction in the number of T cells that are able to respond to TCR ligation compared with WT. This difference is observed with either high or low doses of anti-CD3, although higher levels of anti-CD3 stimulate greater proliferation of LckW97A T cells, and cells that do respond undergo similar rounds of division compared with WT (Fig. 2A). To further examine the activation-induced defect observed in LckW97A T cells, we assessed the levels of secreted IL-2 in stimulated cultures. Analysis of cytokine levels by ELISA indicated a 10-fold reduction in the level of IL-2 in LckW97A culture supernatants compared with WT at 24 h following anti-TCR ligation (Fig. 2B). In addition, we investigated the ability of LckW97A T lymphocytes to express the early activation marker CD69 following TCR-specific activation. Although the level of expression on responding cells is comparable with that on WT T lymphocytes, the percentage of T cells able to induce CD69 is significantly reduced in LckW97A mice following stimulation with various doses of anti-CD3 (Fig. 2C). This difference is still observed at later time points following activation (data not shown). Taken together, these data indicate a defect in the activation of LckW97A T cells following anti-TCR-specific stimulation resulting from a loss of Lck SH3 domain function.

Because the CD28 coreceptor is known to bind the SH3 domain of Lck (30), it is possible that mutation of the Lck SH3 domain might lead to a reduction in costimulatory signaling. However, we found that CD28 receptor engagement enhanced proliferation of LckW97A T lymphocytes so that there were ∼1–1.5-fold more T cells able to respond to stimulation with anti-CD3 Ab alone (Fig. 2A). The enhanced proliferation with CD28 costimulation was observed at both low and high doses of anti-CD3. However, despite CD28 engagement, the population of non-responding cells remains roughly 3-fold higher in LckW97A cultures when compared with WT. To examine the potential effect of the LckW97A mutation on CD28 signaling in isolation from its effect on TCR signaling, the cellular response to CD28 and PMA was examined (Fig. 2A). PMA by itself does not activate primary T cells (30). Our analysis showed that the Lck SH3 domain makes little contribution to CD28 signals that are independent of pathways stimulated by PMA. These studies suggest that the Lck SH3 domain mediates T lymphocyte activation primarily by mediating signaling through the TCR rather than CD28.

**Reduced ERK pathway induction in LckW97A peripheral T cells**

To understand the basis for defective activation of T lymphocytes from LckW97A mice, regulatory pathways critical for activation downstream of TCR signaling were examined. In particular, we wished to examine the MAPK pathway, which we have found to be regulated by Lck SH3 domain function in thymocytes (21). To analyze signaling, we purified and stimulated peripheral LN T cells from LckW97A and WT mice with anti-CD3. Western analysis of lysates probed with anti-PY Abs does not indicate a substantial difference in the pattern of phosphorylation, although several proteins have slightly reduced levels of PY induction (Fig. 3A). To assess intracellular signaling following TCR stimulation, we investigated the activation of proteins involved in the PI and MAPK pathways. Immunoblotting of lysates with anti–phospho-PLCγ1 Ab revealed that phospho-PLCγ1 induction was similar between T lymphocytes purified from LckW97A and WT mice (Fig. 3B). These results suggest that the PI pathway does not require the Lck SH3 domain. To confirm that the PI pathway was not differentially regulated in LckW97A T lymphocytes, we assessed intracellular calcium levels in lymphocytes from C57BL/6 and LckW97A mice following TCR stimulation (Fig. 3E). Like PLCγ1 phosphorylation, there is a similar calcium flux in LckW97A and WT T cells in response to TCR stimulation. In contrast, activation of the MAPK pathway kinases ERK1 and ERK2 was significantly reduced in LckW97A peripheral T cells following anti-CD3 stimulation (Fig. 3B–D). Total levels of ERK1 and -2 are comparable between LckW97A and WT T cells (Fig. 3B). A kinetic analysis showed that the reduction in ERK activation in LckW97A T cells is not due to a delayed response to receptor stimulation (Fig. 3D). In other studies, we found that, in contrast to ERK, induction of p38 MAPK phosphorylation following TCR stimulation was not defective in LckW97A T cells (data not shown). With little change in overall tyrosine phosphorylation, PLCγ1 activation, or calcium mobilization following TCR stimulation, the Lck SH3 domain appears to be selectively required for activation of the ERK signaling pathway. The defect in ERK signaling helps explain the altered activation of T lymphocytes from LckW97A mice.

**LckW97A animals have a significant defect in Th2 Ag-specific immunity**

Given the defects in MAPK pathway induction in LckW97A T cells, and evidence supporting an essential role for this pathway in Th2 immunity (29, 30), we examined the ability of LckW97A mice to respond to immunization with DNP–KLH Ag/adjuvant. Immune responses were determined by monitoring Ag-specific (anti-DNP) Ig serum levels in both LckW97A and WT mice. Baseline serum analysis did not reveal any difference in the overall levels of Ig isotypes between LckW97A and WT mice (data not shown). However, we found significantly reduced serum levels of DNP-specific IgG1 in LckW97A mice compared with WT following immunization (Fig. 4A). In contrast, Ag-specific IgG2b and IgG3 isotypes were comparable between both groups throughout the course of immunization (Fig. 4B, 4C). The particular deficit in IgG1 suggests defective Th2 immunity in LckW97A mice. To determine if the defect in LckW97A immunity was limited to production of the IgG1 isotype or was indicative of a more general defect in the Th2-immune responses in these mice, a parasite infection model was used.

**LckW97A animals have significantly reduced levels of serum IgE and IL-4 following infection with N. brasiliensis**

*N. brasiliensis*, a well-studied model of helminth infection and a strong inducer of Th2 immunity, was used to further examine potential differences in Th immunity in LckW97A mice. Mice were infected with L3 stage parasites, and total serum IgE levels were examined during the course of infection. LckW97A mice have significant reductions in serum levels of IgE compared with C57BL/6 WT mice by day 8 postinfection and continuing throughout the study (day 21) (Fig. 5A). There is a maximum defect in serum IgE on day 14 in LckW97A mice, amounting to a roughly 6-fold reduction compared with WT mice (Fig. 5A). These results indicate that the SH3 domain of Lck acts in a critical fashion in peripheral T lymphocytes to promote Th2-specific immune responses in C57BL/6 mice following both Ag immunization and helminth infection.

IL-4 is important for maintaining Th2 immunity and for the propagation of functional Th2 T cells during parasite infection in mice (25, 31, 32). To investigate whether deficient IL-4 production could explain the observed deficit in IgE levels in LckW97A mice following *N. brasiliensis* infection, serum levels of IL-4 were examined on days 5–12 following infection (Fig. 5B). To assess IL-4, we used an in vivo cytokine capture assay (26), which enabled retention of IL-4 in the serum for improved isolation and
characterization by fluorometric analysis. LckW97A mice appear to have approximately half the available serum IL-4 during the height of the IL-4 response in vivo compared with WT (Fig. 5B). These data demonstrate a significant defect in the ability of LckW97A mice to maintain Th2 immunity and cytokine production during an immune response to parasite infection.

**FIGURE 2.** LckW97A T cell activation is significantly reduced following anti-TCR stimulation compared with WT. **A,** T cell proliferation following in vitro stimulation. CSFE-labeled peripheral LN cells from WT (solid line) or LckW97A (gray line) were stimulated with indicated doses of anti-CD3 Ab with or without 1 µg/ml CD28 Ab or CD28 Ab with 10 ng/ml PMA. Data show fluorescence histograms of CSFE-labeled T cells either unstimulated (filled histogram) or after 48 h of stimulation. Representative of four independent experiments. **B,** IL-2 levels produced by purified T cells stimulated with 1 µg plate-bound anti-CD3 Ab. IL-2 levels in 24-h culture supernatants were measured by ELISA. Mean value (± SD) is shown based on cumulative data from four experiments, total of four to eight mice/group, and analyzed by Student t test (*p* ≤ 0.03). **C,** Induction of CD69 expression following in vitro stimulation. Peripheral T cells were stimulated with indicated doses of anti-CD3 for 4 h and stained with fluorochrome-conjugated anti-CD69 and anti-Thy1. Data show the percent of T cells expressing elevated levels of CD69 (mean fluorescence intensity ≥ 20). Error bars indicate SD; *n* = 3. Statistical analysis performed using Student paired *t* test showed a significant difference between WT and LckW97A samples (*p* ≤ 0.03) for all conditions except unstimulated and 1 µg/ml anti-CD3.
LckW97A animals mount a strong Th1 response to B. abortus immunization

To further characterize the ability of LckW97A mice to mount an effective T cell-mediated response, we used an immunization model for Th1 immunity using heat-killed B. abortus (24). Heat-killed B. abortus stimulates dendritic cells to produce IL-12 and initiates a strong Th1 immune response. Th1 effector function in LckW97A mice was examined at 7 and 10 d post heat-killed B. abortus immunization by analyzing serum levels of IFN-γ using ELISA (Fig. 6, top panel). Our data show that serum levels of IFN-γ in LckW97A mice were comparable to that of WT C57BL/6 mice. To confirm that the expression of IFN-γ was similar in CD4⁺ T cells from Lck⁺ and LckW97A mice, we performed intracellular staining to measure IFN-γ expression following in vitro restimulation of splenocytes from heat-killed B. abortus immunized mice (Fig. 6, bottom panel). Our results show that CD4⁺ T cells from Lck⁺ and LckW97A mice express similarly elevated levels of IFN-γ following heat-killed B. abortus immunization. These findings indicate that although LckW97A mice have a significant defect in Th2 immune responses, their Th1 immune function remains intact.

LckW97A T cells have a reduced ability to polarize exclusively into Th2 T lymphocytes in vitro

Because Th2 immunity is compromised in LckW97A mice, we examined the ability of T cells from LckW97A mice to differentiate into either Th1- or Th2-type cells in vitro. Highly purified CD4⁺ T cells were stimulated with anti-CD3 Ab under conditions that favored differentiation into Th1 or Th2 cells through the addition of IL-12 and anti-IL-4, or IL-4, anti-IL-12, and anti-IFN-γ, respectively. Intracellular cytokine staining showed that LckW97A T cells had the capacity to become either exclusive producers of...
IFN-γ (Th1) or IL-4 (Th2) under biasing conditions (Fig. 7).

However, LckW97AT cells showed a greater tendency to resist Th2-biasing conditions and become IFN-γ producers when compared with the Lck+ T cells (Fig. 7). This effect was still observed with the addition of a CD28 costimulatory signal. These results suggest that although LckW97A T cells are able to differentiate into Th2-type T cells, they are less likely to properly polarize under in vitro conditions that typically lead to the exclusive generation of Th2 cells. LckW97A animals, therefore, have a specific and significant reduction in Th2 immunity, as demonstrated by reduced serum IgE and IL-4 following infection with *N. brasiliensis* compared with WT mice.

In contrast, Th1 immunity appears relatively normal, with comparable serum levels of Ag-specific IgG2b, IgG3 and IFN-γ. Consistent with this, in vitro differentiation studies establish reduced exclusive polarization of LckW97A T cells under Th2-biasing conditions. Thus, the SH3 domain of Lck plays a critical role in

**FIGURE 5.** LckW97A mice have significantly reduced serum IgE and IL-4 following infection with *N. brasiliensis* compared with WT mice. Age and sex-matched mice were infected with 600–700 L3 *N. brasiliensis* larvae. A. *N. brasiliensis*-induced serum IgE levels. Serum was collected on days 0, 11, 14, 16, 18, and upon sacrifice on day 21. Total serum IgE levels were determined by ELISA. Mean values (± SE) are shown based on cumulative data from four experiments. Each time point is based on 12–16 mice/group. B. *N. brasiliensis*-induced serum levels of IL-4. Serum was collected on days 0, 5, 7, 10 and 12 following *N. brasiliensis* infection. A total of 10 μg biotinylated anti–IL-4 Ab BVD4-1D11 was injected i.p. 3 to 4 h before serum collection. IL-4 levels were determined by luminescent ELISA. Mean values (± SE) are shown based on cumulative data from two separate experiments using a total of 4–11 mice/group. Statistical analysis using Student *t* test showed a significant difference between IL-4 serum levels in WT and LckW97A mice on days 7 and 10 (*p* < 0.05).

**FIGURE 4.** LckW97A animals have a significant defect in Ag-specific IgG1 production. Age and sex-matched animals were immunized with 100 μg DNP–KLH in Titer Max Gold adjuvant (Sigma-Aldrich). Serum was collected on days 0 and 14 (preboost) and 21 postboosting with 50 μg DNP–KLH on day 14. Serum levels of DNP-specific IgG1 (A), IgG2b (B), and IgG3 (C) were analyzed by ELISA. Data are shown as the log of the concentrations in micrograms per milliliter. Statistical analysis was performed using Student *t* test (*p* < 0.05). Individual animals are represented by symbols. The total number of animals in each group is indicated in parentheses. Cumulative data from two to four separate experiments is shown.

The Journal of Immunology 3291
Our findings indicate that Lck SH3 domain function is important for the development and/or maintenance of normal T cell numbers in the periphery, as well as T lymphocyte activation following anti-TCR–specific stimulation, although it is not absolutely essential for these processes.

We also examined TCR signaling function in LckW97A T cells to understand the basis for the observed changes in the response to receptor stimulation. Biochemical analysis indicates that activation of the ERK1 and ERK2 MAPKs is defective (Fig. 3). This signaling defect was limited to the ERK pathway, as PLCγ1 activation, calcium mobilization, and the overall pattern of tyrosine phosphorylation were not substantially altered in LckW97A T lymphocytes. These selective reduction in ERK pathway activation in mature T lymphocytes is consistent with previous data from our laboratory and others investigating Lck SH3 domain function in cell lines and thymocytes (21–23). Although the specific target of the Lck SH3 domain that allows it to regulate the MAPK pathway has not been established, Li et al. (22) have recently shown that Lck-deficient J.CaM1.6 cells reconstituted with LckW97A are defective in activation of Raf-1 following receptor stimulation. We also examined the activation of the p38 kinase in LckW97A T cells because the Lck SH3 domain has been reported to recruit the Dlgh1 scaffold protein to the T cell membrane and form a complex leading to activation of p38 (34, 35). However, we did not observe defective p38 activation in LckW97A T cells in response to TCR stimulation. Given the key role of the MAPK pathway in the control of cell proliferation and gene expression, our findings strongly suggest that the altered activation of peripheral LckW97A T cells is due to defective induction of ERK1 and ERK2 following stimulation of the Ag receptor.

Lck has been proposed to play an important role in costimulatory signaling through SH3 domain binding to CD28. This conclusion is based on the observations that a proline-rich region in the cytoplasmic region of CD28 is critical for its costimulatory function (36). Lck can bind to this region, and SH3 domain function of Lck is important for CD28 signaling in cell lines (30). Based on this, we expected that costimulation of LckW97A T cells would be defective. Surprisingly, we found that stimulation of CD28 effectively augmented anti-CD3–induced proliferation in LckW97A T lymphocytes. Furthermore, stimulation with anti-CD28 and PMA failed to reveal any significant defect in the proliferative response of LckW97A T cells to CD28 stimulation. These findings suggest that SH3 domain recruitment of Lck to CD28 is not necessary for CD28 costimulatory function, although it is possible that PMA treatment induced intracellular signals that might otherwise require Lck SH3 domain function. Alternatively, because binding of Lck to the CD28 proline-rich region enhances Lck catalytic activity by displacement of the Lck SH3 domain (30), it is possible that the modest increase in catalytic activity that accompanies mutation of the SH3 domain may replace the role of CD28 binding. It is also possible that the closely related p59Fyn kinase might substitute for Lck in CD28 signaling.

**Discussion**

The mechanisms that regulate the outcome of T lymphocyte activation and the establishment of effector function are not well understood. We present evidence that function of the SH3 domain of an Src family protein tyrosine kinase, p56Lck, is necessary for normal T cell activation and Th2 immunity. These defects correlate with the selective requirement for Lck SH3 domain function in the proper induction of the MAPK pathway following Ag receptor stimulation. Our findings indicate that the Lck SH3 domain provides an important mechanism linking the Ag receptor to intracellular signaling pathways determining the outcome of T lymphocyte activation.

To examine the role of the Lck SH3 domain in mature T cell activation and effector function, we used a knockin mouse model with a mutant Lck allele containing an alanine substitution of a critical tryptophan residue in the SH3 domain (W97A). This mutation is known to disrupt normal ligand binding to the SH3 domain (23, 33). We show that in LckW97A mice, there are modest changes in T cell numbers, particularly the CD8+ T lymphocyte subset, as well as slightly lower CD4 expression on LckW97A T cells (Fig. 1). In vitro activation of LckW97A T cells is substantially altered, as indicated by fewer cells undergoing proliferation and CD69 induction following receptor stimulation, as well as by reduced IL-2 production. However, we found a significant number of cells could be activated, particularly with the addition of CD28 costimulatory signals. These findings indicate that Lck SH3 domain function is important for the development and/or maintenance of normal T cell numbers in the periphery, as well as T lymphocyte activation following anti-TCR–specific stimulation, although it is not absolutely essential for these processes.

The mechanisms that regulate the outcome of T lymphocyte activation and the establishment of effector function are not well understood. We present evidence that function of the SH3 domain of an Src family protein tyrosine kinase, p56Lck, is necessary for normal T cell activation and Th2 immunity. These defects correlate with the selective requirement for Lck SH3 domain function in the proper induction of the MAPK pathway following Ag receptor stimulation. Our findings indicate that the Lck SH3 domain provides an important mechanism linking the Ag receptor to intracellular signaling pathways determining the outcome of T lymphocyte activation.

To examine the role of the Lck SH3 domain in mature T cell activation and effector function, we used a knockin mouse model with a mutant Lck allele containing an alanine substitution of a critical tryptophan residue in the SH3 domain (W97A). This mutation is known to disrupt normal ligand binding to the SH3 domain (23, 33). We show that in LckW97A mice, there are modest changes in T cell numbers, particularly the CD8+ T lymphocyte subset, as well as slightly lower CD4 expression on LckW97A T cells (Fig. 1). In vitro activation of LckW97A T cells is substantially altered, as indicated by fewer cells undergoing proliferation and CD69 induction following receptor stimulation, as well as by reduced IL-2 production. However, we found a significant number of cells could be activated, particularly with the addition of CD28 costimulatory signals. These findings indicate that Lck SH3 domain function is important for the development and/or maintenance of normal T cell numbers in the periphery, as well as T lymphocyte activation following anti-TCR–specific stimulation, although it is not absolutely essential for these processes.

We also examined TCR signaling function in LckW97A T cells to understand the basis for the observed changes in the response to receptor stimulation. Biochemical analysis indicates that activation of the ERK1 and ERK2 MAPKs is defective (Fig. 3). This signaling defect was limited to the ERK pathway, as PLCγ1 activation, calcium mobilization, and the overall pattern of tyrosine phosphorylation were not substantially altered in LckW97A T lymphocytes. These selective reduction in ERK pathway activation in mature T lymphocytes is consistent with previous data from our laboratory and others investigating Lck SH3 domain function in cell lines and thymocytes (21–23). Although the specific target of the Lck SH3 domain that allows it to regulate the MAPK pathway has not been established, Li et al. (22) have recently shown that Lck-deficient J.CaM1.6 cells reconstituted with LckW97A are defective in activation of Raf-1 following receptor stimulation. We also examined the activation of the p38 kinase in LckW97A T cells because the Lck SH3 domain has been reported to recruit the Dlgh1 scaffold protein to the T cell membrane and form a complex leading to activation of p38 (34, 35). However, we did not observe defective p38 activation in LckW97A T cells in response to TCR stimulation. Given the key role of the MAPK pathway in the control of cell proliferation and gene expression, our findings strongly suggest that the altered activation of peripheral LckW97A T cells is due to defective induction of ERK1 and ERK2 following stimulation of the Ag receptor.

Lck has been proposed to play an important role in costimulatory signaling through SH3 domain binding to CD28. This conclusion is based on the observations that a proline-rich region in the cytoplasmic region of CD28 is critical for its costimulatory function (36). Lck can bind to this region, and SH3 domain function of Lck is important for CD28 signaling in cell lines (30). Based on this, we expected that costimulation of LckW97A T cells would be defective. Surprisingly, we found that stimulation of CD28 effectively augmented anti-CD3–induced proliferation in LckW97A T lymphocytes. Furthermore, stimulation with anti-CD28 and PMA failed to reveal any significant defect in the proliferative response of LckW97A T cells to CD28 stimulation. These findings suggest that SH3 domain recruitment of Lck to CD28 is not necessary for CD28 costimulatory function, although it is possible that PMA treatment induced intracellular signals that might otherwise require Lck SH3 domain function. Alternatively, because binding of Lck to the CD28 proline-rich region enhances Lck catalytic activity by displacement of the Lck SH3 domain (30), it is possible that the modest increase in catalytic activity that accompanies mutation of the SH3 domain may replace the role of CD28 binding. It is also possible that the closely related p59Fyn kinase might substitute for Lck in CD28 signaling.
Although LckW97A T cells could respond to receptor and costimulatory signals, it remained possible that they had functional defects, particularly in light of the reduced induction of the MAPK pathway. To examine this, we assessed the in vivo immune response of LckW97A mice to Ag/adjuvant immunization and helminth infection. We found that LckW97A mice have a selective defect in Th2 immunity as characterized by a significant reduction in the serum levels of IgG1, IgE, and IL-4 (Figs. 4, 5). There was no effect observed on preimmune serum Ig levels (data not shown) or the induction of Ag-specific IgG2b or IgG3 isotypes (Fig. 4). In contrast to the effect on Th2 immunity, immunization with the strong Th1 inducer, \(B.\) \textit{abortus}, did not reveal any significant defect in LckW97A mice in the induction of serum IFN-\(\gamma\) or IFN-\(\gamma\)–expressing CD4\(^{+}\) T cells (Fig. 6). These results indicate that Lck SH3 domain function contributes primarily to Th2 immunity, although we cannot rule out that under some circumstances, Th1 immunity may also be impaired. Although the Th2 defect could be due to a failure of LckW97A T lymphocytes to properly develop into Th2 cells from Th0 cells, in vitro differentiation studies indicate that LckW97A T cells are able to differentiate into IL-4–expressing cells under biasing conditions (Fig. 7). However, these studies do not address the survival of Th2 cells, which could be influenced by the strength of ERK signaling. Defective Th2 survival could explain the observation that Ag-specific IgG1 production is reduced in LckW97A mice following secondary immunization but not during the primary response. Alternatively, instead of an effect on Th2 survival, enhanced levels of inhibitory cytokines, such as IFN-\(\gamma\), could antagonize Th2 effector function. This is consistent with our in vitro differentiation studies that show an increased tendency of LckW97A T cells to differentiate into IFN-\(\gamma\) expressors under Th2-biasing conditions, as well as the absence of any detectible serum IgE in LckW97A mice following \(N.\) \textit{brasiliensis} infection despite the presence of IL-4.

A substantial amount of evidence has accumulated showing that TCR signaling influences Th differentiation and function (37). Lck has previously been implicated in Th2 cell differentiation in studies analyzing the effect of expressing a dominant-inhibitory Lck transgene in peripheral T cells (9). This catalytically inactive mutant would be expected to suppress early TCR-signaling events dependent on Lck catalytic activity and reduce the overall strength of TCR signaling. In addition to Lck, other TCR signaling molecules including linker for activation of T cells, the Tec kinases Itk and Rlk, and protein kinase C\(\theta\) have been implicated in Th2 differentiation (10–13). They have been proposed...
to influence Th differentiation by affecting the overall strength of TCR signaling or by specifically regulating the NFAT or NF-κB transcription factors that are required for IL-4 expression and Th2 differentiation.

Our results with LckW97A mice also suggest that normal TCR-signaling function is required for proper Th2 immunity. However, in contrast to other TCR-signaling mutants, the LckW97A mutant does not diminish early TCR signaling events or activation of the PI3 pathway. Instead, our analysis indicates that the Lck SH3 domain specifically links TCR signaling to induction of the ERK signaling pathway. This is consistent with earlier studies showing that perturbation of Ras–MAPK signaling blocked Th2 function (29). Additionally, activation of the MAPK pathway has been implicated in IL-4R signaling and is critical for subsequent propagation of a complete Th2 immune response (29, 38), although a role for Lck in IL-4R signaling has not yet been established. Further studies will be required to establish how Lck SH3 domain function specifically controls Th2 immunity.

In summary, our analysis indicates that Lck SH3 domain function is necessary for normal peripheral T lymphocyte activation and induction of the MAPK pathway following T cell Ag receptor stimulation. Mice with a defective Lck SH3 domain also exhibit a substantial and specific defect in Th2 immunity. These findings suggest that Lck provides a key regulatory function in Th cell development and/or effector function by providing a selective link to activation of the MAPK signaling pathway.

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Disclosures

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