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Fyn Regulates the Duration of TCR Engagement Needed for Commitment to Effector Function

Andrew Filby,* Benedict Seddon,† Joanna Kleczkowska,* Robert Salmond,* Peter Tomlinson,* Michal Smida,* Jonathan A. Lindquist,‡ Burkhart Schraven,‡ and Rose Zamoyska2*

In naive T cells, engagement of the TCR with agonist peptide:MHC molecules leads to phosphorylation of key intracellular signaling intermediates within seconds and this peaks within minutes. However, the cell does not commit to proliferation and IL-2 cytokine production unless receptor contact is sustained for several hours. The biochemical basis for this transition to full activation may underlie how T cells receive survival signals while maintaining tolerance, and is currently not well understood. We show here that for CD8 T cells commitment to proliferation and cytokine production requires sustained activation of the Src family kinase Lck and is opposed by the action of Fyn. Thus, in the absence of Fyn, commitment to activation occurs more rapidly, the cells produce more IL-2, and undergo more rounds of division. Our data demonstrate a role for Fyn in modulating the response to Ag in primary T cells. The Journal of Immunology, 2007, 179: 4635–4644.

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ignals delivered to naive T cells via TCR engagement with peptide:MHC (pMHC) molecules can lead to multiple outcomes depending upon their spatial and temporal context. Thus, the TCR can discriminate self-pMHC engagements, which provide survival signals, from foreign pMHC engagements that stimulate the development of effector function. Biophysical analysis of these interactions has indicated that the duration of TCR engagement, and therefore the period of TCR signaling, is an influential factor in which outcome succeeds (1), with prolonged periods of TCR engagement required to achieve full effector potential and proliferation, while shorter periods of engagement induce a state of partial activation, typified by CD69 expression (2). It has been suggested that TCR:MHC engagement sets up competing positive and negative feedback loops and, depending on which prevails, results in either digital amplification of the signal and activation of the T cell or abortion of the signal and a return to cellular quiescence (3).

The Src family kinase (SFK) Lck is a key initiator of TCR signal transduction, phosphorylating ITAM residues in the CD3 and TCR/ chains, and leading to recruitment and activation of Zap70 and consequent propagation of downstream signals (4). Thus, Lck controls the activation threshold of naive T cells (5) and Lck has been shown to be the target of a regulatory circuit in which positive regulation is through serine/threonine phosphorylation of Lck by ERK, and negative regulation is through dephosphorylation of the active site of Lck, Tyr505, by the phosphatase SHP-1 (6). Additional regulation of Lck is achieved by phosphorylation of its negative regulatory Tyr505, and mutations of this residue lead to enhanced sensitivity of T cells to stimulation (7). However, the dynamics of how Lck activity is regulated by Tyr505 phosphorylation in vivo is not well understood. The cytosolic C-terminal Src kinase (Csk) phosphorylates the SFK regulatory tyrosine when recruited to the plasma membrane (PM) (8). In resting T cells, transmembrane adapter molecules, such as phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG)/Cbp (hereafter referred to as PAG), recruit Csk to phosphorylated tyrosine residues in their cytoplasmic domains, and, upon T cell activation, dephosphorylation of these tyrosine residues leads to Csk release (9, 10). It was suggested that recruitment of Csk by PAG provided a regulatory circuit for controlling T cell activation; however, no defects in T cell development or activation were reported in PAG knockout mice and Csk was still recruited to the PM in the absence of PAG (11, 12); therefore, it remains unclear how regulation of Lck by means of Tyr505 phosphorylation influences naive T cell activation.

The SFK Fyn is also expressed at high levels in T cells and its function is less well understood. The absence of Fyn has only minor consequences, while expression of Lck is both necessary and sufficient for most aspects of T cell function (13). Both Lck and Fyn have been suggested to act as positive mediators of TCR signaling, since the absence of either leads to decreased responses to anti-CD3 stimulation (14). However, differential subcellular compartmentalization of Lck and Fyn in mature T cells (15) suggests they may be involved in divergent outcomes after TCR ligation. Moreover, although Lck is linked to T cell development and proliferation (16), Fyn has been implicated in the generation and maintenance of anergy (17, 18). Furthermore, although both SFKs have significant overlap in their substrates, there are specific targets associated with each. In particular, Fyn has been shown to phosphorylate several negative regulators of T cell signaling, including PAG (19), signalling lymphocyte activation...
molecule-associated protein (20), and Cbl (21), suggesting Fyn may be involved in terminating TCR signals.

In this study, we describe a feedback loop in T cells that influences proliferation and the generation of effector function. We show that Fyn influences the TCR:pMHC contact time required for commitment to full activation and this correlates with changes in phosphorylation of the regulatory tyrosine of Lck which might alter its activity. We confirm that Fyn influences phosphorylation of PAG and recruitment of Csk, suggesting that PAG does indeed play an important, although subtle, regulatory role in T cells. Moreover, we show that Lck might partially regulate itself, since the abundance of Lck influenced the extent of Tyr505 phosphorylation, consistent with the idea that Lck also targets adapters which recruit Csk.

Materials and Methods

Mice

The generation of polyclonal mice expressing an inducible Lck transgene (Lck<sub>Cre/lox</sub>) on a Lck and/or Fyn deficient have been described previously (22) and Fyn<sup>−/−</sup> mice were a gift from Dr. P. Soriano (Fred Hutchinson Research Center, Seattle, WA). These strains were backcrossed to the Rag-1<sup>−/−</sup> F5 TCR-transgenic mouse (23). All mice expressing the inducible Lck transgene were fed doxycycline (dox) in food (1 mg/g for mice on a Lck<sup>−</sup>/background and 3 mg/g for mice on a Lck<sup>−</sup>/Fyn<sup>−</sup>/background) from gestation through to adulthood. To generate either Lck<sup>−</sup> or Lck<sup>−</sup>/Fyn<sup>−</sup> mice, F5 TCR-transgenic mice were fed dox throughout life. Where stated, biotinylated Abs were used for purification of naive T cells along with an optimum dose of A/NT/60-68 influenza virus i.v. Seven days later, recipient mice were sacrificed and LN, spleen, and blood were removed from the dox diet of these animals for at least 1 wk prior to the experiment. All mice were housed in specific pathogen-free conditions and all procedures were conducted in accordance with local and U.K. national guidelines.

In vitro analysis of T cell proliferation and IL-2 production

T cell suspensions were prepared from LN and spleen of F5 or polyclonal mice. Where stated, biotinylated Abs were used for purification of naive T cells by negative selection: anti-B220 (RA3-6B2), anti-CD45d (DX-5), anti-Mac-1 (M1/70), anti-GL3, anti-CD44 (IM7), and anti-i-A<sup>+</sup> (25-2-17) (from BD Biosciences), followed by a 30-min incubation with streptavidin Dynal beads (M-280; Dynal). For proliferation assays, F5 T cells were labeled with CFSE (0.5 μM; Molecular Probes) for 10 min and activated with soluble Ag (in the presence of APC), NP68-loaded H-2<sup>D</sup> MHC dimer molecules (DimerX; BD Biosciences) (with or without purified Lck<sup>−/−</sup> M; Molecular Probes) for 100. The proliferation index (PI) was calculated from the equation

\[
\text{PI} = \frac{\sum \text{AdF} \times \text{AdF}}{\sum \text{AdF}}
\]

for day 0.

In vivo responses to Ag were assessed after transfer of 3 × 10<sup>6</sup> F5 LN cells along with an optimum dose of A/NT/60-68 influenza virus i.v. Seven days later, recipient mice were sacrificed and LN, spleen, and blood were analyzed for F5 cell number, cell phenotype, and intracellular cytokine production after in vitro stimulation with phorbol 12,13-dibutyrate (pDBU) and ionomycin for 4 h in the presence of Brefeldin A as described below.

To determine signal duration for commitment to IL-2<sup>+</sup>, purified CFSE-labeled F5 T cells were spun onto plate-bound NP68-loaded H-2<sup>D</sup><sup>+</sup> molecules (1 μg/ml). Cells were removed, washed three times, and recultured with unlabelled naive F5 cells without Ag in the presence of anti-H-2<sup>D</sup>-Ab 4-aminopyrazole-7-(tr-butyl)-pyrazolo[3,4-d]pyrimidine (PP2) (1 μM; Calbiochem) or diluent were added where indicated. Intracellular IL-2 was measured by incubation in 4 h in pDBU and ionomycin (0.5 μM) with Brefeldin A (1 μg/ml). Cells were fixed in 3% paraformaldehyde, permeabilized using 0.1% Nonidet P-40 for 3 min, and stained with anti-IL-2 (SB6) and IFN-γ (XM1G1.2) Abs (BD Biosciences). IL-2 secretion in intact cells was measured using the commercial IL-2 capture assay (Miltene Biotec).

FACS analysis

Abs for FACS analysis were fluorochrome conjugated: CD69 (H.12F3), CD25 (PC61), CD8a (53-67), and TCRβ (HS7-597). Intracellular phospho-ERK (rabbit mAb; Cell Signaling Technology) staining was performed as described previously (5). A minimum of 30,000 events was collected using a FACS Calibur (BD Biosciences) and data were analyzed with FlowJo software (Tree Star).

Measurement of [Ca<sup>2+</sup>]i

F5 T cells were loaded with 4 μg/ml Indo-1 AM (Molecular Probes) at 37°C for 40 min and washed. Baseline FL4:5 ratio was established on the LSR cytometer (BD Biosciences) and then samples were centrifuged briefly with bone marrow-derived dendritic cells pulsed with 1 μM NP68 or unpulsed bone marrow-derived dendritic cells for controls and returned to the LSR.

Immunoprecipitation and Western blot analysis of pMHC-activated T cells

T cells were centrifuged onto plate-bound pMHC and lysed in radioimmunoprecipitation buffer (Tris (pH 7.4), 140 mM NaCl, 0.5% Nonidet P-40, 2 mM EDTA, 50 mM NaF, 0.1% SDS, 0.5% deoxycholate, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitor mixture (Sigma-Aldrich). Samples were resolved on 10% SDS-PAGE (2.5 × 10<sup>5</sup> cell equivalent/lane) and transferred to Immobilon FL membranes (Millipore). Membranes were pre-blocked for 1 h in 5% BSA and then incubated with the following Abs: anti-PKB pSer916 (D. Cantrell, University of Dundee, U.K.), anti-pErk1/2 and anti-Src pTyr418 (BioSource International). Loading controls were: anti-Zap70 (Signal Transduction), anti-Erk-1 (Santa Cruz Technology), anti-Lck (from A. Magee, Imperial College, London, U.K.), anti-PKB (D. Cantrell), and anti-LAT (M41, from M. Turner, Babraham Institute, Cambridge, U.K.). Abs were diluted in 50% Licor buffer plus 0.1% Tween 20 and incubated overnight at 4°C. Secondary Abs were anti-Rlq Alexa 680 (Molecular Probes), anti-Mlg IR800, and anti-goat IR800 (both Rockland Immunochemicals). Membranes were scanned using a Licor Odyssey machine in the 700- and 800-nm channels and quantified with Licor Odyssey software. Membranes were stripped by incubating them in several changes of 5% nonfat milk in TBS (pH 7.4) for 1 h, incubated for 1 h at room temperature in primary Ab, washed three times with TBS plus 0.01% Tween 20, followed by a 45-min incubation with HRP-conjugated secondary Ab. After additional washing, blots were developed using ECL Detection reagents (Amerham Biosciences) and exposed on Hyperfilm (Amerham). Primary Abs were anti-Csk (C-20; Santa Cruz Biotechnology), anti-PAG (IG-452; ImmunoGlobe) and 30 μl of anti-Rlq beads (Nateuc) overnight. Immunoprecipitations were washed, electrophoresed, and transferred onto nitrocellulose membranes (Amerham Biosciences). Membranes were blocked in 5% nonfat milk in TBS (pH 7.4) for 1 h, incubated for 1 h at room temperature in primary Ab, washed three times with TBS plus 0.01% Tween 20, followed by a 45-min incubation with HRP-conjugated secondary Ab. After additional washing, blots were developed using ECL Detection reagents (Amerham Biosciences) and exposed on Hyperfilm (Amerham). Primary Abs were anti-Csk (C-20; Santa Cruz Biotechnology), anti-PAG (IG-452; ImmunoGlobe), and anti-pTyr (4G10 hybridoma supernatant) and secondary Abs were anti-Mlg-HRP (Dianova) and anti-RlqG-HRP TrueBlot (eBioscience). Whole lysates were probed with rabbit anti-PAG (pY317, provided by Dr. B. Schraven, Institute of Immunology, Otto-von-Guericke University, Magdeburg, Germany), rabbit anti-Fyn (BioSource International), and mouse anti-actin (AC-15; Sigma-Aldrich).

Results

Lck is essential for optimal triggering by pMHC and is opposed by Fyn

Fyn<sup>−/−</sup> mice were backcrossed to Rag-1<sup>−/−</sup> F5 TCR-transgenic mice that recognize NP68 peptide presented by H-2<sup>D</sup>B (23). We also generated F5 Rag-1<sup>−/−</sup> mice lacking endogenous Lck (backcrossed to Lck<sup>−/−</sup> mice) but expressing a tetracycline-inducible Lck transgene, hereafter called Lck<sub>low</sub> because Lck expression in peripheral T cells is lower than endogenous Lck (24, 25). When dox was withdrawn from the diet of these animals for ≥7 days, Lck expression ceased, providing peripheral T cells that were Lck<sup>−/−</sup> Fyn<sup>WT</sup>. Furthermore, by intercrossing Fyn<sup>−/−</sup> and Lck<sup>−/−</sup> strains, we obtained mice in which Lck was inducibly expressed and Fyn was absent (Lck<sub>low</sub> Fyn<sup>−/−</sup>) or, upon dox withdrawal, mice that lacked both SFKs (Lck<sup>−/−</sup> Fyn<sup>−/−</sup>). T cell development was normal in all mouse strains, and the peripheral T cell pool was comparable in phenotype to F5 WT mice. The T cells were predominantly naive although peripheral T cell numbers were reduced in Lck<sub>low</sub> mice (data not shown).

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Stimulation of Fyn−/− F5 T cells with peptide revealed that they were triggered comparably to wild-type (WT) cells, as judged by CD69 up-regulation measured at 24 h after stimulation. In contrast, Lck−/− cells showed a 100-fold reduction in sensitivity to peptide (Fig. 1A). However, when we analyzed later responses, such as proliferation at 72 h, we found Fyn−/− cells to be hyperresponsive over the range of peptide concentrations (Fig. 1B). The different manner in how lack of Fyn vs lack of Lck influences the response to peptide is best illustrated by comparing the profiles of CFSE-labeled cells at a single dose of peptide at 72 h after stimulation (Fig. 1C). Fyn−/− cells were triggered comparably to WT cells, but underwent more rounds of division. In contrast, Lck−/− cells were defective in triggering, since fewer cells entered division, but curiously those cells that did divide had nearly as many division cycles as WT cells. Lck−/− Fyn−/− cells did not respond to pMHC by any of the parameters measured, highlighting that the ability of Lck−/− cells to respond to peptide was mediated by Fyn.

In addition to increased proliferation, substantially more Fyn−/− cells up-regulated CD25 in response to Ag (Fig. 1D) and the amount of CD25 expressed per cell was also increased. A time course following CD25 up-regulation for a single Ag dose showed that at early time points the mean fluorescence intensity (MFI) values of Fyn−/− cells were comparable to those of WT cells. WT cells showed a peak of CD25 expression at 24 h, after which it declined. In contrast, Fyn−/− cells continued to express CD25 with increasing intensity for a further 24 h before expression declined and by 72 h expression was restored to WT levels. In addition, F5 Fyn−/− cells became more effective CTL at 72 h, showing a 10-fold greater efficiency on a per cell basis to kill target cells (Fig. 1D), which correlates with increased expression of granzyme B (data not shown), indicating that they developed superior effector function.

We examined phosphorylation of key proximal substrates induced by stimulation with pMHC dimers to try and establish the cause of the hyperresponsiveness of Fyn−/− F5 T cells. As shown in Fig. 2A, Fyn−/− cells were comparable to WT cells with respect to phosphorylation of Zap70 Y319, a direct target of the SFKs; PKD S916, an indicator of diacylglycerol production (26); and LAT pY171, a target of Zap, confirming that the loss of Fyn had minimal effect on these early phosphorylation events. Phosphorylation of MAPK pERK1/2 was slightly reduced by Western blot, suggesting that loss of Fyn might have more impact on further downstream signaling pathways. The percentage of cells that were positive for pERK was similar between WT and Fyn−/− mice either at shorter (~15 min) (5) or longer (3–4 h) time points (Fig. 2B), while the MFI remained ~50% of WT levels (data not shown). Similar to the effect we showed with CD4 cells (5), loss of Lck had a substantial impact on phosphorylation of Zap and PKD, and a lesser impact on pLAT and pERK. A comparable picture was obtained from measuring pMHC-induced Ca2+ mobilization (Fig. 2C), with Fyn−/− and WT cells responding equivalently, whereas there was a profound reduction in the Ca2+ flux of Lck−/− cells. In keeping with the lack of CD69 and CD25 up-regulation, no phosphorylation of TCR downstream targets or Ca2+ flux was observed in cells that lacked both Fyn and Lck.

**FIGURE 1.** Fyn−/− cells hyperproliferate in response to pMHC. LN cells from Rag-1−/− F5 mice (WT), Rag-1−/− F5 Fyn−/− background (Fyn−/−), and Lck−/− cells obtained from F5 Lck-inducible mice taken off dox food, and cells lacking both Lck and Fyn from Lck-inducible Fyn−/− mice taken off dox food (DKO) were activated with Ag and their responses were assessed. A. The percentage of CD8+ cells that up-regulate CD69+ in response to a titration of peptide at 24 h is shown. B. The PI of CFSE-labeled WT and Fyn−/− cells in response to a titration of NP68 peptide at 72 h. C. FACS histograms of LN T cells proliferating in response to 1 × 10−2 μM NP68 measured at 72 h. The percent divided (%D) and PI are indicated on the graphs. D. The percentage of CD8+ LN WT and Fyn−/− F5 T cells up-regulating CD25 in response to a titration of peptide is shown. E. CD25 expression levels (MFI) on gated CD8+CD25+ cells are shown after activation for different times with APC plus 1 × 10−2 μM NP68. F. CTLs were generated by stimulation with 1 μM NP68 for 72 h and assayed for killing on 51Cr-pulsed EL-4 target cells for 4 h. Values are the mean of triplicate samples ± SD. All data are representative of at least three independent experiments in A, E, and F and five experiments in B and D.
FIGURE 2. Normal tyrosine phosphorylation in Fyn−/− T cells after pMHC stimulation. A, Phosphorylation of key signaling molecules was examined after activation of LN T cells from the different strains of mice with plate-bound NP68 pMHC dimers for the times specified. Western blots of total cell lysates were probed with phospho-specific Abs and loading was controlled by reprobing with antisera to total protein as shown. Images were obtained using the Licor Odyssey infra-red detection system and are representative of two independent experiments. B, The graph shows the percentage of CD8+ pERK+ cells after stimulation with peptide for 4 h. A representative of four experiments with triplicate samples is shown. C, Ca2+ fluxes were measured in T cells loaded with Indo-1. Cells were run on a BD Biosciences LSR analyzer to establish baseline before centrifuging briefly with NP68-pulsed APC at ~50 s. Cells are Indo-1+ gated to exclude unlabeled APC. Triplicate samples were analyzed in three independent experiments.

IL-2 at approximately twice the level of WT cells (cf MFI values; Fig. 3A). This overproduction of IL-2 by Fyn−/− cells resulted from increased transcription of IL-2, confirmed by quantitative PCR (data not shown). Although initial proliferation of CD8 T cells has been shown to be IL-2 independent, IL-2 can sustain CD8 T cell proliferation at later times (28). It was likely, therefore, that increased production of IL-2 was driving the hyperproliferation of Fyn−/− cells. Accordingly, we were able to show that blocking IL-2 with an anti-IL-2 Ab substantially knocked down both hyperproliferation (Fig. 3C) and expression of CD25 (data not shown) in Fyn−/− cells.

Because CD28 signals can influence IL-2 production (29) and Fyn and Lck have been implicated in CD28 signal transduction (30), we asked whether the increased IL-2 production in Fyn−/− cells was down-stream of CD28 rather than the TCR. We stimulated purified T cells with plate-bound NP68-loaded pMHC dimers with or without anti-CD28 Ab to provide costimulation. Inclusion of anti-CD28 Ab during stimulation enhanced production of IL-2 by ~50% for both Fyn−/− and WT cells (Fig. 3C). However, Fyn−/− cells still consistently produced more IL-2 than controls. Note that at this 48-h time point, the increased proliferation of Fyn−/− cells is less apparent then at 72 h. In addition, we found that stimulation in the presence of CTLA-4G reduced the IL-2 MFI equivalently in WT and Fyn−/− cells (data not shown). These data indicate that the absence of Fyn dysregulates IL-2 production by influencing signaling downstream of the TCR rather than by affecting costimulation.

We asked whether the hyperresponsiveness of F5 Fyn−/− cells was apparent after activation in vivo. F5 Fyn−/− and F5 control cells were transferred into Rag−/− recipients along with A/NT/60–68 flu virus, and expansion and cytokine production were measured 7 days later. Both populations up-regulated CD44 and expanded in response to virus. As seen in vitro, F5 Fyn−/− cells showed approximately a 2-fold increase in the number of cells recovered from spleen (Fig. 4A) and also from LN and blood (data not shown). In the absence of virus, there was limited expansion of either population and there was no difference in the number of cells recovered at day 14 (Fig. 4A). In addition, more F5 Fyn−/− cells developed the capacity to make IL-2 in response to virus and the MFI of IL-2-positive Fyn−/− cells was higher than that of WT cells (Fig. 4B), consistent with the phenotypes we observed from activation of Fyn−/− cells in vitro.

Hyperresponsiveness of Fyn−/− cells has not been reported previously; indeed, several groups have reported that polyclonal Fyn−/− cells are hyporesponsive to anti-CD3 stimulation (31–35), although initial triggering of these cells can be assisted by inclusion of anti-CD4 (35) or anti-CD28 (32) Abs. We asked whether the increased proliferation of the F5 Fyn−/− cells was peculiar to this transgenic background and/or stimulation with pMHC. Using microbeads coated with anti-CD3 alone, we showed that F5 and polyclonal Fyn−/− T cells were less responsive than WT cells (Fig. 4C). However, when triggered with beads coated with a 1:1 mix of anti-CD3 and CD8, both F5 and polyclonal Fyn−/− cells proliferated more than WT cells. These results confirm that both the TCR and CD8 need to be engaged for the increased responsiveness of Fyn−/− cells and, given that the coreceptor brings Lck into the signaling complex, suggest that activation of Lck is involved in the hyperproliferative phenotype.

Fyn−/− cells can commit to IL-2 production with reduced pMHC contact time

IL-2 production requires a more sustained exposure of T cells to stimulus than CD69 up-regulation (36). This is thought to reflect
the need for key transcription factors involved in IL-2 production, such as c-Rel to be synthesized de novo and migrate to the nucleus 3–4 h postactivation (2). Moreover, within a population of cells that up-regulate CD69, only a fraction go on to transcribe IL-2 (37), indicating that not every T cell that demonstrably encounters Ag receives sufficient signal to commit to IL-2 production. For F5

Fyn−/− cells, the proportion of cells that produced IL-2 was increased, suggesting that Fyn influenced the ability of cells to cross this second signaling threshold.

Previous studies examined how long T cells needed to contact pMHC to become committed to IL-2 production by removing T cells from the stimulus at defined times and reculturing

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**FIGURE 3.** Fyn−/− cells produce more IL-2 in response to Ag. A. Purified F5 T cells were activated with plate-bound NP68-loaded pMHC dimers and IL-2 secretion was assayed using the IL-2 capture assay. The IL-2 vs CD69 profiles of gated CD8+CD69+ cells are presented and the percentage of positive cells and MFI in the CD69+IL-2+ quadrant is shown. B, WT and Fyn−/− F5 T cells were activated with NP68 peptide with or without IL-2 Ab for 72 h. C, Purified CFSE-labeled F5 T cells were activated using pMHC with or without anti-CD28 and IL-2 was measured by FACS at 48 h. Note that at 48 h the enhanced proliferation of Fyn−/− cells was not as pronounced as at 72 h. MFI values for IL-2+ cells are shown. All data represent at least three experiments.

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**FIGURE 4.** Hyperresponsiveness of Fyn−/− T cells occurs in vivo and requires TCR and coreceptor ligation. Three × 10^6 LN T cells from F5 or F5 Fyn−/− cells were transferred i.v. alone or with A/NT/60-68 flu virus into Rag−/− recipients. Cells were recovered after 14 days for no Ag controls and after 7 days if transferred with virus and stained for intracellular IL-2 after a 4-h recall with pdBu and ionomycin. A, The number of F5 T cells recovered in the spleen (three mice per group). B, Representative staining of intracellular IL-2 gated on F5 T cells. WT cells have 54% in the IL-2+ gate, MFI 89, while Fyn−/− cells have 89% in the IL-2+ gate, MFI 137. C, Purified F5 or naive polyclonal CD8 T cells were CFSE labeled and activated using microbeads coated with CD3 with or without CD8 Ab. Proliferation was measured by CFSE dilution at 72 h. The percent divided (%D) and PI are indicated on the graphs.
Proximal signals are rapidly terminated upon cessation of TCR
IL-2 production (data not shown). These results confirm that
results were obtained if proliferation was measured rather than
pMHC contact time to initiate IL-2 production. Equivalent re-
higher at all time points, indicating that they required less
B produced IL-2 after only 1 h of pMHC contact (Fig. 5
(38), and these higher bands were clearly visible in both WT
and Fyn null cells after 1 h of stimulation and persisted for at
least 9 h before declining by 24 h (Fig. 6B). The total amount
of Lck and the supershifted proportion was similar in WT and
Fyn null cells. Quantitation of Tyr505 phosphorylation relative to
total Lck was performed using the sensitive Licer Odyssey im-
aging system, which provides linear quantitation throughout the
range and showed that there was a small but highly reproducible
decrease in the percentage of pTyr505 in the absence of Fyn
from within 1 h of stimulation that persisted for up to 24 h (Fig.
6C). This was true of both the 56-kDa species and the larger
59/60-kDa species of Lck and was of the same order of mag-
nitude (~2-fold) to the increased IL-2 production (Fig. 3) and
the decreased peptide contact time (Fig. 5B) seen with Fyn null
cells.

The transmembrane adapter molecule PAG is phosphorylated
by Fyn in thymocytes and mature T cells (19). Phosphorylated
PAG recruits Csk to lipid rafts whereupon Csk phosphorylates
the autoinhibitory Tyr505 residue of Lck. We examined the sta-
 tus of the PAG-Csk complexes in F5 T cells in the absence of
Fyn over the time periods in which we observed a decrease in
LckTyr505 phosphorylation. F5 T cells contained constitutively
phosphorylated PAG ex vivo (Fig. 6D) and upon stimulation
transiently lost phosphorylation of Tyr314, the primary residue
for Csk recruitment (10). The peak loss of pTyr314 was in the
order of 0.5–1 h, with rep phosphorylation to ex vivo levels by
4 h. In contrast, Fyn+/− T cells contained very low levels of
total phospho-PAG either before or after activation and pTyr314
was undetectable. Together these data show that although the
hyperresponsive phenotype of Fyn+/− F5 T cells requires shorter
contact time with pMHC, Lck remains active, because the
signals remain PP2 sensitive. During this time period, the
p59 and p60 species of Lck have reduced pTyr505 in the absence
of Fyn, which correlates with the timing of PAG Y314 dephos-
phorylation in WT cells.

Reducing Lck protein in the absence of Fyn exacerbates T cell
dysregulation

Fyn+/− T cells eventually down-regulated IL-2 production
and CD25 expression (Fig. 1), indicating that the response was ul-
timately regulated. Additionally, although phosphorylation of

them in the absence of Ag (36). We adapted this two-step cul-
ture protocol, incubating purified CFSE-labeled F5 T cells from
WT and Fyn null mice with plate-bound pMHC for various times
before extensive washing and transfer to Ag-free wells, giving
a total culture period of 48 h. At transfer, we spiked unlabeled
naive F5 cells into the wells to act as sensors for Ag carryover.
The naive T cells remained unstimulated, showing no IL-2 pro-
duction, and indicating that all Ag was removed during wash-
ing (Fig. 5A). As before, the pMHC contact time required for up-
regulation of CD69 was identical for WT and FynKO cells (Fig.
5B). However, WT T cells required at least 3 h of pMHC ex-
posure to commit to IL-2 production, whereas Fyn null cells
produced IL-2 after only 1 h of pMHC contact (Fig. 5B).

The proportion of Fyn null cells producing IL-2 was consistently
higher at all time points, indicating that they required less
pMHC contact time to initiate IL-2 production. Equivalent res-
ults were obtained if proliferation was measured rather than
IL-2 production (data not shown). These results confirm that
although Fyn null cells phosphorylate early signal transduction
molecules and up-regulate CD69 equivalently to WT cells when
they first encounter Ag, they more readily overcome the thresh-
old for commitment to IL-2 production. Based on this, we hy-
pothesized that Fyn was activating a regulatory pathway that
functioned to temper signal transduction during the normal
course of TCR stimulation with Ag.

Fyn+/− cells continue to signal after removal from pMHC

Proximal signals are rapidly terminated upon cessation of TCR
ligation in WT cells (1), and therefore we explored the possi-
bility that Fyn null cells had reduced requirement for prolonged
pMHC engagement because they failed to terminate signaling
upon removal from Ag. Accordingly, we asked whether con-
tinuous pMHC contact remained coupled to Lck kinase activity
in Fyn null T cells. We repeated the two-step stimulation exper-
iment described above in which WT and Fyn null F5 T cells
were stimulated with pMHC for limited times, but in this case,
upon transfer to the second Ag-free culture, we added the Src
kinase inhibitor PP2. We observed that addition of PP2 to the
Ag-free culture reduced the responses of both Fyn null and WT
cells. Fyn null cells appeared to be more sensitive to PP2 inhib-
ition, with the result that they now required as much pMHC
contact time as WT cells to commit to proliferation (Fig. 6A)
and IL-2 production (data not shown). These data suggest that
the reason prolonged contact with pMHC is required by cells for
commitment to IL-2 production is to provide sustained Lck signaling
and that this occurs more readily in the absence of Fyn.

The ability of PP2 to revert the Fyn null phenotype, along
with the data showing that engagement of the coreceptor was
required to see enhanced responsiveness of Fyn null cells,
strongly suggested that Lck activity contributed to the increased
sensitivity of Fyn null cells. Therefore, we investigated the
phosphorylation status of Lck in the absence of Fyn, focusing
on the status of the regulatory Tyr505, since mutation of this
residue has been shown to lead to increased IL-2 production in
T cells (7). Upon T cell stimulation, Lck migrates at higher
molecular weights due to phosphorylation of serine residues
(38), and these higher bands were clearly visible in both WT
and Fyn null cells after 1 h of stimulation and persisted for at
least 9 h before declining by 24 h (Fig. 6B). The total amount
of Lck and the supershifted proportion was similar in WT and
Fyn null cells. Quantitation of Tyr505 phosphorylation relative to
total Lck was performed using the sensitive Licer Odyssey im-
aging system, which provides linear quantitation throughout the
range and showed that there was a small but highly reproducible
decrease in the percentage of pTyr505 in the absence of Fyn
from within 1 h of stimulation that persisted for up to 24 h (Fig.
6C). This was true of both the 56-kDa species and the larger
59/60-kDa species of Lck and was of the same order of mag-
nitude (~2-fold) to the increased IL-2 production (Fig. 3) and
the decreased peptide contact time (Fig. 5B) seen with Fyn null
cells.
Lck Tyr505 was decreased in the absence of Fyn, there remained a substantial pTyr505 signal, indicating that Csk was still being recruited to Lck, despite the almost complete absence of PAG phosphorylation. Other membrane adaptor molecules have been shown to recruit Csk to the membrane, including Lck-interacting membrane protein (39), SIT (40), Dok (41), and paxillin (42, 43) and some are targets of Lck. Therefore, we asked whether Lck itself influenced membrane recruitment of Csk in the absence of Fyn by reducing the amount of Lck in T cells to see whether this would further impact on Lck Tyr505 phosphorylation.

We used F5 Fyn−/− mice backcrossed with mice expressing an inducible Lck transgene. We have shown previously that CD4 T cells from Lck-inducible mice express 10–20% of WT Lck levels ex vivo even when continuously administered dox (22), and in F5 LckindLck−/− F5 cells (hereafter called LckindLck−/−) mice CD8 T cells expressed ~30% of WT Lck levels. Examination of the status of Lck in these cells revealed several interesting features. First, there was a profound reduction in the phosphorylation of Tyr505 in LckindLck−/− cells compared with Fyn−/− LckWT cells even before stimulation, and, strikingly, it was completely absent on the supershifted 59/60-kDa forms of Lck after Ag stimulation (Fig. 7A). These supershifted forms of Lck were “active” in cells from both mouse strains because they contained substantial phosphorylation of Tyr394, which was broadly similar between the two strains and was slightly decreased in each upon stimulation (Fig. 7A).

We also confirmed the specificity of the Lck pY505 Ab and its lack of reaction with Fyn, since there is no band present in phosphorylation of Tyr505 in LckindLck−/− cells compared with Fyn−/− LckWT cells even before stimulation, and, strikingly, it was completely absent on the supershifted 59/60-kDa forms of Lck after Ag stimulation (Fig. 7A). These supershifted forms of Lck were “active” in cells from both mouse strains because they contained substantial phosphorylation of Tyr505, which was broadly similar between the two strains and was slightly decreased in each upon stimulation (Fig. 7A).

FIGURE 6. Fyn−/− cells signal after pMHC removal. A, F5 cells were activated as in Fig. 3A, but the secondary Ag-free culture was with or without the Src kinase inhibitor PP2, or diluent. As before, proliferation as a function of the time on the pMHC layer is shown; note proliferation was assessed at 48 h. Values are plotted as the mean of triplicate samples ± SD. B, Total cell lysates from cells stimulated with pMHC dimers for the indicated times were probed for Lck pTyr505 and stripped and reprobed for total Lck protein. The pTyr505 Ab is specific for Lck as shown in Fig. 7C. C, Quantitation from three independent experiments showing Lck pTyr505 as a percentage of total Lck. Blots were quantified using the Licor Odyssey system which gives true linear signals throughout the exposure range. Experiment (Expt) 1 is quantification of the blot shown in B and the elevated signal in Fyn−/− cells at 24 h is artificially high from the blemish on the membrane. D, Immunoblots of total cell lysates from cells stimulated with pMHC for the indicated times and probed with Abs for PAG pTyr314, total Fyn, or actin as a loading control. All data are representative of three independent experiments.
cells that lack Lck, i.e., Lck<sup>−/−</sup>Fyn<sup>WT</sup> thymocytes (Fig. 7C, lane 3). Together these results indicate that the presence of both Lck and Fyn influence the phosphorylation status of Lck Tyr<sup>505</sup>, which is reduced after stimulation of T cells that lack Fyn, but if, in addition, expression of Lck is reduced, then phosphorylation of Lck Tyr<sup>505</sup> becomes substantially compromised.

**Discussion**

We have examined the consequences of altering the SFKs Fyn and Lck in primary T cells and have revealed that the balance of signals through these kinases is critical for achieving correctly regulated T cell responses. The absence of Fyn did not affect the early response to Ag but did increase IL-2 production and proliferation at later time points. A key difference was noted after 1 h of stimulation, because Fyn<sup>−/−</sup> cells became Ag independent for progression to cytokine production, whereas WT cells required more sustained contact with Ag to achieve the same level of commitment. We identified that the activity of Lck was important for these effects, because the increased IL-2 production was only observed after stimulation of TCR plus CD8, either by pMHC or cross-linked Abs and not by anti-TCR stimulation alone. The contribution of Lck was also suggested by experiments in which the Lck inhibitor PP2 was shown to inhibit Fyn<sup>−/−</sup> cells from becoming Ag independent earlier than WT cells.

Our data are consistent with a model in which a negative feedback loop regulating the activity of Lck is controlled by the SFKs acting through phosphorylation of the regulatory C-terminal tyrosine residue on Lck. We propose that the feedback operates, in part, through the activity of Fyn phosphorylating the adapter PAG and recruiting Csk, as others have described (19, 44). However, our data suggest that alternative Lck-dependent means of recruiting Csk might operate, since Lck Tyr<sup>505</sup> phosphorylation was present, albeit at reduced levels, in the absence of Fyn. Moreover, reducing expression of Lck (Lck<sup>−/−</sup>Fyn<sup>−/−</sup> cells) further compromised phosphorylation of Lck Tyr<sup>505</sup>. From these data, we postulate that engagement of the TCR plus coreceptor on WT cells activates both Lck and Fyn. Lck primarily stimulates a positive signaling cascade that leads to cytokine transcription and division (4, 5), but also phosphorylates substrates that interact with Csk. At the same time, activation of Fyn promotes Csk recruitment, mainly through phosphorylation of PAG (19). In the absence of Fyn, negative feedback of Lck is reduced and sustained TCR engagement becomes less critical for signal progression. Although we cannot, at this time, exclude that the hyperresponsiveness of T cells in the absence of Fyn was the result of changes in other signaling pathways, none was obviously perturbed in the analysis we undertook.

Fyn has been reported to be associated with the TCR and to be induced by antigen stimuli, and more recently it was shown that the association of Fyn with PAG promotes T cell anergy (17). Lck is primarily associated with the coreceptor although some may be independently associated with the PM and the relative proportion of these two pools seems to vary between individual T cells. In addition, Fyn and Lck have been shown to be segregated in the PM on the basis of differential association with lipid rafts (15), although this segregation does not appear to be absolute. The varied distributions of these two SFKs and how they interact with one another may well influence the initial response to different stimuli (45). For example, the response of cells to antagonist stimuli does not cause relocation of Lck to the supermolecular activation cluster (SMAC) at the interface between TCR and APC (46) and engagement of TCR alone does not activate Lck (47), but probably activates Fyn. In contrast, T cell stimulation by agonist peptide does cause redistribution of Lck to the SMAC (46, 48) and Lck activation (49). However, the formation of SMACs per se is not sufficient to induce a full response, because that requires a sustained period of contact. At early time points, disrupting the TCR-APC interaction causes TCR clustering to break down and signaling to be terminated (1). If T cells initially integrate both positive and negative signals from the SFKs, they become independent of TCR engagement only when the positive signals outweigh the negative, at which point they can commit to division and differentiation.

Interestingly, it has been shown recently that dephosphorylation of PAG and thereby modulating the access of Csk to SFKs influences Ras activation, specifically on endoplasmic reticulum and Golgi membranes (50), which is implicated in sustained Ras activity (51, 52). This raises the intriguing possibility that the point at which TCR signaling becomes independent of pMHC engagement reflects a change in the primary signaling compartment from plasma to intracellular membranes. By implication, T cell commitment to cytokine production and division may be the point at which Ras signaling switches to an intracellular location and becomes sustained. Of note is the report that RasGRPL activates Ras on the Golgi (51) and we have found that activation of RasGRP in

**FIGURE 7.** Lck<sup>−/−</sup>Fyn<sup>−/−</sup> cells have impaired pY505. A, T cells from F5 Fyn<sup>−/−</sup> or dox-fed Lck<sup>−/−</sup>F5 Lck<sup>−/−</sup>Fyn<sup>−/−</sup> (F5 Lck<sup>−/−</sup>Fyn<sup>−/−</sup>) mice were activated with NP68-loaded pMHC dimers for the indicated times and cell lysates were analyzed for pTyr<sup>505</sup> and total Lck or pTyr<sup>394</sup> and total Lck as indicated. The ratio of pLck:tLck is shown for each time point and an actin-loading control is included for the first panel to show that more protein was loaded in the F5 Lck<sup>−/−</sup>Fyn<sup>−/−</sup> lanes to compensate for the lower expression of Lck. B, CFSE-labeled LN cells from dox-fed F5 Lck<sup>−/−</sup>Fyn<sup>−/−</sup> mice were activated with NP68 peptide and division was assessed by FACS at 72 h. The percent divided (%D) and PI values shown are means of triplicate samples. C, Two to 5 × 10<sup>6</sup> thymocytes/lane from the indicated mice were probed with Lck pTyr<sup>605</sup>, stripped, and reprobed with anti-Lck and anti-tubulin for loading. The ratios of pLck:tLck are indicated for each sample; note the pTyr<sup>605</sup> Ab does not react with Lck<sup>−/−</sup>Fyn<sup>WT</sup> cells (lane 3). Data are representative of two experiments.
primary T cells is downstream of Lck and not Fyn (5). Moreover, coincident with this timing is the appearance of 59/60-kDa forms of Lck that have been shown to be products of serine phosphorylation by ERK (38, 53), suggesting colocalization of Lck and ERK. Clearly, phosphatases such as SHP-1 (6) and SHP-2 (51) also influence the phosphorylation status of the adapters and the SFKs themselves, but our data additionally show that the levels of expression of the SFKs can have a significant influence on the balance of signals that determine the outcome of TCR engagement.

Our data implicate Csk as the major player in this feedback loop for the following reasons: first, in F5 Fyn−/− cells, we saw a reduction of Lck pTyr305 compared with WT cells from ~1 h after stimulation; and second, this correlated with the time from which Fyn−/− cells became independent of TCR signals, but still required signals through Lck to commit to proliferation. In contrast, WT cells that have more Lck pTyr305 at this time abort signaling unless they remain in contact with pMHC. Precisely how phosphorylation of pTyr305 regulates Lck activity is not well understood (54); however, mutations at this residue are associated with increased oncogenicity of Lck (55, 56). It has been suggested that the conformational change that occurs when the phosphorylated regulatory tyrosine of the SFKs binds its Src homology 2 domain may influence its interactions through its Src homology 3 domain (57). Certainly, there is not a straightforward correlation with the amount of pTyr305 and Lck kinase activity, since cells lacking CD45 have greatly increased phosphorylation of Lck Tyr305 and yet more Lck kinase activity by in vitro assays along with increased pTyr394 (56). Consistent with this, we did not see a difference in Lck pTyr394 in situations where we had greatly reduced pTyr305 in Lck+/-Fyn−/− cells. Although our data favor a role for Csk, we cannot exclude that changes in activity or availability of CD45, or other phosphatases, also contribute to the Fyn−/− phenotype. It has been shown that CD45 is particularly influential in regulating coreceptor-associated Lck (58–60), but it is unclear how the lack of Fyn might impact on this association.

Csk is the major kinase to regulate Lck and T lineage-specific knockouts show TCR-independent differentiation of CD4 T cells (61, 62), consistent with dysregulated Lck signaling during differentiation. In addition, a dominant-negative form of Csk was shown to cause increased NFAT-AP-1 reporter activity and stronger and more sustained responses to TCR triggering (63). PAG is clearly able to recruit Csk in T cells but there are other adapters, including Lck-interacting membrane protein (39), SIT (40), dok (41), and PAG. These are phosphorylated by Lck and are likely candidates for recruiting Csk to the PM in the absence of PAG. However, it should be noted that unlike monitoring phosphorylation of the inhibitory tyrosine on SFKs, the mere presence of Csk at the PM is not necessarily a good indicator of its activity. For example, a recently described adapter molecule, G3BP, has been shown to recruit Csk to the PM, but its overexpression results in increased T cell responsiveness, suggesting that G3BP inactivates Csk by sequestering it away from lipid rafts (64). PAG−/− mice were shown to have no overt dysregulation of T cell development or function (11, 12) and Csk was still found associated with the PM, albeit at reduced levels in one report (12). We predict that PAG−/− cells should exhibit a similar phenotype to Fyn−/− cells if stimulated in an analogous fashion, because both the nature of the stimulus used to trigger T cells and the readouts used to monitor responses are important to uncover these subtle regulatory pathways. Initial studies that reported hyporesponsiveness of Fyn−/− cells used anti-CD3 to stimulate the cells (31, 34), which does not activate Lck to the extent that occurs when stimulating with anti-CD3 plus CD4/CD8 or Ag (47, 65). Indeed, Fyn−/− cells stimulated with Ag or by anti-CD3/CD4 showed no defects in activation (35), while defects were shown in TCR-transgenic Fyn−/− cells only in response to low-affinity ligands (66). These two studies did not report the increased response to high-affinity ligands that we document here; however, they assessed proliferation with a pulse of [3H]Thymidine incorporation at a time when the difference may not have been apparent. In contrast, our data clearly reveal a role for Fyn as an important modulator of the T cell response to Ag.

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References
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