Normal Development and Activation but Altered Cytokine Production of Fyn-Deficient CD4$^+$ T Cells[1]

Alusha A. Mamchak,* Brandon M. Sullivan,‡§ Baidong Hou,* Linda M. Lee,* Julia K. Gilden,† Matthew F. Krummel,† Richard M. Locksley,*‡§ and Anthony L. DeFranco[2]*

The Src family kinase Fyn is expressed in T cells and has been shown to phosphorylate proteins involved in TCR signaling, cytoskeletal reorganization, and IL-4 production. Fyn-deficient mice have greatly decreased numbers of NKT cells and have thymocytes and T cells with compromised responses following Ab crosslinking of their TCRs. Herein we have addressed the role of Fyn in peptide/MHC class II-induced CD4$^+$ T cell responses. In Fyn-deficient mice, CD4$^+$ T cells expressing the DO11.10 TCR transgene developed normally, and the number and phenotype of naive and regulatory DO11.10$^+$CD4$^+$ T cells in the periphery were comparable with their wild-type counterparts. Conjugation with chicken OVA peptide 323–339-loaded APCs, and the subsequent proliferation in vitro or in vivo of DO11.10$^+$ Fyn-deficient CD4$^+$ T cells, was virtually indistinguishable from the response of DO11.10$^+$ wild-type CD4$^+$ T cells. Proliferation of Fyn-deficient T cells was not more dependent on costimulation through CD28. Additionally, we have found that differentiation, in vitro or in vivo, of transgenic CD4$^+$ Fyn-deficient T cells into IL-4-secreting effector cells was unimpaired, and under certain conditions DO11.10$^+$ Fyn-deficient CD4$^+$ T cells were more potent cytokine-producing cells than DO11.10$^+$ wild-type CD4$^+$ T cells. These data demonstrate that ablation of Fyn expression does not alter most Ag-driven CD4$^+$ T cell responses, with the exception of cytokine production, which under some circumstances is enhanced in Fyn-deficient CD4$^+$ T cells. *The Journal of Immunology,* **2008, 181: 5374–5385.**

Recognition of Ag by the TCR initiates numerous coordinated signaling pathways that ultimately control T cell behavior. TCR signaling is initiated by phosphorylation of the ITAMs present in the ζ-chain and CD3 chains of the TCR by the Src family kinases Lck and Fyn (1, 2). Phosphorylated ITAMs recruit a second type of intracellular tyrosine kinase, ZAP70, leading to its activation. Subsequent signaling reactions are initiated by active ZAP70, Lck, and/or Fyn (1). Lck is associated with the co-receptors CD4 and CD8, which contribute to the activation of conventional α/β T cells by binding to MHC molecules stabilizing the interaction between the TCR and Ag-bound MHC molecules, and by recruiting Lck to the TCR, which promotes TCR-mediated signaling (3, 4). In contrast, Fyn associates with the TCR ζ-chain at low stoichiometry, which may nonetheless allow it to initiate TCR signaling through the phosphorylation of ITAMs within the ζ-chain (5, 6). Other studies have suggested that Fyn also plays a positive role in TCR signaling at subsequent steps through its phosphorylation of SLAP-130 (SH2 domain-containing leukocyte protein of 76-kDa-associated phosphoprotein of 130 kDa)/FYB (Fyn-binding protein) (7), Vav (8), WASp (Wiskott-Aldrich syndrome protein) (9), and Pyk-2 (10), all of which promote cytoskeletal reorganization and the formation of the immunological synapse between T cells and APCs (7, 9). Indeed, two studies utilizing TCR transgenic Fyn-deficient T cells have suggested that in the absence of Fyn, T cell interactions with APCs are weaker (7–9). Additionally, Fyn can phosphorylate PAG (phosphoprotein associated with glycolipid-enriched microdomains), also called Cbp (Csk-binding protein), which acts to down-regulate TCR signaling through its recruitment to the membrane of Csk, a kinase that inhibits both Fyn and Lck through phosphorylation of negative regulatory tyrosines near their C termini (11, 12). Thus, Fyn kinase has been implicated in both the propagation and inhibition of TCR-mediated signaling.

Fyn kinase is also involved in the signaling pathway downstream of the CD2-related cell adhesion molecule SLAM (signaling lymphocyte activation molecule),[3] which promotes T cell differentiation to a Th2 phenotype (13). SLAM associates with the adaptor molecule SAP (SLAM-associated protein), which in turn associates with Fyn and in part signals through it (13–15). The formation of the SLAM/SAP/Fyn complex has been shown to promote T cell secretion of IL-4 (13). Defects in SAP, which is encoded on the X chromosome, lead to the disease X-linked lymphoproliferative (XLP) syndrome in which there are defects in the control of EBV infection and in the generation of germinal centers (16–18). Thus, Fyn kinase is thought to be involved in T cell activation and in differentiation to cytokine-producing effector T cells downstream of both the TCR and SLAM.

An important approach for understanding the unique roles of Fyn in T cell function is the analysis of T cells from Fyn-deficient mice. In the periphery of these mice, conventional α/β T cells are present at normal frequency and number (19, 20), but NKT cells

*Department of Microbiology and Immunology, †Department of Pathology, ‡Department of Medicine, and §The Howard Hughes Medical Institute, University of California, San Francisco, CA 94143

Received for publication November 13, 2007. Accepted for publication August 16, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by The Joseph Marino Research Award from the Crohn's & Colitis Foundation of America (to A.A.M.) and National Institutes of Health Grants P01 AI-35297 (to A.L.D.), AI-26918 (to R.M.L.), and R01 AI-052116 (to M.F.K.).

[2] Address correspondence and reprint requests to Dr. Anthony L. DeFranco, University of California, San Francisco, Box 0414, HSE-1001F, San Francisco, CA 94143. E-mail address: Anthony.Defranco@ucsf.edu

[3] Abbreviations used in this paper: SLAM, signaling lymphocyte activation molecule; CMTMR, 5-(and 6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; DDAO, 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one); [^{3}H]TdR, [*methyl*-^{3}H]thymidine; Ovap$_{323–339}$, chicken OVA peptide 323–339; SAP, SLAM-associated protein; WASp, Wiskott-Aldrich syndrome protein.

are greatly reduced in number (21, 22). This defect may result from failed positive selection of CD1d-dependent NKT cell precursors in the thymus (21, 22) and/or because of the absence of a Fyn-dependent SAP-mediated signal required for the development of these cells (23, 24). In contrast, development of conventional α/β T cells in the thymus appears to be normal in *fyn*$^{-/-}$ mice, with the exception of impaired thymocyte deletion in response to certain superAgs, including Mls-1a (19). Fyn-deficient thymocytes and mature peripheral T cells exhibit striking defects in their in vitro proliferative response to stimulation with anti-CD3 Abs and have substantially decreased anti-CD3-induced signaling reactions, including intracellular calcium mobilization and tyrosine phosphorylation of proteins (19, 20, 25). These observations indicate that Fyn plays an important role in TCR signaling in situations where the co-receptors CD4 and CD8 do not participate and, therefore, Lck is not efficiently recruited to the TCR. However, whether Fyn plays a unique role in TCR signaling in response to peptide-MHC complexes presented by APCs is less clear. Stimulation of AD10 TCR-transgenic Fyn-deficient CD4$^+$ T cells with Ag and APCs in vitro results in relatively normal phosphorylation of signaling components, calcium mobilization, dephosphorylation, and nuclear translocation of NF-AT, as well as IL-2 production (25). In contrast, other studies have reported defects in the interactions of Fyn-deficient T cells with APCs (7–9).

To address the role of Fyn in the physiological response of CD4$^+$ α/β T cells following stimulation of the TCR with peptide in the context of MHC molecules, we bred the *fyn* mutation to the BALB/c genetic background and also introduced a transgene encoding the DO11.10 TCR, which recognizes a peptide from OVA when presented by the BALB/c class II MHC molecule, I-A^d (26). Herein we report that DO11.10 TCR-transgenic Fyn-deficient T cells developed normally, and that their association with APCs, their proliferation in response to Ag stimulation, and their need for costimulation from B7-1 and B7-2 were all similar to Fyn-expressing (wild-type) DO11.10 TCR-transgenic T cells. Moreover, Fyn-deficient CD4$^+$ T cells did not have a defect in their ability to differentiate into IL-4-secreting effector cells following simulation with Ag in vitro, or in response to infection with *Nippostrongylus brasiliensis*. Under certain conditions, however, DO11.10$^+$ Fyn-deficient CD4$^+$ T cells were more potent producers of effector cytokines than were their wild-type counterparts. Combined, these data demonstrate that Ag-driven activation and differentiation to Th2 effector cells are unimpaired in the absence Fyn kinase, and they reveal a previously unappreciated level of redundancy, with respect to Fyn, in Ag-induced CD4$^+$ T cell responses.

Materials and Methods

Mice

BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, MN), Charles River Laboratories, or were bred within our colony. BALB/c Fyn-deficient mice were generated by crossing mice expressing the inactivated Fyn allele, originally described by Soriano et al. (19), on a mixed C57BL/6/129S7/Sv background to BALB/c mice for 6–9 generations (G6–G9) and then to homozygosity for the inactivated Fyn allele. DO11.10 TCR-transgenic BALB/c mice, which express a transgenic TCR that recognizes a peptide from chicken egg OVA peptide 323–339 (Ovap$_{323–339}$) in association with I-A^d (26), were obtained from Dr. A. Abbas (University of California, San Francisco (UCSF)). To generate DO11.10 TCR-transgenic Fyn-deficient mice, G6 Fyn-deficient mice were crossed to the DO11.10 TCR-transgenic mice and were bred to homozygosity for the inactivated *fyn* allele. Once generated, the DO11.10 TCR-transgenic Fyn-deficient mice were maintained within our colony. BALB/c B7-1 and B7-2 double-knockout mice (27) were a gift from Dr. A. Abbas.

The genotype of the DO11.10 TCR-transgenic and Fyn-deficient mice used in these experiments was confirmed by PCR using DNA samples prepared from murine tail sections and primers and PCR conditions as described by Jackson ImmunoResearch Laboratories. The genotype of B7-1- and B7-2-deficient animals was confirmed by the lack of expression of B7 molecules by flow cytometry. All experimental mice were used at 8–13 wk of age and for each experiment mice were matched for sex and age (within 2 wk). Animals were housed in a specific pathogen-free animal facility at UCSF under conditions that meet Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Antibodies and reagents

Anti-CD3ϵ (145–2C11), anti-CD28 (37.51), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD69 (H1.2FE), anti-CD25 (2A3), anti-CD5 (53-7.3), anti-CD62L (MEL-14), anti-CD16/CD32 (2.4G2), anti-B7-1 (16-10A1), anti-B7-2 (24F), anti-SiglecF (E50-2440), anti-CD11b (M1/70), anti-Gr1 (RB6-865), anti-Ter119 (TER-119), anti-NK1.1 (PK136), anti-B220 (RA3-6B2), anti-IL-4 (11B11), anti-IFN-γ (XMG1.2), anti-murine IgE (R35-72), and anti-murine IgE (R35-118) and isotype controls for rat IgG2a,κ (R35-95), mouse IgG1,κ (MOPC-31C), Armenian hamster IgG1,κ (A19-3) unconjugated or conjugated to biotin, FITC, allophycocyanin, PE, PE-Cy7, and PerCp-Cy5.5 as needed were purchased from BD Pharmingen. Anti-CD4-Alexa 700 was purchased from BioLegend. Anti-DO11.10-allophycocyanin or -PE (KJ1-26) and anti-CD4-allophycocyanin-Alexa 750 were purchased from Caltag Laboratories/Invitrogen. Anti-FoxP3-FITC (FJK-16s) was purchased from eBioscience.

Ovap$_{323–339}$ was synthesized and purified by Genmed Synthesis. PMA, ionomycin, and brefeldin A were purchased from Sigma-Aldrich. The intracellular dyes CFSE, Indo-1 AM (indo-1 acetoxymethyl ester), 5-(and 6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR), and 7-hydroxy-9*H*(I,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) were purchased from Molecular Probes/Invitrogen.

Flow cytometry and staining with CFSE

A single-cell suspension was prepared from thymus or spleen for analysis or purification by flow cytometry. In each case, before staining, binding to Fc receptors was inhibited by incubating with an anti-CD16/CD32 Ab. Cells were then stained with different combinations of Abs as indicated. All surface staining was done for 30 min on ice in Ca^{2+}- and Mg^{2+}-free PBS containing 1% FCS with 0.5% sodium azide for analysis, or without sodium azide for cell sorting. For analysis, data were acquired using a FACSCalibur with CellQuest software or LSR II (BD Biosciences). For purification, a MoFlo high-performance cell sorter was used (DakoCytomation).

For intracellular staining for FoxP3, thymocytes or splenocytes were treated with an anti-CD16/CD32 Ab and stained with anti-CD4-PE-Cy7, anti-CD8-PE, and anti-DO11.10-allophycocyanin, or with anti-CD4-PE-Cy7, anti-CD25-PE, and anti-DO11.10-allophycocyanin, respectively. Cells were then fixed, permeabilized, and stained intracellularly with anti-FoxP3-FITC overnight at 4°C using the FITC anti-mouse/rat Foxp3 staining set from eBioscience (no. 71-5775) as per the manufacturer's instructions. To assess cytokine production by intracellular staining for cytokines, lymph nodes cells were surface stained with anti-CD8-FITC and anti-CD4-PE-Cy7 and subsequently fixed, permeabilized, and stained intracellularly with anti-IL-4-allophycocyanin and anti-IFN-γ-PE using a Cytofix/Cytoperm kit from BD Biosciences (no. 554714) as per the manufacturer's instructions.

To assess proliferation by flow cytometry, purified CD4$^+$ or naive DO11.10$^+$ T cells (3×10^7 cells/ml) were labeled with 3 μM CFSE in PBS/0.5% FCS in a 37°C water bath for 8 min. Excess CSFE was removed by the addition of fresh RPMI 1640 containing 10% FCS.

Purification of CD4$^+$ T cells

CD4$^+$ T cells were prepared from the lymph nodes and spleen of mice by negative selection using a CD4$^+$ T cell isolation kit from Miltenyi Biotec, as well as an autoMACS (Miltenyi Biotec), as per the manufacturer's instructions. The purity and activation state of the purified T cells were assessed by flow cytometry. Cells from non-TCR-transgenic mice were prepared for analysis by staining with anti-CD4-allophycocyanin, anti-CD69-FITC, or anti-CD62L-FITC, whereas samples from TCR-transgenic animals were stained with DO11.10-allophycocyanin, anti-CD4-PE-Cy7, and anti-CD69-FITC or anti-CD62L-FITC. FACS analysis revealed purity to be in the range of 90–95% CD4$^+$ T cells, which predominantly expressed high levels of CD62L and were negative for CD69 (data not shown). Eighty to 85% of CD4$^+$ T cells prepared from DO11.10 TCR-transgenic wild-type or Fyn-deficient mice expressed the DO11.10 TCR transgene (data not shown).

Naive DO11.10$^+$ T cells were prepared from the lymph nodes and spleen of DO11.10 TCR-transgenic wild-type mice or DO11.10 TCR-transgenic Fyn-deficient mice. To prepare the spleen samples, a single-cell suspension was made, the RBC were lyzed, and the B220$^+$ cells were

removed through the use of anti-B220 Dynal beads (Invitrogen), as per the manufacturer's instructions. The B220-depleted splenocytes and lymph node cells were then stained with anti-DO11.10-allophycocyanin, anti-CD25-PE, and anti-CD62L-FITC and sorted for live DO11.10$^+$, CD25$^-$, and CD62L^{high} lymphocytes. The typical purity was within the range of 89–95% (data not shown).

In vitro CD4$^+$ T cell stimulation

For stimulation with plate-bound anti-CD3, 96-well flat-bottom tissue culture plates (Corning Costar) were coated with various concentrations of anti-CD3ε in PBS at 4°C overnight. Purified CD4$^+$ T cells from wild-type BALB/c mice or G9 Fyn-deficient mice were plated at a starting cell density of 1×10^5 cells/well in 200 μl of RPMI 1640 (UCSF Cell Culture Facility) supplemented with 10% FCS (Invitrogen), 1 mM HEPES, 1 mM nonessential amino acids, 1 mM pyruvate, 1 mM L-glutamine, and 1 mM penicillin and streptomycin (all of which were obtained from the UCSF Cell Culture Facility) and 5×10^{-5} M 2-ME. Where indicated, 10 ng/ml IL-2 (BD Biosciences) or 2 μg/ml of an anti-CD28 Ab was included in the cultures. CD4$^+$ T cells were cultured at 37°C and 5% CO_2 for 48–72 h. Replicate wells were prepared for proliferation and cytokine production. To assess changes in cell surface marker expression, cells were stimulated with 3 μg/ml plate-bound anti-CD3, harvested at 17–48 h, stained with anti-CD4-Alexa 700, anti-CD25-allophycocyanin, and anti-CD69-PE, and the data were acquired using an LSR II. Proliferation was assessed by addition of 1 μCi [*methyl*-^{3}H]thymidine ([^{3}H]TdR, Amersham Biosciences) incorporation for the last 8 h of culture. The level of [^{3}H]TdR incorporated was measured with liquid scintillation using a TriLux scintillation counter (Wallac).

To assess Ag-induced proliferation, CFSE-labeled Fyn-deficient naive DO11.10$^+$ T cells or CFSE-labeled wild-type naive DO11.10$^+$ T cells were cultured for 40–96 h in 200 μl RPMI 1640 medium, supplemented as described, at 2.5×10^4 cells/well in 96-well U-bottom plates (Corning Costar) with syngeneic sex-matched splenocytes from BALB/c mice, or B7-1 and B7-2 double-knockout BALB/c mice that had been previously treated with mitomycin C (50 μg/ml per 25×10^6 cells/ml for 30 min at 37°C) at a cell density of 2.5×10^5/well and various concentrations of $Ovap_{323–339}$. Proliferation of unlabeled naive DO11.10$^+$ T cells was also assessed by [^{3}H]TdR incorporation during the last 8 h of culture.

For in vitro cytokine production, naive DO11.10$^+$ T cells were cultured in supplemented RPMI 1640 medium at a starting cell density of 2.5×10^5 cells/ml with 1 μg $Ovap_{323–339}$/ml and 2.5×10^6 mitomycin C-treated splenocytes/ml in 24-well flat-bottom plates (Corning Costar). After 96 h, the cells were harvested and the dead cells were removed by centrifugation over Lympholyte-M density gradient (Cedarlane Laboratories) at 2000 rpm for 20 min at room temperature. Live cells were harvested and restimulated at 2.5×10^4/well with 1 μg/ml $Ovap_{323–339}$ and 2.5×10^5 mitomycin C-treated splenocytes in 96-well U-bottom plates for 18 h. The supernatant from each well was harvested and assessed for the presence of IL-4 and IFN-γ by ELISA using BD OptEIA kits for IL-4 and IFN-γ (BD Pharmingen), as per the manufacturer's instructions.

Assessment of calcium mobilization

Splenocytes and lymph node cells from either wild-type or Fyn-deficient mice were pooled and loaded with Indo-1 AM in RPMI 1640 supplemented with 1% BSA (Sigma-Aldrich) and 20 mM HEPES for 1 h at room temperature. Cells were washed and then stained with anti-Gr1-FITC, anti-Ter119-FITC, anti-NK1.1-FITC, anti-CD11b-FITC, anti-B220-FITC, and anti-CD8α-PE-Cy7, with or without the addition of anti-CD3-biotin, for 30 min on ice. Before analysis, 2 μg/ml propidium iodine (Sigma-Aldrich), and in some instances anti-CD4-biotin, was added and the cells were warmed to 37°C for 3 min, after which time calcium mobilization was measured by flow cytometry using an LSR II. Baseline readings were taken for 30 s, after which time 25 μg/ml streptavidin (ImmunoPure streptavidin from Pierce) was added to the cells and measurements were continued. The median intracellular calcium concentration was determined by the ratio of fluorescence 405 nm emission to 530 nm emission over time. Exclusion of PI$^+$FITC$^+$PE-Cy7$^+$ cells from the analysis allowed us to measure the calcium mobilization in CD4$^+$ T cells.

Adoptive transfer and in vivo T cell stimulation

CD4$^+$ T cells from DO11.10 TCR-transgenic mice were purified, the frequency of DO11.10 TCR-expressing cells was determined, and the cells were CFSE-labeled. Two and one-half to 5×10^6 CFSE-labeled CD4$^+$ T cells, containing an identical number of DO11.10$^+$ cells, was transferred into sex-matched recipient BALB/c mice by tail vein injection. Across all experiments the number of transferred DO11.10$^+$ T cells was within the range of $2–4 \times 10^6$ cells/recipient mouse. The following day, recipient mice were immunized s.c. with four 50 μl injections of 1 mg/ml $Ovap_{323–339}$ in CFA. Three days after recipient mice were immunized with Ag, draining (brachial and inguinal) and nondraining (cervical) lymph nodes were harvested from the recipient mice. Lymph node cells were stained with anti-DO11.10-allophycocyanin and the level of CFSE dilution was assessed by flow cytometry. To assess cytokine production, draining and nondraining lymph nodes were harvested 5 days after immunization, the frequency of DO11.10$^+$ cells was ascertained by flow cytometry, and lymph node cells containing $1–1.5 \times 10^4$ DO11.10$^+$ T cells/well were restimulated overnight with 1 μg/ml $Ovap_{323–339}$ in U-bottom plates. The following day the supernatants were harvested and assessed for the presence of cytokines by ELISA.

N. brasiliensis *infection and assessment of inflammation in the lungs*

Third-stage *N. brasiliensis* larvae (L3) were isolated from the cultured feces of infected rats. Wild-type BALB/c mice and G9-*fyn*$^{-/-}$ BALB/c mice were infected with 500 larvae by s.c. injection and given antibiotic water (2 g/L neomycin sulfate and 0.1 g/L polymixin B) for 5 days. Infected mice were sacrificed after 10 days, and the lungs were perfused with 10 ml PBS. Lungs were harvested, crushed, and passed through 70-μm filters to generate single-cell suspensions before staining. Cells were then incubated with 2.4G2, stained with the appropriate Ab cocktail (28), and labeled with the vital dye DAPI (Roche). Counting beads (Caltag Laboratories/Invitrogen) were used to calculate total cell numbers. Data were acquired on a BD LSRII and analyzed using FlowJo software. To assess cytokine production, mediastinal and mesenteric lymph nodes were harvested, a single-cell suspension was prepared, and 5×10^6 cells/ml were cultured for 4 h in supplemented RPMI 1640 containing 100 pg/ml PMA and 1 ng/ml ionomycin, and 10 μg/ml brefeldin A was added for the last 2 h of culture. Cells were assessed for the presence of intracellular IL-4 and IFN-γ by flow cytometry. The concentration of IgE in the serum of infected mice was assessed by ELISA using the mAb R35-72 as a capture Ab and the biotinylated mAb R35-118 for detection. The bound IgE was visualized using streptavidin-alkaline phosphatase.

Assessment of T cell/APC conjugate stability

CFSE-labeled DO11.10$^+$CD4$^+$ T cells (5×10^5) and CMTMR-labeled A20 B cells (5×10^5) were combined and incubated in RPMI 1640 containing 10% FCS 1 mM L-glutamine, 1 mM penicillin and streptomycin, and 5×10^{-5} M 2-ME at 37°C. After 45 min, the samples were agitated and 5×10^5 DDAO-labeled A20 B cells were added. Ninety minutes later, samples were agitated again, fixed with 1% paraformadlehyde, and analyzed by flow cytometry. The percentage of T cell/APC conjugates was determined by gating on CFSE-positive cells, and assessing the frequency of CSFE-positive cells that were also positive for CMTMR or DDAO.

Results

Fyn-deficient CD4$^+$ T cells fail to proliferate, produce effector cytokines, or mobilize calcium in response to stimulation with anti-CD3 Abs

The role of Fyn tyrosine kinase in TCR signaling has been studied by examining the response of Fyn-deficient thymocytes or T cells to anti-TCR Abs, superAg, or Ag presented by APCs (13, 19, 20, 25, 29, 30). These studies were done with T cells isolated from *fyn*$^{-/-}$ mice on a mixed background (129/sv × C57BL/6) or partially backcrossed onto the C57BL/6 background. In our initial experiments, we examined the anti-CD3-induced response of CD4$^+$ T cells from *fyn*$^{-/-}$ BALB/c mice and compared it to that of CD4$^+$ T cells from wild-type BALB/c mice. Stimulation with plate-bound anti-CD3 Ab induced strong proliferation of wild-type, but not of Fyn-deficient, CD4$^+$ T cells (Fig. 1*A*). Similar results were obtained with Fyn-deficient T cells on inbred C57BL/6 background (data not shown). These data are similar to what has been has previously been reported using T cells isolated from less inbred Fyn-deficient mice (13, 25). Stimulation with plate-bound anti-CD3 in combination with IL-2 or anti-CD28 induced a low level of proliferation in Fyn-deficient CD4$^+$ T cells, but their response was always much less than that obtained from wild-type controls (Fig. 1, *B* and *C*). Cytokine production following anti-CD3 stimulation was also assessed, and low levels of IL-4

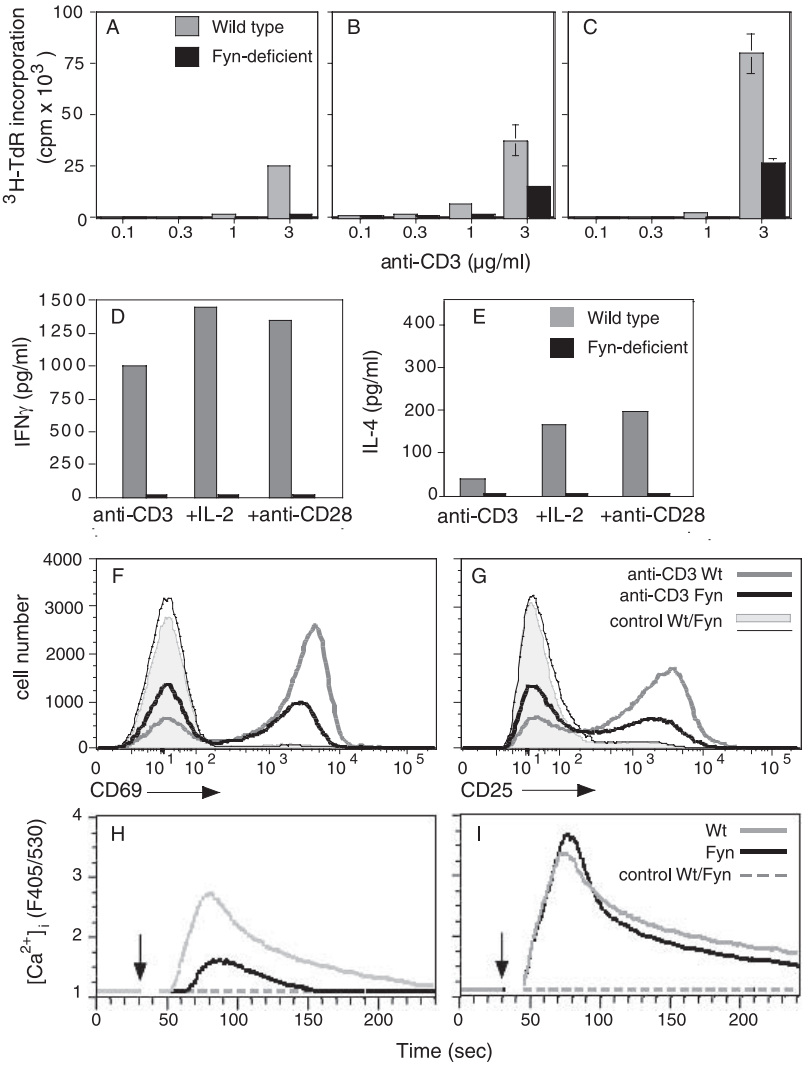

FIGURE 1. Fyn-deficient T cells proliferate poorly, fail to produce cytokines, modestly up-regulate activation markers, and weakly flux calcium in response to stimulation with anti-CD3 Abs. Proliferation of wild-type CD4$^+$ T cells (gray bars) and Fyn-deficient CD4$^+$ T cells (black bars) following stimulation with plate-bound anti-CD3 alone (*A*) or in combination with 10 ng/ml IL-2 (*B*) or 2 μg/ml soluble anti-CD28 (*C*) for 48 h is shown. [^{3}H]TdR incorporation is illustrated as the means and SEM of replicate cultures. Cytokine production was also assessed under the same conditions, and the concentration of IL-4 (*D*) and IFN-γ (*E*) present in the supernatant of wild-type CD4$^+$ T cell cultures (gray bars) or Fyn-deficient CD4$^+$ T cell (black bars) cultures was determined by ELISA. Unstimulated wild-type and Fyn-deficient T cells produced $<$80 pg/ml IL-4 and $<$50 pg/ml IFN-γ (data not shown). Also shown are changes in cell surface expression of CD69 (*F*) and CD25 (*G*) on CD4$^+$ T cells as assessed by flow cytometry following 18 h of stimulation with 3 μg/ml plate-bound anti-CD3 (% CD69$^+$: unstimulated wild-type/*fyn*$^{-/-}$ T cells, 1.5–2%; stimulated wild-type T cells, 78%; and stimulated fyn$^{-/-}$ T cells, 47%. % CD25$^+$: unstimulated wild-type/*fyn*$^{-/-}$ T cells, 14–17%; stimulated wild-type T cells, 81%; and stimulated *fyn*$^{-/-}$ T cells, 55%). *H* and *I*, Calcium mobilization by DO11.10$^+$ transgenic TCR wild-type (gray line) and DO11.10$^+$ Fyn-deficient (black line) CD4$^+$ T cells following stimulation with anti-CD3-biotin (*H*) or anti-CD3-biotin and anti-CD4-biotin (*I*) in the presence of streptavidin. Calcium levels of unstimulated cells or cells stimulated with anti-CD3-biotin alone (data not shown) did not change over time and were similar to the streptavidin alone sample shown (dashed gray line). The data are representative of the results obtained in two separate experiments.

and IFN-γ were detected in the supernatants of wild-type CD4$^+$ T cells following stimulation with plate-bound anti-CD3 alone or in combination with IL-2 or anti-CD28 (Fig. 1, *D* and *E*). In contrast, neither IL-4 nor IFN-γ was present in the supernatants of Fyn-deficient CD4$^+$ T cells that had been stimulated under identical conditions (Fig. 1, *D* and *E*). The absence of cytokine production from Fyn-deficient CD4$^+$ T cells was not surprising given that these cells did not proliferate and therefore presumably could not differentiate into cytokine-producing effector T cells (31). Moreover, the difference between cytokine production of CD4$^+$ T cells from wild-type or Fyn-deficient mice was not due to contaminating NKT cells, as the method used to purify CD4$^+$ T cells specifically removed NKT cells with an anti-CD49b Ab. Stimulation with plate-bound anti-CD3 also induced activation-associated changes in the expression of several cell surface molecules, including CD69 and CD25. We compared the expression of these molecules on Fyn-deficient CD4$^+$ T cells with that of wild-type controls following anti-CD3 stimulation for 17–48 h. Although *fyn*$^{-/-}$ CD4$^+$ T cells did up-regulate CD69 and CD25, their response was always less than that observed for wild-type CD4$^+$ T cells irrespective of the length of stimulation (Fig. 1, *F* and *G*, and data not shown). These data suggest that Fyn-deficient CD4$^+$ T cells receive a weak TCR signal from plate-bound anti-CD3 that is insufficient to fully activate these cells. To compare TCR signal strength in wild-type and *fyn*$^{-/-}$ CD4$^+$ T cells, we measured calcium mobilization following stimulation with anti-CD3-biotin and strepavidin, or with the combination of anti-CD3-biotin, anti-CD4-biotin, and streptavidin. Following stimulation with anti-CD3-biotin and strepavidin, Fyn-deficient CD4$^+$ T cells had a weak calcium signaling response when compared with wild-type CD4$^+$ T cells from BALB/c mice (Fig. 1*H*). However, when *fyn*$^{-/-}$ CD4$^+$ T cells were stimulated with co-crosslinked anti-CD3 and anti-CD4, the calcium response was similar to that obtained for wild-type CD4$^+$ T cells (Fig. 1*I*). Similar results have previously been described using T cells from Fyn-deficient mice on a mixed or partially inbred C57BL/6 background (19, 20, 25, 32). These data show that crosslinking of CD3 alone induced a weak TCR signal in Fyn-deficient CD4$^+$ T cells, which was insufficient to induce proliferation or cytokine production, but was adequate to produce a modest change in the expression of cell surface molecules.

Characterization of T cell development in DO11.10$^+$ Fyn-deficient mice

We wanted to assess how *fyn*$^{-/-}$ CD4$^+$ T cells respond to Ag in a more physiological context in which co-receptors and adhesion molecules participate in the T cell responses. Therefore, mice deficient in Fyn kinase were bred to DO11.10 TCR-transgenic mice, which express a TCR that recognizes Ovap$_{323-339}$ in association with the I-A^d MHC class II molecule (26). Initially, the development of CD4$^+$ T cells in the thymus of

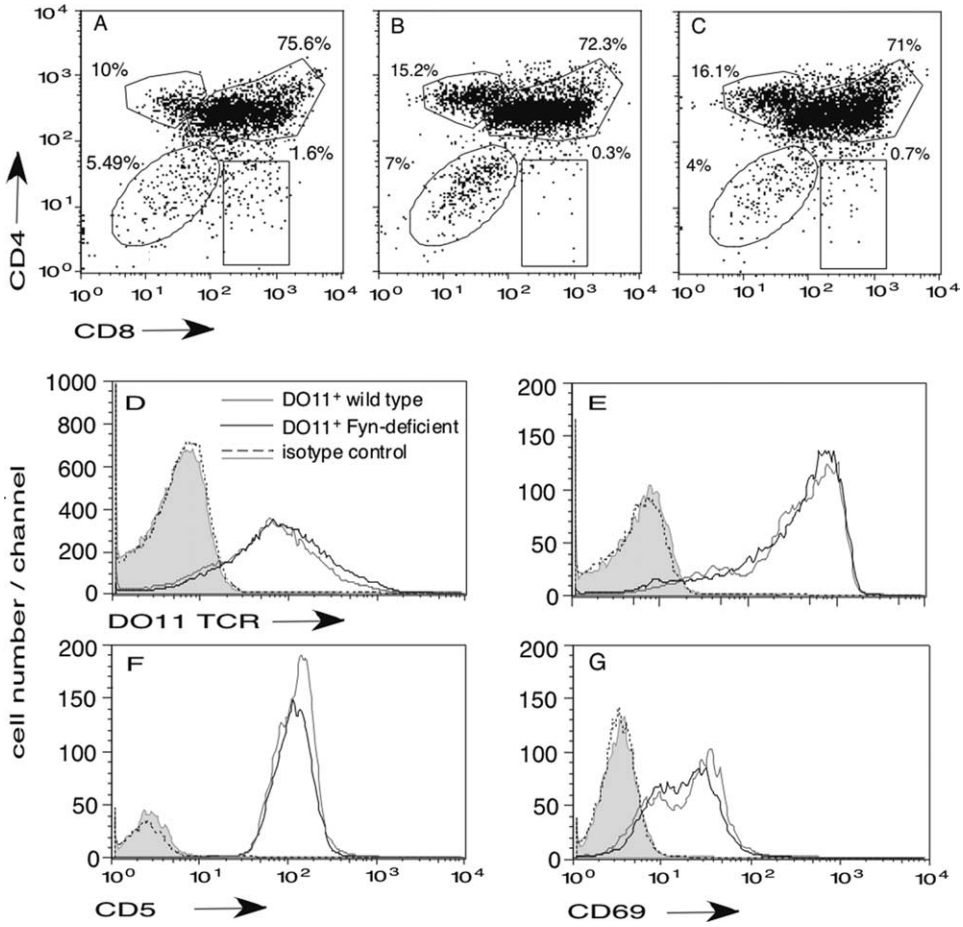

FIGURE 2. Normal development of DO11.10 TCR-transgenic CD4$^+$ T cells in the absence of Fyn kinase. Representative staining for the expression of CD4 and CD8 on thymocytes and the frequency of thymocyte subpopulations from 8-wk-old BALB/c mice (*A*), DO11.10$^+$ wild-type mice (*B*), and DO11.10$^+$ Fyn-deficient mice (*C*) is shown. The mean percentage of CD4 single-positive thymocytes was 9.6% (±0.44% (SD), $n = 3$) for BALB/c mice, 17.1% (±4.3% (SD), $n = 9$) for DO11.10$^+$ wild-type mice, and 16.7% (±2.5% (SD), $n = 8$) for DO11.10$^+$ Fyn-deficient mice. Expression of the DO11.10 TCR is illustrated for CD4$^+$CD8$^+$ thymocytes (*D*) or CD4$^+$CD8$^-$ thymocytes (*E*) from DO11.10$^+$ wild-type mice (open gray histogram) and DO11.10$^+$ Fyn-deficient mice (open black histogram; unstained thymocytes from DO11.10$^+$ wild-type mice, filled gray histogram; and unstained thymocytes from DO11.10$^+$ Fyn-deficient mice, open dashed black histogram). The mean percentage of DO11.10$^+$CD4$^+$CD8$^-$ thymocytes was 82.3% (±8.3% (SD), $n = 9$) for DO11.10$^+$ wild-type mice and 83.0% (±7.4% (SD), $n = 7$) for DO11.10$^+$ Fyn-deficient mice. Also shown are the expression profiles of CD5 and CD69 on DO11.10$^+$CD4$^+$CD8$^-$ thymocytes (*F* and *G*, respectively) from DO11.10$^+$ wild-type mice (open gray histogram) and from DO11.10$^+$ *fyn*$^{-/-}$ mice (open black histogram). *F* and *G* also show isotype control staining (wild-type, filled gray histogram; Fyn deficient, open dashed black histogram). Similar results were obtained in three separate experiments.

DO11.10$^+$ Fyn-deficient mice was examined to determine whether development of CD4$^+$ T cells with the DO11.10 TCR was altered in the absence of Fyn kinase. Thymic cell number (data not shown) and the frequency of double-negative, double-positive, and CD4 single-positive thymocyte subpopulations were comparable in DO11.10$^+$ wild-type mice and DO11.10$^+$ Fyn-deficient mice (Fig. 2, *B* and *C*). Similarly, the expression of CD4, CD8, the DO11.10 TCR, CD3, and the maturation markers CD5 and CD69 were equivalent between the two strains (Fig. 2*B–G* and data not shown). Additionally, intracellular staining for the expression of FoxP3 was used to assess the presence of regulatory CD4$^+$ T cells in the thymus. The frequency of FoxP3$^+$ cells and level of FoxP3 expression were equivalent in DO11.10$^+$CD4$^+$CD8$^-$ wild-type thymocytes and DO11.10$^+$CD4$^+$CD8$^-$ *fyn*$^{-/-}$ thymocytes (mean 0.5 ± 0.16% (SD), $n = 3$ and mean 0.4 ± 0.18% (SD), $n = 4$, respectively, and data not shown). These results indicate that CD4$^+$ T cell development, positive selection, and the formation of Ag-specific regulatory T cells were unaffected by the absence of Fyn kinase.

The effect of Fyn deficiency on the peripheral T cell population in DO11.10 TCR-transgenic mice

In the spleen, the number and frequency of total and transgenic TCR-expressing CD4$^+$ T cells were similar in DO11.10$^+$ wild-type mice and DO11.10$^+$ Fyn-deficient mice (Fig. 3, *A* and *B*, and data not shown). The expression on these cells of various cell surface molecules, including DO11.10 TCR, CD4, CD3, CD28, CD69, CD25, CD62L, and CD44, was assessed, and in all cases the level of expression was almost identical between the two strains of mice (Fig. 3*C–F* and data not shown). Particularly noteworthy, the expression levels of a range of activation markers seen on DO11.10 TCR-transgenic and nontransgenic Fyn-deficient CD4$^+$ T cells were consistent with a naive T cell phenotype (Fig. 3 and data not shown). Similarly, we found that splenic CD4$^+$ T cells from nontransgenic Fyn-deficient BALB/c mice or Fyn-deficient C57BL/6 mice predominantly had a resting T cell phenotype that was similar to that of wild-type control T cells (data not shown). Thus, our results do not support the hypothesis that the loss of signaling from Fyn kinase results in an activated T cell

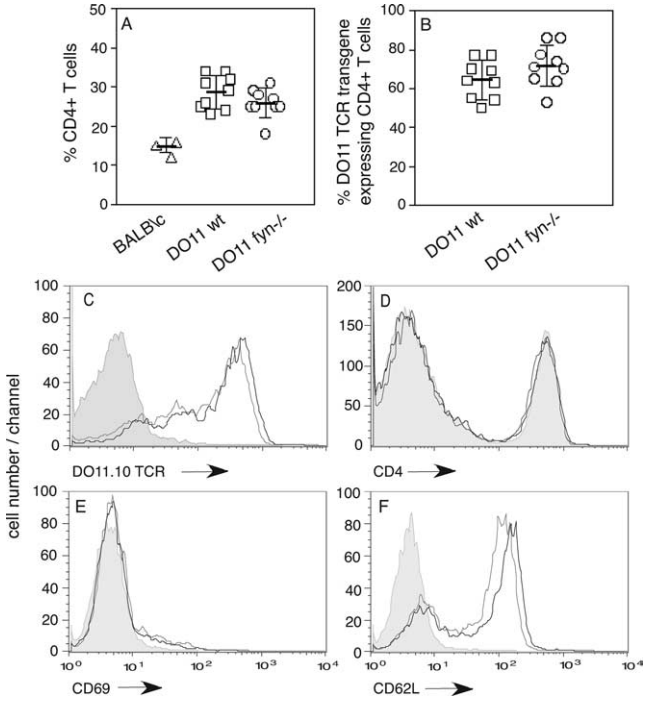

FIGURE 3. Peripheral DO11.10 TCR-transgenic Fyn-deficient $CD4^+$ T cells have a naive phenotype. Spleen cells from 8–10-wk-old BALB/c, $DO11.10^+$ wild-type, and $DO11.10^+$ Fyn-deficient mice were assessed for their expression of various cell surface molecules by flow cytometry. The frequency of splenic $CD4^+$ T cells from BALB/c mice (△), $DO11.10^+$ wild-type mice (□), and $DO11.10^+$ Fyn-deficient (○) is shown (*A*). *B*, Percentage of splenic $CD4^+$ T cells that express the DO11.10 TCR transgene in $DO11.10^+$ wild-type mice (□) and $DO11.10^+$ Fyn-deficient mice (○). In *A* and *B* the means and SD are also shown, and each point represents an individual mouse. The expression of the DO11.10 TCR on $CD4^+$ splenic T cells from TCR-transgenic wild-type mice (open gray histograms) and TCR-transgenic Fyn-deficient mice (open black histogram) is shown (*C*). *D*, Representative staining for the expression of CD4 on splenocytes from BALB/c mice (filled gray histogram), $DO11.10^+$ wild-type mice (open gray histograms), and $DO11.10^+$ Fyn-deficient mice (open black histogram). *E* and *F*, Expression of CD69 (*E*) and CD62L (*F*) on $DO11.10^+CD4^+$ T cells from $DO11.10^+$ wild-type mice (open gray histograms) and $DO11.10^+$ Fyn-deficient mice (open black histogram). While CD62L expression appears to be increased on $DO11.10^+$ $fyn^{-/-}$ T cells (*F*), this observation was not consistent across all $DO11.10^+$ $fyn^{-/-}$ mice assessed. Isotype staining controls (filled gray histograms) are also shown (*C*, *E*, and *F*). The cell surface marker expression profiles are representative of data obtained in three separate experiments.

phenotype, in contrast to the results of an earlier study with Fyn-deficient mice that had been partially backcrossed onto a C57BL/6 background (11). Finally, the frequency of splenic $DO11.10^+CD4^+$ regulatory T cells determined by assessing the expression of FoxP3 was found to be very similar between $DO11.10^+$ wild-type mice (12.5 ± 1.7% (SD), $n = 3$) and $DO11.10^+$ Fyn-deficient mice (9.0 ± 1.2% (SD), $n = 3$). These results were not dissimilar to the frequency of $FoxP3^+CD4^+$ splenic T cells observed in BALB/c mice (14.2 ± 1.9% (SD), $n = 3$). Combined, these data indicate that the DO11.10 TCR-transgenic $CD4^+$ T cells in Fyn-deficient mice resemble their wild-type counterparts in cell number, in the level of spontaneous activation, and in the frequency of $FoxP3^+$ regulatory T cells.

Ag-induced proliferation and cytokine production of $DO11.10^+$ Fyn-deficient $CD4^+$ T cells in vitro

The normal development and phenotype of DO11.10 TCR-transgenic $CD4^+$ T cells from Fyn-deficient mice made it possible to compare their in vitro and in vivo activation to that of their wild-type counterparts. To assess Ag-induced proliferation in vitro, naive $DO11.10^+$ wild-type and $DO11.10^+$ Fyn-deficient T cells were purified by fluorescence-activated cell sorting for $DO11.10^+CD62L^{high}CD25^-$ T cells, CFSE labeled, and stimulated with various doses of $Ovap_{323–339}$ in the presence of mitomycin C-treated splenocytes as a source of APCs. After 40 or 60 h in culture, the TCR-transgenic T cells were harvested and the dilution of CFSE was assessed by flow cytometry. Following 40 h in culture, cell division was not observed from either $DO11.10^+$ wild-type T cells or $DO11.10^+$ Fyn-deficient T cells that had been stimulated with a low dose (30 ng/ml) of $Ovap_{323–339}$ (Fig. 4*A*). However, higher doses of the OVA peptide (100 and 1000 ng/ml) induced one or two cell divisions from a similar proportion of the $DO11.10^+$ wild-type T cells and the $DO11.10^+$ $fyn^{-/-}$ T cells (Fig. 4, *B* and *C*). By 60 h, all of the different $Ovap_{323–339}$ concentrations had stimulated three or four rounds of cell division in both $DO11.10^+$ wild-type T cells and $DO11.10^+$ Fyn-deficient T cells (Fig. 4*D–F*). Similar numbers of $DO11.10^+$ wild-type T cells and $DO11.10^+$ Fyn-deficient T cells had completed each division for each concentration of $Ovap_{323–339}$ and length of time in culture. Incorporation of [^{3}H]TdR was also equivalent (Fig. 4*M*). Thus, under these in vitro conditions, proliferation of $DO11.10^+$ wild-type T cells and $DO11.10^+$ Fyn-deficient T cells was virtually identical, irrespective of Ag dose or length of stimulation.

Although we did not observe any decrease in the in vitro response to Ag of DO11.10 TCR-transgenic Fyn-deficient T cells, it was possible that the response of $DO11.10^+$ Fyn-deficient T cells to Ag was more dependent on costimulation through CD28 than is the response of wild-type $DO11.10^+$ $CD4^+$ T cells. To test this possibility, the proliferation experiments were repeated using splenocytes from B7-1- and B7-2-deficient mice as APCs. In the absence of costimulation provided by CD28, the proliferation of $DO11.10^+$ wild-type and $DO11.10^+$ Fyn-deficient T cells was diminished when compared with stimulation with Ag and B7-expressing splenocytes (Fig. 4*G–M*); however, the Fyn deficiency did not affect the proliferation of $DO11.10^+$ T cells (Fig. 4*G–L*). The similarities of the proliferative responses of wild-type and Fyn-deficient T cells stimulated with $Ovap_{323–339}$ in the presence and absence of costimulation from B7-1 and B7-2 was confirmed by [^{3}H]TdR incorporation (Fig. 4*M*). Additionally, changes to the level of expression of CD69 and CD25 in response to stimulation with $Ovap_{323–339}$ and APCs were assessed and found to be similar for both wild-type and Fyn-deficient $DO11.10^+CD4^+$ T cells, although the response was diminished when B7-deficient splenocytes were used as APCs (data not shown). These results indicate that a deficiency in Fyn kinase did not alter the Ag-specific activation or proliferation of $CD4^+$ T cells in vitro. Additionally, these experiments revealed that the proliferative response of Fyn-deficient T cells was not more dependent on costimulation through B7-1 and/or B7-2 when compared with the response of their wild-type counterparts.

Cytokine production following in vitro priming and restimulation of $DO11.10^+$ Fyn-deficient T cells and $DO11.10^+$ wild-type T cells was also assessed. In contrast to the proliferative response, marked differences were observed for cytokine production from $DO11.10^+$ wild-type T cells and $DO11.10^+$ Fyn-deficient T cells. Supernatants from $DO11.10^+$ Fyn-deficient T cell cultures consistently contained ~2-fold more IL-4 than did those from identical cultures containing $DO11.10^+$ wild-type T cells (Fig. 5*A*). In contrast, the supernatants from cultures containing wild-type T cells typically had more IFN-γ than did those from cultures containing $DO11.10^+$ Fyn-deficient T cells (Fig. 5*B*). For both IL-4 and

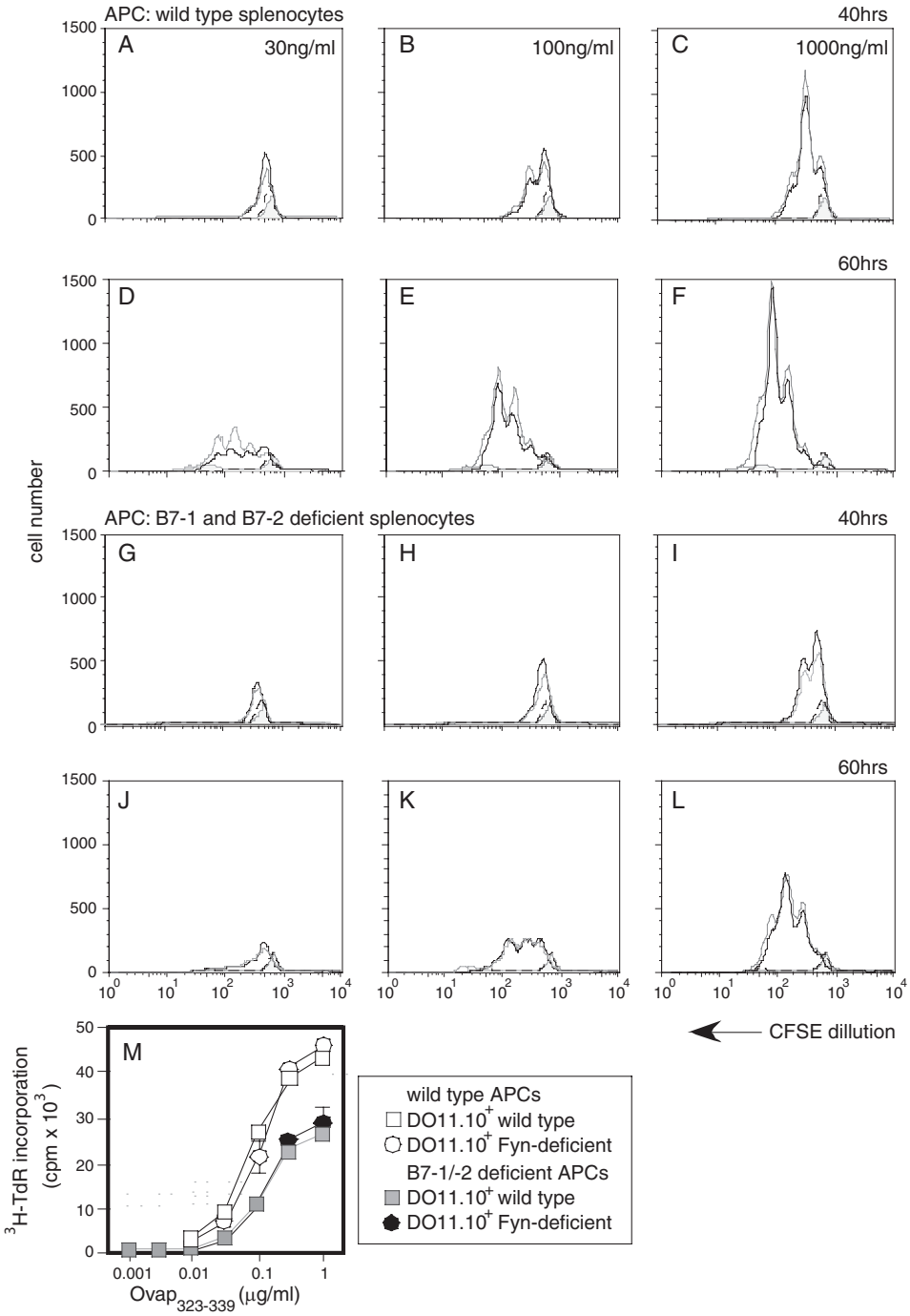

FIGURE 4. Fyn-deficient DO11.10 TCR-transgenic CD4$^+$ T cells proliferate normally in response to Ag. Naive DO11.10$^+$ wild-type and DO11.10$^+$ *fyn*$^{-/-}$ T cells were purified by cell sorting, stained with CFSE, and cultured at a starting cell density of 2.5×10^4 cells/well with 2.5×10^5 mitomycin C-treated syngeneic wild-type (*A–F*) or B7-1- and B7-2-deficient BALB/c splenocytes (*G–L*) and Ovap$_{323–339}$ as indicated. After 40 h (*A–C* and *G–I*) or 60 h (*D–F* and *I–L*) in culture, cells were harvested and stained for DO11.10, and the dilution of CFSE in DO11.10$^+$ cells was assessed by flow cytometry to determine the extent of proliferation. CFSE profiles of Ag-stimulated DO11.10$^+$ wild-type CD4$^+$ T cells (open gray histograms) and of the DO11.10$^+$ Fyn-deficient CD4$^+$ T cells (open black histogram) are shown. Also shown are CFSE profiles of unstimulated DO11.10$^+$ wild-type T cells (filled gray histograms). In replicate cultures, proliferation of DO11.10$^+$ wild-type T cells (squares) and DO11.10$^+$ *fyn*$^{-/-}$ T cells (circles) in response to Ovap$_{323–339}$ and wild-type splenocytes (open symbols) or B7-1/2-deficient splenocytes (filled symbols) was also assessed by [^{3}H]TdR incorporation for the last 8 h of a 48-h culture (*M*). These data were consistent across two separate experiments.

IFN-γ the difference in cytokine production was significant, although to a lesser degree for IFN-γ. Thus, Ag-specific in vitro activation of DO11.10$^+$ Fyn-deficient T cells resulted in the secretion of more IL-4 and less IFN-γ compared with the corresponding activation of DO11.10$^+$ wild-type T cells.

Activation of DO11.10$^+$ Fyn-deficient CD4$^+$ T cells with Ag in vivo

Next, the DO11.10$^+$ wild-type T cells and DO11.10$^+$ Fyn-deficient T cells were stimulated in vivo with Ag in adjuvant. CD4$^+$ T cells were purified from DO11.10$^+$ wild-type mice and DO11.10$^+$ Fyn-deficient mice, and the frequency of DO11.10-positive cells and the expression of activation markers were determined by flow cytometry. Almost all of the purified CD4$^+$ T cells had a naive phenotype similar to that illustrated in Fig. 3. Purified T cells were labeled with CFSE and transferred into naive, sex-matched recipients. Recipient mice were subsequently immunized s.c. with 200 μg of Ovap$_{323–339}$ emulsified in CFA. Three days after immunization, proliferation of the transferred cells was assessed. As observed for Ag stimulation in vitro, the in vivo proliferative responses of DO11.10$^+$ wild-type T cells and DO11.10$^+$ Fyn-deficient T cells induced by immunization with Ovap$_{323–339}$ in CFA were similar (Fig. 6*A*). In response to immunization with Ovap$_{323–339}$ in CFA, many of the DO11.10$^+$ wild-type T cells and DO11.10$^+$ Fyn-deficient T cells present in the draining lymph nodes underwent division, with most cells present in divisions six and seven after 3 days (Fig. 6*A*). In the nondraining lymph nodes, proliferation could also be observed; however, the response was greatly reduced (data not shown). Immunization with Ovap$_{323–339}$ in CFA did not induce proliferation of CFSE-labeled

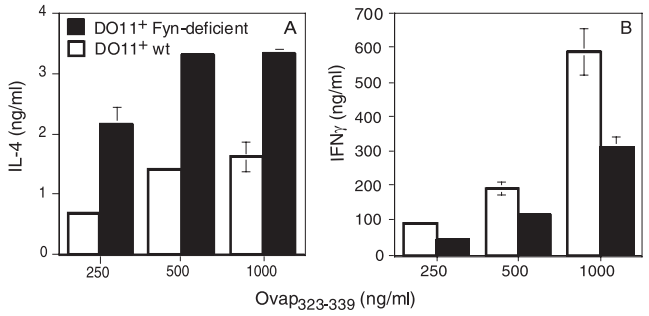

FIGURE 5. DO11.10 TCR-transgenic Fyn-deficient $CD4^+$ T cells more readily differentiate into IL-4-producing cells following in vitro activation with Ag. The concentration of IL-4 (*A*) and IFN-γ (*B*) from cultures of naive DO11.10$^+$ wild-type T cells (open bars) and DO11.10$^+$ *fyn*$^{-/-}$ T cells (filled bars) that had been primed and restimulated in vitro with 1 μg/ml $Ovap_{323-339}$ in the presence mitomycin C-treated syngeneic BALB/c splenocytes is shown. The increased IL-4 production from DO11.10$^+$ Fyn-deficient T cell was shown to be highly significant with Student's *t* test *p*-values of 1×10^{-2}, 3×10^{-6}, and 4×10^{-3} for 1000, 500, and 250 μg of $Ovap_{323-339}$, respectively. The difference in the levels of IFN-γ was also significant, but to a lesser degree than for IL-4, with Student's *t* test *p*-values of 4×10^{-2}, 4×10^{-6}, and 5×10^{-3} for 1000, 500, and 250 μg of $Ovap_{323-339}$, respectively. The concentration of cytokine was assessed by ELISA and is illustrated as the means and SEM. Similar results were obtained in four separate experiments.

DO11.10$^-$$CD4^+$ T cells from wild-type or Fyn-deficient mice (data not shown).

Cytokine production following immunization was also examined. Five days after the recipient mice had been immunized, they were sacrificed and the lymph nodes were harvested. Draining lymph node cells were restimulated in vitro with 1 μg/ml $Ovap_{323-339}$ for 18 h and the supernatants from these cultures were subsequently assessed for the presence of IL-4 and IFN-γ. IFN-γ was detectable in the supernatant from DO11.10$^+$ wild-type T cells or DO11.10$^+$ Fyn-deficient T cells that had been primed in vivo with peptide in CFA (Fig. 6*B*). Interestingly, the supernatants from cultures containing DO11.10$^+$ Fyn-deficient T cells contained more IFN-γ than did the supernatants from their wild-type counterparts (Fig. 6*B*). Neither DO11.10$^+$ wild-type nor DO11.10$^+$ Fyn-deficient T cells from mice that had been primed with $Ovap_{323-339}$ in CFA produced detectable levels of IL-4 (data not shown), consistent with previous work demonstrating that CFA immunization promotes Th1 cytokine production (33, 34). These results, and those illustrated in Fig. 5, indicate that under certain conditions DO11.10$^+$ Fyn-deficient T cells are better cytokine producers than are their wild-type counterparts (Figs. 5 and 6).

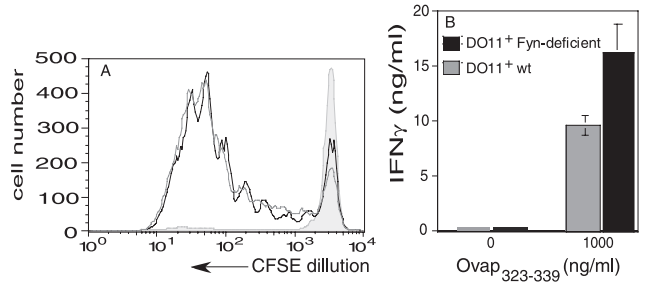

FIGURE 6. In vivo activation with $Ovap_{323-339}$ in CFA of DO11.10$^+$$CD4^+$ wild-type T cells and DO11.10$^+$$CD4^+$ Fyn-deficient T cells reveals similar proliferation but enhanced cytokine production in the absence of Fyn. $CD4^+$ T cells were prepared from DO11.10$^+$ wild-type and DO11.10$^+$ Fyn-deficient mice, CFSE labeled, and $2–4 \times 10^6$ DO11.10$^+$ T cells were transferred into naive sex-matched BALB/c recipient mice by tail vein injection. The following day recipient mice were immunized s.c. with 4×50 μl injections of 1 mg/ml $Ovap_{323-339}$ in CFA. On the third day after immunization, the draining (inguinal and brachial (*A*)) and nondraining (cervical) lymph nodes were harvested, lymph node cells were stained for expression of the DO11.10 TCR, and dilution of CFSE was assessed by flow cytometry. CFSE profiles for DO11.10$^+$ wild-type T cells (open gray histograms) and DO11.10$^+$ Fyn-deficient T cells (open black histograms) are shown. The response of DO11.10$^+$ T cells in nonimmunized recipients is also illustrated (filled gray histogram). Five days after immunization, lymph nodes from recipient mice were harvested and cytokine production was assessed following in vitro restimulation of lymph node cells with 1 μg/ml $Ovap_{323-339}$ and APCs for 18 h. The presence of IL-4 (data not shown) or IFN-γ (*B*) in the supernatants of DO11.10$^+$ wild-type T cell cultures (gray bars) or DO11.10$^+$ Fyn-deficient T cell cultures (black bars) was determined by ELISA. The concentration of cytokine is expressed as the means with the SEM. Similar results were obtained in two separate experiments.

Infection with N. brasiliensis *elicits a strong Th2 response in both Fyn-deficient mice and wild-type mice*

To assess the ability of Fyn-deficient T cells to differentiate into IL-4-secreting cells in vivo, we utilized the *N. brasiliensis* infection model, which induces $CD4^+$ T cell differentiation to Th2 effector cells and inflammation in the lungs characteristic of a Th2 response (35). Cytokine production of $CD4^+$ T cells was assessed 10 days after infection. T cells from the draining lymph nodes of the lung (mediastinal) and gastrointestinal tract (mesenteric) were restimulated in vitro with PMA and ionomycin and cytokine synthesis was assessed by intracellular cytokine staining and flow cytometry. The frequency of IL-4-producing T cells seen in both infected wild-type mice and infected *fyn*$^{-/-}$ mice was higher than the percentage of IFN-γ producing cells, consistent with a primary Th2 response (Fig. 7*A–D*). There was no difference in the magnitude of the response between infected wild-type mice and infected G9 *fyn*$^{-/-}$ mice (Fig. 7*A–D*). We also assessed the concentration of serum IgE, an Ab whose production is dependent on IL-4 (36), in mice that had been infected by *N. brasiliensis*. The sera of both infected wild-type mice and infected *fyn*$^{-/-}$ mice contained increased concentrations of IgE (Fig. 7*E*), whereas IgE could not be detected in the sera of noninfected controls or of infected *rag*$^{-/-}$ mice (data not shown). While the mean serum IgE concentration from infected Fyn-deficient mice was less than that from infected wild-type mice, this difference was not significant (Student's *t* test, $p = 0.3$). Additionally, both wild-type mice and G9 *fyn*$^{-/-}$ mice were able to completely clear the *N. brasiliensis* infection, as worms were undetectable in the small intestine of both strains of mice 10 days after infection (data not shown). Finally, we observed a similar increase in the total number of cells infiltrating the lungs, and specifically in the numbers of eosinophils and $CD4^+$ T cells infiltrating the lungs in infected wild-type mice and infected *fyn*$^{-/-}$ mice (Fig. 7*F–H*). These results clearly indicate that Fyn deficiency does not alter $CD4^+$ T cell recruitment, differentiation into Th2 effector cells, or helper cell function necessary for IgE production during the course of an in vivo response.

Fyn deficiency does not alter the formation or stability of DO11.10$^+$ T cell/APC conjugates

The limited effects on the in vivo responses of Fyn-deficient T cells was quite surprising given the literature indicating that Fyn participates in the cytoskeleton rearrangements involved in the immunological synapse through its phosphorylation of Vav, WASp, and Pyk-2 (7, 9). Therefore, we wanted to assess whether the deficiency in Fyn affected the formation or stability of DO11.10$^+$ T cell/APC conjugates. To do this, we compared the frequency of conjugates formed between CFSE-labeled DO11.10$^+$ wild-type T

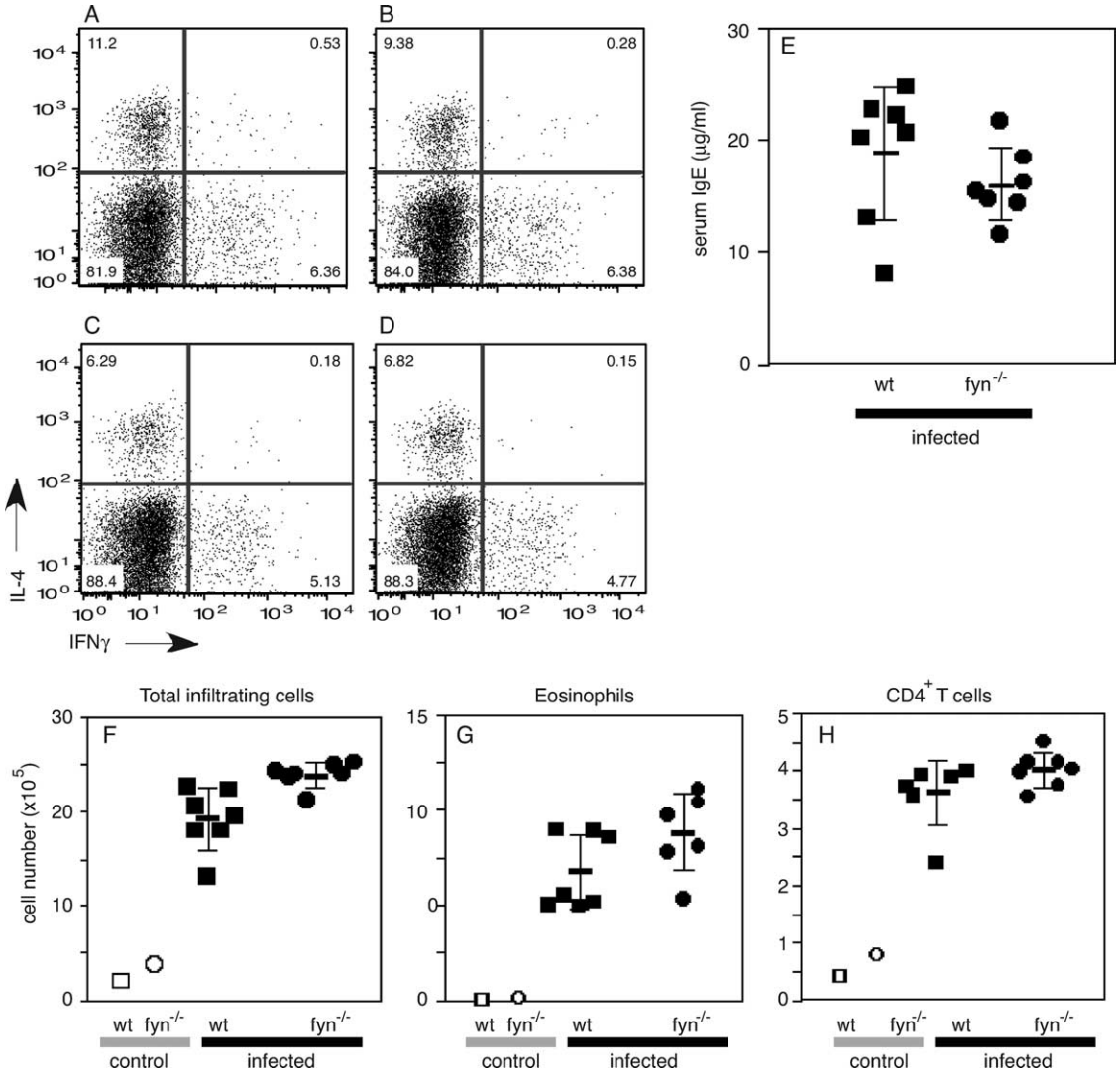

FIGURE 7. *N. brasiliensis* infection elicits a Th2 response in both Fyn-deficient mice and wild-type mice. Ten days postinfection with *N. brasiliensis*, cytokine production by CD4$^+$ T cells in the mediastinal (*A* and *B*) and mesenteric (*C* and *D*) lymph nodes was assessed following in vitro stimulation with PMA and ionomycin. *A-D*, Representative intracellular cytokine staining for IL-4 and IFN-γ from infected wild-type mice (*A* and *C*) and infected *fyn*$^{-/-}$ deficient mice (*B* and *D*). The frequency of IL-4-producing CD4$^+$ T cells in the mediastinal lymph nodes of wild-type mice (mean 12.89 ± 2.9% (SD), $n = 7$) or Fyn-deficient mice (mean 11.9 ± 3.1% (SD), $n = 7$) was comparable. Similarly, the percentage of IL-4-producing cells in the mesenteric lymph nodes was equivalent in wild-type (mean 6.4 ± 2% (SD)) and Fyn-deficient mice (mean 6.4 ± 1.3% (SD)). For IFN-γ, the mean percentage of CD4$^+$ T cells producing was 4.8% (±1.3% (SD), $n = 7$) and 4.4% (±1.9% (SD), $n = 7$) in the mediastinal lymph nodes of infected wild-type mice and infected Fyn-deficient mice, respectively. Comparable results were obtained for the frequency of IFN-γ-producing CD4$^+$ T cells in the mesenteric lymph nodes of wild-type mice (mean 3.9 ± 1.3% (SD)) or Fyn-deficient mice (mean 4.8 ± 2.1% (SD)). *E*, Serum IgE concentration from infected wild-type mice (■) and infected Fyn-deficient mice (●) assessed 10 days postinfection. IgE was not detected in the serum of uninfected control mice (data not shown). *N. brasiliensis* infection also induced inflammation resulting in an increase in the total number of infiltrating cells (*F*), eosinophils (*G*), and CD4$^+$ T cells (*H*) in the lungs when compared with uninfected wild-type (□) and Fyn-deficient controls (○). No significant difference was found in the response of infected wild-type (■) or infected Fyn-deficient mice (●). The means and SD are also shown (*E–H*). In *E–H*, each point represents an individual mouse and the data shown were pooled from two separate experiments.

cells or DO11.10$^+$ Fyn-deficient T cells and Ovap$_{323–339}$-loaded APCs that had been stained with different fluorochromes. We employed a competitive assay measuring the frequency of conjugates between DO11.10 T cells and two populations of APCs that were added to the T cells at different times and/or were loaded with different concentrations of Ovap$_{323–339}$. In this assay, the frequency of conjugates and the ratio of conjugates with the two different APCs provide an indication of the stability of the T cell/APC conjugate. In the absence of peptide only, ~8–10% of DO11.10$^+$ wild-type T cells or DO11.10$^+$ Fyn-deficient T cells formed conjugates with the APC added first (APC1) and fewer formed new conjugates with the APC added second (APC2) (Fig. 8). Loading APC1 with 1 μg/ml Ovap$_{323–339}$ increased the percentage of T cell/APC conjugates by ~3-fold, with little difference being observed in the frequency of conjugates with DO11.10$^+$ wild-type T cells or with DO11.10$^+$ *fyn*$^{-/-}$ T cells (Fig. 8), indicating that DO11.10$^+$ Fyn-deficient T cells are as capable as DO11.10$^+$ wild-type T cells in forming T cell/APC conjugates. When both APCs were loaded with 1 μg/ml Ovap$_{323–339}$, some DO11.10$^+$ T cells that had previously formed conjugates with APC1 switched APCs and formed new conjugates with APC2. Additionally, the total percentage of T cells forming conjugates with APCs was increased (Fig. 8). When APC2 was loaded with a higher concentration of Ovap$_{323–339}$ than APC1, more T cells

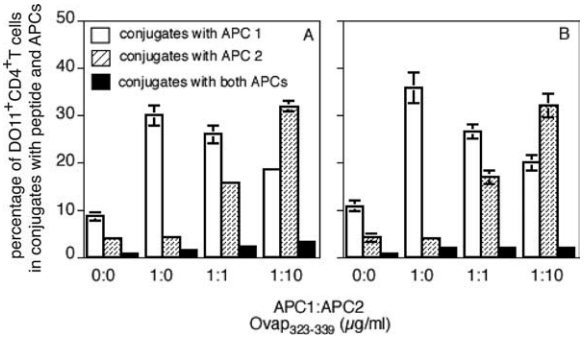

FIGURE 8. Fyn deficiency does not alter the stability of DO11.10$^+$ T cell/APC conjugates. CFSE-labeled DO11.10$^+$ T cells were mixed with CMTMR-labeled A20 B cells (APC1) that had been loaded with various concentrations of $Ovap_{323–339}$, as indicated. After an initial incubation period of 45 min, DDAO-labeled A20 B cells (APC2) loaded with the same or different concentration of $Ovap_{323–339}$ were added. The frequency of T cell/APC conjugates formed 90 min after the addition of APC2 was assessed by flow cytometry. Data are expressed as the mean frequency of CFSE$^+$ T cells in conjugates with APC1 (open bars), APC2 (stippled bars), or both APCs (filled bars). Also shown is the SEM. *A*, Percentage of DO11.10$^+$ wild-type T cell/APC conjugates; *B*, similar data for DO11.10$^+$ Fyn-deficient T cells. Similar results were obtained in two separate experiments.

formed conjugates with APC2, and fewer remained conjugated to APC1; however, no difference was observed between the responses of DO11.10$^+$ Fyn-deficient T cells or of DO11.10$^+$ wild-type T cells (Fig. 8). These data indicate that the formation and stability of T cell/APC conjugates was unaffected by the absence of Fyn kinase and corresponded to the observed minimal effects of Fyn deficiency on T cell responses to Ag in vitro and in vivo.

Discussion

Previous studies have suggested that Fyn is involved in several important T cell signaling pathways, including the initiation and feedback inhibition of TCR-mediated signaling (5, 6, 8, 37, 38), signaling events downstream of the TCR leading to cytoskeletal rearrangement and the formation of stable T cell/APC conjugates (7–9), and signaling of the SLAM/SAP complex, which promotes differentiation to Th2 effector cells (13–15). Indeed, following stimulation with soluble or plate-bound anti-CD3 Fyn-deficient CD4$^+$ T cells have a major defect in proliferation, differentiation into cytokine-producing effector cells, and TCR signaling when compared with wild-type T cells (Fig. 1 and Refs. 19, 20, 25). Therefore, we set out to assess whether Fyn-deficient CD4$^+$ T cells had a defect in their response to Ag in the context of MHC-II. We found that the development and peripheral expansion of DO11.10$^+$ TCR transgenic CD4$^+$ T cells was unaffected by a deficiency in Fyn kinase. The presence of a large number of naive resting Ag-specific T cells in the DO11.10$^+$ Fyn-deficient mice gave us the opportunity to compare the responses of wild-type and Fyn-deficient CD4$^+$ T cells in detail. Surprisingly, Ag-induced proliferation of DO11.10$^+$ Fyn-deficient T cells was comparable to that of DO11.10$^+$ wild-type T cells in vitro and in vivo, even in the absence of costimulation. Additionally, we did not detect a difference in the stability of DO11.10$^+$ T cell conjugation with $Ovap_{323–339}$-loaded APCs between wild-type and Fyn-deficient T cells. Interestingly, the absence of Fyn did not cause a defect in Th2 cytokine production, as has previously been reported in Fyn-deficient T cells and SAP-deficient T cells (13, 29). Thus, Fyn was not essential for the function of SAP in CD4$^+$ T cells. Surprisingly, DO11.10$^+$ Fyn-deficient T cells did produce higher levels of effector cytokines than did DO11.10$^+$ wild-type T cells following Ag-induced activation under certain conditions in vitro or in vivo.

Our assessment of positive selection in the thymus of DO11.10$^+$ Fyn-deficient mice showed that it was unaffected by the absence of signaling though Fyn (Fig. 2 and data not shown). Similarly, positive selection was found to be normal in TCR-transgenic Fyn-deficient mice that expressed either the CD8-selecting H-Y TCR or 2C TCR (30). Given the importance of TCR recognition of self-peptide-MHC complexes at low avidity for positive selection of conventional CD4 or CD8 T cells (39), these results suggest that TCR signaling in response to such ligands is not greatly affected in thymocytes by the absence of Fyn kinase. This is in contrast to the defect observed in Fyn-deficient mice in NKT cell development, which is dependent on the recognition of CD1d molecules bound to lipid Ags (21, 22, 40). One difference between the role of Fyn in thymic development of conventional α/β T cells and NKT cells may reflect the involvement of the CD4 and CD8 co-receptors in the former process and their association with Lck. Recruitment of CD4 or CD8, and thereby Lck, to the TCR efficiently provides Src family kinase function to initiate TCR signaling in α/β T cells (Fig. 1 and Ref. 25). The role of Fyn may be more important in NKT cell development in part because these cells do not utilize CD4 or CD8 as co-receptors. Alternatively, Fyn has been shown to promote NKT cell development following ligation of SLAM family receptors (24). Fyn-deficient mice have also been reported to have a defect in thymic negative selection induced by the superAg Mls-1^a (19). However, this does not seem to be a general defect, as negative selection of Fyn-deficient conventional α/β thymocytes in response to a second superAg, staphylococcal enterotoxin A, is normal (19), as is the negative selection of HY TCR-transgenic CD8$^+$ thymocytes in Fyn-deficient male mice (30).

In the periphery, the vast majority of CD4$^+$ T cells in DO11.10$^+$ Fyn-deficient mice had a naive phenotype (Fig. 3), which is in contrast to previously published results of Yasuda et al., who found evidence for an activated phenotype of CD4$^+$ and CD8$^+$ T cells from Fyn-deficient mice (11). However, we have not seen spontaneous activation of T cells from nontransgenic Fyn-deficient BALB/c mice or from highly inbred nontransgenic Fyn-deficient C57BL/6 mice (data not shown). Therefore, the DO11.10$^+$ TCR-transgenic Fyn-deficient T cells appear to be typical in retaining the naive resting phenotype in the periphery.

Proliferation of peripheral DO11.10$^+$ Fyn-deficient T cells in response to Ag in the context of MHC was almost indistinguishable from the response of DO11.10$^+$ wild-type T cells (Figs. 4 and 6). The similarity of the in vitro and in vivo proliferative responses of DO11.10$^+$ T cells was surprising given the numerous signaling defects that have been described in Fyn-deficient T cells (8, 10, 19, 20, 25, 41). Our data for Ag-driven proliferation of Fyn-deficient DO11$^+$CD4$^+$ T cells was similar to that obtained for Fyn-deficient TCR-transgenic CD8$^+$ T cells that express the 2C TCR (30). However, assessment of Ag-induced CD8$^+$ T cell proliferation from two other Fyn-deficient TCR transgenic mouse strains has produced conflicting results. Utting et al. showed that following stimulation with Ag Fyn-deficient HY$^+$CD8$^+$ T cells proliferate less well than do their wild-type counterparts (30). In contrast, Filby et al. recently showed that Ag-induced proliferation of Fyn-deficient F5 TCR-transgenic CD8$^+$ T cells was more vigorous than for their wild-type counterparts (41). Given these conflicting results it is difficult to form a coherent theory as to the role of Fyn in Ag-induced proliferation of CD8$^+$ T cells, although one possibility is that these data are a reflection of differences in the affinity of TCRs for their respective Ags. The difference between CD4$^+$ and CD8$^+$ T cells could also relate to the fact that CD4 binds more effectively to Lck than does CD8 (2, 42). For this reason, CD8$^+$ T cells may

be more sensitive to signaling defects that result from Fyn deficiency, whereas the greater amount of Lck recruited to CD4 may make Fyn dispensable for TCR signaling in CD4$^+$ T cells. Irrespective of the role of Fyn in CD8$^+$ T cell proliferation, our data clearly show that Fyn-deficient CD4$^+$ T cells proliferate normally in response to Ag under a variety of conditions.

Several studies have shown that Fyn plays a positive role in cytoskeletal remodeling and the formation of stable T cell/APC conjugates (7–9). When stimulated with Ag, AD10$^+$CD4$^+$ Fyn-deficient T cells do not effectively phosphorylate Vav (8, 25), which is required for actin polarization and reorganization promoting the formation of the immunological synapse (43, 44). Correspondingly, AD10$^+$CD4$^+$ *fyn*$^{-/-}$ T cells were reported to form less stable conjugates with Ag-loaded APCs than wild-type AD10$^+$CD4$^+$ T cells (8). In similar experiments, Badour et al. found that OT-II$^+$CD4$^+$ Fyn-deficient T cells formed poor immunological synapses with OVA-presenting APCs, and they provided evidence that this was due to a failure to phosphorylate WASp, resulting in impaired actin polymerization and cytoskeletal rearrangement (9). More recently, OT-I$^+$CD8$^+$ Fyn-deficient T cells were shown to have a defect in the reorganization of the microtubule-organizing center (MTOC) when stimulated with Ag and APCs (7). Reorganization of the MTOC involves Pyk2 and Vav, both of which are phosphorylated by Fyn (8, 10). Despite the reported defects in cytoskeletal reorganization, both AD10$^+$CD4$^+$ Fyn-deficient T cells and OT-I$^+$CD8$^+$ Fyn-deficient T cells produced IL-2 at levels similar to that seen in wild-type controls following Ag stimulation. Moreover, we found that DO11.10$^+$CD4$^+$ Fyn-deficient T cells did not have a defect in the formation of conjugates with Ovap$_{323–339}$-loaded APCs (Fig. 8). The ability of DO11.10$^+$ Fyn-deficient T cells to form stable T cell/APC conjugates is consistent with our observations that their Ag-induced proliferation was normal (Figs. 4 and 6).

Although DO11.10$^+$ Fyn-deficient T cells proliferated normally in response to antigenic stimulation, we did observed a moderate increase in Ag-induced effector cytokine production in some circumstances (Figs. 5 and 6). Similarly, Filby et al. have reported that Fyn-deficient F5 TCR transgenic CD8$^+$ T cells produced more IL-2 following Ag stimulation when compared with wild-type F5$^+$CD8$^+$ T cells (41). However, in our experiments Fyn-deficient CD4$^+$ T cell were not always hyperresponsive with respect to cytokine production, as following infection with *N. brasiliensis* G9 Fyn-deficient mice had a comparable cytokine response to that of wild-type BALB/c mice (Fig. 7). In any case, our data clearly show that Fyn-deficient T cells are capable of differentiating into Th1 or Th2 effector T cells, and that under certain conditions Fyn-deficient T cells are more potent producers of effector cytokines than are their wild-type counterparts (Figs. 5–7).

Previous work has shown that Fyn can play a positive role in Th2 differentiation and IL-4 production through its interaction with SAP, a key signaling adapter associated with several CD2-like adhesion molecules including SLAM (13, 29). Following the homotypic interaction between SLAM molecules, SAP binds to SLAM, and SAP-associated Fyn phosphorylates SLAM, initiating an intracellular signaling pathway that promotes IL-4 production (13). Anti-CD3 stimulation of SAP-deficient T cells or SAP^{R78A} T cells, which have a mutation that prevents the interaction of SAP with Fyn, results in the induction of mRNA for T-bet but not GATA-3, the key transcription factors regulating Th1 and Th2 differentiation, respectively, whereas under the same conditions both T-bet and GATA-3 are produced in wild-type T cells (13, 29). These results suggested that one of the downstream events following the formation of the SLAM/SAP/Fyn complex is the increased expression of GATA-3, a transcription factor that promotes the production of Th2 cytokines (45, 46). While, data from Cannons et al. (29) and Davidson et al. (13) suggested that signaling through Fyn can promote CD4$^+$ T cells to differentiate to IL-4-secreting T cells, our data clearly showed that signaling through Fyn was not obligatory for IL-4 production or Th2 immune responses (Fig. 5 and 7). Surprisingly, we found that under certain conditions Fyn-deficient DO11$^+$CD4$^+$ T cells produced more IL-4 than did wild-type CD4$^+$ T cells (Fig. 5). In agreement with our data, Fyn-deficient CD4$^+$ T cell have previously been shown to be more potent producers of IL-4 following in vitro stimulation with anti-CD3 in the presence of APCs, even in the absence of costimulation though CD28 (47). Furthermore, in a murine model of airway allergy in which mice were sensitized to, and subsequently challenged with, aerosolized OVA Th2-dependent eosinophil infiltration of the lung and the levels of IL-4 and IL-5 in the bronchoalveolar lavage were higher in mice deficient in the hematopoietic form of Fyn, FynT, than in wild-type control mice (48). Combined, these data show that under different conditions naive Fyn-deficient T cells can differentiate into IL-4-producing effector cells secreting as much, or more cytokine, than do wild-type T cells.

This study clarifies the effect of a deficiency in Fyn kinase on Ag-induced CD4$^+$ T cell activation. Through the use of TCR-transgenic Fyn-deficient T cells we have directly assessed the effect of a Fyn deficiency on Ag-induced activation in vitro and in vivo. Our results clearly show that Fyn-deficient T cells formed stable conjugates with Ag-loaded APCs; that Ag-induced proliferation was normal, even in the absence of costimulation; and with respect to Ag-induced cytokine production, Fyn-deficient CD4$^+$ T cells were as good as, or under certain conditions better than wild-type CD4$^+$ T cells. Additionally, we did not observe a defect in IL-4 production by Fyn-deficient CD4$^+$ T cells, demonstrating that the signaling via SLAM/SAP that promotes T cell differentiation to Th2 phenotype can proceed via a pathway that is independent of Fyn kinase. Taken together, these data reveal a previously unappreciated level of redundancy in T cell signaling that allows conventional α/β CD4$^+$ T cells to respond well following Ag recognition in the absence of Fyn kinase.

Acknowledgments

The authors acknowledge Shuwei Jiang for assistance with purification of cells by flow cytometry on the MoFlo, Barbara Sheer for help in preparing mouse tissues, Julia Lyandres for typing the TCR-transgenic and Fyn-deficient mice, members of Dr. Abbas' laboratory for technical advice and reagents, and Mark Lanett for assistance.

Disclosures

The authors have no financial conflicts of interest.

References

1. Chan, A. C., D. M. Desai, and A. Weiss. 1994. The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. *Annu. Rev. Immunol.* 12: 555–592.
2. Zamoyska, R., A. Basson, A. Filby, G. Legname, M. Lovatt, and B. Seddon. 2003. The influence of the src-family kinases, Lck and Fyn, on T cell differentiation, survival, and activation. *Immunol. Rev.* 191: 107–118.
3. Chu, K., and D. R. Littman. 1994. Requirement for kinase activity of CD4-associated p56lck in antibody-triggered T cell signal transduction. *J. Biol. Chem.* 269: 24095–24101.
4. Palacios, E. H., and A. Weiss. 2004. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene* 23: 7990–8000.
5. Samelson, L. E., A. F. Phillips, E. T. Luong, and R. D. Klausner. 1990. Association of the Fyn protein tyrosine kinase with the T cell antigen receptor. *Proc. Natl. Acad. Sci. USA* 87: 4358–4362.
6. Gauen, L. K. T., Y. Zhu, F. Letourneur, Q. Hu, J. B. Bolen, L. A. Matis, R. D. Klausner, and A. S. Shaw. 1994. Interactions of p59Fyn and ZAP-70 with T-cell receptor activation motifs: defining the nature of a signaling motif. *Mol. Cell. Biol.* 14: 3729–3741.
7. Martin-Cofreces, N. B., D. Sancho, E. Fernandez, M. Vicente-Manzanares, M. Gordon-Alonso, M. C. Montoya, F. Michel, O. Acuto, B. Alarcon, and

F. Sanchez-Madrid. 2006. Role of Fyn in the rearrangement of tubulin cytoskeleton induced through TCR. *J. Immunol.* 176: 4201–4207.
8. Huang, J., D. Tilly, A. Altman, K. Sugie, and H. M. Grey. 2000. T-cell receptor antagonists induce Vav phosphorylation by selective activation of Fyn kinase. *Proc. Natl. Acad. Sci. USA* 97: 10923–10929.
9. Badour, K., J. Zhang, F. Shi, Y. Leng, M. Collins, and K. A. Siminovitch. 2004. Fyn and PTP-PEST-mediated regulation of Wiskott-Aldrich syndrome protein (WASp) tyrosine phosphorylation is required for coupling T cell antigen receptor engagement to WASp effector function and T cell activation. *J. Exp. Med.* 199: 99–112.
10. Qian, D., S. Lev, N. S. van Oers, I. Dikic, J. Schlessinger, and A. Weiss. 1997. Tyrosine phosphorylation of Pyk2 is selectively regulated by Fyn during TCR signaling. *J. Exp. Med.* 185: 1253–1259.
11. Yasuda, K., M. Nagafuku, T. Shima, M. Okada, T. Yagi, T. Yamada, Y. Minaki, A. Kato, S. Tani-Ichi, T. Hamaoka, and A. Kosugi. 2002. Cutting edge: Fyn is essential for tyrosine phosphorylation of Csk-binding protein/phosphoprotein associated with glycolipid-enriched microdomains in lipid rafts in resting T cells. *J. Immunol.* 169: 2813–2817.
12. Davidson, D., M. Bakinowski, M. L. Thomas, V. Horejsi, and A. Veillette. 2003. Phosphorylation-dependent regulation of T-cell activation by PAG/Cbp, a lipid raft-associated transmembrane adaptor. *Mol. Cell. Biol.* 23: 2017–2028.
13. Davidson, D., X. Shi, S. Zhang, H. Wang, M. Nemer, N. Ono, S. Ohno, Y. Yanagi, and A. Veillette. 2004. Genetic evidence linking SAP, the X-linked lymphoproliferative gene product, to Src-related kinase FynT in T_H2 cytokine regulation. *Immunity* 21: 707–717.
14. Ma, C., K. Nichols, and S. Tangye. 2007. Regulation of cellular and humoral immune responses by the SLAM and SAP families of molecules. *Annu. Rev. Immunol.* 25: 337–379.
15. Veillette, A. 2006. Immune regulation by SLAM family receptors and SAP-related adaptors. *Nat. Rev. Immunol.* 6: 56–66.
16. Latour, S., R. Roncagalli, R. Chen, M. Bakinowski, X. Shi, P. L. Schwartzberg, D. Davidson, and A. Veillette. 2003. Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. *Nat. Cell Biol.* 5: 149–154.
17. Latour, S., and A. Veillette. 2003. Molecular and immunological basis of X-linked lymphoproliferative disease. *Immunol. Rev.* 192: 212–224.
18. Chan, B., A. Lanyi, H. K. Song, J. Griesbach, M. Simarro-Grande, F. Poy, D. Howie, J. Sumegi, C. Terhorst, and M. J. Eck. 2003. SAP couples Fyn to SLAM immune receptors. *Nat. Cell Biol.* 5: 155–160.
19. Stein, P. L., H. M. Lee, S. Rich, and P. Soriano. 1992. $pp59^{fyn}$ mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell* 70: 741–750.
20. Appleby, M. W., J. A. Gross, M. P. Cooke, S. D. Levin, X. Qian, and R. M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of $p59^{fyn}$. *Cell* 70: 751–763.
21. Eberl, G., B. Lowin-Kropf, and H. R. MacDonald. 1999. Cutting edge: NKT cell development is selectively impaired in Fyn-deficient mice. *J. Immunol.* 163: 4091–4094.
22. Gadue, P., N. Morton, and P. L. Stein. 1999. The Src family tyrosine kinase Fyn regulates natural killer T cell development. *J. Exp. Med.* 190: 1189–1196.
23. Borowski, C., and A. Bendelac. 2005. Signaling for NKT cell development: the SAP-FynT connection. *J. Exp. Med.* 201: 833–836.
24. Griewank, K., C. Borowski, S. Rietdijk, N. Wang, A. Julien, D. G. Wei, A. A. Mamchak, C. Terhorst, and A. Bendelac. 2007. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity* 27: 751–762.
25. Sugie, K., M. S. Jeon, and H. M. Grey. 2004. Activation of naive CD4 T cells by anti-CD3 reveals an important role for Fyn in Lck-mediated signaling. *Proc. Natl. Acad. Sci. USA* 101: 14859–14864.
26. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{lo}$ thymocytes in vivo. *Science* 250: 1720–1723.
27. Borriello, F., M. P. Sethna, S. D. Boyd, A. N. Schweitzer, E. A. Tivol, D. Jacoby, T. B. Strom, E. M. Simpson, G. J. Freeman, and A. H. Sharpe. 1997. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity* 6: 303–313.
28. Voehringer, D., K. Shinkai, and R. M. Locksley. 2004. Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity* 20: 267–277.
29. Cannons, J. L., L. J. Yu, B. Hill, L. A. Mijares, D. Dombroski, K. E. Nichols, A. Antonellis, G. A. Koretzky, K. Gardner, and P. L. Schwartzberg. 2004. SAP regulates T_H2 differentiation and PKC-θ-mediated activation of NF-κB1. *Immunity* 21: 693–706.
30. Utting, O., S. J. Teh, and H. S. Teh. 1998. T cells expressing receptors of different affinity for antigen ligands reveal a unique role for p59Fyn in T cell development and optimal stimulation of T cells by antigen. *J. Immunol.* 160: 5410–5419.
31. Gett, A. V., and P. D. Hodgkin. 1998. Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. *Proc. Natl. Acad. Sci. USA* 95: 9488–9493.
32. Lovatt, M., A. Filby, V. Parravicini, G. Werlen, E. Palmer, and R. Zamoyska. 2006. Lck regulates the threshold of activation in primary T cells, while both Lck and Fyn contribute to the magnitude of the extracellular signal-related kinase response. *Mol. Cell. Biol.* 26: 8655–8665.
33. Shibaki, A., and S. I. Katz. 2002. Induction of skewed Th1/Th2 T-cell differentiation via subcutaneous immunization with Freund's adjuvant. *Exp. Dermatol.* 11: 126–134.
34. Billiau, A., and P. Matthys. 2001. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. *J. Leukocyte Biol.* 70: 849–860.
35. Shinkai, K., M. Mohrs, and R. M. Locksley. 2002. Helper T cells regulate type-2 innate immunity in vivo. *Nature* 420: 825–829.
36. Finkelman, F. D., J. Holmes, I. M. Katona, J. F. Urban, M. P. Beckmann, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann, and W. E. Paul. 1990. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol.* 8: 303–333.
37. Deckert, M., C. Elly, A. Altman, and Y. C. Liu. 1998. Coordinated regulation of the tyrosine phosphorylation of Cbl by Fyn and Syk tyrosine kinases. *J. Biol. Chem.* 273: 8867–8874.
38. Tsygankov, A. Y., S. Mahajan, J. E. Fincke, and J. B. Bolen. 1996. Specific association of tyrosine-phosphorylated c-Cbl with Fyn tyrosine kinase in T cells. *J. Biol. Chem.* 271: 27130–27137.
39. Starr, T. K., S. C. Jameson, and K. A. Hogquist. 2003. Positive and negative selection of T cells. *Annu. Rev. Immunol.* 21: 139–176.
40. Matsuda, J. L., and L. Gapin. 2005. Developmental program of mouse Vα14i NKT cells. *Curr. Opin. Immunol.* 17: 122–130.
41. Filby, A., B. Seddon, J. Kleczkowska, R. Salmond, P. Tomlinson, M. Smida, J. A. Lindquist, B. Schraven, and R. Zamoyska. 2007. Fyn regulates the duration of TCR engagement needed for commitment to effector function. *J. Immunol.* 179: 4635–4644.
42. Ravichandran, K. S., and S. J. Burakoff. 1994. Evidence for differential intracellular signaling via CD4 and CD8 molecules. *J. Exp. Med.* 179: 727–732.
43. Villalba, M., K. Bi, F. Rodriguez, Y. Tanaka, S. Schoenberger, and A. Altman. 2001. Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells. *J. Cell Biol.* 155: 331–338.
44. Hornstein, I., A. Alcover, and S. Katzav. 2004. Vav proteins, masters of the world of cytoskeleton organization. *Cell. Signal.* 16: 1–11.
45. Grogan, J. L., M. Mohrs, B. Harmon, D. A. Lacy, J. W. Sedat, and R. M. Locksley. 2001. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* 14: 205–215.
46. Zhu, J., H. Yamane, J. Cote-Sierra, L. Guo, and W. E. Paul. 2006. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. *Cell Res.* 16: 3–10.
47. Tamura, T., O. Igarashi, A. Hino, H. Yamane, S. Aizawa, T. Kato, and H. Nariuchi. 2001. Impairment in the expression and activity of Fyn during differentiation of naive $CD4^+$ T cells into the Th2 subset. *J. Immunol.* 167: 1962–1969.
48. Kudlacz, E. M., C. J. Andresen, M. Salafia, C. A. Whitney, B. Naclerio, and P. S. Changelian. 2001. Genetic ablation of the src kinase p59FynT exacerbates pulmonary inflammation in an allergic mouse model. *Am. J. Respir. Cell Mol. Biol.* 24: 469–474.