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Th1 cells rolling on selectins trigger DAP12-dependent signals that activate integrin αLβ2

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Abstract

During inflammation, both neutrophils and effector T cells use selectins to roll and integrins to arrest in postcapillary venules. In both cell types, chemokines can transduce signals that convert integrin αLβ2 to a high-affinity conformation, which interacts with ICAM-1 to mediate arrest. In neutrophils, selectins also trigger an immunoreceptor-like signaling cascade that converts integrin αLβ2 to an intermediate-affinity conformation, which interacts with ICAM-1 to slow rolling. It is not known whether selectins induce similar signaling events in T cells. Antigen engagement causes phosphorylation of ITAMs on the TCR; these motifs recruit kinases and adaptors that lead to activation of αLβ2. We found that mouse Th1 cells rolling on P- or E-selectin triggered signals that promoted αLβ2-dependent slow rolling on ICAM-1 in vitro and in vivo. The selectin signaling cascade resembled that used by the TCR, except that unexpectedly, Th1 cells employed the ITAM-bearing protein DAP12, which was not known to be expressed in these cells. Importantly, outside-in signaling through ligand-occupied αLβ2 also required DAP12. Cooperative selectin and chemokine signaling in Th1 cells promoted αLβ2-dependent slow rolling and arrest in vitro and in vivo and migration into antigen-challenged tissues in vivo. Our findings reveal an important function for DAP12 in Th1 cells and a new mechanism to recruit effector T cells to sites of inflammation.

Introduction

Circulating naïve T cells migrate into peripheral lymph nodes where they encounter antigen-presenting cells (1). Antigen recognition by the TCR, in conjunction with costimulatory molecules such as CD28, transduces signals that promote differentiation into effector $CD4^+$

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Authorship

B.S., T.Y., S.R.P., N.Z., and Z.L. performed experiments. B.S., T.Y., and R.P.M. conceived the study, interpreted data, and wrote the manuscript, with input from all authors.

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Disclosures

R.P.M. is a cofounder of Selexys Pharmaceuticals, now part of Novartis AG, and of Tetherex Pharmaceuticals. The remaining authors declare no competing financial interests.

T helper cells and $CD8^+$ T cytotoxic cells. After re-entering the circulation, effector T cells migrate to peripheral sites of inflammation to clear pathogens.

In the multistep paradigm for immune cell recruitment, leukocytes roll on endothelial cells through interactions of selectins with glycosylated ligands (2). Rolling cells encounter immobilized chemokines that initiate signals through G protein-coupled receptors. The signals activate β2 integrins, which then bind to endothelial ligands such as ICAM-1 to mediate arrest and transendothelial migration. This paradigm is well established for homing of naïve T cells to lymph nodes (3). L-selectin on naïve T cells mediates rolling by interacting with mucins on the apical surface of high endothelial venules (HEV). The receptor CCR7 interacts with chemokines on HEV to trigger integrin αLβ2-mediated arrest. A similar paradigm has been suggested for homing of effector T cells to inflammatory sites (1,4). Antigen stimulation in peripheral lymph nodes upregulates glycosyltransferases that enable glycoproteins such as P-selectin glycoprotein ligand-1 (PSGL-1), CD43, and CD44 to interact with P- or E-selectin on endothelial cells in inflamed venules. Antigen stimulation upregulates receptors such as CXCR3 that interact with inflammatory chemokines to activate integrin αLβ2. It has been proposed that high αLβ2 densities on effector T cells permit chemokine-independent arrest (5,6). However, the strength of antigen stimulation varies, and some effector T cells express lower levels of αLβ2 that may not support chemokine-independent arrest (7–9).

For neutrophils, the multistep paradigm has been expanded to include signaling through PSGL-1 and CD44 as they engage P- or E-selectin during rolling (2,10). Selectin signaling converts αLβ2 from a bent, low-affinity conformation to an extended, intermediate-affinity conformation, which interacts reversibly with ICAM-1 to slow rolling velocities (11). Chemokine signaling converts αLβ2 to an extended, high-affinity conformation that causes arrest (12). When chemokine concentrations are limiting, selectin and chemokine signals cooperate to promote αLβ2-dependent slow rolling and arrest (13,14). Engaging PSGL-1 or CD44 on neutrophils triggers a signaling cascade similar to that used by the TCR. Src family kinases (SFKs) phosphorylate the ITAMs on FcRγ and on DNAX activation protein of 12 kD (DAP12), also known as TYRO protein tyrosine kinase-binding protein (TYROBP) (15). The phosphorylated ITAMs recruit spleen tyrosine kinase (Syk) (16), which then recruits the adaptor Src homology domain-containing protein of 76 kD (SLP-76), Tec kinases, and p38 MAPK (13,14,17–20). Other downstream mediators ultimately enable talin-1-dependent integrin activation (13,21,22).

It is not known whether selectin signaling can activate integrins in effector T cells. Antigen stimulation of the TCR activates integrin αLβ2, suggesting that T cells contain at least some of the components for selectin-triggered integrin activation (23). However, the TCR uses ITAMs on its own subunits to propagate signals (23), and the TCR is not known to associate with PSGL-1 or CD44. Other than a few cell lines cloned from $CD4^+CD28^-$ cells in peripheral blood (24), T cells are not thought to express the ITAM-bearing proteins DAP12 and FcRγ found in myeloid cells. Here, we report that mouse Th1 cells rolling on P- or E-selectin triggered signals that promote αLβ2-dependent slow rolling on ICAM-1 in vitro and in vivo. The signaling cascade initiated by selectin-ligand interactions resembled that initiated by antigen binding to the TCR, except that unexpectedly, selectin signaling in Th1

cells required the ITAM-bearing protein DAP12. Notably, outside-in signaling through ligand-occupied αLβ2 also required DAP12. Cooperative selectin and chemokine signaling in Th1 cells promoted αLβ2-dependent slow rolling and arrest in vitro and in vivo and migration into antigen-challenged tissues in vivo. Our findings reveal an important function for DAP12 in Th1 cells and a new mechanism to recruit effector T cells to sites of inflammation.

Materials and methods

Reagents.

Mouse P- and E-selectin-IgM Fc chimeras and the control CD45-IgM Fc chimera were described previously (25). Recombinant mouse ICAM-1 Fc chimera, TNF-α, IL-2, IL-12, IL-4, CXCL10, and CCL5 were from R&D Systems. An ELISA kit to measure mouse interferon-γ, rat anti-mouse IL-4 mAb (clone 11B11), hamster anti-mouse CD3ε mAb (clone 145–2C11), and hamster anti-mouse CD28 mAb (clone 37.51) were from eBioscience. Rat anti-mouse PSGL-1 mAb (clone 4RA10), PE-labeled rat anti-mouse PSGL-1 mAb (clone 2PH1), hamster anti-mouse ICAM-1 mAb (clone 3E2), biotin-labeled rat anti-mouse CD11b (integrin αM subunit) mAb (clone M1/70), biotin-labeled rat anti-mouse CD45R/B220 mAb (clone Ra3–6B2), and biotin-labeled rat anti-mouse CD8α mAb (clone 53–6.7) were from BD Biosciences. FITC-labeled rat anti-mouse CD11a (integrin αL subunit) mAb (clone M17/4), rat anti-mouse CD11a mAb (clone M17/4), FITC-labeled rat anti-mouse CD11b mAb (clone M1/70), rat anti-mouse CD11b mAb (clone M1/70), FITC-labeled rat anti-mouse CD4 mAb (clone GK1.5), FITC-labeled rat anti-mouse CD49d mAb (clone R2–1), PE-labeled rat anti-mouse CD43 mAb (clone S11), PE-labeled anti-mouse CD45RB (clone c36.3–16A), and PE-labeled rat anti-mouse CD44 mAb (clone IM7) were from BioLegend Inc. Purified goat anti-human IgM and purified rabbit anti-mouse phospho-Lck (Tyr 394) polyclonal antibody (pAb) were from Invitrogen. Rat anti-mouse β_2 integrin mAb (clone GAME46), purified rabbit anti-mouse Lck pAb, purified rabbit anti-mouse Fyn pAb and purified rabbit anti-mouse phospho-Src (Tyr 416) pAb were from Cell Signaling Technology. FITC-labeled rat anti-mouse CD4 mAb (clone GK1.5), FITC-labeled rat anti-mouse CD49d mAb (clone R2–1), PE-labeled rat anti-mouse CD43 mAb (clone S11), and PE-labeled rat anti-mouse CD44 mAb (clone IM7) were from BioLegend. Purified goat anti-human IgM and purified rabbit anti-mouse phospho-Lck (Tyr 394) polyclonal antibody (pAb) were from Invitrogen. Rat anti-mouse β_2 integrin mAb (clone GAME46), purified rabbit anti-mouse Lck pAb, purified rabbit anti-mouse Fyn pAb and purified rabbit anti-mouse phospho-Src (Tyr 416) pAb were from Cell Signaling Technology. Rabbit anti-mouse phospho-Fyn pAb was from MyBioSource. Rat anti-mouse E-selectin mAb (clone 9A9) (26) and rat anti-mouse P-selectin mAb (clone RB40.30) (27) were described previously. HRP-conjugated goat anti-rabbit IgG (H+L) pAb was from Thermo Fisher Scientific. Streptavidin microbeads were from Miltenyi Biotec. Piceatannol, filipin III, methyl-β-cyclodextrin, α-cyclodextrin, U73122, SB203580, 4-ethoxymethylene-2-oxazolin-5-one (oxazolone) and fluorescence dyes PKH67 and PKH26 were from Sigma Aldrich. 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyramidine (PP2) and 4-amino-7-phenylpyrazolo[3,4-*d*]pyramidine (PP3) were from CalBiochem. Ficoll-PaquePLUS was

from GE Health Care. Pertussis toxin was from EMD Chemicals. Collagenase A, collagenase B, and recombinant DNase I were from Roche Diagnostics.

Mice.

PSGL-1-deficient mice (*Selplg*$^{-/-}$) were generated as described (25). Wild-type (WT) C57BL/6J (*B6, CD45.2*) mice, Fyn-deficient mice (*Fyn*$^{-/-}$), CD43-deficient mice (*Spn*$^{-/-}$), and CD44-deficient mice (*Cd44*$^{-/-}$) were purchased from The Jackson Laboratory. *Selplg*$^{-/-}$, *Spn*$^{-/-}$, and *Cd44*$^{-/-}$ mice were bred to generate mice lacking CD43 and CD44 (*Spn*$^{-/-}$;*Cd44*$^{-/-}$), PSGL-1 and CD44 (*Selplg*$^{-/-}$;*Cd44*$^{-/-}$), or PSGL-1 and CD43 (*Selplg*$^{-/-}$;*Spn*$^{-/-}$). Rlk/Txk-deficient mice (*Txk*$^{-/-}$) and Itk-deficient mice (*Itk*$^{-/-}$) (28) were provided by Pamela Schwartzberg (National Human Genome Research Institute). DAP12-deficient mice (*Tyrobp*$^{-/-}$) and FcRγ-deficient mice (*Fcer1γ*$^{-/-}$) (29) were provided by Clifford Lowell (University of California at San Francisco). All mice were in the C57BL/6J background. All experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation.

Preparation of Th1 and Th2 cells.

Th1 and Th2 cells were generated in vitro as described previously (30,31). Briefly, CD4$^+$ T cells were isolated by incubating splenocytes with biotinylated antibodies to mouse CD11b, B220, and CD8α, and streptavidin-conjugated microbeads. Purified CD4$^+$ T cells were cultured in plates coated with 10 μg/ml anti-CD3ε and 10 μg/ml anti-CD28 for 2 days in medium containing 4 ng/ml IL-2, 8 ng/ml IL-12, and 0.2 μg/ml anti-IL-4 mAb to promote Th1 cell differentiation or in media containing 4 ng/ml IL-2 and 100 ng/ml IL-4 to promote Th2 cell differentiation. The cells were transferred to uncoated plates and cultured for another 3 days. Th1 cells were further purified with Ficoll-Paque Plus following the manufacturer's instruction. Th1 cells were characterized by measuring interferon γ secreted in the culture medium with ELISA and by examining cell surface expression of CD4, CD11a and CD11b with flow cytometry.

Effector T cells were generated in vivo as described previously (30). Briefly, mice were sensitized with 2% oxazolone on paws, ears, and the shaved abdomen. After six days, the draining lymph nodes were collected, and a single cell suspension was generated by compressing lymph nodes on a 100-μm cell strainer mesh with the plunger of a 1-ml syringe. CD4$^+$ T cells were enriched by negative selection with biotinylated antibodies to mouse CD11b, B220, and CD8a and with streptavidin-conjugated microbeads. The CD4$^+$ T cells were analyzed by flow cytometry after gating out a minor population of dead cells that were distinguished by their forward and side scatter properties. To identify memory cells, the cells were incubated with anti-CD44-FITC and anti-CD45RB-PE. To identify effector T cells expressing interferon γ, CD4$^+$ T cells (0.5×10^6/ml) were added to coverslips containing immobilized E-selectin for 30 minutes at room temperature (19). Non-adherent cells were removed by gentle washing. The adherent cells were fixed with 4% PFA, stained with antibodies to CD4 and interferon γ, and observed with a confocal microscope.

Flow chamber assay.

Leukocyte adhesion under flow was measured as described (19,25). Briefly, goat anti-human IgM (Fc) mAb (10 μg/ml) was absorbed on a demarcated area of 35-mm polystyrene dishes at 4°C overnight. For some experiments, ICAM-1-Fc chimera (20 μg/ml) with or without recombinant CXCL10 (0.2 or 2 μg/ml) was coimmobilized. The coating area was blocked with 2% human serum albumin (HSA) for 2 h followed by capturing of P-selectin-IgM or E-selectin-IgM. In some experiments, the concentration of E-selectin-IgM was increased to capture a three-fold higher density of E-selectin on the dish (19). Th1 cells (10^7/ml in HBSS with 0.5% HSA) were perfused over P-selectin or E-selectin substrates in chambers at a wall shear stress of 1 dyn/cm^2. After 5 minutes, velocities of rolling cells were measured over a 10-second interval using a videomicroscopy system with Element digital image-analysis software (Nikon). Cells that remained stationary over 5 seconds were considered to be arrested. In some experiments, chambers were pretreated with anti-ICAM-1 mAb (20 μg/ml) or cells were pretreated with anti-αLβ2 mAb (20 μg/ml) or isotype-matched control mAb. In other experiments, cells were pretreated with inhibitors to zeta-chain-associated protein kinase 70 (ZAP-70) (piceatannol, 20 μM), SFKs (PP2, 20 μM), p38 MAPK (SB203580, 50 μM), or phospholipase C (PLC) (U73122, 5 μM). For inhibitor-treated cells, the inactive analogue of PP2 (PP3, 20 μM) or an equal volume of DMSO was used as control. In some studies, cells were treated with filipin III or methyl-β-cyclodextrin to disrupt lipid rafts or with the inactive analogue α-cyclodextrin (αCD) or vehicle DMSO as controls (19,32).

Cell adhesion assay under static conditions.

Anti-β_2 integrin mAb or isotype control mAb (20 μg/ml) was immobilized on Costar 48-well plates at 4°C overnight, and the plates were blocked with 2% HSA at RT for 2 hours. Th1 cells (0.5×10^6/ml in HBSS with 0.5% HSA) were seeded on coated plates at 37°C for 30 minutes. The plates were washed gently 5 times, and adherent cells were fixed by 2% paraformaldehyde. Adherent and spreading cells were quantified by microscopy.

Oxazolone-induced contact hypersensitivity and cell competitive homing assay.

The mouse contact hypersensitivity model was prepared as described (31,33). In brief, mice were sensitized by topical application of 2% oxazolone to paws and the abdominal skin at day 0, and were challenged by painting the right ear with 1% oxazolone at day 7. The left ear was painted with solvent vehicle as control. A mixture of differentially labeled Th1 cells from two genotypes (10^7 cells/genotype) was injected into WT mice 16 h after challenge via the tail vein. Three hours after cell injection, peripheral blood was collected from the retroorbital sinus, and red blood cells were lysed in lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, and 1 mM EDTA). After mice were euthanized, ear tissues were digested with collagenase (400 U/ml) and DNase I (10 μg/ml) as described (30), and lymphocytes in ear tissues were purified with Ficoll-Paque Plus. The number of labeled Th1 cells in peripheral blood and ear tissues was counted by flow cytometry.

Intravital microscopy of the cremaster muscle.

Intravital microscopy was performed as described previously (25,33). Briefly, mice were anesthetized by intraperitoneal injection of ketamine and xylazine. The cremaster muscle

was isolated 2 h after intrascrotal injection of 500 ng TNF-α and superfused with thermocontrolled (35°C) bicarbonate-buffered saline. Anti-P-selectin mAb and then anti-β_2 integrin mAb or isotype control mAb (100 μg in 100 μl saline) were injected immediately before injection of labeled Th1 cells. Th1 cells from WT and Rlk/Txk-deficient mice were fluorescently labeled with either CellTracker™ Green CMFDA or CellTracker™ Orange CMRA dye (2.5 μM) for 30 minutes. After washing, 10^7 cells from each genotype were mixed together and injected retro-orbitally into WT mice. The competitive rolling velocities of fluorescently labeled Th1 cells were measured in the same venules using spinning-disc confocal microscopy (21). Visualization of rolling cells was performed with a Nikon Eclipse microscope (E600FN) with a 20X/0.95W XLUM plan F1 water immersion objective, coupled to a confocal light path (Solamere Technology Group) based on a modified Yokogawa CSU-X1 head (Yokagawa Electric Corporation). The spinning disc confocal microscope was driven by National Institutes of Health acquisition software Micromanager. The images were captured by Micromanager 1.4 and analyzed with ImageJ software.

Western blots and signaling assays.

Th1 cells (5×10^6) from WT or DAP12-deficient (*Tyrobp*$^{-/-}$) mice, or bone marrow neutrophils isolated from WT mice (34), were lysed with 1% Triton X-100, 125 mM NaCl, 50 mM Tris pH 8.0, 10 mM EDTA, 2 mM PMSF, 0.1% SDS with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The lysates were analyzed by Western blotting using rabbit antibodies against DAP12 (Millipore), Ly6G (Biorbyt), CD11b (Abcam), or β-actin (Cell Signaling Technology) (19).

To study selectin-induced signaling, Th1 cells from WT or DAP12-deficient mice were incubated in 6-well plates with control CD45-IgM Fc or E-selectin-IgM Fc captured on immobilized anti-human IgM Fc antibody on a rotary shaker at 65 rpm for 10 min at room temperature as previously described (34). In some experiments, selectin engagement was blocked by adding 10 mM EDTA. To analyze signaling induced by engagement of β2 integrins, the cells were incubated in 6-well plates coated with 20 μg/ml of isotype control mAb or anti-β2 integrin mAb (GAME-46) for 10 min at room temperature as previously described (34). The cells were then lysed and analyzed by Western blotting using rabbit antibodies against Fyn, phospho-Fyn, Lck, phospho-Lck, SFK, phospho-SFK (Y416), ZAP-70, phospho-ZAP-70, p38, phospho-p38, phospho-Vav, and β-actin (Cell Signaling Technology) (19). The blots were quantified by densitometry.

RT-PCR.

Total RNA from Th1 cells and bone marrow-derived macrophages of WT and DAP12-deficient mice (10^7 cells per genotype) was prepared with the RNeasy Mini Kit (QIAGEN). The first strand of cDNA synthesis was performed with the SuperScript VI First-Strand Synthesis System (Thermo Fisher Scientific) by using 1 μg of RNA as the template. Transcripts encoding DAP12, CD11a, CD11b, and TCR β subunit were amplified with PCR using 2 μl of cDNA, and PCR products were analyzed by electrophoresis in a 1–2% agarose gel. The primers used for DNA amplification were: TGGGATTGTTCTGGGTGACTTG (sense) and GAGTAGGCATCTGGAATGACCG (antisense) for DAP12; GGTTTGGAGCCGCCATAA CTGC (sense) and GGGATTTCCTCTGGGGAGAACGAC

(antisense) for CD11a; GTGTCA GTGTGTCCCTTGC (sense) and GCATCAATCCAAAGACCTGG (antisense) for CD11b; and TGGGTTGCTCTCTTTCTCCTGG (sense) and GAAGTTCTCTTGGCTTGGTCTGG (antisense) for TCR β subunit.

Statistics.

Data are presented as mean ± SEM. Data were analyzed by unpaired Student *t*-test or by 1-way analysis of variance with the post hoc multiple-comparison test. Values were considered significant at $P < 0.05$.

Results

Th1 cells rolling on P- or E-selectin trigger immunoreceptor-like signals that slow rolling velocities on ICAM-1

We differentiated mouse $CD4^+$ splenocytes into Th1 or Th2 cells in vitro. Th1 cells, but not Th2 cells, secreted interferon γ (Fig. 1A). Th1 cells expressed integrin αLβ2 (CD11a/CD18) but not the myeloid cell integrin αMβ2 (CD11b/CD18) (Fig. 1B). Our Th1 cells consistently expressed moderate surface densities of αLβ2, as noted by other investigators (7–9). In contrast, some groups have reported much higher αLβ2 levels on effector T cells that were prepared by similar methods (5,6).

Th1 cells perfused over immobilized E- or P-selectin rolled with lower velocities when ICAM-1 was coimmobilized (Fig. 1, C and D). Unlike effector T cells with very high αLβ2 densities (5,6), they did not spontaneously arrest on ICAM-1. The slower rolling was mediated by reversible interactions of αLβ2 with ICAM-1, because it was blocked by mAbs to integrin αLβ2 or to ICAM-1. To determine whether similar interactions occur in vivo, we injected fluorescent Th1 cells into mice 2 h after intrascrotal injection of TNF-α, which mobilizes P- and E-selectin in venules of the cremaster muscle (25). The injected cells rolled in these venules (Fig. 1E). Injection of anti-P-selectin mAb limited rolling to E-selectin and did not alter rolling velocities. Subsequent injection of anti-αLβ2 mAb increased rolling velocities, indicating that integrin-ICAM-1 interactions contributed to slow rolling. Rolling Th1 cells did not undergo chemokine-dependent arrest, presumably because they lack receptors for chemokines such as CXCL1 that are expressed in this model (35).

Mouse Th1 cells use only PSGL-1 to roll on P-selectin (36). They use three different ligands to roll on E-selectin: PSGL-1, CD43, and CD44 (30,31,37). Th1 cells from WT mice or from mice lacking any one of these three ligands (*Selplg*$^{-/-}$, *Spn*$^{-/-}$, or *Cd44*$^{-/-}$) rolled with comparable velocities on immobilized E-selectin (Fig. 1F). Cells from all genotypes rolled significantly slower when ICAM-1 was coimmobilized, demonstrating that no single E-selectin ligand is essential to propagate signals that activate αLβ2. The rolling velocities of cells lacking both CD43 and CD44 (*Spn*$^{-/-}$;*Cd44*$^{-/-}$) on E-selectin were similar to those of cells lacking only one of the three ligands. *Spn*$^{-/-}$;*Cd44*$^{-/-}$ cells also rolled slower when ICAM-1 was coimmobilized, demonstrating that PSGL-1 is sufficient to activate αLβ2 (Fig. 1F). A 3-fold higher density of E-selectin supported similar rolling velocities for cells lacking PSGL-1 and either CD44 or CD43 (*Selplg*$^{-/-}$;*Cd44*$^{-/-}$) or (*Selplg*$^{-/-}$;*Spn*$^{-/-}$) (Fig.

1G). *Selplg*$^{-/-}$/*Cd44*$^{-/-}$ cells rolled slower when ICAM-1 was coimmobilized, demonstrating that CD43 is sufficient to activate αLβ2. In contrast, *Selplg*$^{-/-}$;*Spn*$^{-/-}$ cells did not roll slower on ICAM-1. Therefore, E-selectin engagement of CD44 alone does not activate αLβ2 (Fig. 1G).

In neutrophils, the earliest event triggered by engaging selectin ligands is activation of the SFKs Hck, Lyn, and Fgr (15,19). The SFK inhibitor PP2, but not its inactive analogue PP3, blocked integrin-dependent slow rolling of Th1 cells on ICAM-1 coimmobilized with E-selectin (Fig. 2A) or P-selectin (Supplemental Fig. 1A). These data suggest that selectin ligands on Th1 cells must activate SFKs to activate integrin αLβ2. Th1 cells primarily express two SFKs: Lck and Fyn. T cells mature normally in mice lacking Fyn (38,39) but not in mice lacking Lck (40). Th1 cells from Fyn-deficient mice exhibited normal slow rolling on ICAM-1 coimmobilized with E-selectin (Fig. 2A) or P-selectin (Supplemental Fig. 1A). When plated on immobilized E-selectin, but not on a control protein (CD45), WT Th1 cells phosphorylated both Lck and Fyn (Fig. 2B). Chelation of Ca^{2+} with EDTA, which inhibits E-selectin binding to its ligands (41), prevented phosphorylation of Lck or Fyn. Taken together, these data indicate that selectin-induced phosphorylation of either SFK triggers a signaling cascade that leads to integrin activation. In neutrophils, disrupting lipid rafts prevents selectin-triggered phosphorylation of SFKs and slow rolling on ICAM-1 (19). Similarly, disrupting lipid rafts in Th1 cells with methyl-β-cyclodextrin or filipin III prevented slow rolling on ICAM-1 coimmobilized with E-selectin (Fig. 2C) or P-selectin (Supplemental Fig. 1B). Adding serum to restore membrane cholesterol reversed the effects of methyl-β-cyclodextrin. Adding vehicle or the inactive analogue α-cyclodextrin did not prevent slow rolling.

In neutrophils, selectin activation of SFKs leads to phosphorylation of the ITAM proteins DAP12 and FcRγ (15). The ITAMs recruit the tyrosine kinase Syk (16), which recruits other adaptors and kinases that activate mediators such as phospholipase C γ (PLCγ) and p38 MAPK (13,14,17–20). T cells express the Syk-related kinase ZAP-70, as well as PLCγ and p38 MAPK (23). Inhibitors of Syk/ZAP-70 (piceatannol), PLCγ (U73122), or p38 MAPK (SB203580) blocked slow rolling of Th1 cells on ICAM-1 coimmobilized with E-selectin (Fig. 2D) or P-selectin (Supplemental Fig. 1C). These results support the notion that Th1 cells rolling on E- or P-selectin trigger an immunoreceptor-like signaling cascade that enables αLβ2-mediated slow rolling on ICAM-1.

Selectin-induced signaling in Th1 cells requires the ITAM protein DAP12

In T cells, the best-characterized ITAMs are on subunits of the TCR (23). Using three complementary methods, we found that Th1 cells also express the ITAM-containing protein DAP12. As ascertained by flow cytometry, anti-DAP12 antibody stained Th1 cells as well as neutrophils from WT mice but not from DAP12-deficient (*Tyrobp*$^{-/-}$) mice (Fig. 3, A and B). Western blots of cell lysates confirmed DAP12 protein in Th1 cells and neutrophils from WT mice but not from DAP12-deficient mice (Fig. 3C). The presence of DAP12 in Th1 cells was not due to contaminating myeloid cells in the lysate, because antibodies to the myeloid markers Ly6G and the integrin αM subunit (CD11b) reacted only with neutrophil lysate. RT-PCR confirmed mRNA transcripts for DAP12 in both Th1 cells and macrophages from WT

mice but not from DAP12-deficient mice (Fig. 3D). Transcripts for the TCR β subunit were found only in Th1 cells, whereas transcripts for the integrin αM subunit were found only in macrophages, ruling out cell mixing. WT and DAP12-deficient Th1 cells expressed comparable levels of selectin ligands and integrins (Supplemental Fig. 2, A–D). They also expressed CD28 (Supplemental Fig. 2E), which distinguishes them from a T cell line that expresses DAP12 but not CD28 (24).

When plated on immobilized E-selectin, but not on a control protein, Th1 cells from both WT and DAP12-deficient mice phosphorylated SFKs, but only WT Th1 cells phosphorylated the downstream effectors ZAP-70 and p38 MAPK (Fig. 4A). Chelating extracellular Ca^{2+} with EDTA prevented E-selectin from phosphorylating SFKs or its downstream effectors, confirming its specificity. Th1 cells lacking DAP12 failed to roll more slowly on E-selectin and ICAM-1, whereas Th1 cells from WT or FcRγ-deficient mice exhibited normal slow rolling (Fig. 4, B and C). These data demonstrate that DAP12 expressed in Th1 cells is required for selectin-initiated signaling to activate ZAP-70 and other components that lead to activation of integrin αLβ2.

Selectin-induced signaling in Th1 cells requires the Tec family kinase Rlk/Txk

Neutrophils and T cells recruit the adaptor SLP-76 to activated Syk or ZAP-70, respectively (42,43). SLP-76 recruits other effectors that include Tec family kinases (44). In neutrophils, selectin signaling recruits the Tec family kinase Btk to SLP-76 (17,19). Btk then phosphorylates downstream mediators that lead to activation of integrin αLβ2. T cells express three Tec family kinases: Itk, Rlk/Txk, and Tec (45). Unlike Th1 cells from WT and Itk-deficient ($Itk^{-/-}$) mice, Th1 cells from Rlk/Txk-deficient ($Txk^{-/-}$) mice did not exhibit integrin-dependent slow rolling on ICAM-1 coimmobilized with E-selectin (Fig. 4D) or P-selectin (Fig. 4E). Rlk/Txk-deficient Th1 cells rolling on E-selectin in TNF-α-stimulated venules also failed to undergo integrin-dependent slow rolling (Fig. 4F). WT and Rlk/Txk-deficient Th1 cells expressed comparable levels of selectin ligands and integrins (Supplemental Fig. 3, A–E). These results demonstrate that Th1 cells rolling on selectins use the Tec family kinase Rlk/Txk to promote integrin-dependent slow rolling on ICAM-1.

Th1 cells use cooperative DAP12- and chemokine-dependent signals to promote integrin-dependent slow rolling, arrest, and adhesion strengthening

In neutrophils, β2 integrin activation does not require selectin signals when chemokines are abundant (13). However, selectin signals cooperate with limiting chemokine signals to activate integrins in vitro and in vivo (13,14). Th1 cells express CXCR3, a receptor for the chemokine CXCL10 (46). We flowed Th1 cells from WT or DAP12-deficient mice over E-selectin and ICAM-1, with or without CXCL10 coimmobilized at a concentration of 2 μg/ml. At this chemokine concentration, rolling WT Th1 cells rapidly arrested and spread (Fig. 5A). DAP12-deficient Th1 cells also arrested, but fewer cells spread. In both genotypes, pretreating Th1 cells with pertussis toxin (PTx), which disables the $G\alpha_i$ subunit of chemokine receptors, prevented arrest and spreading. Arrested neutrophils initiate integrin outside-in signals that cause spreading, which strengthens adhesion. We plated Th1 cells on immobilized anti-β2 integrin mAb, an ICAM-1 surrogate that induces spreading (29). DAP12-deficient Th1 cells adhered normally but spread less effectively than WT cells (Fig.

5B). This result suggests that integrin outside-in signaling in Th1 cells requires DAP12 to induce spreading. Indeed, when incubated on anti-β2 integrin mAb, both WT and DAP12-deficient Th1 cells activated SFKs (Fig. 5C). Unlike WT Th1 cells, DAP12-deficient Th1 cells did not activate the tyrosine kinase ZAP-70 or the Vav guanine nucleotide exchange proteins, which trigger Rho kinase-dependent actin polymerization (47–49). These results demonstrate that integrin outside-in signaling in Th1 cells requires the ITAM protein DAP12 to activate ZAP-70 and other signaling components that strengthen adhesion.

When CXCL10 was coimmobilized at a 10-fold lower concentration (0.2 μg/ml), WT Th1 cells rolled for longer periods before arresting. They also rolled slower than on E-selectin and ICAM-1 alone (Fig. 5D). PTx treatment prevented the slower, chemokine-dependent rolling, but selectin-triggered slow rolling remained intact (Fig. 5D). PTx treatment prevented arrest (Fig. 5E). DAP12-deficient cells rolled faster than WT cells on E-selectin and ICAM-1, and PTx treatment eliminated slow rolling (Fig. 5D). Fewer DAP12-deficient cells arrested and spread (Fig. 5E). These results demonstrate that, at limiting chemokine concentrations, DAP12-dependent selectin signals cooperate with PTx-sensitive chemokine signals to activate integrin αLβ2.

Th1 cells use cooperative DAP12- and chemokine-dependent signals to promote homing into antigen-challenged tissues

In mice sensitized with oxazolone, Th1 cells are recruited to the ear after local challenge (50). The inflamed ear expresses several chemokines that interact with receptors on Th1 cells (51). We compared the contributions of DAP12- and chemokine-dependent signals to Th1 cell homing in this model of contact hypersensitivity. Equal numbers of differentially labeled WT and DAP12-deficient Th1 cells were incubated with control buffer or with PTx to disable chemokine receptors. The fluorescent cell mixture was intravenously injected into sensitized WT mice 16 h after local challenge. Three hours after injection, flow cytometry was used to quantify the numbers of labeled Th1 cells still circulating in blood or recruited to the challenged ear. The numbers of circulating WT and DAP12-deficient Th1 cells remained equivalent (Fig. 5F). Without PTx treatment, ~60% fewer DAP12-deficient than WT cells entered the ear. PTx treatment reduced recruitment of WT cells by ~60% and eliminated recruitment of DAP12-deficient cells. These results demonstrate that Th1 cells use cooperative DAP12- and chemokine-dependent signals for recruitment into the inflamed ear.

Th1 cells use Rlk/Txk for homing to antigen-challenged tissues but not for integrin outside-in signaling

The reduced homing of DAP12-deficient Th1 cells to the challenged ear could reflect impaired selectin signals that activate αLβ2 as well as defective integrin outside-in signals that strengthen adhesion. Th1 cells rolling on selectins required the Tec kinase Rlk/Txk to induce integrin-dependent slow rolling on ICAM-1 (see Fig. 4). Unlike DAP12-deficient Th1 cells, Rlk/Txk-deficient Th1 cells spread normally when perfused over immobilized E-selectin, ICAM-1, and CXCL10 (Fig. 6A). They also spread normally on immobilized anti-β2 integrin mAb (Fig. 6B), indicating that integrin outside-in signaling does not require Rlk/Txk to strengthen adhesion. Nevertheless, significantly fewer Rlk/Txk-deficient Th1

cells entered the oxazolone-challenged ear (Fig. 6C). These data suggest that defects in selectin-triggered integrin activation are sufficient to reduce Th1 cell recruitment.

$CD4^+$ effector T cells generated in vivo also use DAP12-dependent signals to activate integrin αLβ2 and promote homing into antigen-challenged tissues

In experiments described thus far, we used a widely used method to generate Th1 cells in vitro by stimulating $CD4^+$ splenocytes with anti-CD3ε, anti-CD28, IL-12, and anti-IL-4. To confirm the physiological relevance of our findings, we sensitized WT or DAP12-deficient mice with topically applied oxazolone. After six days, we isolated $CD4^+$ lymphocytes from draining lymph nodes (Supplemental Fig. 4A). Approximately 30% were memory cells, characterized by lower levels of the CD45 RB isoform and higher levels of CD44 (30,52) (Supplemental Fig. 4B). Effector T cells express functional selectin ligands that enable rolling on P- or E-selectin under flow (1,4,30,53,54). Immunofluorescence microscopy revealed that ~60% of $CD4^+$ cells adherent to immobilized E-selectin expressed interferon γ, consistent with a Th1 or Th17 phenotype (55) (Supplemental Fig. 4C). Effector cells generated in vivo from both WT and DAP12-deficient mice rolled on E-selectin, but only WT cells rolled slower on coimmobilized ICAM-1 (Fig. 7A). Pretreating cells with an inhibitor to SFKs (PP2) or to p38 MAPK (SB203580) blocked the slower rolling of WT cells on ICAM-1. A vehicle control (DMSO) or an inactive analogue of PP2 (PP3) had no effect. These data demonstrate that $CD4^+$ effector T cells generated in vivo also exhibit selectin-initiated, integrin-dependent slow rolling that requires SFKs, DAP12, and p38 MAPK.

We perfused $CD4^+$ effector T cells generated in vivo from WT or DAP12-deficient mice over E-selectin and ICAM-1, with or without CXCL10 coimmobilized at a concentration of 2 μg/ml. At this chemokine concentration, rolling WT cells rapidly arrested and spread (Fig. 7B). DAP12-deficient cells also arrested, but fewer cells spread. In both genotypes, pretreating cells with PTx prevented arrest and spreading. When CXCL10 was coimmobilized at a 10-fold lower concentration (0.2 μg/ml), WT cells rolled for longer periods before arresting. They also rolled slower than on E-selectin and ICAM-1 alone (Fig. 7C). PTx treatment prevented the slower, chemokine-dependent rolling, but selectin-triggered slow rolling remained intact (Fig. 7C). PTx treatment prevented arrest (Fig. 7D). DAP12-deficient cells rolled faster than WT cells on E-selectin and ICAM-1, and PTx treatment eliminated slow rolling (Fig. 7C). Fewer DAP12-deficient cells arrested and spread (Fig. 7D). These results with $CD4^+$ effector T cells generated in vivo replicate those with Th1 cells generated in vitro. They confirm that, at limiting chemokine concentrations, DAP12-dependent selectin signals cooperate with PTx-sensitive chemokine signals to activate integrin αLβ2 in Th1 cells.

We injected equal numbers of differentially labeled WT and DAP12-deficient $CD4^+$ effector T cells generated in vivo into oxazolone-sensitized WT mice 16 h after local challenge to the ear. Three hours after injection, flow cytometry was used to quantify the numbers of labeled effector T cells circulating in blood or recruited to the challenged ear. The numbers of circulating WT and DAP12-deficient cells remained equivalent, but ~60% fewer DAP12-deficient than WT cells entered the ear (Fig. 7E). These results confirm that effector T cells

generated in vivo, like those generated in vitro, use DAP12-dependent signals for recruitment into the inflamed ear.

Discussion

We have shown that Th1 cells rolling on selectins triggered an immunoreceptor-like signaling pathway that activated integrin αLβ2 to slow rolling on ICAM-1. Th1 cells expressed the ITAM protein DAP12, which was required for both selectin-induced signaling and integrin outside-in signaling. Selectin and chemokine signals cooperated to promote integrin-dependent Th1 cell arrest, spreading, and migration in vitro and in vivo (Fig. 8).

The selectin signaling cascade in Th1 cells resembled that in neutrophils, although there are cell-type-specific components. Th1 cells used the SFKs Lck and Fyn rather than Fgr, Hck, and Lyn; ZAP-70 rather than Syk; and the Tec kinase Rlk/Txk rather than Btk. In both Th1 cells and neutrophils, P-selectin engages PSGL-1 to initiate signaling (25,36). In neutrophils, E-selectin signals through PSGL-1 or CD44 (19). In Th1 cells, E-selectin signaled through PSGL-1 or CD43, but not through CD44, to enable αLβ2-dependent rolling on ICAM-1.

The signaling role of DAP12 in Th1 cells was unexpected. Other than in a CD28-negative cell line (24), T cells were not thought to express DAP12. All our Th1 cells, prepared by a commonly used protocol, expressed both CD28 and DAP12. Whether other effector T cells express DAP12 warrants reexamination. As in neutrophils (19,56), DAP12 in Th1 cells may colocalize with SFKs and selectin ligands in lipid rafts. Selectin interactions may concentrate raft components, enabling SFKs to phosphorylate DAP12, which in turn recruits ZAP-70 and other components into a signaling complex. We found that Th1 cells, like neutrophils, also used DAP12 to transmit outside-in signals through ligand-occupied β2 integrins. These signals activated Vav proteins, which induce Rho kinase-dependent actin polymerization that strengthens adhesion (47–49). In T cell lysates, integrin αLβ2 was shown to coprecipitate with SFKs and ZAP-70, suggesting direct or indirect associations that could facilitate outside-in signaling (57). Our results demonstrate that DAP12 is an essential component of this complex.

Selectin-induced signaling caused Th1 cells to roll slower on ICAM-1, but not to arrest. This is consistent with conversion of αLβ2 to an extended, intermediate-affinity conformation (11). Selectin-triggered slow rolling on ICAM-1 does not require an intact cytoskeleton (20). One study reported that Th1 cells rolling on P-selectin underwent cytoskeletal-dependent clustering of αLβ2 that mediated arrest on ICAM-1 (9). This unusual response may have been due to high densities of immobilized ICAM-1. Effector T cells rolling on selectins also exhibit chemokine-independent arrest on ICAM-1 if they express very high densities of αLβ2 (5,6). At these densities, low-affinity but high-avidity interactions of αLβ2 with ICAM-1 may permit rapid adhesion strengthening through outside-in signaling. We measured moderate densities of αLβ2 on Th1 cells generated in vitro, which likely resemble those on effector T cells generated by antigen stimulation in vivo. At these densities, chemokine signaling is required to activate αLβ2 to an extended, high-affinity conformation that mediates arrest and adhesion strengthening. We observed that selectin signaling in

$CD4^+$ effector T cells generated in vitro or in vivo enabled limiting concentrations of chemokines to trigger arrest and spreading.

During TCR signaling, activated ZAP-70 recruits the adaptor SLP-76, which recruits Tec kinases and other components into a signaling complex (23). Itk is the predominant Tec kinase for TCR-triggered activation of integrin αLβ2 (28). Its actions include Cdc42-dependent rearrangements of the actin cytoskeleton that strengthen adhesion (44). In neutrophils, selectin signaling activates the Tec kinase Btk, which contributes to integrin-dependent slow rolling on ICAM-1 (17,19). Integrin outside-in signaling also activates Btk, and Btk contributes to actin polymerization events that strengthen adhesion (58). Here we found that selectin-initiated signaling in Th1 cells employed Rlk/Txk rather than Itk to induce αLβ2-dependent slow rolling on ICAM-1. Unlike Itk or Btk, however, Rlk/Txk did not contribute to integrin outside-in signaling that strengthens adhesion.

In vivo, cooperative selectin and chemokine signaling promoted homing of Th1 cells into antigen-challenged ears, supporting the physiological significance of our findings. Homing was partially impaired by deleting DAP12 or Rlk/Txk to block selectin signaling or by treatment with PTx to prevent chemokine signaling. Blocking both pathways prevented homing. Because Rlk/Txk, unlike DAP12, does not mediate integrin outside-in signaling, the defective homing of Rlk/Txk-deficient Th1 cells reveals a key role for selectin signaling when chemokine concentrations are limiting. In an analogous manner, neutrophils use cooperative selectin and chemokine signaling to promote inflammation and thrombosis (13,14).

Our results define an additional mechanism to recruit effector T cells to sites of inflammation, which may guide new therapies for T cell-mediated inflammatory disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

- Th1 cells rolling on selectins trigger DAP12-dependent activation of integrin αLβ2
- DAP12 signaling in Th1 cells facilitates slow rolling, arrest, and migration

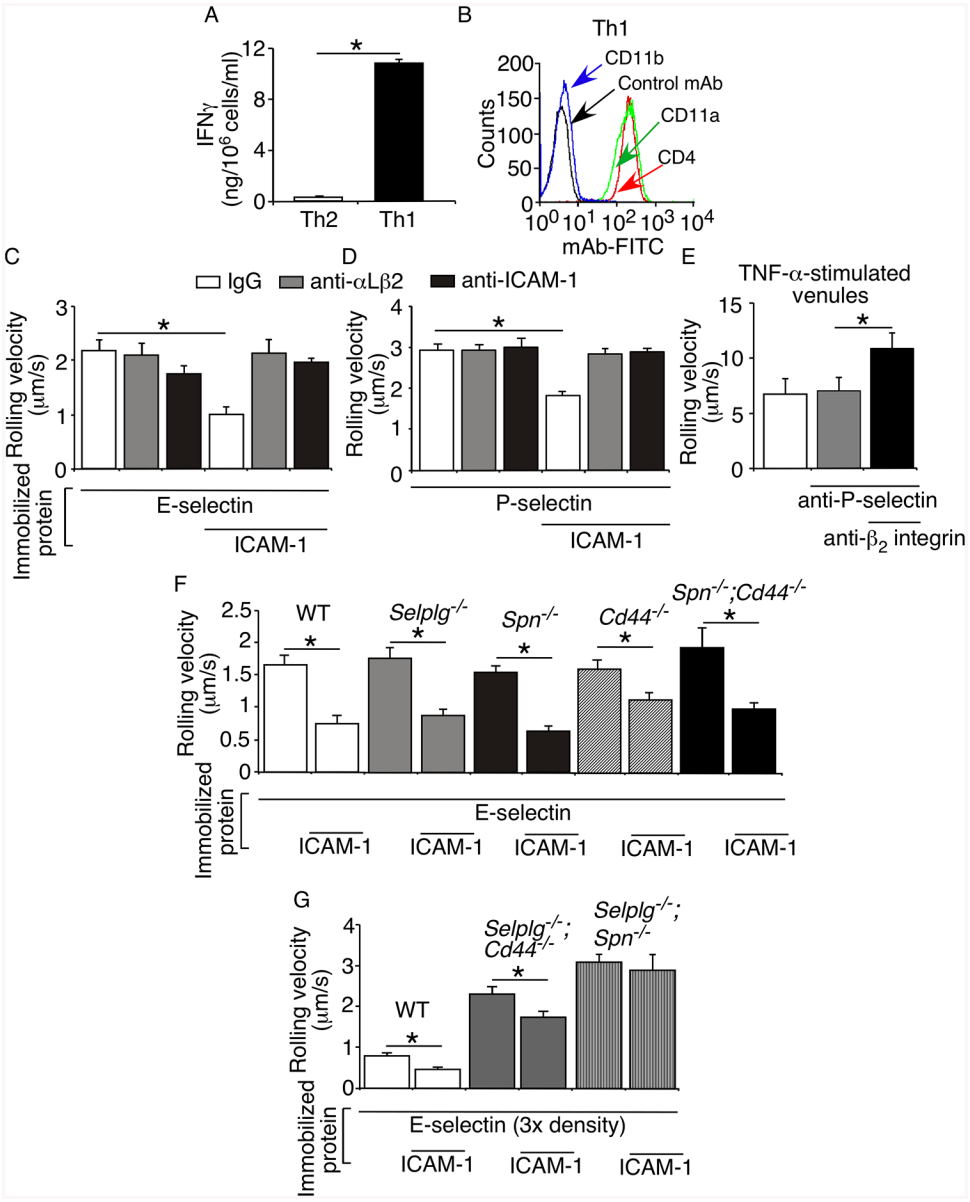

Fig. 1. Th1 cells rolling on P- or E-selectin trigger integrin αLβ2-mediated slow rolling on ICAM-1.

(**A**) Quantification of interferon γ (IFNγ) secreted by Th1 or Th2 cells. (**B**) Representative flow cytometric data for expression of the indicated protein on Th1 cells. **(C and D)** Rolling velocities of Th1 cells on E-selectin or P-selectin with or without coimmobilized ICAM-1 in the presence of an isotype control mAb or a blocking mAb to integrin αLβ2 or to ICAM-1. **(E)** Rolling velocities of Th1 cells in TNF-α-stimulated venules of cremaster muscle, measured before and after injecting a blocking mAb to P-selectin and then a blocking mAb to β2 integrins. **(F)** Rolling velocities of Th1 cells of the indicated genotype on E-selectin with or without coimmobilized ICAM-1. **(G)** Rolling velocities of Th1 cells of the indicated genotype on a three-fold higher density of E-selectin with or without coimmobilized ICAM-1. The data in **A, C, D, F,** and **G** are the mean ± SEM from three experiments. The

data in **B** are representative of three experiments. The data in **E** are the mean ± SEM from five experiments. *, $P < 0.05$.

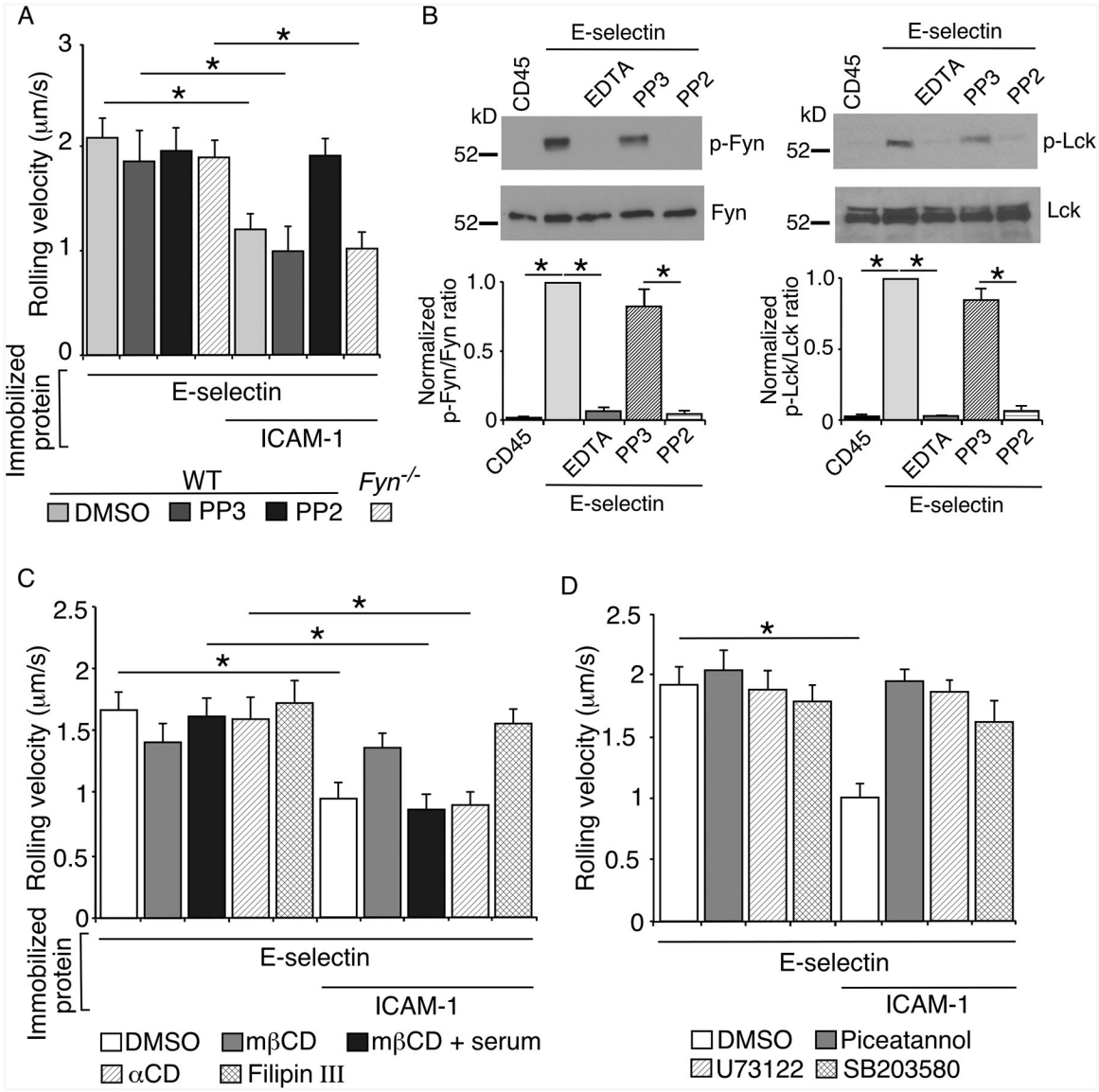

Fig. 2. Th1 cells rolling on E-selectin require SFKs, lipid rafts, ZAP-70, PLCγ, and p38 MAPK to induce integrin αLβ2-mediated slow rolling on ICAM-1.

(**A**) Rolling velocities of Th1 cells from WT or *Fyn*$^{-/-}$ mice on E-selectin with or without coimmobilized ICAM-1. The cells from WT mice were treated with the vehicle control DMSO, the SFK inhibitor PP2, or the inactive analogue PP3. **(B)** *Top*, Th1 cells treated with or without PP3 or PP2 were incubated on immobilized control CD45-IgM or E-selectin-IgM in buffer containing Ca^{2+} or as indicated, EDTA. Lysates were analyzed by immunoblotting with antibodies to total Fyn or Lck or to phosphorylated Fyn or Lck (p-Fyn or p-Lck). *Bottom*, the normalized ratio of p-Fyn/Fyn or p-Lck/Lck was measured by densitometry. The data are the mean ± SD of three experiments. (**C**) Rolling velocities of Th1 cells on E-selectin with or without coimmobilized ICAM-1 in the presence or absence of the vehicle control DMSO, methyl-β-cyclodextrin (mβCD) or its inactive analog α-cyclodextrin (αCD), mβCD plus 15% serum, or filipin III. **(D)** Rolling velocities of Th1 cells on E-selectin with or without coimmobilized ICAM-1 in the presence or absence of the vehicle control DMSO,

the Syk/ZAP-70 inhibitor piceatannol, the PLCγ inhibitor U73122, or the p38 MAPK inhibitor SB203580. The data in **A**, **C**, and **D** are the mean ± SEM from three experiments. *, $P < 0.05$.

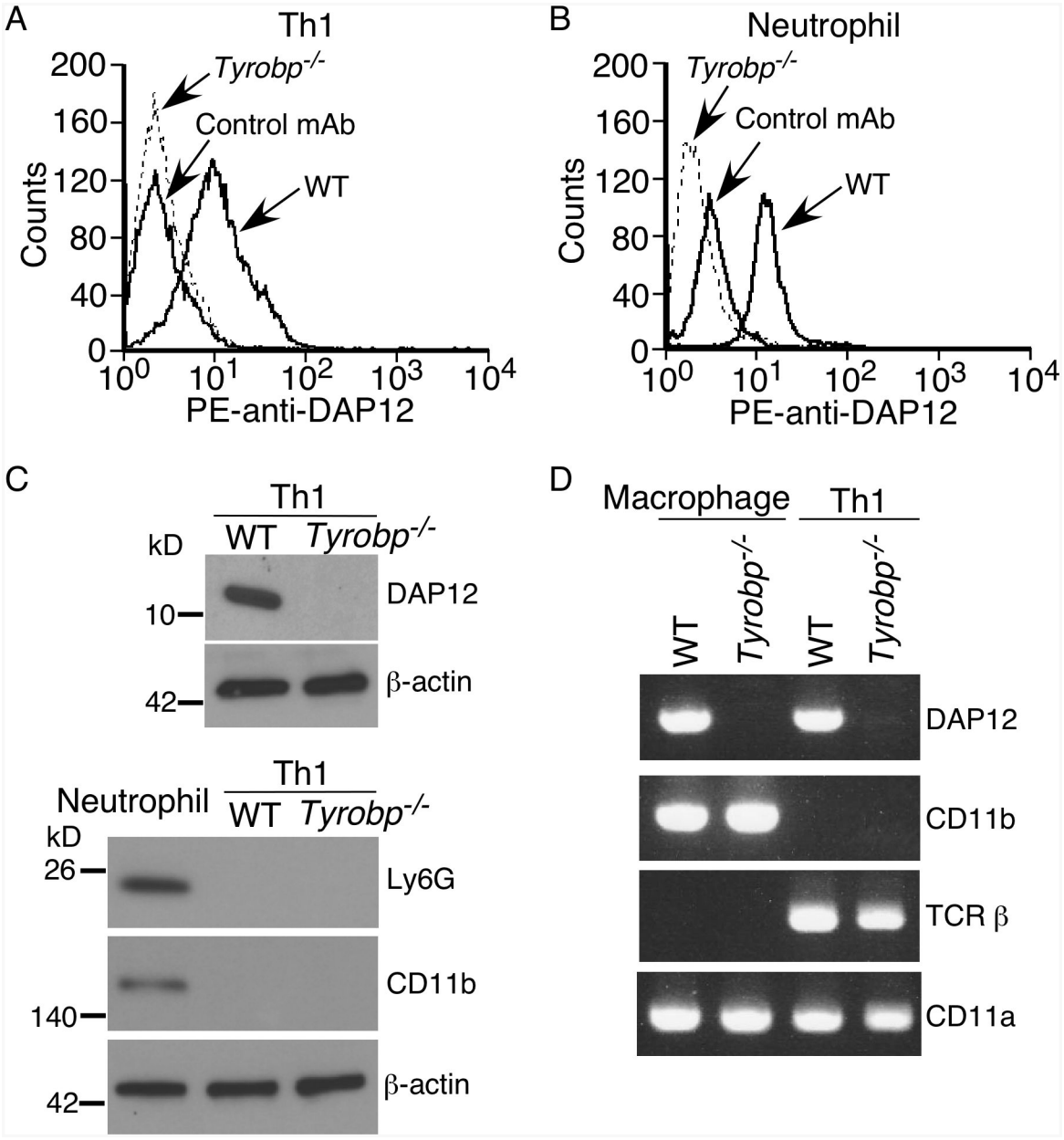

Fig. 3. Th1 cells express the ITAM protein DAP12.

(A and B) Flow cytometric analysis of DAP12 expression on Th1 cells or neutrophils from WT or DAP12-deficient (*Tyrobp*$^{-/-}$) mice. **(C)** Lysates of Th1 cells or neutrophils from WT or *Tyrobp*$^{-/-}$ mice were analyzed by immunoblotting with the indicated antibodies. **(D)** Total RNA was extracted from bone marrow-derived macrophages or Th1 cells from WT or *Tyrobp*$^{-/-}$ mice. RT-PCR was used to amplify cDNA encoding the indicated protein. The amplified products were analyzed by electrophoresis in an agarose gel. The data in **A-D** are representative of at least three experiments.

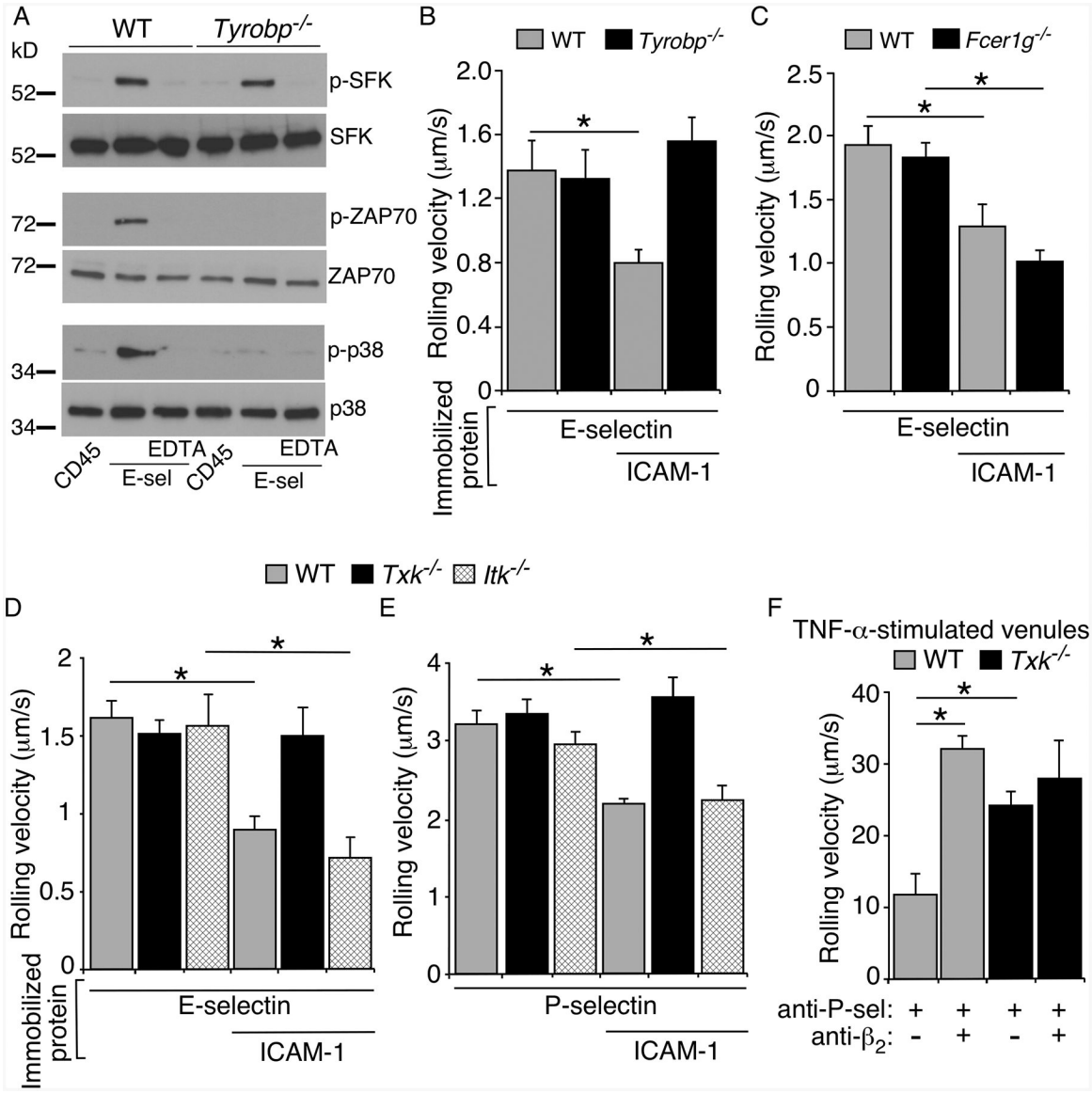

Fig. 4. Selectin-induced signaling in Th1 cells requires the ITAM protein DAP12 and the Tec family kinase Rlk/Txk.

(**A**) Th1 cells from WT or DAP12-deficient (*Tyrobp*$^{-/-}$) mice were incubated on immobilized control CD45-IgM (CD45) or E-selectin-IgM (E-sel) in buffer containing Ca^{2+} or as indicated, EDTA. Lysates were analyzed by immunoblotting with antibodies to total or phosphorylated SFKs, ZAP-70, or p38 MAPK. (**B**) Rolling velocities of Th1 cells from WT or DAP12-deficient (*Tyrobp*$^{-/-}$) mice on E-selectin with or without coimmobilized ICAM-1. (**C**) Rolling velocities of Th1 cells from WT or FcRγ-deficient (*Fcer1g*$^{-/-}$) mice on E-selectin with or without coimmobilized ICAM-1. (**D**) Rolling velocities of Th1 cells from WT, Rlk/Txk-deficient (*Txk*$^{-/-}$), or Itk-deficient (*Itk*$^{-/-}$) mice on E-selectin with or without coimmobilized ICAM-1. (**E**) Rolling velocities of Th1 cells from WT, Rlk/Txk-deficient (*Txk*$^{-/-}$), or Itk-deficient (*Itk*$^{-/-}$) mice on P-selectin with or without coimmobilized ICAM-1. (**F**) Rolling velocities of Th1 cells from WT or Rlk/Txk-deficient (*Txk*$^{-/-}$) mice in

TNF-α-stimulated venules of cremaster muscle of WT mice after injecting a blocking mAb to P-selectin (P-sel) with or without a blocking mAb to β2 integrins. The data in **A** are representative of three experiments. The data in **B-E** are the mean ± SEM from three experiments. The data in **F** are the mean ± SEM of 6–12 cells in each of five experiments. *, $P < 0.05$.

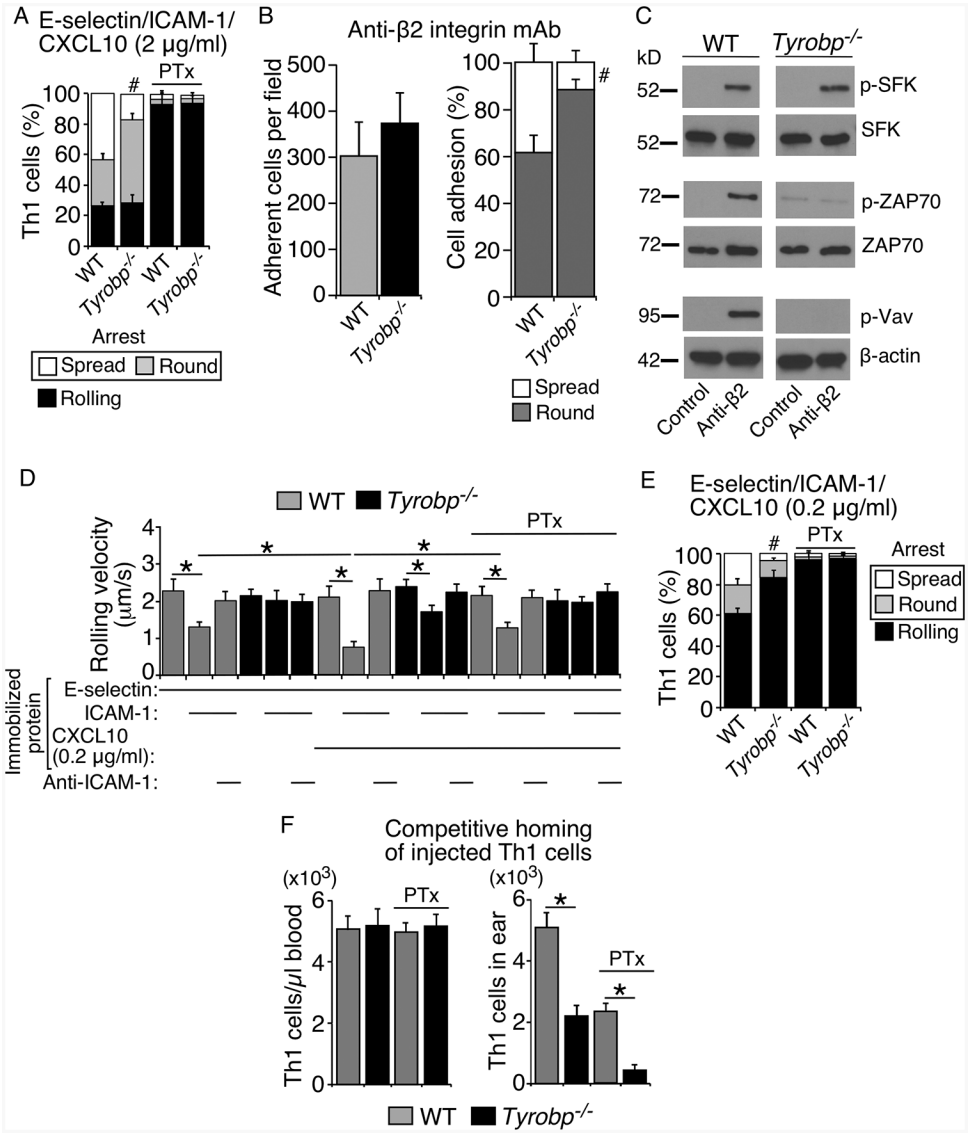

Fig. 5. Th1 cells use cooperative DAP12- and chemokine-dependent signals to promote integrin-dependent slow rolling, arrest, adhesion strengthening, and homing to antigen-challenged tissues.

(**A**) Percentages of untreated or PTx-treated Th1 cells from WT or DAP12-deficient (*Tyrobp*$^{-/-}$) mice rolling, arrested and round, or arrested and spread on coimmobilized E-selectin, ICAM-1, and CXCL10 (2 μg/ml). (**B**) Number of adherent Th1 cells (left) and percentages of adherent round or spread Th1 cells (right) from WT or *Tyrobp*$^{-/-}$ mice on immobilized F(ab')2 fragments of anti-β2 integrin mAb. (**C**) Th1 cells from WT or *Tyrobp*$^{-/-}$ mice were incubated on immobilized F(ab')2 fragments of isotype control IgG or anti-β2 integrin mAb. Lysates were immunoblotted with the indicated antibodies. (**D**) Rolling velocities of untreated or PTx-treated Th1 cells from WT or *Tyrobp*$^{-/-}$ mice on E-selectin coimmobilized with or without ICAM-1 and low-dose CXCL10 (0.2 μg/ml) in the presence or absence of anti-ICAM-1 mAb. (**E**) Percentages of untreated or PTx-treated Th1 cells from WT or *Tyrobp*$^{-/-}$ mice rolling, arrested and round, or arrested and spread on coimmobilized E-selectin, ICAM-1, and low-dose CXCL10 (0.2 μg/ml). (**F**) Equal numbers of differentially

labeled WT and DAP12-deficient (*Tyrobp*$^{-/-}$) Th1 cells were incubated with or without PTx. The 1:1 cell mixture was intravenously injected into oxazolone-sensitized WT mice 16 h after antigen challenge to the ear. Three hours after injection, flow cytometry was used to quantify the numbers of labeled Th1 cells still circulating in blood or recruited to the challenged ear. The data in **C** are representative of three experiments. The data in **A, B, D,** and **E** are the mean ± SEM from three experiments. The data in **C** are representative of three experiments. The data in **F** are the mean ± SEM from five experiments. *, $P < 0.05$; #, $P < 0.05$ for spreading of WT vs. *Tyrobp*$^{-/-}$ cells.

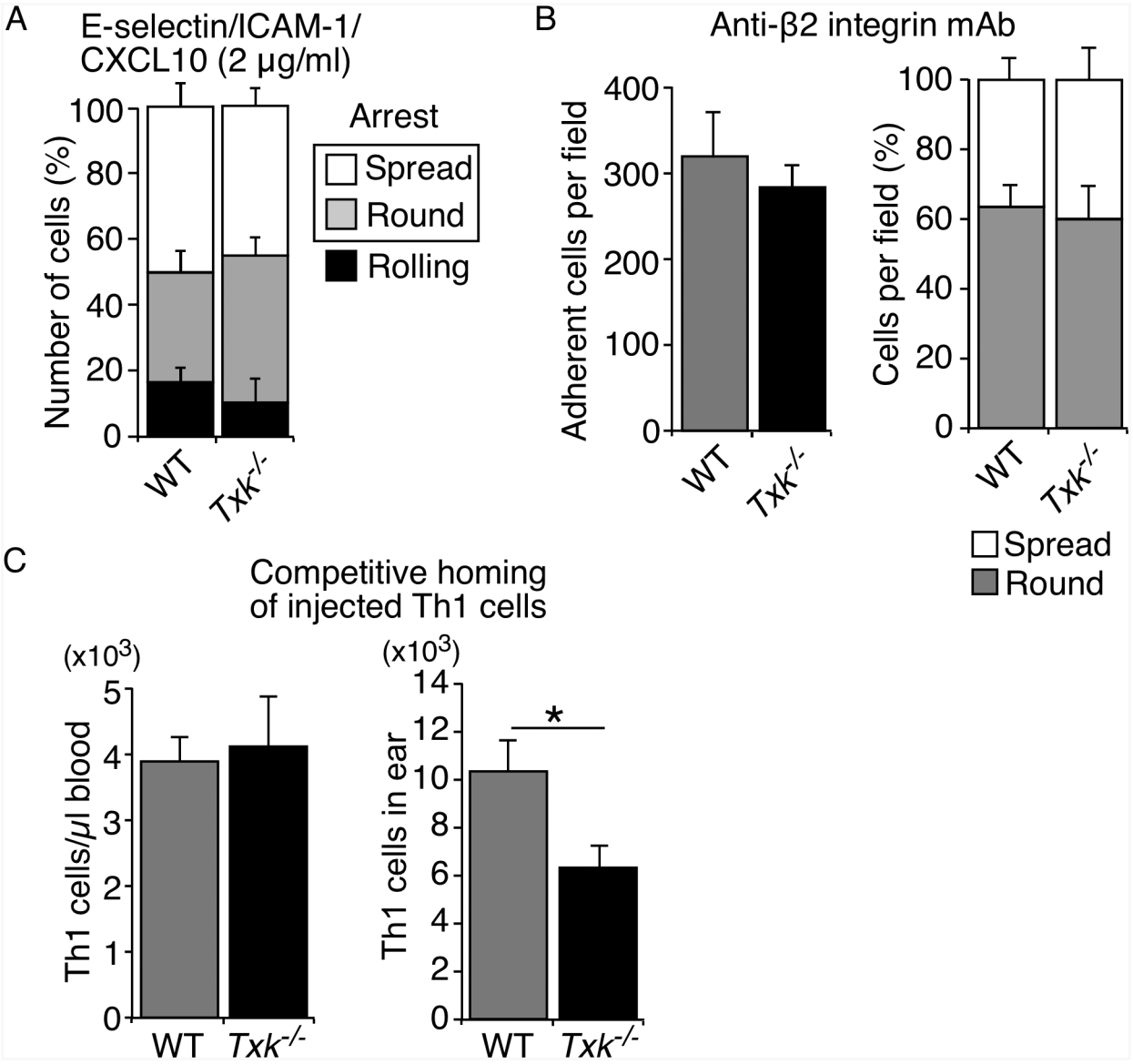

Fig. 6. Th1 cells use Rlk/Txk for homing to antigen-challenged tissues but not for integrin outside-in signaling.

(**A**) Percentages of Th1 cells from WT or *Txk*$^{-/-}$ mice rolling, arrested and round, or arrested and spread on coimmobilized E-selectin, ICAM-1, and CXCL10 (2 μg/ml). (**B**) Number of adherent Th1 cells (left) and percentages of adherent round or spread Th1 cells (right) from WT or *Txk*$^{-/-}$ mice on immobilized F(ab')2 fragments of anti-β2 integrin mAb. (**C**) Equal numbers of differentially labeled WT or *Txk*$^{-/-}$ Th1 cells were intravenously injected into oxazolone-sensitized WT mice 16 h after antigen challenge to the ear. Three hours after injection, flow cytometry was used to quantify the numbers of labeled Th1 cells still circulating in blood or recruited to the challenged ear. The data in **A and B** are the mean ± SEM from three experiments. The data in **C** are the mean ± SEM from five experiments. *, $P < 0.05$.

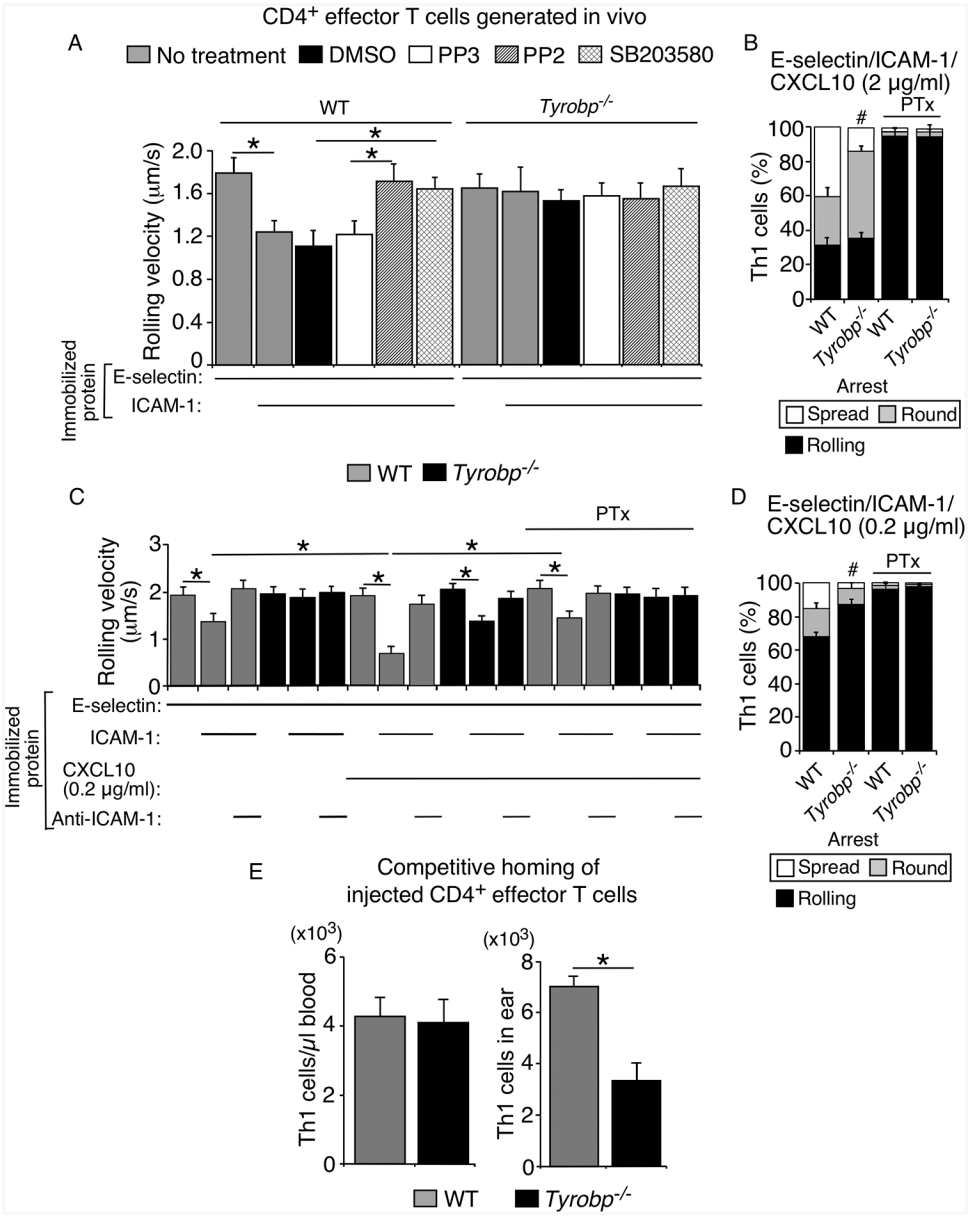

Fig. 7. CD4$^+$ effector T cells generated in vivo also use DAP12-dependent signals to activate integrin αLβ2 and promote homing into antigen-challenged tissues.

(**A**) Rolling velocities of CD4$^+$ effector T cells generated in vivo from WT or DAP12-deficient (*Tyrobp*$^{-/-}$) mice on E-selectin with or without coimmobilized ICAM-1. The cells were treated with or without the vehicle control DMSO, the SFK inhibitor PP2 or its inactive analogue PP3, or the p38 MAPK inhibitor SB203580. (**B**) Percentages of untreated or PTx-treated CD4$^+$ effector T cells generated in vivo from WT or DAP12-deficient (*Tyrobp*$^{-/-}$) mice rolling, arrested and round, or arrested and spread on coimmobilized E-selectin, ICAM-1, and CXCL10 (2 μg/ml). (**C**) Rolling velocities of untreated or PTx-treated CD4$^+$ effector T cells generated in vivo from WT or *Tyrobp*$^{-/-}$ mice on E-selectin coimmobilized with or without ICAM-1 and low-dose CXCL10 (0.2 μg/ml) in the presence or absence of anti-ICAM-1 mAb. (**D**) Percentages of untreated or PTx-treated CD4$^+$ effector T cells

generated in vivo from WT or *Tyrobp*$^{-/-}$ mice rolling, arrested and round, or arrested and spread on coimmobilized E-selectin, ICAM-1, and low-dose CXCL10 (0.2 μg/ml). (**E**) Equal numbers of differentially labeled WT and DAP12-deficient (*Tyrobp*$^{-/-}$) CD4^{+} effector T cells generated in vivo were intravenously injected into oxazolone-sensitized WT mice 16 h after antigen challenge to the ear. Three hours after injection, flow cytometry was used to quantify the numbers of labeled cells still circulating in blood or recruited to the challenged ear. The data are the mean ± SEM from three experiments.

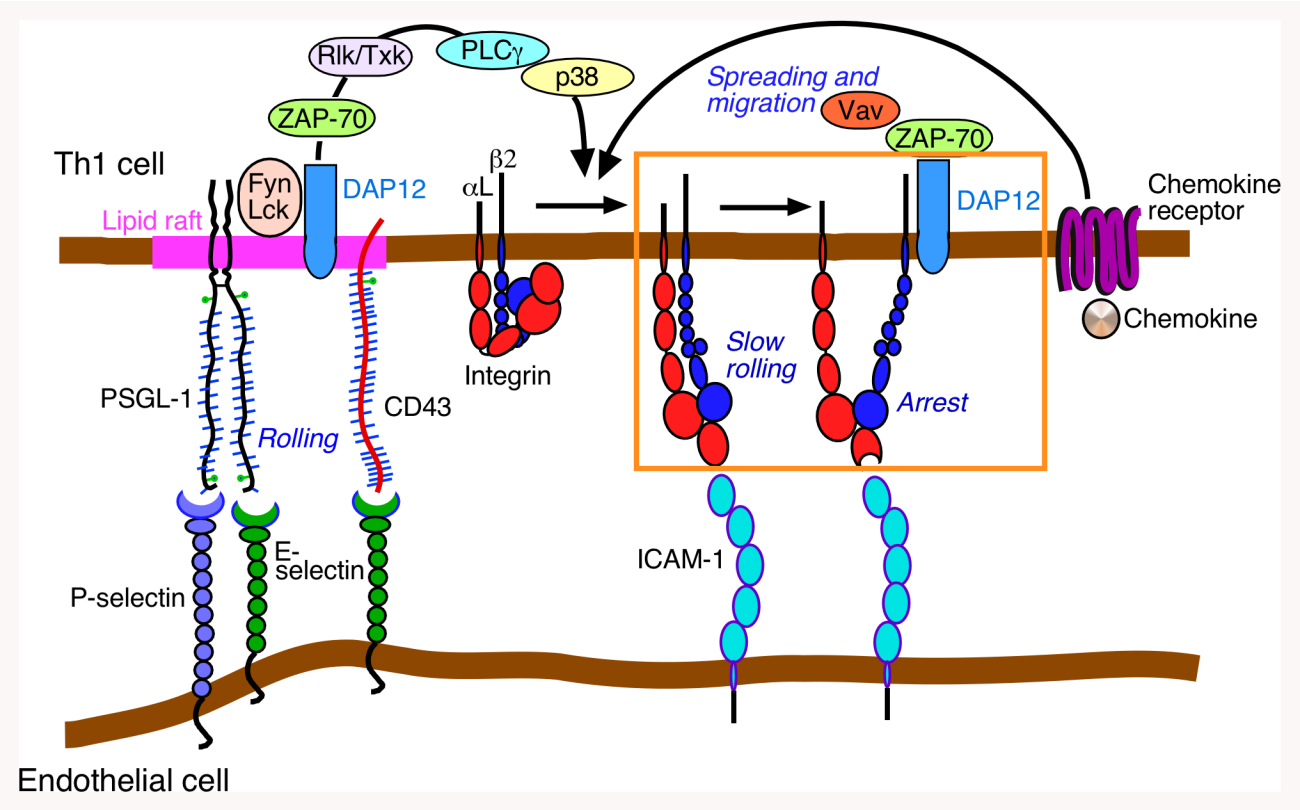

Fig. 8. Selectin, chemokine, and integrin outside-in signaling pathways in Th1 cells.
See Discussion for details.