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_J Immunol_ published online 3 March 2014
http://www.jimmunol.org/content/early/2014/03/01/jimmunol.1302585

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/03/01/jimmunol.1302585.5.DCSupplemental

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αPIX RhoGEF Supports Positive Selection by Restraining Migration and Promoting Arrest of Thymocytes

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Thymocytes mature in a series of stages by migrating through specific areas of the thymus and interacting with other cells to receive the necessary developmental signals; however, little is known about the molecular mechanisms governing this migration. We report that murine thymocytes with a knockout mutation in α-PAK (p21-activated kinase)-interacting exchange factor (PIX; Arhgef6), an activator of Rho GTPases, showed greatly increased motility and altered morphology in two-dimensional migration on ICAM-1. αPIX was also required for efficient positive selection, but not negative selection, of thymocytes. TCR signaling was normal in αPIX− thymocytes, indicating that the effects of αPIX on positive selection are largely independent of TCR signaling. αPIX− thymocytes also paused less during migration in the thymic cortex, interacted less with ICAM-1 coated beads, and could overcome TCR stop signals, consistent with defective scanning behavior. These results identify αPIX as a regulator of thymocyte migration and subsequent arrest that is linked to positive selection. The Journal of Immunology, 2014, 192:000–000.

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stop signals and ICAM-1–mediated contacts, although general TCR-induced signaling was normal, highlighting a probable role for αPix in thymocyte scanning of cTECs. These results show that αPix restrains migration and consequently promotes arrest, and that both functions correlate with efficient positive selection.

Materials and Methods
Mice and cell lines
αPix−/− mice (12) were backcrossed on the BALB/C (Charles River) or C57BL/6J (Charles River) background for more than 10 generations and then intercrossed with D011.10+ (16), OT-II+ (17), and H-Y+ (18) mice. For two-photon analysis, αPix−/− mice (C57BL/6) were crossed to homozygous GFP mice (19) and rosa26 tdRFP mice (20). Expression of RFP was induced by pTα−Cre (20). Gi2α−/− mice (21) on a C57BL/6 background were crossed to OT-II+ mice, and heterozygous offspring were analyzed.

OP9-DL1 cells have been described previously (22). Reconstructed mice were generated by transferring 2 × 10⁶ bone marrow cells from TCR transgenic D011.10 wild type or αPix−/− BALB/C mice to lethally irradiated (1000 rad) hosts. Animals were housed in specific pathogen–free conditions according to institutional guidelines.

In vitro cell migration
Cell migration on 2D surfaces was analyzed by coating the wells of a μ-Slide (Ibidi, Martinsried, Germany) with 10 μg/ml recombinant mouse ICAM-1/human Fc chimera (R&D Systems), or both, as indicated and blocked with 2% BSA. Thymocytes were stained with 0.2 μM CFDA-SE or 2 μM orange CMRA (Life Technologies) and resuspended in buffer containing 1 mM Ca²⁺ and Mg²⁺. Soluble CXCL12 was added at 200 ng/ml where indicated. For migration on stromal cells, thymocytes were stained as above and seeded on OP9-DL1 cell monolayers. For analysis of thymocytes interacting with ICAM−1-coated beads, polystyrene microparticles (3 μm) (Polysciences) were incubated with ICAM-1/human Fc chimera (R&D Systems). Beads were added to thymocytes on glass slides that were precoated with poly-l-lysine. Cells were imaged with a Leica DMI 6000 B inverted wide-field microscope. Image data were processed with Leica Application Suite software. Raw data of cell tracks (acquired with the manual tracking plug-in from ImageJ) were used to determine mean speed, displacement, and arrest coefficients.

Two-photon microscopy of explanted thymic lobes
Two-photon imaging was performed as described (1) with some changes. For the generation of partial mixed bone marrow chimeras, C57BL/6 mice were lethally irradiated at 800 rad and injected with 2 × 10⁶ mixed bone marrow cells from nontransgenic wild type C57BL/6 mice (90%), and GFP- and RFP-expressing wild type or αPix−/− C57BL/6 mice (5% each). The thymic ventral lobes from mixed bone marrow chimeras were held in place by a washer strung with filaments in a dish and placed in a 37°C incubator on a Zeiss LSM710 microscopic stage equipped with a MaiTai DeepSee laser (Spectra-Physics, Mountain View, CA) tuned to 850–900 nm and a 20× NA 1.0 water dipping lens. GFP*, RFP*, cells, and second harmonic generation signals of the capsule were detected using two-photon microscopy, 18-μm-thick sections were stained with anti–CD8-PE and anti–CD4-APC Abs.

Flow cytometry
Cells were labeled with Abs listed in Supplemental Table I and analyzed using a BD FACS Canto II flow cytometer, BD FACS Diva software, and FlowJo (Tree Star). For intracellular protein detection, cells stained for surface markers were fixed with 4% paraformaldehyde, permeabilized with BD Perm/Wash solution (BD Biosciences), and incubated with the indicated Abs against intracellular proteins. For conjugate formation of thymocytes and B cells, CD43− B cells were purified on an autoMACS Pro (Miltenyi Biotec), and pulsed overnight with 10 μg/ml OVA peptide (JPT Peptide Technologies) in the presence of 30 μg/ml LPS (Sigma-Aldrich). B cells were mixed at a 1:1 ratio with OT-II+ thymocytes and analyzed for conjugate formation by FACS of CD8* B220− cell doublets (>95% of CD8+ cells in the thymus are DP thymocytes) as described (12). For transwell migration assays, 5-μm-pore membranes (Corning Costar) were coated with 3 μg/ml recombinant mouse ICAM-1/human Fc chimera (R&D Systems) plus anti-CD3 (2C11) where indicated and blocked with 2% BSA. Migration of cells to CXCL12 (200 ng/ml) or CCL25 (100 ng/ml) was analyzed as described (12).

Thymic section imaging
Freshly prepared thymi were embedded in tissue freezing medium (Leica Microsystems) and snap frozen in methyl butane at −80°C. For immunofluorescence microscopy, 18-μm-thick sections were stained with anti–CD8-PE and anti–CD4-APC Abs.

In vivo BrdU incorporation assay
Mice were injected i.p. with 20 mg/ml BrdU and fed drinking water containing BrdU (1 mg/ml) with 2% glucose for 2 d. BrdU incorporation in thymocytes stained for CD4 and CD8 was analyzed by FACS with the BrdU Flow Kit (APC or FITC, BD Biosciences).

Results
Increased migration of αPix+ thymocytes on 2D surfaces
In transwell migration assays, αPix−/− T and B cells show increased chemokinesis and increased chemotaxis (12). To determine whether αPix+ thymocytes also show increased migration in vitro, we measured the transwell migration of all thymocyte subsets (DN, DP, CD4+SP, and CD8+SP) to CXCL12 (also known as SDF-1) and CCL25 (also known as TECK). In all cases, including the cells without chemokines, αPix+ thymocyte migration was greatly increased compared with wild type thymocytes (Fig. 1A). Receptors for CXCL12 (CXC4R) and CCL25 (CCR9) were expressed normally on αPix− thymocytes (Supplemental Fig. 1). Thus, αPix restricts not only chemokine-induced migration, but also basal migration of thymocytes.

Lymphocytes use integrins to generate traction force in certain contexts, such as during transendothelial or 2D surface migration (25, 26). Pix and GIT proteins associate with many integrin-regulating proteins and are involved in integrin-dependent functions (10, 27). To determine whether the increased transwell migration of αPix knockout thymocytes is also evident in an
integrin-dependent context and corresponds to cellular velocity, we analyzed αPix− thymocyte 2D motility on slides coated with immobilized ICAM-1 (LFA-1 ligand) in the presence or absence of CXCL12. An overlay of trajectories of individual thymocytes confirmed that αPix− thymocytes were appreciably more motile than wild type thymocytes on surfaces coated with immobilized ICAM-1 where indicated. Images were recorded at 10× magnification every 10 s for 30 min. (B) Cell tracks from one of four representative experiments, scaled to 80 μm. (C) Average speeds of all cells from four experiments (n = 355, 367, 341, and 256). (D) Average displacement from origin in 30 min combining four experiments, as in (C), and (E) arrest coefficients of thymocytes from two experiments (n = 100 each). Single-cell data are shown with the median ± interquartile range.*p < 0.05, **p < 0.001, ***p < 0.0001 (Mann–Whitney U test).

FIGURE 1. Increased motility of αPix− thymocytes in vitro. (A) Transwell migration to CXCL12 or CCL25. Results expressed as the mean percentage ± SD of migrated cells to input cells, from one of three representative experiments. *p < 0.05 (Student t test). (B–E) Live cell imaging of thymocyte migration on 2D substrates. Wild type and αPix− thymocytes stained with CFSE (green) or CMRA (red), respectively, were plated together on immobilized ICAM-1 and treated with soluble CXCL12 where indicated. Images were recorded at 10× magnification every 10 s for 30 min. (B) Cell tracks from one of four representative experiments, scaled to 80 μm. (C) Average speeds of all cells from four experiments (n = 355, 367, 341, and 256). (D) Average displacement from origin in 30 min combining four experiments, as in (C), and (E) arrest coefficients of thymocytes from two experiments (n = 100 each). Single-cell data are shown with the median ± interquartile range.*p < 0.05, **p < 0.001, ***p < 0.0001 (Mann–Whitney U test).
and found that it was increased ∼CXCL12, a greater percentage of immobilized ICAM-1 (Fig. 2A). In both the presence and absence of RhoGEF, the cause for PIX expression and basal Rac activation, which is consistent with an RhoGEF, PIX homolog (represenative image, left panel) and cells that changed morphology extensively, showing irregular shapes and forming long protrusions (representative image, right panel). The table shows the percentages of the latter cell type in wild type and aPix− thymocytes. (B) Increased Rac activation in aPix− thymocytes. Rac-GTP was measured in freshly isolated thymocytes using a CRIB domain pull-down assay. Immunoblot analysis of RacGTP (top panels) and total Rac (bottom panels) in aPix− thymocytes was quantified (bar graph) using densitometry and normalized to wild type levels. (C) Increased expression of βPIX in aPix− thymocytes. Levels of βPIX and an unrelated RhoGEF, Lsc used as a loading control, were detected by immunoblot analysis (left panel). βPIX expression was also measured in aPix− DP and CD4+SP thymocytes using flow cytometric analysis of thymocytes stained intracellularly for βPIX and normalized to expression of βPIX in wild type thymocytes (n = 6 each). *p < 0.05 (Student t test), ***p < 0.0001 (Mann-Whitney U test).

FIGURE 2. Increased cytoskeletal-related signaling in aPix− thymocytes. (A) Abnormal morphology of aPix− thymocytes. Thymocytes from wild type and aPix− mice were plated on immobilized ICAM-1 alone or with added CXCL12 and recorded every 10 s by widefield, live cell imaging at 40× magnification for 5 min. Thymocytes were classed in two groups: cells that stayed mainly round during the 5-min observation period showing only small, broad protrusions (representative image, left panel) and cells that changed morphology extensively, showing irregular shapes and forming long protrusions (representative image, right panel). The table shows the percentages of the latter cell type in wild type and aPix− thymocytes. (B) Increased Rac activation in aPix− thymocytes. Rac-GTP was measured in freshly isolated thymocytes using a CRIB domain pull-down assay. Immunoblot analysis of RacGTP (top panels) and total Rac (bottom panels) in aPix− thymocytes was quantified (bar graph) using densitometry and normalized to wild type levels. (C) Increased expression of βPIX in aPix− thymocytes. Levels of βPIX and an unrelated RhoGEF, Lsc used as a loading control, were detected by immunoblot analysis (left panel). βPIX expression was also measured in aPix− DP and CD4+SP thymocytes using flow cytometric analysis of thymocytes stained intracellularly for βPIX and normalized to expression of βPIX in wild type thymocytes (n = 6 each). *p < 0.05 (Student t test), ***p < 0.0001 (Mann-Whitney U test).

Altered signaling to the cytoskeleton in aPix− thymocytes

The morphology and dynamics of aPix− cells in the 2D migration assays appeared qualitatively different from wild type cells. To quantify this finding, we counted cells that displayed dynamic and irregular or asymmetric borders versus cells that remained round on immobilized ICAM-1 (Fig. 2A). In both the presence and absence of CXCL12, a greater percentage of aPix− thymocytes showed irregular borders and appeared to be in constant motion. These data suggest that aPix− thymocytes have an intrinsic tendency to undergo increased shape flux, which can lead to increased migration on substrates that permit traction force. As the increased cytoskeletal dynamics could reflect abnormal activation of GTPases, we tested freshly isolated thymocytes for the presence of Rac-GTP and found that the basal level of Rac activation was indeed significantly higher in the absence of aPIX (Fig. 2B). This finding was unexpected because aPIX is a RhoGEF, and its loss should result in lower levels of Rac activation. We therefore tested whether expression of another RhoGEF, the aPIX homolog βPIX, was affected by the loss of aPIX, and found that it was increased ∼50% in aPix− thymocytes (Fig. 2C). Together, these data show that the absence of aPIX results in higher βPIX expression and basal Rac activation, which is consistent with an increase in cytoskeletal shape changes for aPix− thymocytes.

Impaired positive selection in TCR-transgenic aPix mice

We previously reported that aPix− mice on a mixed C57BL/6 and 129Sv background have normal numbers of thymocytes, including CD4+CD8+ DP thymocytes and CD4+ or CD8+ SP subsets, and reduced numbers of peripheral T cells (12). However, defects affecting thymocyte development are often masked by redundant homeostatic mechanisms to ensure sufficient numbers of thymocytes and T cells (1). We therefore introduced aβTCR transgenes into aPix− thymocytes to limit the TCR repertoire of the thymocyte pool and to reveal any previously hidden developmental defects. Wild type and aPix− mice were crossed to MHC class II-restricted TCR-transgenic mice that give rise to monoclonal CD4+ SP thymocytes, OT-II and DO11.10, on the C57BL/6 and BALB/C backgrounds, respectively. Overall numbers of thymocytes in DO11.10+ aPix− and OT-II+ aPix− TCR-transgenic thymi were slightly increased (Fig. 3D, Supplemental Fig. 2C). However, CD4+SP thymocytes in aPix− mice were ∼3-fold reduced in DO11.10+ mice and ∼2-fold lower in OT-II+ mice (Fig. 3A, 3D; Supplemental Fig. 2A, 2C), parallel with an increase in DP cells. Reductions in TCRβ populations, identified with KJ1-26 mAb for DO11.10 and anti-Vα2 for OT-II, confirmed the reduction of the CD4+SP cell population (Fig. 3B, 3D; Supplemental Fig. 2B, 2C). The average percentage of CD69+KJ1-26+ postselection thymocytes in the DP population was also significantly reduced in aPix− mice, which is consistent with a defect in positive selection (Fig. 3E). Similarly, the numbers of CD5+TCRβ+ DP thymocytes were lower in DO11.10+ aPix− mice (Fig. 3E, Supplemental Fig. 2D). Moreover, upregulation of CD5, which correlates with intensity of TCR-mediated signaling during selection (28), was reduced on DO11.10+ aPix− DP but not CD4+SP thymocytes (Supplemental Fig. 2D). Images of thymic sections from DO11.10+ aPix− mice also showed considerable reductions in medullary areas and increased cortical areas compared with DO11.10+ wild type mice, consistent with the decreased SP/DP ratio (Fig. 3C).

To determine whether the defective output of CD4+SP thymocytes was intrinsic to hematopoietic cells, wild type and aPix− hosts were reconstituted with bone marrow stem cells from DO11.10+ wild type or DO11.10+ aPix− mice (Fig. 3F). The generation of KJ1-26+ SP thymocytes from DO11.10+ aPix− bone marrow–to–wild type hosts was strongly reduced compared with wild type–to–wild type (average of 8.3% versus 23.2%), but chimeras using aPix− mice as hosts and DO11.10+ wild type mice as donors were essentially normal (average of 24.5% KJ1-26+; Fig. 3F, Supplemental Fig. 3A). Numbers of CD69+TCRβ+ postselection DP thymocytes were also considerably reduced among DO11.10+ aPix− donor cells compared with DO11.10+ wild type donor cells (Supplemental Fig. 3B). These reconstitution data showed that radiation-resistant cells in the aPix− thymus are able to support proper thymocyte development and are consistent with a defect in positive selection.

### Table: Proportion of cells with asymmetric morphology dynamics quantified in a 5 minute interval

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>aPix</th>
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<tbody>
<tr>
<td>ICAM-1</td>
<td>23.5%</td>
<td>35.7%</td>
</tr>
<tr>
<td>ICAM-1 + CXCL12</td>
<td>40.5%</td>
<td>67.6%</td>
</tr>
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*Images courtesy of Dr. Jane Doe, Department of Immunology.*
Although wild type thymocytes developed normally in the wild type and αPix− hosts (Fig. 3F), the macrophages and DCs in these mice derive from the wild type bone marrow donor cells. In the αPix− to wild type mice, the macrophages and DCs are also αPix−, and it could be argued that the αPix− defect is not intrinsic to the thymocytes. Therefore, we wanted to assess the development of αPix− thymocytes in an environment that sustains normal development of wild type thymocytes. To do this, we took advantage of the fact that the αPix gene is located on the X chromosome. In female mice, one of the two X chromosomes is randomly inactivated, thus OT-II+ αPix+/− females have both αPix+ and αPix− thymocytes, macrophages, and DCs in the same thymus. We observed a comparable decrease in αPix− CD4+SP and increase in αPix+ DP thymocytes in OT-II+ αPix+/− females (Fig. 3G, Supplemental Fig. 3C), as in the transgenic αPix− mice. Together, these data show that TCR-transgenic αPix− thymocytes have a cell-intrinsic developmental disadvantage at the DP-to-CD4+SP transition.

Normal negative selection in αPix− mice

To measure thymocyte output in the absence of αPIX, we treated mice with a prolonged dose of BrdU and quantified BrdU+ thymocytes. Numbers of BrdU+ DP thymocytes were normal in DO11.10+ αPix− mice, but BrdU+ CD4+SP thymocytes were significantly reduced, indicating that the reduction of CD4+SP thymocytes results from a decreased output from the DP pool of thymocytes (Fig. 4A). We next examined cell death in ex vivo and cultured thymocytes by measuring activated caspase-3+ apoptotic cells and Annexin V+/7-AAD+ dead cells. We found no differences in numbers of wild type and αPix− thymocytes that were cultured for 24 h, either with or without TCR stimulation to induce apoptosis, indicating that signaling from TCR to apoptosis is not impaired by the lack of αPIX (Fig. 4B). The reduction in positively selected thymocytes in αPix− mice implied that there should be increased loss of thymocytes due to death by neglect. However, we tested freshly isolated ex vivo thymocytes and observed a significant increase only in apoptotic cells, but not in dead cells from αPix− TCR-transgenic mice (Fig. 4B), possibly because thymocytes that fail positive selection are rapidly consumed by thymic macrophages and we can detect only the thymocytes that are in earlier stages of apoptosis.
selection of thymocytes specific for the male H-Y Ag presented in male mice (18), but no impairment was observed in αPix− mice (Fig. 4C). Additionally, expression of TCR β-chains (β3, β5, and β11) is low in BALB/C mice because of negative selection (29), but splenic T cells carrying these β-chains or the positively selected β13 chain were present at normal frequencies in αPix− BALB/C mice, indicating that negative selection was operational (Fig. 4D). Together, these data indicate a defect in thymocyte positive selection in αPix− TCR-transgenic mice.

Normal TCR signaling in αPix− thymocytes

We previously reported that TCR signaling to calcium fluxing and ERK activation were normal in αPix− T cells (12). TCR signaling defects can affect positive selection. In particular, intracellular calcium levels regulate migration and arrest of thymocytes during positive selection (3). We therefore assessed TCR-induced calcium fluxing and ERK phosphorylation in DO11.10+ αPix− thymocytes. Both signaling pathways were activated normally in the absence of αPIX (Fig. 5A, 5B). TCR signaling is also required to activate pair formation between T cells and APCs bearing cognate pMHC. In peripheral T cells, αPIX is required for the formation of conjugates, but its role in thymocytes is unknown (12). We mixed wild type B cells pulsed with OVA peptide as APCs with DO11.10+ wild type or DO11.10+ αPix− DP thymocytes and counted pairs formed, but observed no defects resulting from the loss of αPIX (Fig. 5C). In addition, we tested TCR induction of CD69 upregulation in OT-II+ αPix− thymocytes, but found no defects in TCR signaling to CD69 (Fig. 5D).

Increased motility of αPix− thymocytes during positive selection

The increased motility of the αPix− thymocytes in vitro, coupled with the defects in αPix− thymocyte development, led us to investigate αPix− thymocyte migration in vivo in the thymic cortex. To compare wild type and αPix− thymocyte migration in the thymus, we generated chimeric mice containing both GFP-labeled wild type thymocytes and RFP-labeled αPix− thymocytes. Two-photon microscopy was used to image cortical regions in intact,
transplanted thymic lobes (Fig. 6A). Because RFP and GFP transgenes are expressed at much lower levels in immature DP than in mature SP cells, we amplified signals to increase detection, which had the side effect of enhancing autofluorescent signals (yellow) of larger cells that were probably resident dendritic cells or macrophages. However, these cells were easily distinguishable from thymocytes because they were stationary, whereas the thymocytes moved continuously with no significant decline of motility during imaging (Supplemental Video 1). It was immediately apparent that \( \alpha_{\text{Pix}} \) thymocytes were more motile, appearing and disappearing in the plane of focus with higher frequency and covering larger distances within the same time period than wild type thymocytes did (Fig. 6B, Supplemental Videos 1–4). An overlay plot of cell trajectories of cells tracked for 10 min confirmed the increased migration of \( \alpha_{\text{Pix}} \) thymocytes in vivo (Fig. 6C). The mean displacement of \( \alpha_{\text{Pix}} \) thymocytes was larger than that of wild type thymocytes at every time point measured (Fig. 6D). A direct correlation on a plot of displacement against the square root of time indicates a random walk path, typical for cortical thymocytes, and this was comparable for wild type and \( \alpha_{\text{Pix}} \) thymocytes (Fig. 6D).

\( \alpha_{\text{Pix}} \) cortical thymocytes migrated significantly faster than wild type thymocytes did, with a median speed of 6.3 \( \mu \)m/min (interquartile range, 5.3–7.7 \( \mu \)m/min) versus 4.8 \( \mu \)m/min for wild type (interquartile range, 4.1–5.5 \( \mu \)m/min), confirming the increased migration speed in vitro (Fig. 6E). Reversing the genetic GFP and RFP markers for wild type and \( \alpha_{\text{Pix}} \) thymocytes did not change the results of the analysis (data not shown). Thymocytes migrate in a stop-and-go mode; therefore, we tested the duration of stopping in \( \alpha_{\text{Pix}} \) thymocytes. We found that wild type thymocytes paused for a median 17% of the time (instantaneous velocity, 2 m/min), but \( \alpha_{\text{Pix}} \) thymocytes paused for only a median 10% of the time (Fig. 6E). Measurement of arrest intervals showed that \( \alpha_{\text{Pix}} \) thymocytes were more than twice as likely to not stop at all during the 5-min observation interval (Fig. 6F). In addition, most long stops (\( \geq 40 \) s) were made by wild type thymocytes (Fig. 6F). Imaging of the intact thymus confirmed
the in vitro data that αPIX is necessary for restraining thymocyte migration speed and for promoting prolonged rest periods by thymocytes in the cortex. These data also suggest that the reduced stopping ability of αPIX− DP thymocytes likely decreases the chance of productive interactions with cortical APCs delivering positively selecting signals.
Reduced response to stop signals by hypermotile αPix− thymocytes

To investigate αPix− thymocyte arrest further, we used ICAM-1–coated beads as a model for cells that contact thymocytes in the cortex. Wild type thymocytes scanned the surface of the beads extensively for long periods without detaching (Fig. 7A, Supplemental Video 5). Almost all wild type thymocytes (∼95%) stayed in contact with the beads for more than 200 s, but only ∼60% of the αPix− thymocytes interacted for more than 200 s with the beads (Fig. 7A, 7B; Supplemental Videos 5–6). Instead, almost half of the αPix− thymocyte-bead interactions were less than 200 s. The majority of wild type thymocytes-bead interactions lasted as long as 25 min (1500 s), whereas only a small fraction of αPix− thymocytes were capable of such long-lasting interactions (Fig. 7B).

Next, we assessed αPix− thymocyte arrest on a physiologic substrate using a layer of OP9-DL1 stromal cells that can support positive selection (22). Most wild type thymocytes attached to the OP9-DL1 cells via small focal contacts and appeared to swing slowly around the contact point. In contrast, αPix− thymocytes tended to wrench wildly at the attachment site (Fig. 7C, I; Supplemental Video 7). Wild type thymocytes were motile, but only a limited number of cells showed a pronounced migratory morphology or crawled for appreciable distances. In contrast, αPix− thymocytes moved relatively far away or moved the length of several cell diameters before re-adhering (Fig. 7C, 7D, II-IV; Supplemental Videos 8–10). We also observed similar morphological differences between wild type and αPix− on the OP9-DL1 cells as described in Fig. 2, with a greater proportion of αPix− cells appearing angular with long projections or multiple protrusions, or both, shown in a representative time-lapse series of images in Fig. 7D (IV; Supplemental Video 10). αPix− thymocytes were clearly more motile than wild type thymocytes were on the cell layer, with αPix− thymocytes displaying longer migration.
tracks (Fig. 7E) and increased average displacement (Fig. 7F). These data show that stable contact formation between thymocytes and stromal cells is greatly impaired in the absence of αPIX.

To determine the arrest response of αPix−/− thymocytes to TCR stimulation, we tested transwell migration to CXCL12 by DP thymocytes through membranes coated with ICAM-1 and increasing concentrations of anti-CD3 Ab to trigger a TCR-induced stop signal (Fig. 7G). The addition of anti-CD3 did cause reduced migration of wild type and αPix−/− thymocytes in a dose-dependent manner. However, the lowest dose of anti-CD3 blocked greater than 50% of wild type thymocytes from migrating, whereas the majority of αPix−/− cells could still get through. Even at the highest dose of anti-CD3 used, αPix−/− thymocytes were not restrained as fully as wild type, suggesting that the αPix− cell-intrinsic increase in motility can overcome the TCR stop signal.

Discussion

Our study showed that αPIX is an inhibitor of thymocyte migration speeds and is necessary for complete thymocyte arrest on ICAM-1 and stromal cells. We observed a striking alteration in the morphology of migrating αPix−/− thymocytes, suggestive of defects in control of actin dynamics. Although TCR-transgenic thymocytes from αPix−/− mice had defective positive selection, negative selection appeared normal. TCR signaling, an important component of thymocyte maturation, was normal. Thus, the findings presented in this study are consistent with a role for αPIX in restraining thymocyte migration so as to enable pausing on cTECs, two key components of scanning behavior, to collect pMHC signals and become positively selected.

To our knowledge, the deletion of αPIX that we describe in this study is the only mutation to date that results in higher thymocyte migration speeds in the thymus linked to compromised development. PIX proteins have been implicated in signal transduction downstream of many receptors, including G protein-coupled receptors (GPCRs), integrins, and Ag receptors. We studied the migration of αPix−/− thymocytes to receptors from each of these classes: chemokine receptor GPCRs, LFA-1 integrin, and TCR. In wild type thymocytes, GPCRs and integrin ligands enhance migration, and TCR induces migration arrest. In the absence of αPIX, signaling from these receptors to migration was still functional, but the baseline rate of migration was higher in every case. Thus, chemokines and integrin ligands promoted greater migration αPix−/− thymocytes, and TCR activation caused less arrest of αPix− thymocytes.

We found that the deletion of αPIX results in higher expression of βPIX. As PIX proteins and GIT proteins are constitutively bound together in an oligomeric complex, it is likely that βPIX can replace αPIX in the PIX-GIT complex. Thus, a similar replacement can happen upon the loss of GIT2, resulting in formation of a PIX-GIT complex with altered amounts of GIT1. This hypothesis could explain why Git2−/− and αPix−/− mice have different migration phenotypes in vivo. We observed that αPix−/− thymocytes migrate faster than wild type thymocytes in thymic cortex. In contrast, Git2−/− thymocytes have slower overall motility in vivo at areas with a high concentration of CXCL12 (6). The main interactions for GIT proteins are with Arf GTases and paxillin, a key integrin-binding adaptor, and the main interactions for PIX proteins are with PAK, Rac1, and Cdc42. PIX and GIT proteins are also associated with many different additional proteins (10, 11, 30) that probably confer specificity on the PIX-GIT complex at different receptors or in different cell types. For example, GIT proteins downregulate GPCRs; therefore, chemokine signaling may be higher when GIT2 is missing than when αPIX is missing. It is also possible that these specific GIT2-dependent pathways are not apparent in an in vitro chemotaxis assay, in which only one or two specific activators or substrates are tested. Therefore, the differences in the αPIX and GIT2 phenotypes may reflect the different compositions of a βPIX-dominant complex or of a GIT1-dominant complex.

Although the reduction of thymocytes in transgenic TCR αPix−/− mice could have been due to increased negative selection, we found no defects in PIX-mediated negative selection in the H-Y system, which causes apoptosis of H-Y-reactive thymocytes in the cortex (31), or in superantigen-induced deletion of TCR β-chains in BALB/c mice, which occurs in the medulla (32). Surprisingly, the impaired scanning behavior of αPix−/− thymocytes did not compromise negative selection in the medulla or even in the cortex, where it might have been expected that the hypermotility of αPix−/− thymocytes would result in less acquisition of signals, leading to both positive and negative selection. One possible explanation for this could be that the requirements for scanning and pMHC contacts are less stringent for negative selection than for positive selection. Negative selection in the medulla requires regulation of thymocyte migration patterns (7, 33), and thymocytes spend about twice as much time undergoing negative selection (4–5 d) than positive selection in the cortex (2–3 d) (34). If the increased migration of αPix−/− thymocytes results in inefficient, error-prone scanning for pMHC, then the extra time thymocytes spend in the medulla can compensate for their poor performance. In addition, self-peptides in the medulla induce repeated encounters between thymocytes and mTECs and DCs in confined areas (33), which might ensure that αPix−/− thymocytes would eventually encounter any autoactivating peptides. The normal negative selection we observed in the αPix−/− thymus was supported by a lack of evidence of autoimmune disease in these mice, in contrast to CCR7 knockout mice that exhibit extensive autoimmune pathologies (35). Thymocytes from CCR7 knockout mice are unable to migrate to the medulla to undergo negative selection, but the αPix−/− thymocyte hypermotile migratory response to chemokines probably ensures that any αPix−/− CD4+SP thymocytes will succeed in responding to CCL19/21 and crossing the corticomедullary border, preventing the escape of autoimmune thymocytes to the periphery. Supporting the argument that αPIX may be more important for positive rather than negative selection, expression of αPIX mRNA in thymocyte subsets peaks in DP thymocytes undergoing positive selection and declines to lower levels in the postselection and mature SP thymocytes (http://www.immgen.org) (36).

αPIX expression is relatively high in immune cells compared with its homolog, βPIX, whereas βPIX is more broadly expressed in nonimmune tissues (12, 36). βPIX regulates formation of protrusions (14, 37–39), and overexpression of βPIX in fibroblasts and neurons alters the localization of βPIX itself, GIT1, Rac activity, and protrusions from a single leading edge to all around the cell periphery (37, 39). Moreover, increased Rac activity in fibroblasts and epithelial cells correlates with multiple peripheral lamellae, or protrusions, and increased random motility (40). We observed increased expression of βPIX and increased activation of Rac in αPix−/− thymocytes, as well as increased protrusions and shape changes during their migration. Therefore, one possible model for the hypermotility phenotype of αPix−/− thymocytes is that βPIX overcompensates for the loss of αPIX, leading to increased and delocalized Rac activity, driving formation of multiple protrusions all around the cell body and enhancing cell motility. Because immune cells use actin-based protrusions to generate traction force during locomotion (41), an increase in protrusions around the cell periphery would help to propel the thymocytes through the crowded environment of the thymus, similar to how a boat with increased numbers of rowers would
References

Supplemental Figure 1: Normal expression levels of chemokine receptors and LFA-1 in \(\alpha\)Pix\(^{-}\) mice. DP and CD4\(^{+}\)SP thymocytes from wildtype and \(\alpha\)Pix\(^{-}\) (A) Balb/C and (B) C57BL/6 mice were stained for the indicated chemokine receptors and the CD11a/\(\alpha\)L chain of integrin LFA-1. Expression is shown as representative histograms of fluorescence intensities.
Supplemental Figure 2: Reduced CD4⁺SP thymocytes in OT-II⁺ αPix⁻ mice. (A-C) Thymocytes from OT-II⁺ wildtype and OT-II⁺ αPix⁻ mice were stained for CD4, CD8 and the clonotypic TCR alpha chain (Vα2) and analyzed by flow cytometry. (A) Representative dot plots of CD4 and CD8 expression and (B) single-color histograms of Vα2 expression. (C) Total thymocyte counts and percentages of thymocyte populations from all OT-II⁺ wildtype or OT-II⁺ αPix⁻ mice analyzed (n > 5 each) presented as mean values ± SD. (D) CD5 expression on DO11.10⁺ wildtype and DO11.10⁺ αPix⁻ thymocytes. Flow cytometric analysis of CD5 versus TCRβ on DP thymocytes shown as representative dot plots. Cells were gated on early selection CD5⁻/TCRβ⁻ and late selection CD5⁺/TCRβ⁺ DP thymocytes. Average CD5 levels on both populations as well as on CD4⁺SP thymocytes of DO11.10⁺ wildtype and DO11.10⁺ αPix⁻ from one representative experiment (n = 4 each). * p < 0.05; *** p < 0.0001 (Student’s t-test).
Supplemental Figure 3: Flow cytometric analysis of bone marrow chimeric and heterozygous TCR transgenic αPix+/− mice. (A-B) Impaired positive selection of αPix− thymocytes in bone marrow chimeric mice. Wildtype and αPix− hosts were reconstituted with bone marrow stem cells from DO11.10+ wildtype or DO11.10− αPix− mice, as shown in Fig. 3F. Representative dot plots and bar graph of results from all experiments summarized as mean ± SD (n ≥ 3) are shown for (A) CD4 versus CD8 and (B) KJ1-26hi versus CD69 thymocytes. (C) Comparison of thymocyte selection between αPix− and αPix+ cells from an OT-II+ αPix+/− heterozygous female mice. Thymocytes were intracellularly stained for αPIX and gated on αPix+ or αPix− cells. Percentages of DP and CD4+SP thymocytes among αPix− or αPix+ cells, as shown in Fig. 3G, are depicted on representative dot plots of CD4 versus CD8. ** p < 0.001 (Student’s t-test).
### SUPPLEMENTAL TABLE 1: List of antibodies used

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SUPPLEMENTAL MOVIES

Supplemental Movie 1, 2-photon overview of thymus: Overview of a 300 µm x 300 µm cortical area approximately 70 µm below the dorsal capsule of the ventral lobe explanted from partially mixed bone marrow chimeras and imaged by 2-photon microscopy. GFP⁺ wildtype thymocytes are shown in green, RFP⁺ αPix⁻ thymocytes are shown in red, and autofluorescent cells appear yellow due to overlay of green and red.

Supplemental Movie 2, Tracks from 2-photon of wildtype thymocytes: Representative movie cropped from a larger movie showing tracks of wildtype thymocytes (green cells) imaged by 2-photon microscopy.

Supplemental Movie 3, Tracks from 2-photon of αPix⁻ thymocytes: Representative movie identical to Supplemental Movie 2 but showing tracks of αPix⁻ thymocytes (red cells).

Supplemental Movie 4, Tracks from 2-photon compared: Side by side comparison of wildtype and αPix⁻ thymocyte tracks alone, without cells, from Supplemental Movies 2 and 3.

Supplemental Movie 5, ICAM-1-coated beads and wildtype thymocytes: Widefield live cell imaging of a typical wildtype thymocyte contacting and scanning an ICAM-1 coated bead.
**Supplemental Movie 6, ICAM-1-coated beads and aPIX- thymocytes:** Widefield live cell imaging of a typical αPix- thymocyte making rapid and transient contacts with an ICAM-1 coated bead.

**Supplemental Movie 7, WT (green) and aPIX- (red) thymocytes on OP9-DL1 cells I:**
Widefield live cell imaging of CFSE labelled wildtype (small green cells) and CMRA labelled αPix- (small red cells) thymocytes mixed together and seeded on a monolayer of OP9-DL1 stromal cells (large and dimly green adherent cells). Corresponding to Figure 8C I.

**Supplemental Movie 8, WT (green) and aPIX- (red) thymocytes on OP9-DL1 cells II:**
Same as Supplemental Movie 10 but corresponding to Figure 8C II.

**Supplemental Movie 9, WT (green) and aPIX- (red) thymocytes on OP9-DL1 cells III:**
Same as Supplemental Movie 10 but corresponding to Figure 8C III.

**Supplemental Movie 10, WT (green) and aPIX- (red) thymocytes on OP9-DL1 cells IV:**
Same as Supplemental Movie 10 but corresponding to Figure 8D IV.