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Thymocytes are tested for productive rearrangement of the tcrb locus by expression of a pre-TCR in a process termed β-selection, which requires both Notch1 and CXCR4 signaling. It has been shown that activation of the GTPase Ras allows thymocytes to proliferate and differentiate in the absence of a Pre-TCR; the direct targets of Ras at this checkpoint have not been identified, however. Mice with a mutant allele of p110γ unable to bind active Ras revealed that CXCR4-mediated PI3K activation is Ras dependent. The Ras–p110γ interaction was necessary for efficient β-selection–promoted proliferation but was dispensable for the survival or differentiation of thymocytes. Uncoupling Ras from p110γ provides unambiguous identification of a Ras interaction required for thymic β-selection. The Journal of Immunology, 2011, 187: 4667–4675.

T lymphocytes bearing the αβ TCR emerge from the thymus having passed through selective processes (β-selection; positive and negative selection) that collectively serve to select for a functional TCR. The β-selection checkpoint operates on immature thymocytes, which lack expression of CD4, CD8, and the TCRα-chain and are termed double negative (DN). β-selection is initiated within thymocytes, which have successfully rearranged the tcrb locus and express the TCRβ protein together with the pre-TCR and CD3 signaling complex, forming the pre-TCR (1). Within the DN compartment, it is possible to distinguish cells that have undergone, or are yet to undergo, β-selection on the basis of cell-surface phenotype. Thus, DN cells that are surface CD25+CD44loCD27lo are poised to undergo β-selection increase expression of CD27 and have been termed DN3 (2). Among the cellular processes promoted by β-selection are allelic exclusion, proliferation, survival, induction of TCR genes transcription, and differentiation to the CD4+ and CD8+ double-positive (DP) stage. Failure to undergo β-selection results in apoptosis (3).

In addition to the pre-TCR, successful β-selection requires signal transduction by Notch1 and CXCR4 on the surface of thymocytes to be triggered by delta-like 4 and stromal cell-derived factor 1 (SDF1α; CXCL12), which are expressed by thymic stromal cells (4–9). Activation of these receptors promotes downstream signaling cascades necessary for the processes that define β-selection.

Small GTPases of the Ras family act as digital switches for multiple signal transduction cascades with the potential to integrate signals from different receptors (10). The Ras subfamily includes three highly homologous paralogs—H-, K-, and N-Ras—as well as other related proteins, including Ra1, Rap, R-Ras, and TC21. These cycle between an inactive (GDP-bound) and an active (GTP-bound) state under the influence of GAPs and guanine nucleotide exchange factors (11).

Transgenic overexpression of a constitutively active form of H-Ras (12) or the Ras activator RasGRP1 (13) promotes the development of CD4+CD8+ (DP) thymocytes from RAG-mutant precursors that are unable to produce a functional pre-TCR due to lack of VDJ recombination. However, the process of allelic exclusion is not impaired in thymocytes expressing active H-Ras (14). Moreover, among Ras effectors, transgenic expression of an active mutant of the serine/threonine protein kinase Raf-I promoted the development of DP thymocytes from rag-deficient precursors (15). This suggests activation of the Ras pathway may contribute to a subset of the changes accompanying β-selection. However, experiments with dominant-negative Ras or its effectors have not proven informative on their role in β-selection, possibly due to low expression or sp. act. (16).

Among Ras effectors are the catalytic subunits of PI3K enzymes (17). The class IA subfamily of PI3K is comprised of three catalytic subunits termed p110α, β, and δ, whereas the class IB family consists of p110γ. Genetic studies using mouse models have shown that β-selection is mediated, in part, by p110γ in combination with p110α (18–20). This reflects a requirement for p110γ to transmit signals originating in the pre-TCR and a predominantly p110γ-dependent PI3K signal generated by CXCR4 (8). Unopposed activation of the class I PI3K pathway by deletion of the lipid phosphatase PTEN allows the proliferation and development of RAG-deficient thymocytes to the DP stage (21), mimicking some aspects of β-selection.

The class IA and IB PI3Ks are activated by binding to distinct cell-surface receptors. Class I PI3Ks are heterodimers comprised of a catalytic subunit and a receptor coupling regulatory subunit. The class IA regulatory subunits (p85α, p55α, p50α, p85β, and p55γ) bind to YYXM motifs that have been phosphorylated by protein tyrosine kinases, and the class IB regulatory subunits (p101 and p84) bind to the GBγ subunits liberated upon acti-
vation of G-protein coupled receptors (22). It has been demonstrated that during thymocyte development, p110d is required to mediate p110γ activation by CXCR4 (8) and that p85α is required for pre-TCR-mediated PI3K signaling (23).

The generation of mice with defined mutations that affect protein function but not expression promise to greatly further our understanding of gene function (24). In this study, we show that thymocytes harboring a mutation of p110γ that blocked its interaction with Ras were defective when undergoing β-selection in competition with wild-type (WT) thymocytes. When combined with deficiency in p110d, the loss of Ras input into p110γ produced the same phenotype as p110γ/d double-knockout mice. Analysis of cell cycle, apoptosis, and CD4/8 expression revealed that Ras activation of p110γ is selectively required for optimal proliferation during β-selection but does not affect survival or differentiation. We find CXCR4 activates p110γ independently of the pre-TCR by a Ras-dependent mechanism. Thus, p110γ is a key target of Ras signaling that is required for β-selection.

Materials and Methods

Mice

p110d/d−/−, p110γ−/−, and Vav1/2/3−/− mice have all been described previously (25–28). Mice were on the C57BL/6 background and aged 6–13 wk at analysis. For competitive chimera studies, irradiated described previously (25–28). Mice were on the C57BL/6 background and aged 6–13 wk at analysis. For competitive chimera studies, irradiated Rag2−/−/−I2γ−/− mice were injected i.v. with 3 × 10^6 bone marrow (BM) cells at a 1:1 ratio mix of WT(SJL):WT, WT(SJL):p110γ/D, or WT(SJL):p110γ−/− and analyzed 6 wk later. All animal husbandry and experimentation were in accordance with United Kingdom Home Office regulations and subject to local ethical review.

Cell isolation

DN3a and DN3b thymocytes were isolated by FACS. Single-cell thymocyte suspensions were first enriched for DN cells by incubation with biotinylated anti-CD8α Ab followed by streptavidin-coated MACS beads and magnetic depletion prior to staining for DN subsets. DN3a and DN3b cells were isolated as CD4/CD8/CD44/CD11b/NK1.1/Gr1/Ter119/CD98hi and either CD25/CD98hi (DN3a) or CD25/CD98lo (DN3b).

Cell culture

OP9-DL1 cells were maintained as previously described (4). A total of 5 × 10^6 OP9-DL1 cells were cultured in supplemented α-MEM without additional cytokines in 96-well plates that had been previously seeded with 4000 OP9-DL1 cells. In some experiments, DN3a cells were stained before culture with either Cell Trace Violet or Vybrant CFDA SE cell proliferation dyes (Molecular Probes Invitrogen) as per the manufacturer’s instructions. Cells were cultured for up to 72 h before harvesting and analysis by FACS. Cell numbers were determined by reference to the inclusion of a fixed number of microbeads (Spherotech).

FACS analysis

Single-cell suspensions were stained using various combinations of Abs conjugated to fluorochromes for analysis by FACS (LSRII; BD Biosciences). For cell-cycle analysis, cultured cells were harvested, resuspended in ice-cold 70% EtOH, and incubated at −20˚C for at least 30 min. Cells were then washed three times in PBS before incubation with 1 mg/ml RNase at room temperature for 30 min. Propidium iodide was then added at a final concentration of 400 μg/ml. For caspase-3 staining, fixed cells were harvested, fixed, and permeabilized using Cytofix/Cytoperm (BD Biosciences) before staining with PE-labeled Ab to activated caspase-3 (BD Biosciences). Excess Ab was removed by washing before analysis.

Western blot

Lysates from 10^6 thymocytes were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with rabbit anti-p110 (Onyx Pharmaceuticals), and Ab binding was revealed using HRP-conjugated anti-rabbit Ab (DakoCytomation) followed by ECL (Amersham Biosciences). Blots were subsequently probed using mouse anti-β-actin.

Phospho-specific flow cytometry

Unfractionated thymocyte suspensions were resuspended at 10^6 cells/ml in DMEM with 0.1% BSA. Cells were incubated at 37˚C for 20 min before the addition of 10 nM CXCL12. At various time intervals, 10^6 cells were fixed in 2% paraformaldehyde, washed in PBS, and resuspended in 90% ice-cold methanol at −20˚C for at least 30 min. Cells were then washed three times in PBS with 2% FCS before incubation with Abs to surface markers as well as to phosphorylated Akt (clone #4058; Cell Signaling Technology) or phosphorylated Erk (clone #4370; Cell Signaling Technology) for 30 min at room temperature. Phospho-specific Abs were revealed by subsequent incubation with Cy5-conjugated donkey anti-rabbit Ab and FACS. For data analysis, fold induction was calculated as the median fluorescence of stimulated sample/median fluorescence of unstimulated sample. Unstimulated samples showed no change in phospho-Akt or phospho-Erk throughout the time course.

Statistics

Data presented throughout represent the mean and either the SD or SEM (if n ≥ 5). Data were analyzed by one-way ANOVA (or repeated-measures ANOVA for competitive chimera experiments) and statistical significance represented as *p < 0.05, **p < 0.01, and ***p < 0.001.

Results

The interaction of active Ras with p110γ is required for efficient thymic β-selection

To determine whether Ras binding to p110γ is required for T cell development, we studied knockout mice with mutations at five residues of p110γ critical for the binding of activated GTP-Ras (DASAA; T232D, K251A, K254S, K255A, and K256A) (27). Using Western blotting, we found the expression of p110γ^DASAA in thymocytes was equivalent to WT p110γ (Supplemental Fig. 1).

FACS analysis of thymocytes from p110γ^DASAA/DASAA (hereafter termed p110γ^D/D) mice revealed thymic cellularity was reduced in p110γ^D/D mice by ~50%, and this principally reflected a reduction in the number DP thymocytes (Fig. 1A–D). To investigate whether the loss of DP cells was due to a defect at β-selection, we used expression of the H chain of the amino acid transporter CD98 to analyze the preselection DN3a (CD4^+CD25^+CD98^hi) and the postselection DN3b (CD4^+CD25^-CD98^-), the postselection DN3b (CD4^+CD25^-CD98^-), and the DN4 (CD4^+CD25^-CD98^-) populations (Fig. 1B, 1C). Increased CD98 and CD27 expression on DN3 cells has been previously shown to be concomitant with intracellular TCRβ expression (2), and we have found that CD98 and CD27 expression correlate in these populations (Supplemental Fig. 2). We found that the number of DN3a and DN3b cells in p110γ^D/D thymuses was similar to the control, but the post–β-selection CD4^+CD25^-CD98^- DN4 population was reduced (Fig. 1D), suggestive of a partial block at this selection checkpoint.

To examine if β-selection was functional in the p110γ^D/D thymocytes, we used a competitive repopulation assay. Lymphoid-deficient Rag2−/−I2γ−/− mice were reconstituted with a 1:1 ratio of WT BM cells expressing the allotopic marker CD45.1 (B6.SJL) and WT (C57BL/6), p110γ^D/D, or p110γ−/− BM cells (all expressing CD45.2). The resulting lymphoid compartments were analyzed 6 wk after reconstitution and the contribution of each genotype to the lymphocyte reconstitution determined (Fig. 2A). The thymocyte populations were subdivided to enable analysis of progression through β-selection (DN3 to DN4 and small resting DP (CD5^-CD69^)) and positive selection (from DP CD5^-CD69^- to DP CD5^+CD69^) (29). In addition, the single-positive thymocyte and peripheral T cell populations were analyzed. As p110γ is not required for B-lymphocyte development (30), the contribution of each genotype to the reconstitution of peripheral B cells was also determined as a control for hematopoietic reconstitution.

In the B6.SJL/p110γ^D/D mixed chimeras, the proportion of each genotype in the DN3 thymocyte population was equivalent. However, this ratio shifted to a majority of B6.SJL as the cells...
progressed through the DN3 to DN4 and DP CD5+/CD69lo developmental stages, in which ~80% were B6.SJL derived (Fig. 2B). Thus, the p110γDD thymocytes compete poorly with WT cells at the β-selection checkpoint and in the transition from the DN to DP stage. The ratio of B6.SJL/p110γDD remained at ~80% for all subsequent developmental stages, indicating that the p110γDD cells were not at a disadvantage when subjected to further selection pressure. A similar phenotype was observed in the B6.SJL/p110γ−/− mixed chimeras, consistent with the known role for p110γ at this developmental stage.

Previous work has demonstrated that there is functional redundancy between p110γ and p110δ at the β-selection checkpoint (18–20). Therefore, we tested the effect of the p110γDASAA mutation in the context of p110δ deficiency (Fig. 1A–D). Compared to WT and p110γDD single-mutant mice, the number of CD4+CD25hiCD98lo DN3a cells was increased in the double-mutant mice, whereas the DN3b, DN4, and DP populations were all significantly reduced. This phenotype is further evidence for the importance p110γ at the β-selection checkpoint. Taken together, these data indicate that the function of p110γ at the β-selection checkpoint is critically dependent upon the binding of activated Ras to p110γ.

To characterize further the defect in β-selection, we evaluated the ability of DN3a and DN3b thymocytes from WT, p110γDD, and p110γ−/− mice to proliferate and differentiate in vitro following culture on OP9-DL1 stromal cells. It has been reported that, upon coculture with Notch ligand-expressing stromal cells, DN3b thymocytes are more efficient at generating CD4+CD8+ cells than DN3a (2, 31). We found that when cultured under these conditions, p110γDD and p110γ−/− DN3a or DN3b cells failed to generate equivalent numbers of more mature cells expressing CD4 and/or CD8 when compared with the control (Fig. 3). Thus, Ras-mediated regulation of p110γ is necessary to sustain thymocytes during β-selection.

FIGURE 1. Reduced thymic cellularity in mice with a Ras-binding mutant of p110γ. A. Flow cytometric analysis of CD4 and CD8 expression on T lymphocytes in the thymus from WT, p110γDD, and p110γ−/− × p110δ−/− mice. The percentages of CD4+CD8+ DP and CD4−CD8− DN cells are shown. Flow cytometric analysis of the DN thymocytes (identified as Thy1+ and [CD4+CD8+CD44+B220+CD11b+NK1.1/Gr1/ Ter119/γδTCR]−) (B) and the DN3/4 subsets (C). The percentages of CD25hiCD98lo (DN3a), CD25hiCD98hi (DN3b), and CD25lo CD98lo (DN4) are shown. D. Thymic cellularity and the numbers of DN3a, DN3b, DN4, and DP thymocytes for each genotype. Graphs represent the mean and SEM (n = 5). *p < 0.05, **p < 0.001.

The Ras–p110γ interaction regulates proliferation but not differentiation or survival

Both the Ras and PI3K signaling pathways have been implicated in the survival, proliferation, and differentiation that define β-selection, but their exact roles remain unclear. To resolve this, we made use of an in vitro model of β-selection that allows cell division, apoptosis, and differentiation to be evaluated independently. Prior to culture on OP9-DL1 stromal cells, sorted CD25hiCD98lo DN3a cells were labeled with a fluorescent dye to reveal the number of cell divisions. At regular intervals, cells were harvested and analyzed for their division profile and expression of CD4 and CD8 (Supplemental Fig. 3A). Of those cells that had undergone four rounds of division, ~50% expressed the CD8 coreceptor. Expression of the CD4 coreceptor on an equivalent fraction of the cells required one additional cell division (Fig. 4B, Supplemental Fig. 3B). When sorted p110γDD or p110γ−/− DN3a cells were cultured in this manner, we found that they underwent fewer rounds of division at all time points analyzed (Fig. 4A), but the division-linked expression of CD8 and CD4 was preserved (Fig. 4B). Therefore, p110γ is not required for the differentiation program of DN3 cells to become DP.

The reduced proportion of divided cells in the p110γDD and p110γ−/− cultures could be due to increased cell death. To test this, we assessed the proportion of cells undergoing apoptosis in both the undivided and divided populations by staining with an Ab against activated caspase-3 (Fig. 4C). Following 14 h of culture, before cell division had occurred, up to 15% of the cells were caspase-3+, but there was no significant difference in the proportion of cells undergoing apoptosis between the different genotypes. This was also the case for the undivided population at 60 h. In those cells that had undergone at least two divisions, the proportion of caspase-3+ cells was <2%, indicating that once cells had entered the cell cycle, they were less likely to die than those
that remained undivided. These findings show that p110γ is not required for the survival of DN3 cells undergoing β-selection.

To test for changes in proliferation potential, we examined the proportion of cells in G0/G1, S, and G2/M stages of the cell cycle in the undivided and dividing populations. Among the undivided cells, following 60 h of culture, a significantly increased fraction of p110γΔ/Δ and p110γ−/− remained in G0/G1 (Fig. 4D). Similarly, of those WT cells that had divided at least twice, 55% were in G0/G1, whereas 62% of p110γΔ/Δ and 67% of p110γ−/− were found to be in G0/G1. Together, these findings show that the essential role for p110γ is to promote the proliferation of cells upon β-selection and that this signaling pathway is dependent upon Ras.

Ras-mediated p110γ activation is required for CXCR4 signaling

CXCR4, a receptor implicated in β-selection, has previously been shown to activate p110γ in DN3 cells (8). We therefore sought to determine whether active Ras binding to p110γ was necessary for CXCR4 signaling in DN3 cells. Stimulation of WT thymocytes with 10 nM CXCL12 (SDF1α) elicited phosphorylation of protein kinase B (Akt) on serine 473 in DN3 cells (Fig. 5A). This phosphorylation peaked at 1 min and was sustained throughout the short time course of the experiment. The response of p110γΔ/Δ DN3 cells was reduced at all time points compared with WT, although not entirely diminished (Fig. 5A, 5B). This result is similar to that previously observed in DN3 cells lacking p110γ or p101, the regulatory subunit of p110γ in thymocytes (8). We conclude that in DN3 thymocytes p110γ must be activated by active Ras to elicit an optimal CXCR4 PI3K signaling response.

The generation of active Ras in thymocytes is associated with the signaling cascades elicited from the pre-TCR and TCR. To test whether pre-TCR signaling is required for the generation of the Ras-GTP that feeds into the CXCR4–PI3K signaling pathway, we measured the induction of Akt phosphorylation following SDF1α stimulation of Rag2−/− (which fail to form a pre-TCR) or Vav1/2/3−/− DN3 cells in which signaling through the pre-TCR is impaired (Fig. 6). CXCR4-mediated PI3K signaling was intact in the absence of the pre-TCR and was elevated in Vav1/2/3−/− DN3 cells. By contrast, activation of the MAPK pathway by SDF1α, which is also Ras dependent (32, 33), was almost completely ablated in both mutants. These results suggest that there are distinct CXCR4 signaling responses: a Ras-dependent, pre-TCR-independent pathway of PI3K activation and a pre-TCR-dependent pathway required for the MAPK signaling cascade.

CXCR4 signaling is required for an optimal proliferative response at β-selection

To examine the role of CXCR4 in the proliferation of DN3 cells, we cultured WT DN3a cells in the presence of increasing concentrations of the CXCR4 antagonist AMD3100. Inhibition of
CXCR4 reduced the number of division peaks in the cultures (Fig. 7A). However, AMD3100 did not affect the ability of cells to express CD8 and CD4, provided the requisite number of division cycles was reached (Fig. 7B). The defect in cell recovery was not due to an increase in apoptosis, as demonstrated by equivalent proportions of caspase-3+ cells in both the undivided and cycling populations under all culture conditions (Fig. 7C). However, the presence of AMD3100 did increase the proportion of cells in the G0/G1 phase of the cell cycle in a dose-dependent manner (Fig. 7D), most notably at the highest concentration of 10 μM, when statistical significance was reached. Thus, CXCR4 signaling is necessary to overcome a G0/G1 cell-cycle block, but it is not required for cell survival or differentiation.

**Discussion**

The results presented in this manuscript show that Ras activation of p110γ is required for optimal proliferation triggered following the successful generation of a pre-TCR. This is the first study, to our knowledge, to identify a target of Ras signaling required for β-selection and to place it within the context of a signaling
pathway important for thymocyte development. Thus, the p110γ catalytic isoform of PI3K is activated by the chemokine receptor CXCR4 in a Ras-dependent manner. Taken together with our previous results (8), we conclude that in DN3 thymocytes, p110γ must be activated by both Gβγ (via P101) and active Ras to elicit an optimal CXCR4–PI3K signaling response. This signaling pathway specifically promotes proliferation, and, when it is disrupted, a G1/S cell-cycle block is observed. CXCR4 and Ras-mediated activation of p110γ appear not to be essential for survival or differentiation. This constitutes a new Ras-mediated signaling pathway in thymocyte development that is activated independently of the pre-TCR and contributes to pre-TCR–triggered proliferation.

By using in vitro culture to measure cell division, apoptosis, and differentiation in the undivided and dividing cell populations, we have shown that disruption of the CXCR4–Ras–PI3K signaling pathway does not lead to increased apoptosis among DN3 cells. The proportion of apoptotic cells among undivided mutant, AMD3100-treated, and control cultures was similar. However, there are more numerically more apoptotic cells in the cultures treated with PI3K or CXCR4 antagonist because fewer cells have divided. This is consistent with studies in mouse embryonic fibroblasts deficient for H-, N-, and K-Ras, which were unable to proliferate but survived in culture for several weeks (34).

A novel finding in these studies is that the differentiation from the DN3 to DP stage is division linked. That is, the expression of the cd4 and cd8 genes is dependent upon the cells undergoing a defined number of divisions, which is independent of time. This phenomenon has been reported previously for the differentiation of peripheral lymphocytes, including Ig class switching in B cells (35), cytokine secretion in CD4 T cells (36), and perforin and granzyme gene expression in CD8 T cells (37). The rationale for division-linked differentiation is not yet understood, but it is interesting to speculate that it provides a simple means for transcription factors and other critical regulatory proteins either to accumulate, as a result of continued expression over time, or be diluted as a consequence of cell division in the absence of de novo production. Studies by David-Fung and colleagues (38) have shown that many transcription factors that are expressed from the DN1 stage of thymocyte development are rapidly downregulated after β-selection. They propose that this downregulation is commensurate with permanent commitment to the T cell lineage, as transcription factors that promote alternative lineage fates are diluted away during the accompanying burst of proliferation (38).

Our study reveals that the activation of the ERK pathway after CXCR4 stimulation requires the pre-TCR and the Vav family of guanine nucleotide exchange factors. However, the Ras-dependent activation of p110γ following CXCR4 stimulation is independent of the pre-TCR and Vav. Thus, the active Ras required for CXCR4-mediated p110γ activation is generated by CXCR4 independently of the pre-TCR. This raises the question of how the pre-TCR regulates ERK activation downstream of CXCR4 as this is also thought to be dependent upon Ras-mediated activation of Raf. ERK activation by GPCR agonists such as lysophosphatidic acid, bombesin, and carbachol has been shown to occur by the transactivation of the epidermal growth factor receptor (39, 40). The pre-TCR may be required to promote ERK activation by an analogous process in which the pre-TCR and its associated tyrosine kinases fulfill the role of the epidermal growth factor receptor. In this model, trans-activation would require a fully assembled pre-TCR and would not operate in the absence of TCRβ-chain. Furthermore, it is worth considering if functionally and physically distinct nanoclusters of active Ras could explain the independent activation of ERK and p110γ downstream of CXCR4. One pool of active Ras may be pre-TCR dependent and activate the ERK pathway, and a distinct pool of active Ras, which is pre-TCR independent, may activate p110γ. This possibility is supported by the known ability of Ras proteins to signal from distinct internal membrane compartments such as the plasma membrane, Golgi, and endoplasmic reticulum (11, 41).
The second class I PI3K isoform required for efficient β-selection is p110δ, which signals downstream of the pre-TCR (8). Based upon sequence alignments, the predicted target residues required for the interaction with Ras are conserved across the human and mouse p110α, p110δ, and p110γ isoforms (42). Intriguingly, H-, N-, or K-Ras appear not to activate p110δ. Instead, this ability has been attributed to TC21 (43). Although a role for TC21 in the thymus has not been described, peripheral TC21<sup>−/−</sup> T cells show phenotypes similar to those found in p110δ<sup>−/−</sup> T cells (44), prompting the hypothesis that the TC21–p110δ interaction has a biological role in β-selection. To demonstrate this definitively, in addition to examining TC21 mutant mice, a Ras-binding p110δ mutant mouse will need to be generated.

Studies examining Ras–PI3K interactions have focused predominantly upon oncogenesis. Ras activation of p110α has been found to be critical for the formation of lung adenocarcinomas and skin tumors (45). Our findings may also be relevant to understanding malignancy. T cell acute lymphoblastic leukemia (T-ALL) typically presents as the expansion and dissemination of cells that are arrested between the β-selection and DP stages. Mutations that result in enhanced activity of the Notch1 pathway are found in 60% of T-ALL (46). Although Notch1 is considered

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**FIGURE 7.** CXCR4 inhibition affects the proliferative response of DN3 cells. A, Division profiles of sorted WT DN3 cells (CD25<sup>hi</sup>CD98<sup>lo</sup>) cultured on OP9-DL1 in the presence of 0, 0.1, 1, and 10 μM of the CXCR4 antagonist AMD3100. B, Expression of CD8<sup>+</sup> and CD4<sup>+</sup> at each division cycle at 72 h in untreated and cultured supplemented with 0.1, 1, and 10 μM of AMD3100. C, FACS plots show the gates used to identify cells that have divided ≥2 times and those that remain undivided that are either caspase-3 positive or negative. Graphs show aggregate data from three independent experiments. No AMD3100 (<), 0.1 μM (dark gray boxes), 1 μM (light gray boxes), and 10 μM (○). D, DNA content profiles from cells cultured for 60 h. The gating strategy used to identify divided and undivided populations is the same as in Fig. 3D. Graphs show data from three independent experiments where G<sub>0</sub>/G<sub>1</sub> = ■, S = ○, and G<sub>2</sub>/M = gray boxes. For all statistical analyses, a repeated-measures ANOVA test was performed. *p < 0.05, **p < 0.01.
a weak tumor initiator, when combined with constitutively active Ras in mouse models, T-ALL occurs at an increased frequency indicative of synergy between the pathways (47, 48). The mechanism by which this occurs is not yet understood but might involve Ras-mediated activation of PI3K. The constitutive activation of PI3K is a common feature of T-ALL (49), and in T-ALL lines, Notch1 signaling results in the suppression of PTEN, a phosphatase that opposes PI3K enzymatic function (50). Our findings show that Ras is required for the activation of the PI3K p110γ to enhance proliferation. Thus, the combination of activated Notch1 and Ras might establish an amplified feed-forward PI3K signaling response that drives the oncogenic transformation required for T-ALL.

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Disclosures

The authors have no financial conflicts of interest.

References


