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Gα13 Mediates a Signal That Is Essential for Proliferation and Survival of Thymocyte Progenitors

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Abstract

G protein signaling via the Gα12 family (Gα12 and Gα13) has not been well studied in T cells. To investigate whether Gα12 and Gα13 are involved in thymopoiesis, we expressed the regulator of G protein signaling domain of p115RhoGEF to inhibit Gα12 and Gα13 during thymopoiesis. Fetal thymus organ cultures seeded with p115ΔDH-expressing progenitor cells showed impaired thymopoiesis with a block at the $CD4^-CD8^-CD44^-CD25^+$ (DN3) stage. Using Gα13 or Gα12 minigenes, we demonstrated that Gα13, but not Gα12, is required for thymopoiesis. T progenitor cells expressing p115ΔDH showed reduced proliferation and increased cell death. T cell receptor stimulation of the fetal thymus organ cultures did not rescue the block. Overexpression of the antiapoptotic gene Bcl2 rescued the defect in DN3 cells and partially rescued T cell development. Therefore, Gα13-mediated signaling is necessary in early thymocyte proliferation and survival.

Key words: thymopoiesis • G protein • p115RhoGEF • RhoA • RGS

Introduction

Developing T cells undergo a highly regulated process of selection in the thymus before exiting the organ and migrating to secondary lymphoid tissues. Hematopoietic stem cells or common lymphoid progenitor cells move to the fetal liver during early gestation or the bone marrow later in development and then home to the thymus (1, 2) to begin the process of committing to the T cell lineage (3). The most abundant cells in a normal thymus are double positive (DP) thymocytes, which express both CD4 and CD8 receptors. DP cells go through a process of positive selection whereby the TCRα chain rearranges. Only T cells that produce a TCR capable of recognizing either MHC I or MHC II and transducing a signal are able to survive. The surviving thymocytes then proceed through negative selection to eliminate cells that respond too strongly to the presentation of self antigen, preventing the release of autoreactive single positive mature T cells into the periphery (4, 5). However, before thymocytes express CD4 and CD8 and begin the crucial process of selection they must progress through four double negative (DN) stages of development defined by the expression of CD44 and CD25: $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3), and $CD44^-CD25^-$ (DN4) (6–8). DN2 stage cells initiate rearrangement of the TCRβ chain, which is completed during the DN3 stage. TCRβ pairs with the pre–Tα chain to form a functional pre-TCR (9). Successful β chain rearrangement as measured by efficient signaling through the pre-TCR results in significant expansion of the DN3 cells and down-regulation of CD25 as the cells move to DN4 (7, 10). IL-7 and c-kit signaling are also important for survival of DN thymoyctes, whereas Notch signaling is required for differentiation of both DN and DP thymocytes at several stages of development (11). In addition, other signaling pathways, including G protein–coupled receptors (GPCRs), are implicated in controlling thymocyte survival, differentiation, and proliferation (12–14).

G protein–coupled receptors play a key role in enabling T cells to react to a variety of external signals ranging from developmental cues to chemotactic molecules. GPCRs transduce signals through their direct interaction with members of the heterotrimeric G protein families: Gαi, Gαs, Gαq, and Gα12. Research into GPCR function in T cells

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Abbreviations used in this paper: 2-dG, 2-deoxyguanosine; DH, Dbl homology; DN, double negative; DP, double positive; E, embryonic day; FL, fetal liver; FTOC, fetal thymus organ culture; GFP, green fluorescent protein; GPCR, G protein–coupled receptor; LPC, lysophosphatidylcholine; PH, plextrin homology; RGS, regulator of G protein signaling.

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has focused primarily on a subfamily of GPCRs known as chemokine receptors. Chemokine receptor signaling primarily utilizes the Gαi pathway, which plays a particularly important role in both development and inflammation (15). Thymic treatment with pertussis toxin, an inhibitor of Gαi, results in the specific depletion of the DP population (12). Other Gα protein signaling cascades, including the Gα12 family, are also important for peripheral T cell function but remain largely unexplored during thymopoiesis. The Gα12 family consists of two members, Gα12 and Gα13, which have been reported to mediate actin polymerization during T cell activation (16). Differences in function between these two family members are not well defined, although Gα13 knockout embryos die at day 9 due to defects in angiogenesis, whereas Gα12 knockouts are viable (16, 17). Furthermore, recent work showed that G2A knockout mice, a GPCR that is known to work specifically through the Gα13 pathway, develop a late onset autoimmune disorder (18, 19).

The p115RhoGEF protein couples Gα13 signaling to RhoA activation. This protein contains both a regulator of G protein signaling (RGS) domain enabling specific binding to either Gα12 or Gα13, resulting in the activation of their GTPase activity, and a Dbl homology (DH) domain that acts as a guanine exchange factor to specifically activate the small GTPase RhoA (20–22). Both p115RhoGEF and RhoA have been shown to affect T cell function. Peripheral T cells in p115RhoGEF knockout mice hyperproliferate even in the absence of antigenic stimulation (23). Extensive research has demonstrated that RhoA is important in both lymphocyte trafficking and T cell development. Experiments using the transgenic lck C3 transferase mouse, which express the RhoA inhibitor C3 transferase under the control of the lck promoter, revealed a specific requirement for RhoA at the DN2 and DN3 stages for survival (24). C3 transferase expressed under the control of the CD2 promoter, which is active at a later stage of thymocyte development than that of lck, results in a defect in differentiation at DN3 (25).

The clear importance of RhoA in thymocyte development and Gα13's specific link to RhoA activation led us to question whether signaling through Gα13 or Gα12 specifically plays a role in thymopoiesis. To address this question, we constructed a retroviral vector expressing a mutant of p115RhoGEF (p115ΔDH), which lacks the DH domain (amino acids 466–547) and, therefore, cannot activate RhoA signaling. This mutant acts as a dominant negative of p115RhoGEF, able to bind both Gα12 and Gα13 through the RGS domain to prevent them from transducing a signal, thus terminating GPCR signaling (26–29). We transduced fetal liver (FL) progenitor cells with vector control or p115ΔDH retroviruses and seeded the cells into a thymocyte-depleted fetal thymic lobe. Analysis of cells generated from the p115ΔDH seeded mouse fetal thymus organ cultures (FTOCs) showed a developmental block by DN3. Using Gα12 and Gα13 minigenes, we demonstrated that this block was mediated specifically by Gα13. FTOCs seeded with p115ΔDH FL progenitor cells also exhibited reduced prethymocyte proliferation and increased levels of cell death. Experiments using Bcl2-tg FL progenitor cells rescued the defect in DN3, demonstrating a role for Gα13 in mediating a survival signal during early thymopoiesis.

Materials and Methods

Animals. All animals were housed at University of North Carolina-Chapel Hill in a sterile animal facility. FL progenitor cells were harvested from embryonic day (E)14 or E15 fetuses. Fetal thymic lobes were harvested from E15 fetuses. The Institutional Animal Care and Use Committee approved all experiments.

FTOC. FTOC media contains RPMI 1640 (GIBCO BRL) supplemented with L-glutamine, 10% FBS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 1% nonessential amino acid (GIBCO BRL), 10 mM Hepes, 1 mM sodium pyruvate solution (GIBCO BRL), 100 U/ml penicillin, and 100 mg/ml Streptomycin. After dissection, E15 thymic lobes were cultured at 37°C on membranes (Millipore), so that the lobe was in contact with 5% CO_2. The lobes were cultured for 5 d in FTOC media supplemented with 2-deoxyguanosine (2-dG; 1.35 mM; Sigma-Aldrich) (30). The lobes were then washed four times (for 1 h per wash) with PBS/2% FBS and seeded with GFP^+ E14/15 FL progenitor cells. To seed lobes, 10^5 GFP^+ E14/15 FL cells were cocultured in a Terasaki well with a single thymic lobe for 48 h (31). The lobes were washed in PBS/2% FBS and placed onto culture membranes for the indicated times. The media was changed every other day.

Retrovirus Production and Infection. 293T cells were transfected with plasmids encoding HSPG retroviral DNA, VSVg, and gag/pol as described (32). E14/15 FL progenitor cells were cultured for 24 h in FTOC media supplemented with 50 ng/ml SCF, IL-6, and 10 ng/ml IL-11 (Peprotech) before transduction. Retroviral infections by spin inoculation were performed in 2 mL Eppendorf tubes by mixing 2.5×10^6 cells (500 μL FTOC media with cytokines), viral supernatant (500 μL), and 8 μg/ml polybrene. Cells were incubated at 22°C for 20 min and centrifuged (2,000 *g*) for 3 h. Cells were sorted for green fluorescent protein (GFP) expression 48 h after transduction.

FACS. GFP-positive FL progenitor cells were sorted on either a Cytomation MoFlo FACS or Becton Dickinson FACS Advantage. Each lobe was crushed (pestle and eppendorf tube), filtered, thymocytes were counted, washed with PBS/2% FBS, and stained with the indicated antibodies. Total cells harvested per lobe were analyzed using a Becton Dickinson FACS Caliber. Hoechst 33342 staining was performed as described previously (33) and analyzed on a Cytomation MoFlo FACS. Cell death was measured by harvesting FTOC lobes after 2 wk in culture to prevent progression of thymopoiesis beyond DN3 and stained for CD25 and annexin V (34). CD4, CD8, and CD3 antibodies were purchased from Caltag. CD25, CD44, and annexin V antibodies were purchased from BD Biosciences.

Western Blot. Transduced Jurkat cells (10^6 cells) or mouse fetal liver cells (10^5 cells) were lysed and resolved on a 10% SDS-PAGE. Gels were transferred to PVDF membranes (Amersham Biosciences) and blocked with 5% nonfat dry milk. The membrane was probed with αp115RhoGEF (1:1,000), α-rabbit–IgG–HRP (1:10,000) (Amersham Biosciences) and visualized using an ECL Kit (Amersham Biosciences). The αp115RhoGEF antibody was produced in rabbits immunized with recombinant p115RhoGEF protein as described by Hart et al. (35). The blot was stripped and probed with α-actin (Sigma-Aldrich), α-mouse–IgG–HRP.

Results

The p115ΔDH Protein Is Not Cytotoxic when Stably Expressed in Cell Lines and Hematopoietic Progenitor Cells. To investigate the Gα12/13 pathway, we used a dominant negative p115RhoGEF (p115ΔDH) in a retroviral vector. The NH_2 terminus of wild-type p115RhoGEF encodes an RGS domain that functions as a GTPase-activating protein, which specifically inactivates Gα12 and Gα13 signaling (20, 22, 28). The DH domain encodes a guanine nucleotide exchange factor (GEF) specific to RhoA activation and a plextrin homology (PH; reference 36) domain (35) critical to plasma membrane localization. The deletion of the DH domain (amino acids 466–547) in p115RhoGEF (p115ΔDH) inhibits the protein's ability to signal downstream to RhoA (26, 27, 29, 35). The remaining RGS and PH domains enable the mutant to inactivate Gα12/13 signaling and localize properly to the cytoplasmic membrane, respectively. The p115ΔDH construct is expressed from a murine stem cell virus LTR, and transduced cells were monitored through a phosphoglycerate kinase–driven enhanced GFP marker (Fig. 1 a) (37).

Jurkat T cells transduced with p115ΔDH exhibited stable levels of GFP expression over 3 wk in culture (Fig. 1 b). Mouse E15 FL progenitor cells transduced with p115ΔDH retained stable levels of GFP expression at least 1 wk in vitro (Fig. 1 c). Jurkat and mouse fetal liver cells demonstrated stable expression of the p115ΔDH protein at days 21 and 7 postretroviral transduction, respectively (Fig. 1 d). This data demonstrates that expression of p115ΔDH and subsequent inhibition of Gα12/13 signaling does not negatively affect survival and growth of Jurkat T cells or primary mouse FL progenitor cells.

Gα12/13 Signaling Is Critical for Thymocyte Development. Although RhoA, a critical component of many different receptor signaling cascades, including multiple families of G protein–coupled receptors, has been shown to mediate proliferation and survival signals during thymopoiesis, it is not clear which upstream signaling pathways are involved in the process. The heterotrimeric G protein Gα13 has been clearly shown to be a specific activator of RhoA (28, 38). To address the question of whether Gα12/13 signaling played a role in thymocyte development, we expressed the mutant p115ΔDH protein that specifically inactivates Gα12/13 signaling (26–28) in the pHSPG retroviral vector (Fig. 1 a). We used a reconstituted mouse FTOC system. E15 FL progenitor cells were transduced, sorted for GFP expression, and seeded into 2-dG–treated E15 thymic lobes. The lobes were cultured for 3 wk on membranes and analyzed for cell number and expression of GFP, CD4, and CD8. We observed a significant reduction ($P < 0.005$) in the percentage and number of GFP^+ cells in all lobes seeded with p115ΔDH FL progenitor cells but no reduction in the GFP^- population (Fig. 2, a and b). Although the percentages of GFP^+ $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ cells were similar between vector and p115ΔDH (Fig. 2 c), the p115ΔDH-seeded lobes showed an overall decrease in the numbers of GFP^+ thymocytes in each population, significant in $CD4^-CD8^-$, $CD4^+CD8^+$, and $CD4^-CD8^+$ populations (Fig. 2 d). There was no reduction in the number of any of the GFP^- cell populations (Fig. 2 e). This data indicates a critical role for Gα12/13 signaling during early thymocyte development.

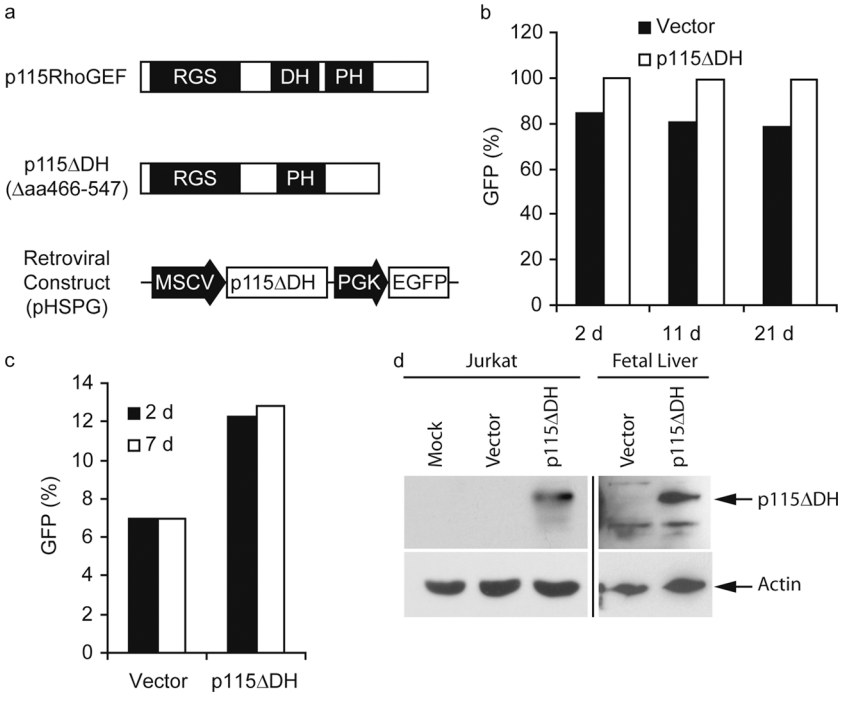

Figure 1. p115ΔDH is stably expressed in both human T cell lines and mouse FL progenitor cells. (a) The DH domain of the p115RhoGEF was deleted to produce p115ΔDH, which contains only the RGS and PH domains. The p115ΔDH was cloned into a retroviral vector, which also encodes enhanced GFP under the control of a phosphoglycerate kinase (PGK) promoter. (b) Flow cytometric analysis of GFP expression in transduced Jurkat T cells at 2, 11, and 21 d posttransduction. (c) Flow cytometric analysis of GFP expression in transduced E14/15 FL progenitor cells at 2 and 7 d posttransduction in culture. (d) 10^5 transduced Jurkat T cells and 10^5 transduced fetal liver cells were analyzed by Western blot for p115ΔDH expression at 21 and 8 d posttransduction, respectively.

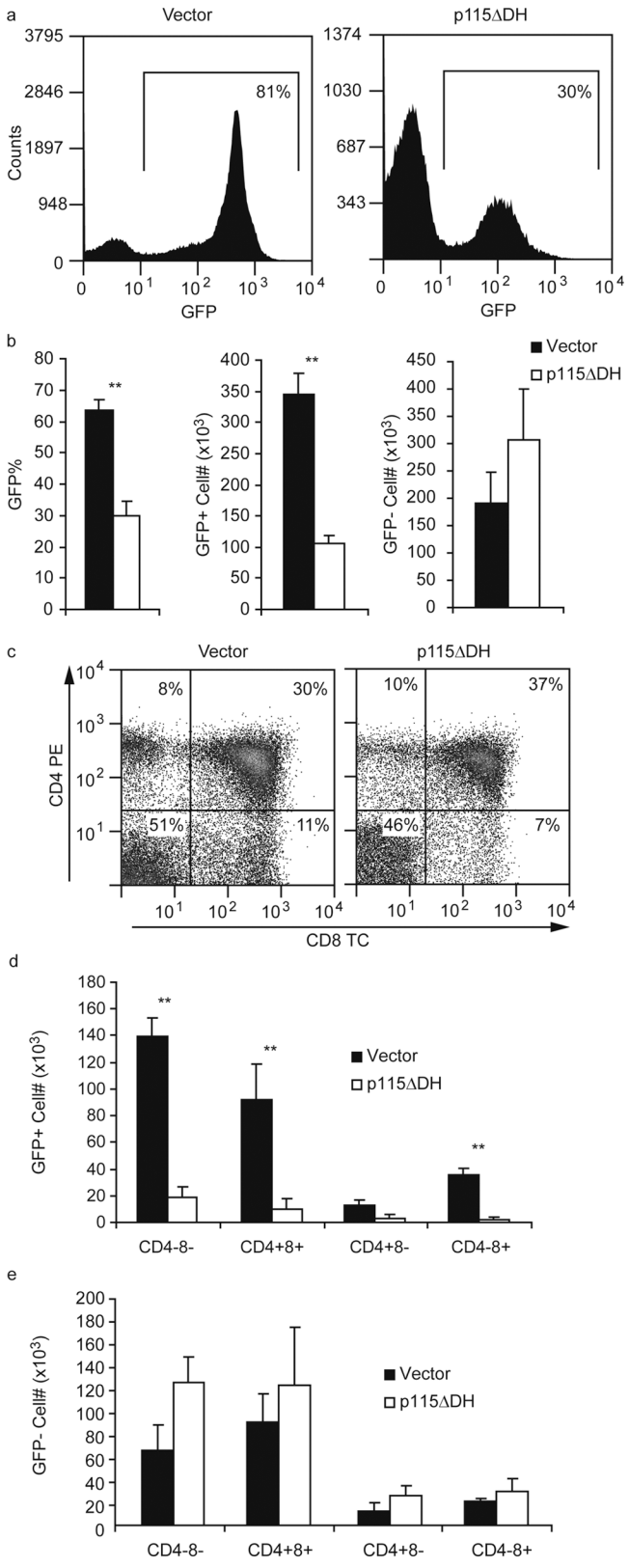

Figure 2. The expression of p115ΔDH inhibits T cell development. 2-dG–depleted E15 thymic lobes were seeded with transduced E14/15 FL progenitor cells and cultured for 3 wk. 24 lobes from four different experiments were analyzed for the percentage (a and b) and the number (b) of GFP^+ and GFP^- cells. The percentage (c) and number of GFP^+ (d) and GFP^- (e) $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ cells generated per lobe were also analyzed. Bars indicate SE; $^{**}P < 0.005$ compared with vector.

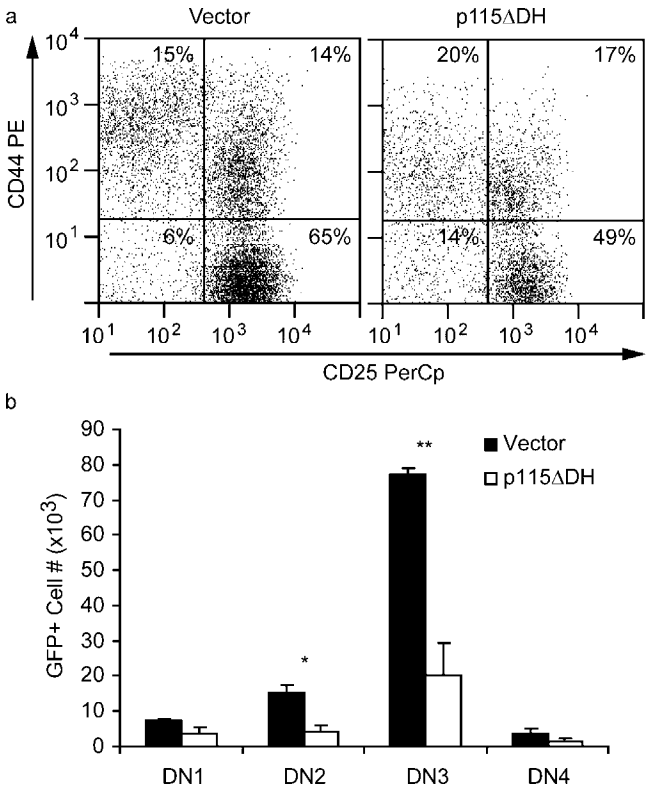

Figure 3. The expression of p115ΔDH inhibits early thymopoiesis. 2-dG–depleted E15 thymic lobes were seeded with transduced E14/15 FL progenitor cells and cultured as above. (a) Thymocytes that were negative for expression of CD3, CD4, and CD8 were analyzed for percentages of $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3), and $CD44^-CD25^-$ (DN4). (b) The total number of DN1, DN2, DN3, and DN4 cells generated per lobe from four lobes for vector or p115ΔDH is summarized. The data is representative of four independent experiments. Bars indicate SE; $^*P < 0.05$; $^{**}P < 0.005$ compared with vector.

Gα12/13 Signaling Is Important for Early Thymopoiesis. When the thymocytes were analyzed for DN subsets, the FTOC system revealed that the expression of p115ΔDH in FL progenitor cells generated a lower percentage of cells preferentially in the DN3 population (Fig. 3 a). However, there was a significant reduction in the number of DN2 and DN3 cells derived from p115ΔDH-seeded FTOC lobes and a general reduction of DN1 and DN4 cells (Fig. 3 b). These results directly demonstrate an essential role for Gα12/13 signaling in early thymopoiesis, most dramatically at the DN3 stage.

Gα13 But Not Gα12 Signaling Is Required for Early Thymopoiesis. The expression of the eleven amino acid COOH terminus, termed minigene, of a Gα protein functions as a specific dominant negative inhibitor of its respective Gα protein by competing for the GPCR binding site (39–45). To determine if Gα12 or Gα13 signaling was specifically required for thymopoiesis, we expressed either a

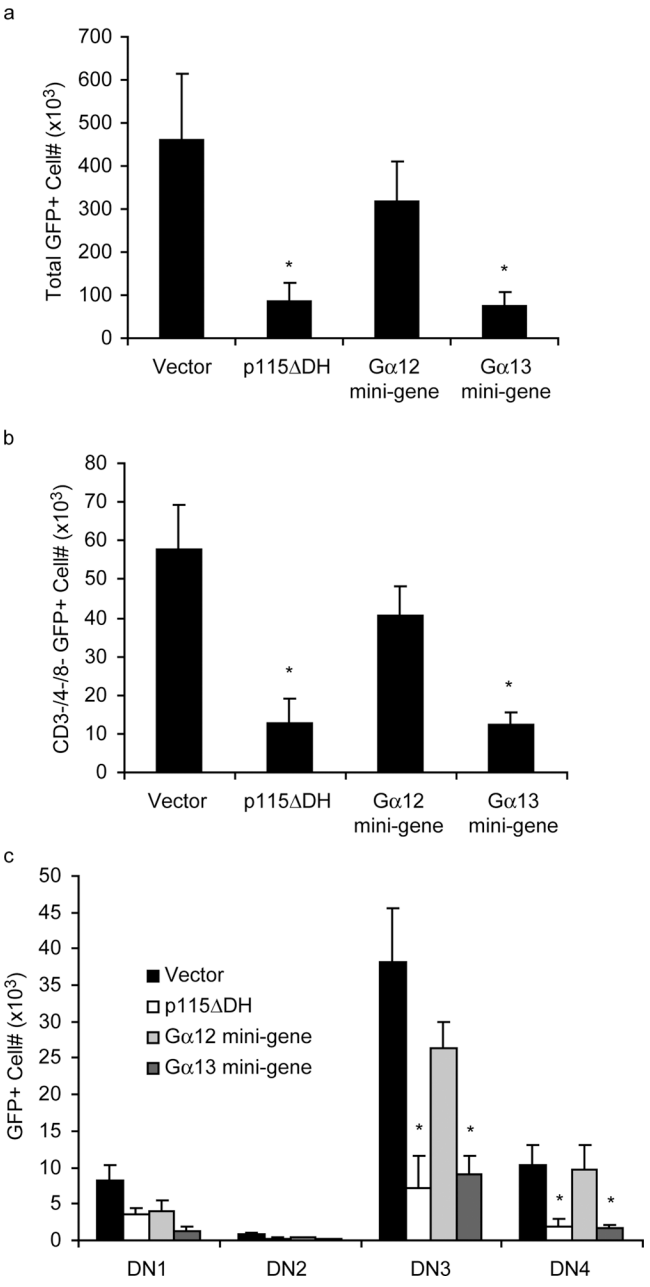

Figure 4. The expression of the Gα13 minigene, but not the Gα12 minigene, inhibits early thymopoiesis. Six FTOC lobes for vector or p115ΔDH were individually analyzed to determine the total number of GFP^+ (a), total $CD3^-CD4^-CD8^-$ (b), and DN1, DN2, DN3, and DN4 (c) cells generated per lobe. The data is representative of two independent experiments. Bars indicate SE; $^*P < 0.05$ compared with vector.

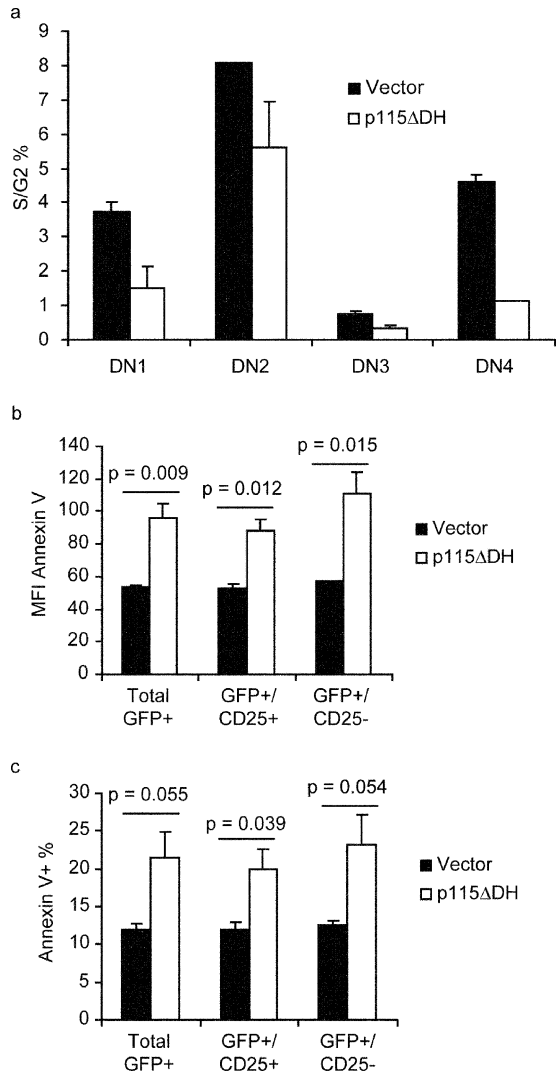

Figure 5. The inhibition of Gα13 signaling results in decreased DN proliferation and increased cell death. 2-dG–depleted E15 thymic lobes were seeded with transduced E14/15 FL progenitor cells. (a) Duplicate pooled samples consisting of six lobes for both vector and p115ΔDH were stained with Hoechst 33342 and CD3/CD4/CD8/CD25/CD44. At least 5×10^5 GFP^+ cells were analyzed to determine the percentage of cells with greater than 2N DNA (S/G2 phases). (b and c) Three lobes for vector or p115ΔDH were harvested after 2 wk in FTOC culture when most thymocytes are between DN1-DN3. Thymocytes were gated on $CD25^+$ (DN2, DN3) or $CD25^-$ (DN1) and individually analyzed for annexin V mean fluorescence intensity (MFI) and percentage of annexin V^+ cells generated per lobe. Bars indicate SE, and p-values are indicated.

Gα12 or Gα13 minigene fused to GFP in the FTOC system. The Gα13 minigene, but not the Gα12 minigene, paralleled the p115ΔDH phenotype by significantly reducing the total number of GFP^+ thymocytes (Fig. 4 a). Early thymocyte populations were also inhibited in the Gα13 minigene reconstituted FTOCs similarly to the phenotype seen using p115ΔDH (Fig. 4, b and c). The combination of the p115ΔDH and Gα13 minigene results suggests that the block in thymopoiesis is a result of the specific inhibition of Gα13 signaling.

The Inhibition of Gα13 Signaling Results in Decreased DN Proliferation and Increased Cell Death. The reduction in the number of prethymocytes derived from the p115ΔDH-seeded FTOCs led us to question whether Gα13 signaling could be involved in proliferation or survival of these cells. Hoechst staining of p115ΔDH-derived thymocyte progenitor cells showed a decrease in the percentage of cells in S/G2 for all DN populations (Fig. 5 a). This decrease dem-

onstrated that inhibition of Gα13 signaling resulted in lowered proliferation of prethymocytes, most obviously in DN1 and DN4 cells. To look at cell death, we harvested fetal liver seeded lobes at 2 wk and stained the cells with annexin V as a marker of apoptosis. At the 2 wk time point, few progenitor cells have progressed past DN3 (unpublished data), allowing us to look at cell death exclusively in prethymocytes. We stained cells with anti-CD25 to separate DN1 from DN2/3. Cells harvested from p115ΔDH-seeded lobes showed an increase in annexin V staining in both $CD25^+$ and $CD25^-$ populations compared with vector (Fig. 5, b and c). This data indicated that the thymic phenotype generated through the inhibition of Gα13 signaling was a combination of reduced DN proliferation and increased cell death.

TCR Stimulation Fails to Compensate for the Loss of Gα13 Signaling in Thymopoiesis. RhoA signaling has been implicated to be downstream of the pre-TCR, though expression of constitutively active RhoA is not sufficient to rescue a defect in pre-TCR signaling (46, 47). In addition, during the DN2/DN3 stages of development the pre-TCR signal serves as a checkpoint for further cell expansion. We asked whether enhancing TCR signaling could compensate for the loss of Gα13 signaling in thymopoiesis. FTOC assays were performed in the presence of anti-CD3 mAb during the last 7 d in culture. Vector control lobes treated with anti-CD3 demonstrated a significant increase in the total number of recovered thymocytes compared with untreated controls. However, p115ΔDH lobes showed no difference between cultures treated with or without anti-CD3 (Fig. 6 a). Individual DN3 and DN4 populations showed significant increases in vector lobes treated with

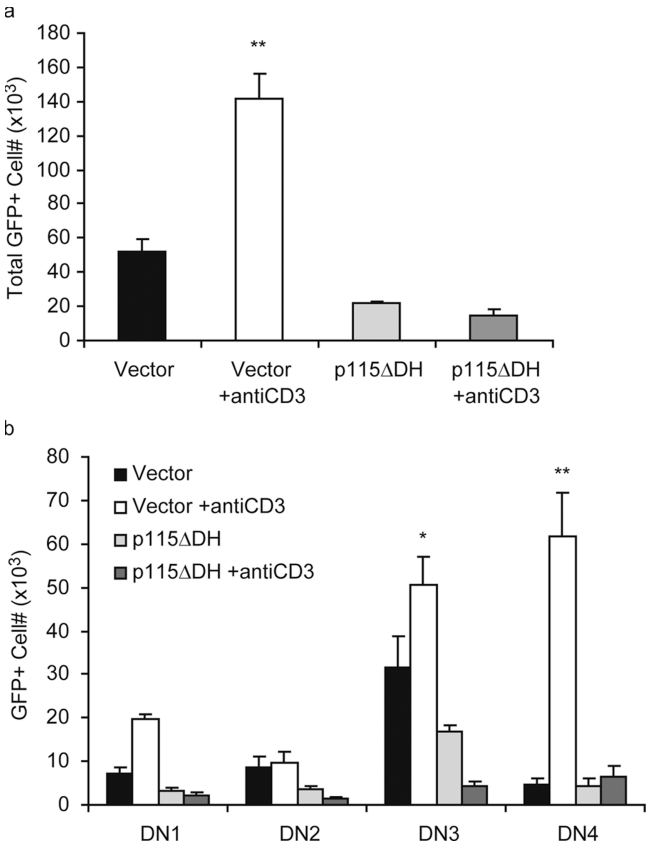

Figure 6. TCR stimulation does not rescue the block in thymopoiesis. 2-dG–depleted E15 thymic lobes were seeded with transduced E14/15 FL progenitor cells and cultured for 2 wk. Lobes were stimulated with 10 μg/ml of anti-CD3 mAb from day 14 to 21 in culture. Fresh media containing anti-CD3 was added every 2 d. Six lobes per sample were individually analyzed to determine the total number of GFP^+ (a) and DN1, DN2, DN3, and DN4 (b) cells generated per lobe. Bars indicate SE; $^*P < 0.05$; $^{**}P < 0.005$ as vector or p115DDH samples compared with unstimulated controls.

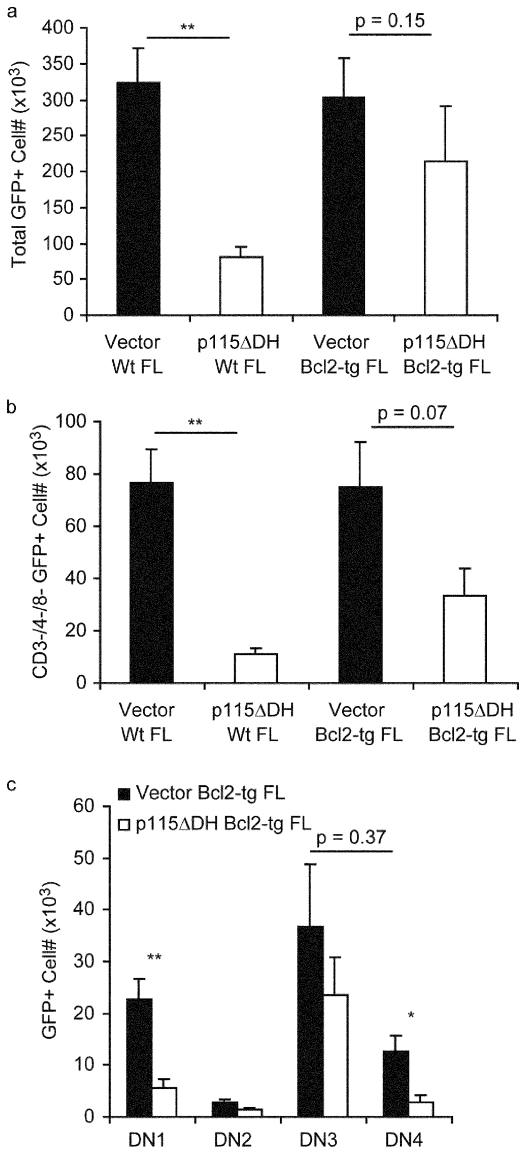

Figure 7. Overexpression of Bcl2 rescues the block in DN3 development. 2-dG–depleted E15 thymic lobes were seeded with wild-type or littermate Bcl2-tg E15 FL progenitor cells transduced with vector or p115ΔDH and cultured for 3 wk. Six lobes per sample were individually analyzed to determine the number of total GFP^+ (a), total $CD3^-CD4^-CD8^-$ (b), and DN1, DN2, DN3, and DN4 (c) cells generated per lobe. Bars indicate SE; $^*P < 0.05$; $^{**}P < 0.005$ compared with vector.

anti-CD3 compared with untreated, but again no increase was observed in p115ΔDH lobes (Fig. 6 b). The block in Gα13 signaling cannot be overcome through exogenous TCR signaling, placing Gα13 signaling in a pathway distinct from the pre-TCR signaling.

Overexpression of Bcl2 Rescues the Block in Gα13 Signaling during Thymopoiesis. The inhibition of RhoA in the lck C3 transferase transgenic mouse model revealed a critical role for RhoA signaling during DN3 cell development, which can be rescued by overexpression of Bcl2 (24). To address whether Gα13 signaling is also acting through a survival pathway, we used Bcl2-tg FL progenitor cells to reconstitute FTOCs. Wild-type littermate controls repeated the initial observation that p115ΔDH inhibited thymopoiesis and specifically reduced the number of both total and DN cells (Fig. 7, a and b). Bcl2-tg FL progenitor cells expressing p115ΔDH showed a partial but significant rescue of total thymopoiesis (Fig. 7 a) and most obviously of DN3 thymocytes (Fig. 7 c). This rescue defines a critical role for Gα13 signaling in the survival of early thymocyte progenitors specifically by the DN3 stage of development. Although expression of Bcl2 rescued DN3 development in p115ΔDH-seeded FTOCs, we still observed a difference in cell number between vector and p115ΔDH in the DN1 and DN4 populations (Fig. 7 c). The inability of DN1and DN4 populations to be rescued by Bcl2 may be explained by a reduction in prethymocyte proliferation (Fig. 5 a). These data demonstrate the critical role of Gα13 signaling in the survival of DN3 cells and in the proliferation of cells during DN1 and DN4.

Discussion

Although previous studies have clearly identified multiple roles for RhoA signaling in thymocyte development (24, 25, 48), it is not clear which upstream signaling pathways are involved in activating RhoA in the thymus. We have shown that blocking Gα13 signaling inhibits thymopoiesis by using a dominant negative mutant of p115RhoGEF (p115ΔDH) to block Gα12 and Gα13 signaling in T cell progenitors. Cells expressing p115ΔDH fail to expand to wild-type levels in FTOC assays. By using Gα12 and Gα13 minigenes (39–45), we were able to demonstrate that Gα13 but not Gα12 was involved in thymopoiesis. In addition, Gα13 signaling was shown to be important for the proliferation and survival of thymocyte progenitors.

The phenotype observed using the p115ΔDH mutant in our FTOC system is similar, although not identical, to the thymic phenotype seen in the lck-C3 transferase transgenic mouse. It has been reported recently that RhoA mediates a p53-dependent checkpoint at the DN3 stage of thymocyte development, which prevents cells with nonproductive TCRβ rearrangements from progressing to the DP stage (47). Though pre-TCR signaling is also important for progressing past the p53-mediated checkpoint, evidence suggests that RhoA signaling acts independently of the TCR signaling pathway to affect survival (47). This is consistent with our finding that stimulation of the pre-TCR with anti-CD3 does not rescue the defect in the Gα13-mediated proliferation and survival signals. Bcl2, a member of a large family of pro- and antiapoptotic factors, has been shown to prevent the death of immature thymocytes in response to many signals including dexamethasone treatment, CD3 stimulation, and radiation (49). By using FL progenitor cells constitutively expressing Bcl2 (50), we were able to partially rescue the effect of blocking Gα13 signaling on thymocyte development, indicating that Gα13 is transducing a survival signal during thymopoiesis.

However, this rescue does not rule out a role for Gα13 in proliferation of cells at different points in development. Hoechst staining of thymocytes derived from p115ΔDH-seeded FTOCs showed a decrease in the proliferation of cells at each DN stage, most obviously at DN1 and DN4. Interestingly, Bcl2 expression rescued the defect in DN3 but not DN1 and DN4 by p115ΔDH. Further evidence for an important role for Gα13 in cell death is the increased annexin V staining in cells expressing p115ΔDH. The lck-C3 transferase mouse has a similar phenotype—large amounts of apoptosis during DN2 and DN3 followed by a defect in the ability of surviving DN4 cells to proliferate (25, 47). Although the lck-C3 transferase mouse also shows an accumulation of cells at DN1 which we do not see, this may be the result of continuous reseeding of the thymus in the transgenic mouse that we are unable to account for in the FTOC system. Additionally, blocking Gα13 signaling does not preclude RhoA activation by other pathways, making differences between the phenotypes unsurprising. Nevertheless, similarities between the phenotypes suggest that a critical Gα13 signal in early thymopoiesis uses RhoA activation to mediate its effect.

RhoA is a clear candidate as a downstream effector for the Gα13 thymocyte survival pathway since biochemical and cell biological evidence has been reported that Gα13 specifically activates RhoA but not Rac or Cdc42 (27, 28, 51). However, potential upstream components that lead to Gα13 activation are not as well established (52). Numerous GPCRs are expressed in lymphoid tissues that can activate RhoA and other Rho family GTPases including Rac and Cdc42, though few of them signal through Gα13 (53, 54). However, the G2A receptor is a GPCR that is expressed predominantly in lymphocytes, including thymocytes (55), and couples specifically to Gα13 (51). Mice with a deletion in G2A develop late onset autoimmunity, indicating a role for the receptor in development and function of T cells (19). Though ligands that bind exclusively to G2A have not been determined, recent work has identified lysophosphatidylcholine (LPC) as a potent activator of the receptor (18). Although it is reported that LPC stimulates chemotaxis of cells expressing G2A and inducing intracellular Ca^{2+} influx, the lysophospholipid is generally believed to be involved in the modulation of immune response. High concentrations (up to 100 μM) of LPC are present in bodily fluids though most of the lipid is probably in an inactive form (55). The expression pattern of G2A on thymocytes, the receptor's known involvement in T cell proliferation and autoimmunity, and the widespread availability of LPC in the body to-

gether makes G2A an attractive candidate for activating the Gα13 signaling pathway in thymopoiesis. Preliminary data in our lab indicated that the addition of LPC to FTOCs stimulated thymocyte production during early thymocyte development (unpublished data). Alternatively, several receptors closely related to G2A that also bind LPC, including the sphingosylphosphorylcholine receptors (55), may be additional candidates relevant to Gα13-mediated survival signaling in early thymocytes.

Two other proteins that are known to be important in early thymocyte survival are IL-7 and c-kit (56). Although both IL-$7^{-/-}$ and IL-$7R\alpha^{-/-}$ mice exhibit impaired thymic cellularity, neither mutation completely blocks thymopoiesis (57, 58). Interestingly, Bcl2 is able to partially rescue the thymocyte phenotype in mice deficient in IL-7 signaling, though the rescue is incomplete (59). Lack of c-kit causes a decrease in thymocyte numbers as early as the DN1 stage (60). Evidence suggests that c-kit is involved in thymocyte proliferation since mice lacking c-kit signaling show a 50% decrease in BrdU incorporation compared with wild-type controls, although a definitive role for c-kit in thymocyte survival has not been established (56). The IL-7 and c-kit signaling pathways can compensate for each other in vivo since mice lacking both c-kit and IL-7 receptor common chain (γc) fail to develop T lymphocytes (56). As described above, we see a partial rescue of thymopoiesis in p115ΔDH-expressing cells in the presence of Bcl2. It is possible that Gα13 signaling may interact with one or both of these receptor kinase signaling pathways to promote survival in developing thymocytes.

Our data shows the first evidence that Gα13-mediated signaling plays an important role in the proliferation and survival of thymocytes during development. This pathway may also have an important role in the T cell response in the periphery. The discovery that Gα13 signaling is able to influence thymocyte maturation not only increases our understanding of T cell development but also opens novel avenues for modulating thymopoiesis and investigating other points in the T cell life cycle where Gα13 might have an important regulatory role.

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