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Requirement of Gai in thymic homing and early T cell development

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Abstract

Demonstration of thymic homing dependent on Gai proteins is one of the keys to determine whether thymic entrance of blood-borne progenitors is a highly selective process. The present study provides compelling evidence of an indispensable role for Gai proteins in this process. Absence of either Gai2 or Gai3 significantly abrogated thymic homing, with an effect of Gai3 being greater than that of Gai2. Pertussis toxin treatment that blocks both Gai2 and Gai3 almost completely blocked thymic seeding in the thymus. Null mutation of Gai3 also hindered bone marrow cell development and thus reduced production of pre-thymic progenitors. In contrast, Gai2 exhibited a more prominent role than Gai3 in guidance of CD4–CD8– double negative (DN) 1 cell migration and early thymic differentiation. The Gai-deficiency-induced defects might be compensated for in part via augmented function of thymic stromal cells so that a nearly normal output of mature T cells could be maintained in these Gai-deficient mice. These studies underscore the importance of Gai in regulating thymic homing and pre-thymic and early thymocyte differentiation.

Introduction

The thymus is the primary site for generating self-tolerant, major histocompatibility (MHC) complex-restricted, and immunocompetent T cells. The process begins with ingress of blood-borne progenitors to the thymus through post-capillary venules near the corticomedullary junction (CMJ) (Donskoy and Goldschneider 1992; Lind et al. 2001). These T cell lineage progenitors migrate outward and then inward, covering several thousand μm within the thymus, during which they undergo a series of developmental events and become mature T cells, capable of emigration to the periphery. This long journey is directed by the cellular action of heterotrimeric Gai-protein coupled receptors (GPCRs) in response to chemokines or bioactive lysophospholipids (Lind et al. 2001; Petrie 2003; Annunziato et al. 2001; Norment and Bevan 2000; Kurobe et al. 2006).

Our earliest appreciation of the role of heterotrimeric Gai proteins in thymocyte emigration stems from the study of *lck*-PTX-Tg mice that express the pertussis toxin (PTX) catalytic S1 subunit under the control of an *lck* promoter (Chaffin and Perlmutter 1991). PTX irreversibly blocks GPCRs' signaling by preventing coupling of Gai proteins to the receptors (Kaslow and Burns 1992). The transgenic mice harbor 2–4 fold more CD4 and CD8 single positive (SP) thymocytes in the thymus than control mice do and have few or no T cells in the peripheral

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lymphoid organs, owing to a defect in thymic emigration, presumably in association with uncoupling of the S1P₁ receptor (Chaffin and Perlmutter 1991; Matloubian et al. 2004). Unfortunately, thymic homing and early thymocyte development could not be evaluated in these animals, since *lck* expression occurs down stream of CD4CD8 double negative (DN) cell differentiation. PTX-sensitive GPCRs, including chemokine receptors CXCR4, CCR7 and CCR9, have consistently been found to play decisive roles in early thymocyte development (Kwan and Killeen 2004; Suzuki et al. 1999; Wurbel et al. 2000; Plotkin et al. 2003; Miszlitz et al. 2004), but direct evidence for G α i involvement in early thymocyte differentiation is still lacking. It also remains elusive whether or not G α i-proteins or any particular GPCR is required for thymic homing.

Among the PTX-sensitive heterotrimeric G proteins, T lymphocytes and thymocytes express primarily G α i2 and G α i3 proteins (Beals et al. 1987; Kim et al. 1988). Unlike *lck*-PTX-Tg mice, G α i2- and G α i3-knockout mice display a nearly normal thymic output, which is somewhat unexpected, given the essential roles of GPCRs in guiding thymocyte migration (Zhang et al. 2005). We show here that deletion of either G α i or G α i3 blocked thymic homing and PTX treatment almost completely abrogated thymic recruitment of blood-borne progenitors, arguing strongly that thymic homing is a G α i-regulated process. Moreover, lack of either G α i2 or G α i3 hinders the migration of DN1 cells and impedes early thymic differentiation, with the effect of G α i2 being stronger than that of G α i3. In contrast, G α i3 has a more pronounced impact in thymic homing and development of pre-thymic progenitors in the bone marrow. These studies underscore the importance of G α i proteins in regulating thymic homing and early thymic development and provide key information in drawing mechanistic similarities between thymic recruitment and lymphocyte homing to peripheral lymphoid tissues.

Materials and Methods

Mice

G α i2-knockout (KO), G α i3-KO, and wild type (WT) control mice on the mixed 129Sv/C57BL/6 background were generated by gene targeting and backcrossed with C57BL/6 (B6) (CD45.2) mice for seven generations as described (Rudolph et al. 1995; Jiang et al. 2001). B6/SJL mice (CD45.1) were obtained from Taconic Farms (Germantown, NJ) and used as recipients for bone marrow transplantation at 5~6 weeks of age. The mice were housed in conventional cages at the animal facilities of the Massachusetts General Hospital in accordance with institutional guidelines.

Flow cytometry analysis

Single-cell suspensions were prepared from thymi, spleens and lymph nodes by mincing the tissues against a 40 μ m cell strainer (BD Bioscience) as described (Thompson et al. 2007). The cells were stained with anti-CD4 and anti-CD8 antibodies (Abs) for CD4⁺ and CD8⁺ T cell analysis. To analyze DN subsets, single-cell suspensions of thymocytes were treated with a cocktail of lineage marker-specific Abs recognizing CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), Mac-1 (M1/70), B220 (TIB 146), Gr-1 (RB6-8C5), and erythroid (TER-119), followed by three consecutive depletions of Ab-bound cells with BioMag goat anti-rat IgG per the manufacturer's instruction. The lineage-depleted cells were reacted with FITC-conjugated Abs against CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), Mac-1 (M1/70), B220 (TIB 146), Gr-1 (RB6-8C5), and erythroid (TER-119) to label residual lineage-positive cells, and anti-CD44 and anti-CD25 Abs to distinguish DN subsets. DN1, DN2, and DN3 cells were determined in gated lineage-negative (lin⁻) cells in the basis of CD44 and CD25 expression on a FACSCalibur cytometer equipped with Cellquest software (BD Bioscience) (Godfrey et al. 1993). A lymphoid forward scatter/side scatter (FCS/SSC) gating was used to exclude other cell lineages, within which greater than 98% cells were CD45.2⁺. Additional Abs used included

anti-CD45.2 (104), anti-CD45.1 (A20), anti-stem cell Ag-1 (Sca-1) (E13-161.7), anti-c-kit (2B8), anti-CXCR4, anti-CCR7, and anti-CCR9 Abs. All Abs were directly conjugated to FITC, PE (phycoerythrin), cy-Chrome (cy5.5), allphycocyanin, PE-Cy7 or biotin, and purchased from BD Pharmingen, with the exceptions of chemokine receptor Abs (BioLegend). Biotinylated Abs were revealed with fluorochrome-conjugated streptavidin. Chemokine receptor expressions on DN subsets were analyzed using a BD FACSAria flow cytometer equipped with BD FACSDiva™ software (BD Bioscience).

Transwell migration assay

Migration assays were performed in a 48-well micro-chemotaxis chamber (NeuroProbe, Gaithersburg, MD). Chemokines CXCL12/SDF-1 (100ng/mL), CCL19 (1µg/mL), CCL21 (1µg/mL) and CCL25 (50ng/mL) were purchased from PeproTech (Rocky Hill) and added to the lower chamber in triplicate in complete RPMI medium (10% fetal calf serum, 2 mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, and 50 µM β-mercaptoethanol). The chemokine concentrations used were found to induce maximal chemotaxis of WT cells in a number of preliminary studies. Transwell membranes with 5-µm pores were carefully placed on top, and 50µl of cell suspension containing 1×10^4 lin⁻ cells prepared from indicated mice were added to the upper chambers. Migration was carried out for 4 hours at 37°C with 5% CO₂. Migrated cells were collected in the lower chambers, pooled from every six wells, and counted for DN1 cell subset analysis. The cells were stained with FITC-conjugated Abs specific for lineage markers, PE-anti-CD44, and biotin-anti-CD25 plus cy-chrome-conjugated streptavidin, and percentages of DN1 subset (CD44^{high}CD25⁻) were determined in gated lin⁻ cell population on a FACSCalibur cytometer as described. Migration of the DN1 subset was compared with or without an indicated chemokine using a chemotactic index (CI), where $CI = (\text{a total number of cells migrating to a chemokine} \times \% \text{ DN1} / \text{the number of cells migrating to the medium control} \times \% \text{ DN1} - 1) \times 100\%$.

Fetal thymus organ culture (FTOC)

Thymic lobes were isolated from E15.5 timed pregnant *Gai2*-KO, *Gai3*-KO and WT mice and cultured on membrane filters (Millipore) in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 50µM 2-mercaptoethanol, 1mM sodium pyruvate, 100U/ml penicillin, 100µg/ml streptomycin, and 1.35 mM 2-deoxyguanosine (Sigma) at 37°C with 5% CO₂ for 5 days as described (Jenkinson et al. 1992). The lobes were then washed and cocultured each with 10^5 DN cells isolated from thymuses of indicated mice in a Terasaki well for 48 hours to allow them to enter the lobes. The lobes were washed, moved to membrane filters, and cultured for another week in the same medium for thymocyte differentiation. The lobes were then collected and minced against a 40 µm cell strainer to prepare a single-cell suspension. Percentages of DP and SP thymocytes were determined by flow cytometry analysis after staining with anti-CD4 and anti-CD8 Abs and compared in the presence or absence of either *Gai* protein.

Competitive repopulation of the thymus

Donor bone marrow cells were isolated from tibias and femurs of indicated knockout mice and age- and sex-matched wild type mice and treated with ACK lysis buffer to remove red blood cells. To prevent potential graft versus-host reaction, bone marrow cells were treated with Abs specific for CD3, B220 (clone TIB 146) and Gr-1 (RB6-8C5) followed by three consecutive depletions of Ab-bound cells with BioMag goat anti-rat IgG per the manufacturer's instruction (Polysciences, Inc.). Sex-matched B6/SJL mice (CD45.1) at 5~6 weeks of age were used as recipients and irradiated with 750 rad 2 hrs before bone marrow transplantation. To the recipient mice, bone marrow cells at a dose of 1×10^7 cells per mouse were intravenously administered on 1:1 ratio of CD45.2+ *Gai2*-KO, *Gai3*-KO or WT bone marrow cells to CD45.1+ cells from

B6/SJL mice. Thymi, spleens and lymph nodes were harvested four weeks later for analysis of DN subsets or CD4⁺ and CD8⁺ T cells by flow cytometry in gated CD45.1⁺ or CD45.2⁺ cells.

Short-term thymic homing

Bone marrow cells from 5 wk-old WT, *Gai2*^{-/-} or *Gai3*^{-/-} mice were labeled for 10 min at room temperature with 10 μ M green dye CFSE (5-carboxyfluorescein diacetate succinimidyl ester) (Molecular Probes). In some samples, WT bone marrow cells were treated with 100 μ g/ml PTX for 2 hrs prior CFSE-labeling. As internal references, WT bone marrow cells were also labeled with 10 μ M red fluorescent dye tetramethylrhodamine isothiocyanate (TRITC) for 10 min in RPMI medium containing 10% fetal calf serum at 37°C with 5% CO₂. Ten million cells of each dye-labeled sample in indicated combinations were mixed and injected intravenously into 4.5-week-old normal mice or irradiated 5-week-old WT mice.

To assay thymic homing of purified progenitors, bone marrow cells isolated from WT and *Gai*-deficient mice were treated with a cocktail of lineage-specific Abs followed by three consecutive depletions of Ab-bound cells with BioMag goat anti-rat IgG as above. The resulting cells were stained with FITC-conjugated Abs specific for various lineage-markers, PE-anti-*c-kit*, allophycocyanin-Cy7-anti-*Sca-1*, and Biotin-anti-CD44 plus cy5.5-streptavidin. The *lin*⁻ *Sca1*⁺ *c-kit*⁺ *CD44*⁺ (LSK) progenitors were sorted on a FACS Aria cell sorter. The sorted LSK cells from WT and *Gai*-deficient mice were labeled with different fluorescence dyes, mixed on 1:1 ratio, and injected into 4.5-week-old normal mice at 10⁵ cells/mouse as above.

The mice were sacrificed two or seven days after the transfer and perfused with 1 x Hanks balanced salt solution lacking Mg²⁺ and Ca²⁺. Blood displaced by perfusion was collected, spun, and treated with ACK lysis buffer to remove red blood cells. Thymi were isolated and single-cell suspensions were prepared as above. The cell samples were scanned by FACS Aria and analyzed using BD FACSDivatm software. Flow cytometric data were expressed as absolute numbers of CFSE- or TRITC-positive cells detected in 500,000 peripheral blood lymphocytes or 2 million thymocytes counted (Rossi et al. 2005).

Histological analysis

Thymuses were fixed with 10% formalin, embedded in paraffin, and sectioned at 4 microns. Sections were stained with hematoxylin (H) and eosin (E) by standard methods and subjected to histological examination.

Statistical analysis

The Student's two-tailed *t* test was used to analyze significance of experimental groups and relevant controls.

Results

Aberrant early thymocyte development in *Gai*-deficient mice

A severe reduction in thymic cellularity was found in newborn *Gai3*^{-/-} mice and to a lesser degree, in *Gai2*^{-/-} mice, despite the absence of gross abnormalities in T cell distribution in the periphery in their adulthood (Rudolph et al. 1995; Huang et al. 2003; Zhang et al. 2005). Absolute numbers of thymocytes were diminished by 12- or 2-fold, respectively, in 3-d (day)-old *Gai3*- and *Gai2*-deficient mice, in comparison with age-matched control mice (Fig. 1A and Table 1). This difference became less prominent with age: there was no significant difference in the absolute number of thymocytes between *Gai2*^{-/-} and control WT mice and only a 2-fold decrease in *Gai3*^{-/-} mice at one week of age (figure 1D), which was no longer

seen after 3 weeks of age. The drastic decreases in thymic cellularity in the neonatal period of *Gai3*-deficient mice had an obvious impact in T cell supply to the periphery, resulting in a 6-fold reduction of T cells in the spleens (Fig. 1B). In agreement with a smaller reduction in thymic cellularity of *Gai2*^{-/-} mice than that of *Gai3*^{-/-} mice, the absolute number of T cells in the spleen was also relatively lower in *Gai2*^{-/-} mice (figure 1B). As the numbers of thymocytes were gradually normalized with age, T cell number in the spleen was also restored to a normal level in both knockouts at 7 days of age (figure 1E). It is not clear at present whether or not this is attributed to both an increased thymic output and homeostatic proliferation in the periphery. Strikingly, despite severely reduced thymic cellularity, T cell development in these animals proceeded normally to a large degree even in newborn mice (Table 1). There was no significant difference in the percentages of CD4⁺ and CD8⁺ SP T cells in these three strains of mice at this age, except for a slight increase in the proportions of CD4 and CD8 SP thymocytes in *Gai3*^{-/-} mice as a result of decreased proportions of DP thymocytes.

A defect in early thymic development can significantly reduce thymic cellularity as has been shown in *CCR7*-deficient mice and *plt/plt* mice (Misslitz et al. 2004; Liu et al. 2005). This, in line with coupling *Gai* proteins to the CXCR4 and *CCR7* receptors, two important chemokine receptors involved in early thymic differentiation, promoted us to evaluate early thymic differentiation in these knockouts. It was found that the proportions of DN1 cells were increased by 5-fold in *Gai2*^{-/-} mice ($p < 0.001$), and 2-fold in *Gai3*^{-/-} mice ($p < 0.001$) at 3 days of age (Table 1). The increment in the proportion of DN1 cells was accompanied by proportional decreases in the percentages of DN2 cells ($p < 0.001$). The results suggest a partial arrest at DN1 developmental stage, which may account partially for reduced thymic cellularity in these two knockouts, as robust proliferation of thymocytes takes place during the transition of the DN-to-DP thymocytes. With gradual normalization of thymic cellularity as the mice were maturing, the difference in the proportions of DN1 and DN2 cells in the presence or absence of either *Gai* dwindled away at 7 days of age (data not shown). Remarkably, in spite of the DN1 blockage, the absolute number of DN1 cells was actually diminished by six-fold in *Gai3*^{-/-} mice when compared to that of WT DN1 cells (Table 1), which may only partially result from a severe reduction in the number of bone marrow cells in the mice (figure 1C).

Decreased ability of *Gai*-deficient progenitors to repopulate the thymus

Thymic hypocellularity seen only in neonatal *Gai2*- and *Gai3*-KO mice but not in adult mice questioned the role of *Gai* in early thymic differentiation, which contradicts well-described function of *Gai* protein-coupled receptors in this process. To address this, competitive repopulation of thymi by bone marrow cells was carried out by mixing equal numbers of CD45.2 WT, *Gai2*^{-/-} or *Gai3*^{-/-} bone marrow cells with CD45.1 WT bone marrow cells and transferring into irradiated B6/SJL (CD45.1) WT mice. Analysis of thymic chimeras, four weeks after transplant, revealed that reconstitution of WT cells was sevenfold or twofold more efficient than reconstitution by *Gai2*- or *Gai3*-deficient cells, respectively ($p < 0.001$, figure 2A and C). When early thymic differentiation was analyzed in the lin-negative population, DN1 cells were found to accumulate at levels 6-fold or 2-fold higher, respectively, in the absence of *Gai2* or *Gai3* than its presence (figure 2B and E). Distinguished from *Gai*-deficient mice, however, these chimeras showed an increase, rather than reduction, in proportions of DN2 cells in the absence of either *Gai* protein (figure 2F). Perhaps, altered production of cytokines and chemokines and largely vacated hemopoietic niches in the thymus after lethal irradiation resulted in migration of DN1 cells independently of *Gai2* or *Gai3*, and DN2 development proportional to the number of accumulated DN1 cells in these chimeras (figure 2F vs. 2E) as implicated by previous studies (Zubkova et al. 2005). The increased proportion of DN1 cells confirms thymocyte differentiation arrest at the DN1 stage in the absence of either *Gai2* or *Gai3*. Furthermore, these chimeras displayed significantly fewer pre-thymic progenitors marked as a Lin⁻Sca-1⁺c-Kit⁺ (LSK) cell subset in the bone marrow in the absence of *Gai3*

(figure 2D), although the mice received equal numbers of bone marrow cells that were comprised of similar proportions of LSK cells in the presence or absence of *Gai3*. The results implicated function of *Gai3* on efficient retention of pre-thymic progenitors in the bone marrow. This may be another factor for a greater reduction of the total number of DN1 thymocytes in newborn *Gai3*^{-/-} mice (Table 1).

A defect in migration of these cells out of the CMJ is likely to account for the increased percentages of DN1 cells, as this vectorial migration is controlled by the CXCR4 and CCR7 receptors, both of which are coupled to either *Gai2* and/or *Gai3* in mature T cells (Thompson et al. 2007). To corroborate this in early developing thymocytes, lin-negative thymocytes were isolated and their chemotactic responses were assayed in the presence of increasing gradients of chemokines SDF-1, CCL19, CCL21, and CCL25. Similar to what has been described in mature T cells, migration of DN1 cells to SDF-1 was impaired in the absence of either *Gai2* or *Gai3* (figure 3A) (Thompson et al. 2007). These *Gai*-deficient DN1 cells responded to CCL21 or CCL19 to a nearly normal degree, but they displayed weakened chemotactic responses to CCL25, a ligand for the CCR9 receptor (Figure 3A). The diminished chemotactic responses, in particular to SDF-1, was not attributed to decreased expression of the specific receptor, since similar levels of CCR7, CCR9 and CXCR4 expression were observed in these cells in the presence or absence of *Gai2* or *Gai3* (data not shown). The results explain DN1 developmental arrest seen in the absence of *Gai2* or *Gai3*, in light of an importance of the CXCR4 receptor in early thymic development (Misslitz et al.2004;Plotkin et al. 2003).

It has been shown that reduced thymic cellularity caused by DN1 developmental arrest is frequently accompanied by altered thymic architecture (Misslitz et al. 2004;Plotkin et al. 2003), due to the fact that thymic stromal cells and early developing thymocytes must communicate with each other to regulate the development of appropriate microenvironments for further thymocyte development (Savino et al. 2002;Lind et al. 2001;Anderson et al. 1996). This was also true for *Gai2*- and *Gai3*-KO mice (figure 3B). Null mutation of *Gai2* or *Gai3* gave rise to a relatively small thymus with medullary zones that were randomly distributing throughout the thymus, in contrast to large confluent medullas in control thymus. While the overall area of the cortex might be comparable between *Gai3*-KO and WT mouse thymi, it was significantly reduced in *Gai2*^{-/-} mice. Conversely, the overall medullary area was decreased in *Gai3*^{-/-} thymus, but it was similar in *Gai2*^{-/-} thymus, when compared with WT thymus. Moreover, in contrast to densely packed lymphoid cells in WT and *Gai2*-deficient cortex, cell density was diminished in the cortex of *Gai3*-deficient thymuses, consistent with thymic hypocellularity in the animals. On higher magnification, the corticomedullary junction in *Gai3*^{-/-} and *Gai2*^{-/-} mice was not as distinct as that of WT mice (Figure 3B, lower panel).

Altered function of thymic stromal cells in the absence of *Gai* proteins

As mentioned earlier, *Gai2*- and *Gai3*-deficient mice exhibit a nearly normal thymic output, despite impairment in early thymic differentiation. To investigate whether altered function of *Gai*-deficient thymic stromal cells plays any role for that, fetal thymic organ culture (FTOC) was set up using WT thymic stromal cells cultivated with lin- progenitor cells isolated from *Gai2*^{-/-} or *Gai3*^{-/-} mice or *vice versa*. We found little difference in the total numbers of T cells when *Gai2*- or *Gai3*-deficient lin- progenitor cells were differentiated in WT thymic lobes, compared to differentiation of WT lin- progenitor cells under similar conditions (Fig 4 A&C). Likewise, the total number of T cells did not differ significantly if WT and *Gai3*-deficient progenitors were cocultured with thymic stromal cells prepared from *Gai3*^{-/-} mice (figure 4B), or WT and *Gai2*-deficient progenitors were cocultured with thymic lobes from *Gai2*^{-/-} mice (Fig 4D). These observations suggest that severe reduction in thymic cellularity in *Gai2*- and *Gai3*-knockouts is unlikely to be caused by decreased proliferation or increased apoptosis of thymocytes.

Interestingly, *Gai3*-deficient thymic stromal cells appear to facilitate DP cell differentiation. Percentages of DP cells developed in *Gai3*-deficient thymic lobes were increased to 45%, from 25% obtained with WT thymic stromal cells, irrespective of *Gai3* expression in *lin*- progenitor cells (Fig 4 right panel, B vs. A and C). The increased differentiation of DP thymocytes concurred with a proportional decrease in DN cell population when compared to the DN cells recovered in the presence of WT thymic stromal cells (figure 4B vs. A). Conceivably, enhanced differentiation of DP thymocytes from DN thymocytes would compensate for early thymocyte developmental arrest and a defect in production of pre-thymic progenitors, giving rise to a nearly normal thymic output in *Gai3*^{-/-} mice, although further investigation is required to demonstrate this. In contrast to *Gai3*^{-/-} thymic stromal cells, *Gai2*^{-/-} thymic stromal cells favored the differentiation of mature SP cells. CD4⁺ and CD8⁺ cell subsets were significantly higher in the presence of *Gai2*-deficient thymic lobes than WT thymic lobes, regardless of whether or not progenitor cells expressed *Gai2* (19% CD4⁺ and 9% CD8⁺ T cells with *Gai2*^{-/-} stromal cells vs. 7% CD4⁺ and 5% CD8⁺T cells with WT stromal cells) (Fig 4D vs. C, right panel). A twofold decrease in the proportion of DP T cells was attributed to increased transition of these cells into SP cells, because the decrease was not concomitant with an increase in the proportion of DN cells in the culture (Figure 4D) (Zhang et al. 2005).

Thymic homing depends on Gai proteins

We next addressed whether *Gai* proteins affected thymic entrance of blood-borne pre-T progenitors by short-term thymic homing assays. The assay directly tracked blood-to-thymic trafficking of intravenously injected, fluorescently labeled bone marrow cells separating from the intrathymic migration as well as progenitor mobilization from bone marrow to blood (Rossi et al. 2005). This is particularly important, because of the disparity of *Gai2* and *Gai3* in development of bone marrow cells (figure 1C). To this end, *Gai2*^{-/-} or *Gai3*^{-/-} bone marrow cells were labeled with the vital green fluorescence dye CFSE, and WT cells were labeled with the vital red fluorescence dye TRITC. Mixtures of these labeled cells on 1:1 ratio were transferred into unmanipulated WT adult mice at 4.5 weeks of age, an age at which the thymus has been reported to be most receptive to settling by blood-borne progenitors (Foss et al. 2001; Porritt et al. 2003; Schwarz et al. 2007). Flow cytometric scanning of donor cells within the thymus, 48 hr after the transfer, revealed that thymic seeding of blood-borne pre-T progenitors to unmanipulated WT adult mice was decreased by more than half in the absence of either *Gai* compared to its presence ($p < 0.01$, figure 5A), with *Gai3* influence of this process greater than *Gai2*, in particular, at one week after bone marrow cell infusion (figure 5C). To exclude that the reduced thymic recruitment was due to altered numbers of progenitors in bone marrows of *Gai*-deficient mice, equal numbers of bone marrow progenitors sorted from WT or *Gai*-deficient mice were labeled with different fluorescence dyes, mixed on 1:1 ratio, and assayed as above in 4.5-week-old normal mice. Similar diminishment in thymic seeding of sorted progenitors as unfractionated bone marrows was observed in *Gai2*^{-/-} or *Gai3*^{-/-} progenitors when compared to WT counterparts (figure 5C).

Importantly, thymic recruitment was reduced to background levels when bone marrow cells were treated with PTX for 2 hr prior to injection (figure 5, A&C), arguing strongly that thymic homing is dependent on *Gai* protein-mediated signaling, similar to thymic emigration (Chaffin and Perlmutter 1991). A possibility that the drastic reduction in PTX-treated cells homing to the thymus resulted from cytotoxic effects of the inhibitor could be ruled out by an increased or comparable number of viable PTX-treated cells in the blood of the animals analyzed in parallel (Figure 5B and D). The increased number of PTX-treated cells in the blood was due to their inefficient homing to bone marrow as a consequence of a blockage in *Gai* activity.

It has been questioned whether the cells entering the thymus during a short-term homing assay are all relevant T lineage progenitors (Schwarz et al. 2007). To address this, we further analyzed

the differentiation of injected, fluorescently labeled bone marrow progenitors within the thymus one week after the injection. In these experiments, we evaluated CFSE-labeled cells within the thymus, because TRIC red dye faded with time and was hardly detectable after one week. Moreover, studies of progenitor differentiation kinetics in the steady-state thymus have identified a maximal number and proportion of DN1 cells around 7~9 days following thymic settling (Porritt, Gordon, and Petrie 2003), after which DN1 cells decline in number and differentiate into DN2 cells. Thus, at 7 days after adoptive transfer, a significant number of CFSE-labeled WT cells were found within the thymus (figure 5D). These cells were phenotypically early T lineage progenitors, with ~65% expressing a high level of *c-kit* (data not shown). In agreement with short-term thymic homing assays, deletion of *Gai2* or *Gai3* prevented 40% or 60% blood-borne progenitors, respectively, from entering the thymus compared with WT bone marrow cells ($p < 0.01$) (figure 5D). PTX treatment of bone marrow cells blocked thymic settling by more than 90%, despite similar numbers of donor cells in the blood. These data provide strong evidence that thymic homing is regulated by a *Gai*-dependent process.

Discussion

T cells are continuously produced in the thymus throughout adult life, but this organ does not harbor long-term self-renewing progenitors. Therefore, the thymus must periodically recruit hematopoietic progenitors from bone marrow via the bloodstream. However, the mechanism by which T progenitors enter the thymus from the blood is poorly defined and the T-lineage progenitor that directly traffics to the thymus from the blood has not been identified to date. The present study, along with several other investigations, suggests that thymic recruitment is very likely analogous to the homing of lymphocytes to other organs in the periphery, requiring selectin-mediated tethering and rolling on endothelial cells, followed by strong adhesion through integrins and transmigration triggered by *Gai*-protein coupled receptor signaling (Wu et al. 1993; Scimone et al. 2006; Rossi et al. 2005). CD44 may be one of the adhesion molecules involved in thymic recruitment as demonstrated by a blockage of this process with a specific Ab for CD44 (Wu, Kincade, and Shortman 1993). The molecule is expressed on bone marrow hematopoietic progenitors and circulating lineage-negative Sca-1^{hi}c-Kit^{hi} (LSK). Likewise, the members of the $\beta 2$ and $\alpha 4$ subfamilies like $\alpha L\beta 2$ and $\alpha 4\beta 1$ integrins have been shown to take part in thymic settling of bone marrow progenitors (Scimone et al. 2006). Besides adhesion molecules, null mutation of platelet selectin (P-selectin) or its glycoprotein ligand-1 (PSGL-1) impeded thymus recruitment (Rossi et al. 2005). P-selectin is expressed by endothelial cells lining the thymic vasculature when thymic niches are vacant, whereas its ligand PSGL-1 is found on bone marrow LSK progenitors and blood LSK cells. Blood progenitors may thus begin with their way to the thymus by interaction of P-selectin on the endothelial cells and PSGL-1 on the blood LSK progenitors (Rossi et al. 2005). As has been well documented in the multiple-step adhesion cascade, after initiating the homing process by adhesion molecule-mediated tethering and rolling on the endothelial cell layer, blood lymphocytes come to a stop only after their cell-surface integrins are activated into a high-affinity conformation by signaling through a *Gai*-coupled receptor (Bargatze and Butcher 1993; Campbell et al. 1998; Warnock et al. 1998). The *Gai*-mediated signaling directs precisely where extravasation takes place. Our investigation shows that lack of either *Gai2* or *Gai3* significantly inhibits the trafficking of pro-thymic progenitors to the thymus and treatment of unfractionated bone marrow cells with PTX almost completely ablates this event. The indispensable role of *Gai* proteins in thymic homing strongly supports mechanistic similarities between thymic and lymphocyte homing.

Our data show a *Gai* protein-mediated mechanism in thymic homing, but the *Gai* protein-coupled receptor(s) involved in the process remains to be yet determined. Identification of the receptor may be a challenge, given the exceedingly small number of progenitors in the blood

and possibly transient expression of the receptor. Blood-borne CCR9⁺ progenitors were shown to preferentially home to the thymus over CCR9-deficient progenitors, and anti-CCR9 antibody could partially block thymic homing (Schwarz et al. 2007; Scimone et al. 2006). An importance of the CCR9 receptor in thymic recruitment is however conflicting with unaltered thymic seeding of CCR7/CCR9-double knockout blood-borne progenitors in the thymus (Liu et al. 2006). There is also no evidence so far indicating that SDF-1, a ligand of the CXCR4 receptor is important for thymic settling. Our data suggest that the GPCR involved in thymic homing is coupled to both G α 2 and G α 3, as is the case with the S1P₁ receptor. The investigation may help to hunt for the specific GPCRs involved in thymic homing and facilitate identification of blood-borne T progenitors that will have enormous implications for ageing, immunodeficiency, bone marrow transplantation, and thymic transplantation.

GPCRs are important at almost every key stage of thymocyte development, ranging from development of pre-thymic progenitors to thymic homing to egress, from intrathymic migration inward to outward, and from early thymocyte differentiations to T cell maturation (Norment and Bevan 2000). These GPCRs, including CCR7, CXCR4, CCR9, and the S1P₁ receptor, are all known to be coupled to PTX-sensitive heterotrimeric G α i proteins (Hamm 2001; Matloubian et al. 2004; Plotkin et al. 2003; Misslitz et al. 2004; Uehara et al. 2002). Remarkably, deletion of either G α i2 or G α i3 has little impact on thymic output and thymocyte development in adult mice, in spite of a defect in early thymic differentiation and thymic homing in newborn mice, suggesting redundant and distinct roles for G α i2 and G α i3 in the signaling of these GPCRs. In accordance with this, lack of either G α i2 or G α i3 doesn't significantly influence the function of the CCR7 receptor or the S1P₁ receptor, and has only a weak effect on the signaling of the CCR9 receptor. The CXCR4 receptor appeared to depend on both G α i2 and G α i3 for a full chemotactic response. Thus, the defect in the early thymic differentiation may be mainly attributed to altered function of the CXCR4 receptor in G α i2- and G α i3-deficient mice. Unfortunately, deficiency in both G α i2 and G α i3 causes a severe growth retardation and lethality around embryonic day 10 (E10.5) (Gohla et al. 2007), which makes it difficult to determine potentially non-redundant function for G α i2 and G α i3 proteins in thymic homing conclusively without using PTX. Moreover, although impairment in thymic homing in G α i2^{-/-} and G α i3^{-/-} progenitors was clearly demonstrated in a competitive environment or in short-time homing assays, it appeared to affect thymocyte development minimally in adult G α i-deficient mice, resembling that seen with P-Selectin-deficient mice (Rossi et al. 2005). Perhaps, a reduced rate of thymic homing can be efficiently compensated for by the expansion of the limited number of progenitors capable of reaching the thymus, consistent with the notion that early progenitor expansion is regulated by the availability of thymic progenitor niches. Alternatively, constitutive or more frequent thymic homing may be driven by unoccupied stromal niches in G α i-deficient thymus, in contrast to periodical thymic recruitment in WT mice (Porritt, Gordon, and Petrie 2003).

Thymocytes migrate outward and then inward, covering thousands of microns of the thymic territory during T cell differentiation. This long journey may be necessary to regulate the rate of T cell generation and to allow adequate output buffering in case any of the steps goes awry under various physiological and pathologic conditions throughout life. G α i proteins may be the most important proteins in this rate-limited control in T cell differentiation in light of their well-described roles in guidance of cell migration.

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Abbreviations used in this paper

CMJ	Corticomedullary junction
DN cells	CD4 ⁻ CD8 ⁻ cells
DP	CD4 ⁺ CD8 ⁺ cells
Gαi	G protein inhibitory subunit α
KO	knockout
lin-cells	lineage-negative cells
mAb	monoclonal antibody
PTX	Pertussis toxin
SP cells	CD4 ⁺ or CD8 ⁺ cells
WT	wild type

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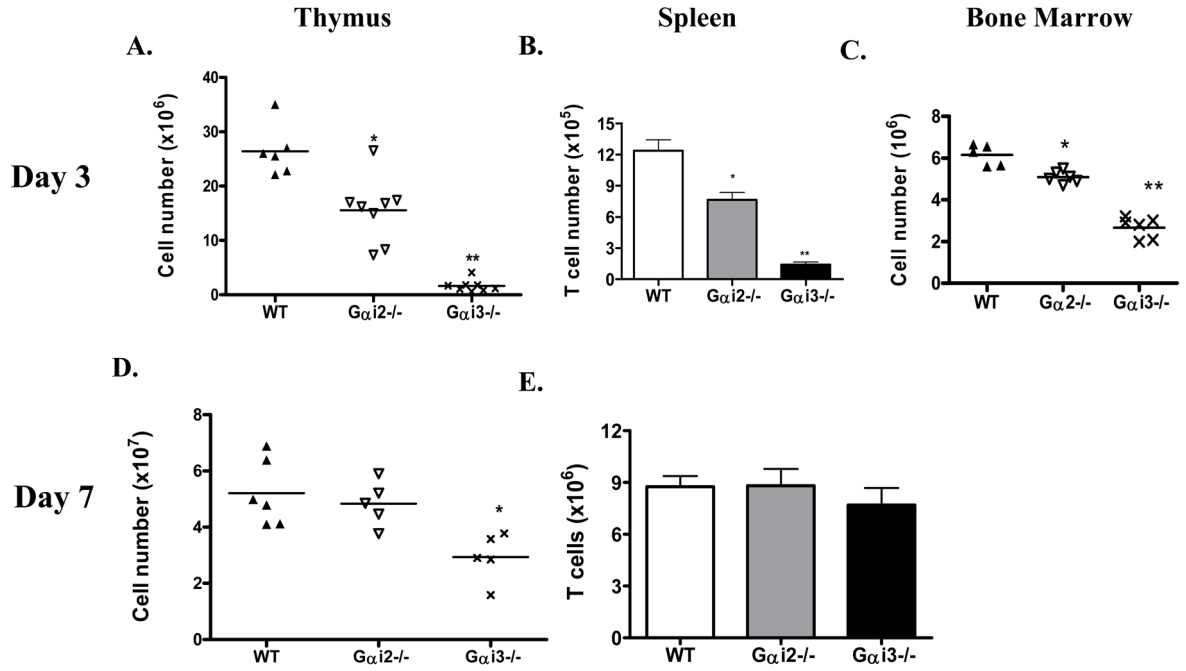


Fig. 1. Abnormal thymocyte development in neonatal $G\alpha i$ -deficient mice. Thymi (**A**), spleen (**B**) and bone marrow (**C**) were isolated from wild type (WT) and $G\alpha i2^{-/-}$ and $G\alpha i3^{-/-}$ mice at 3 days of age. Single cell suspensions were prepared and counted. T cell numbers in the spleen were obtained in the basis of percentages of CD3+ cells identified by flow cytometry. Each symbol in **A**, **C** and **D** relates to data obtained from a single animal and the data in **B** and **E** are the means \pm standard deviation (SD) of 6 (WT), 8 ($G\alpha i2^{-/-}$) and 7 ($G\alpha i3^{-/-}$) mice. *, $p < 0.01$ and **, $p < 0.001$ in the presence or absence of $G\alpha i2$ or $G\alpha i3$.

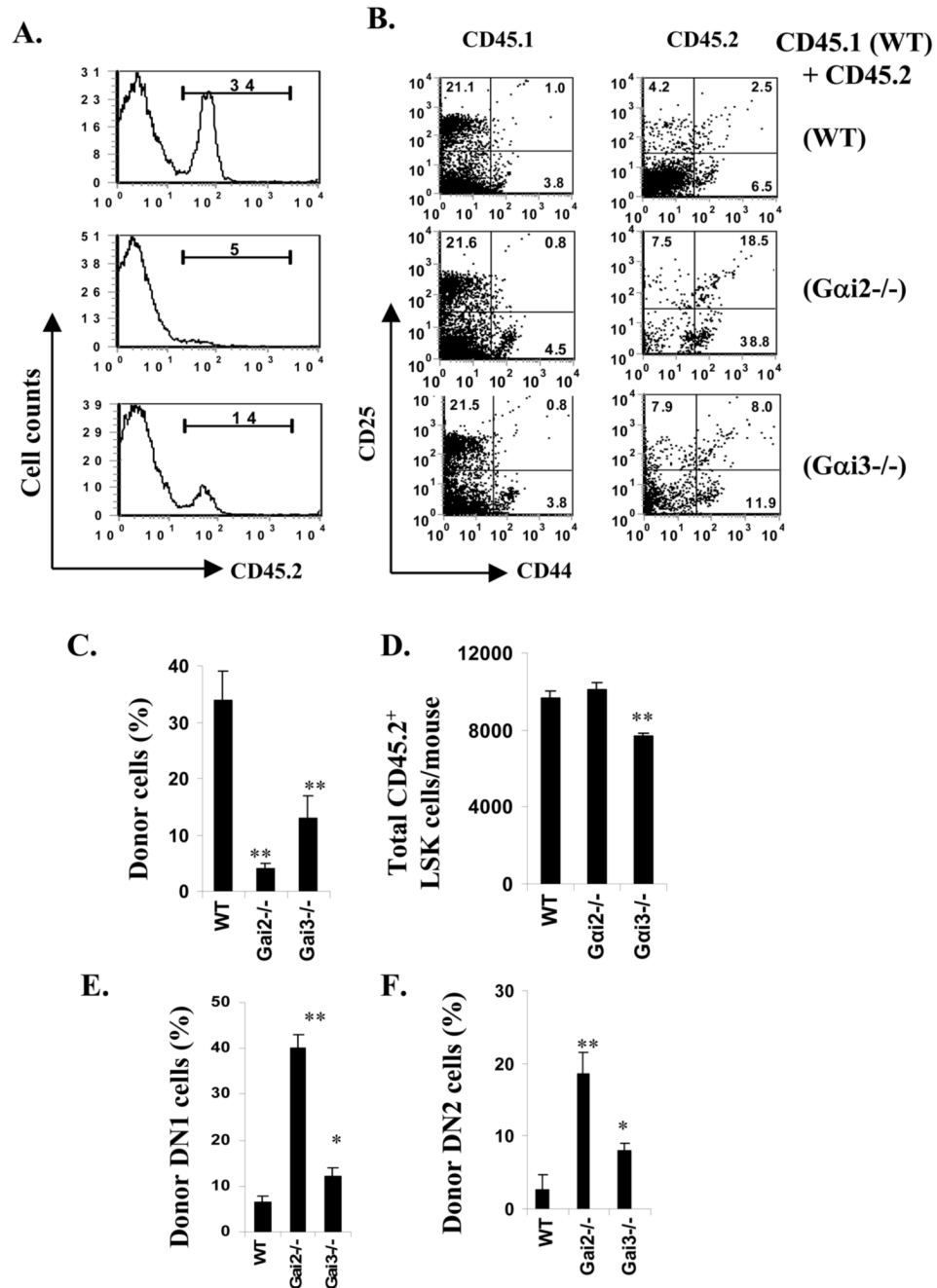


Fig. 2. Requirement of *Gai* protein in early thymic differentiation. Bone marrow chimeras were generated by transfer of equal numbers of CD45.2 WT (upper), *Gai2*^{-/-} (middle) or *Gai3*^{-/-} (low) bone marrow cells with CD45.1 WT bone marrow cells into irradiated B6/SJL (CD45.1) mice, respectively (**A** and **B**). At four weeks after the transfer, donor cells were analyzed by flow cytometry using anti-CD45.2 Ab (**A** and **C**). DN subsets in these chimeras were analyzed in thymocytes in gated CD45.1 or CD45.2 population (**B**, **E** and **F**). The total numbers of pre-thymic progenitors marked as $\text{lin}^- \text{Sca-1}^+ \text{c-Kit}^+$ (LSK) cells per mouse were calculated by the number of LSK cells in 1 million bone marrow cells counted by a FACSAria flow cytometer as detailed in short-term thymic homing assays (**D**). Representative flow cytometric profiles

are shown in **A** and **B**, where the numbers in each gate indicate percentages of positive cells. The data represent the mean percentages \pm SD (**C**, **E** and **F**) or mean counts \pm SD (**D**) of 4 mice in each group.

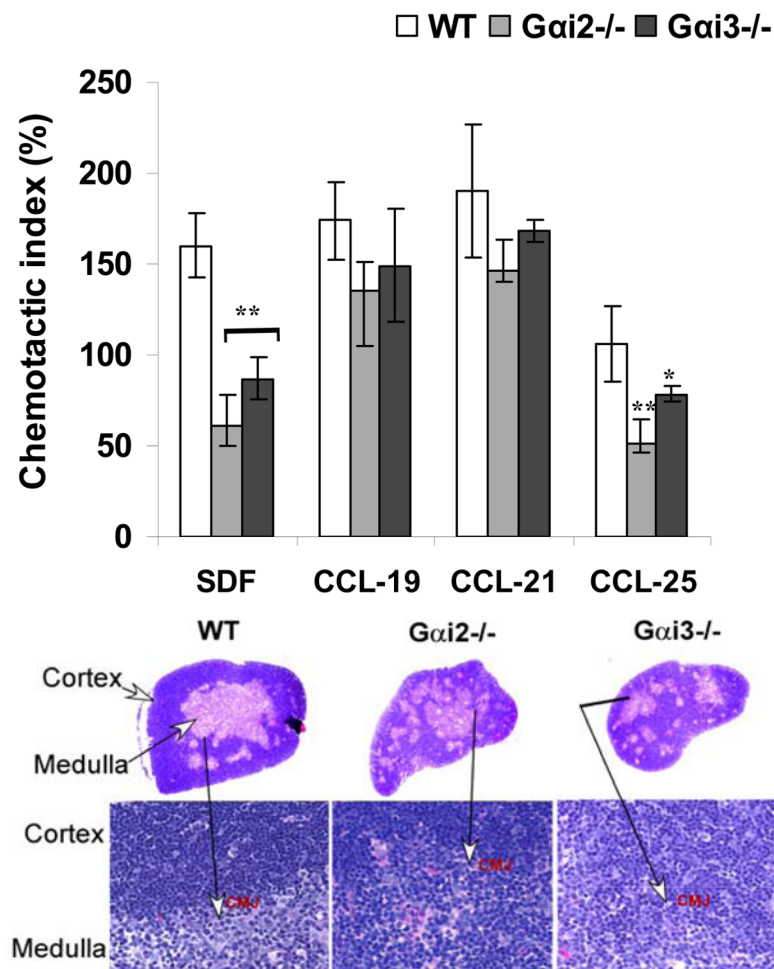


Fig. 3. Altered chemotactic responses of DN1 cells and thymic architecture in the absence of either *Gai2* or *Gai3*. **A.** A defect in chemotactic responses of *Gai*-deficient DN1 cells to SDF-1. Lin⁻ cells were isolated from indicated mice and assayed for 4 hrs at 37C with 5% CO₂ in a 48-well chemotaxis for their responses to various chemokines. DN1 (CD44^{high}CD25⁻) cells were analyzed by flow cytometry in gated lin⁻ cells. Data are presented as the mean chemotactic index \pm SD as defined in materials and methods. Cumulative data from at least five independent experiments with each in duplicate are shown. *, $p < 0.05$ and ** $p < 0.01$ in the presence vs. absence of *Gai2* or *Gai3*. **B.** Altered thymus architecture in *Gai*-deficient mice. Shown are hematoxylin and eosin staining of thymic sections prepared from wild-type (WT), *Gai2*^{-/-} and *Gai3*^{-/-} mice animals at 3 days of age. Note: reduction in the cell density in *Gai3*^{-/-} mice and altered thymic architecture in *Gai2*^{-/-} and *Gai3*^{-/-} mice. Magnification, 5X in the upper panel and the cortico-medullary junction (CMJ) areas are indicated by arrows and enlarged (100 X) at the lower panel. Representative results of five mice in each group.

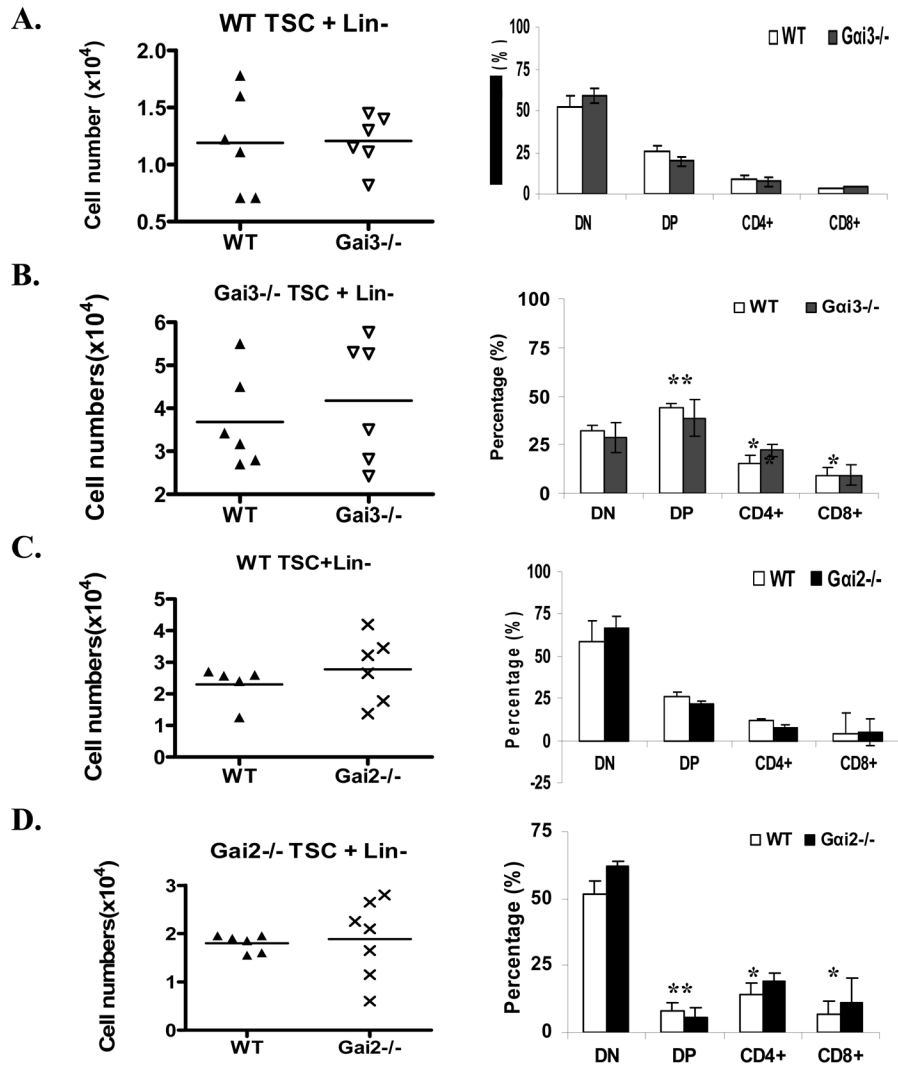
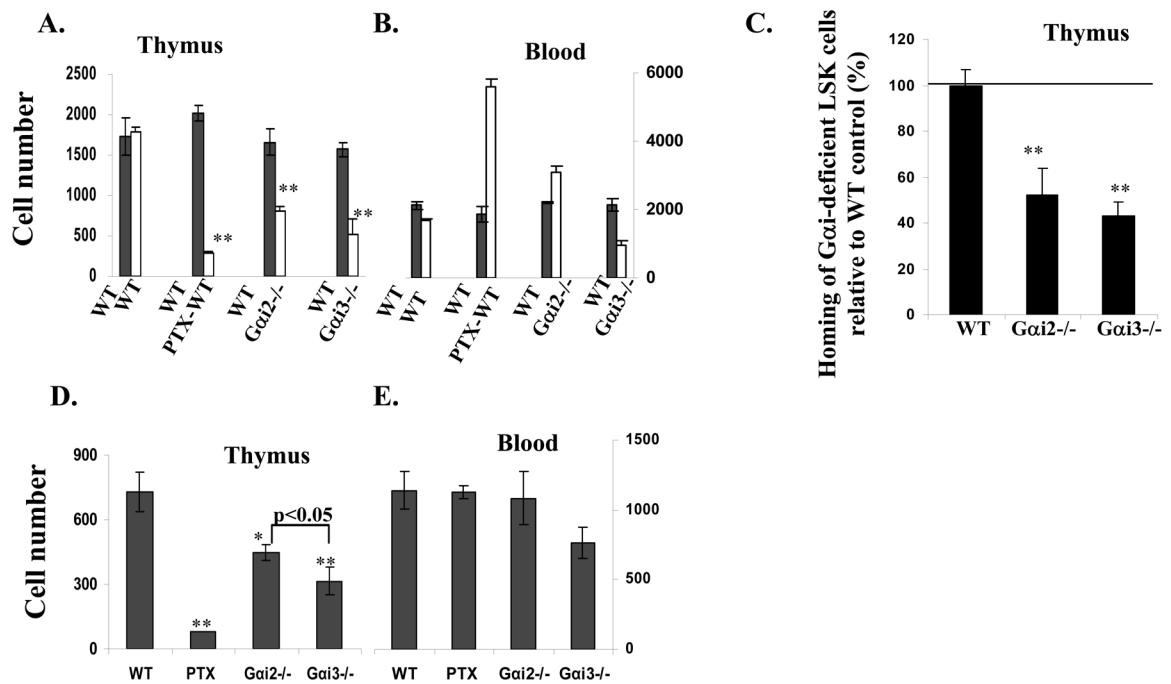


Fig. 4. Augmented thymic stromal function in the absence of either *Gai2* or *Gai3*. Fetal thymic organ cultures (FTOC) were set up, including WT thymic stromal cells (TSC) cocultured with either WT or *Gai3*^{-/-} lin- progenitors (A), *Gai3*^{-/-} TSC with WT or *Gai3*^{-/-} lin- progenitors (B), WT TSC with WT or *Gai2*^{-/-} lin- progenitors (C), and *Gai2*^{-/-} TSC with WT or *Gai2*^{-/-} lin- progenitors (D). Between the two lobes from each fetus, one was co-cultured with WT lin-progenitors and the other with *Gai*-deficient lin-progenitors for comparison in each panel. Thymocytes in single-cell suspensions were counted (left panels) and stained with FITC-CD4 and PE-CD8 Abs, followed by flow cytometric analysis (right panels). Each symbol relates to data obtained from a single lobe. *, p < 0.05 and ** p < 0.01 when *Gai2*^{-/-} or *Gai3*^{-/-} TSC were compared with WT TSC for their ability in support of the differentiation of DP and CD4+ and CD8+ thymocytes.

**Fig. 5.**

Thymic homing depends on Gai proteins. Wild type (WT) bone marrow cells were labeled with TRITC and mixed with an equal number of CFSE-labeled bone marrow cells of indicated phenotypes (**A**, **B**, **D**, and **E**). Or, LSK progenitors were sorted from bone marrows of WT, Gai2^{-/-} and Gai3^{-/-} mice, fluorescently labeled, and mixed as above (**C**). The mixtures were injected intravenously into 4.5-week-old WT mice. Thymi (**A**, **C** and **D**) and blood (**B** and **E**) were analyzed two days (**A**, **B**, and **C**) or seven days (**D** and **E**) later. The data are the means \pm SD of the absolute numbers of labeled cells per thymus (**A** and **D**) or in a million nucleated blood cells (**B** and **E**) of 5 mice in each group. Homing of Gai2- or Gai3-deficient LSK cells relative to WT LSK cells (100%) in the same mouse was shown (**C**). *, $p < 0.05$ and ** $p < 0.01$ in the presence vs. absence of Gai2 or Gai3.

Table 1

Thymocyte numbers and subsets in 3-d-old mice

	WT		Gαi2 ^{-/-}		Gαi3 ^{-/-}	
	%	Cell number (10 ⁶)	%	Cell number (10 ⁶)	%	Cell number (10 ⁶)
Total cells		26.40±4.62		15.57±2.11 [*]		2.13±1.34 ^{**}
CD4 ⁺ CD8 ⁺	80.40±1.97	21.22±3.68	80.08±1.53	12.47±0.24	58.90±14.7	1.25±0.31
CD4 ⁺	12.67±1.77	3.37±0.88	13.21±0.92	2.05±0.14	16.69±4.03	0.36±0.09
CD8 ⁺	3.31±0.48	0.86±0.12	3.39±0.41	0.52±0.05	9.94±6.62	0.21±0.14
	%	Cell number (10 ⁴)	%	Cell number (10 ⁴)	%	Cell number (10 ⁴)
DN1	5.99±1.19	3.43±0.68	31.06±4.41 ^{**}	7.33±1.04 [*]	15.15±1.8 ^{**}	0.58±0.07 ^{**}
DN2	14.92±2.56	8.53±1.46	4.54±0.61 ^{**}	1.27±0.14 ^{**}	7.18±1.26 ^{**}	0.27±0.1 ^{**}
DN3	52.58±2.16	30.08±1.23	40.01±3.99	9.44±0.94 ^{**}	55.41±5.17	2.14±0.19 ^{**}
DN4	11.31±1.46	6.47±0.84	10.73±2.03	2.53±0.48	12.29±4.00	0.47±0.15

* p<0.01; and

** p<0.001 in the presence or absence of either Gαi protein.