Aberrant T-cell ontogeny and defective thymocyte and colonic T-cell chemotactic migration in colitis-prone Gαi2-deficient mice

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doi:10.1111/j.1365-2567.2007.02629.x
Received 16 August 2006; revised 21 December 2006; accepted 22 March 2007.
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Summary

Gαi2-deficient mice, which spontaneously develop colitis, have previously been reported to have an increased frequency of mature, single positive thymocytes compared to wild-type mice. In this study we further characterized the intrathymic changes in these mice before and during overt colitis. Even before the onset of colitis, Gαi2$^{-/-}$ thymi weighed less and contained fewer thymocytes, and this was exacerbated with colitis development. Whereas precolitic Gαi2$^{-/-}$ mice had unchanged thymocyte density compared to Gαi2$^{+/-}$ mice of the same age, this was significantly decreased in mice with colitis. Thymic atrophy in Gαi2$^{-/-}$ mice involved mainly the cortex. Using a five-stage phenotypic characterization of thymocyte maturation based on expression of CD4, CD8, TCRαβ, CD69 and CD62L, we found that both precolitic and colitic Gαi2$^{-/-}$ mice had significantly increased frequencies of mature single-positive CD4^{+} and CD8^{+} medullary thymocytes, and significantly reduced frequencies and total numbers of immature CD4^{+} CD8^{+} double-positive thymocytes compared to Gαi2$^{+/-}$ mice. Furthermore, cortical and transitional precolitic Gαi2$^{-/-}$ thymocytes showed significantly reduced chemotactic migration towards CXCL12, and a trend towards reduced migration to CCL25, compared to wild-type thymocytes, a feature even more pronounced in colitic mice. This impaired chemotactic migration of Gαi2$^{-/-}$ thymocytes could not be reversed by increased chemokine concentrations. Gαi2$^{-/-}$ thymocytes also showed reduced expression of the CCL25 receptor CCR9, but not CXCR4, the receptor, for CXCL12. Finally, wild-type colonic lamina propria lymphocytes migrated in response to CXCL12, but not CCL25 and, as with thymocytes, the chemokine responsiveness was significantly reduced in Gαi2$^{-/-}$ mucosal lymphocytes.

Keywords: thymus; chemokines; mucosal inflammation; animal model

Introduction

T-cell maturation takes place in the thymus, a complex epithelial organ with two distinct anatomical regions, the cortex and the medulla. Haematopoietic stem cells enter the thymus at the corticomedullary junction via the blood, and gradually become lineage-committed under the influence of distinct microenvironments within the thymus. Thymocyte migration within the thymus is crucial for their maturation and chemokines, cell interactions as well as extracellular matrix proteins collectively induce migration and maturation of thymocytes by creating chemotactic gradients and highly specialized microenvironments.

Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disorder of the gastrointestinal tract, comprising ulcerative colitis and Crohn's disease. Defective regulation of T-cell responses to gut flora contribute

Abbreviations: CMF–HBSS, Hanks'balanced salt solution without calcium and magnesium; DN, double-negative; FCS, fetal calf serum; Gαi2$^{-/-}$, Gαi2-deficient; H&E, haematoxylin & eosin; IBD, inflammatory bowel disease; IL, interleukin; IL-1ra, interleukin-1 receptor antagonist; LPL, lamina propria lymphocytes; PBS, phosphate-buffered salin; PTX, pertussis toxin.

to the aetiology of IBD. Thus, severe combined immune-deficient (SCID) mice reconstituted with $CD4^+$ $CD45RB^{high}$ T cells,[1,2] $Tg_\varepsilon 26$ transgenic mice[3] and T-cell receptor-α (TCR-α) chain-deficient mice[4] all develop colitis. The relationship between disease and T-cell maturation in the thymus is, however, poorly understood. The $G\alpha i2^{-/-}$ mouse is a well-established model for colitis[5–7] with T helper type 1 (Th1) driven inflammation with increased levels of interferon-γ (IFN-γ), interleukin-1α (IL-1α), IL-6 and tumour necrosis factor-α (TNF-α) in inflamed tissue.[7] Interestingly, $G\alpha i2^{-/-}$ mice have impaired T-cell development with a significantly increased frequency of mature $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ thymocytes.[5,8] $G\alpha i2^{-/-}$ thymocytes also display increased production of Th1 cytokines (IL-2, IFN-γ and TNF), but not IL-4,[5] as well as increased proliferation[8] when stimulated with anti-CD3, but not when stimulated with phorbol myristate acetate/ionomycin, indicating that the Gαi2-deficiency strengthens TCR-mediated signalling.[8,9] The export of mature thymocytes from the thymus is inhibited in mice transgenic for the catalytic subunit of pertussis toxin, suggesting that the involvement of G-protein-linked receptors is highly important for this event,[10,11] possibly because of the involvement of G-proteins in chemokine signalling.

The responsiveness of thymocytes to different chemokines changes during their maturation process.[12] Thus, thymocyte responsiveness to CCL19 and CCL21, acting via the receptor CCR7, increases during maturation, with cortical thymocytes being unresponsive and medullary single-positive thymocytes being highly responsive.[12] In contrast, cortical, transitional and early medullary thymocytes respond equally well to CCL25, signalling through CCR9, whereas all responsiveness is lost in the most mature medullary thymocytes. Thymocytes of all maturation stages are attracted to CXCL12 with the more immature subsets being somewhat more responsive.[12]

Given the known alterations in the thymus of $G\alpha i2^{-/-}$ mice, as well as the impact of chemokine responsiveness and Gi protein blockade on thymocyte function, we wanted to carefully elucidate the differences in thymocyte maturation using a well-defined phenotypic classification.[13–16] We examined the chemokine responsiveness of thymocytes at defined maturation stages in both precolitic and colitic Gαi2-deficient mice, compared to healthy heterozygotes.

Materials and methods

Mice

Gαi2-deficient ($G\alpha i2^{-/-}$) mice[5] on a 129SvEv × C57BL/6 background (backcrossed four or five generations into 129SvEv and then intercrossed) and on a pure 129SvEv background were used. The animals were kept at the Department of Experimental Biomedicine, Göteborg University. $G\alpha i2^{-/-}$ mice on a 129SvEv × C57BL/6 background were bred using homozygous $G\alpha i2^{-/-}$ males and heterozygous females. Mice on a pure 129SvEv background were bred using heterozygous males and females. Offspring were genotyped by polymerase chain reaction using tail genomic DNA. Gαi2-deficient mice on a mixed 129SvEv × C57BL/6 and a pure 129SvEv background develop a lethal colitis between 10 and 21 weeks and six to 12 weeks of age, respectively. Mice at between four and 21 weeks of age were used throughout the study, and were grouped into precolitic or colitic mice based on clinical appearance including presence/absence of diarrhoea as well as macroscopic and histopathological investigation of the colon and formalin-fixed, haemotoxylin & eosin (H&E)-stained colonic sections, respectively (see below). As the kinetics of colitis development is similar between males and females, both sexes were used throughout the study. Thymocyte properties of $G\alpha i2^{-/-}$ mice within either group were independent of genetic background (data not shown).

All animals were specific-pathogen-free and were maintained in micro-isolator racks with free access to water and standard rodent pellets in accordance with local and national ethical regulations and were health-screened in accordance with recommendations from the Federation of European Laboratory Animal Science Associations (FELASA).

Histopathology

Thymi from six-, 10-, 13-, 18- and 21-week-old-mice were dissected and fixed in 4% buffered formalin (Apoteksbolaget, Gothenburg, Sweden). The entire thymus was sectioned and three 5-μm cross-sections were evenly distributed throughout the thymus then sampled, prepared and stained with H&E according to standard techniques. Cortical and medullary areas were determined using LEICA 1M 1000 IMAGEMANAGER software, where mean areas and ratio of cortex and medulla were calculated from the three thymic lobe cross-sections.

Colonic tissue was fixed in 4% buffered formalin (Apoteksbolaget) and 4–5 μm sections were prepared and stained with H&E according to standard techniques. Colitis was graded in a blinded fashion using a scale of 1–5:[17] where grade 1 is normal mucosa and grade 5 is fulminant inflammation with visible ulcers and fissures, pus at the surface, mucin gland atrophy, and crypt abscesses.

Preparation of cell suspensions

Single cell suspensions were prepared by forcing the whole thymus through a nylon net using a syringe plunger. For chemotaxis assays, thymocytes were preincubated

for 2 × 30 min at 37° in 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) in a Petri dish to remove the adherent cells, such as macrophages and thymic epithelial cells. Colonic lamina propria lymphocytes (LPL) were isolated as previously described[18] with some modifications. Briefly, faecal contents were removed by flushing with saline. The colon was then sealed at one end, everted, filled with phosphate-buffered saline (PBS) and sealed at the other end to expand the crypts. The everted, expanded colons were then washed extensively in Hanks' balanced salt solution without calcium and magnesium (CMF-HBSS; Life Technologies, Paisley, UK) supplemented with 15 mM HEPES (Life Technologies), followed by five 15-min incubations, at 37°, in CMF-HBSS containing 5 mM ethylenediaminetetraacetic acid to remove epithelial cells and intraepithelial lymphocytes. The remaining tissues were incubated for 15 min in RPMI-1640 containing 15 mM HEPES and 10% heat inactivated FCS, followed by three successive 60-min incubations in Collagenase Type XI, 100 U/ml (Sigma, St Louis, MO) dissolved in RPMI-1640 containing 15 mM HEPES and 20% heat inactivated horse serum, yielding LPLs.

Flow cytometry

Single cell suspensions of $0{\cdot}5 \times 10^6$ to 1×10^6 thymocytes/100 μl were stained for flow cytometry with the following monoclonal antibodies: anti-CD45R/B220-phycoerythrin (PE; clone RA3-6B2), anti-CD4-allophycocyanin (APC; clone RM4-5), anti-CD8α-peridinin chlorophyll protein (PerCP; clone 53-6.7), anti-CD8α-fluorescein isothiocyanate (FITC; clone 5H10-1), anti-CD62L-FITC (clone MEL-14), anti-CD69-PE (clone H1.2F3), anti-CD69-FITC (clone FN50), anti-TCR-αβ-FITC (clone H57-597), anti-CXCR4-FITC (clone 2B11/CXCR4) and anti-CCR9-FITC (clone 242503). All antibodies were purchased from BD Pharmingen, San Diego, CA. Analysis was performed on a BD LSR II (BD Biosciences), using FLOW-JO software (Tree star Inc., San Carlos, Ca USA) on a minimum of 20 000 gated thymocytes.

Chemotaxis assays

Following removal of adherent cells, 5×10^6 thymocytes/ml or $3{\cdot}75 \times 10^6$ LPL/ml were placed in the upper well of 5-μm pore, polycarbonate 12-well tissue culture inserts (Corning Incorporated, New York, NY) in 100 μl RPMI-1640 (Gibco, Invitrogen, Burlington, Canada), supplemented with 10% FCS (Gibco, Invitrogen) with 600 μl medium with or without chemokine in the lower well. Cells were incubated for 90 min at 37° in 5% CO_2 whereupon migrated cells were harvested from the lower well, counted and stained for flow cytometric analysis. Chemokines used were recombinant mouse (rm) CXCL12 (SDF-1α), CCL25 (TECK), CCL21 (SLC) and CCL19 (MIP-3β) (R & D Systems, Minneapolis, MN). Chemotactic doses for each chemokine were predetermined in Gαi2$^{+/-}$ thymocytes by testing concentrations ranging from 10 to 200 nM for rmCXCL12 (R & D Systems), 10–300 nM for rmCCL25 (R & D Systems), 10–200 nM for rmCCL21 (R & D Systems), and 1–200 nM for rmCCL19 (R & D Systems) (not shown). In some experiments the effects of stepwise increased concentrations of CXCL12 and CCL25 were compared between Gαi2$^{+/-}$ and Gαi2$^{-/-}$ thymocytes to exclude the possibility of a higher threshold for chemokine responsiveness in Gαi2$^{-/-}$ mice.

Statistical analysis

The Mann–Whitney non-parametric test was used for determination of significant differences. Values of $P = 0{\cdot}05$ were considered to be significant.

Results

Decreased thymic weight and thymocyte numbers in precolitic and colitic Gαi2$^{-/-}$ mice

Thymic atrophy was accompanied by a significant reduction in thymus weight (Fig. 1a) and a significant decrease in thymocyte number (Fig. 1b). Importantly, these changes were apparent before the onset of colitis. The decrease in thymus weight and cell numbers was even more pronounced in Gαi2$^{-/-}$ mice with colitis, in which the thymus was almost undetectable by eye (Fig. 1b). Although the cell density of the thymus of precolitic Gαi2$^{-/-}$ mice remained similar to that of Gαi2$^{+/-}$ mice of the same age, it was significantly reduced in colitic Gαi2$^{-/-}$ mice compared to both precolitic Gαi2$^{-/-}$ mice and Gαi2$^{+/-}$ mice (Fig. 1c).

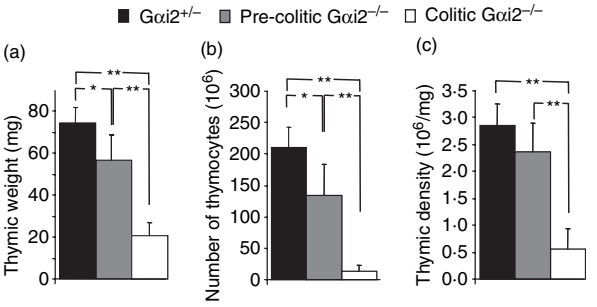

Figure 1. Differences in (a) thymic weight (mg), (b) thymocyte numbers and (c) cellular density (cells/mg) between Gαi2$^{+/-}$ mice ($n = 7$), precolitic Gαi2$^{-/-}$ mice ($n = 6$) and colitic Gαi2$^{-/-}$ mice ($n = 5$) aged 5–11 weeks. Bars represent mean value ± SD where $^*P = 0{\cdot}05$, $^{**}P = 0{\cdot}01$.

Decreasing thymic cortex area with progression of colitis in Gαi2$^{-/-}$ mice

To further analyse the differences between thymi of Gαi2$^{-/-}$ mice and healthy heterozygous mice, the areas of the medulla and cortex were measured in H&E-stained thymic sections. Gαi2$^{-/-}$ and Gαi2$^{+/-}$ mice in five age groups, ranging from 6 to 21 weeks, were examined. The age span represents mild colitis in 6- to 10-week-old mice, mild to moderate colitis in 13- to 18-week-old mice and moderate to severe colitis in 21-week-old mice. The area was measured in two or three sections from three to five thymi per time-point.

Normal thymic involution with age was reflected by a reduced total area in both Gαi2$^{-/-}$ and Gαi2$^{+/-}$ mice with increasing age (Fig. 2). In Gαi2$^{-/-}$ mice, the medulla area was significantly reduced in 13- and 21-week-old mice, compared to 6-week-old mice, in 13-, 18- and 21-week-old compared to 10-week-old-mice, and in 21-week-old compared to 13- and 18-week-old mice. In Gαi2$^{+/-}$ mice, the medulla area was significantly reduced in 13- and 21-week-old mice compared to 6-week-old mice and in 21-week-old compared to 18-week-old mice (data not shown). The size reduction in cortex area in Gαi2$^{+/-}$ mice was, however, not statistically significant in any age group. In contrast, in Gαi2$^{-/-}$ mice the cortex area at 21 weeks of age was significantly reduced compared to that in six-, 10- and 18-week-old mice (Fig. 2). In addition, the cortex area in Gαi2$^{-/-}$ mice was significantly reduced compared to Gαi2$^{+/-}$ mice in 13-, 18- and 21-week-old mice (Fig. 2), whereas the medulla area was not significantly different from that in age-matched Gαi2$^{+/-}$ mice in any age group. To exclude artefacts as a result of level of sectioning within the organ, we also measured the mean ratio of medulla : cortex area in the same sections. The medulla : cortex area ratios in six to 18-week-old Gαi2$^{-/-}$ mice were larger than in age-matched Gαi2$^{+/-}$ mice (not shown). This, together with the cortex and medulla areas, demonstrates that thymic atrophy in Gαi2$^{-/-}$ mice was caused mainly by a decrease in cortex volume.

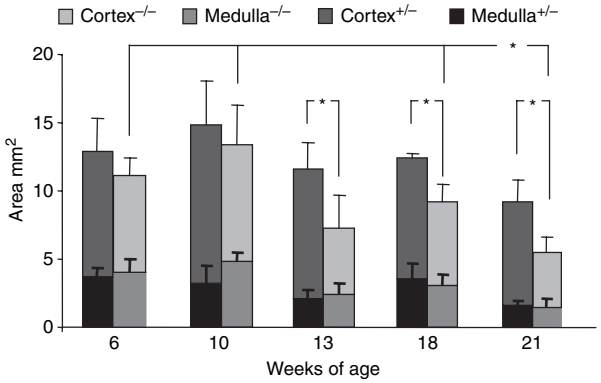

Figure 2. Changes in thymus medulla and cortex area with age. Thymi from 6-, 10-, 13-, 18- and 21-week-old Gαi2$^{-/-}$ (light bars) and Gαi2$^{+/-}$ (dark bars) mice were fixed in 4% buffered formalin and the medullary and cortical area of two or three 5-μm H&E-stained cross-sections per thymic lobe was calculated. Bars represent mean area (mm^2) of medulla or cortex in one lobe ± SD of three to five mice per group. *$P = 0{\cdot}05$.

Fewer cortical immature and more medullary thymocytes in Gαi2$^{-/-}$ mice

Given the anatomical changes of the thymus in Gαi2$^{-/-}$ mice, we next examined the frequencies of immature and mature thymocytes in precolitic and colitic Gαi2$^{-/-}$ mice, compared to healthy heterozygous mice. Thymocytes were analysed by flow cytometry for five phenotypically defined maturation stages[13–16] as follows: (1) CD4$^+$ CD8$^+$ TCR-αβ$^-$ CD69$^-$ (early cortical); (2) CD4$^+$ CD8$^+$ TCR-αβ^{low} CD69$^-$ (late cortical); (3) CD4$^+$ CD8$^+$ TCR-αβ$^+$ CD69$^+$ (transitional between medulla and cortex); (4) CD4$^+$ CD8$^-$ TCR-αβ$^+$ CD69$^+$ CD62L$^{low/-}$ or CD4$^-$ CD8$^+$ TCR-αβ$^+$ CD69$^+$ CD62L$^{low/-}$ (early medullary); and (5) CD4$^+$ CD8$^-$ TCR-αβ$^+$ CD69$^-$ CD62L^{high} or CD4$^-$ CD8$^+$ TCR-αβ$^+$ CD69$^-$ CD62L^{high} (late medullary).[13–16] However, as we found no clear distinction between TCR-αβ^{low} and TCR-αβ$^-$ thymocytes, developmental stages 1 and 2 were analysed together.

In precolitic and colitic Gαi2$^{-/-}$ mice the frequency of immature cortical thymocytes (maturation stage 1–2) was significantly lower compared to Gαi2$^{+/-}$ mice (Fig. 3a). The colitic Gαi2$^{-/-}$ mice also had a significantly lower frequency of transitional thymocytes (maturation stage 3) compared to both precolitic Gαi2$^{-/-}$ mice and Gαi2$^{+/-}$ controls (Fig. 3a, insert), whereas the frequency of CD4$^+$ early medullary thymocytes (stage 4) was not significantly different between Gαi2$^{-/-}$ and Gαi2$^{+/-}$ mice (Fig. 3a, insert). Within the CD8$^+$ population, the colitic Gαi2$^{-/-}$ mice had the highest frequency of stage 4 thymocytes (1·03%), significantly higher than in both Gαi2$^{+/-}$ mice (0·6%, $P = 0{\cdot}044$) and precolitic Gαi2$^{-/-}$ mice (0·15%, $P = 0{\cdot}011$) (not shown). In contrast, both precolitic and colitic Gαi2$^{-/-}$ mice contained significantly higher frequencies of CD4$^+$ late medullary (maturation stage 5) thymocytes compared to Gαi2$^{+/-}$ mice (Fig. 3a). Likewise, in precolitic (6·4 ± 1·6%) and colitic (20·6 ± 11·2%) Gαi2$^{-/-}$ mice the frequency of CD8$^+$ single-positive thymocytes in maturation stage 5 was significantly higher compared to Gαi2$^{+/-}$ mice (1·3 ± 0·6%) ($P < 0{\cdot}0001$ and $P = 0{\cdot}0006$, respectively), and colitic Gαi2$^{-/-}$ mice contained a significantly higher frequency of stage 5 CD8$^+$ thymocytes than precolitic Gαi2$^{-/-}$ mice ($P = 0{\cdot}0011$) (not shown), confirming previous findings.[5,8]

As the total number of thymocytes decreased significantly with colitis development in Gαi2$^{-/-}$ mice (Fig. 1),

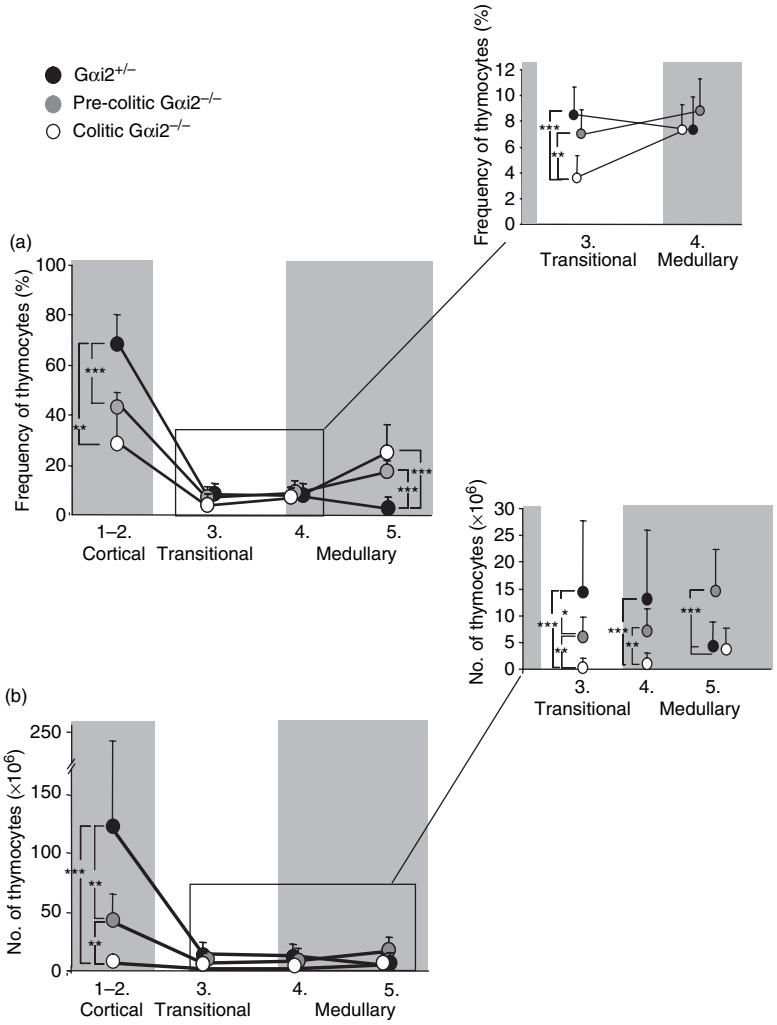

Figure 3. Frequency (a) and total number (b) of immature thymocytes in precolitic and colitic Gαi2$^{-/-}$ mice compared to Gαi2$^{+/-}$ mice. Developmental stages of thymocytes are shown as follows: 1–2, CD4$^+$ CD8$^+$ TCRαβ$^{low/-}$ CD69$^-$ (cortical); 3, CD4$^+$ CD8$^+$ TCRαβ$^+$ CD69$^+$ (transitional between medulla and cortex), 4, CD4$^+$ CD8$^-$ TCRαβ$^+$ CD69$^+$ CD62L$^{low/-}$ (medullary); and 5, CD4$^+$ CD8$^-$ TCRαβ$^+$ CD69$^-$ CD62L^{high} (medullary) as determined by FACS analysis. Results are shown as mean values of: Gαi2$^{+/-}$ ($n = 14$), precolitic Gαi2$^{-/-}$ ($n = 10$) and colitic Gαi2$^{-/-}$ ($n = 6$) ± SD using 5- to 7-week-old-mice.

we also evaluated the total number of thymocytes within the different maturation stages. As with frequencies, both precolitic and colitic Gαi2$^{-/-}$ mice contained significantly decreased total numbers of immature cortical thymocytes (maturation stage 1–2) compared to Gαi2$^{+/-}$ controls. In addition, numbers were significantly reduced in colitic compared to precolitic mice (Fig. 3b). The same was also true for transitional thymocytes, with significantly fewer thymocytes in both precolitic and colitic Gαi2$^{-/-}$ mice, compared to controls, as well as significantly reduced numbers of stage 3 thymocytes in colitic compared to precolitic Gαi2$^{-/-}$ mice. The CD4$^+$ early medullary, stage 4 thymocytes showed the same trend, with fewer cells in Gαi2$^{-/-}$ mice compared to Gαi2$^{+/-}$ controls, although numbers in precolitic mice were not significantly different from controls. In contrast, colitic Gαi2$^{-/-}$ mice contained significantly fewer stage 4 CD4$^+$ thymocytes compared to both precolitic mice and Gαi2$^{+/-}$ controls. The number of CD8$^+$ stage 4 thymocytes in both precolitic ($0{\cdot}14 \times 10^6$) and colitic ($0{\cdot}15 \times 10^6$) Gαi2$^{-/-}$ mice was significantly reduced compared to Gαi2$^{+/-}$ mice ($1{\cdot}15 \times 10^6$) ($P = 0{\cdot}0008$ and $P = 0{\cdot}0066$, respectively) (not shown). The most mature CD4$^+$ thymocytes (stage 5) were found in significantly higher numbers in precolitic Gαi2$^{-/-}$ mice compared to controls and colitic Gαi2$^{-/-}$ mice. Similarly, total numbers of CD8$^+$ stage 5 thymocytes in precolitic Gαi2$^{-/-}$ mice ($5{\cdot}4 \pm 1{\cdot}7 \times 10^6$) were significantly higher than in Gαi2$^{+/-}$ mice ($2{\cdot}7 \pm 2{\cdot}7 \times 10^6$) ($P = 0{\cdot}013$), whereas there was no significant difference in total numbers of CD8$^+$ stage 5 thymocytes between colitic Gαi2$^{-/-}$ mice ($3{\cdot}0 \pm 2{\cdot}7 \times 10^6$) and Gαi2$^{+/-}$ mice. The marked difference in frequency and number of mature, medullary thymocytes in colitic Gαi2$^{-/-}$ mice is a reflection of the reduction in absolute numbers of total thymocytes in these mice.

Impaired migration of Gαi2$^{-/-}$ thymocytes in response to chemokines

Chemokines signal through Gi-linked receptors and are known to be important in thymocyte maturation. Therefore, Gαi2$^{-/-}$ thymocytes were examined for their responsiveness towards chemokines. The chemokine migratory

response was analysed for the five phenotypically defined maturation stages described above.

Cortical, transitional and medullary thymocytes from Gαi2$^{+/-}$ mice all migrated towards CXCL12 (SDF-1α) (Fig. 4a), with the highest migratory responsiveness at the transitional maturation stage. Importantly, cortical, transitional and early medullary thymocytes from precolitic Gαi2$^{-/-}$ mice had a significantly lower migration towards CXCL12 compared to Gαi2$^{+/-}$ thymocytes. CCL25 (TECK) attracted both Gαi2$^{+/-}$ and precolitic Gαi2$^{-/-}$ thymocytes in the transitional and early medullary stages (Fig. 4c), but precolitic Gαi2$^{-/-}$ thymocytes were less responsive to CCL25 (lower mean value), especially during the transitional stage, compared to thymocytes from Gαi2$^{+/-}$ mice, although because of high variance in both groups, this difference did not reach statistical significance (Fig. 4c). CCL21 (SLC) and CCL19 (MIP-3β) induced thymocyte migration in the medullary stages, especially in late medullary thymocytes, whereas cortical thymocytes were relatively unresponsive to these chemokines (Fig. 4b,d). In strong contrast to CXCL12 and CCL25, responsiveness of precolitic Gαi2$^{-/-}$ thymocytes to CCL21 and CCL19 was similar to control thymocytes. Spontaneous migration (chemokinesis) showed no significant differences between Gαi2$^{+/-}$ and Gαi2$^{-/-}$ thymocytes (Fig. 4e). There was, however, a significant difference between spontaneous migration of transitional and early medullary thymocytes compared to cortical and late medullary thymocytes, which is indicative of different constitutive motility in different thymic subset (Fig. 4e).

In mice with colitis, it was very difficult to obtain sufficient numbers of thymocytes to allow for chemotactic analysis. However, in two mice investigated, chemotaxis to CXCL12 and CCL25 was not significantly raised above spontaneous migration to medium alone, indicating that the reduced responsiveness of Gαi2$^{-/-}$ cortical and transitional thymocytes to chemokines was even more pronounced in colitic mice (data not shown).

To exclude the possibility that the reduced responsiveness of Gαi2$^{-/-}$ thymocytes to CXCL12, and to some extent also to CCL25, was due simply to different chemokine concentration optima, we measured responsiveness to a concentration gradient of chemokines. As shown in Table 1, increasing the concentration of CXCL12 to 75,

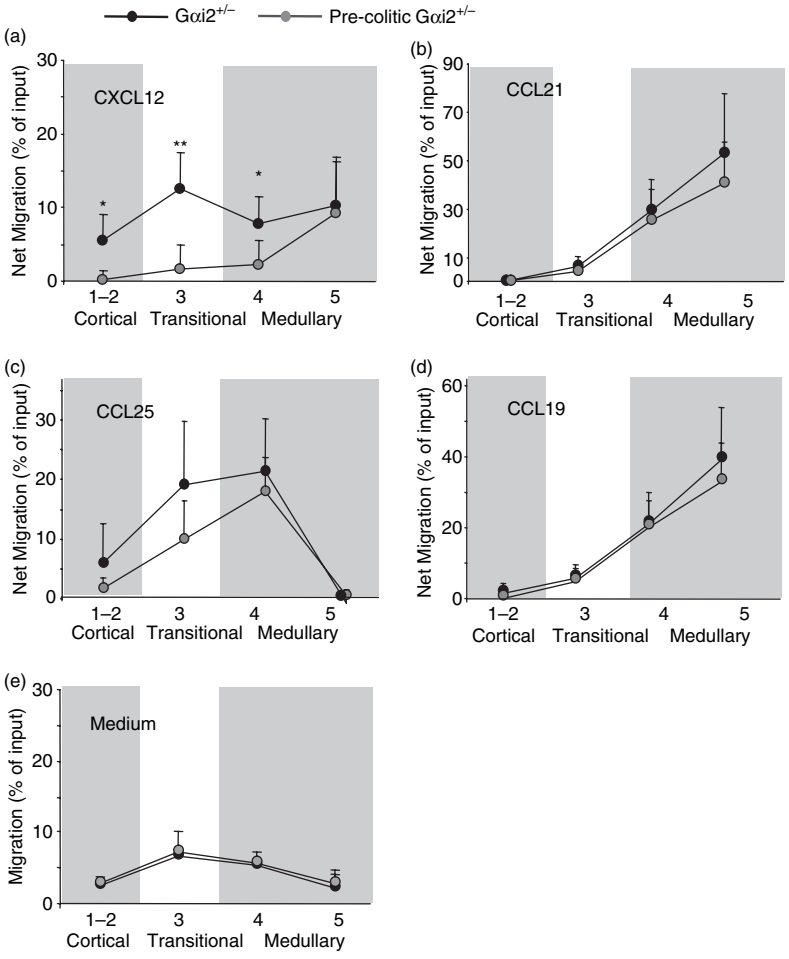

Figure 4. Migration of thymocytes from Gαi2$^{+/-}$ ($n = 6$) and precolitic Gαi2$^{-/-}$ ($n = 6$) mice in response to chemokines during their maturation process. Values are shown as per cent migration of different thymocyte subpopulations in response to (a) CXCL12 (SDF-α) (50 nM), (b) CCL21 (SLC) (100 nM), (c) CCL25 (TECK) (200 nM), and (d) CCL19 (MIP-3β) (10 nM). Background migration (e) (migration to medium alone, in the absence of chemokine) shows spontaneous migration from both Gαi2$^{-/-}$ and Gαi2$^{+/-}$ thymocytes. Developmental stages shown are as follows: 1–2, CD4$^+$ CD8$^+$ TCRαβ$^{low/-}$ CD69$^-$ (cortical); 3, CD4$^+$ CD8$^+$ TCRαβ$^+$ CD69$^+$ (transitional between medulla and cortex); 4, CD4$^+$ CD8$^-$ CD69$^+$ CD62L$^{low/-}$ (early medullary); and 5, CD4$^+$ CD8$^-$ CD69$^-$ CD62L^{high} (late medullary). The cells were counted and stained for four-colour flow cytometry both before and after migration in response to the indicated chemokine as follows: anti-CD4-APC, anti-CD8-PerCP, anti-CD69-PE and anti-CD62L-FITC or anti-TCRαβ-FITC. Results are demonstrated as mean value (% of input) of fluorescence-positive cells ± SD from six independent experiments. Results in (a) to (d) represent total migration minus background migration. $^*P = 0{\cdot}05$; $^{**}P = 0{\cdot}01$ between migrated Gαi2$^{-/-}$ and Gαi2$^{+/-}$ thymocytes.

Table 1. Wild-type and $G\alpha i2^{-/-}$ thymocyte migration in response to different concentrations of chemokines[1]

	Maturation stage							
	1–2: $CD4^+$ $CD8^+$ TCR-$\alpha\beta^{low/-}$ $CD69^-$ (cortical)		3: $CD4^+$ $CD8^+$ TCR-$\alpha\beta^+$ $CD69^+$ (transitional)		4: $CD4^+$ $CD8^-$ $CD69^+$ $CD62L^{low/-}$ (early medullary)		5: $CD4^+$ $CD8^-$ $CD69^-$ $CD62L^{high}$ (late medullary)	
	$G\alpha i2^{+/-}$	$G\alpha i2^{-/-}$	$G\alpha i2^{+/-}$	$G\alpha i2^{-/-}$	$G\alpha i2^{+/-}$	$G\alpha i2^{-/-}$	$G\alpha i2^{+/-}$	$G\alpha i2^{-/-}$
CXCL12								
75 nM ($n = 2$)	18·2 ± 8·0	< 0·0	25·2 ± 12·9	< 0·0	11·7 ± 1·7	< 0·0	10·5 ± 4·4	< 0·0
100 nM ($n = 2$)	18·1 ± 2·2	< 0·0	17·0 ± 3·3	< 0·0	12·9 ± 0·2	< 0·0	15·2 ± 4·5	< 0·0
125 nM ($n = 1$)	16·6	1·2	22·1	3·8	11·6	< 0·0	11·4	1·6
CCL25								
250 nM ($n = 2$)	12·7 ± 4·6	< 0·0	22·4 ± 2·5	< 0·0	26·5 ± 2·8	1·7 ± 1·6	2·9 ± 4·4	< 0·0
300 nM ($n = 2$)	19·8 ± 6·5	< 0·0	34·6 ± 15·7	5·1 ± 2·6	32·7 ± 8·9	6·7 ± 1·7	3·7 ± 3·7	< 0·0

[1]Migration of thymocytes from $G\alpha i2^{+/-}$ ($n = 1$ or $n = 2$) and precolitic $G\alpha i2^{-/-}$ ($n = 1$ or $n = 2$) mice in response to chemokines during their maturation process. Values are shown as per cent migration of different thymocyte subpopulations in response to the indicated concentrations of CXCL12 (SDF-α) and CCL25 (TECK). Background migration (migration to medium alone, in the absence of chemokine) was subtracted from each data point. The cells were counted and stained for four-colour flow cytometry both before and after migration in response to the indicated chemokine as follows: anti-CD4-APC, anti-CD8-PerCP, anti-CD69-PE and anti-CD62L-FITC or anti-TCR-αβ-FITC. Results are demonstrated as mean value (% of input) of fluorescence-positive cells ± SD from two independent experiments.

100 and 125 nM did not reverse the low responsiveness of $G\alpha i2^{-/-}$ thymocytes to this chemokine. In contrast, $G\alpha i2^{+/-}$ thymocytes responded well to all concentrations of CXCL12. With regard to CCL25, all stages of $G\alpha i2^{+/-}$ thymocytes responded well, with some evidence of increasing responsiveness up to 300 nM. However, chemotactic responses of $G\alpha i2^{-/-}$ cells to CCL25 were clearly absent or, in the case of the highest concentration of CCL25, very low.

Reduced expression of CCR9 but not CXCR4 on $G\alpha i2^{-/-}$ thymocytes

To further analyse the mechanism(s) underlying the impaired responsiveness of cortical and transitional $G\alpha i2^{-/-}$ thymocytes to CXCL12 and CCL25, thymocyte expression of CXCR4 and CCR9, the only known receptors for CXCL12 and CCL25, respectively, was investigated. Whereas close to 100% of the more immature thymocytes expressed CXCR4, very few single-positive thymocytes were CXCR4-positive (Fig. 5a). In addition, the intensity of CXCR4 expression was reduced on more mature thymocytes (Fig. 5b). However, neither the frequency of thymocytes expressing CXCR4, nor the intensity of expression, was different between $G\alpha i2^{+/-}$, precolitic and colitic $G\alpha i2^{-/-}$ mice, irrespective of maturation stage (Fig. 5a,b).

Similar to CXCR4, the frequency of $CCR9^+$ thymocytes decreased with increasing maturation stage (Fig. 5c). However, in strong contrast to CXCR4, the frequency of thymocytes expressing CCR9 was significantly reduced in $G\alpha i2^{-/-}$ mice with colitis compared to heterozygous controls, irrespective of maturation stage. The same was also true for precolitic $G\alpha i2^{-/-}$ mice, except for immature $CD4^+$ $CD8^+$ $CD69^-$ (maturation stage 1–2) thymocytes (Fig. 5c). Likewise, the intensity of CCR9 expression was significantly reduced on thymocytes from both colitic and precolitic $G\alpha i2^{-/-}$ mice compared to heterozygous controls, except on the more mature CD8 single-positive thymocytes (Fig. 5d). The median fluorescence intensity of CCR9 staining on $CCR9^+$ thymocytes was, however, not influenced by the maturation stage.

Impaired migration of $G\alpha i2^{-/-}$ colonic lamina propria lymphocytes in response to CXCL12

The chemokines CCL25 and CXCL12 are also expressed in the small and large intestine. CCL25 expression has previously been described as being restricted to the small intestine[19] while CXCL12 is expressed in both small and large intestine.[20–22] Thus, we investigated whether an impaired chemotactic response, similar to that observed in the thymus, was evident also in colonic lamina propria lymphocytes from $G\alpha i2^{-/-}$ mice. Colonic lymphocytes from $G\alpha i2^{-/-}$ mice showed an increased spontaneous migration compared to wild-type mice (data not shown). In agreement with the previously described absence of CCL25 in the large intestine, neither $G\alpha i2^{-/-}$ nor wild-type colonic LPLs were responsive to CCL25 (Fig. 6). In contrast, CXCL12 induced a strong chemotactic response in wild-type colonic lamina propria $CD4^+$ T lymphocytes as well as in B lymphocytes. However, as with thymo-

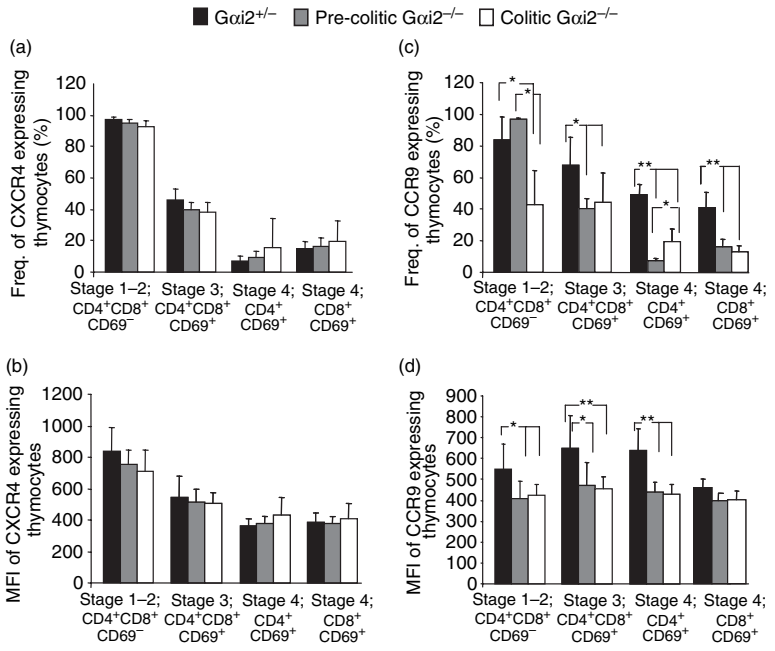

Figure 5. Frequencies (a,c) and median fluorescence intensity (MFI) (b,d) of CCR9 (a,b) and CXCR4 (c,d) expression on thymocytes in maturation stage 1–4 (defined on figure). Cells were stained for four-colour flow cytometry as follows: anti-CD4-APC, anti-CD8-PerCP, anti-CD69-PE and anti-CXCR4-FITC or anti-CCR9-FITC. Gαi2$^{+/-}$ mice were used as controls (CCR9, $n = 11$; CXCR4, $n = 17$); precolitic Gαi2$^{-/-}$ mice (CCR9, $n = 4$; CXCR4, $n = 9$) and colitic Gαi2$^{-/-}$ mice (CCR9, $n = 4$; CXCR4, $n = 6$). Bars represent mean value ± SD where $^{*}P = 0{\cdot}05$ and $^{**}P = 0{\cdot}01$.

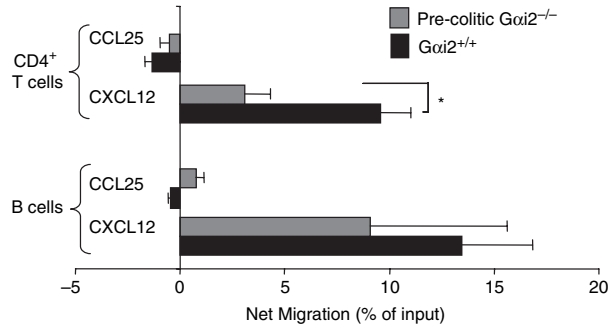

Figure 6. Migration of colonic lamina propria lymphocytes from Gαi2$^{+/+}$ ($n = 8$, two or three mice pooled in three different experiments) and precolitic Gαi2$^{-/-}$ ($n = 3$) mice in response to CCL25 and CXCL12. Values are shown as differences between total chemokine-induced migration minus spontaneous migration of CD4^{+} T lymphocytes and B lymphocytes. Cells were counted and stained for two-colour flow cytometry with anti-CD4-APC and anti-CD45R/B220-PE both before and after migration in response to the indicated chemokine. Results are demonstrated as ratio ± SD from three independent experiments. $^{*}P = 0{\cdot}05$ between Gαi2$^{-/-}$ and Gαi2$^{+/+}$ colonic lamina propria lymphocytes.

cytes, CD4^{+} T lymphocytes from colons of Gαi2$^{-/-}$ mice gave a significantly reduced chemotactic response to CXCL12, compared to wild-type mice (Fig. 6).

Discussion

This study demonstrates that thymic involution is initiated in Gαi2$^{-/-}$ mice even before the onset of colitis, and is exacerbated as the severity of colitis progresses. In addition, we demonstrate that this atrophy is mainly the result of decreased numbers of immature cortical thymocytes. This reduction in immature cortical thymocytes could be because of impaired recruitment of T-cell precursors to the thymus (because both immature and transitional thymocytes responded poorly to CXCL12 and CCL25). In contrast, the number of mature thymocytes was increased in Gαi2$^{-/-}$ mice and their responsiveness to CCL19 and CCL21 was indistinguishable from that in heterozygous mice.

Thymus involution is frequently observed during stress, for example, inflammation, infection, psychological stress, or pregnancy. During inflammation or infection, IL-1 will activate the hypothalamic–pituitary–adrenal axis, leading to increased levels of circulating glucocorticoids.[23] Glucorticoids will, in turn, cause depletion of cortical thymocytes through binding to specific receptors on thymocytes.[24] Increased local intestinal production of IL-1 is a hallmark of the inflammation seen in Gαi2$^{-/-}$ mice and is, in fact, already increased before colitis development.[6,7] In addition, increased circulating levels of IL-1ra are seen at the time of colitis onset, indicating augmented IL-1 production.[25]

Stress-induced thymic involution has also been demonstrated as mediated by reduced migration of precursor T cells from bone marrow to thymus because of reduced responsiveness to chemoattractants released from the thymus.[26–29] The chemokines demonstrated to be involved in the recruitment of T-cell precursors to the fetal thymus are CCL25 and CCL21, whereas anti-CXCL12 antibodies did not significantly affect fetal thymus colonization.[30] However, it should be noted that

the migratory requirements for fetal and postnatal progenitors cannot be assumed to be the same, because fetal progenitors do not migrate outward across the cortex during differentiation, nor do they enter the thymus through blood vessels at the corticomedullary junction, as the fetal organ is neither structured nor vascularized at the time of progenitor seeding.[31] In fact, a study by Plotkin *et al*. demonstrates that CXCL12/CXCR4 signalling is absolutely required for proper localization of early progenitors into the cortex and therefore for successful steady-state differentiation and lineage commitment, and that cells lacking CXCR4 were unable to differentiate past the double-negative (DN for CD4 and CD8) 1 stage.[32]

Importantly, chemokines signal through G-protein-coupled receptors, and irreversible blocking of Gi signalling by pertussis toxin (PTX) affects thymocyte migration.[26,33] As in Gαi2$^{-/-}$ mice, the cellularity of the thymus of pertussis toxin-treated mice was decreased, especially in the cortex. Pertussis toxin-sensitive events were also demonstrated to control the trafficking of single-positive cells across the corticomedullary junction.[34] In agreement, chemotaxis studies on Gαi2$^{-/-}$ thymocytes demonstrated an impaired capacity of these cells to respond to CXCL12 and, to a lesser extent CCL25, compared to Gαi2$^{+/-}$ mice. Both chemokines are important for cortical and transitional thymocyte migration.[12] The reduced capacity to respond to these chemokines is also reflected in the decreased cortex thymocyte count in Gαi2$^{-/-}$ mice. The spontaneous migration in the absence of chemokines, i.e. chemokinesis, was shown to be different within different thymic subpopulations, with the highest spontaneous motility observed in transitional and early medullary thymocytes, but the exact mechanism(s) underlying this behaviour is at present unknown. This finding is not novel; Kim *et al*. have previously demonstrated increased chemokinesis in DN1 compared to DN2–DN4 thymocytes.[35]

Although reduced expression of CCR9 was observed in Gαi2$^{-/-}$ thymocytes (Fig. 5), the migratory response to CCL25 was only marginally affected. We have no clear explanation for this, but there are many examples in the literature showing that the level of receptor expression does not necessarily correlate with migratory potential. There are several mechanism(s) downstream of the receptor that can account for this, e.g. route of receptor signalling, differential usage of signalling intermediates and efficiency of cytoskeleton remodelling.[36–38]

Expression of the only known CXCL12 receptor, CXCR4, was equivalent in Gαi2$^{-/-}$ mice and does not explain the decreased responsiveness to CXCL12. The poor migratory response is thus more likely to be caused by defects in signalling from the receptor caused by the Gi protein deletion. In agreement with our findings, a recent study by Holland *et al*.[37] describes uniform surface expression of CXCR4, but profound differences in CXCR4 activation and signalling following CXCL12 stimulation in invasive versus non-invasive breast cancer cells.[39] More recent investigations have demonstrated that intrathymic CXCL12 exert a CXCR4-mediated chemorepellent activity, contributing to single-positive thymocyte egress from the fetal thymus. Thus, although we found reduced responsiveness to CXCL12 in immature thymocytes only, it cannot be ruled out that the findings of an accumulation of mature single-positive thymocytes in the Gαi2$^{-/-}$ thymus could, at least in part, be the result of poor responsiveness of these mice to CXCL12.[40] Furthermore, although CXCL12$^{-/-}$ and CXCR4$^{-/-}$ mice die *in utero*,[41] studies in mice reconstituted with bone marrow haematopoietic progenitor cells expressing SDF-1(CXCL12) intrakine, which resulted in a dramatic reduction in the expression of CXCR4 in all haematopoietic cells, demonstrated that these mice had a reduced proportion of double-positive, but an accumulation of single-positive thymocytes[41] similar to our observations in Gαi2$^{-/-}$ mice. Somewhat surprisingly, the responsiveness of Gαi2$^{-/-}$ thymocytes to other chemokines, i.e. CCL19 and CCL21, which direct the migration of mature medullary thymocytes, was not different from that in Gαi2$^{+/-}$ thymocytes. This is consistent with the unchanged size of the medullary area, but does not explain the increased numbers of mature thymocytes in Gαi2$^{-/-}$ mice. No peripheral lymphopenia has been observed in Gαi2$^{-/-}$ mice and this would argue against any severe retention of thymocytes in Gαi2$^{-/-}$ mice. Nevertheless, even a small shift in the capacity for mature thymocytes to egress from the thymus could give rise to the observed accumulation of mature thymocytes in Gαi2$^{-/-}$ mice, although only minor changes, if any, would be visible in the periphery – consistent with Zhang *et al.* who did not find significant alterations in thymocyte emigration in Gαi2$^{-/-}$ mice.[8]

The only chemokine to which a significantly reduced responsiveness of Gαi2$^{-/-}$ thymocytes was found was CXCL12. The finding that this chemokine acts mainly on immature (stage 1–3) thymocytes, together with our findings of fewer immature thymocytes in Gαi2$^{-/-}$ mice, support the view that these thymocyte subsets need signals from CXCL12 for proper expansion in the thymic cortex. This impaired signalling response to CXCL12 was also seen in colonic lamina propria lymphocytes, underlining the importance of Gαi2 in signalling from the CXCL12 receptor, CXCR4. It also excludes the possibility that the unresponsiveness to only CXCL12 was the result of the maturation stage of the thymocytes. Thus, a hypothetical model for the selectively impaired chemokine responsiveness in Gαi2$^{-/-}$ mice would be that whereas CCR7, and probably CCR9, are able to compensate for the lack of Gαi2 with, e.g. one of other inhibitory G protein α subunits, CXCR4 has an absolute requirement for Gαi2.

The impaired expansion of CXCL12-dependent immature thymocytes in Gαi2$^{-/-}$ mice would allow for a more

pronounced increase of the more mature, single-positive thymocyte populations. The reliance on Gαi-protein signalling from chemokine receptors is dependent upon the functional status of the cell and will therefore only be utilized for the chemotactic response when concurrent signals favour this function in the cell.[39,42] It is therefore not surprising that we find chemotactically different responses to CXCL12 in different maturation stages of thymocytes.

In the context of autoreactivity, we have previously demonstrated a significantly increased incidence of autoreactive antibodies in $G\alpha i2^{-/-}$ mice[26] and, given the thymic alterations in $G\alpha i2^{-/-}$ mice, an autoreactive T-cell repertoire could also be present in these mice.

The generation of regulatory $CD4^+$ $CD25^+$ T cells, which have been shown to be important in the regulation of IBD, requires high-affinity interactions between thymic cortical epithelial cells and thymocytes.[43,44] Importantly, Faubion *et al*. demonstrated that colitis in $Tg_\varepsilon 26$ transgenic mice was associated with both aberrant thymic development and a paucity of $CD25^+$ Foxp3 expressing T regulatory cells.[34] The significantly reduced thymic size in $G\alpha i2^{-/-}$ mice compared to $G\alpha i2^{+/-}$ mice, which was exacerbated by the development of colitis, could thus give rise to a dysfunctional regulatory T-cell repertoire in the $G\alpha i2^{-/-}$ mice. The frequency and functionality of $CD25^+$ $Foxp3^+$ $CD4^+$ T cells in $G\alpha i2^{-/-}$ mice is presently under investigation.

In conclusion, this study demonstrates how the progression of colitis in Gαi2-deficient mice accelerates thymic involution, especially affecting the thymic cortex. The reduced size of the cortex is also reflected in a decreased frequency of immature cortical thymocytes in $G\alpha i2^{-/-}$ mice which, at least in part, could be explained by reduced responsiveness to CXCL12. This impaired T-cell maturation in $G\alpha i2^{-/-}$ mice could have implications for the peripheral T-cell clonal repertoire and its ability to regulate the normal state of 'physiological inflammation' in the intestinal mucosa.

Acknowlegements

We gratefully acknowledge Maria Sapnara for breeding and genotyping the mice, and Olof Hultgren for valuable comments on the manuscript. This study was supported by The Swedish Research Council (E.H.H. and P.W.B.), The Swedish Cancer Society (E.H.H.), The Astra-Zeneca Research Fund for Gastrointestinal Disorders (E.H.H.), The Nanna Svartz Foundation (E.H.H.), The Sahlgrenska University Hospital Foundation for Clinical research (LUA-ALF) (E.H.H. and P.W.B.), The Royal Society of Arts and Sciences in Goteborg (E.H.H.), The Åke Wiberg Foundation (E.H.H.), The Swedish Society of Medicine (E.H.H.), The Sahlgrenska University Hospital Foundation (K.E. and M.B.), The Anna-Lisa and Bror Björnsson Foundation (R.W.) and The Uppsala University Hospital Foundation for Clinical Research (LUA) (R.W.)

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