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Significant involvement of nuclear factor-κB-inducing kinase in proper differentiation of αβ and γδ T cells

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Summary

Nuclear factor-κB-inducing kinase (NIK) is known to play a critical role in maintaining proper immune function. This is exemplified in the spontaneous mutant mouse lacking functional NIK, alymphoplasia (*aly*), which is simultaneously immune-compromised and autoimmune-prone. To investigate the role of NIK in αβ T-cell repertoire formation, we analysed T-cell development in *aly*/*aly* mice bearing a transgenic T-cell receptor (TCR). Although there were no apparent abnormalities in the mature αβ T cells of non-transgenic *aly*/*aly* mice, the maturation efficiency of idiotype^{high+} T cells in the TCR-transgenic mice was lower in *aly*/*aly* mice compared with those found in *aly*/+ mice, suggesting that the mature αβ T-cell repertoire could be altered by the absence of functional NIK. In one strain of TCR-transgenic *aly*/*aly* mice with a negatively selecting H-2 background, the proportion of CD8^{low+} idiotype^{high+} cells, which are thought to potentially represent the γδ lineage of T cells, was markedly decreased. When the γδ T cells in non-transgenic *aly*/*aly* mice were investigated, the proportion of γδ T cells in the peripheral organs of *aly*/*aly* mice was found to be one-half to one-fifth of those in *aly*/+ mice. Analyses of bone marrow chimera mice indicated that NIK in host cells, rather than in donor cells was important for generating a normal number of peripheral γδ T cells. Collectively, these results suggest that NIK could be involved in thymic positive selection of some αβ T cells and that NIK in non-haematopoietic cells is important for the optimal development and/or maintenance of γδ T cells.

Keywords: nuclear factor-κB-inducing kinase; repertoire formation; T-cell receptor-transgenic mouse.

Introduction

The development of αβ T cells in the thymus is a multistep process that depends crucially on signalling from T-cell receptors (TCRs). In the thymocytes in which successful recombination of the TCR-β gene segments has occurred, pre-TCR complexes composed of the TCR-β chain and the pTα chain are generated, and the ligand-independent triggering of the pre-TCR signal drives the thymocyte differentiation forward to CD4/CD8 double-positive (DP) stages.[1] Further maturation requires signalling from TCR-αβ, but the signalling from TCR-αβ on DP thymocytes should be strictly controlled, because the strength and/or duration of the TCR signalling has to be converted into different signals in quality, leading the cells to different fates. Namely, while apoptosis would be induced in the DP cells bearing TCR-αβ that interact too strongly with self-peptide/MHC molecules, the DP thymocytes with moderate avidity with self-peptide/MHC would survive to mature into CD4 or CD8 single-positive (SP) cells, depending on the classes of MHC molecules with which they have interacted.

Abbreviations: Aly, alymphoplasia; B6, C57BL/6; BM, bone marrow; DP, double positive; LTβR, lymphotoxin β receptor; MHC, major histocompatibility complex; mTEC, medullary thymic epithelial cell; NIK, nuclear factor-κB-inducing kinase; RAG, recombination activating gene; SP, single positive; Tg, transgenic; TCR, T-cell antigen receptor; WT, wild-type

Signalling from TCR activates a number of transcription factors. Among them, nuclear factor-κB (NF-κB) has been demonstrated to be one of the important regulators for thymocyte differentiation.[2] It has been demonstrated that NF-κB activation is observed at β-selection[3] as well as positive and negative selection of αβ T cells.[4–6] Although the PKCθ/Carma1/Bcl10/Malt1 pathways for IκB kinase activation seems pivotal for TCR-induced NF-κB activation in peripheral mature T cells, abrogation of this pathway does not affect TCR-induced NF-κB activation in thymocytes as much as in peripheral T cells.[7–9] These observations indicate that the molecules that mediate TCR-induced NF-κB activation in immature thymocytes may be different from those in mature T cells.

The NF-κB-inducing kinase, NIK, is known to contribute to NF-κB activation,[10] and to play diverse roles in various aspects of homeostasis. Its *in vivo* roles have been investigated mainly using an NIK-deficient mouse[11] and a spontaneous mutant mouse, alymphoplasia (*aly*).[12] The alymphoplasia mouse was isolated as a mutant mouse that lacked all lymph nodes and Peyer's patches,[12] and its causal mutation has been identified on the NIK gene.[13] The mutation is a mis-sense mutation (G855R), which results in defective interaction with IκB kinase α, resulting in impaired phosphorylation of p100.[14] Regarding the role of NIK in TCR signalling in thymocytes, it has been shown that NF-κB activation upon anti-CD3 stimulation was attenuated in *aly*/*aly* thymocytes,[15] suggesting that NIK plays mandatory roles in TCR-mediated NF-κB activation in thymocytes. These results also suggested a possibility that the NIK in thymocytes may be involved in thymic selection, and so in peripheral T-cell repertoire formation.

In *aly*/*aly* mice, however, apparent abnormalities have not been found in T-cell development.[12] The numbers of thymocytes or splenic T cells in *aly*/*aly* mice are normal, and the peripheral $CD4^+/CD8^+$ ratio is almost the same as that in wild-type (WT) mice. Nevertheless, it is still possible that the threshold of positive or negative selection may be shifted by the *aly* mutation, and that the mature T-cell repertoire in *aly*/*aly* mice may be different from that in WT mice. In such a case, the analyses should be performed with a fixed TCR, using TCR transgenic (Tg) mice, to follow the fate of the T cells expressing a particular TCR.

In contrast to the αβ T cells, information on the role of NIK or of NF-κB activation in the development of another subset of T cells, γδ T cells, is sparse. Although the genetic requirements in the development differ between αβ and γδ T cells,[16] it is thought that, like αβ T cells, TCR signalling may be crucial for the maturation of (at least some populations of) γδ T cells in the thymus.[17] Intriguingly, differentiation of thymic γδ T cells has been shown to be affected by the lymphotoxin β (LTβ) signalling upon interaction with DP αβ T cells.[18] Given that NIK is critical in the signal transduction from LTβ receptor (LTβR),[11] it appears quite possible that NIK may play some key roles in the development of γδ T cells, which still remain to be explored.

In the present study, development of αβ T cells and γδ T cells in *aly*/*aly* mice have been investigated using the TCR-αβ Tg mouse, to reveal the roles of NIK in the development of αβ and γδ T cells. The results suggested that the efficiency of the positive selection of at least some of αβ T cells could be affected by the lack of functional NIK. It was also suggested that peripheral maintenance and/or the development of γδ T cells may require functional NIK to be expressed in non-haematopoietic cells.

Materials and methods

Mice

C57BL/6J ($H\text{-}2^b$), DBA/1 ($H\text{-}2^q$), C3H/HeN ($H\text{-}2^k$) mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). B10.S ($H\text{-}2^s$) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The alymphoplasia mice were obtained from Clea Japan, Inc. (Tokyo, Japan), and were bred onto C57BL/6J > 10 times before inter-breeding to produce the *aly*/*aly* mouse or breeding with other strains of mice. The ^{QM11}TCR-Tg mouse, possessing the transgenes for the α and β chains of TCR recognizing $I\text{-}A^k$ as the allo-antigen, was described previously.[19] In some experiments, analyses were performed using ^{QM11}TCR-Tg mice with RAG-2-deficient background.[19] The green fluorescent protein (GFP) -Tg mouse of C57BL/6 background [C57BL/6 TgN (act-EGFP) OsbY01][20] was kindly provided from Dr Masaru Okabe (Osaka University) and was maintained in our animal facility. All mice used in this study were maintained in a specific pathogen-free facility of Kitasato University School of Medicine. The experimental procedure was approved by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine, and all animal experiments were performed following the guidelines of the committee.

Antibodies and reagents

FITC-labelled anti-CD4 antibody (RM4-5), anti-Thy1.2 antibody (53-2.1), and anti-CD25 antibody (PC61) were purchased from BD Pharmingen (San Diego, CA). FITC-labelled antibodies to CD27 antibody (LG.3A10), CD122 antibody (5H4), phycoerythrin-labelled antibodies to γδ TCR (GL3) and CD8 (53-6.7), phycoerythrin-, and phycoerythrin-Cy5-labelled streptavidin were obtained from BioLegend (San Diego, CA). Biotinylated anti-idiotype antibody to ^{QM11}TCR was prepared in our laboratory.[21] Antibodies to CD3 (2C11), and to FcγR II/III (2.4G2) were prepared from hybridomas in the laboratory.

Preparation of bone marrow chimeras

The recipient *aly*/*aly* or *aly*/+ mice were lethally irradiated (8·5 Gy) using an X-ray irradiator MBR-1505R (Hitachi Medico Co., Tokyo, Japan) with a filter (Cu: 0·5 mm, Al: 1 mm). The following day, the recipient mice were reconstituted with $1{\cdot}0 \times 10^7$ of T-cell-depleted bone marrow (BM) cells from GFP-Tg, *aly*/*aly* or *aly*/+ mice. T-cell depletion was conducted by treating the cells with anti-CD4, anti-CD8 and anti-Thy1.2 antibody plus rabbit complement at 37° for 45 min. The chimeric mice were analysed 60–70 days after BM reconstitution.

Flow cytometry

Flow cytometric analyses were performed as described previously.[19] Briefly, 2×10^5 to 10×10^5 cells were stained in and washed with ice-cold Hanks' balanced salt solution containing 0·5% BSA and 0·02% sodium azide. Secondary staining was carried out in the same manner. Stained cells after washing were examined on FACSCalibur (BD Biosciences, Mountain View, CA) with CellQuest software. Cell sorting was performed using FACSAria (BD Biosciences).

ELISA

The concentrations of interferon-γ (IFN-γ) in the culture supernatants were measured by sandwich ELISA, using 'high-binding' EIA/RIA plates (Costar, NY), purified or biotinylated antibodies (Caltag, CA), and horseradish peroxidase-conjugated streptavidin (Thermo Scientific, Rockford, IL). As the substrate for peroxidase, a TMB (3,3′,5,5′-tetramethylbenzidine) liquid substrate system (Sigma, St Louis, MO) was used, and the reaction was stopped by adding the same amount of 0.5 M H_2SO_4. After stopping the reaction, the absorbance at 450 nm was measured.

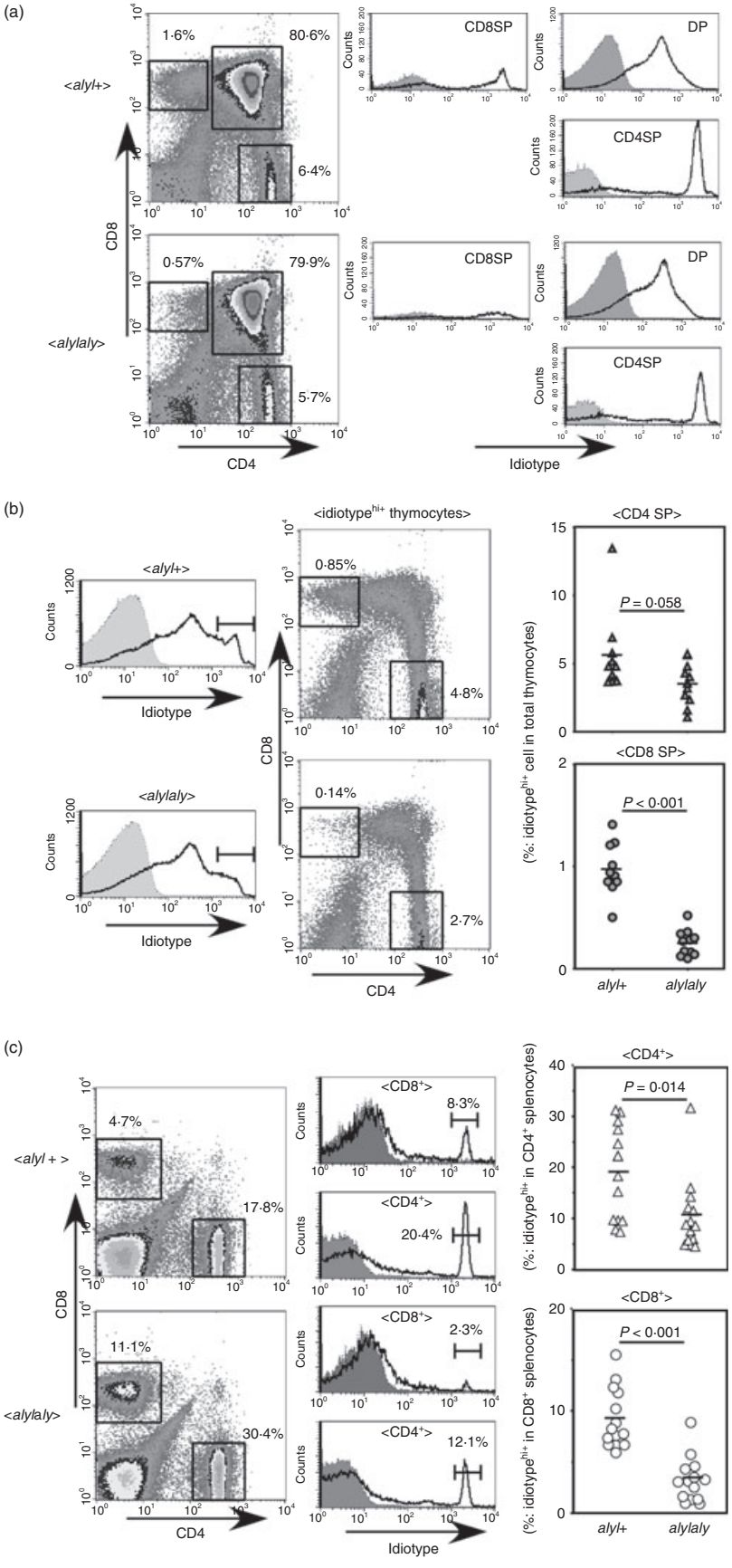
Results

The absence of functional NIK could affect the efficiency of thymic positive selection of T cells expressing a transgenic TCR

The fact that alymphoplasia mice have a normal number of T cells suggests that the absence of NIK may not have a significant impact on T-cell development. However, we examined the possibility that the *aly* mutation could affect the threshold of thymic selection by investigating the differentiation of T cells expressing a fixed, transgenic TCR. The TCR-Tg mouse used here was the ^{QM11}TCR-Tg mouse that we developed and have reported on previously.[19] In this TCR-Tg system, several different selecting MHC molecules have been identified. Among these, class I MHC D^q/L^q and class II MHC I-A^q were identified to be the positively selecting elements to drive Tg-TCR^+ T cells to differentiate $CD8^+$ or $CD4^+$ T cells, respectively.[19] Hence, on an H-2^q background, selecting MHC molecules of both class I and class II are available simultaneously. Indeed, in this situation, differentiation of idiotype^{high+} cells into both CD4SP and CD8SP cells could be observed.[19]

We crossed *aly*/*aly* mice with the ^{QM11}TCR-Tg mice and examined whether the differentiation of the idiotype^{high+} cells could be influenced by the *aly* mutation. As shown in Fig. 1a,b, in H-2^q, ^{QM11}TCR-Tg, *aly*/*aly* mice, the efficiency of positive selection of idiotype^{high+} cells was decreased compared with that in *aly*/+ mice. The effect was more pronounced for differentiation into CD8SP cells than into CD4SP cells. It should be noted, however, that differentiation of idiotype^{high+} cells into CD4SP was also less effective in *aly*/*aly* mice, although it was not statistically significant. The same tendency was also observed in splenic mature T cells. Notably, the reduction of the proportion of idiotype^{high+} cells in *aly*/*aly* mice was statistically significant for both $CD4^+$ and

Figure 1. Inefficient positive selection of ^{QM11}T-cell receptor transgenic (^{QM11}TCR-Tg) T cells in *aly*/*aly* mice. (a) The T cells expressing ^{QM11}TCR can be positively selected by class I MHC (D^q/L^q) or class II MHC (I-A^q) to mature into CD8 single-positive (SP) or CD4SP T cells, respectively. Expression of idiotype on CD4SP, CD8SP or CD4/CD8 double-positive (DP) thymocytes of H-2^q (expressing both D^q/L^q and I-A^q) *aly*/+ (upper panels) and *aly*/*aly* (lower panels) mice are shown. Right panels indicate the expression of idiotype on CD4SP, CD8SP and DP thymocytes. Shaded histograms indicate negative control staining. A representative set of results from the analyses of > 10 mice [as in (b)] is shown. (b) Idiotype^{high+} cells in thymocytes from H-2^q *aly*/+ (upper panels) and *aly*/*aly* (lower panels) mice were gated as indicated to show their expression of CD4 and CD8. The numbers indicate the percentage CD4SP idiotype^{high+} or CD8SP idiotype^{high+} cells in total thymocytes. In the right hand graphs, the percentages of CD4SP idiotype^{high+} cells (top) and CD8SP idiotype^{high+} cells (bottom) in total thymocytes from H-2^q, ^{QM11}TCR-Tg, *aly*/+, or *aly*/*aly* mice are shown. In the left panels, shaded histograms indicate negative control staining. Mice aged from 8 to 18 weeks were analysed. The *P* values were determined by two-tailed Student's *t*-test. (c) The splenocytes from H-2^q *aly*/+ (upper panels) and *aly*/*aly* (lower panels) mice were analysed for idiotype expression on $CD4^+$ and $CD8^+$ T cells. A representative set of results for the expression of CD4 and CD8 (left panels) and the expression of idiotype on $CD4^+$ or $CD8^+$ T cells (middle panels) is shown. In the left panels, the numbers indicate the percentage of $CD4^+$ or $CD8^+$ T cells in total splenocytes. In *aly*/*aly* mice, B-cell survival is impaired[12] owing to defective B cell activating factor belonging to the tumor necrosis factor family (BAFF) signalling, which may lead to relative increase of T-cell proportion in the spleen of *aly*/*aly* mice. In the middle panels, the shaded histograms show negative control staining, and the numbers indicate the percentages of idiotype^{high+} cells among $CD4^+$ or $CD8^+$ T cells. Fourteen mice (6–13 weeks old) for each strain were analysed and the results are shown in the right hand graphs.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CD8
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$CD8^+$ T cells (Fig. 1c), whereas the total T-cell number was not decreased in *aly*/*aly* mice (data not shown).

These results were also reproduced when analysed on a RAG-2-deficient background (see Supplementary material, Fig. S1). The number of idiotype^{high+} cells either of CD4SP or CD8SP mature thymocytes were smaller in *aly*/*aly* mice compared with those in *aly*/+ mice. This was also true for the number of $CD4^+$ or $CD8^+$ T cells in the spleen.

These results indicate that NIK plays an important role in the optimal positive selection of at least some subsets of T cells, and so that the $\alpha\beta$ T-cell repertoire in the *aly*/*aly* mouse may be different from that in the normal mouse.

The effects of *aly* mutation on the negative selection of T cells expressing the transgenic TCR

We next examined the impact of *aly* mutation on the negative selection in the ^{QM11}TCR-Tg system. In this setting, idiotype^{high+} cells were negatively selected by two different MHC molecules, one of which was I-A^k (original specificity of ^{QM11}TCR), and the other was the H-2^s class I MHC molecule.[19,21] Negative selection of idiotype^{high+} cells in ^{QM11}TCR-Tg mice was observed therefore in two strains of mouse; H-2^{bxk} and H-2^s. The total number of thymocytes in *aly*/*aly* mice of either H-2^{bxk} or H-2^s background was not different from that observed in *aly*/+ mice (Fig. 2a), where the proportion of CD4/CD8 DP subset was dramatically decreased in both *aly*/+ and *aly*/*aly* mice (Fig. 2b, data not shown), implying that negative selection of the cells specific to the antigens expressed in the thymus may be operated properly in *aly*/*aly* mice. Unexpectedly, however, it was noticed that in the H-2^s background, the CD8^{low+} idiotype^{high+} cells were markedly reduced in *aly*/*aly* mice (Fig. 2c). The CD8^{low+} idiotype^{high+} cells were also reported in other TCR-Tg mice,[22,23] and were demonstrated to exist in any H-2 backgrounds, although their existence is eminent particularly in negatively selecting background. Although the mechanism of their differentiation has not been fully clarified, it was suggested that these cells might be one of the populations that represents the $\gamma\delta$ lineage of T cells in the TCR-Tg setting.[24] In our system, this population was deleted in the H-2^{bxk} background, probably because of co-receptor-independent recognition of I-A^k by the ^{QM11}TCR,[25] and therefore the effect of the *aly* mutation on the generation of this population in the H-2^{bxk} background could not be investigated. Nevertheless, the examination of mice on an H-2^s background indicated that the generation of the CD8^{low+} idiotype^{high+} cells may require functional NIK.

The proportion and the number of $\gamma\delta$ T cells, but not $\alpha\beta$ T cells, in the peripheral organs were decreased in *aly*/*aly* mice

The observation of impaired generation of CD8^{low+} idiotype^{high+} cells in the periphery of the H-2^s, ^{QM11}TCR-Tg, *aly*/*aly* mice led us to investigate the development of $\gamma\delta$ T cells in the non-transgenic *aly*/*aly* mouse. In the thymus, despite the total cell number being normal[12] (data not shown), the proportion of $\gamma\delta$ T cells was slightly reduced in *aly*/*aly* mice compared with that in *aly*/+ mice (Fig. 3a). However, the reduction in *aly*/*aly* mouse of the

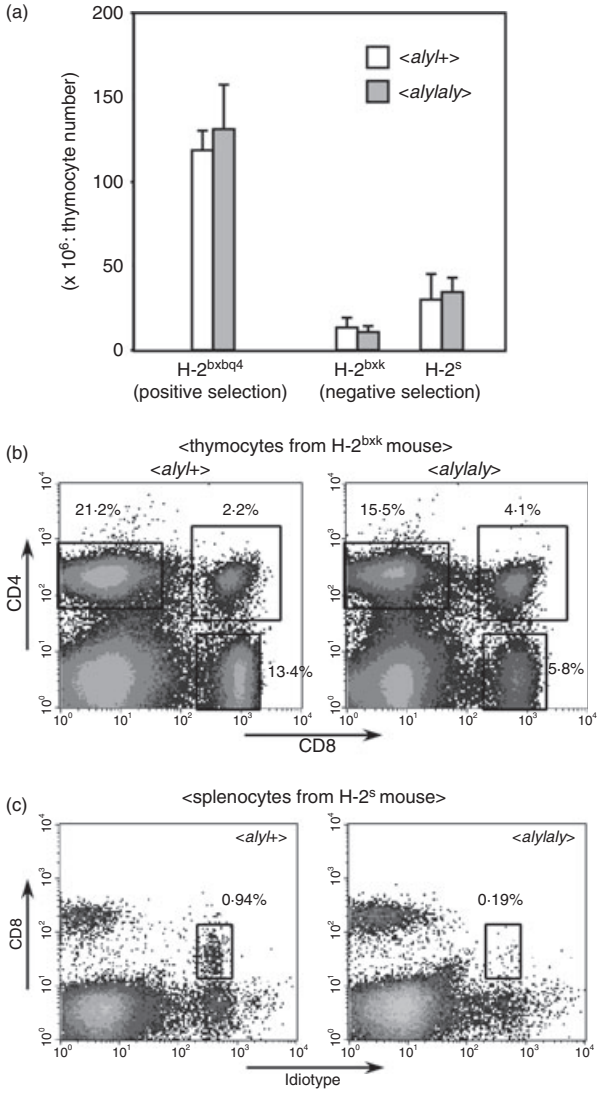

Figure 2. Negative selection of ^{QM11}T-cell receptor transgenic (^{QM11}TCR-Tg) T cells occurred normally, but CD8^{low+} idiotype^{high+} cells failed to develop in *aly*/*aly* mice. (a) The thymocyte numbers of ^{QM11}TCR-Tg, *aly*/*aly* or *aly*/+ mice with indicated H-2 backgrounds are shown. Five to six mice aged 8 to 10 weeks for each strain were analysed. Error bars represent SD. (b) The expression of CD4 and CD8 on the thymocytes from ^{QM11}TCR-Tg, *aly*/*aly* (right) or *aly*/+ (left) mice with negatively selecting H-2^{bxk} background is indicated. A representative set of results from the analyses of > 10 mice is shown. (c) The expression of CD8 and idiotype on the splenocytes from ^{QM11}TCR-Tg, *aly*/*aly* (right) or *aly*/+ (left) mice on an H-2^s background are shown. A representative set of results from analyses of > 10 mice is shown. The percentage of CD8^{low+} idiotype^{high+} cells in *aly*/*aly* mice varied from 1/20 to 1/3 of that in *aly*/+ mice.

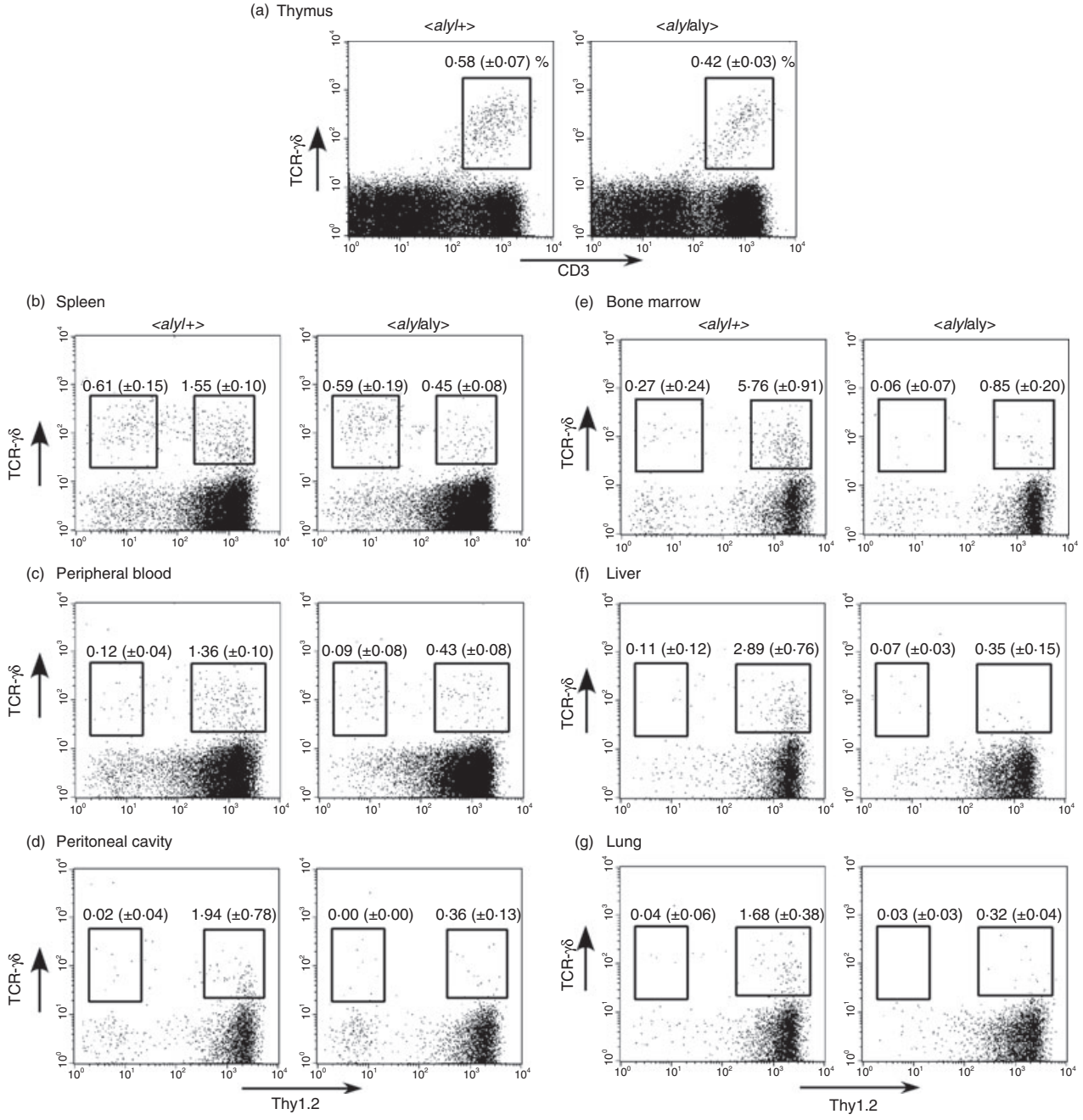

Figure 3. Reduced proportion of $\gamma\delta$ T cells in *aly/aly* in comparison with that of *aly/+* mice. (a) The average percentages ($\pm$ SD) of $\gamma\delta$ T cells in $CD3^{high+}$ cells in thymi from *aly/aly* or *aly/+* mice (10 times backcrossed to C57BL/6, 9–11 weeks old, $n = 4$) are demonstrated. The difference between *aly/aly* and *aly/+* mice was statistically significant ($P = 0{\cdot}0029$). (b–g) The mice analysed in (a) were examined for the expression of $\gamma\delta$ T-cell receptor (TCR) and Thy1.2 on $CD3^+$-gated cells in the indicated organs, and the average percentages $\pm$ SD in $CD3^+$ cells are indicated. The difference between *aly/aly* and *aly/+* mice was statistically significant in every organ indicated ($P < 0{\cdot}001$).

$\gamma\delta$ T cells, especially of $Thy1.2^+$ cells, was more evident in peripheral tissues, such as the spleen, peritoneal cavity, bone marrow, liver and lungs (Fig. 3b–g). This also held true in most, but not all, tissues when the absolute numbers of $\gamma\delta$ T cells were compared (Fig. 4a). In contrast, as shown in Fig. 4(b), the number of $\alpha\beta$ T cells did not decrease in the *aly/aly* mouse, or rather, it significantly increased in some tissues, such as liver and lung, which might be due to pathogenic infiltration of $CD4^+$ $\alpha\beta$ T cells.

The peripheral $\gamma\delta$ T cells could be divided into specific subsets according to their expression of activation markers or cell surface molecules.[26] We next assessed whether the decrease of peripheral $\gamma\delta$ T cells in *aly/aly* mice may

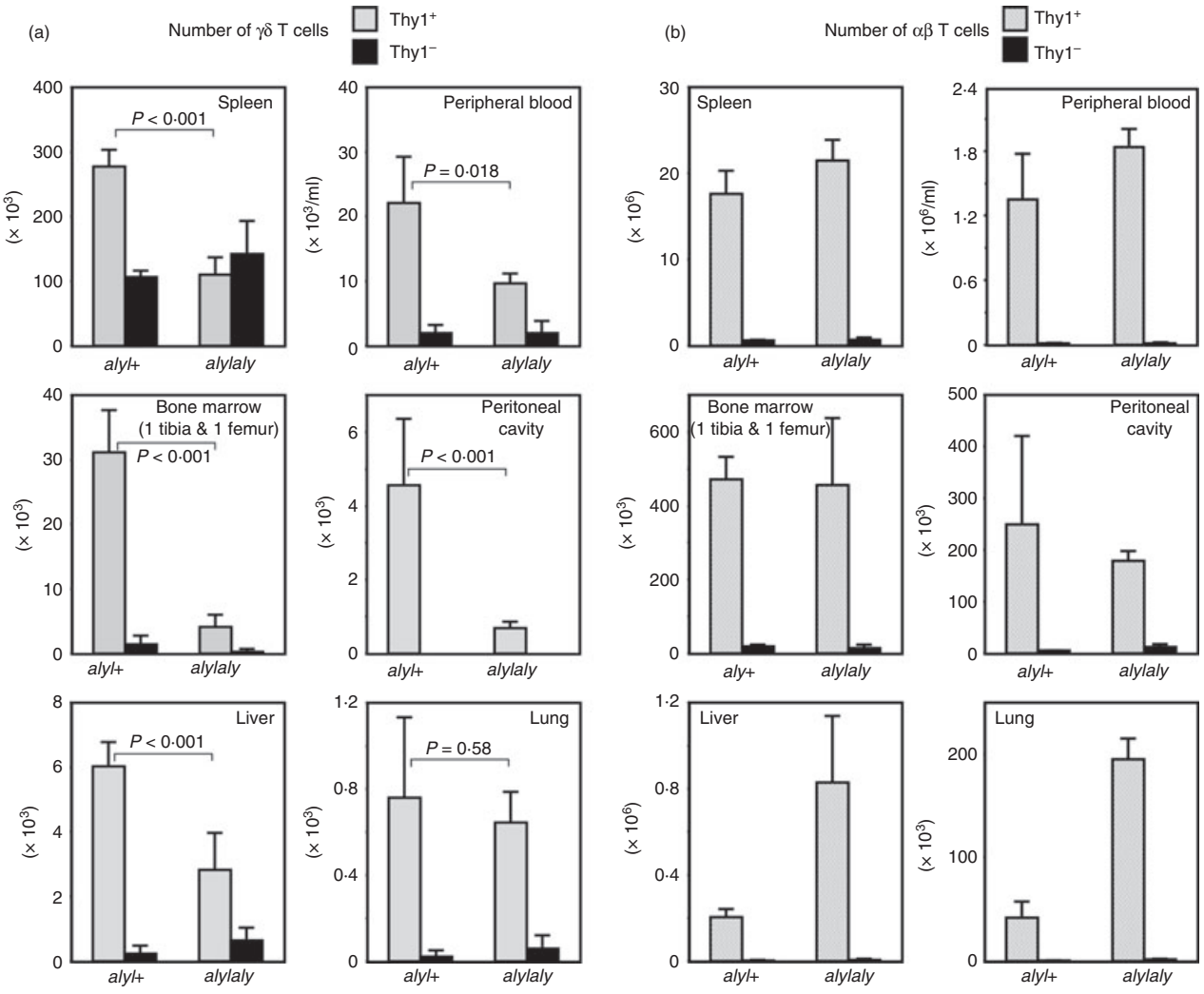

Figure 4. Absolute number of $\alpha\beta$ or $\gamma\delta$ T cells in *aly*/*aly* and *aly*/+ mice in the peripheral organs. The leucocytes were harvested from indicated organs from the mice analysed in Fig. 3, and the expression of CD3, T-cell receptor $\alpha\beta$ (TCR-$\alpha\beta$), TCR-$\gamma\delta$ and Thy1.2 on them was examined. Total numbers of $\gamma\delta$ (a) or $\alpha\beta$ (b) T cells from indicated organs from *aly*/*aly* or *aly*/+ mice are shown. Error bars represent SD.

result from impaired generation of some particular $\gamma\delta$ subsets, by comparing the expression of CD27, CD25, or CD122 on $\gamma\delta$ T cells in the peripheral blood of *aly*/*aly* mice with those of *aly*/+ mice. As shown in Fig. 5, the proportion of cells positive for these markers was almost the same, suggesting that the reduction of the $\gamma\delta$ T cells in *aly*/*aly* mice may be independent of expression for these markers.

NIK in host cells may be necessary for maintaining a normal number of $\gamma\delta$ T cells in the periphery

We next prepared the BM chimera mice to determine whether functional NIK in haematopoietic or host cells was important for the maintenance of a normal number of $\gamma\delta$ T cells. For this purpose, the GFP-Tg mouse of B6 background was used to discriminate the donor cells from host cells. We developed the GFP-Tg, *aly*/*aly* or *aly*/+ mice and the BM cells from these mice were injected intravenously into non-Tg *aly*/*aly* or *aly*/+ mice that had been irradiated (8·5 Gy) the day before transfusion of donor cells. After 9 to 10 weeks, the number and proportion of GFP$^+$ $\gamma\delta$ T cells in several organs from those chimeric mice were analysed. For unknown reasons, in the BM chimeras, the number or proportion of peripheral $\gamma\delta$ T cells was not statistically different in some sites other than peritoneal cavity or BM between the [GFP × *aly*/*aly* → *aly*/*aly*] mice and the [GFP × *aly*/+ → *aly*/+] mice, although there was still a tendency for the $\gamma\delta$ T cells to be reduced in the [GFP × *aly*/*aly* → *aly*/*aly*] chimera compared with the [GFP × *aly*/+ → *aly*/+] chimera (Fig. S2, data not shown). In Fig. 6, the number and proportion of the GFP$^+$ $\gamma\delta$ T cells in the peritoneal cavity and the BM of each chimera are shown. Both the number

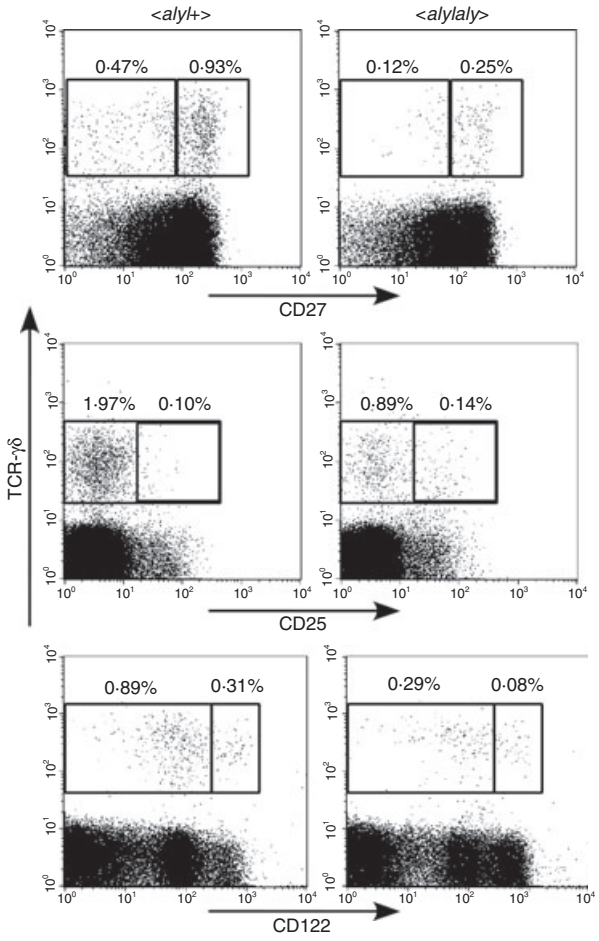

Figure 5. The majority of peripheral blood $\gamma\delta$ T cells in *aly/aly* mice were $CD27^{high+}$, $CD25^{-}$, $CD122^{low+}$, as were in *aly/+* mice. Expression of CD25, CD27 or CD122 on $\gamma\delta$ T cells in peripheral blood of *aly/aly* and *aly/+* mice was investigated and the percentages of the cells positive or negative for each marker, in $CD3^{+}$ cells are shown. Representative results obtained from three mice are indicated.

and the proportion of $\gamma\delta$ T cells were significantly reduced when *aly/aly* mice were used as hosts, as compared with those in *aly/+* recipient mice, even when *aly/+* donor cells were transplanted. By contrast, no significant differences in $\gamma\delta$ T-cell number or proportion was found between the mice receiving *aly/+* BM cells and the mice receiving *aly/aly* donor cells. These results indicate that NIK in non- haematopoietic cells, rather than $\gamma\delta$ precursor cells, is important for supporting a normal number of $\gamma\delta$ T cells in the periphery.

Impaired IFN-γ production in $CD27^{+}$ $\gamma\delta$ T cells in aly/aly mice

To assess whether *aly* mutation would have any impact on the function of $\gamma\delta$ T cells, IFN-γ production from splenic $\gamma\delta$ T cells was examined. As it was shown that among splenic $\gamma\delta$ T cells, $CD27^{+}$ $\gamma\delta$ T cells were the main sources of IFN-γ,[27] we sorted $CD27^{+}$ $\gamma\delta$ T cells and stimulated the cells *in vitro* with plate-bound anti-CD3ε antibody. As shown in Fig. 7, the amount of IFN-γ secreted from *aly/aly* cells was smaller than that from normal *aly/+* cells, suggesting that NIK may contribute not only to the development and/or peripheral distribution of $\gamma\delta$ T cells but also to maximal production of IFN-γ from them.

Discussion

Nuclear factor-κB-inducing kinase, which was originally identified as a kinase mediating the signal from tumour mecrosis factor receptor, Fas or interleukin-1 receptor,[10] is expressed in various types of cells, and plays a critical role in non-canonical pathways of NF-κB activation.[28] The crucial role of NIK in the maintenance of proper immune function was highlighted by analyses of the alymphoplasia mouse, which was revealed to be defective in its ability to mount immune responses against allogeneic cells[12] or viruses.[29] On the other hand, the *aly/aly* mouse was also shown to develop autoimmune diseases in exocrine organs, such as lacrimal gland, lung, liver and salivary glands.[30] Hence, in the *aly/aly* mouse, immune responses to foreign antigens seem to be impaired whereas those to autoantigens are more prone to be invoked compared with WT mouse. These observations indicate that the T-cell repertoire may not be appropriately formed in *aly/aly* mice. In fact, it was demonstrated that autoimmune diseases could be transferred to RAG2 knockout mouse by adoptively transferring a T-cell-enriched fraction of spleen from *aly/aly* mice.[30] Nonetheless, no apparent abnormality was observed either in the thymocytes or splenic T cells in *aly/aly* mice.[12]

It has been shown that negative selection is defective in the *aly/aly* mouse, owing to impaired formation of medullary thymic epithelial cells,[31] which were implicated in the deletion of auto-reactive T cells, especially the cells specific for peripheral tissue antigens. NIK is downstream of CD40 or RANK signalling and Akiyama *et al.*[32] demonstrated that defective RANK/CD40-mediated signalling in medullary thymic epithelial cell precursor cells is responsible for impaired medullary thymic epithelial cell formation in *aly/aly* mouse.

The examination of negative selection in the ^{QM11}TCR-Tg, *aly/aly* mouse disclosed that elimination of T cells recognizing an auto-antigen expressed in the thymus could be properly carried out in *aly/aly* mice, which is consistent with a previous observation in the HY-TCR Tg mice.[31] Hence, the defective self-tolerance in T cells of *aly/aly* mice appears to be limited against peripheral tissue antigens, particularly those in exocrine organs, although its underlying mechanism is at present unclear. In addition to imperfect negative selection in *aly/aly* mice, in the present study, we have demonstrated that

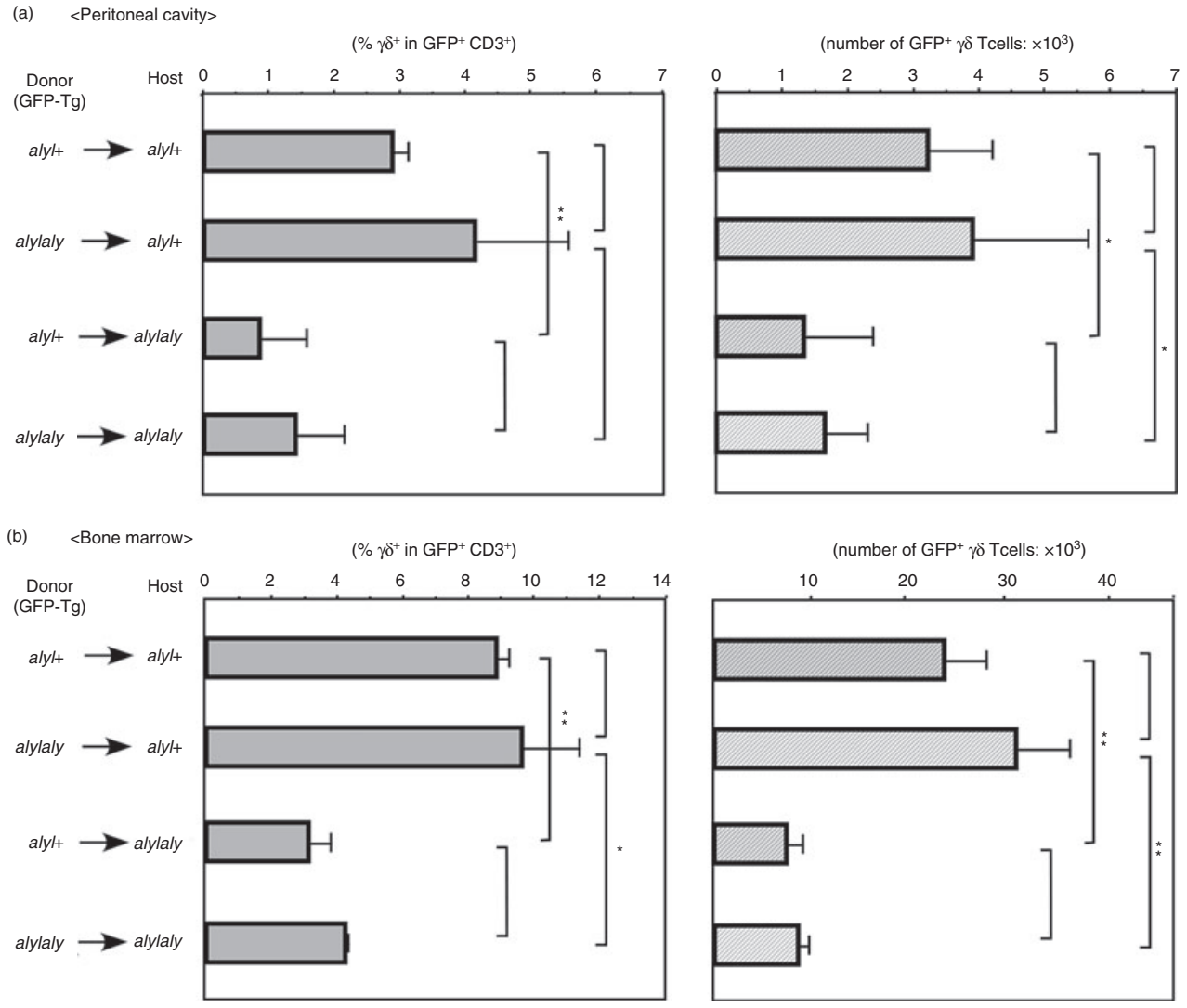

Figure 6. Nuclear factor-κB-inducing kinase (NIK) in non-haematopoietic cells is important for maintaining a normal number of γδ T cells in peritoneal cavity and bone marrow. Bone marrow cells from green fluorescent protein transgenic (GFP-Tg) *aly/aly* or GFP-Tg *aly/+* (B6 background) were injected into 8·5 Gy irradiated *aly/aly* or *aly/+* mice (1×10^7/mouse). Nine to 10 weeks later, percentage of GFP^+ γδ T cells in GFP^+ $CD3^+$ cells and absolute number of GFP^+ γδ T cells in peritoneal cavity (left) and in bone marrow (right) were analysed. The average numbers ± SD from four recipients are shown. $^*P < 0.05$, $^{**}P < 0.01$.

positive selection in at least some subsets of T cells was indeed affected in *aly/aly* mice and suggested that NIK could be involved in shaping the αβ T-cell repertoire.

In the ^{QM11}TCR-Tg system, where both classes of selecting MHC molecules are available simultaneously, the differentiation of DP thymocytes into CD8SP was more severely affected than was CD4SP differentiation by the *aly* mutation. This is partly consistent with a previous study by Jimi *et al.*,[4] demonstrating that the inhibition of NF-κB activity in thymocytes by transgenic expression of a 'super-repressor' form of IκBα, repressed the CD8SP cell differentiation, but had little impact on CD4SP cell differentiation in TCR-Tg mice. Substantial influence of CD4SP differentiation observed in the ^{QM11}TCR-Tg mouse could be due to the differences of TCR specificity, or it might be attributable to some functions of NIK other than NF-κB activation, as described below. We are currently preparing mice to develop BM chimeras so that we could determine whether impaired positive selection in *aly/aly* mice would be restored by transferring *aly/+* BM cells.

In the TCR-Tg system, some unusual populations have been found, one of which is the $CD8^{low+}$ $idiotype^{high+}$ cells.[22,23] Although these cells were initially thought to be an artefact of the TCR transgenic system, detailed analyses of these cells suggested that they might represent γδ T-cell lineage.[24] This notion was further corroborated by the findings by Pennington *et al.*,[33] who demonstrated

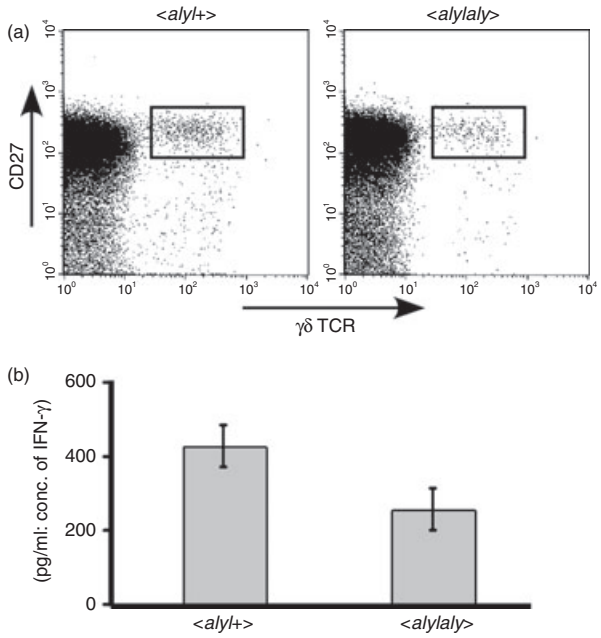

Figure 7. Impaired interferon-γ (IFN-γ) production from CD27^{high+} $\gamma\delta$ T cells in *aly/aly* mouse. (a) The expression of CD27 and T-cell receptor (TCR) -$\gamma\delta$ on splenocytes from *aly/aly* or *aly/+* mice are shown. (b) CD27^{high+} $\gamma\delta$ T cells in the spleen of *aly/aly* or *aly/+* mice were sorted with cell sorter. Twenty thousand cells per well were stimulated with plate-bound anti-CD3 antibody (10 μg/ml) in a microtitre plate for 18 hr, and the concentration of IFN-γ in the supernatants was determined by ELISA. A representative result is shown. The average value of the ratio of IFN-γ secreted from *aly/aly* versus that secreted from *aly/+* cells in three similar experiments was 0·55, and was statistically significant (P = 0·025).

that these cells expressed '$\gamma\delta$-biased genes'. Although the putative association between these two populations needs to be examined further in future studies, defective appearance of these populations in the periphery were also observed in our system of *aly/aly* mice.

Regarding the role of NIK in the development of $\gamma\delta$ T cells, it was previously shown by Nanno *et al.*[34] that the proportion of intestinal intraepithelial $\gamma\delta$ T cells in *aly/aly* mice is smaller than that in *aly/+* mice. This observation has been herein extended to show that the reduction of $\gamma\delta$ T cells is more prominent in other peripheral organs. Thymic differentiation of $\gamma\delta$ T cells has been shown to be regulated in *trans* by DP $\alpha\beta$ thymocytes through stimulating the LTβR on $\gamma\delta$ T cells;[18] for this reason it was speculated that the development of $\gamma\delta$ T cells may be influenced by the mutation of NIK which mediates signalling from LTβR.[10] Unexpectedly, however, the BM chimera experiment suggested that NIK in host cells rather than donor cells is important for maintaining a normal number of $\gamma\delta$ T cells in the peritoneal cavity and in the BM. This observation argues against the possibility that the impaired generation of $\gamma\delta$ T cells in *aly/aly* mice may be the result of the impaired signalling from LTβR in $\gamma\delta$ precursor. Rather, this result may indicate that the proper generation of $\gamma\delta$ cells would require the intact thymic structure, as was observed in NKT cells.[35] Alternatively, considering that the reduction of $\gamma\delta$ T cells was more profound in the periphery than in the thymus, the cells in the peripheral tissue may require NIK expression for maintenance of a normal number of $\gamma\delta$ T cells. It is also possible that more prominent reduction of $\gamma\delta$ T cells in the periphery may result from a defect in emigration from the thymus.[36] However, the impaired ability of IFN-γ production from splenic CD27$^+$ $\gamma\delta$ T cells from *aly/aly* mice may still possibly be due to defective trans-differentiation by LTβ stimulation.

The developmental pathways or the molecules involved in the differentiation of $\gamma\delta$ T cells have not yet been clarified in comparison to those of $\alpha\beta$ T cells. There are numbers of genes that were shown to be crucial in $\gamma\delta$ T-cell development, but most of these are also mandatory for $\alpha\beta$ T-cell differentiation.[16] In terms of this, NIK may be a rare molecule whose absence affects the appearance of $\gamma\delta$ but not $\alpha\beta$ T cells. Furthermore, very few molecules have been described that need to be expressed in non-haematopoietic cells in contributing to proper $\gamma\delta$ T-cell generation.

Although further investigation is essential to understand the molecular basis of NIK involvement in the maintenance of $\gamma\delta$ cells, the function of NIK to support $\gamma\delta$ T-cell differentiation could be independent of the non-canonical pathway of NF-κB activation, as it was shown that in mice deficient for either NF-κB2 or RelB, the proportion and number of $\gamma\delta$ T cells remained unaltered.[37] A similar situation has been reported that while the number of CD4$^+$ FoxP3$^+$ regulatory T cells is diminished in the *aly/aly* mouse, no such regulatory T-cell reduction was observed in NF-κB2-deficient mice.[38,39] In this context, some studies have indicated that, in addition to NF-κB activation, NIK could participate in other signalling pathways, such as signal transducer and activator of transcription 3[40] or mitogen-activated protein kinases.[41] Defining the pathways through which NIK takes part in $\gamma\delta$ T-cell differentiation would lead to uncovering a novel function of NIK, which is being attempted in our ongoing study.

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Disclosures

The authors declare no financial or commercial conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Defective positive selection of T cells expressing ^{QM11}T-cell receptor.

Figure S2. Impaired generation of $\gamma\delta$ T cells in *aly/aly* host mice of bone marrow chimera.

