Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

NF-κB-inducing kinase contributes to normal development of cortical thymic epithelial cells: its possible role in shaping a proper T-cell repertoire

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doi:10.1111/imm.13186
Received 19 June 2019; revised 2 March 2020; accepted 3 March 2020.
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Summary

Nuclear factor (NF)-κB-inducing kinase (NIK) is known to be a critical regulator of multiple aspects of the immune response. Although the role of NIK in the development of medullary thymic epithelial cells (mTECs) has been well documented, the impact of NIK on the differentiation and function of cortical thymic epithelial cells (cTECs) remains ambiguous. To investigate the possible involvement of NIK in cTEC differentiation, we have compared the gene expression and function of cTECs from a NIK-mutant mouse, *aly*mphoplasia (*aly*/*aly*) with those of cTECs from wild-type (WT) mice. Flow cytometric analyses revealed that expression levels of MHC class II, but not MHC class I or other TEC markers, were higher in *aly*/*aly* cells than in WT cells. Notably, the proportion of MHC class II^{hi+} cTECs was elevated in *aly*/*aly* mice. We also demonstrated that expression of *Ccl5* mRNA in the MHC class II^{hi+} subset of *aly*/*aly* cTECs was decreased compared with that in WT cells, implying an abnormal pattern of gene expression in *aly*/*aly* cTECs. Analyses of bone marrow chimera using *aly*/*aly* or *aly*/+ mice as hosts suggested that Vβ usage and CD5 expression on WT T-cells were altered when they matured in *aly*/*aly* thymi. These results collectively indicate that NIK may be involved in controlling the function of cTEC in selecting a proper T-cell repertoire.

Keywords: cortical thymic epithelial cells; NF-κB-inducing kinase; T-cell repertoire; thymic positive selection.

Introduction

In the thymus, a highly diverse set of T-cell antigen receptors are generated through random recombination of gene segments. A subset of T-cell clones with appropriate specificities are selected from within this diversity. Through this process, self-tolerant, immunocompetent T-cells, which are expected to effectively recognize foreign antigens in the context of either one type of the MHC molecules expressed in the thymus, are able to mature and emigrate to the periphery. Thymic epithelial cells (TECs) play pivotal roles in this process of T-cell clonal selection.[1,2]

Each of the two anatomically compartmentalized regions of the thymus (the cortex and medulla) contains epithelial cells with different properties. Although TECs in the cortex (cortical thymic epithelial cells, cTECs) and those in the medulla (mTECs) arise from a common precursor,[3,4] they differentiate to have distinct functions and characteristics. While cTECs are known to mediate T-lineage specification and positive selection of potentially immune-competent clones, mTECs are exclusively devoted to ensuring self-tolerance,[5–7] where autoimmune regulator (AIRE) and forebrain embryonic zinc finger-like protein 2 (Fezf2) cooperate in inducing tolerance to tissue-restricted antigen (TRA)-specific T-cell clones.[8–11]

It has been well established that development of AIRE^{+} mTECs requires activation of a non-canonical pathway of nuclear factor (NF)-κB, in which NF-κB-inducing kinase (NIK) plays a non-redundant role.[12–17] NIK is likely to be

Abbreviations: cTEC, cortical thymic epithelial cell; mTEC, medullary thymic epithelial cell; NIK, nuclear factor-κB-inducing kinase

involved in signalling via some members of the TNFR family, such as CD40, RANK or LTβR, all of which synergistically transduce prerequisite signals to foster production of mature, functional mTECs.[18–21] Analyses of a spontaneous mutant mouse line, *aly*mphoplasia, (*aly*/*aly*), which possesses a loss of function point mutation in the NIK gene,[22,23] have uncovered the essential roles of NIK in multiple levels of T-cell response.[16,24] These mutant mice have disorganized thymi, with very few mTECs, and display autoimmune symptoms with infiltration of lymphoid cells into several organs, especially exocrine systems such as the liver, pancreas, lung, salivary gland or lacrimal gland.[13,25–27] As Sekai *et al.*[27] have clearly demonstrated, autoimmunity in *aly*/*aly* stems from lack of mTEC progenitors, leading to impaired deletion of some auto-reactive T-cell clones, although negative selection of T-cells specific for ubiquitous antigens may still occur.[28,29]

While the critical importance of NIK in the development of mTEC, and thus in self-tolerance, has been extensively explored, the role of NIK in the development and function of cTECs remains obscure. Because *aly*/*aly* mice have a normal number of mature T-cells, it is generally presumed that NIK may be dispensable for normal cTEC function. However, given that cTECs express some TNFR family molecules during their ontogeny, such as CD40 or LTβR,[6,21] it is possible that NIK might play a role in preserving proper cTEC function. Indeed, our previous study demonstrated that the efficiency of positive selection for particular T-cells expressing a transgenic T-cell receptor (TCR) was reduced in mice with an *aly*/*aly* background, indicating a possibility that despite the normal number of mature T-cells, the T-cell repertoire in *aly*/*aly* mice could be different from that in wild-type (WT) mice.[29] The current study was performed in an attempt to delineate whether *aly*/*aly* cTECs are entirely normal in their ability to select appropriate T-cell clones, by comparing gene expression and function of *aly*/*aly* cTECs with those of WT cells. The results suggest that NIK may be important for differentiation of normal cTECs, which select proper T-cell repertoires, as well as mTECs.

Materials and methods

Mice

C57BL/6J (H-2^b) mice were purchased from CLEA Japan (Tokyo, Japan). The *alymphoplasia*[22] line was originally obtained from CLEA Japan, and mice were bred onto a C57BL/6J background more than 10 times before intercrossing heterozygotes. Male *aly*/*aly* were bred with female *aly*/+ to produce *aly*/*aly* and *aly*/+ offspring, and *aly*/*aly* and *aly*/+ mice from the same litters were used in each experiment. RAG-2 (−/−) mice[30] in a C57BL/6J background were kindly provided by Dr Yoichi Shinkai (Riken, Advanced Science Institute). The mice used in this study were maintained in a specific pathogen-free facility at the Kitasato University School of Medicine. The Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine approved experimental procedures, and all animal experiments were performed following the guidelines of the committee.

Antibodies and reagents

FITC-labelled anti-CD8 and anti-CD5 antibodies (Abs), and PE-labelled anti-CD44 Abs were purchased from BD BioScience (San Diego, CA). Allophycocyanin (APC)-labelled anti-CD4 Abs and PE-labelled anti-FoxP3 Abs were from eBioscience (San Jose, CA). PE/Cy7-labelled anti-CD326 Abs and anti-TCRβ Abs, PE-labelled anti-CD45.2 Abs and anti-NK1.1 Abs, Alexa Fluor 647-labelled Ly51 Abs, APC/Cy7-labelled anti-CD69 Abs, anti-IA/IE Abs and anti-K^b Abs, and PerCP/Cy5.5-labelled anti-CD62L Abs were obtained from BioLegend (San Diego, CA). FITC-labelled UEA (*Ulex europaeus* Agglutinin) −1 was purchased from Vector Laboratories (Burlingame, CA). An anti-mouse TCR Vβ Screening Panel (BD Bioscience) was used to examine Vβ usage in mature T-cells. The anti-idiotype antibody to TCR_{QM11}, ID11 was prepared and biotinylated in the laboratory.[31] The antibody to FcγR II/III (2.4G2) was prepared from culture supernatants of hybridomas in the laboratory and was used to block non-specific staining.

Isolation of thymic epithelial cells

Thymic epithelial cells were prepared as described previously.[32] Briefly, thymi from *aly*/*aly* or *aly*/+ mice, 6–12 weeks old, were treated with 0·05% [w/v] Liberase TH and 100 U/ml DNase I (Roche Diagnostics, Indianapolis, IN) to disperse epithelial cells in the medium. All cells were stained with fluorescence-labelled antibodies for flow cytometric analyses. For sorting TECs, anti-Thy1.2 beads (BD Bioscience) and a magnet were used to remove thymocytes and enrich TECs in the cell suspension.

Flow cytometric analyses and cell sorting

About one million cells were stained in 50 μl of 2.4G2 culture supernatant containing a predetermined concentration of fluorescence-labelled antibodies. After 30 min of incubation on ice, cells were washed with ice-cold Hank's balanced salt solution containing 0·5% bovine serum albumin, and 0·02% sodium azide. Washed cells were analysed with FACSVerse or FACSCalibur (BD Bioscience), and acquired events were analysed with FlowJo software. Cell sorting was performed with FACSAria (BD Biosciences). Dead cells stained with 7-AAD (BioLegend) were gated out during the analyses or sorting of the cells.

Preparation of bone marrow chimeras

Irradiation bone marrow chimeras were prepared by methods previously described.[29] Briefly, RAG-2 (−/−) *aly*/*aly* or *aly*/+ mice of C57BL/6J background, or RAG-2 (−/−) mice with H-2^q background were lethally irradiated (8·5 Gy). On the next day, mice were injected intravenously with $1{\cdot}0 \times 10^7$ T-depleted bone marrow cells from WT C57BL/6J mice or TCR_{QM11}-Tg mice of *aly*/*aly* or *aly*/+ background. Chimeric mice were analysed more than 8 weeks after transfusion.

Quantitative polymerase chain reaction

Quantitation of mRNA was performed as described previously.[32] Briefly, total RNA was extracted with TRIzol reagent (Life Technologies, Carlsbad, CA). Single-strand DNA (ssDNA) was prepared from 500 ng of total RNA by reverse transcription (Takara, Otsu, Japan). An aliquot of ssDNA was used as a polymerase chain reaction (PCR) template to quantify the mRNA. Quantitative PCR was performed with SYBR Premix Ex Taq (Takara) using the Bio-Rad real-time PCR system, CFX96/384 (Bio-Rad, Hercules, CA). The mRNA amount was normalized with *Hprt* mRNA. The primer sequences were as follows: *Ccl5* (forward): 5′-CGC ACCTGCCTCACCATA-3′; *Ccl5* (reverse): 5′-TCCT TCGAGTGACAAACACG -3′; *Ccrl1* (forward): 5′-TTGCC TGACTCAGAGAGGTC-3′; *Ccrl1* (reverse): 5′-CTGA CTGGTTCAGCTCCAGA-3′; *Cd205* (forward): 5′-CCTG GAGAAAAGTCCATTGC-3′; *Cd205* (reverse): 5′-CCAAGC TGATGAGCCCTAAG-3′; *Cd83* (forward): 5′-CGCAG CTCTCCTATGCAGTG-3′; *Cd83* (reverse): 5′-GAAGGAGC TGTTTTGCTTGC-3′; *Ctsl* (forward): 5′-TCTGTTGC TATGGACGCAAG-3′; *Ctsl* (reverse): 5′-TTCACTTCCC-CAGCTGTTCT -3′; *Cx3cl1* (forward): 5′-CGCGTTC TTCCATTTGTGTA-3′; *Cx3cl1* (reverse): 5′-TAGCTGA-TAGCGGATGAGCA-3′; *Dll4* (forward): 5′-AGGTGCCAC TTCGGTTACAC-3′; *Dll4* (reverse): 5′-AGCTGGGTGTCT-GAGTAGGC-3′; *Hprt* (forward): 5′-TGGATACAGGCCA-GACTTTG-3′; *Hprt* (reverse): 5′-AACTTGCGCTCA TCTTAGGC-3′; *Il-7* (forward): 5′-TGCTGCCTGTCACAT-CATC-3′; *Il-7* (reverse): 5′-CGGGCAATTACTATCAG TTCC-3′; *Prss16* (forward): 5′-TACAGATAGTCTTGCG-CAGC-3′; *Prss16* (reverse): 5′-GCTGGGAAAAAGGAC ACTGA-3′; *Psmb11* (forward): 5′-GGCAAAAGCTTGT GGAATCT-3′; *Psmb11* (reverse): 5′-ATTATCGCG-CAGTCTTCTGG-3′.

In vitro recall response to ovalbumin

Endotoxin-free ovalbumin (OVA; Hyglos GmbH, Bernried, Germany) was precipitated with alum and injected into hind foot pads of chimeric mice at 100 μg/mouse. Nine days later, inguinal and popliteal lymph nodes were harvested, and 3×10^5 lymph node cells/well were incubated with the indicated concentration of OVA for 4 days. Proliferative responses were assessed by incorporation of [^{3}H]-thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) during the last 16 hr.

Statistical analysis

Results are expressed as the mean ± SD for each group of mice. Statistics were determined using unpaired, two-tailed Student's *t*-test.

Results

Reduced efficiency of positive selection for NIK-sufficient T-cells expressing a transgenic TCR when matured in *aly*/*aly* hosts

In our prior study, we investigated the impact of NIK deficiency on T-cell repertoire formation, by utilizing TCR-Tg mice to follow the fate of T-cells expressing a particular TCR, called TCR_{QM11}.[31] In this TCR_{QM11}-Tg setting, both classes of selecting MHCs, D^qL^q and $I\text{-}A^q$, have been identified. In mice with the H-2^q haplotype, differentiation of idiotype^{hi+} cells into CD8$^+$ and CD4$^+$ subsets can be observed simultaneously.[31] Analyses of mature T-cells from TCR_{QM11}-Tg in the *aly*/*aly* background revealed that the proportion of idiotype^{hi+} cells among the CD4$^+$ and CD8$^+$ populations was decreased compared with the proportion in the *aly*/+ background, indicating that the efficiency of positive selection for TCR_{QM11} was somehow diminished in the *aly*/*aly* background.[29]

To examine the effect of loss of NIK function in thymic stromal cells on impaired positive selection, we prepared bone marrow chimeric mice by transfusing bone marrow cells from NIK-sufficient TCR_{QM11}-Tg into *aly*/*aly* or *aly*/+ mice with H-2^q RAG-2 (−/−) background. We found a tendency for the proportion of idiotype^{hi+} cells among either CD4SP or CD8SP mature thymocytes to be smaller in *aly*/*aly* hosts than in *aly*/+ hosts, although the differences were not statistically significant (Fig. S1). These results led us to investigate the possibility that *aly*/*aly* cTECs may be selecting a different set of T-cell clones than WT cTECs.

NIK deficiency resulted in severely impaired mTEC differentiation, but did not apparently affect development of normal proportions of cTECs in the thymus

It has previously been shown that in an *aly*/*aly* thymus, ER-TR5$^+$ mTECs were sparse, while the frequency of ER-TR4$^+$ cTECs seemed normal.[26] We then analysed the expression of several cell surface molecules on TECs by flow cytometry to compare them between *aly*/*aly* and *aly*/+. As shown in Fig. 1, the overall percentage of TECs (CD45$^-$, CD326$^+$ cells) was lower in *aly*/*aly* thymus compared with that in *aly*/+ thymus. The number of TECs in a thymus was also decreased in *aly*/*aly*

mice to about one-third of that in *aly*/+ mice ($3{\cdot}1 \times 10^5 \pm 1{\cdot}0 \times 10^5$ in *aly*/+, $1{\cdot}2 \times 10^5 \pm 0{\cdot}5 \times 10^5$ in *aly*/*aly* mice, $n = 5$). The reduced proportion and the number of TECs in the thymus were largely due to severely impaired differentiation of mTECs, which bind to UEA-1 lectin, in the *aly*/*aly* background, and the percentage of cTECs, expressing a higher level of Ly-51, within a thymus was similar between *aly*/*aly* and *aly*/+ (Fig. 1b). These results corroborated the previous result, which indicated that NIK may be dispensable for differentiation of cTECs in normal numbers.

The cTECs from *aly*/*aly* mice contained a larger subset of MHC class II^{hi+} cells and exhibited higher expression of MHC class II, but not MHC class I, than cTECs from *aly*/+ mice

In comparing cTECs from *aly*/*aly* and *aly*/+ mice, we noticed a difference in the pattern of MHC class II expression. Similarly to mTECs, which are divided into two subsets depending on their expression level of MHC class II, cTECs could also be separated into two subsets, i.e. MHC class II^{hi+} (MHCII^{hi}) and MHC classII^{lo+} (MHCII^{lo}) populations.[33,34] We observed that the proportion of the MHCII^{hi} subset was higher in *aly*/*aly* cTECs than in *aly*/+ cTECs (Fig. 2). The MHCII^{hi} cells showed higher expression of Ly-51, and the difference in composition of this cTEC subset between the two mice was more evident when the cells were displayed by expression of MHC class II and Ly-51 (Fig. 2b). Thus, despite the conclusion that *aly*/*aly* thymi contained a normal proportion of CD326$^+$, Ly-51$^+$ cTECs, their subset composition may be different from that of WT cTECs.

We also noticed that the overall MHC class II expression level was higher in *aly*/*aly* cTECs than in *aly*/+ cTECs. This held true for both MHCII^{hi} and MHCII^{lo} subsets of cTECs (Fig. 3a). In contrast, MHC class I or CD326 expression levels of *aly*/*aly* and *aly*/+ cells were indistinguishable. Furthermore, in contrast to MHC class II, expression levels of Ly-51 in either MHCII^{hi} or MHCII^{lo} subsets of cTECs in *aly*/*aly* were almost identical to those in *aly*/+ cTECs (Fig. 3b). These results thus suggested that NIK in cTECs could be specifically involved in regulating MHC class II expression.

Differential gene expression between MHCII^{hi} and MHCII^{lo} subsets of cTECs and reduced expression of *Ccl5* mRNA in MHCII^{hi} cTEC from *aly*/*aly* cells

We next attempted to assess whether there were any differences in gene expression between *aly*/*aly* and *aly*/+ cTECs, other than MHC class II molecules. Considering that the proportion of MHCII^{hi} and MHCII^{lo} subsets of cTECs were different between *aly*/*aly* and *aly*/+ cells, and that gene expression patterns may differ between the two

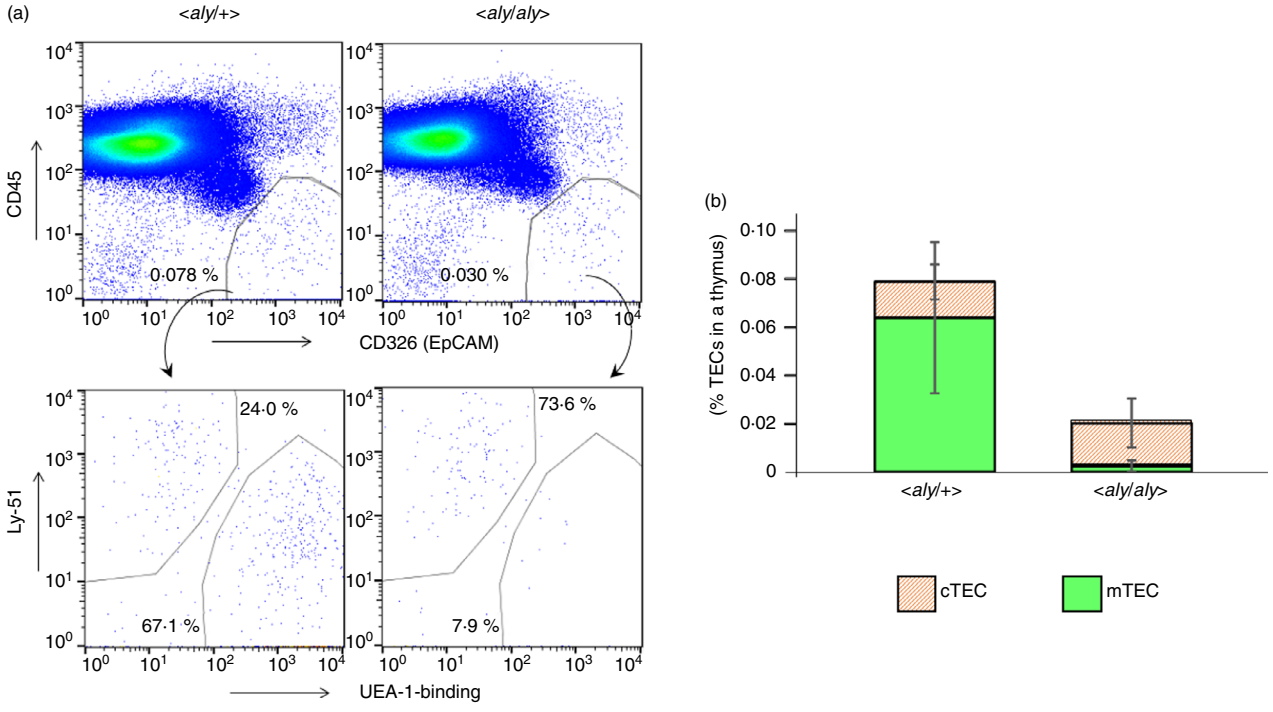

Figure 1. Generation of normal proportion of cortical thymic epithelial cells (cTECs) in the *aly*/*aly* thymus. (a) Thymi from *aly*/*aly* or *aly*/+ mice were treated with Liberase and DNase I to dissociate and release TECs into the medium, and cells were analysed by flow cytometry. In the lower panels, CD45$^-$, CD326$^+$ TECs were divided by their expression of Ly-51 and UEA-1 ligand into Ly-51^{hi} cTECs and UEA-1-bound mTECs. (b) The average percentage of cTECs (hatched) and medullary (m)TECs (shadowed) in a thymus from an *aly*/*aly* or *aly*/+ mouse are shown. ($n = 5$).

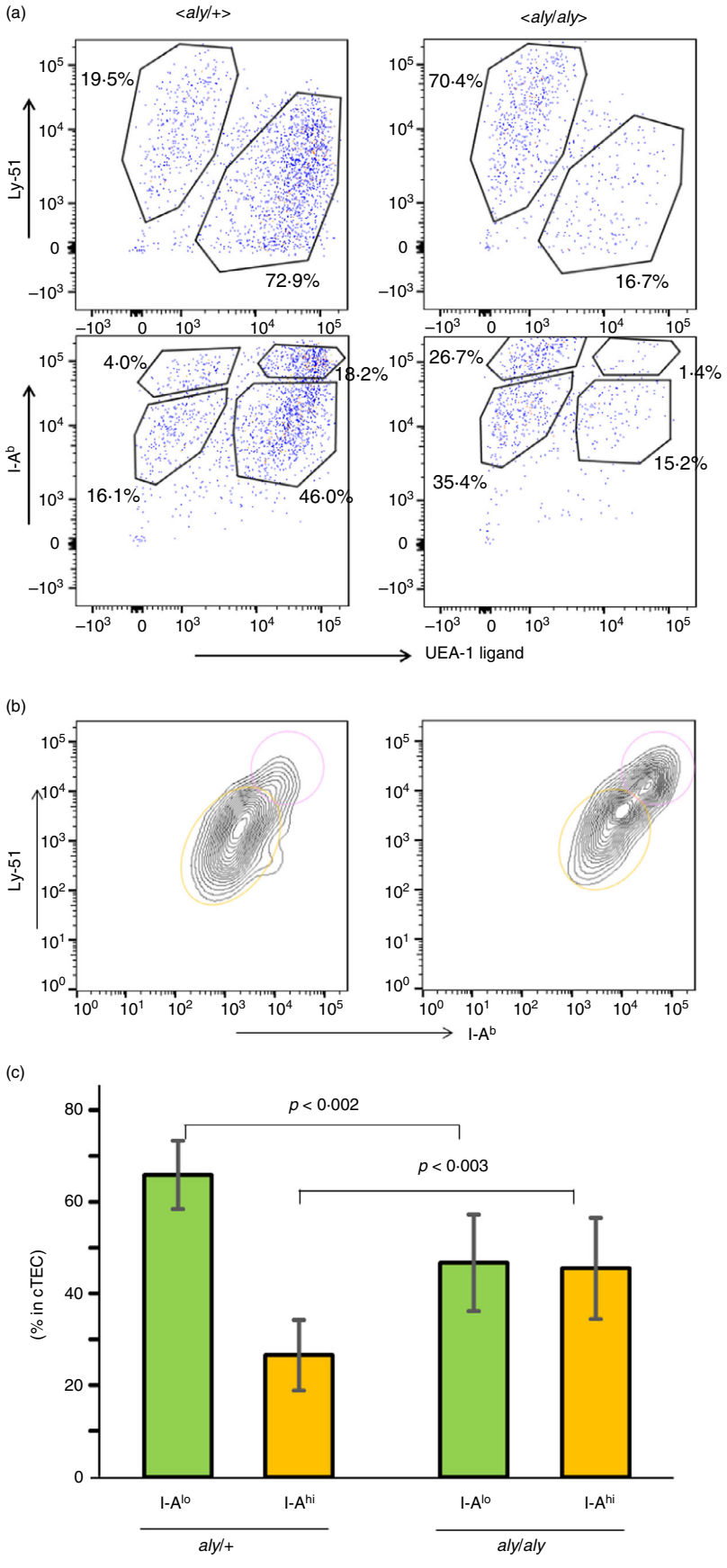

Figure 2. The increased proportion of MHCII^{hi} subset of cortical thymic epithelial cells (cTECs) from *aly/aly* mice. (a) CD45^{-}, CD326^{+} TECs in the thymi from *aly/aly* (right) or *aly/+* (left) mice were analysed for their expression of Ly-51, UEA-1-ligands and MHC class II. (b) The expressions of Ly-51 and MHC class II on Ly-51^{hi} cTEC from *aly/aly* (right) and *aly/+* (left) mice are shown by contour plots. The two circles indicate two distinct populations. (c) The proportions of two cTEC subsets, divided by their MHC class II expression level, in *aly/aly* or *aly/+* are shown (n = 7).

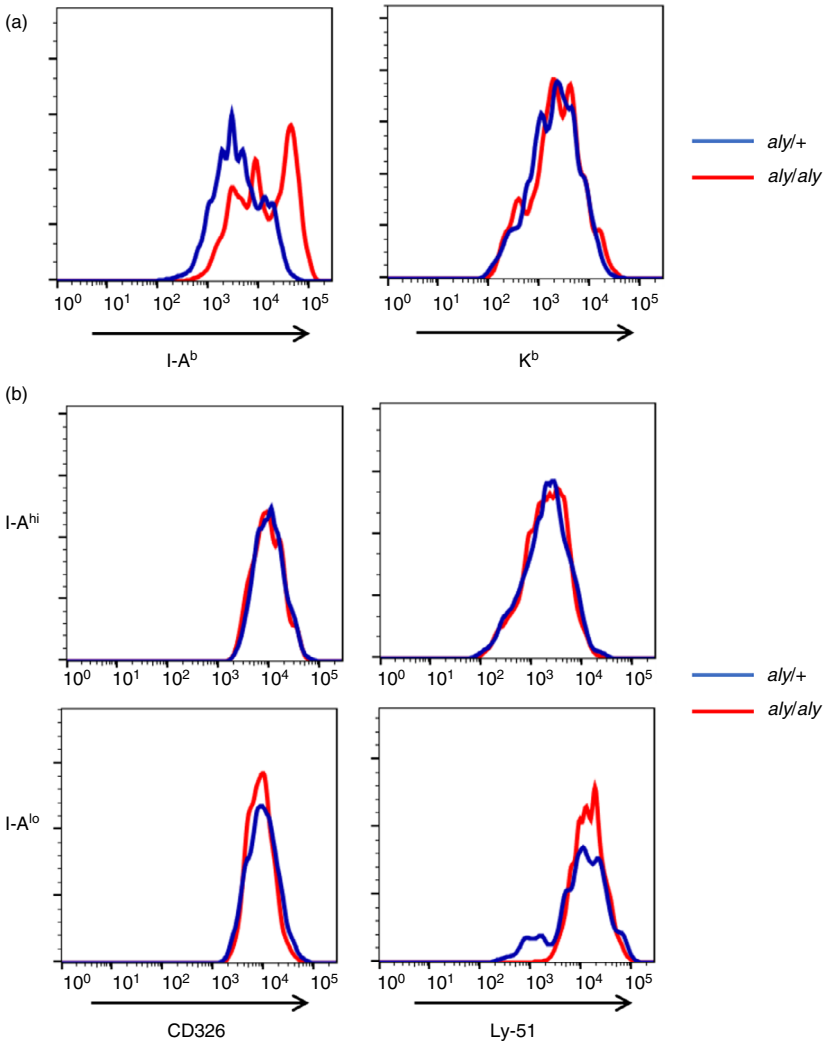

Figure 3. Elevated expression of MHC class II, but normal levels of expression of MHC class I and thymic epithelial cells (TECs) markers on cortical TECs from *aly/aly* thymi. (a) Expression of MHC class II (left) or MHC class I (right) on cTECs from *aly/aly* (red) or *aly/+* (blue) mice were examined, and a set of representative results from several independent experiments are shown. (b) The expression of CD326 (left) or Ly-51 (right) on MHC II^{hi+} (upper) or MHC II^{lo+} (lower) subsets of cTECs are demonstrated.

subsets,[33–35] these two cTEC components were separately sorted from *aly/aly* or *aly/+* thymi, and mRNA levels of several molecules known to be expressed in cTECs were quantified. As shown in Fig. 4, the gene expression pattern was substantially different between $MHCII^{hi}$ and $MHCII^{lo}$ cells. Comparison of *aly/aly* and *aly/+* cells revealed no significant differences in expression level of most genes examined. One exception was the expression of *Ccl5* (or RANTES) in $MHCII^{hi}$ subsets, which was markedly lower in *aly/aly* cells than in *aly/+* cells. Although the physiological relevance of this decreased expression of *Ccl5* in *aly/aly* cTECs is currently unclear, these results may indicate that gene expression in *aly/aly* cTECs is different from that in *aly/+*.

Altered Vβ usage in mature T-cells from WT mice when differentiated in *aly/aly* thymus

To address the possibility that altered gene expression in *aly/aly* cTECs could affect their properties, we compared the functionality of *aly/aly* cTECs with that of *aly/+* cells to examine whether or not NIK-mutated cTECs were functionally normal. Because the principal function of cTECs is to positively select an appropriate T-cell repertoire, we investigated the impact of NIK deficiency in cTECs on the Vβ usage of mature T-cells that had been fostered by them.

Between *aly/aly* and *aly/+* mice, we found some differences in the Vβ usage of mature T-cells from the thymus and spleen (Fig. S2). To dissect specifically the effect of NIK in thymic stromal cells, we prepared bone marrow chimera in which WT bone marrow cells were infused into *aly/aly* or *aly/+* mice of a RAG2-KO background. The proportion of $CD69^+$ cells in CD4/CD8 double-positive thymocytes was similar between the two chimeric mice, and the number of positively selected thymocytes in *aly/aly* thymus was also similar to that in *aly/+* thymus (data not shown). Then, mature T-cells from the thymi of those chimeras were analysed for their Vβ usage. Because *aly/aly* mice are known to have a defect in generating normal numbers of $FoxP3^+$ Tregs and $NK1.1^+$ NKT cells,[13,26] these cells were excluded in the analyses to compare the Vβ usage among conventional T-cells. As shown

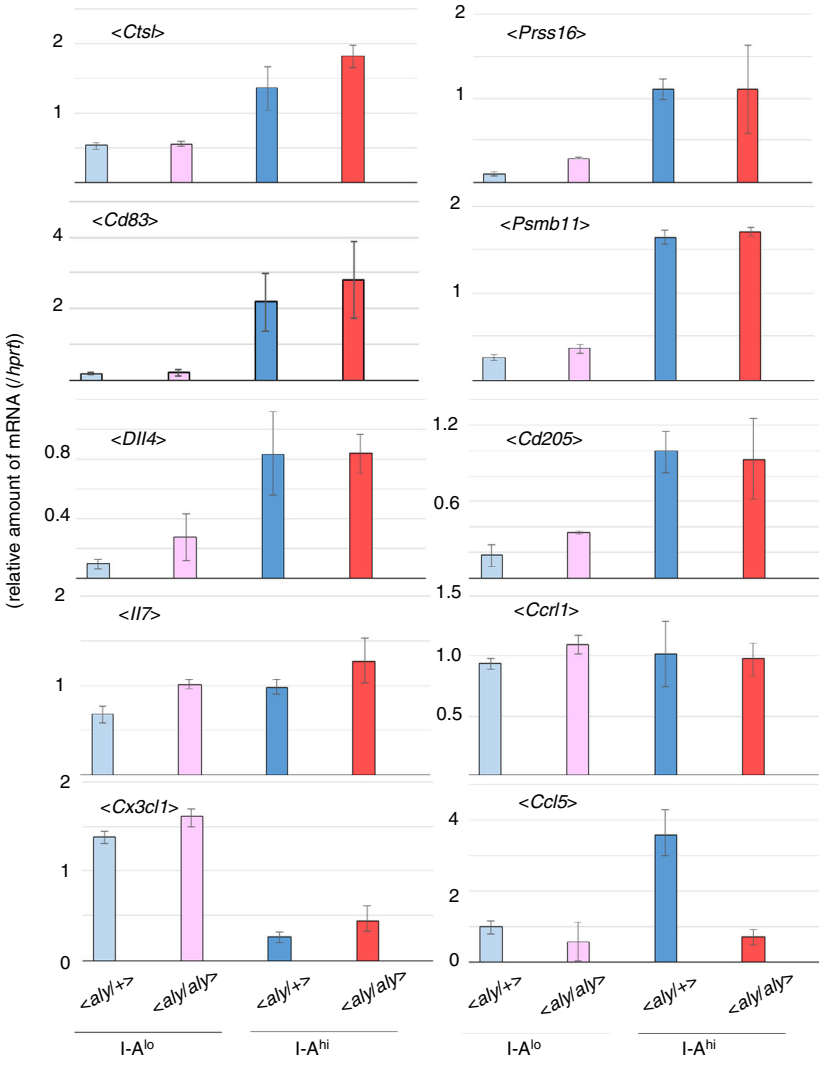

Figure 4. Expression of *Ccl5* mRNA was defective in the MHCII^{hi} subset of cortical thymic epithelial cells (cTECs) in *aly*/*aly* mice. MHC II^{hi+} and MHC II^{lo+} populations were sorted by FACS, and mRNA expression level of indicated genes was quantitated by quantitative polymerase chain reaction (qPCR) with normalization to *Hprt* transcripts. A representative set of results from several independent experiments for each gene is shown.

in Fig. 5, some Vβs were still differentially employed in mature T-cells of *aly*/*aly* hosts compared with those in *aly*/+ hosts. These results indicate that the repertoire of T-cells selected by NIK-defective cTECs may not be the same as the normal ones.

Reduced expression level of CD5 on mature T-cells differentiated in *aly*/*aly* thymi

The intensity of TCR signalling in thymocytes during thymic selection correlates with the level of CD5 expression of matured T-cells.[36–38] We examined the CD5 expression level on mature T-cells from *aly*/*aly* mice to evaluate the effect of NIK-deficient TECs on the CD5 expression level of T-cells that had been positively selected by them. CD5 expression by mature T-cells in *aly*/*aly* mice was slightly, but significantly, reduced compared with that in *aly*/+ mice (Fig. S3). The absence of NIK activity in thymic stroma may be partly responsible for it, because, as shown in Fig. 6, similar results were obtained in bone marrow chimera in which *aly*/*aly* hosts received WT bone marrow cells. Thus, WT T-cells matured in *aly*/*aly* thymi expressed lower levels of CD5 than WT T-cells matured in *aly*/+ hosts. Among CD4 single-positive mature thymocytes, the reduction of CD5 was detected at relatively immature CD62L^{lo}/CD69^{hi} population in *aly*/*aly* hosts, and the difference in the CD5 expression between the two hosts was larger at more mature CD62L^{hi}/CD69^{lo} stage (Fig. S4). These results suggest that the reduction in CD5 expression by *aly*/*aly* mature T-cells may be attributable, at least in part, to NIK deficiency in cTECs, and that thymocytes may receive weaker signals when they are positively selected by *aly*/*aly* cTECs.

Wild-type T-cells matured through positive selection in *aly*/*aly* thymus can respond almost normally to a foreign antigen

It is currently thought that TCR specificity of positively selected T-cell clones may be affected by the intensity of TCR signals.[36] Our observation that CD5 expression

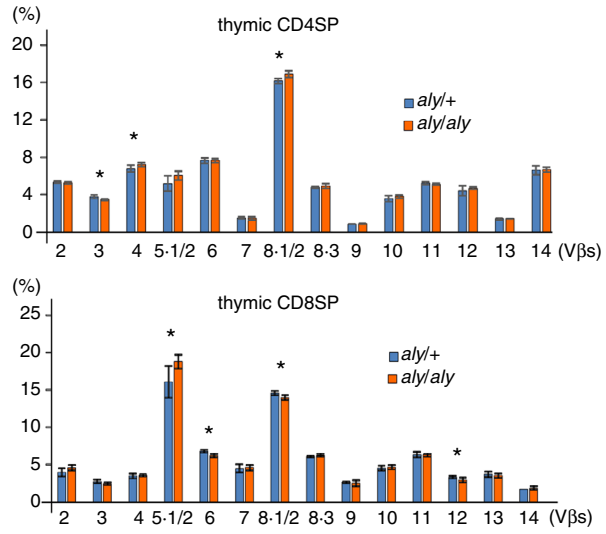

Figure 5. Comparison of T-cell receptor (TCR) Vβ usage between mature thymocytes developed in *aly/aly* thymi, and those in *aly/+* thymi. Bone marrow chimeric mice were prepared using wild-type (WT) B6 mice as donors, and *aly/aly* or *aly/+* mice with a RAG-2 (−/−) background as recipients. CD4 single-positive (upper) or CD8 single-positive (lower) TCRβ^{hi+} mature thymocytes from these chimeras were analysed for the percentage of cells expressing the indicated Vβ by flow cytometry. The analyses were performed for FoxP3^{-}/NK1.1^{-} cells. Asterisks show statistically significant differences ($P < 0{\cdot}05$, $n = 5$).

levels were reduced on WT T-cells that were differentiated in an *aly/aly* thymus raises the possibility that *aly/aly* cTECs fail to select appropriate T-cell clones capable of efficiently responding to foreign antigens. To investigate this possibility, we transferred splenocytes from bone marrow chimera of [WT → RAG2-KO, *aly/aly*, or RAG2-KO, *aly/+*] into RAG2-KO mice and immunized them with OVA antigen to examine *in vitro* recall responses. As shown in Fig. 7, WT T-cells that had been differentiated in an *aly/aly* host could substantially respond to the immunized soluble antigen, indicating that in spite of some phenotypical or functional differences, *aly/aly* cTECs may still preserve the ability to positively select T-cells capable of responding to foreign antigens.

Discussion

In this study, we have suggested that NIK, a well-documented kinase critically required for the differentiation of mTECs, may also be involved in proper differentiation and function of cTECs. This conclusion was supported by the following results.

1 The efficiency of positive selection of TCR-Tg T-cells was altered when bone marrow cells of NIK-sufficient TCR-Tg were transferred into NIK-mutant *aly/aly* hosts in the positive selecting H-2 background.
2 There were differences in the subset composition and gene expression in cTECs between *aly/aly* and WT mice.
3 The CD5 expression level on WT T-cells that had been developed through positive selection by *aly/aly* cTECs was significantly reduced compared with that in WT host.
4 A subtle, but statistically significant difference was detected in some Vβs expressed on the thymocytes of bone marrow chimera between *aly/aly* and WT hosts.

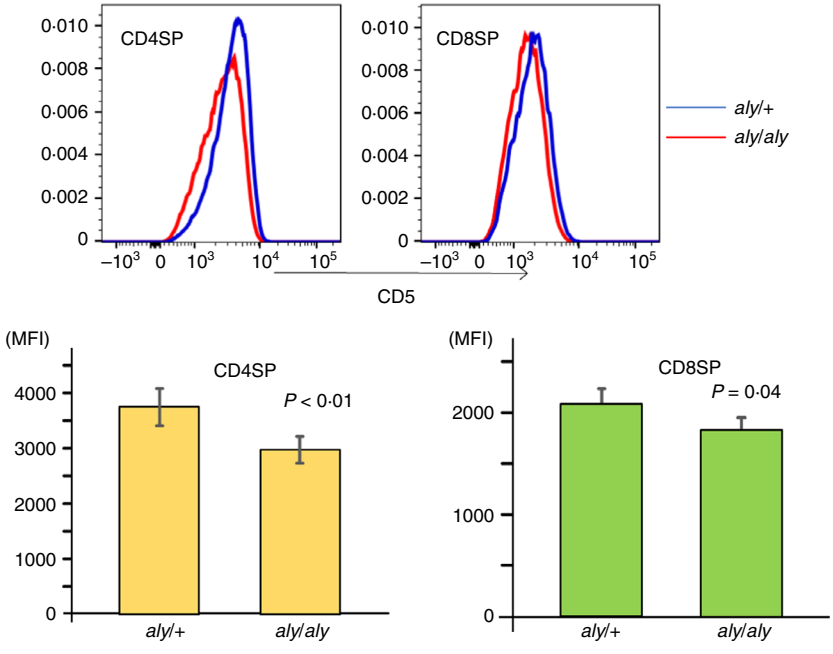

Figure 6. Decreased CD5 expression on wild-type (WT) T-cells matured in an *aly/aly* thymus. Expression of CD5 on TCRβ^{hi+} mature thymocytes where FoxP3^{+} Tregs and NK1.1^{+} NKT cells were gated out was compared between bone marrow chimeric mice of [WT B6 → *aly/aly* RAG-2 (−/−)] and [WT B6 → *aly/+* RAG-2 (−/−)] ($n = 5$). Red or blue lines indicate the CD5 expression on thymocytes in *aly/aly* or *aly/+* host, respectively. The graphs below show the mean fluorescence intensities.

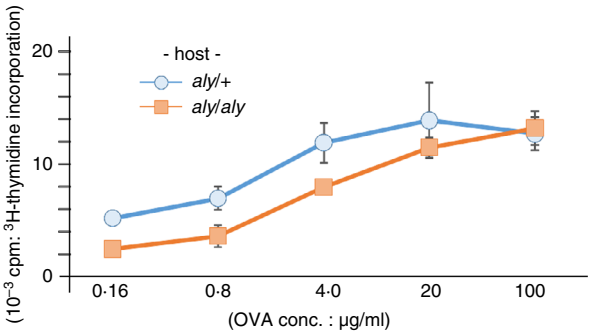

Figure 7. The repertoire of wild-type (WT) T-cells shaped through maturation in an *aly/aly* thymus was immune-competent to respond to a foreign soluble antigen. Splenocytes from [WT B6 → *aly/aly* RAG-2 (−/−)] or [WT B6 → *aly/+* RAG-2 (−/−)] bone marrow chimeras were transferred into RAG-2 (−/−) mice, and recipient mice were immunized with ovalbumin (OVA)/CFA. Nine days later, cells in draining lymph nodes were cultured in the presence of indicated concentrations of OVA. Proliferative responses were examined by measuring ^{3}H-labelled thymidine incorporation.

These results indicate NIK may be involved in the differentiation of cTECs and that NIK-defective *aly/aly* cTECs may not be functionally normal, inducing weaker TCR signals than WT cTECs during positive selection, which could affect T-cell repertoire selection. Given that the number of mature T-cells in *aly/aly* mice is not decreased, *aly/aly* cTECs may retain the ability to select a normal number of T-cells. Consistent with this notion, the number of CD69^{+} DP cells in the thymi from bone marrow chimera of *aly/aly* hosts receiving WT cells was similar to that in WT hosts (data not shown).

In comparison with mTECs, information is relatively limited on the precise differentiation steps or molecular requirements for maturation of cTECs. Because impairment of the non-canonical pathway of NF-κB activation resulted in a conspicuous phenotype of dysregulated mTEC formation and autoimmunity,[13,14,27,39–42] the function of molecules involved in this signalling pathway has been focused onto their role in mTEC differentiation. In contrast to the maturation requirements of mTECs, NIK is thought to be dispensable for cTEC maturation. The presence of apparently normal numbers of cTECs in the *aly/aly* thymus has been suggested by histological examination,[26] and is corroborated here by flow cytometric analyses. The current study, however, also uncovered some phenotypic differences between *aly/aly* and *aly/+* cTECs. For instance, we observed that *aly/aly* cTECs contain an increased proportion of the MHCII^{hi} subpopulation than do heterozygote cTECs, although the relationship between the two subsets segregated according to their expression levels of MHC class II and Ly-51 has yet to be fully addressed. The expression level of MHC class II or CD80 divides mTECs into two populations, in which MHCII^{hi} cells contain an AIRE^{+} subset, and MHCII^{lo} include the precursor cells of mature MHC II^{hi}, as well as a post-AIRE population.[43,44] Analogous application of this dichotomy to cTECs would suggest that some MHCII^{hi} cTECs may be derived from MHCII^{lo} cTECs. This inference may align with our observation that MHCII^{hi} cTECs express several important genes related to the cTEC function of antigen presentation in larger amounts than MHCII^{lo} cells. Given that the MHCII^{lo} TEC population may also contain the progenitor of mTECs,[33] it is tempting to speculate that blockade of their transition to the mTEC lineage by NIK deficiency might lead to accumulation of MHCII^{hi} cTECs.

It has been found that the expression level of MHC class II is augmented on *aly/aly* cTECs, while the expression level of MHC I or other TEC markers on *aly/aly* cTECs is almost identical to that of *aly/+* cTECs. Because expression of some genes implicated in MHC class II expression, such as *Ctsl*, *Prss16* or *Cd83*, in *aly/aly* cTEC was not perturbed, the molecular mechanism for enhanced MHC class II expression on *aly/aly* cTECs remains unclear. The expression level of MHC class II on peripheral APCs such as B-cells or dendritic cells was not elevated in *aly/aly* mice (data not shown), which might imply that NIK has a role in regulating MHC class II expression, specifically in cTECs.

The present study also revealed that chemokine *Ccl5* gene expression in the MHCII^{hi} subset of cTECs was impaired. Ccl5 may play a role in migration of thymocytes within the thymus, as thymocytes reportedly express Ccr3 or Ccr5,[45,46] both of which are receptors for Ccl5. Ccl5 has been shown to be required for optimal *in vivo* T-cell responses to foreign soluble antigens.[47] Our results indicate that the T-cell repertoire could be modified in the absence of Ccl5 in the thymus. Although the physiological relevance of altered expression of MHC class II or *Ccl5* in *aly/aly* cTECs has yet to be revealed in future studies, these observations may suggest that the gene expression pattern in *aly/aly* cTECs is different from that in WT cTECs, and therefore that *aly/aly* cTECs may not be entirely normal.

The differential gene expression in *aly/aly* cTECs appears to correlate with their dysregulated activity. Analyses of bone marrow chimera revealed that there were differences in Vβ usage and CD5 expression levels on NIK-sufficient thymocytes differentiated in *aly/aly* thymi, compared with those in *aly/+* thymi, indicating that selected sets of mature T-cell clones in *aly/aly* thymi could be different from those in WT mice. However, despite the significant difference in CD5 expression between *aly/aly* and WT hosts, the difference in Vβ usage was very small. This was surprising for us considering also the fact that we have not been able to exclude the effect of impaired negative selection in *aly/aly* thymi on the Vβ usage in thymic mature T-cells. Thus, in future studies, this should formally be analysed in mice where negative selection is normally mediated via mTEC,

for example, by supplementing mTEC precursor cells in *aly*/*aly* thymi.[27]

One of the principal purposes of thymic positive selection may be to prepare a collection of T-cell clones with suitable potential to react with as yet unseen foreign antigens in the context of self MHCs. From this perspective, the T-cell repertoire formed by *aly*/*aly* cTECs may not be 'defective', as WT T-cells matured in *aly*/*aly* thymi mounted a substantial response, at least to a foreign antigen, OVA. Nevertheless, recent studies have indicated that altered signalling during thymic selection could affect the reactivity of selected T-cells,[48] and it was suggested that this could affect their differentiation into, for example, memory or effector T-cells.[48,49] Further investigations will be required to elucidate the effect of positive selection of T-cells by *aly*/*aly* cTECs on long-term reactions, especially secondary memory responses.

Acknowledgements

The authors are grateful to Drs Michiyuki Kasai (Kochi Medical School, Japan) and Yan Xing (University of Minnesota) for helpful advice on preparation of thymic epithelial cells, and to Dr Yoichi Shinkai (Riken, Advanced Science Institute, Japan) for providing us with RAG-2 (−/−) mice. This work was partly supported by a Grant-in-Aid for Scientific Research (KAKENHI) (19K07715 to KE).

Disclosures

The authors declare no financial or commercial conflicts of interest.

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Supporting Information

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

Figure S1. Inefficient development of idiotype^{hi+} T-cells in TCR_{QM11}-Tg mice with an *aly*/*aly* background may be ascribed to the absence of NIK activity in non-haematopoietic cells rather than in thymocytes.

Figure S2. Comparison of Vβ usage in mature T-cells between *aly*/*aly* and *aly*/+ mice.

Figure S3. Comparison of CD5 expression on thymic mature T-cells from *aly*/*aly* and *aly*/+ mice.

Figure S4. Reduced expression of CD5 on thymic CD4SP cells matured in *aly*/*aly* thymi.

