Alymphoplasia (*aly*)-type Nuclear Factor κB–inducing Kinase (NIK) Causes Defects in Secondary Lymphoid Tissue Chemokine Receptor Signaling and Homing of Peritoneal Cells to the Gut-associated Lymphatic Tissue System

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Abstract

Alymphoplasia (*aly*) mice, which carry a point mutation in the nuclear factor κB–inducing kinase (NIK) gene, are characterized by the systemic absence of lymph nodes and Peyer's patches, disorganized splenic and thymic architectures, and immunodeficiency. Another unique feature of *aly/aly* mice is that their peritoneal cavity contains more B1 cells than normal and *aly/+* mice. Transfer experiments of peritoneal lymphocytes from *aly/aly* mice into recombination activating gene (RAG)-$2^{-/-}$ mice revealed that B and T cells fail to migrate to other lymphoid tissues, particularly to the gut-associated lymphatic tissue system. In vivo homing defects of *aly/aly* peritoneal cells correlated with reduction of their in vitro chemotactic responses to secondary lymphoid tissue chemokine (SLC) and B lymphocyte chemoattractant (BLC). The migration defect of *aly/aly* lymphocytes was not due to a lack of expression of chemokines and their receptors, but rather to impaired signal transduction downstream of the receptors for SLC, indicating that NIK is involved in the chemokine signaling pathway known to couple only with G proteins. The results showed that the reduced serum levels of immunoglobulins (Igs) and the absence of class switch to IgA in *aly/aly* mice are due, at least in part, to a migration defect of lymphocytes to the proper microenvironment where B cells proliferate and differentiate into Ig-producing cells.

Key words: RAG-$2^{-/-}$ mice • peritoneal cavity cell transfer • lamina propria • IgA plasma cells • chemotaxis assay

Introduction

Recent studies indicate that the development of structurally and functionally normal lymphoid organs is a complex process involving several members of the TNFR superfamily. Mice deficient for lymphotoxin (LT)[1] α (1, 2) or LTβR (3), each of which interacts with membrane LT heterotrimer (LTα1β2) and other ligands, such as LIGHT (4), are characterized by a severe defect in the formation of LNs, a complete absence of Peyer's patches (PPs) and a profound disturbance in the organization of the spleen, including the absence of germinal centers (GCs) and follicular dendritic cell (FDC) network. Mice deficient for either TNF (5) or TNFRI (6, 7) show a defect in the organization of the spleen (loss of GCs and FDC network), but retain the ability to form LNs or PPs. Studies on the mechanism of disturbed LN formation by bone marrow (BM) transplantation (8) or administration of the soluble LTβR-Ig fusion protein during pregnancy (9, 10) have shown that there is a developmental window during which LT-producing cells must interact with LT-sensitive cells to trigger the formation of LNs and PPs. In LTα$^{-/-}$ mice, formation of FDC clusters and GCs were restored by transplantation of normal BM cells (11) and subsequently, LTα-producing

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[1]*Abbreviations used in this paper: aly*, alymphoplasia; BLC, B lymphocyte chemoattractant; BLR1, Burkitt's lymphoma receptor 1; BM, bone marrow; CCR, CC chemokine receptor; ELC, EBV-induced molecule 1 ligand chemokine; FDC, follicular dendritic cell; GALT, gut-associated lymphatic tissue; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GC, germinal center; LT, lymphotoxin; LP, lamina propria; MLN, mesenteric LN; NF-κB, nuclear factor κB; NIK, NF κB–inducing kinase; PEC, peritoneal cavity; PP, Peyer's patch; RAG, recombination activating gene; SLC, secondary lymphoid tissue chemokine.

B cells were shown to provide the essential signal for induction and maintenance of the lymphoid architecture necessary for GC formation (12, 13).

Alymphoplasia (*aly*) mice, which carry a natural point mutation in the gene encoding nuclear factor κB–inducing kinase (NIK) (14), represent another model for the abnormal development of lymphoid organs, being characterized by the systemic absence of LNs and PPs, disorganized splenic architecture, and immunodeficiency (15). Because *aly*-type NIK affects the signaling pathway of LTβR (14), which is exclusively expressed by nonlymphoid cells (16, 17), it is natural to interpret that the *aly/aly* phenotypes are due to the defect of LTβR signaling in non-BM–derived cells. However, *aly/aly* mice have unique features that are not shared by $LT\beta R^{-/-}$ and $LT\alpha^{-/-}$ mice: disturbed thymic structure, depressed T cell functions, and reduced numbers of mature B cells in BM, spleen, and peripheral blood (15, 18, 19). Furthermore, spleen, mesenteric LNs (MLNs), and PPs remain atrophic when *aly/aly* BM cells are transferred to irradiated wild-type mice (15). In contrast, $LT\alpha^{-/-}$ BM cells have no homing defect to secondary lymphoid organs (8). The results suggest the presence of a homing defect in *aly/aly* lymphocytes.

Another feature of *aly/aly* lymphocytes that suggests their migration defect is a higher B1/B2 cell ratio in their peritoneal cavity (PEC) than normal mice (14). More than 20 years ago, Husband and Gowans (20) suggested a link between PEC cells and antigen-specific B cells in the lamina propria (LP) of the rat small intestine. Approximately half of the IgA plasma cells in the LP of intestine appeared to be derived from PEC cells, suggesting that frequent migration of lymphocytes may take place between PEC and GALT (21, 22). Although chemokines responsible for PEC lymphocyte migration to secondary lymphoid organs have not been identified, several chemokines are shown to be involved in the constitutive homing of B lymphocytes to their proper locations within lymphoid organs. Mice with targeted disruption of the gene for Burkitt's lymphoma receptor 1 (BLR1; also known as CXC chemokine receptor 5 [CXCR5]), lack functional GCs in the spleen with impaired B lymphocyte recirculation (23). The ligand for BLR1, termed B lymphocyte chemoattractant (BLC) or B cell attracting chemokine 1 (BCA-1), is shown to promote migration of B lymphocytes in lymphoid follicles (24, 25). Another chemokine, secondary lymphoid tissue chemokine (SLC; also known as 6Ckine, Exodus-2, or thymus-derived chemotactic agent 4 [TCA-4]), stimulates the chemotaxis of naive T, memory T, and B cells (26–29). Recently, targeted disruption of the gene for CC chemokine receptor (CCR)7, one of the receptors for SLC, revealed that this receptor is important for T, B, and dendritic cells to migrate to the proper microenvironments, to initiate an antigen-specific immune response, and to establish a functional architecture of the secondary lymphoid tissues (30).

Here, we report that *aly/aly* mice had no IgA^+ B cells in LP of small intestine, and that *aly/aly* PEC lymphocytes have a migration defect to the gut-associated lymphoid tissue (GALT) system as well as spleen. We have also demonstrated that the *aly* mutation in the NIK gene blocks signaling from the receptors for SLC, providing the first demonstration that NIK is involved in signal transduction of seven transmembrane–type receptors.

Materials and Methods

Mice. *aly/aly*, *aly/+*, and recombination activating gene (RAG)-$2^{-/-}$ mice were maintained under specific-pathogen free conditions and $LT\alpha^{-/-}$ mice were kept in conventional conditions at the animal facility of the Department of Medical Chemistry, Kyoto University, and used between 10 and 14 wk of age.

Cell Preparation and Adoptive Transfer. PEC cells were harvested by flushing the peritoneum with 10 ml of RPMI 1640 medium containing 2% FCS, washed twice, and resuspended in PBS. A total of 1.3×10^7 cells from *aly/aly* or *aly/+* mice, and 3×10^7 cells from $LT\alpha^{-/-}$ mice, were injected intraperitoneally into RAG-$2^{-/-}$ recipient mice.

Flow Cytometry Analysis. The following antibodies were used: FITC-conjugated anti-B220 (PharMingen), anti-IgM (Southern Biotechnology Associates), anti-CD3 (PharMingen), PE-conjugated anti-IgA (Southern Biotechnology Associates), and anti–Mac-1 (PharMingen). All analyses were performed on a FACSCalibur™ (Becton Dickinson). Data were obtained on 2×10^4 viable cells, as determined by forward light scatter intensity and propidium iodide exclusion.

Quantification of Plasma Cells and T Cells. Recipient mice were killed 3–6 wk after transfer. RAG-$2^{-/-}$ mice injected with $LT\alpha^{-/-}$ PEC cells were analyzed 12 wk after transfer. A standard procedure was used to prepare single cell suspensions from PEC, spleen, BM obtained from two femurs, and MLNs and LP of small intestine. The percentages of IgA plasma cells in the LP of the small intestine and MLNs were determined by FACS®. The percentages of plasma cells in all lymphoid tissues were determined after cytocentrifuge preparation (Cytospin 3; Shandon, Inc.), intracytoplasmic staining with FITC-labeled anti-IgM, anti-IgA, and anti-IgG (PharMingen), and counterstaining with DAPI (Wako Pure Chemicals) to visualize nuclei. The slides were examined with a fluorescence microscope. The total number of T cells present in each organ was calculated from the frequency estimated by immunofluorescence analysis and the total number of cells recovered in each organ.

ELISA. Total IgM, IgA, and IgG in the sera were measured by a modification of the sandwich ELISA described previously (31). In brief, microtitration plates (Corning) were coated with goat anti–mouse IgM (Southern Biotechnology Associates), goat anti–mouse IgA (Cappel Laboratories), or anti–mouse IgG (Kirkegaard & Perry Laboratories) antibodies and were blocked with BSA. After incubation with the serum samples, alkaline phosphatase–conjugated goat anti–mouse IgM and IgG antibodies (Southern Biotechnology Associates), or goat anti–mouse IgA antibody (Zymed Laboratories) was applied. The plates were washed and developed using *p*-nitrophenyl phosphate (Sigma Chemical Co.). Absorbency was measured using an ELISA reader.

Chemotaxis. Cell suspensions were prepared from spleen and PEC, and were incubated for 2 h in RPMI 1640 medium supplemented with 10% FCS, with or without 20 μg/ml LPS (Sigma Chemical Co.). Chemotaxis assays were performed as described (32), using 10^6 total cells per 5-μm transwell (Corning Costar Corp.). Cells that migrated to the lower chamber were

enumerated by collecting events for a fixed time (60 s) under a constant sheath pressure on a FACSCalibur™. By counting a 1:10 dilution (in the case of SLC) and 1:20 dilution of input cells (for BLC) in the same way, we determined the absolute number of cells that transmigrated. To determine which subsets of cells were migrated, the cells from the lower chamber were stained with antibodies (PharMingen) as follows: FITC-conjugated anti-IgM in combination with PE-conjugated anti–Mac-1 and/or PE-conjugated anti-CD5 for PEC cells; FITC-conjugated anti-B220 in combination with PE-conjugated anti-CD3 for spleen cells.

Northern Blot Analysis. 5–10 μg of total RNA from spleen, PEC cells, and small intestine was subjected to electrophoresis, transferred to Hybond N^+ membranes (Amersham Pharmacia Biotech), and probed with randomly primed ^{32}P-labeled mouse cDNA probes for chemokines BLC, SLC, and EBV-induced molecule 1 ligand chemokine (ELC), and chemokine receptors BLR1 and CCR7. Filters were rehybridized with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. All probes were cloned PCR products from wild-type splenic RNA. For chemokine receptor expression, RNA was prepared from unstimulated or LPS-stimulated splenic and PEC cells (20 μg/ml LPS for 2 h).

Electrophoretic Mobility Shift Assay. PEC cells from 10-wk-old *aly/aly* and *aly/+* mice kept in specific-pathogen free conditions were stimulated with SLC (500 ng/ml) for 15 and 30 min. Nuclear extracts were prepared according to the protocol described (33). Consensus and mutant double-stranded oligonucleotides encoding NF-κB and Oct-1 binding sequence were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara Biotech). For competition and supershift assay, cold or mutant oligonucleotide, or 1 μg of antibodies (Santa Cruz Biotechnology) was added to the reaction. The samples were loaded onto 6% nondenaturing polyacrylamide gel and run in 0.25× Tris-borate buffer. The resultant DNA–protein complexes were detected by autoradiography.

Results

Peritoneal Lymphocytes from aly/aly Mice Fail to Generate IgA Plasma Cells in the GALT System of RAG-2$^{-/-}$ Mice. Two unique features of *aly/aly* mice, abundant B1 cells in the PEC (Fig. 1, A and B) and the complete absence of the B cell populations, $B220^+IgM^+$ small lymphocytes, and $B220^-IgA^+$ plasma cells (our unpublished results) in LP of the small intestine (Fig. 1, C and D) led us to suspect that *aly/aly* PEC cells may have a migration defect to LP, although the lack of IgA plasma cells in LP can be partially explained by the lack of PPs, which normally contains the B2 cell precursors of plasma cells (20, 34–37). Therefore, we transferred PEC cells from *aly/aly* and *aly/+* mice into the PEC of RAG-2$^{-/-}$ mice and analyzed the lymphoid tissues 3 and 6 wk after the transfer. First, FACS® analyses revealed that no $B220^-IgA^+$ cells could be detected in LP or MLNs of RAG-2$^{-/-}$ mice transferred with *aly/aly* peritoneal lymphocytes. By contrast, *aly/+* peritoneal B cells injected into RAG-2$^{-/-}$ mice migrated to MLNs and intestinal LP, where they gave rise to IgA plasma cells (Fig. 2, A and B). In agreement with these results, the PEC of RAG-2$^{-/-}$ mice injected with *aly/aly* lymphocytes contained more B1 cells than the PEC of RAG-2$^{-/-}$ mice injected with *aly/+* lymphocytes (Fig. 2 C). The absence of IgA plasma cells reflects a homing defect to the GALT system and not a defect in class switching because *aly/aly* lymphocytes can produce IgA by in vitro culture in the presence of cytokines. When 4×10^5 PEC cells were cultured for 7 d with IL-5 (100 U/ml), the Ig levels (ng/ml) in the culture supernatants were as follows: IgM (784.37 ± 193.38), IgG (200.55 ± 14.75), and IgA (10.04 ± 2.91) for *aly/aly* PEC cells; and IgM (825.66 ± 245.32), IgG (232.94 ± 30.01), and IgA (13.03 ± 4.32) for *aly/+* PEC cells. Inter-

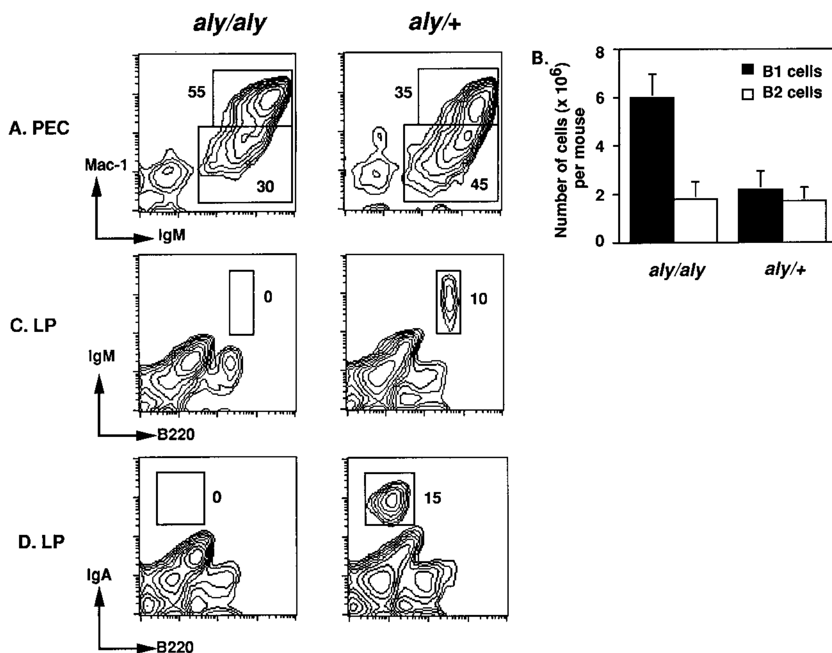

Figure 1. Abundant B1 cells in the PEC and complete absence of B cell populations in LP of *aly/aly* mice. (A) Flow cytometric analysis of PEC cells from *aly/aly* and *aly/+* mice. A total of 2×10^4 cells were analyzed for IgM in combination with Mac-1. The numbers are the percentages of B1 ($IgM^+Mac\text{-}1^+$) and B2 ($IgM^+Mac\text{-}1^-$) cells. (B) Total numbers of PEC cells in *aly/aly* and *aly/+* mice, as calculated by (percentage of indicated cells) × (number of viable cells). (C and D) Flow cytometric analysis of LP cells of *aly/aly* and *aly/+* mice, stained with FITC-conjugated anti-B220 and PE-conjugated anti-IgM or anti-IgA. The numbers are the percentages of B cells ($B220^+IgM^+$) and plasma cells ($B220^-IgA^+$).

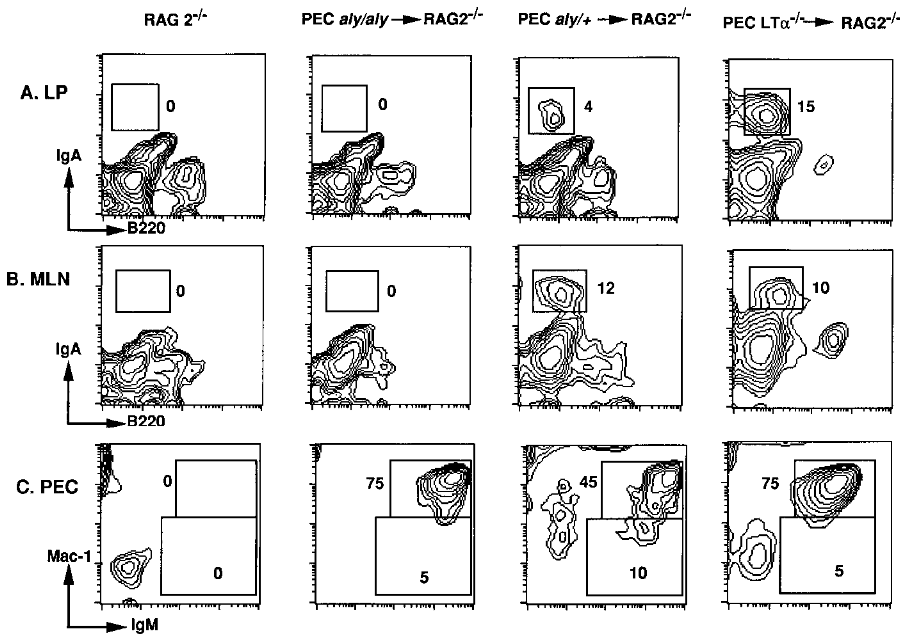

Figure 2. Transfer of PEC cells from *aly/aly* mice fails to generate IgA plasma cells in GALT of RAG-$2^{-/-}$ mice. Flow cytometric analysis of LP cells (A) and MLNs (B) derived from RAG-$2^{-/-}$ mice, RAG-$2^{-/-}$ mice injected with 1.3×10^7 PEC cells from *aly/aly* and *aly/+* mice, and 3×10^7 PEC cells from $LT\alpha^{-/-}$ mice, at 6 and 12 wk, respectively, after the transfer. A total of 2×10^4 cells were analyzed for B220 in combination with IgA. The numbers are the percentages of IgA plasma cells ($B220^-IgA^+$). (C) RAG-$2^{-/-}$ mice, RAG- $2^{-/-}$ mice that were injected intraperitoneally with PEC cells from *aly/aly* and *aly/+* mice 3 wk previously, and RAG-$2^{-/-}$ mice at 12 wk after injection with $LT\alpha^{-/-}$ PEC cells. A total of 2×10^4 cells were analyzed for IgM in combination with Mac-1. The numbers are the percentages of B1 ($IgM^+Mac\text{-}1^+$) and B2 ($IgM^+Mac\text{-}1^-$) cells.

estingly, $LT\alpha^{-/-}$ PEC cells did not show any homing defect to the GALT system (Fig. 2, A and B).

FACS® results were further confirmed and extended by staining of fixed cytocentrifuge preparations of LP and MLN lymphocytes for IgM, IgA, and IgG. As shown in Table I, reconstitution of IgA or IgG plasma cells in LP of the small intestine was not observed when PEC cells from *aly/aly* were transferred to RAG-$2^{-/-}$ mice, whereas LP of RAG-$2^{-/-}$ mice injected with *aly/+* PEC cells contained many IgA plasma cells and a few IgG plasma cells. On the other hand, we did not observe any IgM plasma cells in LP of normal C57BL/6 or BALB/c mice (data not shown), or RAG-$2^{-/-}$ mice transferred with PEC cells from *aly/aly* or *aly/+* mice (Table I).

No IgM, IgA, or IgG plasma cells could be identified in MLNs of RAG-$2^{-/-}$ mice injected with *aly/aly* PEC cells. On the contrary, MLNs of RAG-$2^{-/-}$ mice injected with *aly/+* PEC cells contained IgA, IgM, and IgG plasma cells, and the percentage of IgA plasma cells was at least two times higher than that in LP. These results strongly suggest that the absence of IgA plasma cells in the LP of *aly/aly* mice is due not only to the lack of PPs, but also to a defect of PEC cells to repopulate the GALT system with IgA plasma cells.

Migration Defect of aly/aly PEC Cells Is Not Restricted to Either GALT System or B Cells. 3 wk after transfer, the spleen or BM of RAG-$2^{-/-}$ mice transferred with *aly/aly* peritoneal B cells contained very few IgM or IgG and no IgA plasma cells (Table I, and data not shown). On the contrary, spleen of RAG-$2^{-/-}$ mice injected with *aly/+* peritoneal B cells contained many IgM or IgG plasma cells, and less but a significant number of IgA plasma cells, whereas their BM contained <0.5% each of plasma cells expressing various isotypes (Table I, and data not shown). Although

Table I. *Impaired Generation of Plasma Cells in RAG-$2^{-/-}$ Mice Transferred with aly/aly PEC Cells*

	Percent IgM		Percent IgA		Percent IgG	
	aly/aly	aly/+	aly/aly	aly/+	aly/aly	aly/+
LPLs (3 wk)	0	0	0	2.33 ± 0.81	0	<1
LPLs (6 wk)	0	0	0	5.66 ± 1.36	0	<1
MLN (3 wk)	0	<1	0	7.86 ± 1.20	0	<1
MLN (6 wk)	0	3.66 ± 3.12	0	11.66 ± 3.93	0	<1
Spleen (3 wk)	<1	2.27 ± 0.55	0	<1	0	1.66 ± 0.57
Spleen (6 wk)	<1	7.10 ± 3.35	0	1.37 ± 0.47	<1	3.33 ± 1.15

Fixed cytocentrifuge preparations of LP lymphocytes (LPLs), MLNs, and spleen cells from RAG-$2^{-/-}$ mice injected with PEC cells from *aly/aly* and *aly/+* mice were stained for intracytoplasmic IgM, IgA, and IgG, and counterstained with DAPI to visualize nuclei. The percentage of plasma cells in total cells represents mean ± SE from three transfer experiments.

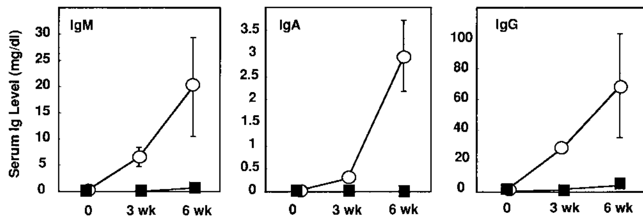

Figure 3. Isotype of serum Ig in RAG-$2^{-/-}$ mice injected with PEC cells from *aly/aly* (■) and *aly/+* (○) mice. Serum Ig levels were determined 3 or 6 wk after transfer, by isotype-specific ELISA. Results represent mean ± SE from three separate experiments.

IgM-secreting cells in normal mice generally represent <1% of the total number of splenic cells, in RAG-$2^{-/-}$ mice injected with *aly/+* lymphocytes the IgM plasma cells represented ~7% and >10% of all splenic cells by 6 and 12 wk, respectively, after the transfer (Table I, and data not shown). The presence of high numbers of IgM-secreting B cells in RAG-$2^{-/-}$ mice transferred with *aly/+* PEC cells and yet reduced numbers of $B220^{+}IgM^{+}$ cells (1/3 of those in normal mice) in spleen suggests that peritoneal B cells may contain precursors to plasma cells or differentiate rapidly to plasma cells after transfer.

The difference in the numbers of plasma cells between RAG-$2^{-/-}$ mice injected with *aly/aly* and *aly/+* PEC cells was also reflected in the serum levels of Igs, as shown in Fig. 3. In RAG-$2^{-/-}$ mice, 6 wk after transfer of *aly/aly* PEC cells, the serum titers of IgM and IgG were very low compared with those injected with *aly/+* PEC cells, and the IgA levels were not detected, even after 6 wk.

The percentages and numbers of *aly/aly* T cells that migrated to LP and MLNs 3 wk after the transfer into RAG-$2^{-/-}$ mice were four to six and two times less, respectively, than those of *aly/+* T cells (Fig. 4, A and B, and data not shown). Although T cells from *aly/aly* PEC have a migration defect to the GALT system, their homing to spleen and BM was unaffected (Fig. 4 B). These results suggest that chemokines involved in homing of B and T cells to spleen may be different from those necessary for their migration to the GALT system. Alternatively, if the same chemokines are involved, they probably use different signaling pathways.

Both Chemokines and Their Receptors Are Expressed in aly/aly Mice. To explore the cause of the migration defect of *aly/aly* PEC cells, we measured mRNA expression levels of chemokines and their receptors. We first studied the mRNA expression of SLC, ELC (also known as macrophage inflammatory protein 3β), and BLC in the spleen and small intestine, to which PEC cells were shown to migrate in RAG-$2^{-/-}$ mice. As shown in Fig. 5 A, SLC mRNA was expressed in the spleen and small intestine of RAG-$2^{-/-}$

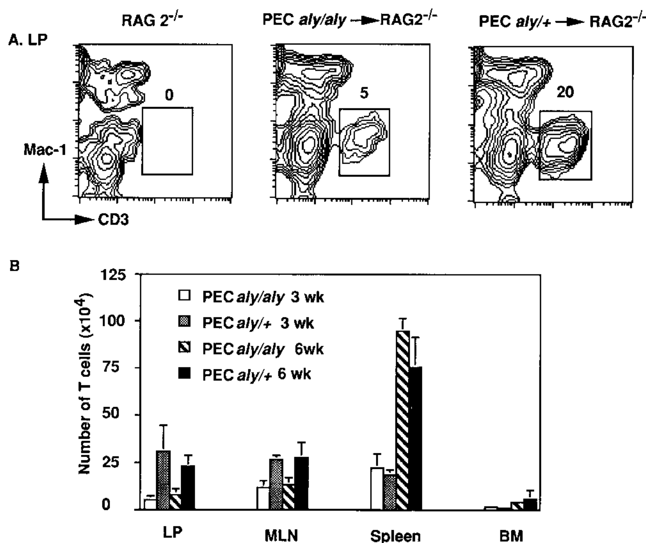

Figure 4. Migration defect of *aly/aly* peritoneal T cells to the GALT system. (A) Flow cytometric analysis of LP cells derived from RAG-$2^{-/-}$ mice and RAG-$2^{-/-}$ mice transferred with 1.3×10^7 PEC cells from *aly/aly* and *aly/+* mice 6 wk previously. A total of 2×10^4 cells were analyzed for CD3 in combination with Mac-1. The numbers are the percentages of T ($CD3^{+}Mac\text{-}1^{-}$) cells. (B) Numbers of T cells recovered 3–6 wk after transfer, calculated by multiplying total cell number with the percentage of T cells in indicated lymphoid tissues. Results represent mean ± SE from three different experiments.

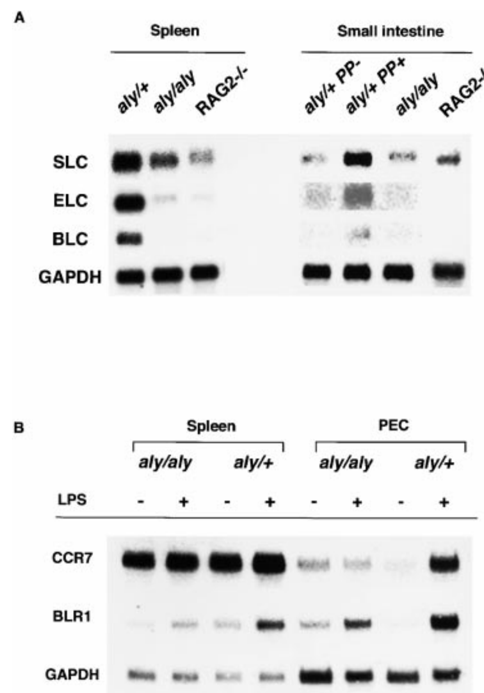

Figure 5. Chemokine and chemokine receptor expression in *aly/aly* mice. (A) Chemokine expression in spleen and small intestine of RAG-$2^{-/-}$, *aly/aly*, and *aly/+* mice. Northern blot analysis of total RNA from spleen and small intestine of RAG-$2^{-/-}$ mice and *aly/aly* mice, and small intestine with or without PPs from *aly/+* mice, and probed to detect expression of SLC, ELC, and BLC. (B) Chemokine receptor expression in spleen and PEC cells from *aly/aly* and *aly/+* mice. LPS stimulation markedly increased the expression of CCR7 and BLR1 RNA in peritoneal cells of *aly/+* but not *aly/aly* mice. Northern blot analysis of total RNA from unstimulated and LPS-stimulated (20 μg of LPS for 2 h) splenic and PEC cells and probed to detect expression of CCR7 and BLR1. GAPDH hybridization indicates amount of total RNA loaded in each lane.

mice and *aly/aly* mice, albeit at lower levels than in *aly/+* mice. ELC mRNA expression levels were lower in spleen and absent or lower in small intestine of both RAG-$2^{-/-}$ and *aly/aly* mice compared with *aly/+* mice. BLC mRNA expression was drastically reduced in spleen and absent in small intestine of RAG-$2^{-/-}$ and *aly/aly* mice. Therefore, among the chemokines constitutively expressed in secondary lymphoid organs, SLC mRNA was found most abundantly expressed in RAG-$2^{-/-}$ mice, suggesting that this chemokine could be important for homing of injected lymphocytes to the lymphoid tissues of RAG-$2^{-/-}$ mice.

We also found that both PEC and spleen cells of *aly/aly* and *aly/+* mice expressed at least detectable levels of CCR7 and BLR1 (Fig. 5 B). BLR1 mRNA expression levels of spleen and PEC cells from both *aly/aly* and *aly/+* mice were upregulated after LPS stimulation. On the other hand, CCR7 mRNA expression was not affected except for *aly/+* PEC cells, probably because CCR7 mRNA was abundantly expressed in spleen cells even without in vitro activation.

Reduced Chemotactic Activities of SLC and BLC on Resting and LPS-activated aly/aly Peritoneal Lymphocytes. Based on the mRNA expression profiles of chemokines and their receptors, we assayed chemotactic responses of *aly/aly* and *aly/+* PEC cells to two chemokines: SLC and BLC. As shown in Fig. 6 A, peritoneal B1 cells from *aly/aly* mice did not respond to SLC at all, whereas those from *aly/+* mice responded to SLC. *aly/aly* peritoneal B2 and T cells also showed very weak chemotactic responses to SLC compared with those from *aly/+* mice (Fig. 6, B and C). Interestingly, splenic B and T cells of *aly/aly* mice showed a normal chemotactic response to SLC (Fig. 6, D and E). BLC induced a very weak chemotactic response in peritoneal B1 and B2 cells of *aly/aly* mice. On the other hand, BLC induced a strong chemotactic response in peritoneal B1 and B2 cells and splenic B cells of *aly/+* mice, as well as in splenic B cells of *aly/aly* mice (Fig. 6, F–H). As expected, BLC showed limited activity toward T cells (data not shown).

LPS activation was found to enhance the chemotactic response to SLC and BLC of peritoneal but not of splenic B cells (Fig. 6). Although *aly/aly* peritoneal B lymphocytes showed an increase in the chemotactic response to SLC after LPS stimulation, their responses were still weaker than those of nonstimulated *aly/+* peritoneal B cells. By contrast, LPS stimulation augmented the BLC responsiveness of *aly/aly* PEC B cells to similar levels as nonstimulated *aly/+* PEC B cells, in agreement with upregulation of BLR1 mRNA by LPS stimulation (Fig. 5 B). The increased responsiveness of activated PEC B cells to SLC and BLC did not reflect an increase in the spontaneous mobility of B cells, as there was no difference in the migration frequency in the absence of chemokines between activated and nonactivated B cells.

NIK Is Downstream of SLC Receptor Signaling. *aly/aly* PEC cells failed to migrate to the GALT system as well as to the spleen. The in vivo migration defect of *aly/aly* PEC cells correlates well with the in vitro defective chemotactic response of *aly/aly* PEC cells to SLC and BLC. This migration defect cannot be explained by the lack of chemokines or their receptors, suggesting that the signal downstream of chemokine receptors might be affected in *aly/aly* mice. Therefore, we examined whether chemokine-induced NF-κB activation is affected by the *aly* mutation of NIK. We

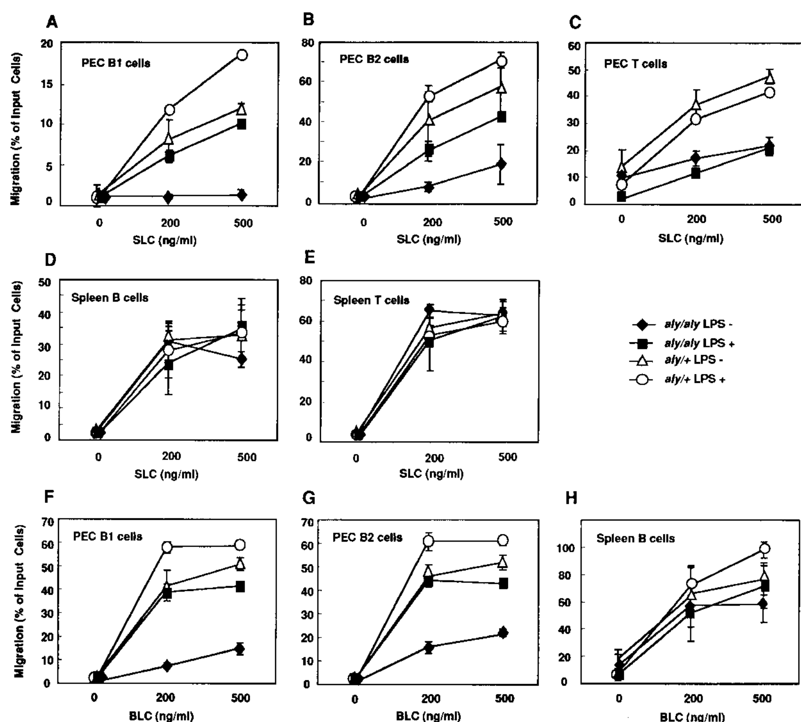

Figure 6. Decreased chemotactic activity of SLC and BLC on resting and acutely activated PEC cells from *aly/aly* mice compared with *aly/+* mice. The number of input and migrating cells of each subtype was determined by immunostaining and flow cytometry. Results are expressed as the percentage of input cells of each subtype migrating to the lower chamber of a Transwell filter. Graphs show migration of: (A) PEC B1 cells; (B) PEC B2 cells; (C) PEC T cells; (D) spleen B cells; (E) spleen T cells, towards SLC; (F) PEC B1 cells; (G) PEC B2 cells; and (H) spleen B cells towards BLC. PEC cells and spleen cells were preincubated with medium alone (LPS−) or LPS at 20 μg/ml for 3 h. Data points represent the mean ± SE for experiments performed in triplicate. Each experiment was repeated at least three times.

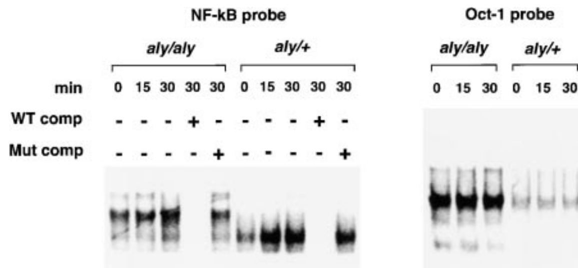

Figure 7. Impaired activation of NF-κB by SLC in *aly/aly* PEC cells. Nuclear proteins (6 μg) from unstimulated and SLC (500 ng/ml) stimulated PEC cells for 15 or 30 min, were incubated with unlabeled (cold competitor, WT comp) and mutant (Mut comp) NF-κB probe for 20 min. The samples were then incubated with ^{32}P-labeled NF-κB sequence for 20 min, followed by separation on polyacrylamide gel and analysis by autoradiography. The Oct-1–specific probe was used as internal control.

stimulated PEC cells from *aly/aly* and *aly/+* mice with SLC, and the activation of NF-κB was determined by the gel shift assay of nuclear extracts, 15 or 30 min after stimulation. As shown in Fig. 7, SLC stimulation did not induce activation of NF-κB in *aly/aly* PEC, but increased the levels of NF-κB in the nuclei of *aly/+* PEC. The same stimulation did not affect the amount of Oct-1 transcription factor in the nuclear extract of either *aly/aly* or *aly/+* PEC cells. Although the SLC-induced activation of NF-κB was not observed in nuclei of *aly/aly* PEC cells, the NF-κB complexes were present even before SLC stimulation. What was different from *aly/+* PEC cells was not the constitutive level, but rather the species of the NF-κB complexes. The supershift assay (Fig. 8) indicated that the NF-κB complexes in *aly/aly* PEC cells consisted mostly of p50-p50 homodimers and p50-p65 heterodimers because anti-p65 and anti-p50 mAbs formed supershifted bands in *aly/aly* nuclear extracts. On the other hand, the NF-κB complexes in *aly/+* PEC cells contained mainly p50-p50 homodimers. These results clearly demonstrate that NIK acts downstream of the signaling pathway of the receptors for SLC, leading to activation of the NF-κB complex. In addition, the *aly*-type mutation in the NIK gene affects this signaling pathway, resulting in the migration defect of *aly/aly* PEC cells.

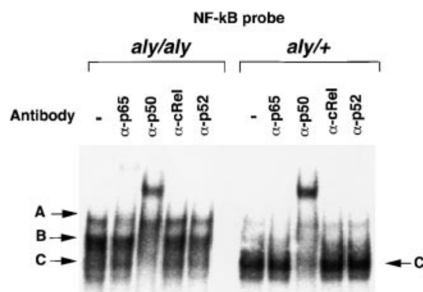

Figure 8. The NF-κB complexes are different in PEC cells from *aly/aly* and *aly/+* mice. Nuclear extracts from PEC cells stimulated for 30 min with SLC were incubated with the indicated antibody followed by ^{32}P-labeled NF-κB sequence. The samples were migrated on polyacrylamide gels and analyzed by autoradiography. (A) p65-cRel/p65-RelB complex, (B) p50-p65/p50-RelB complex, and (C) p50-p50 complexes.

Discussion

The absence of secondary lymphoid tissues such as LNs and PPs in LTα$^{-/-}$, LTβR$^{-/-}$, and *aly/aly* mice is now clearly explained by the defect in the common signaling pathway using LTβR and NIK (14). Because NIK appears to be ubiquitously expressed in the mouse (our unpublished data), and the *aly/aly* phenotype is more severe than that of LTβR$^{-/-}$ mice, we considered the possibility that the NIK mutation affects the signaling pathways of other receptors in lymphoid as well as nonlymphoid cells. We have shown by in vivo and in vitro experiments that *aly/aly* PEC cells have a defect that affects their homing capacity, especially to the GALT system. The in vivo migration defect of *aly/aly* PEC cells correlated well with the in vitro impaired chemotactic response toward SLC and BLC. We found that SLC stimulation did not activate NF-κB in *aly/aly* PEC cells, whereas the same stimulation increases the nuclear NF-κB in *aly/+* PEC cells, thus demonstrating that NIK is located downstream of the signaling pathway through the receptors for SLC, and that *aly*-type NIK affects this pathway. NIK is known to participate in the signaling cascade responsible for NF-κB activation through receptors of the TNF and IL-1R/toll-like receptor (TLR) families (38–44) including LTβR, TNFR, CD40, and CD95. In this study, we have shown that the receptors for SLC also use NIK for NF-κB activation. In agreement with this, we found that overexpression of CCR7 in 293T cells induced NF-κB activation in an NIK-dependent manner (our unpublished data). Chemokine receptors including BLR1 and CCR7 are seven transmembrane receptors that are coupled with G proteins for signal transduction (45–48). This is the first report that a G protein–coupled receptor is also involved in NF-κB activation through NIK.

It has been reported that except for the mucosal addressin cell adhesion molecule 1 (MAdCAM-1) in the spleen, the expression of lymphocyte adhesion molecules and their cognate receptors is similar in *aly/aly* and *aly/+* mice (49). In this study, we have shown that *aly/aly* lymphocytes also have normal expression levels of chemokine receptor mRNAs in spleen and PEC cells. The reduced levels of chemokine expression in *aly/aly* mice were probably due to the severe defect of stromal cells that have been shown to produce chemokines necessary for B and T compartmentalization in the spleen (50). At the same time, the lymphoid cell depletion, in particular B cell depletion in spleen of *aly/aly* mice, may also contribute to the reduced level of BLC, as it is known that B lymphocytes are important for induction of BLC expression, by providing LTα1β2 and possibly TNF (50). Although the chemokine mRNA expression was reduced in spleen and small intestine of *aly/aly* compared with *aly/+* mice, the chemokine mRNA expression levels were higher in *aly/aly* mice than in RAG-2$^{-/-}$ mice, which can support homing of normal peritoneal B and T cells to the GALT system and spleen. These results suggest that *aly/aly* PEC cells may have an intrinsic defect that affects their homing capacity, especially to the GALT system. However, we cannot completely ex-

clude alternative explanations, that the failure of PEC cell migration to lymphoid tissues is due to extrinsic defects such as differences in cytokines released by T cells and macrophages of *aly/aly* and *aly/+* mice, or differences in the ability of *aly/aly* and *aly/+* lymphocytes to induce upregulation of chemokines by stromal cells.

The inability to recover Ig levels by *aly/aly* PEC cells correlates well with the observation that *aly/aly* PEC B cells gave rise to a few IgM and IgG plasma cells in the spleen, and no plasma cells in the GALT system of RAG-$2^{-/-}$ mice. Therefore, the migration capacity of B cells appears to be closely related to their ability to produce antibodies that were affected in *aly/aly* mice. We found that *aly/+* PEC B and T cells migrate to all lymphoid tissues of RAG-$2^{-/-}$ mice. PEC B cells that migrated to the GALT system differentiated mainly to IgA plasma cells, whereas those migrated to spleen expressed predominantly IgM and IgG. We detected a large number of $B220^{-}IgA^{+}$ cells in MLNs of RAG-$2^{-/-}$ mice transferred with *aly/+* PEC cells. Though in normal mice $B220^{-}IgA^{+}$ plasma cells could be found mainly in LP and represent <1% of the lymphocytes in MLNs (our unpublished data), in RAG-$2^{-/-}$ mice this population represented ~12% of the lymphocytes in MLNs, 6 wk after transfer. The absolute numbers of IgA plasma cells were also two times higher in MLNs than in LP of these mice. This observation suggests that PEC B cell proliferation and differentiation to IgA plasma cells may take place in the MLNs, although we cannot exclude the possibility that this phenomenon is restricted to the RAG-$2^{-/-}$ environment, which is probably devoid of normal regulatory mechanisms.

We still do not know which chemokines are involved in PEC cell migration in vivo. As *blr1*$^{-/-}$ mice, which lack PPs and therefore the B2 cell precursors of plasma cells, have normal IgA-producing cells in LP (23), the impaired chemotactic response to BLC is unlikely to be the major cause of the *aly/aly* PEC cell migration defect to the GALT system. Regardless of which chemokines or chemokine receptors are involved in the PEC cell migration, it is likely that NIK is involved in their signaling pathways.

Interestingly, the in vitro chemotactic response of splenic cells to SLC and BLC was very similar between *aly/aly* and *aly/+* mice, indicating that chemotactic responses of splenic and PEC cells are regulated differently. The difference could be explained first by the different microenvironments in spleen and PEC, which could affect the activation status of cells partially reflected in the chemokine receptor expression levels. Supporting this notion, LPS stimulation upregulated the levels of chemokine receptor mRNA expression and increased the migration capacity of peritoneal but not splenic cells (Fig. 5 B, and Fig. 6). Second, it is also possible that splenic and PEC cells use different chemokine receptors, and the pathway involving NIK is important only for PEC cells migration.

This study offers an explanation for their complex and severe *aly* phenotype, showing that the migration defect of lymphocytes to the proper places is also responsible for the immunological abnormalities in *aly/aly* mice.

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