Development of TCR $\alpha\beta$ CD8 $\alpha\alpha$ Intestinal Intraepithelial Lymphocytes Is Promoted by Interleukin-15-Producing Epithelial Cells Constitutively Stimulated by Gram-Negative Bacteria *via* TLR4

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The microbes present in the intestine have a strong influence on the development and maturation of lymphoid organs. The cross-talk mechanisms between intestinal intraepithelial lymphocytes (i-IEL) and noninvasive microbes are still poorly understood. The influence of microbes and lipopolysaccharides on the development of i-IEL, especially the TCR $\alpha\beta^+$ CD8 $\alpha\alpha$ subset, was investigated using the different TLR4-mutant mouse strains C3H/HeJ, BALB/lps^d, and C57BL/10ScCr. Intestinal epithelial cells (i-EC) from TLR4-mutant strains did not express interleukin (IL)-15 mRNA, while IL-15 mRNA expression in i-EC from the corresponding wild-type, C3H/He, BALB/c, and C57BL/10ScSn mice was detected. The development of TCR $\alpha\beta^+$ CD8 $\alpha\alpha$ cells in i-IEL significantly decreased in TLR4-mutant mice compared with the corresponding wild-type mice, while other T cell subsets in i-IEL showed similar percentages in the TLR4-mutant and wild-type mice. Adult thymectomized (ATx-) and lethally irradiated C3H/HeJ mice reconstituted with T cell-depleted bone marrow cells from C3H/He mice showed a significantly lower percentage of TCR $\alpha\beta$ CD8 $\alpha\alpha$ i-IEL than ATx-C3H/He mice after transfer of C3H/HeJ BM cells. The percentage of TCR $\alpha\beta$ CD8 $\alpha\alpha$ i-IEL and IL-15 mRNA expression in i-EC from BALB/lps^d mice did not increase during *Salmonella typhimurium* infection but was significantly enhanced during *Listeria monocytogenes* infection. Our findings suggest that LPS induces IL-15 production by i-EC, resulting in the development of TCR $\alpha\beta$ CD8 $\alpha\alpha$ i-IEL.

Key words intestinal intraepithelial lymphocyte; TCR $\alpha\beta$ CD8 $\alpha\alpha$; interleukin-15; TLR4; intestinal epithelial cell

The intestinal mucosa is a large interface between the epithelium and the external environment and contributes to defense mechanisms protecting against the invasion of microbes. In addition, the flora of noninvasive microbes present in the intestine have a strong influence on the development and maturation of lymphoid organs, especially lymph nodes involved in mucosal immunity. The activation of the innate immune response can be a prerequisite for the triggering of an adaptive immune response. Recently, it has been demonstrated that Toll-like receptors (TLRs), a family of mammalian homologues of *Drosophila* Toll, are important molecules for the pattern recognition of microbes in innate immunity.^{1,2} Epithelial cells constituting the intestinal mucosa also express TLRs^{3,4} and potentially produce cytokines by constitutive stimulation with noninvasive microbes or released bacterial products and components. It is a logical assumption that the cytokines produced stimulate the development of lymphoid tissues.

Poltorak *et al.*⁵ found that TLR4 is the signaling receptor for lipopolysaccharide (LPS), a representative molecule of the outer membrane of Gram-negative bacteria, and that in two types of LPS-nonresponder mouse strains, one strain, C57BL/10ScCr (B10Cr), had a deletion of the TLR4 gene and the other, C3H/HeJ, had a missense mutation in the third exon of TLR4, resulting in the exchange of proline to histidine at position 712 of the polypeptide chain.⁵ Intestinal epithelial cells (i-EC) expressing TLR4 on the cell surface appear to be capable of receiving constitutive stimulation by noninvasive Gram-negative bacteria and seem to secrete cytokines that are important for the development of lymphocytes implicated in mucosal immunity. Intestinal intraepithelial lymphocytes (i-IEL), localized between i-EC, are phenotypically and functionally distinct from T cells present in the

periphery.^{6,7} i-IEL consist of approximately 60% TCR $\gamma\delta$ T and 40% TCR $\alpha\beta$ T cells. Most of the TCR $\gamma\delta$ T cells seem to develop extrathymically,^{8–11} and TCR $\alpha\beta$ T cells express the CD8 $\alpha\alpha$ homodimer. TCR $\alpha\beta$ CD8 $\alpha\alpha$ cells produce little interferon (IFN)- γ and display a low level of cytotoxicity when activated through TCR in the presence of exogenous interleukin (IL)-2.¹² The cross-talk mechanisms between i-IEL and noninvasive microbes are still poorly understood.

The T cell growth factor IL-15 binds to IL-2R β and the common γ (γ c) chains and shares some activities with IL-2 despite the lack of sequence homology in both cytokines.^{13–15} IL-2 is mainly produced by activated T cells (Th1 cells), while IL-15 is secreted by different types of cells present in various tissues. IL-15 plays a pivotal role in the development of i-IEL from progenitors.^{16–20} Expression of IL-15 mRNA was seen at detectable levels in i-EC^{21,22} and promoted proliferation and survival of TCR $\gamma\delta$ and CD8⁺ TCR $\alpha\beta$ i-IEL.^{12,23} Interestingly, not only IL-2R $\beta^{-/-}$ but also IL-15R $\alpha^{-/-}$ mice had a significant reduction of CD8 $\alpha\alpha$ i-IEL numbers,^{24,25} suggesting that IL-15 regulates extrathymic development of i-IEL, in particular CD8 $\alpha\alpha$ cells. Takano *et al.* showed that the level of IL-15 mRNA expression in macrophages after *Escherichia coli* infection was up-regulated in C3H/He but not in C3H/HeJ mice.²⁶ These observations stimulated us to investigate the role of noninvasive Gram-negative bacteria and potentially released LPS for the development of i-IEL, especially CD8 $\alpha\alpha$ cells. Using three types of LPS-nonresponder strains, C3H/HeJ, BALB/lps^d, and B10Cr, the potential contribution of LPS to the development of i-IEL was investigated. Unresponsiveness to LPS in BALB/lps^d mice originates from the mutation of TLR4 in C3H/HeJ mice. In this study, we found that Gram-negative bacteria strongly influence IL-15 production by i-EC stimu-

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lated with LPS *via* TLR4 and that IL-15 plays a pivotal role in the development of TCR $\alpha\beta$ CD8 $\alpha\alpha$ i-IEL.

MATERIALS AND METHODS

Mice Female C3H/He and C3H/HeJ mice were obtained from Japan SLC Inc. (Shizuoka, Japan). BALB/c mice were purchased as breeding pairs from Jackson Laboratories (Bar Harbor, ME, U.S.A.). Breeding pairs of BALB/lps^d, C57BL/10ScSn (B10Sn), and B10Cr mice were obtained from the Max-Planck-Institute for Immunobiology (Freiburg in Breisgau, Germany). All mice were raised in the animal facility of Kitasato University under specific pathogen free (SPF) conditions and fed autoclaved food and water before use. They were used at 7 to 10 weeks of age. For infections, mice were housed in a special room (level 2 class). The experiments described in this article were performed in adherence with the guidelines of the National Institutes of Health on the use of experimental animals. Approval of the Animal Use Committee of Kitasato University School of Science was obtained prior to initiating the experiments.

Isolation of i-IEL, i-EC, and Peritoneal Exudate Cells i-IEL and i-EC were prepared as follows: the small intestine was removed from mice, and fecal contents were flushed with 20 ml of PBS. After the Peyer's patches had been cut away, the small intestine was opened longitudinally, cut into 1-cm pieces, and shaken vigorously in 30 ml of RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with penicillin 100 U/ml, streptomycin 100 μ g/ml, 5×10^{-2} mM 2-ME, sodium bicarbonate, and 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO, U.S.A.) at 37 °C for 30 min. After removing cell debris through gauze and remaining intestine by decantation, the cells were centrifuged through a 25–45–67.5% discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient at 2200 rpm at 20 °C for 20 min. i-EC and i-IEL were collected at the interface of 25–45% and 45–67.5%, respectively.

Peritoneal exudate cells (PEC) were harvested with Hank's solution (Nissui), washed twice, and resuspended in 10% FBS RPMI 1640 medium.

Flow Cytometry Analysis The following anti-mouse mAbs (PharMingen, San Diego, CA, U.S.A.) were used: anti-CD3 (145-2C11), anti-CD4 (RM4.5), anti-CD8 α (53-6.7), anti-CD8 β (53-5.8), anti-TCR β (H57-597), anti-TCR $\gamma\delta$ (GL3), anti-V β 8 (F23.1), anti-V β 11 (RR3-15), anti- $\alpha_E\beta_7$ (2E7), and anti-Thy1.2 (53-2.1). PE-conjugated anti-mouse TLR4/MD2 mAb (MTS510) was purchased from eBioscience (San Diego, CA, U.S.A.).

After treating with anti-Fc γ R2/III mAb (2.4G2) for 15 min 4 °C to block nonspecific binding to Fc γ R, the cells were incubated for 30 min 4 °C with FITC-, PE-, and biotin-conjugated mAbs and washed with a staining medium (PBS with 1% bovine serum albumin and 0.1% Na₂S₂O₃). Biotinylated cells were labeled with Cy-Chrome-streptavidin (PharMingen) for 30 min 4 °C. Labeled cells were washed three times, resuspended in staining medium, and analyzed on a EPICS ELITE (Beckman Coulter, Inc., Fullerton, CA, U.S.A.).

Measurement of Cytokine mRNA Expression Total RNA was isolated from i-IEL or i-EC using Trizol reagent (Gibco Laboratories, Grand Island, NY, U.S.A.). Each RNA

sample (5 μ g) was reverse-transcribed with 50 pmol of random hexamers, 0.5 mM dNTP mix, 10 mM DTT, and 200 units of SUPER SCRIPT II (Gibco) in a volume of 20 μ l at 37 °C for 60 min for synthesis of first-strand cDNA. The PCR reaction mixture (50 μ l) consisted of 2 μ l of cDNA product, 5 μ l of 10 $\times$ PCR buffer, 0.2 mM of dNTP mix, 20 pmol of forward and reverse primers, and 2.5 units of *Taq* polymerase (Takara, Kyoto, Japan). PCR amplification was performed in a Gene Amp PCR System 9600 (Perkin-Elmer Cetus, Norwalk, CT, U.S.A.) programmed for denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 30 s. Before the first cycle, a denaturation step at 94 °C for 7 min was included, and after the final cycle the extension step at 72 °C was prolonged to 4 min. PCR products separated by electrophoresis in 1.8% agarose gels were stained with ethidium bromide and visualized under UV light. The specific oligonucleotide primers used in PCR amplification were as follows: IL-7 sense, 5'-AAA TGC AGC TGA CTG CTG CC-3'; IL-7 antisense, 5'-TCT CCA GTC TAA AAC AGG AC-3'; IL-15 sense, 5'-GGA AGG CTG AGT TCC ACA TC-3'; IL-15 antisense, 5'-AGG GAG ACC TAC ACT GAC AC-3'; β -actin sense, 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'; and β -actin antisense, 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'.

Measurement of V Gene Segment Total RNA was extracted using Trizol reagent from TCR $\gamma\delta$ i-IEL purified to more than 98% by the EPICS ELITE cell sorting system. First-strand cDNA synthesis was performed using γ -chain C region (C γ) primers (5'-CTT ATG GAG ATT TGT TTC AGC-3') or δ -chain C region (C δ) primers (5'-CGA ATT CCA CAA TCT TCT TG-3') in 20 μ l of reaction mixture for reverse-transcription. PCR amplification was as described above. The V primers were as follows: V γ 1/2, 5'-ACA CAG CTA TAC ATT GGT AC-3'; V γ 2, 5'-CGG CAA AAA ACA AAT CAA CAG-3'; V γ 4, 5'-TGT CCT TGC AAC CCC TAC CC-3'; V γ 5, 5'-TGT GCA CTG GTA CCA ACT GA-3'; V γ 6, 5'-GGA ATT CAA AAG AAA ACA TTG TCT-3'; V γ 7, 5'-AAG CTA GAG GGG TCC TCT GC-3'; V δ 1, 5'-ATT CAG AAG GCA ACA ATG AAA G-3'; V δ 2, 5'-AGT TCC CTG CAG ATC CAA GC-3'; V δ 3, 5'-TTC CTG GCT ATT GCC TCT GAC-3'; V δ 4, 5'-CCG CTT CTC TGT GAA CTT CC-3'; V δ 5, 5'-CAG ATC CTT CCA GTT CAT CC-3'; V δ 6, 5'-TCA AGT CCA TCA GCC TTG TC-3'; V δ 7, 5'-CGC AGA GCT GCA GTG TAA CT-3'; and V δ 8, 5'-AAG GAA GAT GGA CGA TTC AC-3'.

PCR products were separated by electrophoresis on 1.8% agarose gel and transferred to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The Southern blots of γ PCR products were hybridized with a C γ probe (5'-TTC AGC AAC AGA AGG AAG GAA AAT AGT-3'), and those of δ PCR products were hybridized with a C δ probe (5'-CTG AGA CTT ATA GTC ACC TC-3'). The C γ and C δ probes were labeled with digoxigenin (DIG) using a DIG Luminescent Detection Kit (Roche, Mannheim, Germany). The membranes were incubated in hybridization buffer with 10 pmol of DIG-labeled probe and 100 μ g/ml of heat-denatured salmon sperm DNA for 16 h at 37 °C. After hybridization, the membranes were washed in 2 $\times$ SSC for 15 min at 37 °C. Each band of PCR product was detected immunologically using alkaline phosphatase-conjugated anti-DIG mAb and then visualized with the chemiluminescence

substrate CSPD using the DIG Luminescent Detection Kit. Light emission was recorded on X-ray films.

Generation of ATxBMT Mice Adult thymectomized, lethally irradiated, and bone marrow-transferred (ATxBMT) mice were generated using a modified method described by Yada *et al.*²⁷⁾ In brief, 8-week-old female C3H/He and C3H/HeJ mice were anesthetized by administration of pentobarbital sodium solution and thymectomized. Four weeks after thymectomy, the mice were exposed to lethal X-ray irradiation of 9.5 Gy, followed by intravenous transfer of 1×10^7 T cell-depleted bone marrow cells obtained from C3H/He or C3H/HeJ mice. T cell depletion from bone marrow cells was achieved by complement-dependent lysis using anti-Thy1.2 mAb (Meiji Institute of Health Science, Tokyo, Japan) and Low-Tox Guinea Pig Complement (Cedarlane Laboratories, Hornby, Canada). This procedure effectively depleted $>98\%$ of CD3⁺ cells from the bone marrow cells (data not shown).

Bacterial Strains and Infection *Salmonella typhimurium* Δ aroA (SR-11, χ 3181)²⁸⁾ was kindly provided by Dr. P.A. Gulig (Department of Immunology and Medical Microbiology, University of Florida College of Medicine, Gainesville) through Dr. H. Matsui (Kitasato Institute for Life Sciences, Kitasato University) and grown in Tryptone-Soya broth (Nissui). Bacteria were incubated at 37 °C with shaking until an OD₅₉₅ of 0.4–0.5 was reached, then washed and resuspended in PBS. *Listeria monocytogenes* was grown in Brain Heart Infusion broth (Gibco). Following 6 h of fasting, BALB/lps^d mice were orally infected with 1×10^9 colony-forming units (CFU) of *S. typhimurium* Δ aroA or 4×10^8 CFU of *L. monocytogenes* after treatment with 50 μ l of 10% NaHCO₃ in water. i-IEL and i-EC were isolated 14 d after infection and used for flow cytometry analysis or RT-PCR, respectively.

Statistical Analysis The statistical significance of the data was determined using Student's *t*-test and Dunnett's test. Differences with a *p* value of less than 0.05 were considered significant.

RESULTS

IL-15 mRNA Expression and TLR4-MD2 Expression in i-EC Since both IL-7 and IL-15 are γ c-dependent cytokines and stimulate the development of i-IEL from progenitor cells,¹⁶⁾ IL-15 and IL-7 mRNA expression in i-EC prepared from untreated mice was compared. None of the i-EC from LPS-nonresponder C3H/HeJ, BALB/lps^d, and B10Cr mice showed detectable levels of IL-15 mRNA expression (Fig. 1). On the other hand, the IL-7 transcription was at similar levels in both types of all i-EC prepared from both types of untreated mice (data not shown). Next, we determined whether TLR4-MD2 complex is expressed on the cell surface of i-EC. TLR4-MD2 was detected on i-EC and PEC obtained from B10Sn mice (Fig. 2). No expression of the TLR4-MD2 complex was observed on those cells from B10Cr mice. These results indicate that although IL-7 and IL-15 stimulate i-IEL development, only IL-15 produced by i-EC is responsible for i-IEL development as a consequence of constitutive stimulation *via* TLR4 with LPS from noninvasive Gram-negative bacteria.

No Difference in TCR $\alpha\beta$ and TCR $\gamma\delta$ Subsets in i-IEL between LPS-Responder and -Nonresponder Mice

Fig. 1. Expression of IL-15 mRNA in i-EC from LPS-Responsive C3H/He, BALB/c, and B10Sn Mice or LPS-Nonresponder C3H/HeJ, BALB/lps^d, and B10Cr Mice

Total RNA was extracted from i-EC of LPS-responder and -nonresponder mice. Expression of IL-15 and β -actin mRNA was measured by RT-PCR. i-EC were contained in less than 3% CD3⁺ cells. (A) Results show one representation of three experiments. (B) Ratio of the intensity of IL-15 and β -actin mRNA represent mean $\pm$ S.D. for three sets of experimental data. **p* < 0.05, ****p* < 0.001 (Student's *t*-test).

Fig. 2. Cell Surface Expression of TLR4-MD2 Complex on i-EC

i-EC and PEC from B10Sn (thick line) or B10Cr (thin line) were analyzed by flow cytometry after staining with mAb against TLR4-MD2 complex. i-EC were contained less than 3% CD3⁺ cells.

Since IL-2R β ^{-/-} and γ c^{-/-} mice have decreased cell numbers of TCR $\gamma\delta$ i-IEL and completely lack TCR⁺ i-IEL, respectively,¹⁶⁾ it has been suggested that the development of both TCR $\alpha\beta$ and TCR $\gamma\delta$ cells among i-IEL critically depends on the amount of IL-15 capable of binding to IL-2R β and γ c chains. As failure of IL-15 mRNA expression was observed in LPS-nonresponder mice (Fig. 1) and the total cell numbers of i-IEL was almost the same in both types of mouse strain (data not shown), flow cytometry was used to analyse whether the percentage of TCR $\alpha\beta$ and TCR $\gamma\delta$ subsets in i-IEL is similar. The total ratio of CD3⁺ i-IEL was similar in all mice tested (Table 1). Comparing the percentages of TCR $\alpha\beta$ or TCR $\gamma\delta$, similar values were detected in between BALB/c and BALB/lps^d mice or between B10Cr and B10Sn mice. Only C3H/HeJ mice, however, had a lower ratio of TCR $\alpha\beta$ i-IEL than in C3H/He mice, compensated for by a higher percentage of TCR $\gamma\delta$ i-IEL.

Next, we examined whether there is a difference in the V gene usage of TCR $\gamma\delta$ i-IEL between C3H/He and C3H/HeJ mice. V gene usage in total RNA extracted from sorted

Table 1. Percentage of TCR⁺ i-IEL in LPS-Responsive and Hyporesponsive Mice^{a)}

Strain	CD3 ⁺	CD3 ⁺ gated	
		TCRαβ	TCRγδ
C3H/He	84.9±5.5	63.5±2.6	29.5±4.0
C3H/HeJ	82.8±4.7 ^{ns}	50.0±5.7***	40.1±5.7***
BALB/c	90.0±1.7	63.0±2.9	32.1±1.0
BALB/lps ^d	88.8±1.5 ^{ns}	65.7±6.4 ^{ns}	30.0±6.1 ^{ns}
B10Sn	85.0±5.6	41.0±3.0	45.3±6.1
B10Cr	80.9±3.5 ^{ns}	41.2±5.6 ^{ns}	41.7±4.6 ^{ns}

a) The percentages of TCR⁺ i-IEL subsets from C3H/He, BALB/c, and B10Sn mice and LPS-nonresponder C3H/HeJ, BALB/lps^d, and B10Cr mice were determined by flow cytometry. Percentages represent arithmetic mean±S.D. of 5–10 mice/group. ns: not significant. ****p*<0.001 (vs. the corresponding LPS-responders, Student's *t*-test).

Fig. 3. Vγ or Vδ Usage of TCRγδ i-IEL

Total RNA was extracted from FACS-purified TCRγδ i-IEL from C3H/He or C3H/HeJ mice. First-strand cDNA was synthesized using Cγ or Cδ primers and amplified by PCR with Cγ or Cδ primers and various Vγ or Vδ primers, respectively. The Southern blots of γ PCR or δ PCR products were hybridized with an oligonucleotide probe for pan Cγ or Cδ internal-2, respectively.

TCRγδ i-IEL was analyzed with RT-PCR. The gene expression of Vγ and Vδ did not change between C3H/He and C3H/HeJ mice (Fig. 3). A similar result was also found in B10Sn and B10Cr mice (data not shown). The similarity of the percentage of TCRαβ and TCRγδ subsets in i-IEL from LPS-responder and -nonresponder mice suggests that factors other than IL-15 are involved in their cell development.

Difference in Percentage of TCRαβ CD8αα i-IEL IL-15 promotes CD8αα i-IEL development, and infections with *S. choleraesuis* and *E. coli* increase IL-15 mRNA expression in mice.^{26,29)} We found failure of IL-15 mRNA expression in i-EC from LPS-nonresponder mice (Fig. 1). Taken together, these observations suggest that the percentage of TCRαβ CD8αα cells in i-IEL would be changeable due to responsiveness to LPS followed by cytokine secretion. Downregulation of TCRαβ CD8αα cells was detected in i-IEL from all LPS-nonresponder C3H/HeJ, BALB/lps^d and B10Cr mice (Fig. 4). Significant differences in percentages (decreased percentage between both types) of TCRαβ CD8αα i-IEL between combinations of C3H/He and C3H/HeJ (63.2±6.8/44.6±12.1; 70.5%), BALB/c and BALB/lps^d (42.5±1.0/38.5±0.5; 90.6%), and B10Sn and B10Cr mice (57.5±4.8/45.1±6.8; 78.4%) were observed. This result indicates that impairment of IL-15 production due to unresponsiveness of i-EC to LPS via TLR4 might cause decreased development of TCRαβ CD8αα cells. In particular, the percentage of TCRαβ CD8αα i-IEL in C3H/HeJ mice was markedly decreased compared with that of C3H/He mice. Moreover, the percentage of Vβ8 and Vβ11 in TCRαβ CD8αα i-IEL from C3H/HeJ mice did not differ from that from C3H/He mice (Table 2).

Percentage of TCRαβ CD8αα i-IEL in BMT Mice To demonstrate that the development of TCRαβ CD8αα i-IEL is due to unresponsiveness of IL-15-producing i-EC to LPS,

Fig. 4. Influence of LPS on the Percentage of TCRαβ CD8αα i-IEL

i-IEL from LPS-responsive or LPS-nonresponsive mice were analyzed by flow cytometry after staining with mAb against TCRαβ, CD8α, and CD8β. TCRαβ⁺ cells were gated to analyze the CD8αα subset. A representative set of results (individual, A, and the mean±S.D. of 5–10 mice/group, B) is shown. **p*<0.05, ***p*<0.01, ****p*<0.001 (Student's *t*-test).

Table 2. Percentage of TCR Vβ8 and Vβ11 in TCRαβ CD8αα i-IEL^{a)}

	TCRαβ CD8αα gated	
	Vβ8	Vβ11
C3H/He	29.2±11.1	1.47±0.36
C3H/HeJ	23.5±5.3 ^{ns}	1.76±0.03 ^{ns}

a) The percentages of TCR Vβ8 and Vβ11 in TCRαβ CD8αα i-IEL from C3H/He and C3H/HeJ mice were determined by flow cytometry. Percentages represent arithmetic mean±S.D. of 5 mice/group. ns: not significant (vs. the corresponding LPS-responders, Student's *t*-test).

adult thymectomized and lethally irradiated C3H/He (ATx-C3H/He) and ATx-C3H/HeJ mice received an intravenous transfer of T cell-depleted bone marrow cells (BMT) obtained from C3H/HeJ and C3H/He, respectively. Isolated i-IEL from the resulting ATxBMT mice were analyzed 5 weeks after BMT. The percentage of TCRαβ CD8αα i-IEL in ATx-C3H/HeJ mice receiving C3H/He bone marrow was approximately half of that in ATx-C3H/HeJ mice reconstituted with C3H/He BM (Fig. 5). This result indicates a potential role of i-EC from LPS-responder mice in the development of TCRαβ CD8αα i-IEL.

Percentage of TCRαβ CD8αα i-IEL and IL-15 mRNA Expression after Bacterial Infection The results obtained so far suggest that LPS upregulates the transcription of IL-15 and thereby the development of TCRαβ CD8αα i-IEL. Increased levels of IL-15 mRNA were detected in mice after

Fig. 5. Percentage of TCRαβ CD8αα i-IEL in ATxBMT Mice

Adult thymectomized (ATx) and lethally irradiated ATx-C3H/He and -C3H/HeJ mice received an intravenous transfer (BMT) of T cell-depleted bone marrow cells from C3H/HeJ and C3H/He mice, respectively. The donor-derived i-IEL isolated from ATxBMT mice were analyzed by flow cytometry. Results represent mean±S.D. of 5 mice/group.

Fig. 6. IL-15 mRNA Expression after Bacterial Infection

BALB/c mice or BALB/lps^d mice were orally infected with 1×10^9 CFUs of *S. typhimurium* ΔaroA or 4×10^8 CFUs of *L. monocytogenes* after treatment with 50 μl of 10% NaHCO₃ in water. i-EC were isolated 14 d after infection. Total RNA was extracted from i-EC, and expression of IL-15 and β-actin mRNA was determined by RT-PCR. i-EC were contained less than 3% CD3⁺ cells.

infections with Gram-positive *L. monocytogenes* and Gram-negative *S. choleraesuis*.^{29,30} To confirm whether bacterial infections increase the IL-15 transcription and development of TCRαβ CD8αα i-IEL, 4×10^8 CFUs of *L. monocytogenes* or 1×10^9 CFUs of *S. typhimurium* ΔaroA were orally inoculated in LPS-nonresponder BALB/lps^d mice. i-IEL and i-EC were isolated on day 14 after infection. The percentage of CD3⁺ i-IEL was similar in both infections (data not shown). In LPS-responder BALB/c mice, the level of IL-15 mRNA expression was similarly increased in both infections (Fig. 6). However, in BALB/lps^d mice, an increased level of IL-15 mRNA expression was detected in i-EC from *L. monocytogenes*-infected mice, but only a slight transcription was seen in i-EC from mice infected with *S. typhimurium* ΔaroA. Moreover, in *L. monocytogenes*-infected BALB/lps^d mice the ratio of TCRαβ CD8αα i-IEL increased significantly, whereas no significant change was observed in *S. typhimurium* ΔaroA-infected animals (Fig. 7).

DISCUSSION

In this study we demonstrated that noninvasive Gram-negative bacteria and LPS potentially influence the development of TCRαβ CD8αα i-IEL as a consequence of IL-15 expression in i-EC after LPS signaling via TLR4. Impairment of responsiveness to LPS is responsible for deletion or dysfunction of TLR4 expressing on different cell types. B10Cr mice

Fig. 7. Percentage of TCRαβ CD8αα i-IEL after Bacterial Infection

BALB/lps^d mice were orally infected with 1×10^9 CFUs of *S. typhimurium* ΔaroA or 4×10^8 CFUs of *L. monocytogenes* after treatment with 50 μl of 10% NaHCO₃ in water. i-IEL were isolated 14 d after infection. i-IEL were stained with mAbs against TCRαβ, CD8α, and CD8β. TCRαβ⁺ cells were gated and the CD8αα subset was analyzed. The data represent arithmetic mean±S.D. of 5 mice/group. ns: not significant. **p* < 0.05 (Dunnett's test).

have deletion of the TLR4 gene and C3H/HeJ and BALB/lps^d mice carry a mutation at a site of the intracellular protein domain which is critical for signaling. Under usual conditions, these mice show unresponsiveness to LPS. After priming with live *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), C3H/HeJ mice become sensitive to LPS and succumb to endotoxin shock.³¹ In contrast, B10Cr mice never become sensitive to LPS after priming and are unresponsiveness to IL-12 due to deletion of a gene coding for the STAT4-binding site.³² There is a distinct difference in terms of *Nramp1* (natural resistance-associated macrophage protein 1) or host resistance locus *Bcg/Ity/Lsh* between C3H/HeJ and BALB/lps^d mice, although the unresponsiveness to LPS of BALB/lps^d mice originates from that of C3H/HeJ mice. C3H/HeJ mice show higher natural resistance of macrophages against *Salmonella typhimurium* ΔaroA infection than BALB/lps^d mice (unpublished data). The functional backgrounds of the three LPS-nonresponder mouse strains are different, although unresponsiveness to LPS is the common phenotype. The percentages of TCRαβ⁺ and TCRγδ⁺ cells in CD3-gated i-IEL were significantly higher in C3H/HeJ mice than in C3H/He mice. In the other two pairs BALB/c and BALB/lps^d or B10Sn and B10Cr mice, the percentages of TCR⁺ i-IEL subsets did not change. It arose from the question of why a significant upregulation of TCRγδ cell population occurs in only C3H/HeJ mice (Table 1).

The percentage of TCRαβ CD8αα i-IEL in C3H/He mice was higher than in the other LPS-responsive mice (Fig. 4), because C3H/He mice are high responders to LPS compared with the weaker reacting BALB/c and B10 mice.³³ Consequently, the percentages of TCRαβ⁺ and TCRγδ⁺ cells in CD3-gated i-IEL may be significantly changed only in C3H/HeJ mice.

The development of natural killer cells and certain subsets of i-IEL depends on IL-2 and/or IL-15, which induce signaling by binding to the IL-2Rβ chain expressed on these cells.³⁴ IL-15 mRNA expression causes proliferation of γδ T cells in mice infected with *S. choleraesuis*.³⁵ We also demonstrated impairment of IL-15 mRNA expression in i-EC from all LPS-nonresponder mice tested (Fig. 1). In addition, development of TCRαβ CD8αα i-IEL decreased significantly in normal LPS-nonresponder mouse strains housed

under SPF conditions (Figs. 1, 4). Taken together, as a constituent of the outer membrane of noninvasive Gram-negative bacteria, LPS constitutively signals to i-EC via TLR4 for IL-15 production. This conclusion was supported by an increased percentage of TCR $\alpha\beta$ CD8 $\alpha\alpha$ i-IEL after infection with Gram-positive *L. monocytogenes* but not with Gram-negative *S. typhimurium* Δ aroA (Fig. 6A). Mitani *et al.* reported that the expression of IL-15 mRNA in i-EC increased at the early stage of oral infection with *L. monocytogenes*.³⁰⁾ In our study, LPS-hyporesponsive BALB/lps^d mice did not express IL-15 mRNA in i-EC after infection with *S. typhimurium*, while IL-15 mRNA expression was detected in *L. monocytogenes*-infected mice (Fig. 6B).

In cell reconstitution experiments, the percentage of TCR $\alpha\beta$ CD8 $\alpha\alpha$ i-IEL in ATx-C3H/HeJ mice reconstituted with C3H/He-BM was approximately half that in ATx-C3H/He mice received cell transfer of C3H/HeJ-BM (Fig. 5). This result suggests that the development of TCR $\alpha\beta$ CD8 $\alpha\alpha$ cell depends on IL-15 produced by i-EC of recipient mice, because the development of TCR $\alpha\beta$ CD8 $\alpha\alpha$ i-IEL was significantly lower in ATx-C3H/HeJ mice that received BM transfer after lethal irradiation.

In Gram-positive *L. monocytogenes* infection, TLR2 functions as the signaling receptor to activate transcription of the IL-15 gene via NF- κ B.^{36,37)} In addition, IL-15 synthesis was upregulated by invasion of *L. monocytogenes* into i-EC, which was mediated by the *Listeria* surface proteins internalin A and B.³⁸⁾ We also confirmed upregulation of IL-15 mRNA expression in i-EC followed by increased development of TCR $\alpha\beta$ CD8 $\alpha\alpha$ in BALB/lps^d mice infected with *L. monocytogenes* (Fig. 6). The lower percentages of TCR $\alpha\beta$ CD8 $\alpha\alpha$ in i-IEL from all LPS-nonresponder strains (Fig. 4) are attributed to the absence of NF- κ B activation via TLR4 by LPS, while the activation of the TLR2 and NF- κ B pathway normally functions even if BALB/lps^d mice are infected with *L. monocytogenes*. In Gram-negative *S. typhimurium* infection, however, no significant increase in TCR $\alpha\beta$ CD8 $\alpha\alpha$ i-IEL development and very weak IL-15 mRNA expression in i-EC was detected in BALB/lps^d mice (Fig. 6). This slight increase in IL-15 mRNA expression might have been caused by a TLR4-independent mechanism. However, the low level was obviously not sufficient to affect the significant development of TCR $\alpha\beta$ CD8 $\alpha\alpha$ i-IEL. If the activation of the TLR2/NF- κ B pathway normally functioned in BALB/lps^d mice, IL-15 mRNA expression should be comparable to that in BALB/c mice by constitutive stimulation with Gram-positive bacteria as constituents of microflora. As shown in Fig. 1, IL-15 mRNA expression was lower in i-EC from BALB/lps^d than from BALB/c mice. In infected mice, IL-15 mRNA expression upregulated in i-EC from *L. monocytogenes*- but not *S. typhimurium*-infected BALB/lps^d mice compared with noninfected controls (Fig. 6). Since IL-15 is mainly produced by stimulation with *Listeria* surface proteins internalin A and B,³⁸⁾ upregulation of IL-15 mRNA in i-EC from *Listeria*-infected BALB/lps^d mice seems to be due to stimulation with such *Listeria* proteins.

In conclusion, constitutive stimulation by Gram-negative bacteria and LPS triggers IL-15 synthesis by i-EC and promotes the development of TCR $\alpha\beta$ CD8 $\alpha\alpha$ i-IEL.

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