Molecules and Cells

Microbial Colonization at Early Life Promotes the Development of Diet-Induced CD8αβ Intraepithelial T Cells

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Intraepithelial lymphocytes (IELs) develop through the continuous interaction with intestinal antigens such as commensal microbiome and diet. However, their respective roles and mutual interactions in the development of IELs are largely unknown. Here, we showed that dietary antigens regulate the development of the majority of CD8αβ IELs in the small intestine and the absence of commensal microbiota particularly during the weaning period, delay the development of IELs. When we tested specific dietary components, such as wheat or combined corn, soybean and yeast, they were dependent on commensal bacteria for the timely development of diet-induced CD8αβ IELs. In addition, supplementation of intestinal antigens later in life was inefficient for the full induction of CD8αβ IELs. Overall, our findings suggest that early exposure to commensal bacteria is important for the proper development of dietary antigen-dependent immune repertoire in the gut.

Keywords: antigen free, dietary antigen, germ free, intraepithelial T cells, microbiota

INTRODUCTION

A single layer of the epithelium covers small intestinal lumen and facilitates efficient uptake of nutrients. At the same time, resident memory populations of intraepithelial lymphocytes (IELs) continuously scan the epithelial layer to protect the host from the pathogenic infection (Sheridan and Lefrancois, 2010; Sheridan et al., 2014). Consequently, IELs occupy the largest number of lymphocytes in the body, and it is estimated that about two third of whole lymphocytes reside in IEL (Cheroutre et al., 2011; Kunisawa et al., 2007; McDonald et al., 2018). However, how these IELs interact with both commensal microbiome and dietary antigens is not well defined.

IELs are comprised of similar number of TCRαβ or TCRγδ T cells, and CD8αβ IELs outnumber CD8αα and CD4 positive subsets of TCRαβ IELs (Regnault et al., 1994). The number of IELs gradually increases after birth via continuous interaction with microbial and dietary antigens (Latthe et al., 1994; Williams et al., 2004), and oligoclonal repertoire of CD8αβ IELs is shaped in adult period (Regnault et al., 1994; Williams et al., 2004). TCRγδ IELs participate in tissue-repairing process by secreting IL-22, IL-10 or TGFβ. On the other hand, CD8αβ IELs express IFNγ, TNFα, IL-4 and/or IL-17 and exert protective effects upon systemic or oral infection with pathogenic organisms such as *Listeria monocytogenes* or *Toxoplasma gondii* (Buzoni-Gatel et al., 1999; Chardes et al., 1994; Lee et al., 2018; Lepage et al., 1998; Pope et al., 2001; Sheridan et al., 2014) and commensal segmented filamentous bacteria (SFB) (Ivanov et al., 2009; Umesaki et al., 1999). At the same time, commensal *Lactobacillus reuteri* can promote the

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generation of immunoregulatory CD4 and CD8αα double-positive IELs (Cervantes-Barragan et al., 2017). However, abnormal activation of IELs can cause inflammatory disorders such as food allergy, celiac disease and inflammatory bowel disease including Crohn's disease and ulcerative colitis (Abadie et al., 2012; Bol-Schoenmakers et al., 2011; Catalan-Serra et al., 2017; Hu and Edelblum, 2017; Mercer et al., 2009; Regner et al., 2018). Hence, maintaining a proper balance between tolerance and the effector response for both commensal microbiome and dietary antigens is important to maintain healthy homeostatic condition.

Dietary antigens play critical roles in the development and induction of IELs. Continued feeding with the antigen-minimized diet to germ-free (GF) mice caused a gradual reduction in the numbers and cytotoxic effects of CD8αβ IELs (Kawaguchi-Miyashita et al., 1996). In SPF mice, protein-free diet supplemented with equivalent amounts of amino acid arrested the development of TCRαβ IELs after weaning (Menezes et al., 2003). These two reports clearly demonstrated the importance of dietary proteins particularly in the development of CD8αβ IELs in GF and SPF conditions respectively. However, the effect of commensal microbiome in the development of diet induced CD8αβ IELs has not been explored. To address this issue, we deprived commensal microbiota and/or dietary antigens and examined the generation of CD8αβ IELs before or after weaning. As a result, we found that in the absence of commensal flora, the development of CD8αβ IELs specific to dietary antigens was significantly delayed. On the other hand, in the absence of dietary antigens, microbiota could induce only about 10% of the CD8αβ IELs compared to those of SPF mice, indicating that intestinal bacteria cannot substitute dietary antigens. Interestingly, delayed supplementation of microbial and dietary antigens was very inefficient for the induction of CD8αβ IELs. Taken together, these results indicate that the presence of commensal microbiota in early life is essential for the proper development of diet-induced CD8αβ IELs at later time points.

MATERIALS AND METHODS

Mice and diet

C57BL/6 (B6) mice were purchased from Jackson Laboratory and maintained in a SPF animal facility at POSTECH. GF B6 mice were kindly provided by Drs. Andrew Macpherson (Bern Univ., Switzerland) and David Artis (Univ. Pennsylvania, USA) and maintained in sterile flexible film isolators (Class Biological Clean Ltd., USA) in GF mouse facility at the POSTECH. We regularly checked the sterility of GF mice by the absence of bacterial colonies in the culture experiment using their fecal pellets. The offspring of GF B6 mice was weaned and raised with an AF diet ad libitum, which contains ultra-filtered low molecular chemically defined elements supplemented with soybean oil containing oil-soluble vitamin A, D3, K and E (Kawaguchi-Miyashita et al., 1996; Kim et al., 2016; Pleasants et al., 1986). Dietary components, wheat plus wheat midds (wheat), corn plus corn gluten meal (corn), soybean meal or brewers yeast were purchased from Envigo. Each dietary component was dissolved in AF diet, autoclaved and fed to SPF or GF mice. Normal chow diet of SPF and GF mice was from Purina and Envigo respectively, and the latter was autoclaved for sterilization. Unless it is specified, all mice with 6-10 weeks old were used for the experiments according to the protocols approved by the Institutional Animal Care and Use Committees (IACUC) of the POSTECH.

Cell preparation

After carefully removing all the Peyer's patches from the small intestine, large and small intestines were cut open longitudinally to expose the luminal side and then cut into ~5mm pieces. These fragments were incubated with DPBS (WelGene) containing 5% FCS and 1 mM EDTA (WelGene) at 37℃ with agitation for 30 min. The supernatant was filtered through a 40 μm cell strainer in order to collect the epithelial populations. Cells were then resuspended in 40% Percoll (GE Healthcare) and overlaid with a 75% Percoll layer for the isolation of lymphocytes.

Flow cytometry

Cell suspensions were prepared and pre-blocked with anti-CD16/CD32 (93). For cell surface staining, the following fluorescent monoclonal antibodies (mAbs) were used: anti-CD45.2 (104; 30-F11), anti-TCRβ (H57-597), anti-TCRγδ (GL3), anti-CD4 (RM4-5), anti-CD8α (53-6.7) and anti-CD8β (YTS 156.7.7). For intracellular staining of IL-17A (eBio17/37), cells were stimulated by PMA (81 nM) and ionomycin (1.34 μM) for 4 h, fixed and permeabilized with kits from BD Biosciences. Dead cells were excluded by labeling with propidium iodine or ghost viability dye (Tonbo). Cells were stained with mAbs for 20 min at 4℃. Samples were analyzed with the LSRFortessa, LSR II or FACSCanto II (BD Biosciences) and analyzed by FlowJo software (Tree-Star).

Immunohistochemistry

Jejunum from SPF, GF or AF mice was cut longitudinally and embedded in OCT compound (Sakura). The tissues were cut in 5 μm pieces for frozen section, and the sections were fixed with methanol at -20℃. The slide sections were pre-blocked with anti-CD16/CD32 (93). For immunofluorescence staining, the slides were stained with primary anti-CD11c (N418, biotin) and IgA (C10-3, Alexa Fluor 488) overnight at 4℃ and secondary streptavidin (Alexa Fluor 594) for 2 h at room temperature. The stained slides were then mounted using ProLong GoldTM anti-fade reagent (Thermo Fisher) with DAPI. Slides were analyzed for immunofluorescence using a confocal microscope (Zeiss LSM700).

Statistical analysis

Data are presented as mean ± SD. Prism software (GraphPad) was used for statistical analyses. One-way ANOVA and unpaired two-tailed *t* test were used for data analyses and the generation of p-values.

RESULTS

Commensal microbiota are essential for the timely development of diet-induced CD8αβ IELs

The IELs develop with continuous interaction between host

immunity and luminal antigens such as commensal microbiome and dietary foods (Helgeland et al., 2004; Menezes et al., 2003; Regnault et al., 1994; Williams et al., 2004). To investigate the role of dietary antigens on the development of CD8αβ IELs, we used antigen-free (AF) diet system that we previously developed, in which the protein component of diet was fully replaced by individual amino acids (Kim et al., 2016). When SPF mice were weaned onto AF diet, there was about 90% reduction of CD8αβ IELs in adult period, indicating IELs are mainly generated in response to dietary antigens (Fig. 1A). This is consistent with previous report that showed GF mice fed with AF diet gradually lose CD8αβ IELs (Kawaguchi-Miyashita et al., 1996), and SPF mice fed with amino-acid diet failed to develop IELs after weaning (Menezes et al., 2003). Next, we investigated the role of microbial antigens for the generation of diet-induced CD8αβ IELs by comparing SPF and GF mice (Fig 1B). The development of TCRαβ and TCRγδ IELs was found to be minimal in 2 week-old mice both in SPF and GF condition. Under SPF conditions, weaning the 3 week-old mice onto normal chow diet rapidly induced the development of CD8αβ IELs, which eventually reached a plateaus at 6 weeks (Fig. 1B). However, in GF mice, the developmental kinetics of CD8αβ as well as TCRγδ IELs were significantly delayed compared to those of SPF mice (Fig. 1B). In addition, consistent with previously reports (Chung et al., 2012; Imaoka et al., 1996; Umesaki et al., 1993), microbiome depletion in adult mice by treatment with broad-spectrum antibiotics for 2 weeks dramatically reduced the number of CD8αβ IELs (data not shown). Therefore, the intestinal microbiome is required not only for the timely development of CD8αβ IELs but also for the maintenance of them.

Microbial and dietary antigens play respective roles in effector differentiation of IELs

By analyzing SPF and GF mice fed with standard diet (STD) or AF diet (AFD), we could examine the effect of either intestinal microbiota or dietary antigens during the development of CD8αβ IELs. In GF mice fed with AFD (AF mice), CD8αβ IELs only could see endogenous self-antigens during their development. In the thymus and spleen, the total numbers of TCRαβ T cells showed no significant differences amongst SFP, GF and AF mice, indicating that microbial or dietary antigens do not influence overall lymphocyte development (Fig. 2A). However, AF mice had significantly fewer numbers of TCRαβ T cells in IEL and lamina propria (LP). Compared to SPF mice, GF mice had thicker and more extended intestines, but they were normal in AF mice, probably due to the liquid nature of the AFD (Supplementary Fig. S1A). Consistent with the loss of CD8αβ IELs, GF and AF mice also had defect of other immune components including $CD11c^+$ cells or IgA-secreting cells in the small intestine (Supplementary Fig. S1B). Therefore, luminal antigens are responsible not only for the development of IELs, but also for dendritic cells or B cells in the small intestines.

Small intestinal IELs are the local resident memory population in the epithelial region (Anderson et al., 2014; Cheroutre et al., 2011). To examine whether the small number of IELs detected in AF mice is not blood contamination from circulation, we intravenously injected FITC-conjugated anti-CD45.2

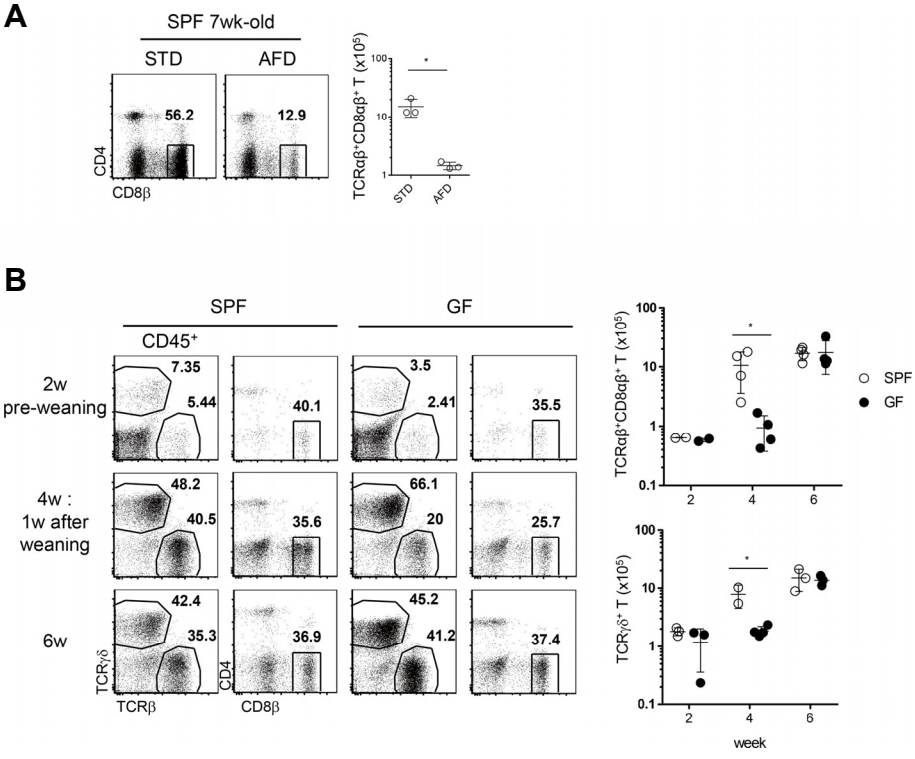

Fig. 1. Commensal microbiota are essential for the timely development of diet-induced CD8αβ IELs. (A) Three-week old SPF mice were weaned onto either standard diet (STD) or antigen-free diet (AFD) and analyzed for the development of $CD8\alpha\beta^+$ IELs. Shown are representative dot plots of gated $TCR\alpha\beta^+$ cells (left). Graph shows their absolute numbers (right). (B) Representative dot plots show IELs with indicated phenotype in 2, 4 or 6 week-old SPF or GF mice (left). Graph shows absolute numbers of $CD8\alpha\beta^+$ IELs (right upper) and TCRγδ IELs (right lower) at indicated time points. Numbers indicate the frequencies of cells in adjacent gates. Each dot represents an individual mouse and horizontal bars indicate mean values. $*P < 0.05$ (unpaired t-test). Error bars indicate SD. IEL, intraepithelial lymphocytes.

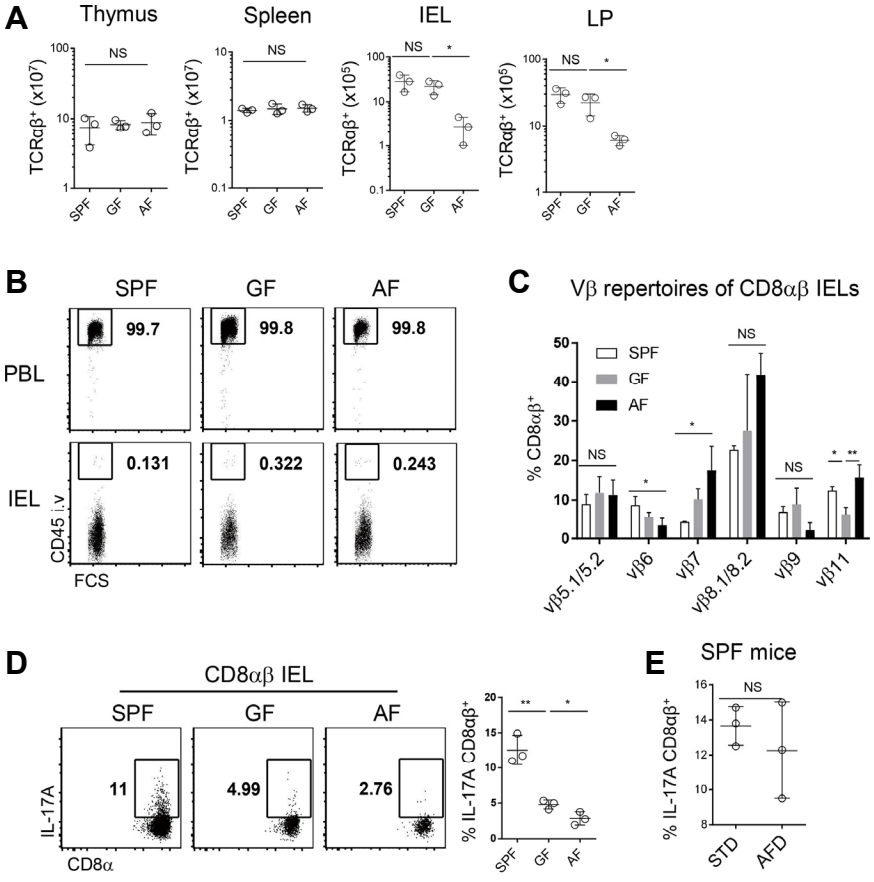

Fig. 2. Microbial and dietary antigens play respective roles in effector differentiation of IELs. (A) The absolute numbers of TCRαβ$^+$ T cells from thymus, spleen, IEL or LP were compared amongst SPF, GF and AF mice. (B) Mice were injected with FITC-conjugated anti-CD45 antibody and sacrificed after 5 min. Representative dot plots show the frequency of intravascular cells. (C) CD8αβ$^+$ IELs were analyzed for their frequencies of Vβ 5.1/5.2, Vβ6, Vβ7, Vβ8.1/8.2, Vβ9 and Vβ11 TCRs in the small intestine. (D) IELs harvested from the indicated mice were stained for IL-17A after stimulation with PMA and ionomycin for 4 h. Representative dot plots show the frequencies of IL-17A$^+$ cells in gated CD8αβ$^+$ IELs from the small intestine (left). The graph shows their frequencies (right). (E) IELs were harvested from SPF mice fed either STD or AFD and analyzed IL-17-producing CD8αβ$^+$ IELs as in (D). Graph shows their frequencies. Numbers indicate the frequency of cells in adjacent gates. Each dot represents an individual mouse and horizontal bars indicate mean values. $*P < 0.05$, $**P < 0.01$ (one-way ANOVA). NS, non-specific. Error bars indicate SD. IEL, intraepithelial lymphocyte; LP, lamina propria

antibody and labelled circulating hematopoietic cells (Anderson et al., 2012; 2014). IELs from SPF, GF and AF mice were all uniformly CD45.2$^-$, whereas blood circulating cells were all CD45$^+$ (Fig. 2B), thereby showing that the small number of IELs we analyzed from GF and AF mice is not the part of circulating cells in blood. We also analyzed TCR Vβ usages in CD8αβ IELs to see if there is any skewing of their repertoire and found that they were largely comparable amongst SPF, GF and AF mice (Fig. 2C). Although there were some significant changes in Vβ6, 7, and 11 usages, and we did not analyze their individual TCR sequences, we concluded that GF and AF mice have similar but small number of CD8αβ IEL populations.

IL-17-producing CD8 T cells (Tc17) are induced by commensal microbiota (Tajima et al., 2008) in the gut. To determine whether microbiota and dietary antigens also influence IL-17 expression in IELs, we analyzed IL-17-producing cells in CD8αβ IELs from SPF, GF and AF mice after PMA and ionomycin stimulation. We found that the frequencies of IL-17-secreting CD8αβ IELs were decreased in GF mice and further decreased in AF mice compared to those of SPF mice (Fig. 2D). Therefore, both microbiome and dietary antigens contribute the development Tc17. We further analyzed Tc17 in SPF mice fed with either STD or AFD to test the role of microbiome in this process. We found that the frequency of Tc17 was not different between them (Fig. 2E), indicating that microbiome can compensate the absence of dietary antigens for the induction of Tc17. However, as there was more than ten-fold decrease of total CD8αβ IEL number when SPF mice fed with AFD (Fig. 1A), these findings suggest that dietary antigens induce the development of CD8αβ IELs, whereas intestinal microbiome contribute for the functional maturation of them.

Wheat protein is not sufficient for the full induction of CD8αβ IELs

Based on our findings that dietary antigens, rather than intestinal microbiome, induce CD8αβ IELs in the small intestine, we wondered if there is the specific dietary component that induces them. To address this issue, we added wheat, corn, soybean or yeast extracts that were components of STD to AFD under SPF condition (Fig. 3A). When the mice were analyzed at 5 weeks after feeding, both wheat alone and a combination of corn, soybean and yeast comparably increased the proportions and total numbers of CD8αβ IELs to a level that of STD-fed mice (Fig. 3A). Next, we asked if these components also induce CD8αβ IELs in the absence of commensal microbiota. When GF mice were weaned onto

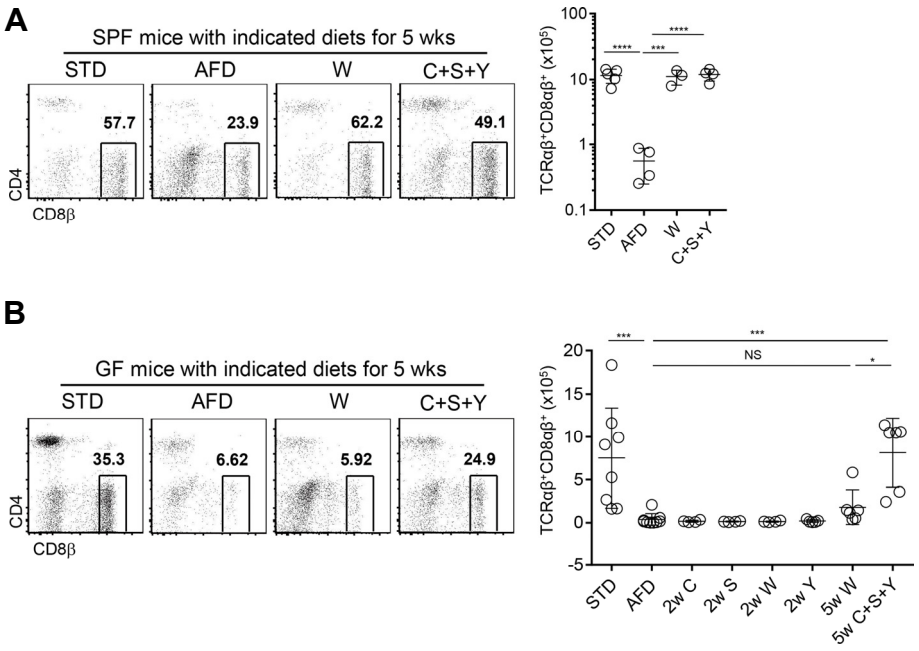

Fig. 3. Wheat protein is not sufficient for the full induction of CD8αβ IELs. (A) SPF mice were fed with standard diet (STD), antigen-free diet (AFD), wheat (W) or a mixture of corn, soybean and yeast (C+S+Y) diets for 5 weeks from weaning. Representative dot plots show frequencies of CD8αβ IELs (left), and graph shows absolute numbers of CD8αβ IELs in each group (right). (B) GF mice were fed with STD, AFD, W or a mixture of C+S+Y diets for 2 or 5 weeks from weaning. Representative dot plots show frequencies of CD8αβ IELs in 5 week fed mice (left). The graph shows the absolute numbers of CD8αβ IELs in indicated condition (right). Numbers in dot plots indicate the frequency of cells in adjacent gates. Each dot in graph represents an individual mouse and horizontal bars indicate mean values. $*P < 0.05$, $***P < 0.0001$, $****P < 0.0001$ (one-way ANOVA). NS, non-specific. Error bars indicate SD. IEL, intraepithelial lymphocyte

single wheat, corn, soybean or yeast components, they failed to develop CD8αβ IELs after 2 weeks (Fig. 3B). However, the combination of corn, soy and yeast promoted the development of CD8αβ IELs after 5 weeks, whereas wheat alone was not sufficient to induce them. These results indicate that even single dietary component can induce the development of CD8αβ IELs in the presence of microbiome, but various sources of dietary antigens are required for the development of CD8αβ IELs in the absence of intestinal bacteria.

Microbial colonization in early life is critical for CD8αβ IEL development

Finally, we investigated whether there is a critical time window for IEL development, during which intestinal microbiome should be introduced to promote the development of CD8αβ IELs. AF mice were simultaneously exposed microbial and dietary antigens by transferring 3 or 6 week-old AF mice to SPF cages, and we analyzed the induction of CD8αβ IELs after 2 weeks (Fig. 4). As expected, 3 week-old AF mice successfully generated CD8αβ IELs after conventionalization for 2 weeks (Fig. 4). Interestingly, however, 6 week-old AF mice were significantly less efficient for the generation of CD8αβ IELs although they moderately increase in the number of CD8αβ IELs (Fig. 4). This finding suggests that there is specific time window that intestinal bacteria and dietary antigens together are able to efficiently promote the development of CD8αβ IELs. Unfortunately, we did not test the condition that SPF mice fed with AFD and switched into STD at different time points, which can demonstrate the role of microbiome for the early and later supplementation of dietary antigens.

DISCUSSION

In this report, we evaluated the effects of microbiome and dietary antigens in the generation of CD8αβ IELs separately by using GF mice and an AF dietary regimen. We generated conditions that are deficient for microbial antigens, dietary antigen and both of them by using GF mice fed with STD, SPF mice fed with AF diet and GF mice fed with AF diet respectively. In these mice, we compared the development of CD8αβ IELs and showed that they are mainly induced by dietary antigens, and commensal flora are required for their timely development and functional maturation. One potential caveat of this research would be that SPF mice with AF diet have different microbial repertoire from those with STD due to the change of nutritional environment. However, we previously showed that microbial load was not different between SPF mice fed with STD and AFD (Kim et al., 2016), although there were minor changes of the specific kinds of intestinal flora. We think it is very unlikely that such a trivial alteration results in dramatic change of host T cell differentiation.

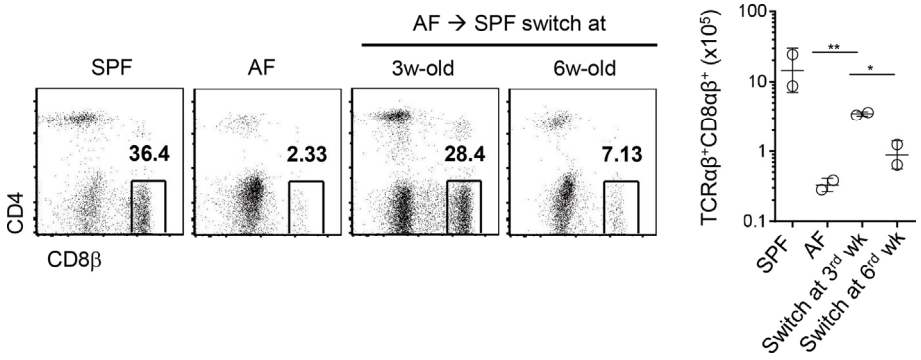

Fig. 4. Microbial colonization in early life is critical for CD8αβ$^+$ IEL development. Three or 6 week-old AF mice were switched to the SPF condition for 2 weeks. IELs were harvested and analyzed for CD8αβ$^+$ T cells. Representative dot plots show the frequencies of the CD8αβ$^+$ IELs in the indicated mice after gating CD45$^+$TCRγδ$^-$TCRαβ$^+$ cells (left). The graph shows the absolute numbers of TCRαβ$^+$CD8αβ$^+$ IELs (right). Numbers in dot plots indicate the frequency of cells in adjacent gates. Each dot represents an individual mouse and horizontal bars indicate mean values. $*P < 0.05$, $**P < 0.01$ (unpaired t-test). Error bars indicate SD. IEL, intraepithelial lymphocyte

We also tested individual dietary sources that induce CD8αβ IELs. Wheat alone could induce normal number of CD8αβ IELs in SPF condition, but not in GF condition even after feeding for 5 weeks (Fig. 3). However, combined corn, soy and yeast extracts could induce normal number of CD8αβ IELs in the GF condition after feeding for 5 weeks. Because the individual dietary components were fed together with AFD regimen, it is unlikely that nutritional change influenced the result. Rather it is possible that diverse sources of proteins can overcome the need of intestinal bacteria. In the future study, the detailed analysis of their ingredients in wheat and a complex of corn, soy and yeast extract would define specific molecules required for the maturation of CD8αβ IELs.

The microbiome is required for the early development of the mucosal immune system (Cebra, 1999), and GF mice have less IELs than SPF mice (Chung et al., 2012). In our experiment, we also confirmed that the density of IELs in our GF mice was much lower than that of SPF mice (data not shown). So far, however, the input timing of intestinal antigens has not been tested. In this study, we compared the effect of early (3 weeks) and late (6 weeks) time points of antigenic input in the development of CD8αβ IELs (Fig. 4). Interestingly, when the intestinal antigens were provided at later time point, the induction of CD8αβ IELs was less efficient, indicating there is critical time window that the host can generate CD8αβ IELs in response to intestinal antigens. CD8αβ IELs have a distinct TCR repertoire and are generated from naïve CD8αβ T cells when they encounter cognate intestinal antigens in the mesenteric LN or Peyer's patch (Cheroutre et al., 2011). Therefore, one possible explanation of this phenomenon is that young individual has more of these precursors than old ones or they have different propensity to generate IELs. In future experiments, we will examine the development of CD8αβ IELs when diet and microbial antigens are separately introduced to further address the role of microbiome for the development of diet-induced CD8αβ IELs.

During delivery, the mother's vaginal normal flora is transmitted to the neonates. After birth, diverse microorganisms are rapidly introduced to intestinal immune cells (Maynard et al., 2012). For this reason, infants born by Caesarean section show higher susceptibility to food allergies at early life and to atopic disease at later time points (Eggesbo et al., 2003; 2005). Various dietary antigens such as soy, wheat, nuts, peanuts and eggs are common in childhood and continuously challenged until adulthood. Our results indicate that if proper microbiomes are not established before exposure to these dietary antigens, development of intestinal T cells and their potential regulatory functions could be inefficient.

Kawaguchi-Miyashita *et al* first reported the dramatic effect of dietary antigens in the development of CD8αβ IELs (Kawaguchi-Miyashita et al., 1996); in that report, they developed antigen-minimized mice by feeding AFD to adult GF mice and showed αβ T cells, but not γδ T cells, gradually decrease. However, they did not investigate the role of microbiome. On the other hand, the current understanding of IEL development is mostly limited to microbial antigens, and the effects of dietary antigens had not been well recognized (Chung et al., 2012; Imaoka et al., 1996; Umesaki et al., 1993). In this report, we confirmed again that the majority of CD8αβ IELs is induced by dietary antigens and additionally showed that the number of CD8αβ IELs is dramatically increased after weaning, but they require commensal bacteria.

Our result showed the unexpected role of commensal microbiota in IEL development. Mechanistically, it is possible that microbiota provide immunological signals that facilitate the development of CD8αβ IELs or metabolize dietary components and make them more immunogenic. Furthermore, the luminal antigens are likely to complement with each other, and dietary changes can trigger intestinal dysbiosis. However, we excluded this possibility by comparing the GF and AF conditions, in which commensal flora are absent and only dietary antigens are different. In the future, it would be interesting if we can define specific interactions between dietary components and intestinal bacteria that can enhance

the development of IELs in the host.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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