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Antigen presentation by small intestinal epithelial cells uniquely enhances IFN-γ secretion from CD4$^+$ intestinal intraepithelial lymphocytes

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ABSTRACT

Small intestinal epithelial cells (sIECs) express major histocompatibility complex class II molecules even in a normal condition, and are known to function as antigen presenting cells (APCs) at least in vitro. These findings raised the possibility that sIECs play an important role in inducing immune responses against luminal antigens, especially those of intestinal intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs). We herein showed that antigenic stimulation with sIECs induced markedly greater secretion of interferon-gamma (IFN-γ) by CD4$^+$ IELs, but not interleukin (IL)-4, IL-10 and IL-17 although the proliferative response was prominently lower than that with T cell-depleted splenic APCs. In contrast, no enhanced IFN-γ secretion by CD4$^+$ LPLs and primed splenic CD4$^+$ T cells was observed when stimulated with sIECs. Taken together, these results suggest that sIECs uniquely activate CD4$^+$ IELs and induce remarkable IFN-γ secretion upon antigenic stimulation in vivo.

1. Introduction

Intestinal epithelial cells (IECs) provide a crucial barrier function and separate a myriad of microbial and food antigens in the intestinal lumen from the gut-associated lymphoid tissue. IECs also have an important role in regulation of the gut immune system, secreting a wide variety of cytokines and chemokines, either constitutively or inducibly upon recognition of microbial components [1]. Moreover, IECs are located in contact with numerous T lymphocytes, both IELs within the epithelium and LPLs present in the underlying lamina propria and modulate function of these T cells directly or indirectly [1–3]. IECs express major histocompatibility complex (MHC) class II molecules and function as antigen presenting cells (APCs) at least in vitro [4,5]. Luminal antigens mainly cross the intestinal mucosa via specialized epithelial cells, M cells, overlying the Peyer's patches [6]. However, a transcellular route through cytoplasm of IECs is also expected. Numerous reports have demonstrated the capacity of IECs to incorporate proteins and process antigens internally [5,7]. Furthermore, sIECs derived from rats after oral administration of peptides induced antigen specific proliferation of T cells in vitro [8]. These findings strongly suggest that IECs incorporate luminal antigens and present them to CD4$^+$ IELs or LPLs in vivo.

IELs existing adjacent to IECs are unique populations mainly consisting of T cells. Numerous reports have shown the difference between IELs and systemic T cells in subset composition, phenotypes and immunological functions [9]. IELs not only exert cytotoxicity against pathogens, but also play an important role in immune regulation [10]. The expression of MHC class II molecules on IECs is induced in an IFN-γ dependent manner [11] and several subsets of IELs constitutively express IFN-γ [12], suggesting that IELs are indispensable for MHC class II expression on IECs.

Normal human colonic epithelial cells (cECs) exhibit low level expression of MHC class II [13,14] and its expression is enhanced in a diverse group of pathological conditions including inflammatory bowel disease (IBD) [14] and graft versus host disease [15]. IBD patients derived cECs preferentially stimulate peripheral blood CD4$^+$ T cells to proliferate and secrete IFN-γ [13]. These observations suggest that aberrant activation of CD4$^+$ T cells by cECs is one of the major factors for the chronic inflammation in IBD. Unlike the weak expression of MHC class II on normal cECs, almost all the sIECs apparently express class II molecules on their surface even in a normal condition both in humans and mice [11,16]. However, although surrounded by numerous CD4$^+$ T cells and luminal antigens, sIECs do not normally elicit active immune responses nor induce chronic inflammation. It has been suggested that

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$CD4^+$ T cells stimulated by sIECs become anergy state because of the lack of surface expression of CD80 and CD86 [16] or may function as regulatory T cells [17]. But the precise function of $CD4^+$ T cells that interact with sIECs still remains to be elucidated.

Hitherto, systemic $CD4^+$ T cells or T cell clones have been often used for antigen presentation analysis by IECs. In addition, although many investigators have examined the T cell proliferation induced by IECs associated with polarity of MHC class II or antigen processing [4,5], their effector function has not been studied well. In the present study, we attempted to analyze cytokine secretion of $CD4^+$ IELs or LPLs stimulated with sIECs to reveal the physiological significance of constitutive expression of MHC class II molecules on sIECs.

2. Materials and methods

2.1. Animals

Female BALB/c ($H\text{-}2^d$) mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Female OVA specific TCR-transgenic DO11.10 mice on a BALB/c background ($H\text{-}2^d$) were kindly provided by Prof. H. Ishikawa (Keio University, Tokyo, Japan). Pregnant BALB/c mice were purchased from Sankyo Laboratories (Tokyo, Japan). Guidelines formulated by the University of Tokyo were followed for the care and use of animals.

2.2. Antibodies and reagents

Anti-mouse CD16/CD32 mAb (2.4G2), biotinylated rat IgG2b isotype control mAb (A95-1), fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 mAb (H129.19) and phycoerythrin (PE)-Cy5-conjugated streptavidin were purchased from BD Biosciences (San Diego, CA). Biotinylated anti-mouse MHC class II mAb (M5/114.15.2) was purchased from eBioscience (San Diego, CA). Recombinant mouse IFN-γ and mouse IL-2 were purchased from R&D Systems (Minneapolis, MN).

2.3. Primary culture of sIECs and a sIEC line, MoS13

Primary cultured sIECs were prepared from small intestines of fetal BALB/c mice as previously described [18]. A fetal sIEC line, MoS13 was established in our laboratory as in the same way as aMoS7, an adult sIEC line [19]. In brief, primary cultured fetal sIECs were infected with retroviruses recombined with the COS-7 cells derived SV40 large T oncogene, and immortalized cells were subcloned. sIECs were maintained as described previously [19]. Primary cultured sIECs and MoS13 were treated with IFN-γ (100 ng/ml) for 72 h before coculture with $CD4^+$ T cells. This condition was sufficient for inducing MHC class II molecules on MoS13 cells confirmed by flow cytometry (data not shown). When sIECs were cocultured with $CD4^+$ T cells, all the cells were cultured in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Sigma, St.Louis, MO), 0.03% L-glutamine and 50 μM 2-ME. All cultures were performed at 37 °C in a humidified 10% CO_2 incubator.

2.4. Cell preparation

$CD4^+$ T cells were purified from splenocytes, IELs and LP cells by using anti-CD4 magnetic beads and MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). To obtain IELs, small intestines were removed, washed and turned inside-out, and then shaken at 135 rpm for 45 min at 37 °C in Hanks' balanced salt solution (HBSS, Invitrogen, Carlsbad, CA) (5% FBS). Cell suspensions were then separated on a Percoll density gradient (GE Healthcare, Buckinghamshire, England). A discontinuous density gradient (30%, 44% and 70%) was used. Cells layered between 44% and 70% fractions were collected as IELs. $CD4^+CD8\alpha^-$ IELs and $CD4^+CD8\alpha^+$ IELs were purified by using anti-CD4 magnetic beads, FITC-conjugated anti-mouse CD4 mAb, anti-FITC MultiSort Kit (Miltenyi Biotec) and anti-CD8α magnetic beads. LP cells were obtained from small intestines as described previously [20]. Splenic $Thy1^-$ cells were purified from splenocytes of BALB/c mice by using anti-Thy1.2 magnetic beads, and used as control APCs. To prepare primed splenic $CD4^+$ T cells, splenic $CD4^+$ T cells (1×10^6) from DO11.10 mice were stimulated with mitomycin C (Sigma)-treated BALB/c $Thy1.2^-$ splenocytes (2×10^6) in the presence of 0.5 μM OVA323–339 peptide in 6-well plates. Culture medium was exchanged for RPMI 1640 containing 10 ng/ml IL-2 every 2–3 days. After 8–10 days, viable T cells were separated by using mouse lymphocytes separating buffer (JIMRO, Gunma, Japan) and used for assays.

2.5. Proliferation assay and measurement of cytokine

OVA specific TCR-transgenic mice derived $CD4^+$ T cells (3×10^4) were cocultured with mitomycin C-treated APCs (splenic APCs or IFN-γ-treated MoS13 or primary cultured sIECs, 3×10^4 respectively) in 96-well U-bottom plates in the presence of OVA323–339 peptide. To stimulate BALB/c mice derived $CD4^+$ IELs, Staphylococcal Enterotoxin B (SEB, Toxin Technology, Sarasota, FL) was used as antigens. Cultures were incubated for 96 h and pulsed with 37 kBq of [^{3}H]-thymidine for the last 24 h. The incorporated radioactivity was measured with a Betaplate scintillation counter (Wallac Oy, Turku, Finland). Culture supernatants were collected 72 h later and concentration of cytokines was measured by sandwich ELISA. IFN-γ and IL-4 were determined as described previously [21]. IL-10 and IL-17 were measured by using BD OptEIA Kit (BD Biosciences) and DuoSet (R&D Systems) respectively according to the manufacturer's instructions.

2.6. Flow cytometry

Cells were washed in HBSS containing 1% FBS and 0.1% sodium azide, and incubated with anti-CD16/CD32 mAb for 5 min at 4 °C, followed by reaction with biotinylated anti-MHC class II mAb or isotype control for 20 min at 4 °C, and subsequently stained with PE-Cy5-conjugated streptavidin for 20 min at 4 °C. Flow cytometry analysis was performed using a FACS LSR and CellQuest software (BD Biosciences).

2.7. Statistics

Student's two-tailed *t*-test was used for the comparison of group values. Significance was analyzed using MS-EXCEL (Microsoft, Redmond, WA), and values of $p < 0.01$ were considered significant.

3. Results

3.1. IFN-γ-treated MoS13 cells induce antigen specific proliferation of $CD4^+$ IELs

Due to the difficulty in obtaining a large number of primary cultured IECs, we used a murine sIEC line (MoS13) established in our laboratory to reveal the physiological significance of MHC class II expression on sIECs. As shown in Supplemental Fig. S1, MoS13 cells display a polygonal shape, a typical morphological characteristic of epithelial cells (panel A), and express an epithelial cell marker, cytokeratin (panels B) but not express a mesenchymal cell marker, vimentin (panels C). Tight junction proteins, ZO-1 and occludin are also found in the cell–cell contact region of MoS13 cells (panels D

and E of Supplemental Fig. S1, respectively). These results indicate that MoS13 cells have a character of epithelial cells.

First, we analyzed surface MHC class II expression on MoS13 cells. As shown in Fig. 1A, MHC class II was not detected on non-treated MoS13 cells, and evidently observed on MoS13 cells after IFN-γ treatment (left panel). In contrast, splenic Thy1$^-$ cells which mainly consist of B cells constitutively expressed MHC class II without any stimulation and were considered to be suitable as a positive control APC (right panel of Fig. 1A). It was noted that IFN-γ-treated MoS13 cells had a prominently lower level of MHC class II than splenic Thy1$^-$ cells, as assessed by the value of mean fluorescence intensity (MFI = 134.8 ± 23.9 or 2650.2 ± 777.6, respectively).

To analyze the antigen specific activation of T cells by sIECs, TCR-transgenic mice derived CD4$^+$ T cells expressing the same MHC haplotype as MoS13 cells derived from BALB/c mice were used for this study. As shown in Fig. 1B, proliferative response was not observed when CD4$^+$ IELs derived from DO11.10 mice expressing a transgenic TCR specific for OVA323–339 peptide were cultured alone or cocultured with non-treated MoS13 in the presence of specific peptide. In contrast, IFN-γ-treated MoS13 cells were able to induce proliferation of CD4$^+$ IELs in an antigen dose-dependent manner (Fig. 1B). However, correlated with the surface expression levels of MHC class II on MoS13 and splenic APCs shown in Fig. 1A, the proliferative response of IELs stimulated with MoS13 was apparently weak as compared with that of splenic APCs (Fig. 1B). These results indicate that IFN-γ-treated MoS13 cells expressing MHC class II molecules on their surface are able to stimulate CD4$^+$ IELs in an antigen-specific manner, but the T cell stimulation capacity of sIECs is expected to be weak compared with professional APCs.

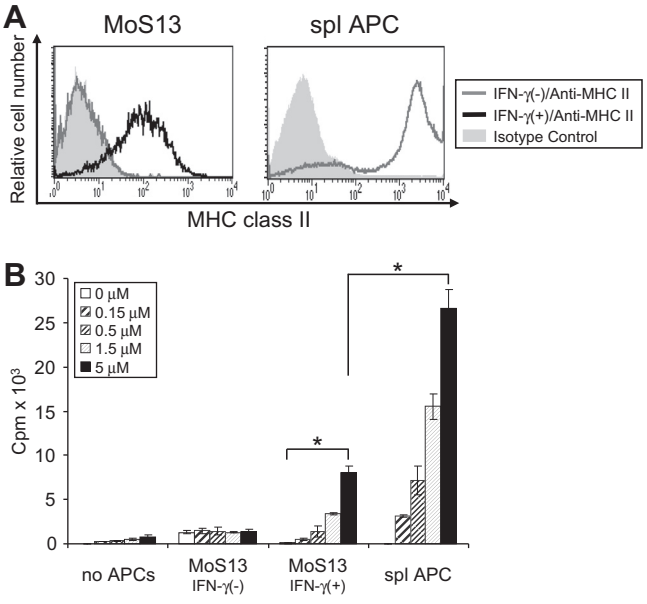

Fig. 1. IFN-γ-treated MoS13 cells induce antigen specific proliferation of CD4$^+$ IELs. (A) Surface expression of MHC class II molecules on MoS13 or T-cell-depleted splenocytes was analyzed by flow cytometry. Left panel, MHC class II histograms obtained by staining non-treated MoS13 cells (gray line) or MoS13 cells incubated with IFN-γ for 72 h (black line); right panel, MHC class II histograms obtained by staining splenic APCs (gray line). The gray areas in each histogram show back ground fluorescence obtained by staining of isotype-matched control. (B) CD4$^+$ IELs from DO11.10 mice were cocultured with non-treated or IFN-γ-treated MoS13 cells or splenic APCs or without APCs for 96 h with the indicated concentrations of OVA323–339 peptide. Proliferation was assessed by incorporation of [^{3}H]-thymidine uptake. Values shown are means ± SD of determinations from triplicate cultures, compared cpm in MoS13 to that in splenic APCs (*$p < 0.01$). Data shown are representative of three independent experiments with similar results.

3.2. sIECs induce markedly enhanced IFN-γ secretion by CD4$^+$ IELs

Next, we investigated cytokine secretion of CD4$^+$ IELs stimulated with MoS13 cells. In contrast with the weak proliferative induction by MoS13 shown in Fig. 1B, MoS13 cells induced markedly enhanced IFN-γ secretion by CD4$^+$ IELs as compared with splenic APCs at any concentration of OVA peptide (Fig. 2A). Moreover, time-course analysis showed that IFN-γ secretion by CD4$^+$ IELs stimulated with MoS13 was greater over the tested time intervals from 48 h to 120 h than that with splenic APCs (data not shown). On the other hand, the amounts of IL-4 and IL-10 secreted by IELs stimulated with MoS13 were less than those with splenic APCs (Figs. 2B and C), and almost the same amount of IL-17 was detected when activated by MoS13 or splenic APCs (Fig. 2D).

As CD4$^+$ IELs used above contained both CD4$^+$CD8α$^-$ and CD4$^+$CD8α$^+$ subsets, we further examined IFN-γ secretion following purification of CD4$^+$CD8α$^-$ IELs and CD4$^+$CD8α$^+$ IELs. We also examined whether MoS13 cells could induce enhanced IFN-γ secretion from CD4$^+$ T cells other than IELs. IELs and LPLs are known to exhibit a phenotype of antigen-primed memory/effector T cells [10]. Therefore, we attempted to quantify the amount of IFN-γ secreted from CD4$^+$ LPLs or OVA-primed splenic CD4$^+$ T cells. As shown in Fig. 3, CD4$^+$CD8α$^-$ IELs and CD4$^+$CD8α$^+$ IELs cocultured with MoS13 secreted significantly more amount of IFN-γ than those stimulated with splenic APCs, respectively. In contrast, the induction of IFN-γ by MoS13 was weaker than splenic APCs in CD4$^+$ LPLs, and IFN-γ secretion from primed splenic CD4$^+$ T cells was hardly induced when cocultured with MoS13 (Fig. 3). These findings indicate that induction of markedly enhanced IFN-γ secretion is uniquely observed between sIECs and CD4$^+$ IELs but not LPLs and primed splenic T cells.

To exclude the possibility that the results shown above was just due to the coculture system in which an artificially immortalized sIEC line and TCR-transgenic mice derived IELs were used, primary cultured sIECs and BALB/c mice derived IELs were used instead. As shown in Fig. 4A, antigenic stimulation with IFN-γ-treated primary cultured sIECs also induced significantly more amount of IFN-γ by CD4$^+$ IELs than splenic APCs, while the proliferative response was apparently lower than that with splenic APCs (data not shown). Furthermore, coculture of BALB/c mice derived CD4$^+$ IELs with MoS13 in the presence of super antigens, SEB which bind to both α chain of MHC class II and specific Vβ regions of TCR, also resulted in greatly enhanced IFN-γ secretion as compared with splenic APCs (Fig. 4B). These data strongly suggest that antigenic stimulation with sIECs generally induce greatly enhanced IFN-γ secretion from CD4$^+$ IELs.

4. Discussion

In the present study, we provide the new evidence that sIECs induce markedly enhanced IFN-γ secretion from CD4$^+$ IELs although the induction of proliferative response is prominently weak. Furthermore, we show that the enhancement of IFN-γ secretion by sIECs is uniquely observed in IELs but not in other CD4$^+$ T cells.

Our current effort is to clarify the mechanism how sIECs enhance IFN-γ secretion by CD4$^+$ IELs. We focused on cytokines and B7 and TNF family molecules, well-known T cell-cosignaling ligands, and investigated the expression pattern of these genes in MoS13 cells and splenic APCs. Among cytokines, IL-12 family (IL-12, IL-23 and IL-27), IL-7, IL-15 and IL-18 have been already reported to function in enhancing IFN-γ secretion [22,23]. As a result of RT-PCR analyses, we showed that mRNA expression of IL-18, B7-H3, B7-H4 and 4-1BBL in MoS13 cells was much higher than those of splenic APCs (Supplemental Fig. S2). However, addition of blocking mAbs against them to the coculture of CD4$^+$ IELs with MoS13

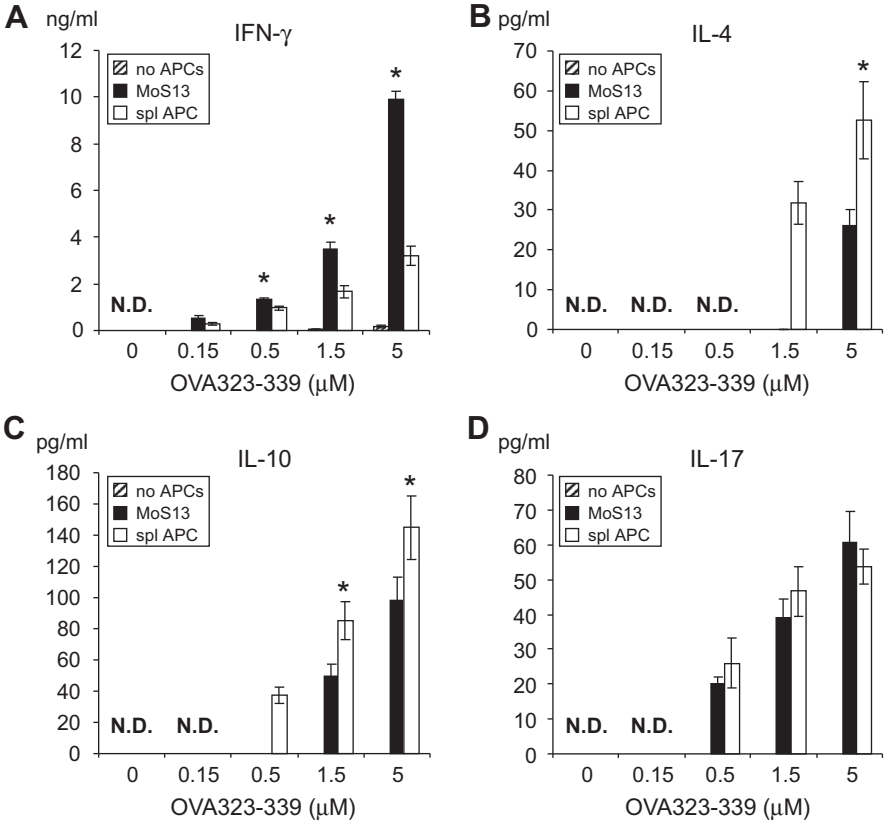

Fig. 2. MoS13 cells induce markedly enhanced IFN-γ secretion by $CD4^+$ IELs. $CD4^+$ IELs from DO11.10 mice were cocultured with IFN-γ-treated MoS13 cells or splenic APCs or without APCs with the indicated concentrations of OVA323–339 peptide. The amounts of IFN-γ (A), IL-4 (B), IL-10 (C) and IL-17 (D) in the culture supernatants were measured by ELISA. Values shown are means ± SD of determinations from triplicate cultures, compared the amount in MoS13 to that in splenic APCs ($^*p < 0.01$). ND means undetectable. Data shown are representative of three independent experiments with similar results.

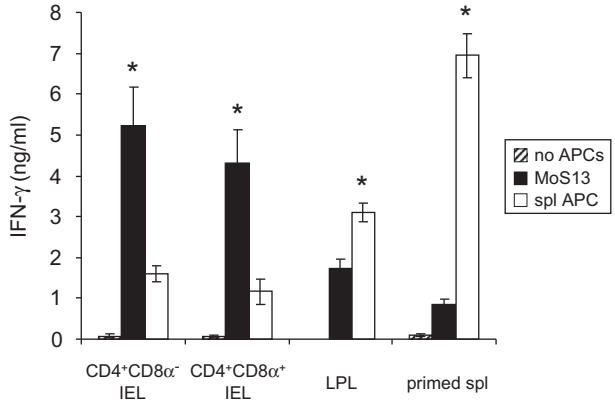

Fig. 3. The vigorous induction of IFN-γ secretion by MoS13 cells is uniquely observed in $CD4^+$ IELs. $CD4^+CD8\alpha^-$ IELs, $CD4^+CD8\alpha^+$ IELs, $CD4^+$ LPLs, or primed splenic $CD4^+$ T cells from DO11.10 mice were cocultured with IFN-γ-treated MoS13 cells or splenic APCs or without APCs in the presence of 5 μM OVA323–339. The amounts of IFN-γ in the culture supernatants were measured by ELISA. Values shown are means ± SD of determinations from triplicate cultures, compared the amount in MoS13 to that in splenic APCs ($^*p < 0.01$). Data shown are representative of three independent experiments with similar results.

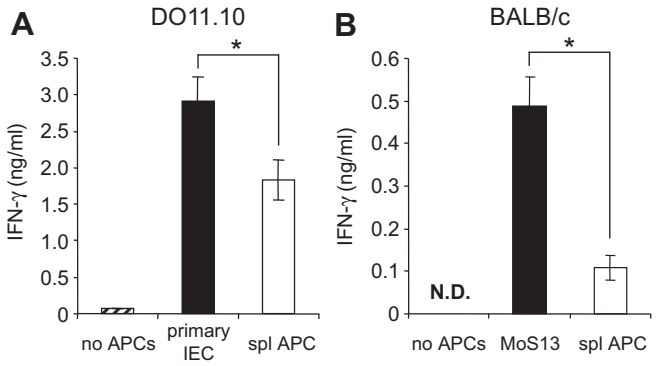

Fig. 4. $CD4^+$ IELs stimulated with sIECs generally secrete a great amount of IFN-γ. (A) $CD4^+$ IELs from DO11.10 mice were cocultured with IFN-γ-treated primary cultured sIECs or splenic APCs in the presence of 5 μM OVA323–339 peptide. (B) $CD4^+$ IELs from BALB/c mice were cocultured with IFN-γ-treated MoS13 cells or splenic APCs in the presence of 10 mg/ml SEB. The amount of IFN-γ in the culture supernatants was measured by ELISA. Values shown are means ± SD of determinations from triplicate cultures. * shows points of significant difference ($p < 0.01$). ND means undetectable. Data shown are repeated twice (A) and three times (B) with similar results.

cells hardly inhibited IFN-γ secretion (data not shown). These results suggest that sIEC derived factors involved in the enhancement of IFN-γ secretion from IELs are novel and yet undefined molecules.

Almost all the sIECs apparently express MHC class II molecules on their surface both in humans and mice, suggesting that sIECs are constitutively exposed to IFN-γ in vivo [11,16]. In contrast with the physiological condition, sIEC lines such as MODE-K and aMoS7 normally cultured in an IFN-γ-free condition do not express class II on their surface, and IFN-γ pretreatment is necessary for the antigen presentation analysis of IECs [19,24]. Since we were concerned that the vigorous induction of IFN-γ secretion from $CD4^+$ IELs by sIECs was due to the effect of IFN-γ pretreatment, we also

examined IFN-γ secretion by $CD4^+$ IELs stimulated with IFN-γ-pretreated splenic APCs. In contrast to sIECs, IFN-γ pretreatment of splenic APCs hardly affected the amount of IFN-γ secreted from IELs (data not shown).

IFN-γ secreted from IELs possibly affects IECs or IELs themselves. The effects of IFN-γ on sIECs are assumed to enhance expression of both MHC class I and II molecules, and to enhance MHC class I antigen presentation by controlling proteolytic cascade [25]. These effects are required to defense against pathogens. Anti-inflammatory effects of IFN-γ were also reported. IFN-γ down-regulates IL-8 production by human cECs [26]. For T cells, IFN-γ suppresses proliferation of Th2 cells, and inhibits differentiation of naive $CD4^+$ T cells into Th17 cells [27]. IFN-γ also serves to limit the IL-17-producing T cell population in vivo after infection with mycobacteria [28]. Immunosuppressive function of IFN-γ was also shown by inducing nitric oxide synthase and indoleamine-2,3-dioxygenase in APCs [29]. It is possible that these immunomodulatory effects of IFN-γ contribute to suppress allergic and inflammatory responses against incorporated luminal antigens, and it is needed to investigate in vivo allergy or infection models using sIEC specific MHC class II knockout mice to reveal the physiological significance of MHC class II on sIECs.

It is noteworthy that IFN-γ specific enhancement by sIECs is uniquely observed in $CD4^+$ IELs, but not in LPLs and primed splenic T cells. There was an intriguing report showing that sIEC membranes down-regulated proliferative and cytokine responses of IELs, but not those of splenic T cells [30]. This evidence also indicates that the interactions between sIECs and IELs are different from those between sIECs and systemic T cells. Our present findings reveal the novel unique interaction between sIECs and IELs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.024.

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