Table 1
The number of $CD3^+$ cells detected in the jejunum

Mouse groups	Diet	$CD3^+$ cells (N/villi) (mean ± SD)
SPF OVA23-3	EW ($n = 5$)	$35.7 \pm 10.1^{\alpha}$
	CN ($n = 4$)	8.4 ± 5.5
RAG-2 gene-KO OVA23-3	EW ($n = 4$)	$43.6 \pm 12.5^{\alpha}$
	CN ($n = 4$)	4.9 ± 0.9
GF OVA23-3	EW ($n = 5$)	$14.6 \pm 6.3^{\beta}$
	CN ($n = 6$)	4.3 ± 0.4
GF BALB/c	EW ($n = 4$)	3.4 ± 0.9

$^{\alpha}$Significantly increased in comparison to controls ($p < 0.05$).
$^{\beta}$Significantly increased in comparison to controls and EW-diet fed GF BALB/c ($p < 0.05$).

Table 2
Cytokine production by $CD4^+$ T cells harvested from the MLN of SPF OVA23-3 mice

Mouse groups (diet) and OVA concentration	IL-4 (pg/mL)	IFN-γ (ng/mL)
EW (OVA 1.0 mg/mL)	96.3	3.6
EW (OVA 0 mg/mL)	< 5	3.8
CN (OVA 1.0 mg/mL)	< 5	64.9
CN (OVA 0 mg/mL)	< 5	6.7

$CD4^+$ T cells harvested from the MLN of SPF OVA23-3 mice were cultured with OVA (1.0 or 0 mg/mL) and APC. Culture supernatants were assessed for Th1 and Th2 cytokines.

4. Conclusion

Analysis of inflammation in OVA23-3 mice and RAG-2 gene KO mice strongly suggests that antigen-specific Th2 type T cells and inflammatory cells primed by Th2 type responses play important roles in the development of the disease. Analysis of GF OVA23-3 mice indicated that intestinal T cells could directly respond to dietary antigens and cause inflammation without intestinal microflora. However, in the presence of indigenous bacteria, T cells could mediate a more severe inflammatory response. Our TCR-Tg system should be an informative system to evaluate the effect of probiotics, prebiotics, and other food factors on antigen-specific T cell responses.

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CD4$^-$c-kit$^-$CD3ε$^-$IL-2Rα$^+$ Peyer's patch cells are a novel cell subset which secrete IL-5 in response to IL-2: implications for their role in IgA production

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In this study, we examined which cell population contributes to IL-5 production by Peyer's patch (PP) cells. Thy1.2$^-$ fraction of PP cells, but not those of splenocytes, secreted IL-5 in response to IL-2. We found that CD3ε$^-$IL-2Rα$^+$ cells purified from the Thy1.2$^-$B220$^-$ fraction of PP cells secreted IL-5 when stimulated with IL-2. CD3ε$^-$IL-2Rα$^+$ cells were subdivided into CD4$^+$ and CD4$^-$ populations or c-kit$^+$ and c-kit$^-$ populations, and only the CD4$^-$ and c-kit$^-$ CD3ε$^-$IL-2Rα$^+$ cells secreted IL-5 in response to IL-2. CD3ε$^-$IL-2Rα$^+$ cells did not express NK cell-markers and exhibited a lymphoid morphology. We have therefore identified CD3ε$^-$IL-2Rα$^+$ cells as a unique lymphoid population that are not classified into conventional IL-5-producing cell populations, such as T cells, mast cells and NK cells. Depletion of CD3ε$^-$IL-2Rα$^+$ cells from PP resulted in reduced IL-5 production. Furthermore, IgA secretion by B cells was increased when PP B cells were cocultured with CD3ε$^-$IL-2Rα$^+$ cells. Taken together, these results suggest that the novel subset of CD4$^-$c-kit$^-$CD3ε$^-$IL-2Rα$^+$ PP cells are capable of secreting a high level of IL-5 in response to IL-2, contribute markedly to IL-5 production and help IgA secretion by B cells.

Key words: IL-5 / Peyer's patch / IgA

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1 Introduction

IL-5 is mainly produced by Th2 cells and was originally recognized by its activity as a B cell growth factor [1], an IgA-enhancing factor [1–3], and as a differentiation or an activation factor for eosinophils [4–6]. IL-5 also induces growth and IgA and IgM production by B1 cells [7]. Analyses of IL-5 transgenic mice, IL-5 gene deficient (IL-5$^{-/-}$) and IL-5Rα$^{-/-}$ mice show that IL-5 is critically involved in the development and activation of B cells and eosinophils [8–12].

Several reports showed that IL-5 production from certain cells was also induced by IL-2 stimulation [13–16]. Recent studies showed that a human allergic CD4$^+$ T cell clone produced IL-5 in response to IL-2 [16] and that human NK cells can be induced to produce IL-5 following costimulation with gamma-irradiated melanoma cells and IL-2 [17]. These studies strongly suggest that IL-2 induces both T cells and non-T cells to produce IL-5.

Large numbers of IgA-producing cells, which are the first line of defense against luminal pathogens and bacteria, exist in the gut mucosa. Further, in allergic conditions, eosinophils infiltrate into these sites. These observations suggest that IL-5 is an important cytokine in regulating immunologic and allergic reactions in the gut mucosa. One of the representative organs of the gut-associated lymphoreticular tissue is the Peyer's patch (PP), which is an organized peripheral lymphoid tissue distributed along the intestinal tract and thought to be a major inductive site of IgA Ab [18, 19].

In the present study, we first examined the effect of exogenous IL-2 on IL-5 production by PP cells and found that PP cells produce IL-5 in response to IL-2 in a dose-dependent manner. We then isolated various cell types from PP and stimulated each to identify which population produces IL-5 in response to IL-2. Here we show that CD4$^-$CD3ε$^-$IL-2Rα$^+$ cells isolated from PP, which are unique and not previously classified as IL-5-

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Abbreviations: PP: Peyer's patch **SPL:** Spleen

producing cells such as CD4⁺ T cells or NK cells, secrete IL-5 when stimulated with PMA+A23187 or IL-2. We also suggest that CD4⁻CD3ε⁻IL-2Rα⁺ cells help B cells to secrete IgA.

2 Results

2.1 PP cells produce IL-5 in response to IL-2 in a dose-dependent manner

We have observed that freshly isolated PP cells derived from OVA-specific TCR-transgenic mice produced higher levels of IL-5 than splenocytes when stimulated with OVA in primary cultures (manuscript in preparation). Many studies have been published on the role and source of IL-5 in allergic conditions; however, few have discussed IL-5 production in the initiation of immune responses, though it has been thought to be an important cytokine for the mucosal immunity [20]. Since PP is thought to be a major inductive site for immune responses against luminal Ag, we chose to study IL-5 production by PP cells to identify which cell population may be responsible for IL-5 production at the beginning of immune responses.

It has been reported that IL-5 gene transcription and protein synthesis were also induced upon stimulation with exogenous IL-2 in human PBMC or in T cell clones from allergic patients [13–16]. We therefore speculated that exogenous IL-2 may also induce PP cells and splenocytes to produce IL-5 in mice, and hence we initially analyzed whether IL-2 might affect the IL-5 production by these cells. As shown in Fig. 1, PP cells from BALB/c mice produced IL-5 in response to IL-2 in a dose-dependent manner, but no IL-5 production was observed in splenocytes stimulated with IL-2. Both PP cells and splenocytes also produced IFN-γ and IL-6 in response to IL-2 (Fig. 1).

Since Mori et al. [16] reported that human T cell clones produced IL-5 in the presence of IL-2, we tested whether naive PP CD4⁺ T cells could produce IL-5 by the stimulation with IL-2. Analysis of CD4⁺ T cells showed that stimulation with immobilized anti-CD3 and anti-CD28 mAb induced IL-5 production by naive PP CD4⁺ T cells, whereas IL-2 alone failed to induce IL-5 production (Fig. 2A). Similar results were obtained from analyses of naive spleen (SPL) CD4⁺ T cells (data not shown). We hypothesized that non-T cells in PP may be responsible for producing IL-5 in response to IL-2 and therefore we depleted Thy1.2⁺ cells from PP cells by negative immunomagnetic selection with MACS and examined the Thy1.2⁻ fraction for its response to IL-2. Fig. 2B shows that the Thy1.2⁻ fraction of PP cells produced IL-5 in

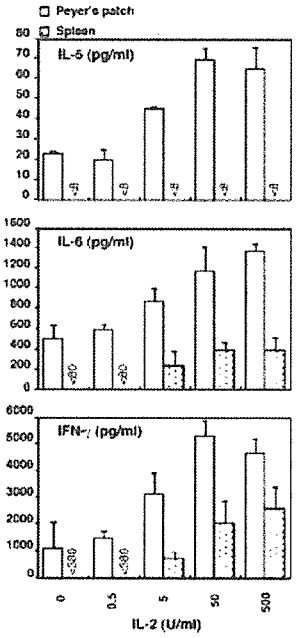

Fig. 1. PP cells produce IL-5 in response to IL-2 in a dose-dependent manner. PP cells and splenocytes were prepared by dispase treatment and cultured in a 96-well plate (1×10^6 cells/well) with variable concentrations of IL-2. Culture supernatants were collected at 48 h and examined for secreted cytokines using the respective cytokine-specific ELISA. The reults are expressed as the mean of triplicate cultures ± SD. Data are representative of four independent experiments.

response to IL-2 in a dose-dependent manner. The Thy1.2⁻ fraction of PP cells produced IL-6 and both the Thy1.2⁻ fraction of PP cells and splenocytes also produced IFN-γ in response to IL-2 (Fig. 2B). These results suggested that non-T cells that produced IL-5 in response to IL-2 exist in PP, but not in SPL.

2.2 CD3ε⁻IL-2Rα⁺ cells produce IL-5

To identify which cell population may produce IL-5 we separated the components of PP cells and stimulated each population. In another experiment we found that IL-5 production by the Thy1.2⁻ fraction of PP cells was strongly induced by PMA+A23187 compared to IL-2 (data not shown). In contrast, we could not detect IL-5 production by the Thy1.2⁻ fraction of splenocytes even though they were stimulated with PMA+A23187 (data not shown). We therefore adopted PMA+A23187 as the cell-stimulation method to observe IL-5 production by PP cells. Since the Thy1.2⁻ fraction of PP cells contains B cells, Mϕ and DC, we separated and stimulated each

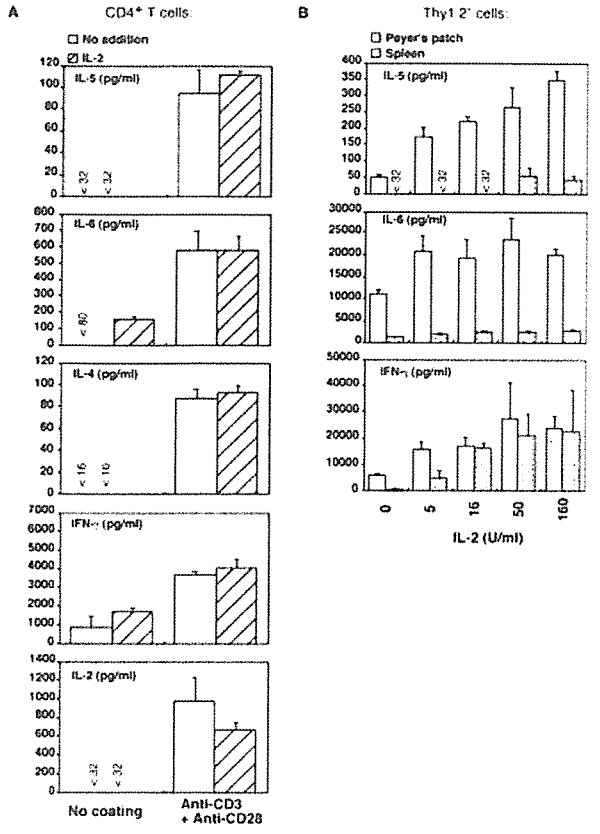

Fig. 2. Non-T cells from PP produce IL-5 in response to IL-2. $CD4^+$ T cells were isolated from PP or SPL by MACS, cultured in a 96-well plate (4×10^5 cells/well) and stimulated by immobilized anti-CD3 (3 μg/ml) and anti-CD28 Ab (5 μg/ml). In some cultures IL-2 (50 U/ml) was added (A). Thy1.2$^-$ cells were isolated from PP or SPL by negative selection of MACS and cultured with variable concentrations of IL-2 (B). Culture supernatants were collected at 48 h and examined for secreted cytokines using the respective cytokine-specific ELISA. The results are expressed as the mean of triplicate cultures ± SD. Similar results were obtained from more than three independent experiments.

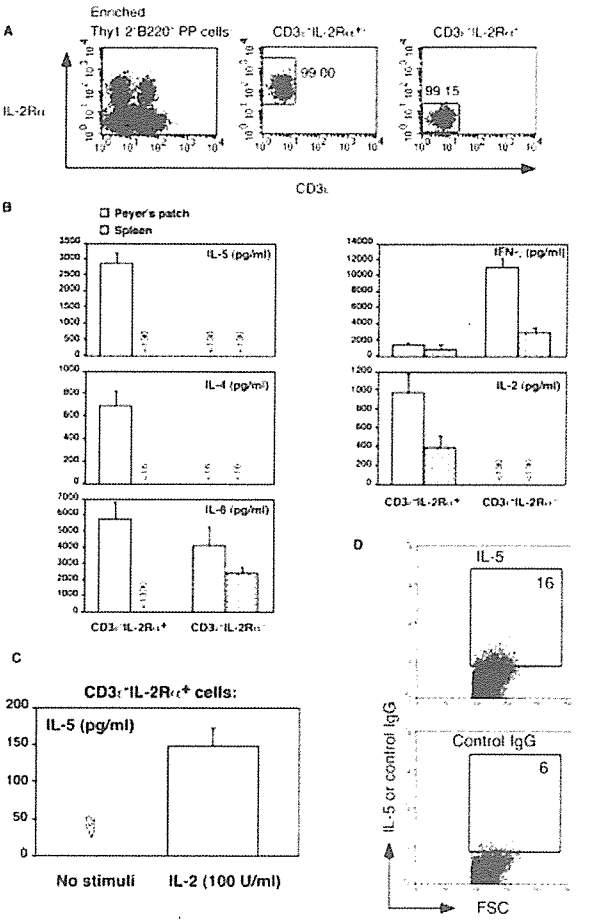

Fig. 3. $CD3\varepsilon^-IL\text{-}2R\alpha^+$ cells from PP produce IL-5. Thy1.2$^-$B220$^-$ cells were isolated from PP or SPL by negative selection of MACS and stained with anti-CD3ε and anti-IL-2Rα mAb. Then $CD3\varepsilon^-IL\text{-}2R\alpha^+$ cells and $CD3\varepsilon^-IL\text{-}2R\alpha^-$ cells were isolated by FACS sorting (A). These cells were cultured in a 96-well plate (5×10^4 cells/well) with 50 ng/ml of PMA plus 250 ng/ml of calcium ionophore A23187 (B). PP $CD3\varepsilon^-IL\text{-}2R\alpha^+$ cells were cultured in the presence or absence of 100 U/ml of IL-2 (C). Culture supernatants were collected at 48 h and examined for secreted cytokines using the respective cytokine-specific ELISA. The results are expressed as the mean of triplicate cultures ± SD. Purified PP $CD3\varepsilon^-IL\text{-}2R\alpha^+$ cells were stimulated with PMA and ionomycin and intra-cellular IL-5 was stained (D). Cells were gated on FSC and SSC to define the lymphoid population. Similar results were obtained from more than three independent experiments.

with PMA+A23187; however, we could not detect IL-5 production among these cells (data not shown), even though the Thy1.2$^-$B220$^-$ fraction of PP cells from which Mϕ or DC were separated produced IL-5 when stimulated with PMA+A23187 (data not shown). These results suggested that another cell population with an ability to produce IL-5 might exist in PP.

We hypothesized that because IL-2 activated cells to produce IL-5 they might express the IL-2R α chain (IL-2Rα), which forms a high affinity IL-2R with β and γ chain [21]. We therefore examined the expression of IL-2Rα on the Thy1.2$^-$B220$^-$ fraction from both PP cells and splenocytes and found about 10% of PP Thy1.2$^-$B220$^-$ cells and 5% of SPL Thy1.2$^-$B220$^-$ cells expressed IL-2Rα (Fig. 3A and data not shown). Next we purified $CD3\varepsilon^-IL\text{-}2R\alpha^+$ cells or $CD3\varepsilon^-IL\text{-}2R\alpha^-$ cells from the Thy1.2$^-$B220$^-$ fraction of PP cells or splenocytes and cultured each in the presence or absence of PMA+A23187. PP $CD3\varepsilon^-IL\text{-}2R\alpha^+$ cells produced IL-5, IL-4, IL-6, IFN-γ and IL-2 when

they were stimulated with PMA+A23187 (Fig. 3B). In contrast, we could not detect IL-5, IL-4 or IL-6 production by SPL CD3ε⁻IL-2Rα⁺ cells stimulated with PMA+A23187 (Fig. 3B). These results suggested that CD3ε⁻IL-2Rα⁺ cells existed in both PP and in SPL, however, only PP CD3ε⁻IL-2Rα⁺ cells could produce IL-5 in response to PMA+A23187 stimulation. Further analysis revealed that PP CD3ε⁻IL-2Rα⁺ cells produced IL-5 in response to exogenous IL-2 (Fig. 3C). Thus we defined CD3ε⁻IL-2Rα⁺ cells as the IL-2-responsive IL-5-producing population in PP. Cytostaining analysis revealed that approximately 10% of the PP CD3ε⁻IL-2Rα⁺ cells expressed IL-5 (Fig. 3D).

2.3 CD3ε⁻IL-2Rα⁺ cells are a unique subset of cells that secrete IL-5

It has been reported that Th2 cells, Tc2 cells, γδ T cells, mast cells, NK cells [17], NKT cells [22] or eosinophils [23] may produce IL-5. To identify the lineage of PP CD3ε⁻IL-2Rα⁺ cells, expression of surface molecules on PP CD3ε⁻IL-2Rα⁺ cells were analyzed by FCM. As shown in Fig. 4, CD3ε⁻IL-2Rα⁺ cells do not express TCRαβ, TCRγδ or Pan-NK cells marker DX5 [24]. Furthermore, we analyzed NK1.1 expression on PP CD3ε⁻IL-2Rα⁺ cells from C57BL/6N mice after confirming that PP CD3ε⁻IL-2Rα⁺ cells from C57BL/6N mice produced IL-5 when stimulated with PMA+A23187 (data not shown). CD3ε⁻IL-2Rα⁺ cells were found not to express NK1.1 molecules (Fig. 4). We therefore concluded that CD3ε⁻IL-2Rα⁺ cells are not T cells, NK cells or NKT cells.

To examine whether or not CD3ε⁻IL-2Rα⁺ cells are eosinophils, immunohistochemical analysis was carried out. This analysis revealed that CD3ε⁻IL-2Rα⁺ cells exhibit a lymphoid morphology, are small in size and have a relatively large nucleus to cytoplasm ratio (Fig. 5), thus indicating they differ from eosinophils, DC and Mϕ.

It was also revealed that almost all CD3ε⁻IL-2Rα⁺ cells from BALB/c mice express IL-7Rα and lack expression of CD8, B220, CD11b and CD23 (Fig. 4). The expression patterns of these and other cell surface molecules are shown in Table 1. We obtained similar results for the expression of surface molecules on CD3ε⁻IL-2Rα⁺ cells when we prepared PP cells by crushing PP mechanically (by non-enzymatic technique). CD3ε⁻IL-2Rα⁺ cells were further divided into c-kit⁺ and c-kit⁻ populations, or CD4⁺ and CD4⁻ populations (Fig. 4). To identify which subset of CD3ε⁻IL-2Rα⁺ cells produce IL-5, each subset was isolated and stimulated with PMA+A23187 or IL-2. As a result we found that c-kit⁻CD3ε⁻IL-2Rα⁺ cells and CD4⁻CD3ε⁻IL-2Rα⁺ cells produced IL-5 when stimulated with PMA+A23187 (Fig. 6A, B). Furthermore, the c-

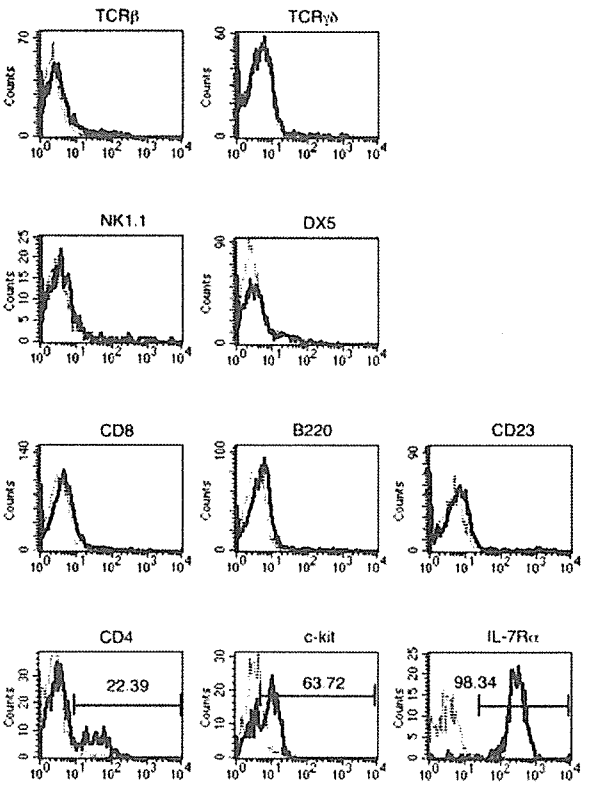

Fig. 4. Surface phenotypes of PP CD3ε⁻IL-2Rα⁺ cells. Thy1.2⁻B220⁻ fraction of PP cells were isolated from BALB/c mice or C57BL/6N mice by negative selection of MACS and stained with anti-CD3ε, anti-IL-2Rα and various mAb against surface molecules as indicated. Expression of TCRβ and TCRγδ, DX5, NK1.1, CD23, CD4, CD8, B220, c-kit, IL-7Rα (filled line) and isotype-matched mAb (solid line) of gated PP CD3ε⁻IL-2Rα⁺ cells are presented. Similar results were obtained from more than three independent experiments.

kit⁻CD3ε⁻IL-2Rα⁺ cells and CD4⁻CD3ε⁻IL-2Rα⁺ cells produced IL-5 in the presence of IL-2 (Fig. 6C). Conversely we could not observe IL-5 production by c-kit⁺CD3ε⁻IL-2Rα⁺ cells or CD4⁺CD3ε⁻IL-2Rα⁺ cells. Since CD3ε⁻IL-2Rα⁺ cells that have a potential to produce IL-5 do not express c-kit, these cells are distinguishable from mast cells. Taken together, these results strongly suggest that CD3ε⁻IL-2Rα⁺ cells are not classified with Th2 cells, Tc2 cells, γδ T cells, mast cells, NK cells, NKT cells or eosinophils and that CD3ε⁻IL-2Rα⁺ cells are a unique subset of cells that produce IL-5.

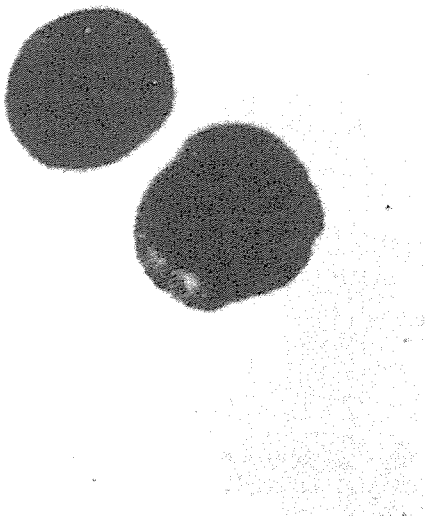

Fig. 5. Morphology of CD3ε⁻IL-2Rα⁺ cells. Purified PP CD3ε⁻IL-2Rα⁺ cells were stained with Diff-Quik.

Table 1. Surface expression of molecules on PP CD3ε⁻IL-2Rα⁺ cells

Molecules	Expression
Thy1.2	Low to –
sIgD	–
sIgM	–
Mac-1	–
CD11c	–
Syndecan-1	–
B7-1	–
B7-2	–
CD40	–
CD44	+
LFA-1	+
CD62L	–
CD69	+
Fas	–
Integrin α4	Low to –
Integrin β7	+

2.4 CD3ε⁻IL-2Rα⁺ cells are a major source of IL-5 production in PP

To assess the influence of the CD3ε⁻IL-2Rα⁺ cells upon IL-5 production by PP cells we compared cytokine production by PP cells depleted of CD3ε⁻IL-2Rα⁺ cells with control PP cells upon stimuli. We prepared non-treated PP cells and Ab-stained PP cells as controls which were treated with the mAb described in Sect. 4.3. The latter control was included since it was considered possible that T cells and other cells might be activated by anti-CD3 mAb when depleting CD3ε⁻IL-2Rα⁺ cells. Since IL-5-producing CD3ε⁻IL-2Rα⁺ cells lack B220 (Fig. 4), we therefore depleted B220⁻CD3ε⁻IL-2Rα⁺ cells to observe the effect of these cells upon cytokine production by PP cells. Using this method, the proportion of B220⁻CD3ε⁻IL-2Rα⁺ cells in PP is diminished from 1.5% to 0.35%. IL-5, but not other cytokine production, was significantly reduced by the depletion of the CD3ε⁻IL-2Rα⁺ cells when stimulated with IL-2 or anti-CD3 mAb (Fig. 7A). IL-5 and IL-6 production by CD3ε⁻IL-2Rα⁺ depleted PP cells was also significantly reduced when stimulated with PMA+A23187 (Fig. 7B). In this case IL-5 production by CD3ε⁻IL-2Rα⁺ depleted PP cells was reduced by one-third (1,500 pg/ml to 530 pg/ml) compared with Ab-stained control PP cells. We also tested whether FACS sorting might affect cytokine production by PP cells but we did not find any effect of this procedure (data not shown). These results suggest that PP CD3ε⁻IL-2Rα⁺ cells contribute markedly to production of IL-5 and minimally to IL-6, but do not contribute to the production of IFN-γ and IL-2.

2.5 CD3ε⁻IL-2Rα⁺ cells enhance IgA production by PP B cells

Finally, we examined whether CD3ε⁻IL-2Rα⁺ cells might affect Ig-production by B cells. When B cells isolated from PP were cocultured with c-kit⁻CD3ε⁻IL-2Rα⁺ cells in the presence of LPS and IL-2, IgA, but not IgM, IgG1 and IgG2a, concentration in culture supernatants were significantly increased (Fig. 8). These observations indicate that CD3ε⁻IL-2Rα⁺ cells secrete IL-5 and help B cells to differentiate into IgA-producing plasma cells.

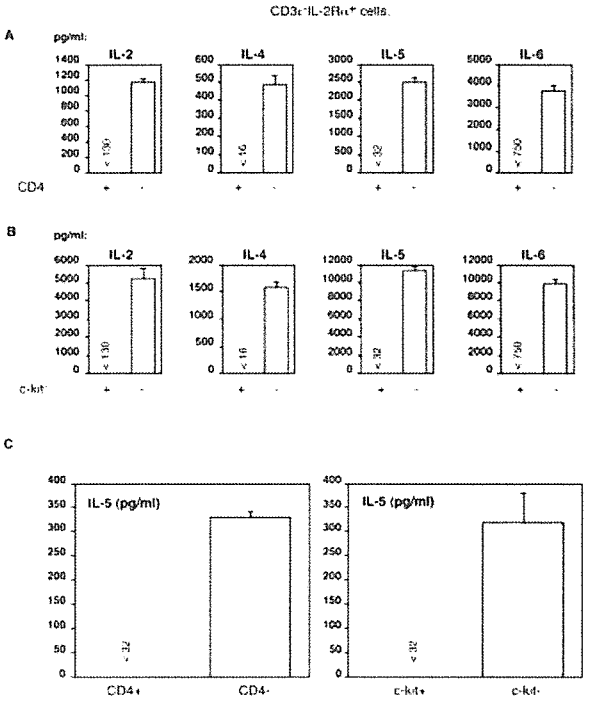

Fig. 6. CD4⁻CD3ε⁻IL-2Rα⁺ cells and c-kit⁻CD3ε⁻IL-2Rα⁺ cells produce IL-5 in response to IL-2. CD3ε⁻IL-2Rα⁺ cells were further divided into CD4⁺ and CD4⁻ populations or c-kit⁺ and c-kit⁻ populations by FACS sorting. CD4⁺ or CD4⁻ CD3ε⁻IL-2Rα⁺ cells (4×10^4 cells/well, A), c-kit⁺ or c-kit⁻ CD3ε⁻IL-2Rα⁺ cells (2×10^4 cells/well, B) were cultured in a 96-well plate. These cells were stimulated with PMA (50 ng/ml) plus A23187 (250 ng/ml, A, B) or IL-2 (250 U/ml, C). Culture supernatants were collected at 48 h and examined for secreted cytokines using the respective cytokine-specific ELISA. The results are expressed as the mean of triplicate cultures ± SD. Similar results were obtained from more than two independent experiments.

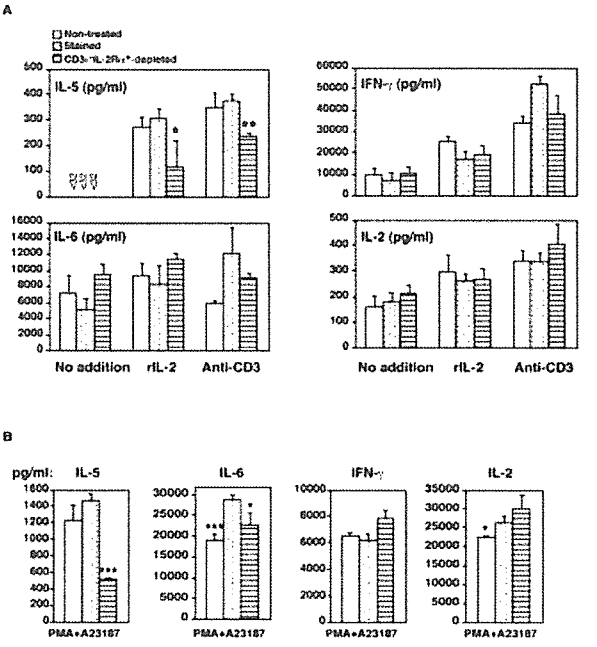

Fig. 7. CD3ε⁻IL-2Rα⁺ cells contribute to IL-5 production by PP cells. PP cells were stained with FITC-conjugated anti-B220, PE-conjugated anti-CD3ε and biotinylated anti-IL-2Rα Ab followed by streptavidin-conjugated RED 670. The B220⁻CD3ε⁻IL-2Rα⁺ cells were depleted from PP cells by FACS sorting. Non-treated PP cells, Ab-stained PP cells and B220⁻CD3ε⁻IL-2Rα⁺ depleted PP cells were cultured in a 96-well plate (1×10^6 cells/well) with or without IL-2 (50 U/ml), anti-CD3 Ab (1 μg/ml) (A), or PMA (50 ng/ml) plus A23187 (250 ng/ml) (B). Culture supernatants were collected at 48 h and analyzed for secreted cytokines using the respective cytokine-specific ELISA. The results are expressed as the mean of triplicate cultures ± SD. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ when compared with Ab-stained control PP cells. Similar results were obtained from three independent experiments.

3 Discussion

In this study we focused on IL-5 production by PP cells, and have shown that PP CD4⁻c-kit⁻CD3ε⁻IL-2Rα⁺ cells produce IL-5 in response to IL-2. Our *in vitro* analysis to assess an effect of CD4⁻c-kit⁻CD3ε⁻IL-2Rα⁺ cells on IL-5 production by PP cells showed that IL-5 production by PP cells was markedly reduced when CD3ε⁻IL-2Rα⁺ cells were depleted, even though normal numbers of naive CD4⁺ T cells or other immunocytes were present. Based on our analyses, when we reduced CD3ε⁻IL-2Rα⁺ cells from PP from 1.5% to 0.35% there was a decrease in IL-5 production from 1,500 pg/ml to 530 pg/ml. This indicates that approximately 80% of the total IL-5 produced by PP cells stimulated with PMA+A23187 is derived from CD3ε⁻IL-2Rα⁺ cells. Furthermore, comparing the ability of different cell types to produce IL-5 has shown that 4×10^5 naive PP CD4⁺ T cells produced 95 pg/ml of IL-5 (4.75×10^{-5} pg/cell) when stimulated with immobilized anti-CD3 and anti-CD28 mAb (Fig. 2A), whereas 2×10^4 PP c-kit⁻CD3ε⁻IL-2Rα⁺ cells produced 320 pg/ml of IL-5 (3.2×10^{-3} pg/cell) when stimulated with IL-2 (Fig. 6C). Though the stimulation methods differ between CD4⁺ T cells and c-kit⁻CD3ε⁻IL-2Rα⁺ cells, it remains a significant observation that CD3ε⁻IL-2Rα⁺ cells produce approximately 50 times more IL-5 than naive CD4⁺ T cells. Thus it is considerable interest that CD3ε⁻IL-2Rα⁺ cells are a major source for IL-5 production in Peyer's patches.

It is thought that IL-5 is produced by Th2 cells, Tc2 cells, γδ T cells, mast cells, NK cells [17], NKT cells [22], eosin-

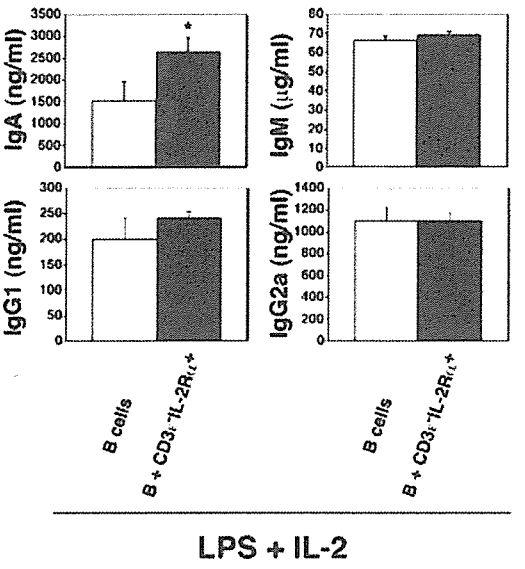

Fig. 8. CD3ε⁻IL-2Rα⁺ cells enhance IgA production by PP B cells. B220⁺ B cells were prepared from PP cells by MACS and cultured in a 96-well plate (2×10^5 cells/well) with (filled bar) or without (open bar) PP c-kit⁻CD3ε⁻IL-2Rα⁺ cells (2×10^4 cells/well) purified by FACS sorting in the presence of LPS (20 μg/ml) plus IL-2 (250 U/ml). Culture supernatants were collected at 7 days and analyzed for secreted Ab by ELISA. The results are expressed as the mean of triplicate cultures ± SD. *, $p < 0.05$ when compared to cultures without PP c-kit⁻CD3ε⁻IL-2Rα⁺ cells. Similar results were obtained from two independent experiments.

ophils [23], and epithelial cells [25]. Characterization of CD3ε⁻IL-2Rα⁺ cells shows that these cells do not belong to the T lineage because they lack CD3ε, TCRβ and TCRγδ. The lack of expression of CD23, c-kit, DX5 and NK1.1 suggests that CD3ε⁻IL-2Rα⁺ cells are not mast cells, NK cells or NKT cells. Moreover, immunohistochemical analysis reveals that CD3ε⁻IL-2Rα⁺ cells show a lymphoid morphology, therefore eliminating the possibility that they are eosinophils or epithelial cells with the potential to produce cytokines. Thus CD3ε⁻IL-2Rα⁺ cells do not belong to a previously characterized population with an ability to produce IL-5.

Yoshida et al. [26] reported that embryonic intestinal CD3⁻IL-7Rα⁺ cells, which they call PP inducers, play an essential role in PP organogenesis and that administration of anti-IL-7Rα mAb to mice during gestation disrupted PP development in the progeny. CD3⁻IL-7Rα⁺ cells are subdivided into CD4⁺ or CD4⁻ populations, and further study showed that CD4⁺CD3⁻IL-7Rα⁺ cells were able to activate VCAM-1⁺ICAM-1⁺ mesenchymal cells through LTβR to express adhesion molecules and chemokines [27]. A more recent report by Finke et al. [28] revealed that CXCL13 signaling via its receptor CXC chemokine receptor 5 (CXCR5) activated α4β1 expression on fetal CD4⁺CD3⁻ cells and induce PP development. CD4⁺CD3⁻ cells are also considered critical for secondary lymphoid tissue development including LN [29, 30] and nasopharyngeal-associated lymphoid tissue [31]. CD4⁺CD3⁻ cells are thought to be derived from fetal liver [32, 33] and migrate to developing LN [34] or intestine [33]. The migrated CD4⁺CD3⁻ cells can give rise to APC, NK cells and follicular cells but not T or B cells [33, 35]. More recently, Kim et al. [36] described a CD4⁺CD3⁻ population in adult mice which is able to support the survival of CD4 T cells. Although these populations are phenotypically similar to the CD4⁻CD3ε⁻IL-2Rα⁺ cells described here, it differs from our CD4⁻CD3ε⁻IL-2Rα⁺ cells since CD4 is expressed on these cells. On the other hand, the function of CD4⁻CD3⁻IL-7Rα⁺ cells is still unclear, though Yoshida et al. [33] suggested that these cells may have the potential to give rise to NK cells. It is possible that CD4⁻CD3⁻IL-7Rα⁺ cells in the embryonic intestine and CD4⁻CD3ε⁻IL-2Rα⁺ cells described here may represent related populations, although they do have some differences such as expression of IL-2Rα and c-kit. They may be distinct subsets, or alternatively they may be derived from a common precursor that colonizes the PP and matures to lose c-kit expression and gain IL-2Rα expression and the ability to produce cytokines in adult. Clearly, more investigations are needed to determine whether or not they are related populations.

Although at present the function of our CD4⁻CD3ε⁻IL-2Rα⁺ cells *in vivo* is unclear, our *in vitro* data (Fig. 8) suggests that these cells enhance IgA secretion by B cells. IL-5 induces maturation and differentiation of sIgA⁺ B cells into IgA-producing plasma cells, therefore it may be possible that CD3ε⁻IL-2Rα⁺ cells secrete IL-5 in response to IL-2 and help B cells to produce IgA. We also observed that CD3ε⁻IL-2Rα⁺ cells prepared from mesenteric LN secrete IL-5 in response to IL-2 (data not shown). Since PP and mesenteric LN have been considered to be inductive sites for initiation of secretory IgA immune responses in the gastrointestinal tract, CD3ε⁻IL-2Rα⁺ cells may help IgA induction at these sites. B220⁻CD3ε⁻IL-2Rα⁺ cells also exist in the gut lamina propria, known for the effector site of IgA and IgM Ab, by FACS analysis (data not shown). B1 cells, which express IL-5Rα spontaneously on their surface and respond to IL-5 to mature or differentiate into Ab-secreting plasma cells, exist in these sites so it is possible that CD3ε⁻IL-2Rα⁺ cells help in the Ab production by lamina propria B1 cells. We are now currently studying whether B220⁻CD3ε⁻IL-2Rα⁺ cells of the lamina propria could have a potential to produce IL-5 and might affect IgA or IgM production by the lamina propria B1 cells.

We observed IL-5 production by CD3ε$^-$IL-2Rα$^+$ cells in response to IL-2 (Fig. 3C and 6C). IL-2 is a principal cytokine secreted by naive CD4$^+$ T cells, so it could be considered that CD4$^-$CD3ε$^-$IL-2Rα$^+$ cells rapidly produce IL-5 in response to IL-2, which may be produced by naive CD4$^+$ T cells at the beginning of immune responses. The fact that we could not detect IL-5 production by CD3ε$^-$IL-2Rα$^+$ cells stimulated with anti-CD3 mAb, whereas IL-5 production by PP cells was significantly reduced by a depletion of CD3ε$^-$IL-2Rα$^+$ cells when stimulated with anti-CD3 mAb, are compatible with this hypothesis (Fig. 7A and data not shown).

In summary we have described a unique subset of lymphoid cells in PP from adult mice. The population is characterized by a high potential to produce IL-5, by the expression of IL-2Rα and IL-7Rα, and by an absence of CD3ε, CD4 and c-kit expression. Our data also indicate that CD4$^-$CD3ε$^-$IL-2Rα$^+$ cells are not T cells, NK cells, NKT cells, mast cells or eosinophils, all of which are capable of producing IL-5. We observed that CD4$^-$CD3ε$^-$IL-2Rα$^+$ cells produced IL-5 in response to IL-2, and that elimination of these cells from PP resulted in a marked reduction of IL-5 production *in vitro*. Furthermore, CD4$^-$CD3ε$^-$IL-2Rα$^+$ cells increased IgA secretion by B cells. Our hypothesis is that CD4$^-$CD3ε$^-$IL-2Rα$^+$ cells produce IL-5 in response to IL-2 produced by naive CD4$^+$ T cells and help IgA secretion by B cells in gut-associated lymphoreticular tissue.

4 Materials and methods

4.1 Mice

Female BALB/c and C57BL/6N mice (6–8 weeks old) were purchased from CLEA Japan (Tokyo, Japan).

4.2 Ab

The following Ab were used for staining or stimulating cells. Anti-IL-7Rα Ab (A7R34) was a kind gift from Dr. Ishikawa (Keio University School of Medicine). Anti-CD11c Ab (N418), anti-CD62L Ab (MEL-14) and anti-CD16/32 Ab (2.4G2) were purified from hybridoma culture supernatant in our laboratory. Anti-IL-2Rα (7D4), anti-CD3ε (145–2C11), anti-CD4 (H129.120), anti-CD28 (37.51), anti-Thy1.2 (30-H12), anti-B220 (RA3–6B2), anti-c-kit (2B8), anti-NK1.1 (PK136), anti-CD23 (B3B4), anti-TCR-αβ (H57–597), anti-CD8α (53–6.7), anti-B7–1 (16–10A11), anti-B7–2 (GL1), anti-CD40 (3/23), anti-CD44 (IM7), anti-LFA-1 (2D7), anti-Fas (Jo2), anti-β7 (M293) and anti-Syndecan-1 (281–2) were purchased from BD PharMingen (San Diego, CA). Anti-Mac-1 (M1/70; Caltag, San Francisco, CA), anti-DX5 (DX5 [24]; eBioscience, San Diego, CA), anti-TCR-γδ (GL3; Cedarlane, Ontario), anti-sIgD (11–26; Southern Biotechnology Associates, Birmingham, AL), anti-sIgM (1B4B1; eBioscience) and anti-α4 (R1–2; Cedarlane) were also purchased. Upon flow cytometric analysis, we checked that these Ab were able to detect surface antigens expressed on positive control cell populations. Surface phenotype of CD3ε$^-$IL-2Rα$^+$ cells was analyzed using a FACSort with CellQuest software (BD Biosciences, Mountain View, CA). Before staining, Fcγ R was blocked using 2.4G2 (anti-CD16/CD32).

4.3 Cell preparation

Both PP and SPL were digested with collagenase Type I (Sigma, St. Louis, MO) and single-cell suspension was prepared. CD4$^+$ T cells, B220$^+$ B cells and Thy1.2$^-$ cells were isolated using magnetic beads (MACS, Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's protocol. CD3ε$^-$IL-2Rα$^+$ cells were prepared from the Thy1.2$^-$B220$^-$ fraction of PP and SPL in parallel as follows. Thy1.2$^-$B220$^-$ cells were isolated by MACS and were incubated with mAb. After washing, stained cells were analyzed using a FACSort or CD3ε$^-$IL-2Rα$^+$ cells were purified using a FACS Vantage (BD Biosciences). Further, CD4$^+$CD3ε$^-$IL-2Rα$^+$ cells, CD4$^-$CD3ε$^-$IL-2Rα$^+$ cells, c-kit$^+$CD3ε$^-$IL-2Rα$^+$ cells or c-kit$^-$CD3ε$^-$IL-2Rα$^+$ cells were sorted using a FACS Vantage. The purity of sorted cells was routinely > 98%.

To deplete IL-5-producing CD3ε$^-$IL-2Rα$^+$ cells, we incubated PP cells with FITC-conjugated anti-B220, PE-conjugated anti-CD3ε and biotinylated anti-IL-2Rα Ab, followed by streptavidin-conjugated red 670 (Life Technologies, Gaithersburg, MD). B220$^-$CD3ε$^-$IL-2Rα$^+$ cells were then depleted from PP cells using a FACS Vantage.

In some experiments, we used dispase (grade II, Boehringer Mannheim, Mannheim, Germany) for collagenase to prepare PP cells and splenocytes, or PP cells were prepared by crushing PP mechanically (by non-enzymatic technique).

4.4 Cell culture

Cells were cultured at various concentrations as indicated in the figure legends in RPMI 1640 containing 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 0.03% L-glutamine and 0.2% $NaHCO_3$ for 48 h or 7 days. For cell stimulation, 50 U/ml or 250 U/ml human rIL-2 (Takeda, Tokyo, Japan), 50 ng/ml PMA (Wako, Osaka, Japan) plus 250 ng/ml calcium ionophore A23187 (Wako), 1 μg/ml anti-CD3ε Ab or 20 μg/ml LPS (Sigma) were added. To stimulate CD4$^+$ T cells plate-bound anti-CD3ε Ab (3 μg/ml) and anti-CD28 Ab (5 μg/ml) were used. The culture supernatants were collected and kept frozen until use.

4.5 ELISA

Cytokine levels and Ab titers in culture supernatants were determined by ELISA as described [37].

4.6 Cytostaining

Purified PP $CD3\varepsilon^-IL\text{-}2R\alpha^+$ cells were stimulated with PMA and ionomycin for 24 h and Golgi Stop™ (BD PharMingen) was added in the final 6 h. The cells were collected and fixed with 4% paraformaldehyde/PBS for 15 min. After washing, the cells were permeabilized in PBS/0.5% BSA/0.5% saponin/0.2% azide. The cells were then incubated with PE-conjugated anti-IL-5 Ab (TRFK5, BD PharMingen) or PE-conjugated isotype-matched IgG (BD PharMingen). After washing carefully, stained cells were analyzed using a FACSort.

4.7 Immunohistochemistry

Freshly purified $c\text{-}kit^-CD3\varepsilon^-IL\text{-}2R\alpha^+$ cells were centrifuged at 800 rpm for 5 min onto glass slides and air-dried. The cells were then stained with Diff-Quik (International Reagents, Kobe, Japan).

4.8 Statistics

Data were expressed as the mean ± SD and compared by the Student's *t*-test.

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Candida albicans and *Saccharomyces cerevisiae* induce interleukin-8 production from intestinal epithelial-like Caco-2 cells in the presence of butyric acid

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Abstract

Intestinal epithelial cells (IEC) are important in initiation and regulation of immune responses against numerous foreign substances including food, microorganisms and their metabolites in the intestine. Since the responses of IEC against yeasts have not yet been well understood, we investigated the effects of *Candida albicans*, *Saccharomyces cerevisiae*, and their cell wall components on interleukin-8 (IL-8) secretion by the IEC-like Caco-2 cells. Live cells of both yeast species stimulated Caco-2 cells to produce IL-8 only in the presence of butyric acid, which is a metabolite produced by intestinal bacteria. *S. cerevisiae* zymosan and glucan also enhanced IL-8 secretion. Treatment of Caco-2 cells with butyric acid increased the expression of mRNAs coding for Toll-like receptor 1 (TLR1), TLR6 and dectin-1, which recognize zymosan. *C. albicans* induced more IL-8 secretion and also decreased transepithelial electrical resistance more rapidly than *S. cerevisiae*. These results suggest that both yeasts in the intestine stimulate the host's mucosal immune systems by interacting with IEC.

Keywords: *Candida albicans*; *Saccharomyces cerevisiae*; Caco-2 cells; Interleukin-8; Butyric acid

1. Introduction

Intestinal epithelial cells (IEC), which line the inner surface of the intestinal lumen, function as the physical barrier of this organ. These cells are exposed to many foreign substances, including food, microorganisms and their metabolites. IEC are also important in the initiation and regulation of mucosal immune responses to foreign substances [1].

Many kinds of microorganisms, including yeasts, reside in the human intestine. In healthy humans, *Candida albicans* is a commensal yeast, but in immunosuppressed patients it becomes an opportunistic pathogen that causes mucosal candidiasis [2]. This species of yeast is also thought to participate in atopic dermatitis [3] and asthma [4]. In contrast, *Saccharomyces cerevisiae* is not a commensal yeast, although it may be transiently present in the intestine following oral ingestion. *S. cerevisiae* is recognized as nonpathogenic and is utilized for food production and as a probiotic [5,6]. Live *S. cerevisiae* cells have been found in the intestinal tract of gnotobiotic mice and human feces after oral ingestion [7]. By being present in the human intestine, *C. albicans* and *S. cerevisiae* may react with IEC and stimulate the mucosal immune system.

It has been reported that the production of cytokines, such as interleukin-1β (IL-1β), IL-6, IL-8, IL-15, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α), by IEC and IEC-like cell lines increases in response to both pathogenic [8–10] and

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nonpathogenic [11–13] bacteria. While recent studies have shown that *C. albicans* enhances the production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) by oral and vaginal epithelial cells [14,15], the response of IEC to yeasts is not known.

These responses are believed to be induced by the binding of foreign substances to receptors on IEC, among which are the Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns (PAMPs). To date, 10 of these receptors, termed TLR1 to TLR10, have been identified, and seven of them have been shown to recognize specific PAMPs. Of these, TLR2, in cooperation with TLR1 or TLR6, has been found to recognize zymosan on yeast cells and peptidoglycan on Gram-positive bacteria [16,17]. Four other receptors, TLR3, TLR4, TLR5 and TLR9 have been shown to recognize double-stranded RNA, lipopolysaccharide on Gram-negative bacteria, bacterial flagellin, and unmethylated CpG DNA, respectively [18]. An IEC-like cell line, Caco-2 is known to express mRNAs and/or proteins of TLR1, TLR2, TLR4, TLR5 and TLR6 [19–21]. In addition to TLRs, other pattern recognition receptors (PRRs) are shown to participate in responses to yeast components. Dectin-1, which binds to β-glucan contained in zymosan, mediates cytokine production by macrophage in response to zymosan [22–24].

Butyric acid is produced during carbohydrate fermentation by intestinal bacteria such as *Clostridium* spp., *Eubacterium* spp. and *Butyrivibrio* spp. [25–27]. Butyric acid has been reported to inhibit the proliferation and induce differentiation and apoptosis in the IEC-like cell lines, HT29 and Caco-2 [28,29]; hence, its presence in the intestine may have profound health consequences for the host. Because IEC are exposed to butyric acid, the synergistic action of yeasts with this metabolite may have some effects on the immune responses of the intestinal lumen. We therefore investigated whether *C. albicans*, *S. cerevisiae*, or their cell wall components induces IL-8 production by Caco-2 cells in the presence or absence of butyric acid. We also examined whether butyric acid and yeast cells alter the expression of mRNAs coding for TLRs and dectin-1.

2. Materials and methods

2.1. Microorganisms

Candida albicans JCM 1542^T and *S. cerevisiae* JCM 7255^T, purchased from the Japan Collection of Microorganisms (JCM; Wako, Japan), were grown in YM broth (Difco Laboratories, Detroit, MI) at 30 °C for 3 days. Cell suspensions of each yeast were aliquoted, washed three times with phosphate-buffered saline (PBS) by centrifugation at $1000 \times g$ at 4 °C for 10 min, resuspended in the same buffer, and kept at 4 °C for no more than 3 h prior to use. The number of viable cells in each suspension was estimated by the pour-plate count method using Potato dextrose agar (Eiken Chemical, Tokyo, Japan).

Killed organisms were prepared by heating cell suspensions in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) for 30 min at 65 °C. To confirm that all treated cells were killed, a 100 μl aliquot containing 1×10^8–3×10^8 colony-forming units (CFU) ml^{-1} prior to treatment was added to a Potato dextrose agar plate and incubated at 30 °C for 2 days.

2.2. Culture of Caco-2 cells

Caco-2 (RCB0988) cells [30,31] were purchased from the Riken Cell Bank (Tsukuba, Japan) and cultured in DMEM, supplemented with 1 mM L-glutamine (Wako Pure Chemical Industries, Osaka, Japan), 10 mM HEPES (Dojindo Molecular Technologies, Kumamoto, Japan) and 15% heat inactivated fetal bovine serum (FBS; PAA Laboratories, Linz, Austria), in 6-well culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) at 37 °C in an atmosphere of 5% CO_2. For cytokine assays and transepithelial electrical resistance (TER) measurements, 1.5×10^5 cells of passages 20–35 in 0.5 ml of medium were seeded onto a 12-mm Millicell-HA filter (Millipore, Bedford, MA). Each filter was placed into a well of a 24-well culture plate (Becton–Dickinson Labware) containing 0.5 ml medium, and the filters were cultured for 6–7 days in the same medium, changing the medium every day. For mRNA assays, 6.0×10^5 cells in 2.5 ml of medium were seeded onto a 45-mm filter. Each filter was placed in a well of a 6-well culture plate (Becton–Dickinson) containing 2.5 ml medium. The cells were incubated for 4 days in media containing various concentrations of butyric acid (0–20 mM) (Wako Pure Chemical Industries), with adjustments of the media to pH 7.4 with sodium bicarbonate. The media were refreshed every day. Caco-2 cells were subsequently co-cultured with various concentrations of *S. cerevisiae* or *C. albicans* cells or with various concentrations of the *S. cerevisiae* cell wall components, zymosan, glucan or mannan (all from Sigma, St. Louis, MO) in 0.5 ml (12-mm filter) or 2.5 ml (45-mm filter) medium, with or without butyric acid, which was added to the apical side of the filter. An identical volume of fresh medium without yeast cells or cell wall components was added to the basolateral side of the filter. Yeast cells and cell wall components were left in each well throughout the 3, 7, or 24 h co-culture period.

2.3. Measurement of secreted IL-8 and TER

For cytokine assays, basolateral supernatants were collected after co-culture for 7 or 24 h, centrifuged for 5 min at 1000*g*, divided into aliquots, and stored below −20 °C until assayed. Secreted IL-8 was determined using a human IL-8 enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Woburn, MA). TER of Caco-2 cell monolayers was assayed using a Millipore electrical cell resistance meter and probe (Millipore).

2.4. Assay of IL-8, TLR and dectin-1 mRNAs

Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) from each Caco-2 cell culture before (0 h) or after co-culture with yeasts for 3 or 7 h. An aliquot of each RNA was treated with amplification grade DNase I (Invitrogen), and cDNA was synthesized using Omniscript reverse transcriptase (Qiagen, Hilden, Germany) and oligo $d(T)_{12-18}$ primers (Amersham Bioscience, NJ). The LightCycler real-time PCR system (Roche, Mannheim, Germany) was utilized for PCR amplification of the cDNAs, using a QuantiTect SYBR Green PCR Kit (Qiagen) and oligonucleotide primer pairs specific for IL-8, TLR1, TLR2, TLR6, dectin-1 and GAPDH mRNA (Nihon Gene Research Laboratories, Sendai, Japan). The primer sequences, PCR conditions and expected amplicon size are shown in Table 1. The relative concentration of each message in the cell cultures was calculated using a standard curve for each, generated from serially diluted known standards, and the level of each transcript was normalized to that of GAPDH in the same sample. The specificity of each PCR primer pair was confirmed by observing a single peak by melting curve analysis with the LightCycler system (Roche) and by a single band of expected size by electrophoresis in 2% agarose gels (Agarose L 03, Takara Bio, Otsu, Japan), using a DNA ladder of 100–1500 bp (Takara Bio) as the molecular weight standards.

2.5. Statistical analysis

Statistical analysis was performed using SPSS software (Statistical Package for Social Sciences, Chicago, IL). When group variances were not significantly different, the statistical significance of differences between means was calculated using the two-sided Dunnett method for multiple comparisons. When the group variances were unequal, the statistical significance of differences between means was calculated using the Dunnett T3 method. Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Candida albicans enhances IL-8 secretion from Caco-2 cells only in the presence of butyric acid

Caco-2 cells were co-cultured with or without *C. albicans* (3.2×10^5 CFU ml^{-1}) for 24 h in the presence or absence of butyric acid to determine whether this species of yeast alters IL-8 secretion. Butyric acid increased dose-dependently IL-8 secretion, which was further enhanced when *C. albicans* was included in the culture medium (Fig. 1). Optimum IL-8 secretion, in the presence or absence of *C. albicans*, was observed in media containing 10 mM butyric acid (Fig. 1).

3.2. Saccharomyces cerevisiae also enhances IL-8 production by Caco-2 cells in the presence of butyric acid

We subsequently compared the ability of *C. albicans* and *S. cerevisiae* to induce IL-8 secretion from Caco-2 cells. Caco-2 cells were co-cultured with 1.6×10^7–1.6×10^3 CFU ml^{-1} *C. albicans* or 3.8×10^7–3.8×10^3 CFU ml^{-1} *S. cerevisiae*, with or without butyric acid. *S. cerevisiae* as well as *C. albicans* had no significant effect on IL-8 secretion from Caco-2 cells cultured in the

Table 1
Primer sequences and amplification conditions

Target gene	Primer sequence (5′ – 3′)		Annealing temperature (°C)	Extension time (s)	Amplicon size (bp)
GAPDH	Sense	tgaacgggaagctcactgg	63	30	307
	Antisense	tccaccaccctgttgctgta			
IL-8	Sense	tggctctcttggcagccttc	63	30	238
	Antisense	tgcacccagttttccttggg			
TLR1	Sense	ccaaggaaaagagcaaacgtg	60	15	130
	Antisense	gcagcaatatcaacaggaggaa			
TLR2	Sense	tgcggaagataatgaacacc	60	15	140
	Antisense	gatcccaactagacaaagactg			
TLR6	Sense	aagcaaacgtgggctctt	56	20	156
	Antisense	cgactgtactattcaccatcatcc			
Dectin-1	Sense	tcaatgtaagaggaagggtg	52	15	127
	Antisense	gccaagctctctaaacattt			

The amplification protocol consisted of an initial heat activation step at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at the temperature shown for 20 s, and extension at 72 °C for the time shown.

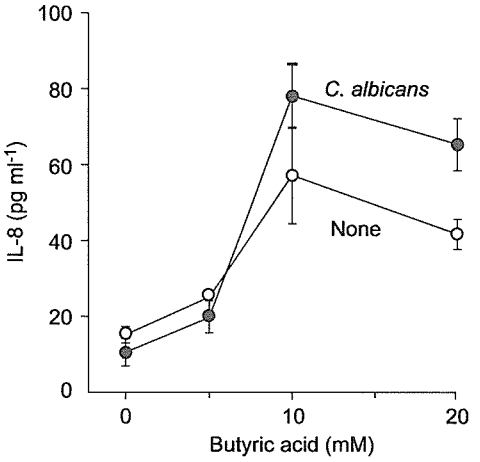

Fig. 1. IL-8 secretion from Caco-2 cells in response to intact *C. albicans* in the presence of various concentrations of butyric acid. Caco-2 cells were seeded at 1.5×10^5 cells well^{-1} on a 12-mm Millicell-HA filter and pre-cultured for 4 days in DMEM, supplemented with 1 mM L-glutamine, 10 mM HEPES and 15% heat inactivated fetal bovine serum, also containing 0–20 mM butyric acid. The cells were cultured for 24 h in the presence or absence of 3.2×10^5 CFU ml^{-1} *C. albicans* in 0.5 ml of fresh, identical medium, which was added to the apical side of the filter. IL-8 secreted into the basolateral supernatants (0.5 ml) was measured by ELISA. Data are presented as means ± SD of triplicate cultures. Data shown are representative of at least three independent experiments.

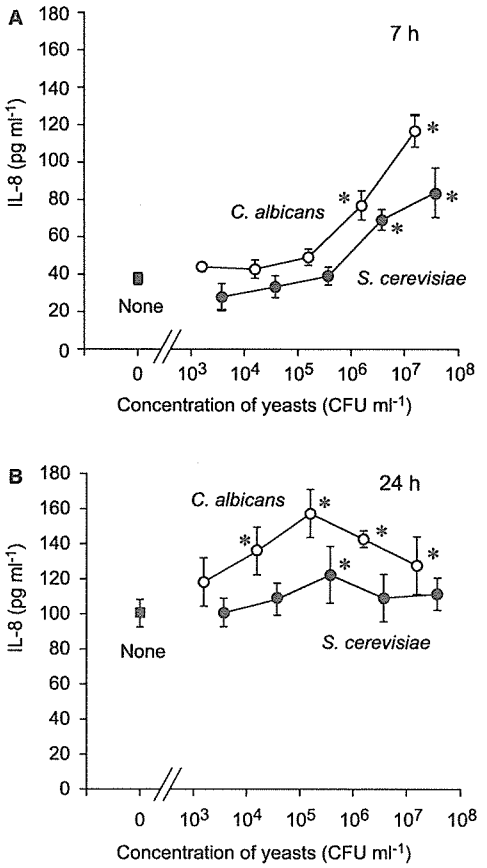

Fig. 2. IL-8 secretion from Caco-2 cells in response to intact *C. albicans* and *S. cerevisiae* in the presence of butyric acid. Caco-2 cells were seeded at 1.5×10^5 cells well^{-1} on a 12-mm Millicell-HA filter and pre-cultured for 4 days in DMEM, supplemented with 1 mM L-glutamine, 10 mM HEPES and 15% heat inactivated fetal bovine serum, also containing 10 mM butyric acid. The cells were cultured for 7 (A) or 24 h (B) in the presence or absence of *C. albicans* (1.6×10^7–1.6×10^3 CFU ml^{-1}) or *S. cerevisiae* (3.8×10^7–3.8×10^3 CFU ml^{-1}) in 0.5 ml of fresh, identical medium, which was added to the apical side of filter. IL-8 secreted into the basolateral supernatants (0.5 ml) was assayed by ELISA. Data are presented as means ± SD of five cultures. Data shown are representative of at least three independent experiments. $^*P < 0.05$ compared with untreated cells.

medium without butyric acid after co-culture for 7 or 24 h (data not shown). In the presence of 10 mM butyric acid, however, *C. albicans* concentrations of at least 1.6×10^6 CFU ml^{-1}, and *S. cerevisiae* concentrations of at least 3.8×10^6 CFU ml^{-1} induced dose-dependent secretion of IL-8 after 7 h (Fig. 2A). In contrast, after 24 h, maximal IL-8 secretion was seen at 1.6×10^5 CFU ml^{-1} *C. albicans* and at 3.8×10^5 CFU ml^{-1} *S. cerevisiae* (Fig. 2B). Heat-killed cells of both species had no effect on IL-8 secretion (data not shown).

To determine whether yeast-induced enhancement of IL-8 secretion is accompanied by increased levels of IL-8-specific mRNA, we assayed IL-8 message in these cultures by quantitative real-time PCR. Although Caco-2 cells pre-cultured with butyric acid for 4 days in the absence of yeast (0 h) showed enhanced levels of IL-8 mRNA, the addition of *C. albicans* (3.8×10^7 CFU ml^{-1}) or *S. cerevisiae* (1.4×10^7 CFU ml^{-1}) for 3 or 7 h further increased the IL-8 mRNA production (Table 2).

3.3. TER of Caco-2 cell monolayers is decreased by C. albicans more than by S. cerevisiae

We also compared the effects of *C. albicans* and *S. cerevisiae* on TER of Caco-2 cell monolayers cultured in the medium containing 10 mM butyric acid. After 7 h in culture, higher concentrations of *C. albicans* ($\geq 1.6 \times 10^4$ CFU ml^{-1}), but not *S. cerevisiae*, significantly decreased TER (Fig. 3A). After co-culturing for 24 h, *S. cerevisiae* had some effect, but the effect of *C. albicans* was still significantly greater (Fig. 3B).

3.4. Saccharomyces cerevisiae cell wall components also enhance IL-8 secretion

We assayed the effect of the *S. cerevisiae* cell wall components, zymosan, glucan and mannan (0, 10, 100 and 1000 μg ml^{-1}), to induce IL-8 secretion by Caco-2 cells in the presence of 10 mM butyric acid. We found that zymosan and glucan each had a significant, dose-dependent effect on IL-8 secretion after co-culture for 7 and 24 h. In contrast, mannan did not enhance IL-8

Table 2
Effect of butyric acid and intact yeast cells on expression of IL-8 mRNA in Caco-2 cells

Butyric acid	Microorganism (CFU ml^{-1})		IL-8		
			0 h	3 h	7 h
0 mM	None		1.0	1.0	1.5
0 mM	*C. albicans*	(6.0×10^6)	–	1.2	1.2
0 mM	*S. cerevisiae*	(2.2×10^6)	–	0.6	1.6
10 mM	None		37	42	33
10 mM	*C. albicans*	(6.0×10^6)	–	221	663
10 mM	*S. cerevisiae*	(2.2×10^6)	–	130	435

Caco-2 cells, seeded at 6×10^5 cells well^{-1} on a 45-mm Millicell-HA filter, were pre-cultured for 4 days in medium with or without 10 mM butyric acid and cultured for 0, 3, or 7 h in the presence or absence of *C. albicans* or *S. cerevisiae* in 2.5 ml of identical medium, which was added to the apical side of the filters. Data are shown as expression of IL-8 mRNA relative to that in Caco-2 cells solely pre-cultured for 4 days in medium without butyric acid. The level of each transcript was normalized to that of GAPDH in the same sample. The results shown are representative of four independent experiments.

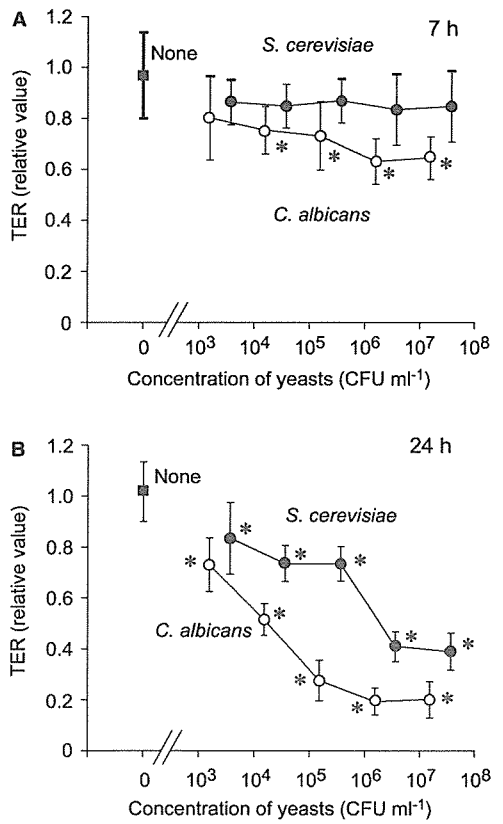

Fig. 3. Effects of intact yeasts on transepithelial electrical resistance (TER) of Caco-2 monolayers. Caco-2 cells were seeded at 1.5×10^5 cells well^{-1} on a 12-mm Millicell-HA filter and pre-cultured for 4 days in DMEM, supplemented with 1 mM L-glutamine, 10 mM HEPES and 15% heat inactivated fetal bovine serum, also containing 10 mM butyric acid. The cells were cultured for 7 (A) or 24 h (B) in the presence or absence of *C. albicans* (1.6×10^7–1.6×10^3 CFU ml^{-1}) or *S. cerevisiae* (3.8×10^7–3.8×10^3 CFU ml^{-1}) in 0.5 ml of fresh, identical medium, which was added to the apical side of the filter. The ratios of TER before and after culture with yeast are shown. Data are presented as means ± SD of five cultures. Data shown are representative of at least three independent experiments. $^*P < 0.05$ compared with untreated cells.

secretion (Fig. 4). When these cells were pre- and co-cultured in medium in the absence of butyric acid, however, 7 or 24 h culture with zymosan, glucan or mannan had no effect on IL-8 secretion (data not shown).

3.5. Butyric acid enhances TLR1, TLR6 and dectin-1 mRNAs

Since cytokine response to zymosan has been shown to be mediated by TLR2 with either TLR1 or TLR6 [16,17] and dectin-1 [22–24], we assayed the effects of butyric acid and yeast cells on the levels of TLR and dectin-1 mRNAs. In the absence of butyric acid and in the absence of yeast stimulation (0 h), Caco-2 cells expressed TLR1, TLR2, TLR6 and dectin-1 mRNAs as shown by RT-PCR (Fig. 5), and quantitative RT-PCR using the LightCycler system showed that the expression of each message was not affected by stimulation with yeast cells for 7 h (Table 3). In contrast, culture of Caco-2 cells for 4 days in medium containing 10 mM butyric acid without stimulation with yeasts (0 and 7 h) increased expression of TLR1, TLR6 and dectin-1 mRNA, but had no effect on TLR2 message (Table 3). These results shown in Table 3 suggest that, in the presence of 10 mM butyric acid, stimulation of Caco-2 cells with yeast cells decreased TLR1 and TLR6 mRNAs, but increased dectin-1 mRNA. Data from repeated experiments, however, showed that, when stimulated with yeasts, the relative amounts of TLRs and dectin-1 mRNA increased or decreased less than threefold compared with expression in unstimulated Caco-2 cells (data not shown).

4. Discussion

Our finding, that both *C. albicans* and *S. cerevisiae* enhance the production of IL-8 by IEC-like Caco-2 cells in the presence of butyric acid suggests that some yeast

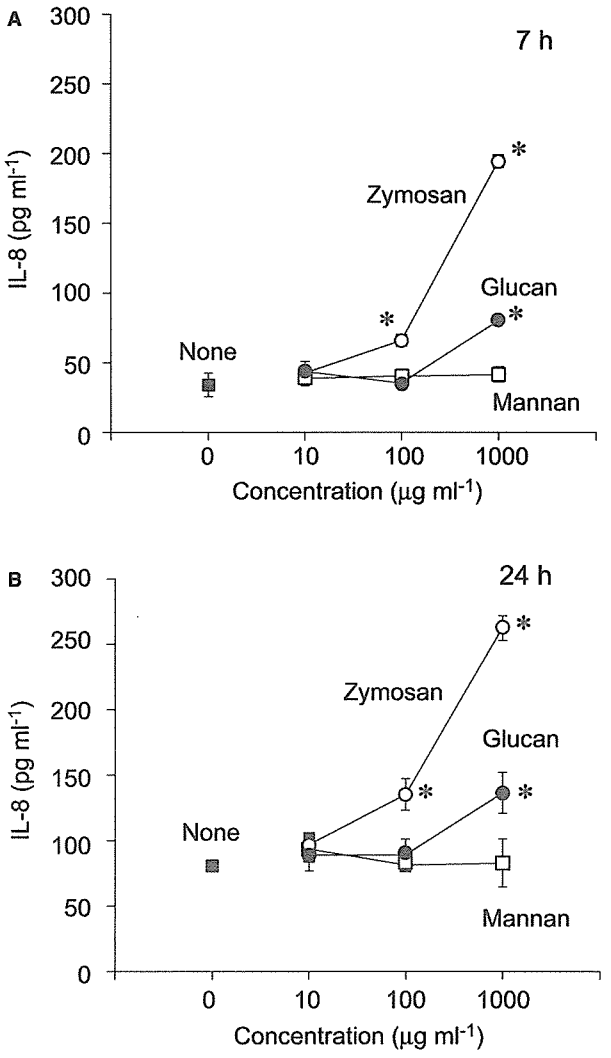

Fig. 4. IL-8 secretion from Caco-2 cells in response to *S. cerevisiae* cell wall components in the presence of butyric acid. Caco-2 cells were seeded at 1.5×10^5 cells well^{-1} on a 12-mm Millicell-HA filter and precultured for 4 days in DMEM, supplemented with 1 mM L-glutamine, 10 mM HEPES and 15% heat inactivated fetal bovine serum, also containing 10 mM butyric acid. The cells were cultured for 7 (A) or 24 h (B) in the presence or absence of various concentrations of zymosan, glucan or mannan, in 0.5 ml identical medium added to the apical side of filter. IL-8 secreted into the basolateral supernatants (0.5 ml) was assayed by ELISA. Data are presented as means ± SD of triplicate cultures. Data shown are representative of at least three independent experiments. $^*P < 0.05$ compared with untreated cells.

cells stimulate the intestinal immune system by inducing cytokine secretion from IEC. The observation that *S. cerevisiae* cells enhanced IL-8 production, albeit to a lesser extent than *C. albicans*, suggests that both opportunistic and nonpathogenic yeasts in the intestine stimulate the immune system via cytokine secretion from IEC. Under normal conditions, the weak stimulation of the host's mucosal immune system by nonpathogenic microorganisms may have favorable effects

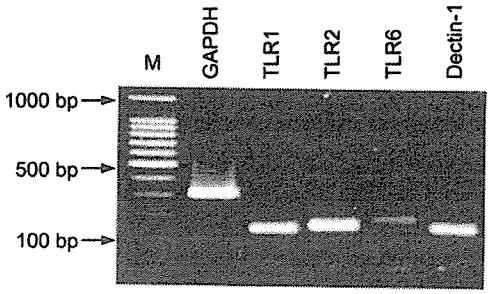

Fig. 5. RT-PCR assay of TLR1, TLR2, TLR6, and dectin-1 mRNAs in Caco-2 cells cultured in DMEM, supplemented with 1 mM L-glutamine, 10 mM HEPES and 15% heat inactivated fetal bovine serum, in the absence of butyric acid and in the absence of yeast stimulation (0 h). Lane M, 100 bp DNA ladder. Expression of mRNA for GAPDH, a housekeeping gene, was also detected as a positive control.

on the development and maintenance of this immune system.

Candida albicans is known to produce phospholipomannan, mannoprotein, proteinases, and high-affinity iron permease, all of which are considered virulent factors [32–34]. In addition, *C. albicans* has been shown to form germ tubes in response to environmental factors, such as temperature, pH, hemin, or some serum components [35–37]. These substances produced by *C. albicans* and/or these morphological changes are thought to be involved in yeast adhesion to host cells, cytokine production, and/or yeast pathogenicity. In this study, we confirmed that *C. albicans* germinated during the 7 and 24 h co-culture period (data not shown). The substances expressed by, or the morphological changes induced in, *S. cerevisiae* may be different from those in *C. albicans*, which may account for the different effects of these yeasts on cytokine secretion and TER in Caco-2 cells. The more rapid decrease in TER induced by *C. albicans* compared with *S. cerevisiae* suggests that the former induces a greater degree of damage on Caco-2 cells.

We found that the yeast cell wall component, zymosan, enhanced IL-8 secretion from Caco-2 cells. The zymosan used in our study was supposed to expose glucan to the outer side, according to the product information [38,39]. Zymosan has been reported to be recognized by TLR2, together with either TLR1 or TLR6 [16,17]. We also found that glucan enhanced IL-8 secretion. Since the inflammatory response of macrophages to β-glucan has been reported to be inhibited in a dominant-negative mutant of MyD88, a mediator of signaling through the Toll-like receptor/interleukin-1 receptor-like (TIR) domain, glucan can be considered to induce cytokine responses through TLRs [40]. However, β-glucan is also known to bind to receptors other than TLRs, including dectin-1, complement receptor 3 and scavenger receptor [23,24,41,42]. Thus, the glucan in zymosan may have been recognized by TLRs and other receptors, such as dectin-1, leading to enhanced IL-8 secretion from Caco-2 cells.

Table 3
Effect of butyric acid and intact yeast cells on TLR and dectin-1 mRNA levels in Caco-2 cells

Butyric acid	Microorganism (CFU ml^{-1})		Relative mRNA expression index							
			TLR1		TLR2		TLR6		Dectin-1	
			0 h	7 h	0 h	7 h	0 h	7 h	0 h	7 h
0 mM	None		1.0	1.2	1.0	1.1	1.0	2.4	1.0	0.2
0 mM	*C. albicans*	(2.4×10^6)	–	1.8	–	1.8	–	2.0	–	2.1
0 mM	*S. cerevisiae*	(2.4×10^6)	–	1.4	–	0.8	–	2.8	–	0.4
10 mM	None		24	38	0.4	0.6	49	66	7.3	4.7
10 mM	*C. albicans*	(2.4×10^6)	–	14	–	0.3	–	22	–	15
10 mM	*S. cerevisiae*	(2.4×10^6)	–	26	–	0.7	–	32	–	11

Caco-2 cells, seeded at 6×10^5 cells well^{-1} on a 45-mm Millicell-HA filter, were pre-cultured for 4 days in medium with or without 10 mM butyric acid cultured for 0 or 7 h in the presence or absence of *C. albicans* or *S. cerevisiae* in 2.5 ml of identical medium, which was added to the apical side of the filters. Data are shown as expression of each specific mRNA relative to that in Caco-2 cells pre-cultured for 4 days in medium without butyric acid. The level of each transcript was normalized to that of GAPDH in the same sample. The experiments were repeated at least three times.

On the other hand, intact yeast cells contain glucan in the inner layer of cell walls. We found that intact cells enhanced IL-8 secretion. Since some TLRs are expressed on vesicle membranes as well as on the cell surface [43,44] and recruited to phagosomes [16,17], intact yeast cells may have been taken up by Caco-2 cells and degraded, thus exposing their inner cell wall components, including glucan, stimulating TLR signaling inside Caco-2 cells. It is also possible that components other than glucan on the surface of yeast cells may induce various responses of mammalian cells. *C. albicans* phospholipomannan has been reported to be an important TLR2 ligand [45], and *C. albicans*- and *S. cerevisiae*-derived mannan have been shown to activate TLR4 and CD14 on human monocytes [46]. Furthermore, other components of yeast cells may bind to other PRRs, eliciting immune responses such as cytokine production. Further work is needed to determine the mechanism of yeast recognition by mammalian cells and the ensuing induction of cytokine responses.

We found that butyric acid increased the expression of TLR1, TLR6 and dectin-1 mRNAs in Caco-2 cells (Table 3). Thus, the increased expression of these molecules may be responsible for the enhanced response of Caco-2 cells to the yeast cells caused by butyric acid. Otherwise, butyric acid may affect intracellular signaling pathways downstream of some PRRs, leading to the enhancement of cytokine production. In the presence of butyric acid, stimulation of Caco-2 cells with *C. albicans* or *S. cerevisiae* either increased or decreased the expression of TLR1, TLR6 and dectin-1 mRNAs within a three-fold range, compared with expression in Caco-2 cells not stimulated with yeasts. Since the expression of TLR mRNA is known to be regulated by stimulation with the lipopolysaccharide, lipoarabinomannan and certain cytokines [47–49], it is possible that yeast stimulation directly altered mRNA expression levels. Otherwise, these alterations in mRNA expression in the presence of yeasts found in our experiment may be caused by slight differences in the characteristics of the Caco-2 cells used in each experiment, and/or analytical errors in the real-time RT-PCR method. Further experiments are required to determine the effects of yeast cells on the PAMP receptor expression.

The concentration of butyrate in the human large intestine has been estimated at 5–24 mM, depending on daily food intake, and higher than its concentration in the small intestine [50,51]. In Caco-2 cells, butyrate has been reported to affect the cell cycle, alkaline phosphatase activity, cell migration, cytokine secretion, and TER, depending on the extent of differentiation [28,52–56]. There have been conflicting reports on the effect of butyrate on IL-8 production by Caco-2 cells, with some groups reporting enhancement [53,54] and others reporting inhibition [55,56]. We confirmed that pre-culture of Caco-2 cells with 10 mM butyric acid for 4 days but not for 1 day enhanced the subsequent IL-8 secretion from Caco-2 cells stimulated with yeast cells in the presence of 10 mM butyric acid. It seems that the effects of butyric acid on cytokine secretion from IEC vary depending on the concentration of butyric acid, incubation time, and extent of IEC differentiation. Further analyses are necessary to determine the effects of butyric acid on Caco-2 cells.

Live yeast cells induced IL-8 secretion, but heat-killed cells of both species had no effect. This confirms previous findings that live *C. albicans* cells, but not heat-killed cells, induce IL-1α, IL-1β, IL-8, TNF and GM-CSF production by oral epithelial cells [14,15]. The inability of heat-killed yeasts to induce cytokine production may be due to the modification and/or destruction of the substance(s) responsible for cytokine induction, or by the absence of metabolite(s) secreted from live cells that participate in cytokine production. The induction of IL-8 by zymosan and glucan, which are extracted under harsher conditions than those we used to kill yeasts, suggests that our heat treatment may have destroyed labile substance(s) on the surface of these cells, without exposing any internal active substance(s) that retain the ability to induce cytokine production.

IL-8 is a chemokine, or chemoattractant cytokine, which has been shown to recruit neutrophils and trigger the firm adhesion of monocytes to vascular endothelial cells [57,58]. Although IL-8 secretion is often detected in proinflammatory responses, the ability of yeasts to enhance IL-8 secretion from IEC does not necessarily mean that yeasts in the intestine are promoters of inflammation. Triggering of the immune system by nonpathogenic microorganisms in the absence of inflammation may serve to enhance host defenses against occasional pathogenic microorganisms. For example, it has been reported that probiotic *Streptococcus thermophilus* and nonpathogenic *E. coli* induce IL-8 production from IEC line HT29/19A [13]. In the presence of underlying leucocytes, nonpathogenic *Lactobacillus sakei* has been shown to induce IL-8 mRNA expression by Caco-2 cells [11]. In addition, a commensal oral bacterium, *Fusobacterium nucleatum*, was found to induce IL-8 production, independent of inflammation, from gingival epithelial cells [59]. Thus, our finding, that *S. cerevisiae* induce IL-8 secretion weakly, does not suggest that this yeast provoke inflammation; rather, the microorganism may serve to prime the intestinal immune system against pathogenic organisms.

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