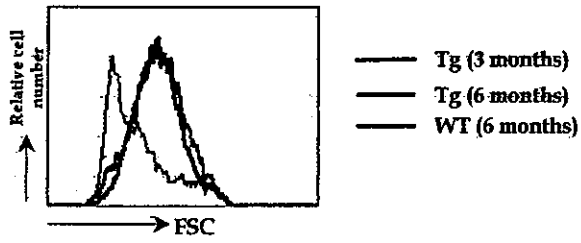
(A) Cell Sizing Analysis of SI-LPL



B) Cytokine Profiles of Expanded CD8+ T cells

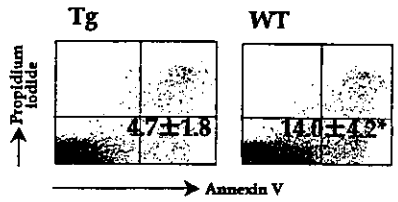
Phenotype	Tissue		Age	IFN-γ	TNF-α	IL-2
CD8αβ	SI	Tg	6 mo	65.2 ±7.5#	44.5 ±5.4#	2.5 ±1.7
		WT	6 mo	9.5 ±7.0	6.9 ±5.6	3.6 ±2.5
		Tg	3 mo	31.0±8.1*	20.1±8.1*	3.9±1.7
		WT	3 mo	10.1±0.6	8.6±2.3	4.2±1.0
CD8αα	SI	Tg	6 mo	15.3 ±14.1	11.0 ±7.4	6.5 ±2.9
		WT	6 mo	3.1±0.3	4.8±2.3	2.7 ±0.3
		Tg	3 mo	8.6±4.2	9.8±4.2	6.2±3.0
		WT	3 mo	3.0±0.1	4.6±1.1	2.9±0.2
CD8αβ	MLN	Tg	6 mo	9.7 ±0.3	5.3 ±1.9	0.5 ±0.2
		WT	6 mo	3.0 ±1.6	3.8 ±2.0	1.3 ±0.2
		Tg	3 mo	13.1±4.0	6.9±1.8	0.6±0.1
		WT	3 mo	3.0±0.6	4.3±0.9	1.2±0.1

Mean Percentage ±SEM, n=6
*p<0.05 (Tg 3 mo vs WT 3 mo)
# p<0.05 (Tg 6 mo vs WT 6 mo)

FIGURE 5. Characterization of pathological CD8αβ+ T cells developed in T3b-IL-15 Tg mice with small intestinal inflammation. Analysis of cell size of LP lymphocytes (LPL) in SI (*A*) and cytokine synthesis pattern (with percentage of positive cells) of expanded CD8αβ+ and CD8αα+ T cells in LP of the SI (SI-LP) and MLN (*B*) isolated from T3b-IL-15 Tg and WT mice. Lymphocytes isolated from the SI of Tg mice showed larger and more blastic morphology than those of WT mice. As the disease progressed, high numbers of IFN-γ- and TNF-α-secreting cells were detected in the CD8αβ+ T cells of SI-LP, but not in the CD8αβ+ T cells of MLN. The data are representative of six independent experiments. The numerical data of cells are shown as mean ± SE below each histogram (n = 6). *, $p < 0.05$ (Tg 3 mo vs WT 3 mo); #, $p < 0.01$ (Tg 6 mo vs WT 6 mo).

disturbed in intestinal inflammatory diseases (49–52). In T3b-IL-15 Tg mice, we found that intestinal CD8αβ+NK1.1+ T cells, under the influence of overexpressed IL-15, circumvented AICD-induced apoptosis and thus, continuously expanded in the intestine. In addition, the IL-2/IL-2Rα-independent nature of CD8αβ+NK1.1+ T cells further contributed to the avoidance of the AICD pathway. Thus, it appears that locally produced IL-15 disrupted the balance between proapoptotic IL-2 and anti-apoptotic IL-15, thus favoring the propagation of pathogenic CD8αβ+NK1.1+ T cells.

(A) Annexin V-PI double Staining



(B) Flowcytometric TUNEL Analysis

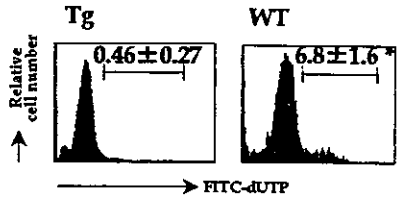


FIGURE 6. Assessment for apoptosis in LP lymphocytes (LPL) of SI isolated from T3b-IL-15 Tg and WT mice. *A* and *B*, FACS analysis performed by annexin V-propidium iodide double staining and by TUNEL staining, respectively. Lower numbers of apoptotic cells are present in T3b-IL-15 Tg mice. FACS results shown are representative of three independent experiments. Data are shown as mean ± SE. *, $p < 0.05$.

The restriction of inflammation to the SI in our model contrasts with the selective involvement of the LI in various other intestinal disease models (42, 48–52). In the colitis models, a role has been suggested for the bowel microflora (52, 53), but this does not seem to be a plausible explanation for small intestinal disease in our model. In fact, when we decontaminated the digestive tract with broad spectrum antibiotics, we found that the bacterial flora were diminished while the immunopathologic changes in the jejunum of T3b-IL-15 Tg mice remained, suggesting that bacterial microflora have a very minimal role in intestinal disease in this murine model.

Involvement of the SI is one characteristic feature of Crohn's disease. Thus, the preferential expansion and activation of CD8αβ+ NK1.1+ T cells in the SI of T3b-IL-15 Tg mice may be relevant to the pathology of this human inflammatory bowel disease. However, the complexity of the biological effects of IL-15 prevents us from ascribing the intestinal inflammation and death in our Tg mice to a single factor. Although abundant Th1-type cytokine synthesis was noted in intestinal CD8αβ+ NK1.1+ T cells of the diseased T3b-IL-15 Tg mice, low levels of Th1-type cytokine production were observed from expanded CD8αβ+ NK1.1+ T cells in MLN of the same mice. Analysis of the TCR Vβ repertoire usage of the CD8αβ+ T cells in MLN and small intestinal LP did not show any drastic skewing, but enhancement of selective

TCR Vβ usage was noted by FACS analysis (our unpublished observation). This result excludes the possibility that IL-15 production stimulated simple polyclonal expansion of CD8$\alpha\beta^+$ NK1.1$^+$ T cells and the subsequent production of IFN-γ and TNF-α. Instead, the induction and expansion of CD8$\alpha\beta^+$ NK1.1$^+$ T cells by Ags of unknown specificity may be involved.

In summary, we have established a new and novel model of small intestinal inflammation by the selective overexpression of IL-15 in the murine gastrointestinal tract, using the transgene construct T3^b-hIL-2–15 FLAG. IL-15-induced CD8$\alpha\beta^+$ T cells expressing NK1.1$^+$, CD69$^+$, and CD122 (IL-2Rβ)$^+$ appear to be critical in the pathogenesis of the small intestinal lesions in the T3^b-IL-15 Tg mice. Furthermore, this unique subset of CD8$\alpha\beta^+$ T cells preferentially produced Th1-type cytokines, and the preferential expansion of these Th1-type CD8$\alpha\beta^+$ NK1.1$^+$ T cells could be partly attributed to the anti-apoptotic activity of IL-15. The finding of small intestinal inflammation in T3^b-IL-15 Tg mice suggests that CD8$^+$ T cells and IL-15 are potential targets of therapy in chronic inflammatory diseases of the SI, such as Crohn's disease.

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Nasal vaccination induces the ability to eliminate *Candida* colonization without influencing the pre-existing antigen-specific IgE Abs: a possibility for the control of *Candida*-related atopic dermatitis

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Abstract

In some cases of atopic dermatitis (AD), a possible pathological contribution to disease development by *Candida albicans* (*C. albicans*) has been suggested. AD patients with severe symptoms showing positive capsulated hydrolic carrier polymer radioallergosorbent test (CAP-RAST) against *C. albicans* demonstrated significantly higher levels of serum IgE Abs than did AD patients with mild symptoms. Based on the clinical facts, we have postulated that elimination of *C. albicans* by mucosal vaccination may lead to the restoration of severe symptoms in AD patients. For this purpose, we have developed an allergic murine model. Mice which were systemically challenged with *C. albicans*-associated antigen, manganese superoxide dismutase (MnSOD) or secreted aspartic proteases 2 (SAP2) , together with alum, exhibited hyper IgE Abs. Systemically primed mice were then immunized with MnSOD or SAP2 plus cholera toxin (CT) as mucosal adjuvant through the nasal route. Interestingly, nasally immunized mice showed increased levels of *Candida* Ag-specific IgA Ab in fecal and nasal washes as well as in saliva samples but unchanged levels in Ag-specific IgE responses. Consistent with the Ab levels, high numbers of *Candida* Ag-specific IgA Ab-forming cells were induced in mononuclear cells isolated from intestinal lamina propria, nasal passages and salivary glands of nasally vaccinated mice with Ag plus CT. Furthermore, nasal immunization using MnSOD or SAP2 together with CT resulted in the elimination of colonized *C. albicans* from the intestinal tract. These results also suggest a potential role of mucosal vaccination in the control of *C. albicans* in patients with allergic diseases, including AD, although more research is needed to establish this therapeutic approach for mucosal vaccination.

Keywords: Atopic dermatitis; *Candida albicans*; Common mucosal immune system; IgA; Nasal immunization

1. Introduction

Atopic dermatitis (AD) is an inflammatory skin disease associated with atopic diathesis which runs a course of remission and exacerbation [1]. It has been proposed that hypersensitivity to some environmental (e.g. house dust, mites) or food allergens plays an important role in the onset of AD [2]. Since a considerable percentage of AD patients have strong immediate-type skin reactions and specific IgE Ab titers to *Candida albicans* (*C. albicans*), the role of *C. albicans* in the aggravation of AD has been a focus of investigation [1,3,4]. A previous study demonstrated that AD was also often accompanied by the multiplication of *Candida* in the intestinal tract [5]. Consequently, this AD patient group possessed high levels of *Candida*-specific IgE antibodies (Abs) and was likely to develop serious symptoms because of the simultaneous exaggeration of the Th2-type but the failure of the delayed type reaction to *Candida* [4].

Several studies have reported the relationship between indigenous bacteria and mucosal tissue or skin associated diseases. Our group previously reported that an intestinal microenvironment consisting of foods and bacterial flora such as *Bacteroides vulgatus* is considered to be a causative factor for inflammatory bowel disease [6]. Moreover, the human *Staphylococcus aureus* is a major cause of community- and hospital-acquired skin and respiratory symptoms [7]. It has also been demonstrated that some immunological relationship exists between these two surfaces, skin and mucosal tissues, where low amounts of secretory IgA (S-IgA) Abs were found in tears and on skin surfaces of AD patients [8,9]. Therefore, an obvious and interesting strategy

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would be to induce an effective *Candida*-specific immune response at the mucosal barrier where the colonization of *C. albicans* occurs which may lead to the alteration of disease condition at skin surfaces in AD patients. Anti-fungal drugs have been shown to improve the symptoms of AD patients [10,11]; however, treatment with anti-fungal drugs easily provokes the Jarisch–Herxheimer response [12]. In order to develop an alternative treatment, we have postulated that mucosally induced *C. albicans*-specific immune responses would be able to eliminate *C. albicans* effectively and safely from the gastrointestinal tract of the AD patient.

The purpose of this study was to examine whether the mucosal vaccination can induce *C. albicans*-specific S-IgA responses without affecting the existing hyper-IgE condition. In addition, we have also evaluated the efficacy of the mucosal vaccine at eliminating pre-colonized *C. albicans* from the gastrointestinal tract. Based on the results obtained by this study, we have suggested the possibility that application of nasal vaccination with *C. albicans* antigen such as manganese superoxide dismutase (MnSOD) or secreted aspartic proteases 2 (SAP2) plus cholera toxin (CT) as mucosal adjuvant could be employed for the control of the number of *C. albicans* in patients with severe AD.

2. Materials and methods

2.1. Mice

Balb/c female mice (Japan Clea Co., Tokyo, Japan) at 6–7 weeks of age were housed in the experimental animal facility at the Research Institute for Microbial Disease, Osaka University. All mice received sterilized food (certified diet MF; Oriental Yeast Co., Osaka, Japan) and autoclaved tap water.

2.2. Immunization

In order to establish a sensitized animal model, Balb/c mice were intraperitoneally challenged with MnSOD or SAP2 (100 μg) and an equal volume of alum (PIERCE, IL, USA) at days 0 and 14. Mice were then nasally administered with a 20 μl aliquot (10 μl per nostril) containing MnSOD or SAP2 (10 μg) together with CT (0.5 μg; List Biologic Laboratories, Campbell, CA) at days 63, 70, and 77 for the induction of Ag-specific mucosal immune responses. MnSOD and SAP2 were kindly provided by Takara Shuzo Co. (Kyoto, Japan).

2.3. Detection of Ag-specific Abs

Serum, saliva, fecal extract, and nasal wash were obtained as described previously [13,14]. The Ab titers in serum and external secretions were determined by ELISA as described elsewhere [13,14]. Briefly, plates were coated with MnSOD or SAP2 (100 ng/ml of PBS) and blocked with 2% bovine serum albumin (BSA) in PBS. After washing, serial dilutions of samples were added in duplicate. Following incubation, the plates were washed and peroxidase-labeled goat anti-mouse μ, γ, or α heavy chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added. The color development was done with 3,3′,5,5′-tetramethyl-benzidine (TMB; Moss Inc., Pasadena, CA). Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an OD at 414 nm of 0.1 greater than background after 15 min of incubation. In no case did non-immunized mice give titers greater than $\log_2$ of 5 in sera and $\log_2$ of 2 in external secretions.

2.4. ELISA for total and Ag-specific IgE Abs

For detection of total and Ag-specific IgE Ab titers in sera, the ELISA method was adopted as described previously [15]. In brief, the immunoplates (Nalge Nunc International, Naperville, IL, USA) were coated with purified rat anti-mouse IgE mAb (R35-72; BD PharMingen, San Diego, CA) and incubated overnight at 4 °C. After blocking with 2% BSA in PBS, serial dilutions of serum samples and mouse IgE mAb (27–74; BD PharMingen) as a standard were added and incubated for 4 h at room temperature. After extensive washing, biotinylated rat anti-mouse IgE mAb (R35-118; BD PharMingen) for total IgE Ab or biotinylated MnSOD or SAP2 for Ag-specific IgE Ab were added, respectively. Then, peroxidase-labeled anti-biotin mAb (Vector Laboratories, Burlingame, CA) was added and the color reaction was developed with TMB (Moss Inc.) for total IgE Ab and with Super Signal ELISA Pico Chemiluminescent Substrate (PIERCE) for Ag-specific IgE Ab.

2.5. Enzyme-linked immunospot (ELISPOT) assay

Spleens were aseptically removed and single cell suspensions were obtained. The nasal passage, salivary gland, and small intestine were carefully excised and mononuclear cells were obtained as described previously [14]. To assess the number of MnSOD or SAP2-specific Ab-forming cells (AFC), an ELISPOT assay was performed [14]. In brief, 96-well nitrocellulose plates (Millititer HA; Millipore, Bedford, MA) were coated with MnSOD or SAP2 (10 μg/ml of PBS) and blocked with 10% FCS in PBS. The blocking solution was discarded, cells at various dilutions were added, and the cell suspensions were incubated for 4 h at 37 °C in 5 % CO_2 incubator. The detection Ab consisted of peroxidase-conjugated goat anti-mouse μ, γ, or α heavy chain-specific Abs (Southern Biotechnology Associates). Following overnight incubation, plates were washed with PBS and spots were then developed by addition of 3-amino-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer containing H_2O_2 (Moss Inc.). Plates were incubated at room temperature for 15–20 min and washed with water, and AFCs were counted with the aid of a stereomicroscope.

2.6. Culture and colonization of C. albicans TIMM1768

C. albicans TIMM1768 [16] was kindly provided by the Research Center for Medical Mycology of Teikyo University (Tokyo, Japan) and maintained at 4 °C with monthly transfer on *Candida* isolation agar (Difco, Detroit, MI). For colonization in the gastrointestinal tract, mice were fed with autoclaved tap water containing Ampicillin (1.0 mg/ml, Nakarai tesque, Kyoto, Japan) and Kanamycin (0.2 mg/ml, Nakarai tesque) for disruption of normal microflora from 4 days before inoculation [17]. *C. albicans* TIMM1768 was harvested from the broth cultures by centrifugation, washed in PBS, and diluted to 5×10^6 cells/ml of PBS. Antibiotic-treated mice were challenged intra-gastrointestinally via a plastic catheter (Fuchigami, Kyoto, Japan) in a volume of 200 μl (1×10^6 cells) per mouse. Colonization of *C. albicans* TIMM1768 was confirmed after 3 weeks of inoculation the method described elsewhere [18].

2.7. Quantification of colony-forming units (CFU) in fecal extracts

Quantification of *Candida* colonies in feces was performed by the plate-dilution method using *Candida* isolation agar supplemented with chloramphenicol (50 mg/ml, Nakarai tesque) [19]. Extracts from fecal samples were obtained by adding weighed pellets to PBS (1 ml per 100 mg of fecal sample) and were then applied to the Agar plate. Following incubation at 37 °C for 48 h, *Candida* colonies were counted. A minimum of five mice per group were tested and each experiment was repeated at least three times.

2.8. Immunosuppression

Mice were treated with cortisone acetate (1.0 mg, Sigma, St. Louis, MO) and cyclophosphamide (6.0 mg, Wako, Osaka, Japan) intraperitoneally as described elsewhere [20].

2.9. Data analysis

Data were expressed as mean ± S.D. and evaluated by the *t*-test for unpaired samples using a Statview II statistical program (SAS Inc., Cary, North Carolina, USA) designed for the Macintosh computer. *p*-values of less than 0.05 were assumed to be statistically significant.

3. Results

3.1. Nasal immunization by Candida Ag plus CT induced Ag-specific IgA Abs without influencing the pre-existing IgE responses in systemically primed mice

In order to investigate the possible role of mucosal vaccination in the control of *Candida*-associated immune responses, we have first established a murine model in which mice have high levels of *Candida* Ag-specific IgG and IgE Abs in their systemic compartments. For this purpose, groups of mice were challenged with *Candida* Ags, such as MnSOD or SAP2, plus alum, a well-known potent Th2 stimulator [21]. As one might expect, *Candida* Ag-specific IgG and IgE Ab titers in the sera of the challenged mice were significantly higher than those of the naïve PBS group (data not shown). However, we could not find any allergic symptoms (e.g. diarrhea or skin hypersensitivity etc.) in these mice.

We next performed a series of experiments to examine the effect of nasal immunization using MnSOD or SAP2 together with CT as mucosal adjuvant in these pre-sensitized mice with high IgG and IgE Abs. Interestingly, the levels of *Candida* Ag-specific IgA Ab were significantly more increased in sera of mice nasally immunized with MnSOD or SAP2 plus CT than in mice nasally administered with PBS alone (Fig. 1). Consistent with the Ab levels, increased numbers of MnSOD or SAP2-specific IgA AFC were induced in mononuclear cells isolated from spleen of mice nasally immunized with *Candida* Ag plus CT as mucosal adjuvant. However, no significant difference was seen in the IgG Ab titers of serum and IgG AFCs of spleen between the group nasally immunized with Ag plus CT and that immunized with PBS alone. These results clearly indicate that nasal vaccination induces *Candida* Ag-specific IgA Ab responses in the systemic compartment of these pre-sensitized mice.

3.2. Induction of Ag-specific mucosal IgA responses by nasal immunization with C. albicans Ag plus CT in systemically primed mice

Since *Candida* invade through and colonize at mucosal tissues [19], it is important to induce effective immune responses at the mucosal surface for the prevention of and/or removal of *Candida* colonization in patients with AD. Therefore, we next examined if nasal vaccination would induce the *C. albicans* Ag-specific Ab responses in the mucosal compartments. Interestingly, significantly higher levels of Ag-specific IgA responses were detected in nasal wash, saliva, and fecal extracts of mice nasally administered with MnSOD or SAP2 plus CT than in those of mice administered with PBS alone (Fig. 2A). Consistent with the Ab levels, high numbers of Ag-specific IgA AFC were detected in mononuclear cells isolated from nasal passages, salivary glands, and the intestinal lamina propria of mice nasally immunized with *Candida* Ag plus CT (Fig. 2B). These results strongly suggest that nasal immunization with *C. albicans*-derived Ags together with CT can induce Ag-specific IgA Ab responses in the systemic and mucosal compartments of pre-sensitized mice with high levels of *Candida* Ag-specific IgG and IgE responses.

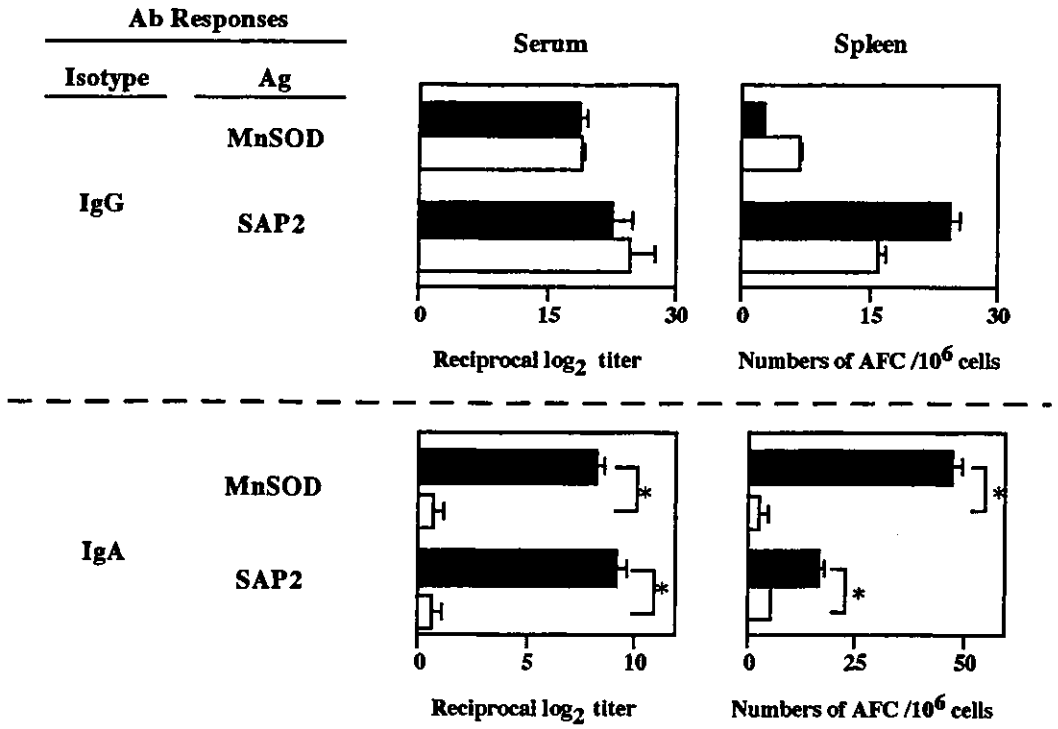

Fig. 1. Nasal immunization with MnSOD or SAP2 together with CT as mucosal adjuvant induces Ag-specific IgA Abs in serum of *Candida* pre-sensitized mice. Mice were intraperitoneally challenged with 100 μg of MnSOD or SAP2 plus alum at days 0 and 14. Then, mice were nasally administered with 10 μg of MnSOD or SAP2 together with 0.5 μg of CT as mucosal adjuvant at days 63, 70, and 77. One week after final immunization, mice were sacrificed. The levels of Ag-specific IgG and IgA Ab in serum and the numbers of Ag-specific IgG and IgA Ab-forming cells (AFC) in spleen of mice nasally immunized with MnSOD or SAP2 plus CT (■) or PBS alone (□) were determined by ELISA and ELISPOT, respectively. Bars represent the mean ± S.D. in each group. Each group consisted of five mice and the data are representative of three separate experiments. $P < 0.05$ (denoted by *) when compared with PBS group.

3.3. Nasal immunization with Candida Ag and CT did not influence the pre-existing levels of IgE Abs

To determine if nasal administration enhances IgE Ab responses, we have assessed both total and Ag-specific IgE Ab levels using ELISA. The levels of both total and Ag-specific IgE Abs in serum were determined before and after nasal immunization (days 62 and 85). As shown in Fig. 3, nasal administration with MnSOD plus CT influenced neither total nor Ag-specific IgE Ab titers. Although two courses of systemic challenge with MnSOD in alum induced high levels of IgE Abs, the nasal immunization did not affect these pre-existing IgE Ab contents. Taken together, our findings indicate that nasal immunization with *C. albicans*-derived Ag together with CT induced mucosal and systemic IgA Ab responses and that it did so without any enhancement of pre-existing IgE Ab levels.

3.4. Ag-specific immune responses induced by nasal immunization with MnSOD or SAP2 plus CT eliminated the pre-colonized C. albicans in the gastrointestinal tract and protected mice from infection

In order to assess the efficiency of nasal immunization, a strain of *C. albicans* TIMM1768 that has been shown to colonize the gastrointestinal tract of mice was used in this study (Fig. 4A). After colonization of the gastrointestinal tract by *C. albicans* TIMM1768, MnSOD- and SAP2-specific IgG and IgA Abs were slightly increased in serum but not saliva or fecal extracts (Fig. 4B and C). Mice were then nasally administered with MnSOD or SAP2 plus CT and *C. albicans* Ag-specific IgG and IgA Ab titers were determined. Interestingly, three rounds of nasal immunization using MnSOD or SAP2 together with CT elicited significant levels of Ag-specific IgA Abs in saliva and fecal extracts (Fig. 4B and C). From 1 week after final nasal immunization, CFU were monitored using the fecal extracts from each group. The numbers of CFU were significantly lower in groups of mice nasally immunized with MnSOD or SAP2 plus CT than in those administered OVA plus CT or PBS alone (Fig. 4D). As one might expect, fecal samples from the non-colonized group did not contain *C. albicans* (data not shown).

It is well established that immunosuppression with cortisone acetate and cyclophosphamide on the *C. albicans*-colonized mice results in penetrative growth by the filamentous form of *C. albicans* in the gastrointestinal tract and in subsequent dissemination to the kidney and liver [20]. To investigate the effects of nasal immunization on the infection of *C. albicans*, mice persistently colonized with

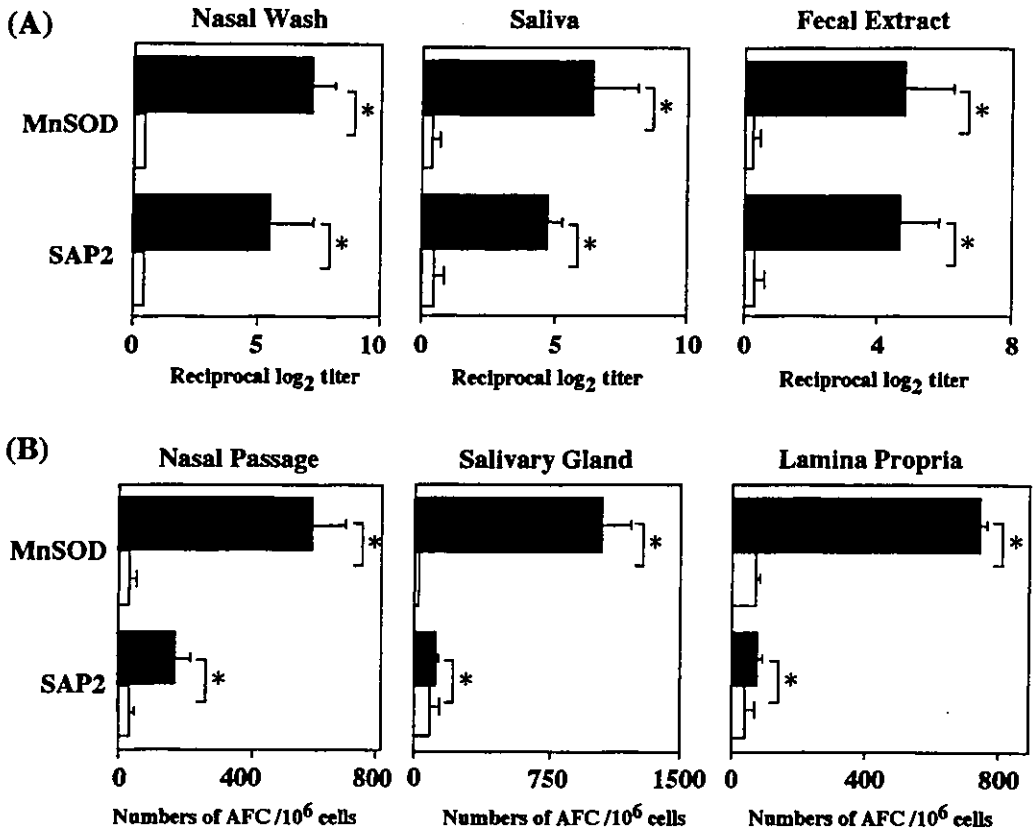

Fig. 2. Nasal immunization with MnSOD or SAP2 together with CT induces Ag-specific IgA Ab responses in mucosal compartments. The levels of Ag-specific IgA Ab in mucosal secretions and the numbers of Ag-specific IgA Ab-forming cells (AFC) in mucosal tissues of mice nasally immunized with MnSOD or SAP2 plus CT (■) or PBS alone (□) were determined by ELISA and ELISPOT, respectively. Detailed immunization methods were described in the legend of Fig. 1. Bars represent the mean ± S.D. in each group. Each group consisted of five mice and the data are representative of three separate experiments. $P < 0.05$ (denoted by *) when compared with control group.

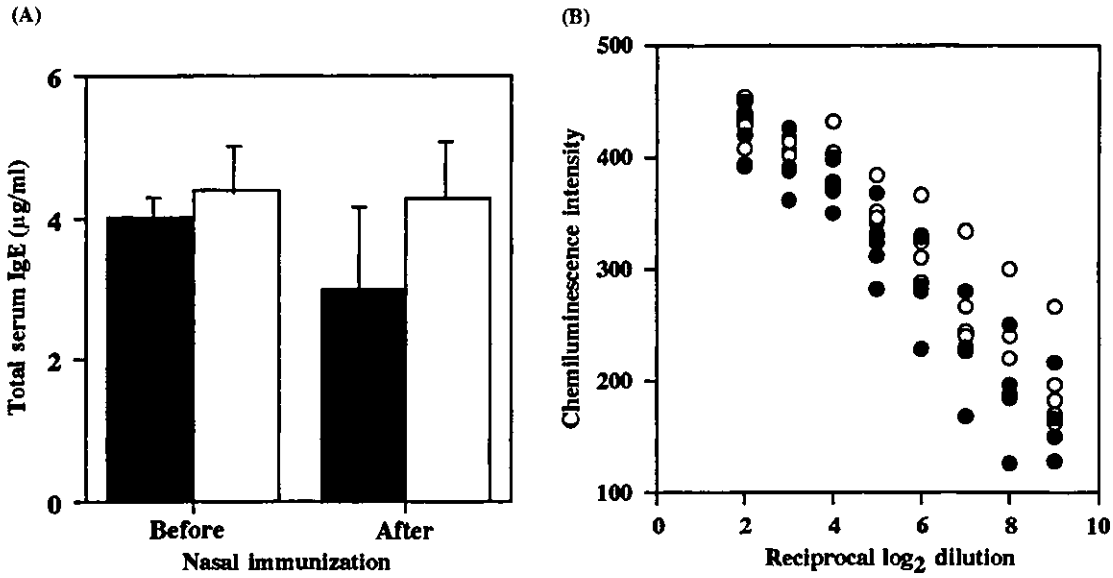

Fig. 3. Nasal immunization with MnSOD and CT did not influence the levels of pre-existing IgE Abs. The IgE Ab levels of both total (A) and MnSOD-specific (B) in serum of mice nasally immunized with MnSOD plus CT (■) or (●) or PBS alone (□) or (○) were determined by ELISA. Detailed immunization methods were described in the legend of Fig. 1. Bars represent the mean ± S.D. in each group. Each group consisted of five mice and the data are representative of three separate experiments.

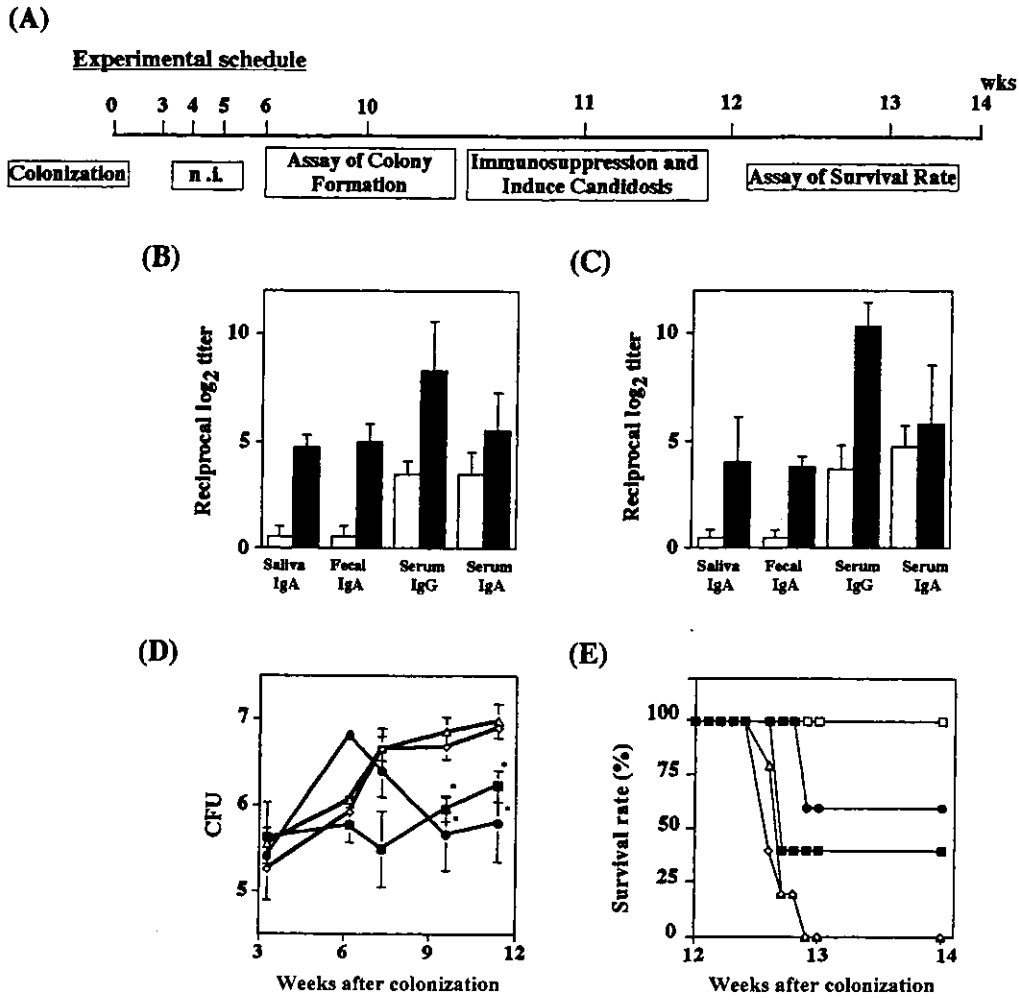

Fig. 4. Nasal immunization with MnSOD or SAP2 plus CT resulted in the removal of *C. albicans* from the intestinal tract of pre-colonized mice. Mice were pretreated for 4 days with drinking water containing Ampicillin and Kanamycin for disruption of normal microflora and then orally inoculated with *C. albicans* TIMM1768 (5×10^6 cells). After 3–5 weeks of inoculation, each group of mice received nasal immunization with MnSOD (B) or SAP2 (C) together with CT. At weeks 3 (□) and 6 (■), serum and mucosal secretions were obtained from each group and then MnSOD- or SAP2-specific IgG and IgA Ab levels were examined by ELISA. In order to assess the influence of nasal immunization on CFU of intestinal *C. albicans* (D), the groups of mice were nasally immunized with MnSOD (■), SAP2 (●) or OVA (◇) together with CT or PBS alone (△) and extracts of fecal samples were obtained. Persistently colonized mice were treated with cortisone acetate and cyclophosphamide intraperitoneally at 12 weeks and then survival rates were monitored following nasal immunization [MnSOD (■), SAP2 (●) or OVA (◇) together with CT or PBS alone (△)] (E). Groups of uninoculated mice immunosuppressed with cortisone acetate and cyclophosphamide were used as controls (□). All control groups survived. The mice were monitored daily for death. Bars represent the mean ± S.E. (B,C) and S.E. (D) in each group. Minimum of five mice per group were tested, and each experiment was repeated at least two times. $P < 0.05$ (denoted by *) when compared with PBS alone group.

C. albicans TIMM1768 were immunosuppressed with cortisone acetate and cyclophosphamide (Fig. 4A). Interestingly, groups of mice nasally administered with MnSOD or SAP2 plus CT, which demonstrated low levels of CFU, showed significantly higher survival rate than did mice nasally immunized with OVA plus CT or PBS alone (Fig. 4E). Overall, these results indicate that mucosal immunization with *Candida* Ags, MnSOD or SAP2 together with the mucosal adjuvant CT is capable of inducing effective immunity which leads to the removal of *C. albicans* and protection from candidosis.

4. Discussion

Although it is still controversial whether colonization by *C. albicans* contributes to the symptoms associated with AD [22], it has been reported that an aberrant immune response to *Candida* may be related to the severe stage of AD. A previous study has shown that there is a correlation between the severity of AD symptoms and IgE-RAST titers of *Candida* [23] and that these symptoms can often be mitigated by oral treatments with anti-fungal agents [10,11]. Further, AD patients suffering from a multiplication of

Candida in the intestinal tract [5] have also been shown to exhibit low levels of IgA responses in mucosal secretions (unpublished data from Prof. Ikezawa, Yokohama City University, Medical School, Yokohama, Japan). Therefore, it is possible that an immuno-therapy which could induce mucosal IgA Ab but not IgE Ab responses might prove to be beneficial for the control of intestinal *Candida* in AD patients with low levels of Ag-specific IgA. In this regard, we first established the sensitized murine model with *C. albicans* Ag and examined whether nasal immunization using *Candida* Ag plus CT could induce mucosal immune responses without influencing the pre-existing IgE Abs. Secondly, we adopted the colonization method of *C. albicans* in mouse intestinal tracts and examined whether mucosally induced *Candida* Ag-specific immune responses could remove *C. albicans* from intestinal tracts. The present study has demonstrated that nasal administration of *Candida* antigens together with CT induces IgA production in the mucosal compartments of pre-sensitized mice and protects these mice from colonization and infection by *C. albicans*. These findings provide fresh evidence that nasal vaccination might potentially play an important role in the development of a mucosal immuno-therapy for the control of intestinal microflora in patients with the severe form of AD.

The mucosal immune system employs tightly regulated dynamic mucosal intra- and inter-nets consisting of inductive and effector sites for the induction of an appropriate immunological homeostasis between the host and mucosal environments [24]. The common mucosal immune system (CMIS), which interconnects the inductive (e.g. Peyer's patch or nasopharyngeal-associated lymphoid tissue; NALT) and effector (e.g. intestinal lamina propria and nasal passage) tissues for the induction of the IgA response, is well characterized [25]. Thus, a large number of previous studies have provided evidence pointing to the feasibility of developing a mucosal vaccine by utilizing the CMIS [14,26–28]. Nasal immunization, like other mucosal routes, offers several advantages when compared with parenteral immunization. For example, administration is safer and more comfortable for the patient. Further, lower doses of proteins are required to induce antigen-specific Ab responses when compared with other mucosal routes (e.g. oral), decreasing the cost of vaccination. To elicit mucosal IgA responses comparable to those induced by oral immunization, only 5–10% of the quantity of vaccine is required in nasal administration [25,29]. In addition, the doses used in nasal immunization tend to induce lower total and Ag-specific IgE levels in serum [30]. Taken together, these results suggest that nasal immunization deserves greater attention as a potential new and effective antigen delivery regimen for the control of AD-associated mucosal destruction.

In this study, we demonstrated that significant *Candida* Ag-specific IgA Ab responses were induced in multiple mucosal secretions such as saliva, nasal wash, and fecal extracts in groups of mice nasally given *Candida* Ag (i.e. MnSOD or SAP2) together with CT as mucosal adjuvant. Moreover, nasal immunization of *Candida* Ag and CT in *Candida* pre-sensitized mice failed to increase total and Ag-specific IgE levels in serum. It has been reported that internal use of anti-fungal drugs leads to the improvement of AD patients suffering with severe symptoms [10]. Total IgE Abs in serum and the *Candida*-specific RAST value were dramatically decreased in these patients, and disease condition was also improved [10]. However, internal use of an anti-fungal has several disadvantages, e.g. destruction of normal microflora, transient aggravation and an efficacy which is only temporary. Mucosal vaccination, however, might be able to rebuild an appropriate mucosal immunity against *Candida* in patients with AD. Thus, it might prevent adhesion of *C. albicans* at the mucosal epithelium. Eventually, immunological homeostasis between intestinal immunity and *C. albicans* could be restored and *C. albicans*-specific IgE Ab could also be decreased for the gradual alleviation of AD symptoms.

Here, we have used two forms of *C. albicans*-associated pathogenic components, MnSOD and SAP2 [18,31–34], as vaccine Ags. In the gastrointestinal tract, the host recognizes *C. albicans* and starts producing reactive oxygen species (ROS) to interrupt fungal growth. Once *C. albicans* invades a host, MnSOD of *C. albicans* plays a pathological role by suppressing ROS activity which had been produced by host neutrophils, macrophages and others [31,32]. Further, SAPs are also widely believed to play a pathological role during the infection process of *C. albicans* [35]. SAP iso-enzymes associated with adhesion of *C. albicans* are expressed in the mucosal tissue [33]. When a mutation was inserted into the SAP region, the infection ability of *C. albicans* was dramatically reduced [36]. These results suggest a critical role for SAP2 in the virulence of *C. albicans* [36]. The fact that MnSOD protects *C. albicans* from the attack of ROS and that SAP2 helps *C. albicans* to adhere to epithelial cells suggests that enhancing levels of anti-MnSOD or anti-SAP2 IgA Abs in the intestinal secretions by nasal vaccination may act to modulate colonization and replication of *C. albicans* in the gastrointestinal tract.

Our present findings demonstrate that nasal vaccination with MnSOD or SAP2 and the mucosal adjuvant CT induces Ag-specific IgA Ab responses which are capable of eliminating *C. albicans* from the intestinal tract. Several recent studies demonstrated that vaccination with *C. albicans* extracts is effective for elimination of *C. albicans* infection [16,37–40]. For example, mice vaccinated with *C. albicans* L-mannan by intravenous injection exhibited fewer vaginal CFU of *C. albicans* [37]. It was also shown that oral candidiasis was effectively suppressed by oral immunization with formalin-fixed *C. albicans* vaccine [39]. In addition, systemic immunization with *C. albicans* membrane Ag in incomplete Freund's adjuvant induced resistance to systemic candidiasis [16]. New results presented by our study offer an alternative immunization regimen for the induction of *Candida*-specific protective immunity.

Vaccination through the mucosal tissues has been shown to be an effective and novel method for the induction of Ag-specific immune responses in both systemic and mucosal compartments [41]. To effectively adopt the mucosal immunization regimen, co-administration of mucosal adjuvant such as CT and heat labeled toxin (LT) have been shown to be necessary [14,26–29,40]. Our present results provide supportive evidence for a new therapeutic approach using nasal administration of MnSOD or SAP2 together with CT for the control of AD. However, CT has potent diarrhea-inducing properties and is unsuitable for use in humans. Thus, the general consensus could be that it is difficult to adapt this immunization regimen for mucosal immuno-therapy for AD patients. In order to overcome this obstacle, our group as well as others have been focusing on developing a mutant form of bacterial toxin which retains adjuvant activity but not toxicity. For example, the newly developed non-toxic mutant CT (mCT) can be considered as a new form of the mucosal adjuvant. Our group has developed mCTs with a mutation in the ADP-ribosyltransferase cleft of CT-A subunit which causes a loss of diarrhoeagenicity but a retention of adjuvanticity when used systemically [29] and nasally [14]. The combination of *pneumococcal* surface protein A (PspA) and mCT induced protective PspA-specific Abs, and therefore should be considered as a candidate adjuvant for vaccinating humans against *pneumococci* [26]. To further explore the potential of AD mucosal immuno-therapy, our next efforts will be focused on determining whether mCTs can be used as a mucosal adjuvant for the induction of *Candida*-specific IgA Ab responses for the elimination of pre-colonized *Candida* from the intestinal tract.

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INFLAMMATION AND INFLAMMATORY BOWEL DISEASE

Development of antigen induced colitis in SCID mice reconstituted with spleen derived memory type CD4+ CD45RB+ T cells

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Background and aims: Enteric bacterial and/or food antigens may be crucial in the development of colitis but little is known of the exact mechanism of antigen specific reactions in this condition. The aim of this study was to determine whether systemically primed antigen specific CD4+ T cells containing both CD45RB^{high} and CD45RB^{low} populations participate as a pathogenic subset that in turn leads to inflammatory reactions selectively in the large intestine.

Methods: SCID mice were reconstituted with splenic CD4+ CD45RB+ T cells or CD4+ CD45RB^{low} T cells isolated from donor mice systemically primed with ovalbumin (OVA) plus CFA. The reconstituted mice were then fed OVA for several weeks.

Results: Reconstitution of SCID mice with OVA primed splenic CD4+ T cells, containing populations of CD45RB^{high} and CD45RB^{low}, resulted in the development of colitis by 4–5 weeks following repeated administration of oral OVA. Histopathological study revealed thickened wall, inflammatory cell infiltration, crypt elongation, and loss of goblet cells in the large intestine. The CD4+ CD45RB^{low} population of cells extracted from the affected large intestine secreted high levels of interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) at the protein and mRNA levels. Administration of neutralising antibodies to TNF-α, but not to IFN-γ, prevented the development of colitis. Furthermore, adoptive transfer with OVA primed splenic CD4+ CD45RB^{low} T cells evoked severe colitis.

Conclusions: These results demonstrate that systemically primed activated/memory CD4+ CD45RB^{low} T cells can mediate the development of specific antigen induced colitis in SCID mice, and also that TNF-α is critical in the induction of this type of colitis. Our results contrast with those from studies in some colitis models in which CD45RB^{low} T cells appeared to prevent colitis through secretion of immunosuppressive cytokines.

Manipulation of genes for specific cytokines, cytokine receptors, and immunological molecules can result in chronic inflammation, preferentially in the large but not the small intestine, of experimental animals.[1–4] Several hypotheses are proposed to explain this regional disease development. Firstly, differences in the microbial environment—for example, the much greater population of bacteria in the colon—may favour the development of colonic inflammation; indeed, experimental colitis has often been abrogated under germ free conditions or after treatment with antibiotics.[5 6] Secondly, the mucosal immune environment differs between the small and large intestine.[7–9] For example, the frequency of CD4+ and αβ T cells as well as expression of lymphocyte function associated antigen 2 (LFA-2) and L-selectin are higher in the large intestine than in the small intestine.[7–9] Thus large intestinal lymphocytes may differ from small intestinal lymphocytes in their immunological responses to oral and/or enteric bacterial antigens. Thirdly, a specific cross talk immune pathway may exist between the systemic compartment (for example, the spleen) and the large intestine. In this regard, our results and those of others have shown that selected populations of T cells, derived from systemic tissue, induced localised inflammatory responses in the large intestine.[10–12]

The results obtained by different experimental models of colitis indicate that antigens derived from food and/or bacterial flora may be involved in the induction of localised inflammation.[13–15] For example, CD4+ T cells isolated from C3H/HeJBir mice, which spontaneously develop colitis, had significant proliferative and cytokine responses following exposure to protein antigens of enteric bacteria.[13] Also, we demonstrated that colitic T cell receptor (TCR)αα$^{-/-}$ mice suffered from food sensitisation, and their colonic T and B cells were reactive to food proteins such as soy bean and wheat[14] whereas TCRα$^{-/-}$ mice fed an elemental diet without antigenic proteins did not develop colitis.[15] The enteric bacterial species present in the TCRα$^{-/-}$ mice fed an elemental diet also differed substantially from those in mice fed a normal diet.[15] Taken together, these results indicate that enteric bacterial and/or food antigens may be crucial in the onset of colitis; however, little is known of the exact origin and role of antigen specific CD4+ T cells in the development of inflammation in the large intestine.

Our recent and a separate study demonstrated that adoptively transferred antigen primed splenic CD4+ T cells obtained from GFP transgenic donor mice preferentially migrated into the large but not the small intestine.[11] These results together with those of other studies[10] suggest that a unique and important immunological cross talk system exists between the spleen and large intestine. This system may influence the development of colitis. Thus a major aim of this study was to determine whether systemically primed antigen specific CD4+ T cells containing both CD45RB^{high} and CD45RB^{low} populations participate as a pathogenic subset that

Abbreviations: TNF-α, tumour necrosis factor α; IFN-γ, interferon γ; OVA, ovalbumin; TCR, T cell receptor; PBS, phosphate buffered saline; mAb, monoclonal antibody; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction; ICAM-1, intercellular adhesion molecule 1; TGF-β, transforming growth factor β; CFA, complete Freund adjuvant; LFA, lymphocyte function associated antigen; KLH, keyhole limpet haemocyanin.

in turn leads to inflammatory reactions selectively in the large intestine. To this end, SCID mice were reconstituted with splenic $CD4^+$ $CD45RB^+$ T cells or $CD4^+$ $CD45RB^{low}$ T cells isolated from donor mice systemically primed with ovalbumin (OVA) plus Complete Freund Adjuvant (CFA). The reconstituted mice were then fed OVA for several weeks. We found that spleen derived activated/memory $CD4^+$ $CD45RB^{low}$ T cells of the Th1 type from OVA primed mice and tumour necrosis factor α (TNF-α) were crucial in the development of colitis in the reconstituted SCID mice after OVA feeding.

METHODS

Mice

BALB/c and SCID mice of the same background were purchased from Japan Clea Co. (Tokyo, Japan). $STAT4^{-/-}$ mice of the BALB/c background were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). All mice were housed in the experimental animal facility at the Research Institute for Microbial Diseases, Osaka University, and fed sterilised food (certified diet MF; Oriental Yeast Co., Osaka, Japan) and tap water ad libitum. All mice were aged 6–8 weeks at the beginning of the experiments.

Immunisation and adoptive transfer

BALB/c mice were primed by the subcutaneous route with 1 mg of OVA (fraction V; Sigma Chemical Co., St Louis, Missouri, USA) or 100 μg of keyhole limpet haemocyanin (KLH) (Sigma) in 100 μl of CFA (Difco Laboratories, Detroit, Michigan, USA), respectively. One week after systemic challenge, the spleen was removed aseptically and a single cell suspension was prepared by mechanical dissociation methods, as described previously.[16] FACS separated $CD4^+$ $CD45RB^+$ T cells or $CD4^+$ $CD45RB^{low}$ T cells ($4–5\times10^5$) were resuspended in 200 μl of phosphate buffered saline (PBS) and adoptively transferred to SCID mice by tail vein injection. One week after the reconstitution, the recipient SCID mice were repeatedly challenged with 50 mg of OVA or 5 mg of KLH by gastric intubation three times a week. Body weight of naive and reconstituted SCID mice was measured every week. After 8–10 oral administrations, the spleen, and small and large intestines were removed aseptically.

Histological evaluation

For the histopathological study, the small and large intestines from recipient SCID mice were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin for evaluation of general histopathology changes and stained with periodic acid-Schiff for identification of goblet cells. The degree of inflammation in the microscopic sections of the colon was graded semiquantitatively in a blinded manner using a scoring system of 0 to 4 (0, no inflammation; 1, very mild inflammation; 2, mild inflammation; 3, moderate inflammation; 4, severe inflammation, with infiltration of mononuclear cells, crypt elongation, crypt abscesses, loss of goblet cells, and thickening of the colon wall).[17]

Neutralising monoclonal antibody treatment

SCID mice were injected intraperitoneally with 100 μg of anti-mouse interferon γ (IFN-γ) monoclonal antibody (mAb) (XMG1.2; Pharmingen, San Diego, California, USA), anti-mouse TNF-α mAb (MP6-XT3; Pharmingen), or isotype matched rat IgG2b mAb (R35-38; Pharmingen) once a week for the entire period of the experiments. The mAb treatment was started one week before the adoptive transfer.

Isolation of mononuclear cells

Intraepithelial lymphocytes and lamina propria lymphocytes from the small and large intestine were isolated as described previously.[14 18] In brief, intestinal tissues were digested using RPMI medium containing 1 mM EDTA and a collagenase (type IV 0.5 mg/ml of RPMI 1640; Sigma) after removal of Peyer's patches and colonic patches in the 37°C incubator. The single cell suspensions were pooled, washed, and placed on a discontinuous 40% and 70% Percoll gradient (Pharmacia, Uppsala, Sweden). After centrifugation for 20 minutes at 600 *g*, the cells were collected from the interface.

FACS analysis and cell sorting

Isolated mononuclear cells from the spleen, small intestine, and large intestine were preincubated with an Fc blocking mAb (2.4G2; Pharmingen) for 15 minutes on ice. The following Abs were purchased from Pharmingen and used in this study: FITC conjugated antimouse CD4 (L3T4), CD2 (RM2-5), CD11a (CL891F) mAbs, and PE conjugated antimouse CD48 (BCM1), CD69 (H1.2F3), CD44 (1M7), CD54 (3E2), and CD45RB (16A) mAbs. Two colour analysis was performed in flow cytometry analysis using a FACS Calibur (Becton Dickinson, San Jose, California, USA). In some experiments, the flow cytometry sorting separation was performed by FACS Vantage (Becton Dickinson) using FITC conjugated anti-CD4 and PE conjugated anti-CD45RB mAbs.

In vitro OVA specific proliferative responses

Mononuclear cells isolated from the spleen and large intestine of the reconstituted SCID recipients were suspended in RPMI 1640 medium (Sigma) containing 10% heat inactivated fetal calf serum, HEPES buffer, L-glutamine, penicillin, and streptomycin. The cells were cultured in the presence of 1 mg/ml of OVA for four days, as described previously.[16] To measure antigen specific proliferation, 0.5 μCi of [^{3}H]thymidine (ICN, Costa Mesa, California, USA) was added for the final 18 hour incubation and the amount of [^{3}H]thymidine incorporation was determined by scintillation counting.

Intracellular cytokine assay

For intracellular cytokine analysis, mononuclear cells isolated from the spleen and the small and large intestines were cultured with complete RPMI medium containing 10% fetal bovine serum, soluble anti-CD28 mAb (37.51, 2 μg/ml), and recombinant interleukin (IL)-2 in 24 well flat bottomed plates coated with anti-CD3ε mAb (145–2C11, 10 μg/ml) for 16 hours.[11 19] GolgiStop (2 μM/ml; Pharmingen) was added during the final four hours of incubation, and cytoplasmic staining was then performed using Cytofix/Cytoperm Kits (Pharmingen). FITC conjugated antimouse CD45RB (16A) and PE conjugated antimouse IFN-γ (XMG1.2), TNF-α (MP6-XT22), IL-4 (BUD4-1D11), IL-10 (JES5-16E3), and antirat IgG1 (R3-34) mAbs for isotype control were used. Labelled cells were analysed by flow cytometry analysis using FACS Calibur (Becton Dickinson). Unless otherwise indicated, mAbs were purchased from Pharmingen.

Quantitative RT-PCR method

A quantitative reverse transcription-polymerase chain reaction (RT-PCR) was employed for the assessment of cytokine specific mRNA expression by different subsets of $CD4^+$ T cells according to a method described previously.[11] Total RNA was extracted using TRIzol reagent (Life Technologies, Gaithesburg, Maryland, USA), and 5 μg/ml of extracted RNA was subjected to RT reaction using Superscript II reverse transcriptase (Life Technologies). The cDNA from 10 ng of RNA was used for each cytokine specific PCR (for example, IFN-γ, TNF-α, IL-4, and IL-10). Rapid cycle DNA amplification was performed by a LightCycler (Boehringer Mannheim GmbH, Mannheim, Germany) with the double strand specific dye SYBER Green I.[20] The conditions of the PCR cycle used were as follows: initial denaturation at 95°C for two minutes, followed by 45 cycles of denaturation at 95°C for 0 seconds and combined annealing-extension at 55°C for five seconds and 72°C for 10 seconds. Cycle to cycle fluorescence emission readings were plotted on the computer screen for

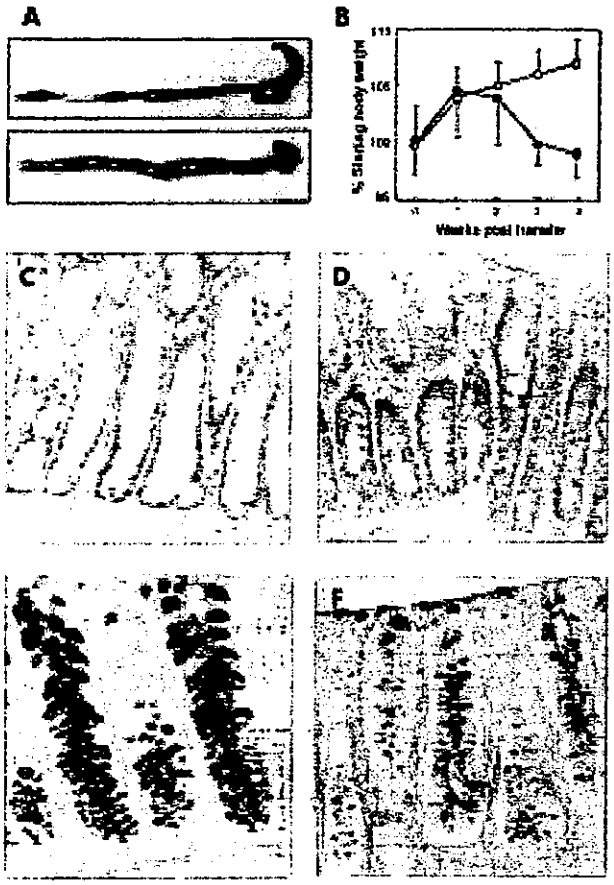

Figure 1 Induction of colitis in SCID mice by reconstitution with ovalbumin (OVA) primed splenic CD4⁺ CD45RB⁺ T cells. BALB/c mice were immunised by subcutaneous injection with OVA in complete Freund adjuvant. One week after systemic challenge, FACS separated CD4⁺ CD45RB⁺ T cells obtained from the spleens were adoptively transferred to SCID mice of the same background mice (5×10^5 cells per mouse). Control SCID mice received splenic T cells from non-primed BALB/c mice. Beginning one week after the reconstitution, the recipient SCID mice were repeatedly challenged with 50 mg of OVA by gastric intubation. After four weeks of oral challenge, tissue sections were taken from the proximal, middle, and distal regions of the large intestine and stained with haematoxylin and eosin (C, D) or periodic acid Schiff staining (E, F). (A) Gross view of the large intestine of control SCID mice without colitis (upper) and reconstituted mouse with colitis (lower). (B) Change in body weight in control (open circle) and reconstituted (filled circle) SCID mice. (C, E) Histology of a control SCID mouse without colitis. (D, F) Histology of the proximal large intestine of a reconstituted SCID mouse with a severe pathological lesion. Periodic acid-Schiff staining shows decreased numbers of goblet cells in the proximal region of the large intestine from a diseased mouse (F) compared with similar tissue from a control mouse (E). Original magnification ×100–200.

continuous monitoring of PCR product using LightCycler software.[20]

Data analysis

Data are expressed as mean (SEM) and were evaluated by the Mann-Whitney U test for unpaired samples using a Statview II statistical program (SAS Inc., Cary, North Carolina, USA) designed for the Macintosh computer. p values less than 0.05 were assumed to be statistically significant.

RESULTS

Systemically primed splenic CD4⁺ T cells induce weight loss and provoke inflammation in the large intestine of the SCID mice after specific antigen feeding

To investigate whether antigen specific CD4⁺ T cells derived from the systemic immune compartment play a pathogenic

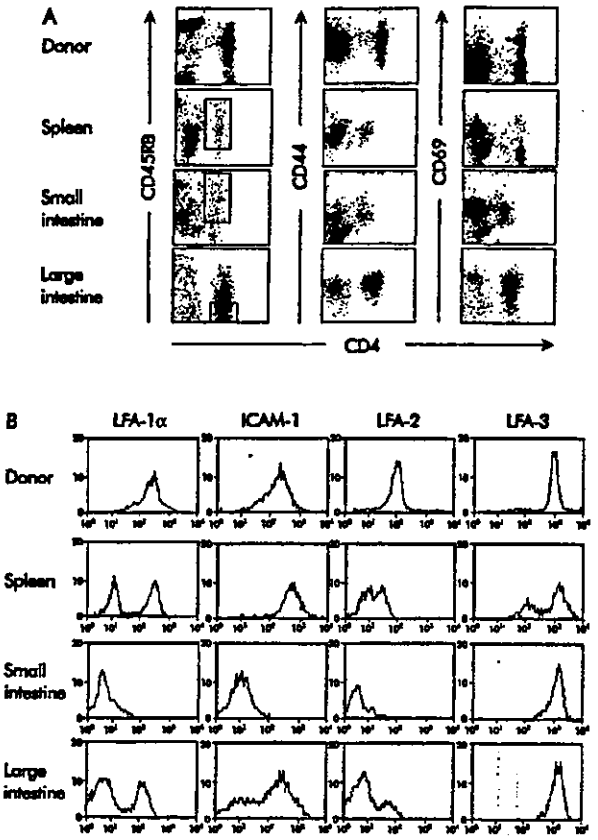

Figure 2 Expression of an activated/memory phenotype (A) and adhesion molecules (B) by the CD4⁺ T cells isolated from spleen, and small and large intestines of the reconstituted SCID mice after oral challenge with ovalbumin. The small boxes in the dot plot of the FACS data indicate the phenotype of the activated/memory type population (for example, CD45RB^{low}, CD44^{high}, and CD69^{high}). These data are representative of three independent experiments containing 3–5 mice per group. Detailed immunisation methods are described in the legend to fig 1. ICAM-1, intercellular adhesion molecule 1; LFA, lymphocyte function associated antigen.

role in the large intestine, splenic CD4⁺ T cells taken from mice systemically primed with OVA containing populations of both high and low expression levels of CD45RB molecule (CD45RB^{high} and CD45RB^{low}) were adoptively transferred to SCID mice which were then fed OVA for four weeks and sacrificed. We found that the mice had lost weight, had loose stools, and had gross colitis (fig 1A, B). Histological evaluation of the spleens and small and large intestines revealed that lesions were restricted to the large intestine with the most severe lesions being located in the proximal colon. Prominent inflammatory cell infiltration, crypt elongation, and crypt abscesses were present (fig 1D), and the number of goblet cells was decreased (fig 1F) compared with the control group (fig 1C, E).

The CD4⁺ CD45RB^{low} population was selectively expanded in the large but not the small intestine

To define the phenotype of mononuclear cells from the spleen, small intestine, and large intestine of the reconstituted SCID mice, flow cytometry analysis was performed using mAbs specific for various surface-membrane molecules (fig 2). Higher numbers of CD4⁺ T cells were found in the large intestine than in the spleen and small intestine of the recipient SCID mice following repeated oral administration of OVA (for example, the frequency of CD4⁺ cell subset: spleen 16%, small intestine 25%, and large intestine 73%). In addition, the large intestinal CD4⁺ T cells contained high

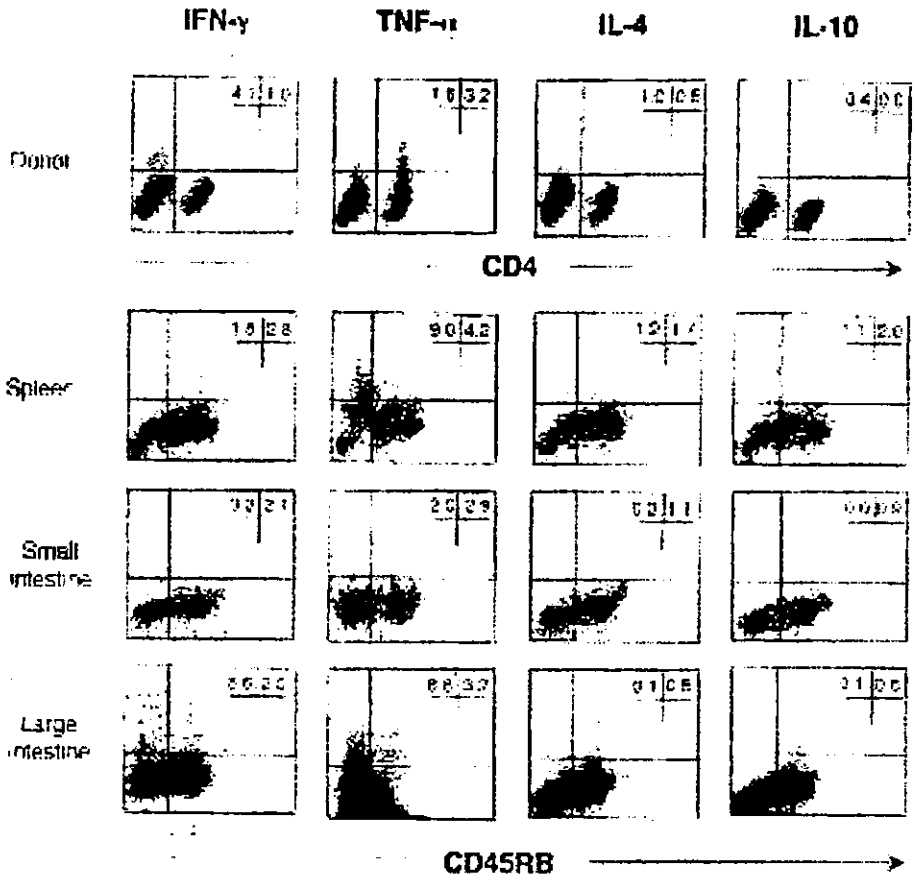

Figure 3 Analysis of cytokine production by ovalbumin primed large intestinal CD4⁺ and CD45RB^{low} T cells. Details are described in the methods section. The main quadrant line represents staining with isotype control monoclonal antibody. These data are representative of two independent experiments containing 3–5 mice per group. TNF-α, tumour necrosis factor α; IFN-γ, interferon γ; IL, interleukin.

numbers of CD45RB^{low} CD44^{high} CD69^{high} T cells which are well known phenotypes of activated/memory type T cells (fig 2A). Also, mononuclear cells isolated from the large intestine expressed high levels of LFA-1α, intercellular adhesion molecule 1 (ICAM-1), and LFA-2 compared with those of the small intestine (fig 2B). Neither large intestinal nor small intestinal mononuclear cells expressed L-selectin, $\alpha_4\beta_7$ or $\alpha_{im}\beta_7$ (data not shown). The expression pattern of adhesion molecules in mononuclear cells isolated from the spleen was similar to that in cells from the large intestine (fig 2B). As it is well established that increased expression of LFA-1, LFA-2, LFA-3, and ICAM-1 is a marker of activated/memory type cells,[21 22] our data demonstrate that activated/memory type CD4⁺ T cells preferentially migrated to and were expanded in the large intestine.

CD45RB^{low} subsets of the large intestine produced predominant Th1 type cytokines

To determine the profile of cytokine synthesis of CD4⁺ CD45RB⁺ T cells isolated from the spleen, small intestine, and large intestine of the recipient SCID mice, we performed intracellular cytokine staining and cytokine specific RT-PCR assays. Analysis of cytokine production at the single cell level revealed that a significant number of large intestinal CD45RB^{low} subsets expressed IFN-γ compared with those from the small intestine (fig 3). Furthermore, large numbers of TNF-α secreting cells were also detected in the CD45RB^{low} population of the large intestine and spleen (fig 3). In contrast, no IL-4 or IL-10 secreting cells were detected in the spleen, small intestine, or large intestine of recipient SCID mice (fig 3). When the cytokine specific RT-PCR assays were performed, colonic CD4⁺ T cells were found to possess high levels of mRNA for IFN-γ and TNF-α; however, no mRNA for IL-4 and IL-10 was detected (table 1). Taken together, these findings suggest that the development of colitis in reconstituted SCID mice is caused by the spleen derived colonic CD4⁺ CD45RB^{low} T cells producing IFN-γ and TNF-α in the colon.

Administration of neutralising Ab to TNF-α but not to IFN-γ protected the development of colitis in SCID mice reconstituted with systemically primed splenic CD4⁺ CD45RB^{low} T cells

To directly examine the role of Th1 type cytokines in the development of colitis, reconstituted SCID mice were treated

Table 1 CD4⁺ T cells obtained from the large intestines of SCID mice produce predominant interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α)

Cytokines	Location of CD4⁺ T cells	Fluorescence units (at 520 nm)*		
		30 cycle	35 cycle	40 cycle
IFN-γ	SP	2.0	10.0	13.0
	LI	8.0	16.5	18.5
TNF-α	SP	ND	ND	ND
	LI	22.5	33.5	40.0
IL-4	SP	ND	ND	ND
	LI	ND	ND	ND
IL-10	SP	ND	ND	ND
	LI	ND	ND	ND

*Reverse transcription-polymerase chain reaction methods were performed by the LightCycler (Boehringer Mannheim).
ND, not detectable; SP, spleen; LI, large intestine; IL, interleukin.

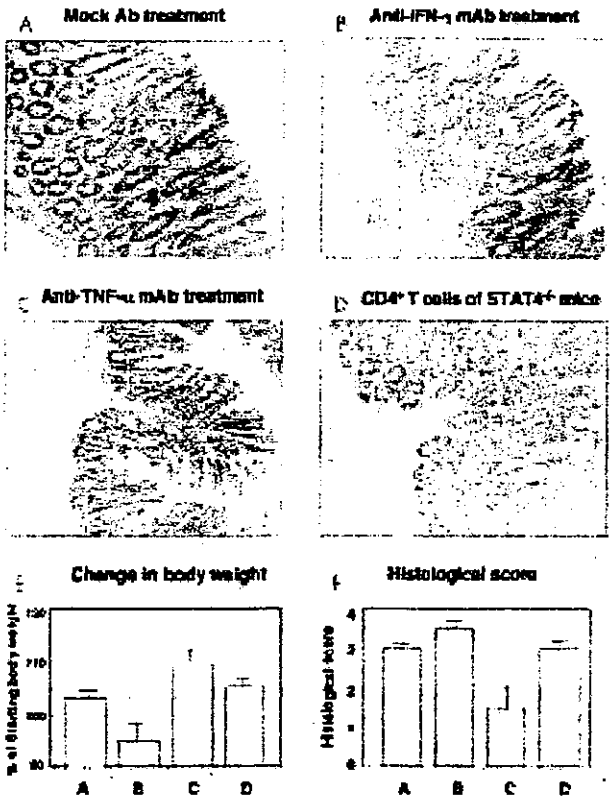

Figure 4 Effects of administering mock antibody (Ab) (A) or neutralising Abs to interferon γ (IFN-γ) (B) or tumour necrosis factor α (TNF-α) (C) to SCID mice reconstituted with splenic CD4+ CD45RB+ T cells of systemically primed BALB/c mice. Histological sections of the proximal large intestine are shown. (D) Large intestine of SCID mice reconstituted with splenic CD4+ CD45RB+ T cells of systemically primed STAT4-/- mice. (E) Percentage starting body weight in each group corresponding to those of (A–D). (F) Histological score graded semiquantitatively from 0 to 4. Details of histological score and immunisation schedules are described in the methods and in the legend to fig 1, respectively. Original magnification ×100.

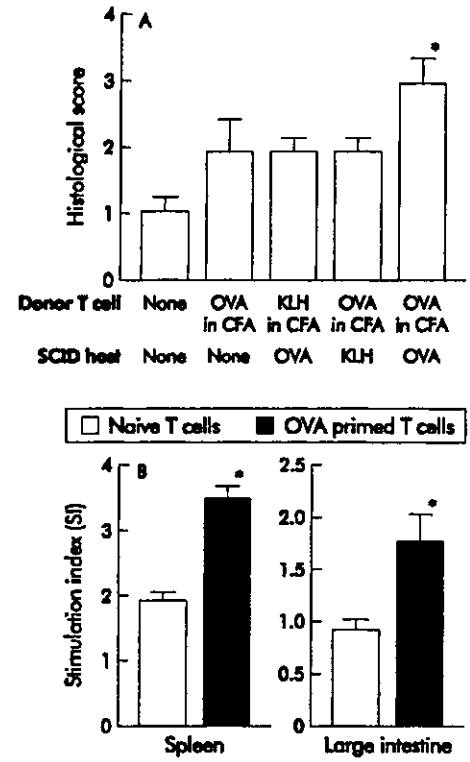

Figure 5 Role of ovalbumin (OVA) specific CD4+ T cell responses in the development of colitis. (A) Histopathological score of the proximal large intestine of the reconstituted SCID mice. Donor splenic CD4+ CD45RB+ T cells were isolated from BALB/c mice systemically primed with OVA or keyhole limpet haemocyanin (KLH) in complete freund adjuvant (CFA) and then adoptively transferred to each group of SCID recipient via tail vein injection. After one week of the reconstitution, SCID recipients were fed relevant (OVA) or irrelevant (KLH) proteins. Details of histological scoring are described in the methods section. (B) Assessment of OVA specific T cell proliferation by mononuclear cells isolated from SCID mice reconstituted with naive splenic CD4+ CD45RB+ T cells or OVA primed splenic CD4+ CD45RB+ T cells. Stimulation index is expressed as [^{3}H]thymidine incorporation by cells incubated with OVA divided by [^{3}H]thymidine incorporation by cells in controls, incubated without OVA. Levels of [^{3}H]thymidine incorporation of control wells were 500–1000 cpm. *$p<0.05$ versus group of SCID mice reconstituted with naive CD4+ CD45RB+ T cells.

with neutralising Abs to IFN-γ or TNF-α. Neutralising Abs to TNF-α but not to IFN-γ completely prevented clinical and histological evidence of colitis. Histologically, the proximal large intestine of anti-IFN-γ Ab treated SCID mice had prominent crypt elongation, crypt distortion, reduced numbers of goblet cells, and inflammatory cell infiltration (fig 4B, F) whereas these signs of disease were absent in mice treated with anti-TNF-α Ab (fig 4C). Also, mice treated with anti-IFN-γ lost weight but mice treated with anti-TNF-α did not (fig 4E). As expected, colitis was normally developed in the mock Ab treated recipient SCID mice (fig 4A, F). As neutralisation with anti-IFN-γ Ab did not protect against the development of colitis, we next tested whether the STAT4 signalling cascade is involved in the development of colitis. Donor CD4+ T cells were obtained from the spleens of STAT4$^{-/-}$ mice following subcutaneous priming with OVA in CFA. Reconstitution of SCID mice with the splenic CD4+ T cells resulted in colitis (fig 4D, F), and the level of disease severity was similar to that seen in the SCID mice reconstituted with CD4+ T cells from the primed wild type mice (fig 1D). Thus the lack of IFN-γ inducing pathway did not prevent the development of colitis by CD4+ CD45RB^{low} T cells. Taken together, our data suggest that TNF-α rather than IFN-γ is crucial for the development of the colitis studied here.

Antigen specific reactions accelerate the development of colitis

To clarify if antigen specific responses are involved in the development of colitis, SCID mice were reconstituted with splenic CD4+ CD45RB+ T cells isolated from donor mice systemically primed with OVA or KLH in CFA. After four weeks of oral administration with the relevant or irrelevant protein, the severity of colitis was compared. Severe colitis developed in SCID mice reconstituted with OVA primed T cells following oral challenge with OVA (fig 5A). In contrast, mild colitis developed in the reconstituted SCID mice following oral challenge with irrelevant protein or PBS alone; their histological scores were lower than those of SCID mice orally challenged with relevant protein (fig 5A).

To further investigate involvement of antigen specific T cell responses in the development of colitis in SCID recipients, mononuclear cells were isolated from the spleens and large intestines of SCID mice, and OVA specific T cell proliferative responses were then examined. High levels of OVA specific T cell proliferation were seen in the spleen and large intestine of the SCID recipient reconstituted with the OVA primed CD4+ CD45RB+ T cells following repeated oral administration of relevant antigen—that is, OVA (fig 5B). On the other hand, mice reconstituted with naive T cells had low levels of T cell proliferation. These results further suggest that antigen specific reactions could accelerate the development of colitis.

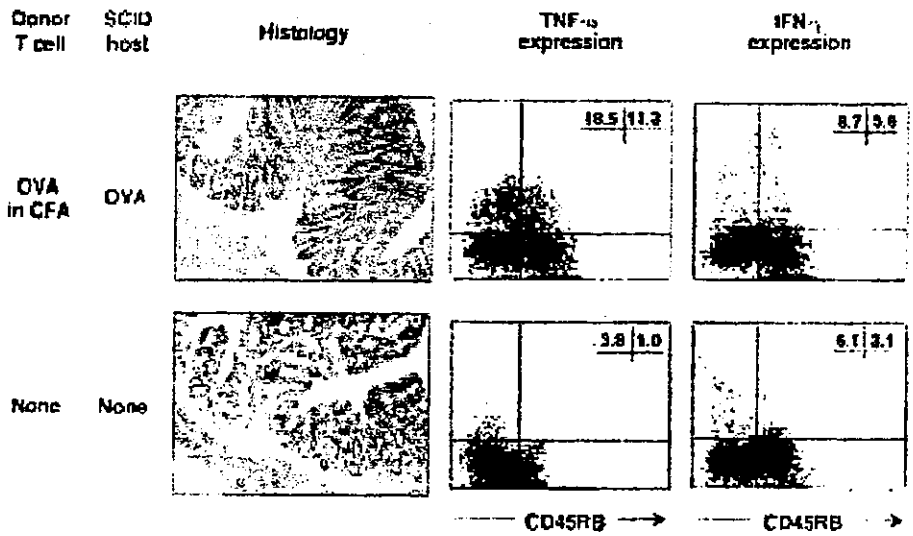

Figure 6 Pathological role of ovalbumin (OVA) primed CD4⁺ CD45RB^{low} T cells in the development of colitis. Histopathological analysis of the proximal large intestine was performed by haematoxylin and eosin staining, and Th1 type cytokine expression by the large intestinal mononuclear cells was determined by intracellular cytokine staining. SCID mice were reconstituted with unprimed or OVA primed splenic CD4⁺ CD45RB⁺ T cells. Beginning one week after reconstitution, SCID mice were repeatedly challenged by gastric intubation with 50 mg of OVA. Original magnification ×100. TNF-α, tumour necrosis factor α; IFN-γ, interferon γ; CFA, complete Freund adjuvant.

Reconstitution of OVA primed CD45RB^{low} CD4⁺ T cells leads to colitis in SCID recipients

To directly ascertain a pathogenic role for CD4 CD45RB^{low} T cells in the development of colitis, SCID mice were reconstituted with splenic CD4⁺ CD45RB^{low} T cells isolated from OVA primed mice. Following repeated oral administration of OVA, histological score of the large intestine and intracellular cytokine expression by large intestinal mononuclear cells were examined. The SCID mice adoptively transferred with OVA primed splenic CD4⁺ CD45RB^{low} T cells developed severe colitis characterised by infiltration of inflammatory cells, crypt elongation, loss of goblet cells, and thickened large intestinal wall (fig 6). Most interestingly, increased numbers of TNF-α secreting cells were detected in SCID recipients reconstituted with OVA primed splenic CD4⁺ CD45RB^{low} T cells compared with those of SCID mice adoptively transferred with non-primed CD4⁺ CD45RB^{low} T cells (fig 6). In contrast, no significant difference was seen in IFN-γ secreting cells between the two groups (fig 6). These results further implicate TNF-α in the development of colitis in this model. In summary, TNF-α producing antigen specific activated/memory CD4⁺ CD45RB^{low} T cells play a critical role in the development of colitis.

DISCUSSION

CD4⁺ T cells can be grouped into naive and memory subsets based on expression of CD45RB^{high} and CD45RB^{low}, respectively.[23] These two distinct subsets of CD4⁺ T cells seem to be the key for induction of experimental colitis. Previous studies demonstrated that SCID mice reconstituted with CD4⁺ CD45RB^{high} T cells from naive wild type mice developed colitis.[12 24–27] It has been proposed that the pathogenesis of this colitis is disturbance in the cytokine balance between the Th1 type cytokines such as IFN-γ or TNF-α and inhibitory cytokines such as IL-10 or transforming growth factor β (TGF-β).[4] In support of this concept, cotransfer of CD4⁺ CD45RB^{low} T cells with CD4⁺ CD45RB^{high} T cells prevented the development of colitis,[4 26] a result which suggested that naive spleen derived CD4 CD45RB^{low} T cells contain regulatory T cells producing suppressive cytokines, including IL-10 and/or TGF-β. In contrast, other studies have shown that the adoptive transfer of CD4⁺ T cells containing both CD45RB^{high} and CD45RB^{low} into the SCID mice resulted in a chronic and lethal inflammatory bowel disease.[28–30]

In the present study, SCID mice receiving spleen derived CD4⁺ CD45RB⁺ T cells from mice systemically primed with OVA lost weight and developed localised inflammation in the large intestine 4–5 weeks following oral administration of OVA (fig 1). As CD4⁺ CD45RB^{low} T cells from the spleens of systemically primed mice were predominantly found in the diseased region of the large intestine (fig 2), we further sought to clarify their cytokine synthesis pattern. When CD4⁺ T cells isolated from the disease region were cultured with anti-CD28 and IL-2 in anti-CD3 precoated wells, high production of IFN-γ and TNF-α was noted (fig 3). Interestingly, reconstitution with OVA primed splenic CD4⁺ CD45RB^{low} T cells into recipient SCID mice resulted in the development of severe colitis and the numbers of TNF-α secreting cells correlated with the severity of colitis (fig 6). Thus it appears that antigen primed splenic CD4⁺ CD45RB^{low} T cells can behave as a pathogenic subset via production of Th1 type cytokine (for example, TNF-α) rather than exerting a suppressive effect in this colitis model.

Uncontrolled activation and proliferation of colonic CD4⁺ T cells is believed to play a critical role in the development of colitis.[31] This notion is supported by the observation that adoptive transfer of SCID mice with concanavalin A activated splenic CD4⁺ T cells accelerated the development of colitis compared with transfer of non-activated CD4⁺ T cells.[32] In addition, colonic CD4⁺ T cells isolated from colitic SCID mice had higher numbers of proliferating cell nuclear antigen positive cells than those from SCID mice without colitis.[29] More recently, reconstitution with large (activated) CD4⁺ T cells resulted in early (6–12 weeks) and severe colitis in SCID mice while small (resting) CD4⁺ T cells developed a late onset (12–16 weeks) colitis.[32 33] In agreement with these data, our present study revealed that activated/memory type CD4⁺ CD45RB^{low} T cells from OVA primed spleen mediated the development of colitis in recipient SCID mice on repeated oral exposure with antigen. It is still unclear how oral antigen can selectively activate colonic CD4⁺ T lymphocytes. Perhaps splenic CD4⁺ pathogenic precursor T cells are activated by systemic challenge with OVA plus CFA to expand and become activated/memory CD4⁺ CD45RBlow T cells capable of producing IFN-γ and TNF-α. Also, continuous oral challenge with an identical antigen might preferentially recruit to and further stimulate these activated/memory Th1 type CD4⁺ T cells in the large intestine for the development of colitis. In

support of this view is the observation that colonic CD4+ T cells from the reconstituted SCID mice and donor splenic CD4+ T cells had an identical phenotype of adhesion molecules (fig 2B). Furthermore, high levels of antigen specific proliferative responses were detected in mononuclear cells isolated from the spleen and large intestine of SCID mice reconstituted with OVA primed splenic CD4+ T cells (fig 5B).

Several recent studies demonstrated that signal transduction via STAT molecules are critical in the development of colitis,[33–36] and IL-12/STAT-4 mediated Th1 responses were involved in Crohn's disease.[37] Furthermore, mice transgenic for STAT-4 developed severe colitis after immunisation with DNP-KLH plus CFA, suggesting that STAT-4 signalling is a critical intracellular pathway for the pathogenesis of colitis.[34] On the other hand, reconstitution with CD4+ CD45RB^{high} T cells isolated from STAT4$^{-/-}$ mice showed mild but definite colitis in reconstituted RAG2$^{-/-}$ and SCID mice.[36] In agreement with these data, we found that SCID mice that received CD4+ T cells obtained from STAT4$^{-/-}$ mice developed colitis at a later time (>20 weeks) compared with colitis in animals that received CD4+ T cells from wild type mice.[33] In the present study, the onset of colitis in SCID mice was not influenced by the actions of STAT-4 signalling as adoptive transfer of CD4+ CD45RB+ T cells from systemically primed STAT4$^{-/-}$ mice resulted in colitis (fig 4). These results suggest that Th1 type proinflammatory cytokines, which have a strong pathogenic effect for the development of colitis, are not controlled by IL-12/STAT4 signalling.

Several studies have attempted to elucidate the role of IFN-γ and TNF-α in the onset of colitis. Previous studies showed that treatment with either anti-IFN-γ or anti-TNF-α Abs protected against the development of colitis in reconstituted SCID mice.[12] In contrast, our present study revealed that administration of neutralising Abs to TNF-α, but not to IFN-γ, protected against the development of colitis. In another study, administration of anti-TNF-α Abs attenuated disease progression in murine[38] and human colitis.[39] Moreover, colitis did not develop in RAG2$^{-/-}$ mice reconstituted with CD4+ CD45RB^{high} T cells obtained from TNF$^{-/-}$ mice even although increased levels of IFN-γ expression were noted in the colons,[40] and comparably severe colitis was seen in SCID mice reconstituted with CD4+ CD45RB^{high} T cells of IFN-γ$^{-/-}$ mice.[36] These studies together with our results provide supportive evidence that neutralisation of TNF-α could be beneficial in the treatment of antigen induced colitis.

Our present results demonstrate that the antigen primed splenic CD4+ CD45RB^{low} T cell population is a pathogenic rather than a protective subset when these T cells are continuously exposed to orally administered antigen. These results reveal that spleen derived, antigen specific activated/memory CD4+ T cells can mediate the development of localised inflammation in the large intestine. Furthermore, TNF-α produced by these activated/memory CD4+ T cells appears to be crucial in the development of this colitis.

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