RESULTS

*Binding of HCV-derived peptides to HLA-A*0201 molecules*

Twenty-four HCV-derived peptides (Table 1) that have previously been defined as CTL epitopes (2,4, 10,27,28,32,34) were analyzed for their ability to bind to HLA-A*0201 molecules. The peptide-binding assay shows the binding affinity of a peptide for HLA-A*0201 molecules by measuring the stabilization of HLA-A*0201 on the TAP-deficient cell line T2 (26) pulsed with each peptide (21). According to the BL_{50} values shown in Table 1, the peptides examined were classified into three categories: 16 high binders displaying BL_{50} values less than 100 μM; 5 medium binders displaying BL_{50} values ranging from 100 to 200 μM; and 3 low binders displaying BL_{50} values of more than 200 μM. These data indicated that most of the epitopes were high binders. In particular, Core-132, Core-178, NS4-1666, NS4-1769, and NS5-2252 showed extremely high binding affinities for HLA-A*0201 molecules. On the other hand, E1-257 and E2-726 showed quite low binding capacities for HLA-A*0201 molecules.

Half-lives of peptide–class I complexes

Sixteen high binders were further evaluated by complex stability assay (Table 1). It might be expected that the long half-life of the peptide–MHC class I complexes should increase the cell surface concentration of the peptide, thereby increasing the avidity of the cell–cell interaction. An epitope present at high cell surface concentration might be able to better stimulate epitope-specific T cells. Interestingly, half-lives of the peptide–MHC class I complexes at 37oC were found to vary among the 16 high binders (Table 1). In the cases of Core-35, NS3-1406, NS3-1585, and NS4-1789, the half-life of the complexes was more than 24 h. In contrast, the half-lives of complexes associated with NS4-1666, NS4-1920, and NS5-2145 were only 2.9, 2.7, and 2.5 h, respectively. These data indicate that the stability of the peptide–MHC class I complexes is not always correlated with the binding affinity of a peptide for MHC class I molecules.

Induction of CTLs specific for HCV-derived epitopes

We next investigated the induction of CTLs specific for each epitope, using HHD mice. Mice were immunized with either Adex1SR3ST or Adex1CA3269 via the intraperitoneal route of inoculation. Adex1SR3ST and Adex1CA3269 are replication-defective recombinant adenoviruses that express the HCV structural proteins (Core, E1, and E2) and the HCV nonstructural proteins (NS3, NS4, and NS5A) under the control of the CAG promoter, respectively (15,17,32). After 2 weeks, spleen cells were harvested and stimulated *in vitro* with syn-

TABLE 2. DETECTION OF CYTOLYTIC ACTIVITIES AND QUANTITATION OF IFN-γ-SECRETING $CD8^+$ T CELLS IN RESPONSE TO EACH PEPTIDE DERIVED FROM HCV

	Lysis (%)[a]		*ICS*[b]			*Lysis (%)*		*ICS*	
Peptide	*(+)*	*(–)*	*Mi*	*Mc*	*Peptide*	*(+)*	*(–)*	*Mi*	*Mc*
Core-35	3.4	5.5	0.22	0.00	Core-132	47.7	7.0	6.83	0.02
Core-178	12.5	5.0	0.00	0.01	E1-220	3.9	5.1	0.04	0.02
E1-257	72.1	10.0	0.08	0.03	E1-363	42.9	6.3	0.08	0.02
E2-686	62.0	11.9	2.36	0.01	E2-726	73.2	9.9	0.04	0.05
E2-728	7.5	4.6	0.00	0.03	NS3-1073	29.1	5.5	0.11	0.01
NS3-1131	2.1	2.5	0.05	0.03	NS3-1169	15.2	4.0	0.00	0.05
NS3-1406	82.8	12.2	0.02	0.02	NS3-1585	48.4	9.8	0.02	0.00
NS4-1665	59.4	8.2	0.05	0.00	NS4-1671	85.3	9.7	0.17	0.01
NS4-1769	40.2	9.5	0.07	0.04	NS4-1789	57.4	9.8	0.02	0.04
NS4-1807	63.6	10.0	0.08	0.02	NS4-1851	27.6	7.1	0.00	0.02
NS4-1920	44.7	9.4	0.31	0.02	NS5-1992	13.4	4.5	0.74	0.03
NS5-2145	11.6	7.6	0.05	0.09	NS5-2252	17.7	7.4	0.08	0.02

[a]Spleen cells of immunized mice were stimulated *in vitro* with each peptide, and CTL assays were performed. Data are shown as percent specific lysis of target cells pulsed with (+) or without (–) each peptide at an *E*:*T* ratio of 150.

[b]ICS, intracellular cytokine staining; spleen cells of mice immunized with Adex1SR3ST or Adex1CA3269 (Mi), or control mice injected with Adex1w (Mc), were stimulated *in vitro* with each peptide for 5 h. ICS was then performed by staining for cell surface CD8 and antigen-induced intracellular IFN-γ in spleen cells. Data indicate percentages of intracellular IFN-γ-positive cells in $CD8^+$ cells.

geneic spleen cells pulsed with each peptide. Spleen cells of mice immunized with the wild-type adenovirus Adex1w were stimulated *in vitro* with peptide-pulsed cells as well, and were used as negative controls. One week later, CTL assays were performed at various effector:target (*E:T*) ratios. As shown in Fig. 1 and Table 2, 13 peptides including Core-132, E1-257, E1-363, E2-686, E2-726, NS3-1406, NS3-1585, NS4-1666, NS4-1671, NS4-1769, NS4-1789, NS4-1807, and NS4-1920 elicited strong peptide-specific CTL responses. Eleven of them were either high binders or medium binders of HLA-A*0201 (Tables 1 and 3). However, E1-257 and E2-726 exhibited low binding affinities for HLA-A*0201 molecules (Tables 1 and 3). On the other hand, five peptides, Core-35, E1-220, E2-728, NS3-1131, and NS5-2145, failed to induce peptide-specific CTLs (Fig. 1 and Table 2). Interestingly, all these peptides were high or medium binders with only one exception, E2-728, which was a low binder (Tables 1 and 3). These data strongly suggest that the high binding affinity for MHC class I molecules may be necessary but not sufficient for the immunogenicity of CTL epitopes. Any significant CTL response was not observed in spleen cells of mice injected with Adex1w after *in vitro* stimulation with each peptide (data not shown).

Quantitation of IFN-γ-producing CD8$^+$ T cells in response to HCV-derived peptides

To further examine the immunogenicity of HCV-derived peptides, frequencies of HCV epitope-specific CD8$^+$ T cells were examined by staining for the cell surface CD8 molecule and antigen-induced intracellular IFN-γ in the spleen cells of immunized mice. Because the cells were stimulated *in vitro* with an appropriate peptide for only 5 h, the possibility of substantial *in vitro* expansion of responder cells was precluded (3). As shown in Table 2 and Fig. 2, high frequencies of IFN-γ-producing CD8$^+$ T cells were detected for Core-132 (6.83%) and E2-686 (2.36%), which belong to the high binder and medium binder groups, respectively (Tables 1 and 3). As shown in Fig. 1 and Table 2, both peptides induced high

TABLE 3. CLASSIFICATION OF TWENTY-FOUR HCV-DERIVED CTL EPITOPES ON THE BASIS OF CURRENT DATA

		Type I Antigens[a]		
Peptide	*Affinity*[b] *(high/medium)*	*Lysis*[c] *(high/medium)*	*ICS*[d] *(high/medium)*	*Stability*[e]
Core-132	27.5	40.7	6.81	3.6
NS4-1920	57.1	35.3	0.29	2.7
NS3-1073	66.9	23.6	0.10	8.1
NS4-1671	104.3	75.6	0.16	NT
E2-686	156.9	50.1	2.35	NT
		Type II Antigens		
Peptide	*Affinity (high/medium)*	*Lysis (high/medium)*	*ICS (low/ND)*	*Stability*
NS4-1665	27.0	51.2	0.05	2.9
NS4-1769	28.3	30.7	0.03	13.4
NS4-1789	45.3	47.6	ND	24<
NS3-1585	49.6	38.6	0.02	24<
NS4-1851	68.8	20.5	ND	4.8
NS3-1406	76.7	70.6	ND	24<
E1-363	130.7	36.6	0.06	NT
NS4-1807	172.5	53.6	0.06	NT
		Type III Antigens		
Peptide	*Affinity (low)*	*Lysis (high)*	*ICS (medium)*	*Stability*
E1-257	416.4	62.1	0.05	NT
E2-726	607.7	63.3	ND	NT

(continued)

TABLE 3. CLASSIFICATION OF TWENTY-FOUR HCV-DERIVED CTL EPITOPES ON THE BASIS OF CURRENT DATA (*CONT'D*)

Type IV Antigens

Peptide	*Affinity (high)*	*Lysis (low/ND)*	*ICS (medium)*	*Stability*
NS5-1992	37.8	8.9	0.71	10.2
Core-35	42.5	ND	0.22	24<

Type V Antigens

Peptide	*Affinity (high/medium)*	*Lysis (low/ND)*	*ICS (low/ND)*	*Stability*
NS5-2252	19.8	10.3	0.06	11.9
Core-178	23.0	7.5	ND	17.7
NS5-2145	51.2	ND	ND	2.5
NS3-1169	60.0	11.2	ND	9.7
NS3-1131	60.3	ND	0.02	5.5
E1-220	166.6	ND	0.02	NT

Type VI Antigens

Peptide	*Affinity (low)*	*Lysis (ND)*	*ICS (ND)*	*Stability*
E2-728	204.5	ND	ND	NT

[a]Type I: High or medium in the affinity, high or medium in the lysis, and high or medium in the ICS. Type II: High or medium in the affinity, high or medium in the lysis, and low or ND in the ICS. Type III: Low in the affinity, and high in the lysis. Type IV: High in the affinity, low or ND in the lysis, and medium in the ICS. Type V: High or medium in the affinity, low or ND in the lysis, and low or ND in the ICS. Type VI: Low in the affinity, ND in the lysis and ICS.

[b]BL_{50} in peptide-binding assays. High, less than 100 μM; medium, 100–200 μM; low, more than 200 μM.

[c]Percentage of specific lysis of target cells pulsed with each peptide minus percentage of specific lysis of target cells pulsed with no peptide at an $E:T$ ratio of 150 in CTL assays. High, more than 30%; medium, 20–30%; low, less than 20%; ND, not detected.

[d]ICS, intracellular cytokine staining; percentage of intracellular IFN-γ-positive cells in $CD8^+$ T cells in mice immunized with Adex1SR3ST or Adex1CA3269 minus that in control mice injected with Adex1w. High, more than 1.0%; medium, 0.1–1.0%; low, less than 0.1%; ND, not detected.

[e]Data of complex stability assays. NT, not tested.

levels of peptide-specific CTL responses. On the other hand, modest numbers of IFN-γ-producing $CD8^+$ T cells were observed in response to Core-35 (0.22%), NS3-1073 (0.11%), NS4-1671 (0.17%), NS4-1920 (0.31%), and NS5-1992 (0.74%) (Table 2). None of the remaining peptides significantly induced IFN-γ-producing $CD8^+$ T cells (Table 2).

DISCUSSION

In the current study, a number of HLA-A*0201-restricted, HCV-derived peptides were evaluated by examining the peptide-binding affinity for MHC class I molecules, the stability of the peptide–MHC complexes, killing activities of peptide-induced CTLs and frequencies of intracellular IFN-γ-positive $CD8^+$ T cells. On the basis of these results, 24 peptides tested have been classified into 6 types (Table 3).

Among 24 peptides, 15 peptides containing type I, II, and III epitopes induced high or medium killing activities of peptide-specific CTLs. On the other hand, the remaining nine peptides from types IV to VI could not induce good CTL responses (Table 3), although all the peptides had previously been defined as epitopes for HCV-specific CTLs (2,10,27,32). Thirteen of the 15 peptides that stimulated good peptide-specific CTL responses exhibited high or medium binding affinities for HLA-A*0201 molecules (Table 3, types I and II), whereas only 2 of them were low binders (Table 3, type III). On the other hand, type IV and type V peptides did not elicit any effective CTL response although they were high or medium binders of HLA-A*0201. These data confirm that high binding affinity for MHC class I mol-

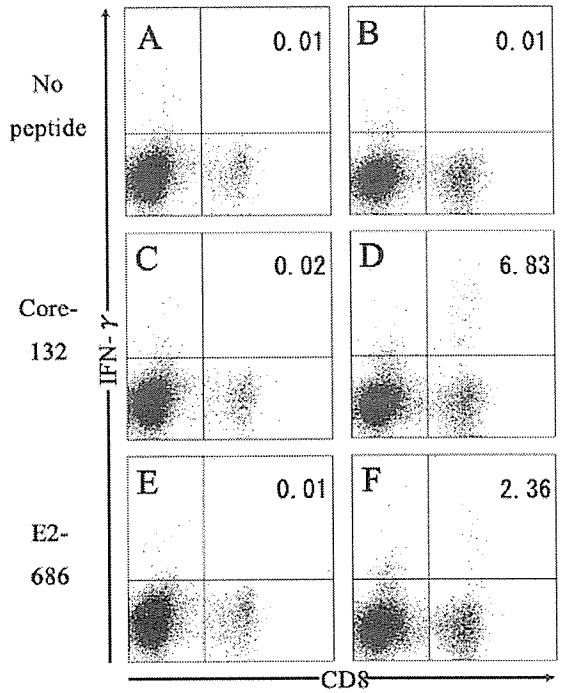

FIG. 2. Intracellular IFN-γ staining of CD8$^+$ T cells in response to HCV-derived peptides. Mice were injected with p3XFLAG-IL-12 and immunized intraperitoneally with 5×10^7 PFU of either Adex1w (**A**, **C**, and **E**) or Adex1SR3ST (**B**, **D**, and **F**). One week later, spleen cells were prepared and cultured with or without (**A** and **B**) either Core-132 (**C** and **D**) or E2-686 (**E** and **F**) for 5 h. After stimulation, cells were stained for their surface expression of CD8 (x axis) and intracellular IFN-γ (y axis). Numbers shown indicate the percentages of CD8$^+$ cells that are positive for intracellular IFN-γ. Data shown are representative of three independent experiments. Three mice per group were used in each experiment, and spleen cells of all mice per group were pooled.

ecules is essential but not sufficient for a peptide to be highly immunogenic. Because the mice in this study were immunized with adenovirus expressing HCV proteins, there could be a number of factors influencing immunogenicity here, including the fact that HCV proteins and other adenoviral proteins are necessarily expressed together in the immunization process. Therefore, finding high-affinity binders that do not express high-level CTL killing may not be due solely to binding affinity. One possible explanation is that there might be potential competition for multiple epitopes including HCV epitopes in the antigen processing and presentation. Further, it must be considered that murine versus human processing, presentation, and, ultimately, immunodominance may differ. It is obvious that a low capacity for binding to HLA-A*0201 should explain why E2-728 (Table 3, type VI) was so poorly immunogenic. In contrast to the binding affinity, the stability of peptide–MHC class I complexes was not correlated with killing activities of peptide-induced CTLs.

Surprisingly, only a limited number of peptides could induce high or medium frequencies of IFN-γ-producing CD8$^+$ T cells (Table 3, types I and IV), although the sensitivity of this assay might have been too low. Gruener *et al.* (9) reported that HCV-specific CD8$^+$ T lymphocytes in HCV-infected individuals showed reduced synthesis of IFN-γ as well as TNF-α after *in vitro* stimulation, compared with responses to Epstein–Barr virus and cytomegalovirus, and suggested that this behavior of HCV-specific CTLs might contribute to viral persistence through failure to effectively suppress viral replication. Furthermore, HCV-specific CTLs derived from chronically infected patients displayed impaired effector functions including IFN-γ production and peptide-specific cytotoxicity when compared with HCV-specific CTLs of recovered patients (33). These data strongly suggest that IFN-γ secreted by HCV-specific CTLs plays a crucial role in the clearance of HCV. In this regard, type I and type IV epitopes in Table 3 might be preferable. Overall, our data suggest that five peptides classified in the type I epitope (Table 3) should be useful candidates for a peptide-based HCV vaccine for the reasons described above. In particular, four peptides including Core-132, E2-686, NS4-1671, and NS4-1920 should be suitable because their amino acid sequences are highly conserved [(11); and our unpublished data]. However, it must be taken into account that there may be differences between the immunogenic variation observed in HLA class I transgenic mice and that in humans, mainly because the antigen processing and presentation may differ between them.

There is almost no doubt that the early appearance of escape mutation, particularly within regions encoding CTL epitopes, is closely associated with the establishment of HCV chronicity. Several studies of escape mutation in HCV-infected chimpanzees (7) and humans (6,22,29,31) clearly indicated that HCV-specific CTLs exerted immune selection pressure on viral mutation. This implies that strong HCV-specific CTL responses may result in generation of HCV escape variants and HCV chronicity. However, some HCV-derived epitopes seem to stay conserved despite consistently detectable CTL responses. This may presumably reflect the balance between the strength of immune responses and the ability of virus to escape. For the development of a CTL-based, HCV vaccine, delivery of immunodominant multiple epitopes to increase the breadth of the immune response, rather than strong but narrowly focused epitopes, should be considered. On this point, the extensive analyses of CTL epitopes such as in the current study might be of great significance.

In conclusion, we investigated 24 kinds of HLA-A*0201-restricted, HCV-derived peptides for their immunogenicity. Among 24 peptides, 5 were categorized as type I epitopes (Table 3), which showed good binding affinity for MHC class I molecules and effectively induced peptide-specific, IFN-γ producing CTLs. This study might provide important information in the design of HCV vaccine that could induce vigorous and broad $CD8^+$ T cell-mediated responses.

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Breakdown of Mucosal Immunity in Gut by 2,3,7,8-Tetraclorodibenzo-*p*-dioxin (TCDD)

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Abstract

Objectives: Mucosal immunity plays a pivotal role for body defense against infection and allergy. The aim of this study was to clarify the effects of 2,3,7,8-tetraclorodibenzo-*p*-dioxin (TCDD) on mucosal immunity in the gut.

Methods: Fecal IgA level and oral tolerance induction were examined in TCDD-treated mice. Flow cytometric and histological analyses were also performed.

Results: Single oral administration of low dose 2,3,7,8-TCDD resulted in a marked decrease in IgA secretion in the gut without any effects on the cellular components of gut-associated lymphoid tissues (GALT) including Peyer's patches (PPs) and mesenteric lymph nodes (LNs). Decreased IgA secretion by TCDD was not observed in aryl hydrocarbon receptor (AhR)-deficient mice. Flow cytometric analysis revealed that IgA^+ B cells in PPs and the mesenteric LNs remained unchanged in the TCDD-treated mice. An immunofluorescence study also demonstrated that a significant number of cytoplasmic IgA^+ cells were present in the lamina propria of the gut in the TCDD-treated mice. Furthermore, oral tolerance induction by ovalbumin (OVA) was impaired in the TCDD-treated mice and OVA-specific T cell proliferation occurred in the peripheral lymphoid tissues including the spleen and LNs.

Conclusions: These results suggest that a relatively low dose of TCDD impairs mucosal immunity in the gut and induces systemic sensitization by oral antigens.

Key words: TCDD, mucosal immunity, IgA, oral tolerance, allergy

Introduction

TCDD has been reported to exert a variety of adverse effects on immune responses including antibody production and cytotoxic T lymphocyte (CTL) generation (1–5). Exposure to TCDD also results in decreased resistance to several infectious agents (6–8). However, the immunological effects of dioxins on mucosal immunity in the gut have not been intensively examined to date, despite the fact that most dioxin exposures occur in the digestive tract. Intestinal mucosal immunity is characterized by massive IgA secretion into the gut lumen and the induction of oral tolerance against large amounts and different types of dietary antigens. Both intestinal IgA and oral tolerance play a pivotal role in body defense to protect against pathogens and to prevent systemic allergic sensitization by oral antigens (9–11). We previously demonstrated that mucosal immunity in the gut was impaired in a (New Zealand Black × New Zealand White) F1 hybrid mouse strain (BWF1), a murine model for systemic lupus erythematosus (SLE) (12, 13). Aged BWF1 mice developing lupus nephritis showed defective IgA secretion in the gut and increased susceptibility to bacterial infection. Oral tolerance was also impaired and orally administered antigens induced systemic allergic sensitization in the

Abbreviations: OVA, ovalbumin; PPs, Peyer's patches; LNs, lymph nodes; CTL, cytotoxic T lymphocytes; BWF1, (New Zealand Black × New Zealnad White) F1 hybrid; SLE, systemic lupus erythematosus; HRP, horse radish peroxidase; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanate; CFA, complete Freund's adjuvant; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; TGF, transforming growth factor; GVH, graft versus host reaction.

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respiratory tract in these mice. On the other hand, it is well recognized that the incidence of allergic diseases has been increasing over the past several decades in developed countries and that environmental factors are more involved in "increasing the incidence of disease" than genetic factors (14–16). These environmental factors include increased degree of air pollution, and increased amounts of dust mites, dietary antigens, and environmental chemicals, among others (17, 18). We hypothesize that environmental chemicals that disrupt mucosal immunity in the gut would result in allergic sensitization by oral antigens and could be a critical environmental factor in the increase in the incidence of allergic diseases.

We found that the administration of low-dose TCDD resulted in defective IgA secretion in the gut in an AhR-dependent manner and in the breakdown of oral tolerance. Antigen-specific systemic sensitization was established in TCDD-treated mice and the pathological significance of impaired mucosal immunity by TCDD in allergic diseases is discussed.

Materials and Methods

Mice

Specific pathogen-free C57BL/6J mice, originally obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), were maintained under SPF conditions in our animal facility at The University of Tokyo. Female mice aged 6–8 wk were intragastrally administrated with TCDD (Daiichikagaku Co., Tokyo, Japan) in corn oil. TCDD at 1 μg/kg was administrated to mice except in the dose-response experiment. All experiments except dose-response curve in Fig. 1 All animal experiments complied with the standards contained in the guidelines for the use and care of laboratory animals in the University of Tokyo. AhR deficient mice were originally generated from a C57BL/6 background by Schmidt et al. (19) and kindly provided by Dr. C. Tohyama (The University of Tokyo) with a permission of Dr. R. E. Peterson (University of Wisconsin). The AhR genotype was determined by RT-PCR analysis using specific primers as described by Benedict et al. (20). Five mice 6–8 wk old were used for each experimental group. No growth retardation was observed in the adult mouse colony.

Cell preparation

Mice were sacrificed under ether anesthesia to prepare cell suspensions from lymphoid organs. Peritoneal B1 cells were purified using MACS® magnetic beads (Miltenyi Biotech.) from whole peritoneal cells. Briefly, T cells, macrophages and B2 cells were depleted by incubating with a biotinylated mAb cocktail (anti-Thy1.2, anti-F4/80 plus anti-CD23 mAbs) followed by incubation with streptavidin-conjugated magnetic beads. Splenic B2 cells and $CD4^+$ T cells were also isolated using MACS beads conjugated with anti-mouse B220 or anti-mouse CD4 mAb. Cell purity was more than 90% throughout the experiments.

ELISA for fecal IgA

One hundred milligrams of fecal pellets was placed into 1.5 ml microcentrifuge tubes: 1 ml (10 volumes, w/v) of PBS was added and the tubes were incubated at room temperature for 15 min. The fecal samples were vortexed, left to settle for 15 min, revortexed until all materials were suspended, then centrifuged at 12,000 rpm for 10 min. The supernatant was removed and stored at −80°C or immediately tested using ELISA kit for IgA (Bethyl Laboratories, Montgomery, TX). Microtiter plates were coated with goat anti-mouse IgA affinity purified antibody and incubated for 60 min. Plates were washed with PBST (PBS containing 0.05% Tween 20) and each well was blocked with 200 μl of 50 mM Tris (pH 8.0) containing 0.15 M NaCl and 1% BSA for 30 min. After washing with PBST, 100 μl each of the test samples and standards was added per well and incubated for 60 min. Horse radish peroxidase (HRP)-labeled goat anti-mouse IgA-Fc specific Ab was added to each well and incubated for 60 min. Color was developed with a HRP substrate (3,3',5,5'-tetramethyl benzidine) for 30 min and read at 450 nm with using an Emax® precision microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

Flow cytometry

Fluorescein-isothiocyanate (FITC)-conjugated anti-CD4 (GK1.5), anti-CD5 (53-7.3RRH) and anti-CD11b (M1/70); Phycoerythrin (PE)-conjugated anti-CD8α (53-6.7), anti-CD11c (HL-3), and anti-B220 (RA3-6B2); and allophycocyanin (APC)-conjugated anti-B220 (RA3-6B2) mAbs were purchased from PharMingen (San Diego, CA). Lymphoid cells were stained with 1) FITC-conjugated anti-CD4, PE-conjugated anti-CD8 and APC-conjugated anti-B220 mAbs, or 2) FITC-conjugated anti-CD5 and PE-conjugated anti-B220 mAbs, or 3) FITC-conjugated anti-CD11b and PE-conjugated anti-CD11c mAbs and analyzed on an Epics Elite® cell sorter (Coulter Electronics, Hialeah, FL).

Immunofluorescence study

PPs, mesenteric LNs, and spleen tissue samples were embedded in Tissue-Teck® embedding compound (Miles Inc., Elkhart, IN) and frozen in liquid nitrogen. Six-micron-thick cryostat sections were incubated with FITC-conjugated anti-mouse IgA Ab, PE-conjugated anti-B220 Ab, and APC-conjugated anti-collagen type IV Ab (Biomedical Technology, Inc.) and observed under a fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Induction of oral tolerance

The induction of systemic unresponsiveness to OVA (Sigma Chemical Co., St. Louis, MO) was performed as described previously (7). Briefly, mice were given 25 mg of OVA in 250 μl of PBS by gastric intubation on Day 0. Control mice received PBS. On Day 7 and 21, mice were immunized and challenged subcutaneously (s.c.) with 100 μg of OVA in 100 μl of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI). The OVA specific Ab level in the serum was measured 7 days after the second s.c. immunization.

OVA-specific serum Abs by ELISA

Anti-OVA Ab titers in serum samples were determined by ELISA. Briefly, ELISA plates (Corning Incorporated Life

Sciences, Acton, MA) were coated overnight at 4°C with 1 mg/ml OVA in PBS. Blocking was performed with 200 μl of 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. Serial dilutions of serum in 1% BSA/PBS were prepared and 100 μl was added per well in duplicate. Following incubation at 37°C for 4 h, HRP-labeled goat anti-mouse IgG-Fc specific Abs (Bethyl Laboratories) were added and incubated overnight at 4°C. Color was developed with 1.1 mM 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma Chemical Co.) in 0.1 M citrate-phosphate buffer (pH 4.2) containing 0.01% H_2O_2.

OVA induced cell proliferation assay

Twenty-five milligrams of OVA in 250 μl of PBS was administered intragastrally three times in the next week after the TCDD treatment (Day 0). Two weeks after the last administration, the spleen and lymph nodes (axillar, pulmonary, mesenteric, renal, and inguinal) were removed aseptically. Single-cell suspensions were obtained using fine-mesh screens (Cell Strainer, Becton Dickinson, Franklin Lakes, NJ). Cells (4×10^5) were cultured in the presence of OVA or keyhole limpet hemocyanin (KLH) (200 μg/ml) for 5 days at 37°C in a 5% CO_2 atmosphere. Each well was pulsed with ^{3}H-thymidine (1 μCi/ml) for the last 18 h of the culture. Cells were then harvested onto a glass filter and radioactivity was determined using a liquid scintillation counter.

Results

Decreased IgA secretion in the gut by oral administration of TCDD

To investigate the immunological effects of TCDD on the intestinal mucosa, C57BL/6 mice were intragastrally administered various doses of TCDD and the fecal IgA level was determined by ELISA. The fecal IgA level was dose-dependently decreased in mice treated with TCDD (Fig. 1A). Note that relatively low doses of TCDD (0.1 μg/kg and 1.0 μg/kg) significantly inhibited IgA secretion into the gut lumen. The fecal IgA level returned to the normal level by 4 weeks after the administration of 1 μg/kg TCDD (Fig. 1B). To determice whether the inhibitory effect of TCDD on IgA secretion in the gut is mediated by AhR, which is a specific receptor for TCDD, the fecal IgA level in AhR-deficient mice administered with a TCDD (1 μg/kg) was examined. The inhibitory effect of TCDD on IgA secretion in the gut was totally abrogated in AhR-deficient mice with a C57BL/6 background, whereas heterozygous littermates and C57BL/6 mice showed a marked decrease in IgA secretion in the gut (Fig. 2).

Flow cytometric analysis

FACS analysis revealed that there was no significant change in the cellular compartments ($CD4^+$, $CD8^+$, $B220^+$, and $CD11b^+11c^+$ cells) in PPs, and in the thymus, spleen, and LNs in mice treated with 1 μg/kg TCDD (Fig. 3A–E). The percentages of $CD11b^+CD11c^+$ cells, a cell surface phenotype for myeloid type dendritic cells, in PPs, and in the mesenteric LNs and spleen were 0.25±0.07, 0.25±0.07, and 0.6±0.01 in the TCDD treated mice and 0.3±0.01, 0.2±0.01, and 0.55±0.07 in the control mice, respectively. The absolute number of each subset

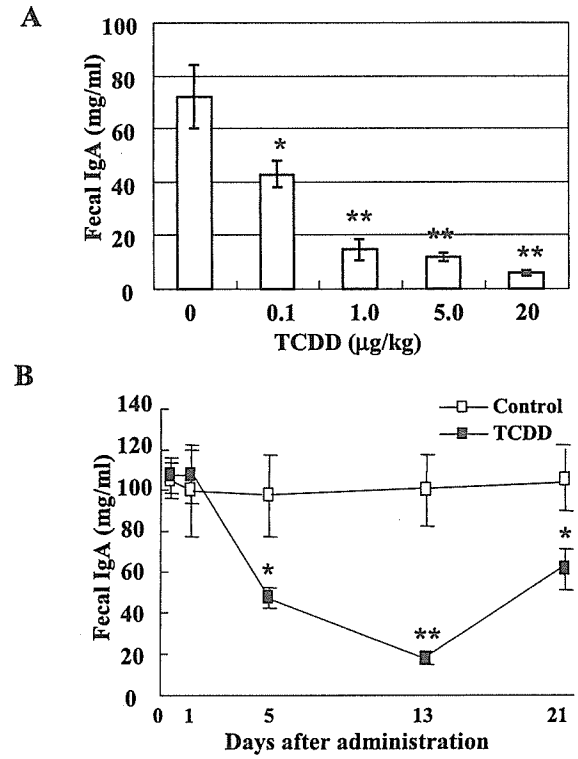

Fig. 1 TCDD suppression of IgA secretion in gut. A. Dose-dependent IgA suppression by TCDD. C57BL/6 mice (n=5) were intragastrally administered TCDD (0.1, 1.0, 5, 20 μg/kg) and the fecal IgA level was determined by ELISA 1 wk after TCDD treatment. Monoclonal IgA Ab was used as a control. The mean concentrations±SD are presented in each graph. A representative result from four independent experiments is presented. B. Kinetic study of IgA suppression by TCDD. Mice (n=5) were given 1 μg/kg TCDD (filled squares, ■) or corn oil (open squares, □). Feces were collected on days 1, 5, 13, and 21 and the fecal IgA level was determined by ELISA on the same plate on the same day.

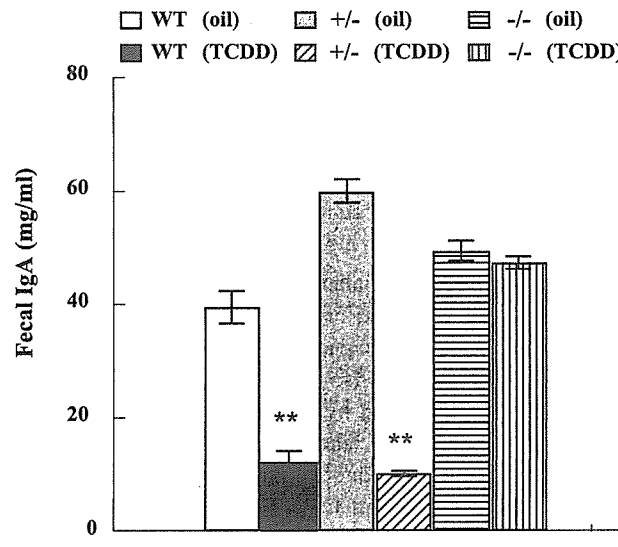

Fig. 2 AhR-dependent suppression of IgA secretion in TCDD-treated mice. TCDD (1 μg/kg) or corn oil was given to C57BL/6 (WT), AhR heterozygous (+/–), and AhR-deficient (–/–) mice and the fecal IgA level was determined by ELSA 1 wk after TCDD treatment (n=5). A representative result from two independent experiments is presented. Statistical analysis was performed using the Student's *t*-test. ** $p<0.001$.

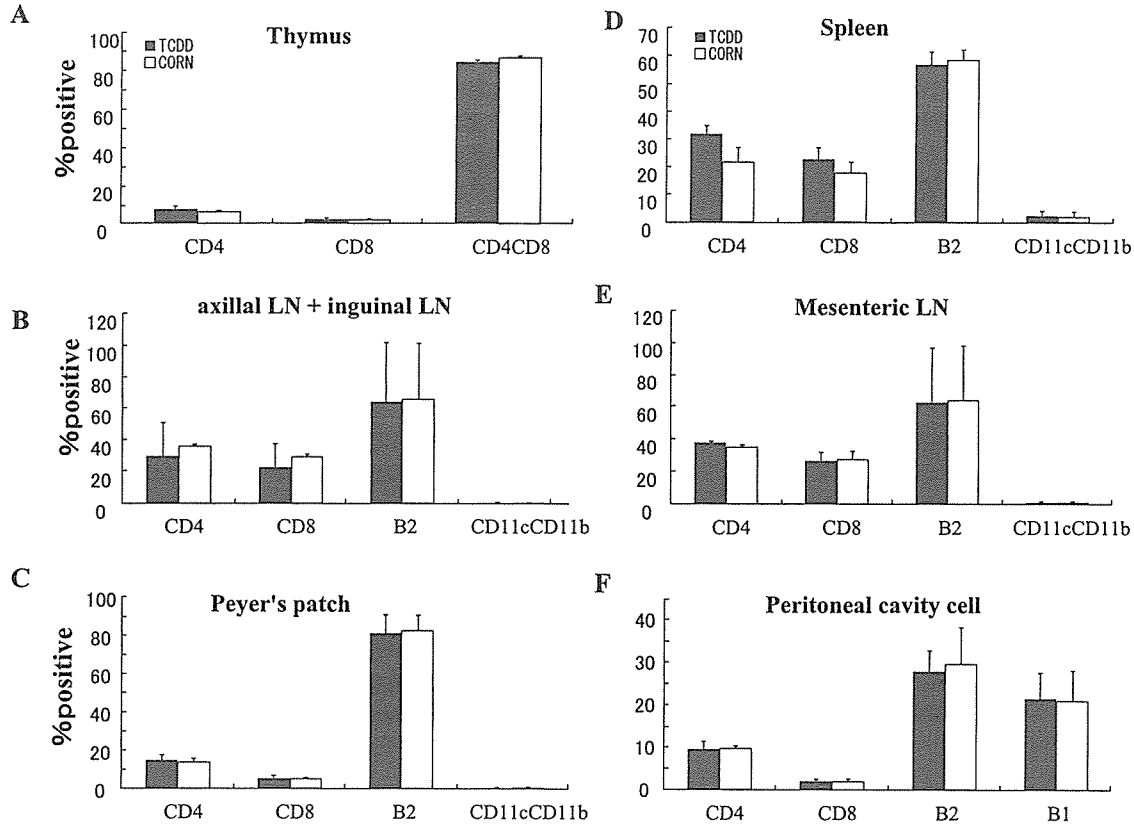

Fig. 3 FACS analysis on lymphoid tissues. Cells from the thymus, spleen, and LNs were stained with a mixture of FITC anti-CD4, PE anti-CD8, and APC-anti-B220 Abs or FITC anti-CD11b and PE anti-CD11c Abs and analyzed using a flow cytometer. Peritoneal cells were stained with FITC anti-CD5 and PE-B220 antibodies to discriminate B1 ($CD5^+B220^{int}$) and B2 ($CD5^-B220^{high}$) cells. The mean percentages±SD are presented in each graph (n=4). A representative result from three independent experiments is presented.

was not changed either (data not shown). Mean fluorescence intensities for CD4, CD8 and B220 molecules also remained unchanged in TCDD-treated mice (data not shown). Note that the percentage of peritoneal B1 ($CD5^+B220^+$) cells remained unchanged in the TCDD-treated mice (Fig. 3F).

No histological change and presence of IgA^+ cells in TCDD-treated mice

Hematoxylin-Eosin (H-E) staining showed that the intestinal mucosa remained unchanged in the TCDD-treated mice (Fig. 4A). To determine whether defective IgA secretion in the gut can be attributed to a decreased percentage of IgA^+ cells in the gut-associated lymphoid tissues (GALT), FACS analysis and an immunofluorescence study were performed. FACS analysis on the B cells in PPs and the mesenteric LNs revealed that the frequency of IgA^+ cells remained unchanged in the TCDD-treated mice (Fig. 4B). The immunofluorescence study also demonstrated that a significant number of cytoplasmic IgA^+ cells (plasma cells) were present in the lamina propria of the gut in the TCDD-treated mice as well as in the control mice (Fig. 4C).

Effect of TCDD on oral tolerance

Oral tolerance is historically and originally described as the antigen specific inhibition of antibody production by oral preadministration of protein antigen. As shown in Fig. 5A, OVA-specific IgG production was suppressed in mice that had been orally administered OVA before systemic immunization, demonstrating that oral tolerance was induced in the control mice. The oral administration of KLH did not inhibit OVA-specific IgG production (data not shown). In contrast, the suppression of IgG production was partially abrogated in the TCDD-treated mice, suggesting a breakdown of oral tolerance. To examine the effect of TCDD treatment on antigen uptake, mice were administered 1 mg of Alexa488-labeled OVA one week after TCDD treatment. Alexa488-labeled OVA was incorporated into the subepithelial dome (SED) of PPs, a major site for antigen uptake in the gut, similarly in the TCDD-treated and control mice (Fig. 5B).

Consistent with the impaired oral tolerance in the TCDD-treated-mice, lymphocytes in PPs, and in the axillar, inguinal, and cervical LNs, and the spleen of TCDD-treated mice antigen-specifically proliferated in vitro in the presence of OVA, whereas KLH stimulation did not induce cell proliferation (Fig. 6).

Discussion

We have demonstrated in this study that the oral administration of a relatively low dose of TCDD results in a marked decrease in IgA secretion in the gut. However, FACS analysis revealed that the number of IgA^+ B cells was not decreased in the mesenteric LNs and PPs in the TCDD-treated mice. An immunofluorescence study also showed that a significant

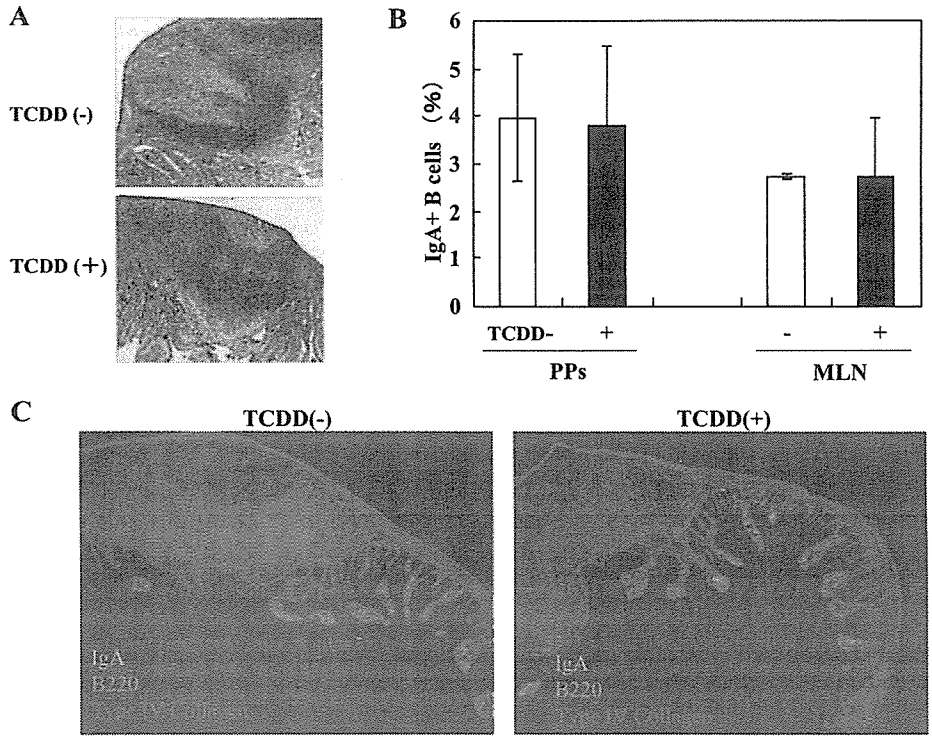

Fig. 4 Histological change in intestinal mucosa and presence of IgA$^+$ cells in TCDD-treated (1 μg/kg) mice. A. Hematoxylin-Eosin (H-E) staining of intestinal mucosa of TCDD-treated mice showed no pathological changes such as inflammation or necrosis (×100). B. Cells prepared from PPs or mesenteric LNs were stained with FITC-anti-IgA, PE-anti-B220, and APC-anti-CD19 Abs. The percentages of IgA$^+$B220$^+$ cells among CD19$^+$ cells are presented (n=3). C. Cryosections of intestinal mucosa were stained with FITC anti-IgA, PE-anti-B220, and APC anti-collagen Type IV Abs and examined under a confocal laser scanning microscope (×100). Many IgA$^+$ cells (green) were observed in the intestinal lamina propria in the TCDD-treated mice as well as in the control mice.

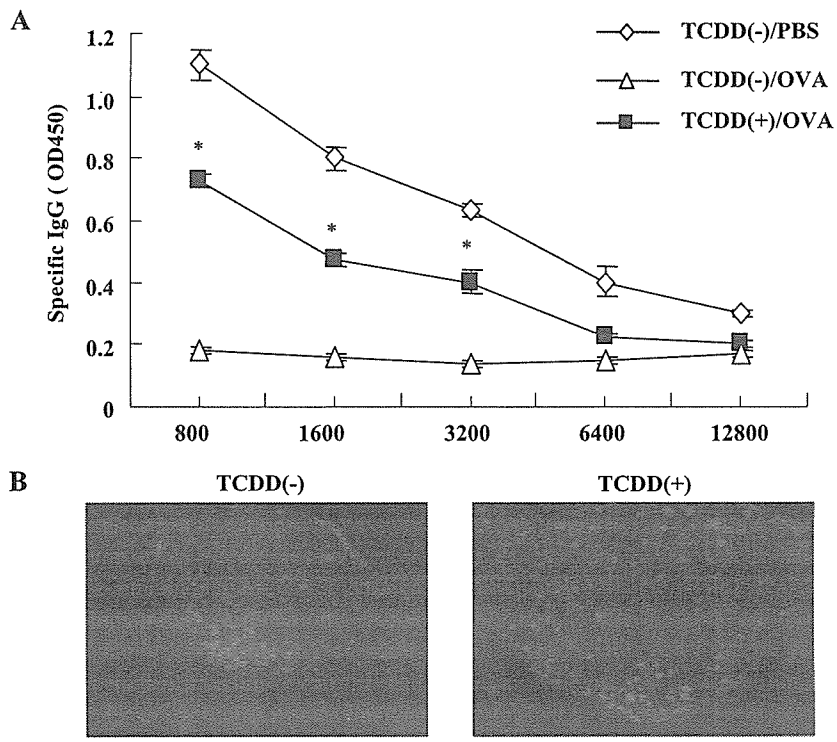

Fig. 5 Breakdown of oral tolerance and systemic sensitization by oral antigens and intact antigen uptake in TCDD-treated (1 μg/kg) mice. A. Mice were treated with TCDD (filled squares, ■) or corn oil (open triangles, △) on Day 0 and orally administered 25 mg of OVA on Days 7 and 14. Then, mice were immunized s.c. with 100 μg of OVA in CFA on Days 21 and 28 (n=4). Mice immunized with OVA plus CFA were used as positive controls (open diamonds, ◇). The serum concentration of OVA-specific IgG on Day 35 was determined by ELISA. The results are expressed as mean±SD. Representative data from three experiments are presented. * $p<0.001$. B. Alexa488-labeled OVA was administrated intragastrally and cryostat sections prepared 2 h after administration were analyzed under a fluorescence microscope (×100). The labeled OVA localized mainly in the subepithelial dome (SED) of PPs both in the TCDD-treated and control mice.

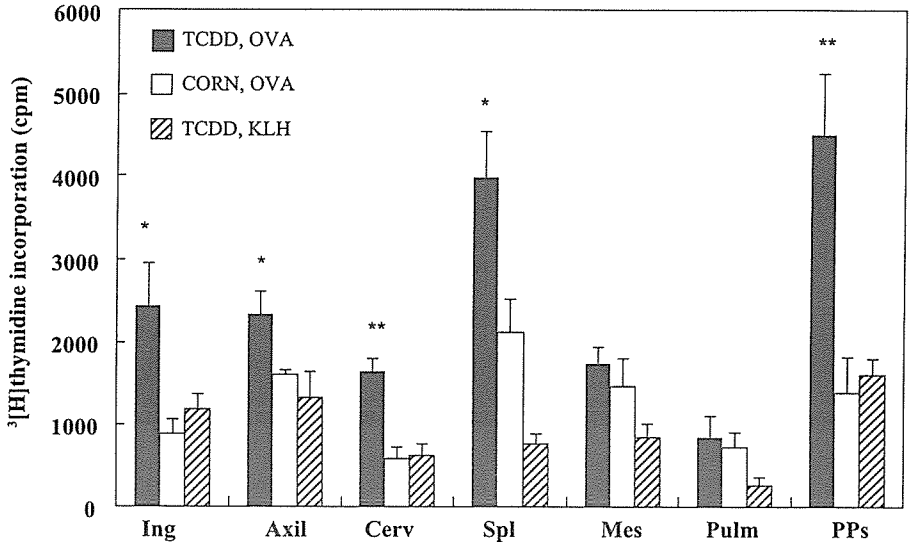

Fig. 6 Systemic sensitization with orally administered OVA by TCDD pretreatment (1 μg/kg). Mice were treated with TCDD on Day 0 and then mice were intragastrally given 25 mg of OVA twice on Day 7, 10 and 14. On Day 28, single-cell suspensions were obtained from the spleen (Spl), and inguinal (Ing), axillar (Axil), mesenteric (Mes), and pulmonary (Pulm) lymph nodes, and PPs and stimulated in vitro with OVA or KLH (200 μg/ml) at 37°C for 5 days. Cell proliferation was measured by ^{3}H-thymidine incorporation as described in Materials and Methods. Results are presented as means±SD (n=4). Representative data from three experiments are presented. * $p<0.05$, ** $p<0.01$.

number of IgA^+ cells were present in the lamina propria of the gut in the TCDD-treated and control mice. Cellular subsets of immunocompetent cells in lymphoid tissues including PPs, and the spleen, thymus, and peripheral LNs, remained unchanged.

It has recently been reported that the constitutively AhR causes selective loss of peritoneal B1a ($CD5^+B220^{low}IgM^{high}$) cells (21), suggesting that B1 cells are sensitive cellular targets for TCDD. B1 cells are a specialized cell population distinguished from conventional B cells (B2 cells) by their origin, cell surface phenotype, unique tissue distribution and capacity for self renewal; these cells have also been considered to be involved in autoantibody production in the development of autoimmune diseases (22). It is also believed that approximately half of the IgA^+ cells in the intestinal lamina propria are derived from B1 cells and B1 cells play a pivotal role in innate mucosal immunity in the gut (23, 24). In this study, however, the number and frequency of B1 cells in the peritoneal cavity were not decreased in the TCDD-treated mice. The expression of constitutively active AhR during ontogeny may result in different effects on B1 cells from those observed in our study. The mechanisms for defective IgA secretion in the gut remain to be elucidated. It is possible that TCDD affects the synthesis of secretory component or the secretion machinery of intestinal epithelial cells.

The breakdown of oral tolerance by TCDD is another interesting finding in this study. Oral tolerance is historically and originally described as the antigen-specific inhibition of systemic IgG production by oral preadministration of protein antigens (10, 11). Many studies have been performed using an experimental protocol similar to that used in this study to demonstrate the presence or absence of oral tolerance. However, the precise mechanism for oral tolerance still remains to be clarified (25). It was previously reported that regulatory T cells producing transforming growth factor (TGF)-β and/or IL-10 were induced in PPs by the oral administration of protein antigens (26, 27). However, the classical idea for the pivotal role of PPs in oral tolerance has been challenged by several studies demonstrating that oral tolerance could be induced independently of PPs (28, 29). It was also demonstrated that the spleen plays an important role in oral tolerance (30, 31). These results are in agreement with the idea that mesenteric LNs and the spleen are critical lymphoid organs functioning as induction sites for oral tolerance although they do not exclude the physiological role of PPs. Accumulating evidence also suggests that dendritic cells (DCs) play a pivotal role in oral tolerance (32–34). However, the frequency of $CD11c^+$ cells remained unchanged in PPs, and the mesenteric LNs and spleen before and after the TCDD treatment. Funatake et al. (35) have recently demonstrated that TCDD generates $CD25^+CD4$ T cells with a regulatory function in a graft versus host (GVH) reaction. However, FACS analysis showed only a marginal increase in the frequency of $CD25^+CD4$ T cells in PPs, and the mesenteric LNs and spleen (6.3±0.28, 12.3±0.28, and 8.95±0.21 in the TCDD-treated mice and 4.25±0.35, 11.4±0.21, and 7.6±0.28 in the control mice, respectively). The route of antigen trafficking may be another factor affecting the immune response in the gut as indicated by our recent work on a murine model for SLE (13). However, alexa488-labeled OVA was incorporated into the subepithelial dome (SED), a major site for antigen uptake, similarly in the TCDD-treated and control mice. Functional analysis on DCs with different localizations is under way to elucidate the mechanism involved in oral tolerance disruption by TCDD.

As a result of the breakdown of oral tolerance, T cells in PPs, and in the axillar inguinal, and cervical LNs were sensitized by the orally administered OVA. It is known that patients with atopic dermatitis show a high frequency of food allergy and that dietary allergens such as egg albumin often turn out to be the allergen in the skin of these patients, indicating the existence of immunological cross talk between

the intestinal mucosa and the skin. It is considered that microbial infection, excessive antibiotic administration, and the early onset of a weaning diet among others are the causes of systemic allergic sensitization to oral antigens in infants. Our findings suggest that TCDD may be a possible candidate for such disruptors of mucosal immunity. Although 1 μg/kg TCDD is far more than the human adult daily intake, breast-fed newborn infants take in 15–20 times more TCDD than the tolerable daily intake (TDI). Furthermore, the immature mucosal immune system of newborn infants may be much more sensitive to TCDD exposure. The effects of TCDD on mucosal immunity in the gut in newborn infants will be further clarified in the near future.

Collectively, we have demonstrated that a relatively low dose of TCDD results in the breakdown of intestinal mucosal immunity and systemic sensitization by oral antigens in mice. The immunological health effects of environmental chemicals such as dioxins should be assessed on the basis of mucosal immunity in the gut. This may also provide a new insight for understanding environmental factors responsible for the increased incidence of allergic diseases in recent decades.

Acknowledgments

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Aberrant B1 cell trafficking in a murine model for lupus

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TABLE OF CONTENTS

1. ABSTRACT

B lymphocyte chemoattractant (BLC/CXCL13) is ectopically and highly expressed in the target organs such as the thymus and kidney in aged (NZB x NZW)F1 (BWF1) mice, a murine model for SLE. Ectopic expression of BLC/CXCL13 was attributed to mature myeloid DCs infiltrating in the target organs. DCs were also increased in the peripheral blood in aged BWF1 mice and differentiated into BLC/CXCL13- producing DCs in the presence of TNF-α or IL-1β, but not IFN-α or IFN-γ. BLC/CXCL13 expression in mature myeloid DCs was confirmed in bone marrow derived-DCs generated *in vitro* in the presence of GM-CSF and TNF-α. B1 cells expressed higher level of CXCR5 and migrated towards BLC/CXCL13 much better than B2 cells. B1 cells failed to home to the peritoneal cavity and preferentially recruited to the target organs in aged BWF1 mice developing lupus nephritis. B1, but not B2 cells possessed a potent antigen presenting activity in allo-MLR and activated autologous thymic CD4 T cells in the presence of IL-2. $CXCR5^+$ CD4 T cells were also increased in aged BWF1 mice and they enhanced IgG production by B1 cells *in vitro*. These results suggest a possible involvement of aberrant B1 cell trafficking in activation of autoreactive CD4 T cells and autoantibody production in the target organs during the development of lupus, providing a new insight for the pathogenesis of B1 cells in lupus.

2. INTRODUCTION

B1 cells are a specialized cell population that is distinguished from conventional B cells (B2 cells) by their origin, antigen specificity, cell surface phenotype, and unique tissue distribution (1-3). B1 cells were first described as Ly-1 $(CD5)^+$ B cells and considered to be involved in the development of autoimmune diseases because increased frequency of B1 cells was observed in particular autoimmune-prone mice including (NZB x NZW)F1 (BWF1) mice. In consistence with this idea, it was demonstrated that elimination of B1 cells from the peritoneal cavity by injection of distilled water decreased anti-dsDNA Ab production and pathogenic changes in the kidney in BWF1 mice (4). It was also described that down-regulation/elimination of B1 cells by administration of anti-IL-10 antibody (5, 6) delays the onset and disease severity in BWF1 mice.

B1 cells produce IgM antibodies (Ab) with broad specificity including for self antigens (7) and they are able to undergo isotype switching and somatic hypermutation (8, 9), suggesting that B1 cell can contribute to produce pathogenic high-affinity IgG autoantibodies. On the other hand, B1 cells possess a potent antigen presenting activity comparable to dendritic cells (10, 11). Lanzavecchia *et al.* (12) reported that B cells could efficiently uptake antigens through their surface immunoglobulin receptors, process, and then present to specific T cells in a MHC-restricted manner. These findings may indicate that B1 cells are involved in the pathogenesis of murine lupus as APCs to activate auto-reactive T cells.

In human, elevated levels of $CD5^+$ B cells have been documented in patients with autoimmune disorders including Sjogren's syndrome, rheumatoid arthritis and SLE (13-15). However, several evidences also suggest minor role of $CD5^+$ B cells in the development of SLE (16, 17). Due to different frequency and localization of $CD5^+$ B cells among the species (18), and also to possible conversion of CD5 expression during B cell activation (19), the pathological significance for $CD5^+$ B cells in the development of SLE still remains controversial. In this article, we highlight possible roles of aberrant B1 cell trafficking in the pathogenesis of murine lupus.

3. ABERRANT HIGH EXPRESSION OF BLC/CXCL13 BY MYELOID DCS IN THE TARGET ORGANS IN AGED BWF1 MICE DEVELOPING LUPUS NEPHRITIS

BWF1 mice spontaneously develop systemic autoimmune disorders characterized by production of a variety of IgG autoantibodies and massive deposition of immune complexes in glomeruli in the kidney (20, 21). A marked mononuclear cell infiltration in the target organs including the kidney and lung is another characteristic in aged BWF1 mice. More than 95 % of the BWF1 female mice die from renal failure before 12 months of age. RT-PCR analysis revealed that BLC/CXCL13 expression was markedly increased in the target organs including the kidney and the thymus in aged BWF1 mice developing lupus nephritis (Figure 1A) (22). Quantitative PCR analysis revealed that BLC/CXCL13 gene expression in the thymus and kidney of aged BWF1 mice is 1500 and 500 times higher, respectively compared to that in young mice. BLC protein was also highly expressed in the cellular infiltrates in these organs with a reticular pattern of staining (Figure 1B). Some, but not all blood vessels in the thymic medulla were heavily stained with anti-BLC mAb. BLC expression in the thymus of similarly-aged NZB and NZW mice was much lower than that in aged BWF1 mice (data not shown). It is generally accepted that BLC/CXCL13 is expressed by follicular dendritic cells (FDCs) in B cell follicles of the secondary lymphoid tissues (23, 24). However, aberrant BLC/CXCL13 expression in the target organs in aged BWF1 mice was attributed to mature myeloid DCs. $CD11b^+CD11c^+$ cells were markedly increased in the spleen and thymus and purified $CD11b^+CD11c^+$ cells preferentially expressed BLC/CXCL13 mRNA (Figure 1C). It was also demonstrated that $CD11b^+CD11c^+$ cells were increased in the peripheral blood in aged BWF1 mice and differentiated into BLC/CXCL13 expressing-mature DCs in the presence of TNF-α (Figure 1D, E) (25). Both TNF-α and IL-1β, but neither IFN-α nor IFN-γ could induce BLC gene expression in bone marrow-derived immature DCs generated in the presence of GM-CSF (Figure 1F). Since TNF-α and IL-1β expression is in fact enhanced in the target organs in aged BWF1 mice (26, our unpublished data), it is likely that peripheral blood DCs differentiate into mature DCs expressing BLC/CXCL13 in the target organs during the development of lupus.

4. BLC/CXCL13 CHEMO-ATTRACTS MORE B1 CELLS THAN B2 CELLS

BLC/CXCL13 chemo-attracted more B1 cells than B2 cells presumably due to higher expression of CXCR5 on B1 cells (Figure 2A, B) (22). Preferential chemotaxis of B1 cells towards BLC was also observed in other mouse strains including NZB, NZW, BALB/c, and C57BL/6 (data not shown). Although SLC/CCL21 and SDF-1/CXCL12 also have weak chemotactic activity for B1 cells, there was no difference in chemotaxis by these chemokines between B1 and B2 cells (unpublished data).

5. DEFECTIVE B1 CELL HOMING TO THE PERITONEAL CAVITY AND PREFERENTIAL LOCALIZATION IN THE TARGET ORGANS

It has been recently reported that BLC is essential for B1 cell homing to the peritoneal cavity via omentum milky spots (27). In CXCL13 deficient mice, few B1 cells were present in the peritoneal and pleural cavity, but present in the spleen. CFSE labeled-B1 cells failed to migrate to the peritoneal cavity in CXCL13 deficient mice when injected i.v. Interestingly, B1 cells failed to home to the peritoneal cavity in aged BWF1 mice developing lupus nephritis while B1 cell homing to the peritoneal cavity was intact in young BWF1 mice (28). When CFSE-labeled B1 cells were i.v. injected into young BWF1 mice, a number of labeled cells accumulated in the omentum milky-spots while only few cells were observed in aged BWF1 mice (Figure 3A). B1 cell accumulation in the omentum milky-

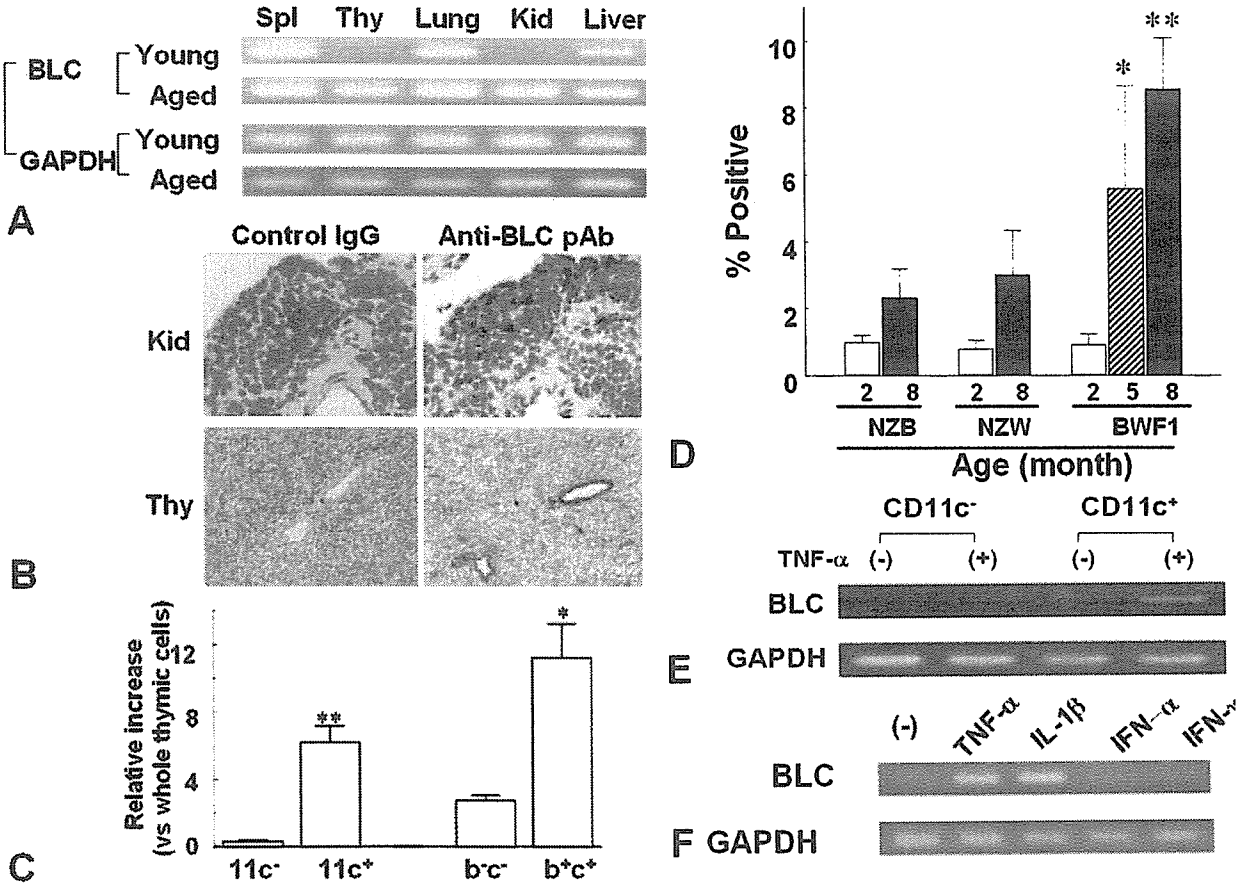

Figure 1. Ectopic high BLC expression by DCs in aged BWF1 mice. A. RT-PCR analysis was performed on total cellular RNAs obtained from the spleen, thymus, lung, kidney, and liver of young and aged BWF1 mice. B. Cryosections from kidney and thymus of aged BWF1 mice were stained with goat anti-BLC pAbs followed by biotinylated rabbit anti-goat pAb and HRP-labeled streptavidin. Representative pictures from one of three aged BWF1 mice are presented. (C) Quantitative RT-PCR analysis for BLC expression on $CD11c^+$ cells purified MACS® magnetic beads coupled with anti-CD11c mAb and FCM sorted-$CD11b^+CD11c^+$ cells obtained from aged BWF1 thymus. BLC quantity in each purified cell population was expressed relative to normalized BLC quantity in whole thymic cells. D. Increased DCs in the peripheral blood. PBLs from young and aged NZB, NZW, and BWF1 mice were stained with FITC-labeled anti-CD11b and PE-labeled anti-CD11c Abs and analyzed on EPICS flow cytometer. The mean ± SD for $CD11b^+CD11c^+$ cells were presented (n=3 for NZB and NZW mice; n=7 for 2 and 8 month-old BWF1 mice, n=5 for 5 month old BWF1 mice). Statistical analysis was performed by Student's t test. * $p<0.03$, ** $p<0.0002$ as compared to the 2 month old value. E. TNF-α induces BLC expression in DCs. $CD11c^+$ and $CD11c^-$ cells obtained from PBLs of aged BWF1 mice were cultured in the presence or absence of TNF-α for 3 days and RT-PCR analysis for BLC expression was performed. F. Induction of BLC expression in bone marrow derived-DCs by TNF-α and IL-1β, but not by IFN-α and IFN-γ. Bone marrow cells were cultured in the presence of GM-CSF for 6 days with removal of floating cells at day 3. TNF-α (10 ng/ml), IL-1β (10 ng/ml), IFN-α (100U/ml), or IFN-γ (100U/ml) for 3 days was added at day 6 and cultured for additional 3 days. RT-PCR analysis for BLC expression was performed.

spots was intact in similarly aged BALB/c mice, suggesting that the defective B1 cell homing to the peritoneal cavity in aged BWF1 mice was not merely an age-related phenomenon. It was also demonstrated that the number of peritoneal macrophages in the peritoneal cavity, a major cell source for BLC/CXCL13, was markedly decreased in aged BWF1 mice compared to those in young BWF1 mice (28).

Instead of homing to the peritoneal cavity, B1 cells injected i.v. preferentially migrated to the target organs including the kidney, thymus, and lung in aged BWF1 mice. When the mixture of CFSE-labeled B1 cells and CMTMR-labeled B2 cells were injected i.v. into the same aged BWF1 mice, more B1 cells were recruited in the cellular infiltrates in the target organs than B2 cells (Figure 3B). In consistence with these results, the frequency of B1 cells in total B cells in the target organs in aged BWF1 mice was significantly higher than that in secondary lymphoid tissues (28). These results suggest that aberrant high expression of BLC/CXCL13 in the target organs of aged BWF1 mice and decreased number of BLC producing

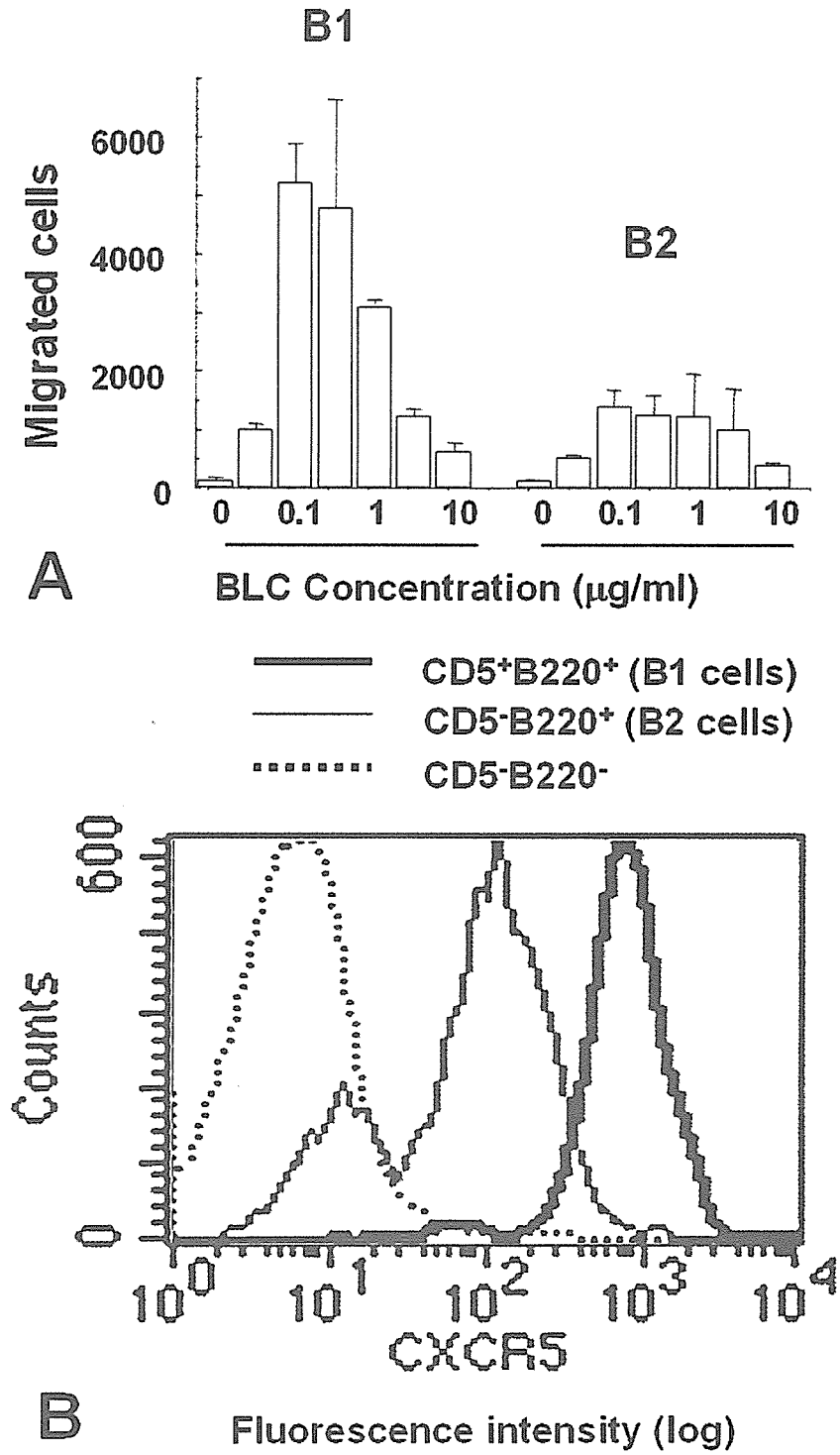

Figure 2. Preferential chemotaxis of B1 cells towards BLC. A. Mixture of spleen and peritoneal cells from young BWF1 mice were stained for FITC-labeled CD5 and PE-B220 and sorted into B1 and B2 cells on an EPICS ELITE® cell sorter and then subjected to BLC chemotaxis assay. Migrated cells were counted on a flow cytometer under the constant flow rate. B. Higher CXCR5 expression on B1 than B2 cells. Mixture of spleen and peritoneal cells from young BWF1 mice were stained with anti-CXCR5, anti-CD5, and anti-B220 mAbs and then analyzed for CXCR5 expression on gated B1 or B2 population. Mean fluorescence channels for B1 and B2 cells were 796 ± 18 (n=3) and 113 ± 9.0 (n=3), respectively ($p<0.001$). Staining on $CD5^-$ $B220^-$ cells was presented as a negative control.

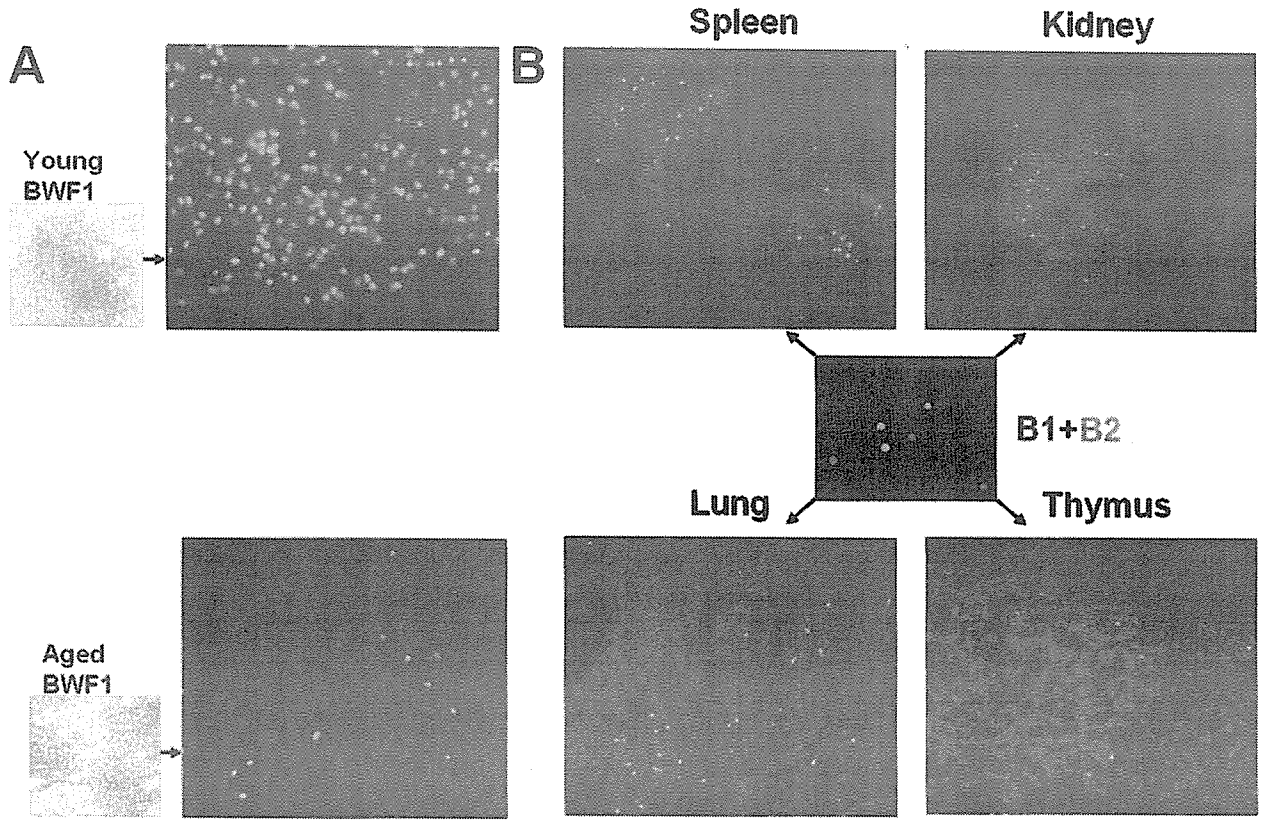

Figure 3. Failure of B1 cells homing to the peritoneal cavity and migration to the target organs in aged BWF1 mice. A. Decreased B1 cell accumulation to the omentum milky-spots in aged BWF1 mice. Four million CFSE labeled-peritoneal B1 cells were injected i.v. into young and aged BWF1 mice. Omentum milky-spots were analyzed 24 hr after injection under confocal laser scanning microscope (x100). B. Preferential B1 cell recruitment to non-lymphoid target organs including the kidney and lung. B1 and B2 cells were labeled with CFSE (green) and CMTMR (red), respectively and the 1:1 mixture of B1 and B2 cells was injected i.v. into the same aged BWF1 mice. Mice were sacrificed 24 hr after injection and analyzed under confocal laser scanning microscope (x200). Cellular infiltrates were highlighted using biotin-conjugated B220 mAbs (blue).

peritoneal macrophages result in defective B1 cell homing to the peritoneal cavity and preferential B1 cell trafficking to the target organs.

6. POTENT ANTIGEN PRESENTING ABILITY OF B1 CELLS

Mohan *et al.* (10) reported splenic B1a cells possessed potent antigen-presenting capability in NZM2410 lupus-prone mice. Peritoneal B1 cells obtained from BWF1 as well as BALB/c mice also induced a good alloreactive response in MLR when used as antigen presenting cells while B2 cells showed poor antigen presenting ability (Figure 4A) (11). Antigen presenting ability of B1 cells was as potent as that of splenic DCs and is not restricted to B1 cells derived from autoimmune mice. Peritoneal B1 cells expressed significantly higher levels of costimulatory molecules including CD80, CD86, and ICAM-1 than splenic B2 cells while the level of MHC class II expression was similar between B1 and B2 cells (Figure 4B).

7. B1 CELL MIGRATION TO THE THYMUS MAY RESULT IN AUTOREACTIVE CD4 T CELL ACTIVATION

Thymic post-capillary venules (PVS) were markedly enlarged in aged BWF1 mice and a number of B cells were infiltrated in the enlarged PVS (Figure 5A) (11). Approximately 15 % of infiltrated $B220^{+}$ cells are CD5 positive. The boundary between thymic PVS and parenchyma became unclear and some B cells were readily detected in the thymic medulla. BLC protein and PNAd were co-localized on luminal surfaces of HEV-like blood vessels in enlarged PVS. Recruitment of B1 cells to the enlarged thymic PVS and transmigration across the basement membranes to the thymic medulla was observed when CFSE labeled-B1 cells were injected i.v. into aged BWF1 mice (Figure 5B).

It is of great interest what would happen to tolerance induction when B1 cells which have a potent antigen presenting activity aberrantly migrate to the thymus. When thymocytes labeled with CFSE were cultured with B1 or B2 cells in the presence of IL-2, small,