

Sub-compartmentalization of the gastrointestinal (GI) immune system determined with microbeads that differ in release properties

Richard Ivan Cronkhite*, J. Gabriel Michael¹

Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, Cincinnati, OH 45267, USA

Received 25 June 2003; received in revised form 25 November 2003; accepted 16 December 2003

Abstract

Immunization of two specific regions of the murine GI tract of two types of mice with ovalbumin (OVA) encapsulated in microbeads with two different pH-sensitive coatings allowed a more precise analysis of this compartment of the mucosal immune system. Acute, chronic and pervasive immunization protocols were utilized in an attempt to stimulate specific types of immunity. Chronic immunization potentiated antibody isotypes influenced by type 2 T helper cells (T_H2). Pervasive immunization of both regions of the GI tract mimicked chronic immunization, stimulating high levels of OVA-reactive IgE. Acute immunization was best able to potentiate isotypes influenced by type 1 T helper cells (T_H1) and a sequential segregated immunization protocol allowed the targeting of T_H1-like memory responses. © 2004 Elsevier Ltd. All rights reserved.

Keywords: Vaccine; Gastrointestinal; Microbeads

1. Introduction

Compartmentalization within the immune system allows complex cellular interactions dependent on antigen concentration, the site(s) of antigen presentation, and the frequency of exposure to antigens to differentiate between pathogens and food [1–5]. The mucosa contains a greater number of mucosal mast cells (MMC) which aid T_H2 responses, while the submucosa contains a greater number of connective tissue mast cells (CTMC) which aid T_H1 responses [6–8]. Dendritic cells that breach the GI barrier can deliver antigens to the mesenteric lymph nodes (MLNs) and spleen [9]. The upper small intestine (SI), the duodenum and jejunum, contains IELs with a greater proliferative capacity and fewer lymphoid follicles than the lower SI or ileum [1,10]. The lower SI contains a greater number of lymphoid follicles than the upper SI as well the Peyer's patches in most species [1,11]. These lymphoid structures are the sites where T cells, B cells, antigen presenting cells, and mast cells cooperate to induce antibody production [12–14]. They also produce cytokines that favor IgA production and tolerance: IL 4, IL 10 and TGF- β [14,15].

Both penetration and/or invasion of the epithelial layer and protection of antigens from digestion influence immune responses at the mucosa [16,17]. Ingested biochemicals are generally poor immunogens because the GI tract rapidly degrades antigenic structure in digestion [16]. Peptide antigens presented directly to the T_H2 cells and intraepithelial lymphocytes (IELs) of the GI tract induce tolerance and inhibit IgE production [18–20]. In fact, oral tolerance can be abrogated by simply delivering a sufficient quantity of native antigen to the mucosa of the SI, that would normally be degraded by the stomach [21]. Large antigens, such as heat killed *Salmonella*, induce a greater T_H2 response with relatively low levels of IgG_{2a} while invasive live *Salmonella* induce T_H1 responses measurable by DTH [22,23]. Even though bacteria-bound LPS will induce IgG_{2a} when administered systemically, it induces predominantly IgG₁ when delivered perorally (p.o.) [22].

The induction of T_H1 responses is desired for the production of blocking antibodies in allergy treatments and for immunity to some pathogens. Germinal centers (GCs) require T_H1 cytokines as well as *bcl-6* gene expression, and are efficiently induced by T and B cells which have previously encountered antigen [24–26]. IgE production requires IL 4 and CD40 signaling, but not GCs [18,26–28]. One approach that might be useful for immune therapy in allergic disease or vaccination against some pathogens could employ the induction of GCs in the GI tract [29]. Since naive B cells

* Corresponding author. P.O. Box 2728, Cincinnati, OH 45201, USA. Tel.: +1-513-621-0990.

E-mail address: richardivancronkhite@dialup4less.com (R.I. Cronkhite).

¹ Retired.

are relatively short-lived and most B cells recirculate much more slowly than T cells, most recirculating lymphocytes are T cells or antigen-stimulated “memory” B cells [30–32]. Therefore, a sequential segregated immunization protocol that targets different regions of the GI tract for the induction of GCs might be useful for inducing T_H1 responses by stimulating relocated memory lymphocytes. Differences in TGF- β production in specific regions of the GI tract may also aid in the induction of T_H1 responses. While TGF- β inhibits IgE responses by direct action on B cells as well as the selection of T_H3 cells over T_H2 cells, it does not inhibit activated T_H1 cells [15,33,34].

In this study, native OVA antigen was precisely delivered to the upper SI (duodenum/jejunum) and/or the lower SI (ileum) of two different types of mice using microbeads constructed of two different methacrylic copolymers which dissolve at different pH values. The immunization protocols employed can be described as acute, chronic, or pervasive. Acute immunization delivered single doses of antigen to one site in the SI. Chronic immunization delivered antigen on multiple days. Pervasive immunization of the SI was accomplished by utilizing the two different preparations of microbeads at the same time. Acute immunization proved to be the most effective method of inducing antibodies influenced by T_H1 cytokines, IgG_{2a}. This was most successful in BDF₁ mice when memory cells were specifically targeted at new immunization sites using a sequential segregated immunization protocol. A greater T_H1 response, which avoided high titers of IgE, was induced in BDF₁ mice in this manner. Although chronic immunization, six doses in 12 days, or at times even three times in 3 days, produced higher levels of OVA-reactive antibodies than single doses of antigen, it was inferior at producing T_H1 -skewed responses. Multiple immunizations of the same region of the GI tract eventually selected a T_H2 response with high levels of IgE. Furthermore, pervasive immunization of the GI tract induced high titers of IgE. An analysis of systemic immune responses alone or after mucosal priming complemented this analysis. Systemic immunization with OVA demonstrated that this response was isolated from the mucosal immune system. High titers of IgG_{2a} were induced in this manner. Even IgA was induced separately and independently of previous mucosal immunizations. These results suggest that effective vaccines and treatments for allergies can be developed for the GI tract which would not interfere with conventional immunotherapy.

2. Materials and methods

2.1. Antigen preparation and analysis

Highly purified OVA (Grade V, Sigma, St. Louis, MO, Cat. No. A3014) was encapsulated into microbeads with the two different methacrylate copolymers, Eudragit L30D and FS30D by Degussa Rohm Polymers, Darmstadt, Germany

and Glatt Laboratory, Ramsey, NJ, respectively. This technology has been previously described [22,35,36]. Briefly, this procedure utilizes a water soluble system with a pH dependent coating (Eudragit) which protects antigen during passage through the acidic environment of the stomach. Soluble antigen is spray coated onto a sugar/starch core using a Wurster bottom spray process. The antigen coated cores are then enteric coated with an aqueous dispersion of the pH sensitive methacrylic acid copolymer (Eudragit, Rohm Polymers). The pH of dissolution of each microbead preparation was determined by rocking each of them in phosphate buffered saline (PBS) solutions of different pH values and then sampling the supernatants at various times, in triplicate. The protein concentration in each sample was determined using the Pierce BCA protein assay reagent (Rockville, IL) and compared to identical samples mechanically disrupted by grinding to obtain a percentage of protein release.

2.2. Animals and immunizations

Ten to 20 weeks old female BALB/c and BDF₁ mice were obtained from the National Cancer Institute (Bethesda, MD). These mice were housed in an American Association for Laboratory Animal Care-approved quarters under specific pathogen-free conditions with a defined diet at the University of Cincinnati College of Medicine. Experimental groups consisted of six or more mice. Pools of sera were made from the same number of mice per group. Prior to oral immunization the mice were fasted for 2 h. The mice were then anesthetized with isoflurane and 10 or 20 mg of microbeads were placed in the back of their mouth after it had been acidified with water. The mice were allowed to swallow the beads with more acidified water, and then placed back in their cages for 2 h more before allowing them to have food. The microbeads are large enough (0.75 mm in diameter) to ensure that each dose is administered completely. For oral gavage, 0.5 ml of a 1 mg/ml solution of OVA (Grade V, Sigma, St. Louis, MO, Cat. No. A3014) was administered to anesthetized mice in PBS, pH 7.4. For i.p. injections, three mice were injected with 0.2 ml of a 250 mg/ml solution of grade II OVA (Sigma, St. Louis, MO Cat. No. A5253) resuspended in sterile saline by vortexing. Sera were obtained by retro-orbital bleeding into heparin-saline on the following days: day 21 post primary mucosal immunization, d14 post chronic “primary” immunization, d7 post booster mucosal immunization, and d7 post i.p. immunizations.

2.3. ELISAs and analysis of sera

ELISA assays were performed as previously described with some modifications [37]. Briefly, sera were diluted in microfuge tubes using phosphate buffer containing 0.05% Tween 20 and 5% gelatin. This was further diluted into OVA-coated (Grade V, Sigma, St. Louis, MO, Cat. No. A3014) microtiter plates containing the same diluent. After

60 min of incubation at 37 °C, the plates were washed five times and alkaline phosphatase conjugated isotype-specific antibodies in the same diluent were added. Rabbit (Rb) anti-mouse(m) IgM, Rb anti-mIgA, Rb anti-mIgG₁, Rb anti-mIgG_{2a} and Rb anti-GAM (specific for mouse IgG, IgM and IgA) were obtained from Zymed laboratories (South San Francisco, CA). After 60 min incubation at 37 °C, the plates were washed as above and substrate, 4-nitrophenylphosphate (Boehringer–Mannheim), was added at a final concentration of 1 mg/ml. After an appropriate period of incubation, 50 µl of 1N NaOH was added and the absorbance was read at 405 nm in a Biorad EIA reader. All titers were calculated in the following manner. Each absorbance value is divided by the absorbance of a standard control serum that was treated in the same manner on each plate and expressed as the proportion of this control. The titer was established as the reciprocal of the last dilution that gave a proportion of the control of 0.2 or greater.

The levels of IgE were determined in a slightly different manner. Plates were coated with Rat anti-mouse IgE (RαMIgE) (Pharmingen, Cat. No. 553413). The plates were then blocked with phosphate buffer containing 0.05% Tween 20 and 5% gelatin. After washing the plates, the samples of diluted sera were added as above and incubated 60 min at 37 °C. The plates were then washed and biotin-conjugated OVA was added. After a 60 min incubation period, the plates were washed again and streptavidin-conjugated horse radish peroxidase was added (Pharmingen 13047E). The plates were incubated for another 60 min, washed, and tetramethylbenzidine dihydrochloride reagent in 0.05 M phosphate citrate buffer with H₂O₂ was added (Sigma, St. Louis, MO, Cat. No. T-3405). The reaction was stopped with 50 µl of 2N H₂SO₄ and the absorbance was read at 450 nm. Titers were calculated in the same manner as described above for the other isotypes using a standard control serum. This assay was shown to be specific for IgE, except when OVA-reactive IgM was present. IgM sticks non-specifically to the RαMIgE coated plates; therefore, the level of OVA-reactive IgE could not be accurately determined near the titer of OVA-reactive IgM.

Analysis of the data in this manner allowed the sera to be directly compared to each other. Although the titer of each isotype of anti-OVA antibody is not directly comparable to the titer of the other isotypes for an exact concentration difference, the relative differences between samples are directly comparable. This is because of the differences in avidity and concentration of the various isotypes as well as differences in the immunoassays for the detection of IgE and non-IgE isotypes.

2.4. Statistical analyses

Sera analyses were performed in triplicate for each dilution of each serum or sera pool. Statistical analyses were performed using a Mann–Whitney *U*-test on an IBM compatible computer (Anderson Bell).

3. Results and discussion

3.1. Antigen release from Eudragit coated microbeads is pH-dependent as determined in vitro

To determine the pH at which each microbead preparation released OVA, the two preparations containing OVA (w/w) L30D/1% OVA or FS30D/1% OVA were rocked horizontally at room temperature in PBS solutions of different pH values. Release of OVA was determined by measuring the amount of protein released into the supernatants after various periods of rocking. Fig. 1 demonstrates that protein was released from L30D microbeads in PBS buffers of pH values of 6.0 or greater. 100% release of OVA from the L30D/OVA occurred within 60 min at pH 7.0 and within 90 min at pH 6.5, but was not released at pH values 5.5 for at least 240 min. Buffers with a pH value of 8 or greater caused 100% release of OVA from the FS30D/OVA microbeads within 30 min of rocking, and nearly 100% release within 90 min at a pH of 7.5 (Fig. 2). However, pH values <6.5 did not cause release of the antigen in at least 240 min. The actual pH in the PBS buffer decreases approximately 0.5 pH units due to dissolution of the carboxylic acid-containing copolymer. Therefore, L30D/OVA should deliver antigen to the upper SI or duodenum/jejunum (pH = 6.4) and FS30D/OVA should deliver OVA to the lower SI or ileum (pH = 7.4) [11].

3.2. Antigen release in vivo

The specific region of the GI tract in which OVA was released from each microbead preparation could easily be determined by establishing the positions and conditions of the 0.75 mm microbeads in the mouse GI tract at different times after p.o. administration. These preparations are biodegradable and safe for administration of antigens to the GI tract as has been well described [35,36,38–40]. The distances of the beads from the stomach at various times after oral administration are depicted in Table 1. Antigen release occurred within the first 9 cm of the SI (duodenum/jejunum) for the L30D/OVA preparation and 23–31 cm (mid to terminal ileum) for the FS30D/OVA preparation. These results were very reproducible. Beads were never found undissolved after 0–9 cm from the stomach for L30D/OVA or dissolved less than 23 cm from the stomach for FS30D/OVA. Furthermore, X-ray photography demonstrated that L30D-coated microbeads loaded with Barium Sulfate passed unaltered through the stomach and completely dissolved in the upper SI (data not shown).

3.3. Sub-compartmentalization of the GI immune system as determined by oral administration of OVA-containing microbeads encapsulated with L30D or FS30D

Measurement of serum antibodies reflects the induction of systemic antibodies as well as mucosal antibodies since antibody produced at mucosal sites is transported into the

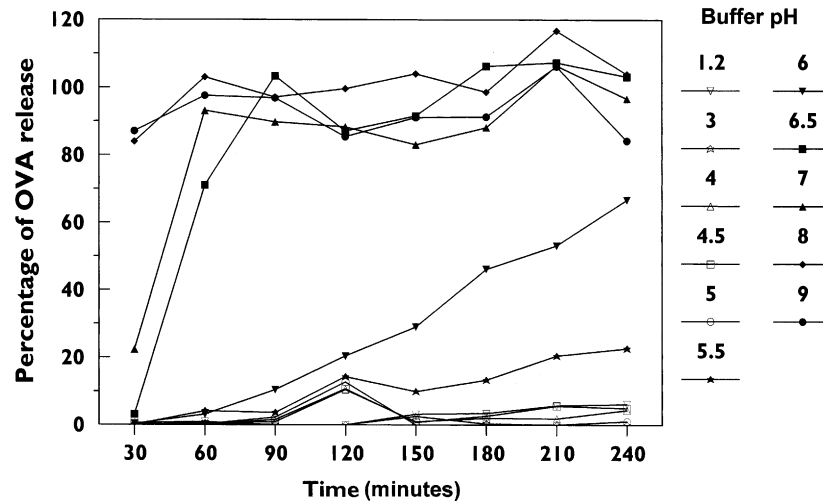


Fig. 1. Dissolution of L30D/OVA in buffers of different pH. Fifty milligrams of L30D/OVA was rocked in PBS of different pH values. Protein concentration in the supernatant was determined at the times indicated as described in Section 2. The results are expressed as the mean of triplicate measurements of the proportion of the protein released compared to an equivalent sample mechanically disrupted.

blood; whereas antibodies secreted at mucosal sites do not normally reflect serum responses [41,42]. Since immunization of the SI with Eudragit-coated microbeads containing OVA has been shown to induce OVA-specific mucosal IgA as well as serum antibodies [40], we chose to measure the anti-OVA antibody response in the sera in this first study of the sub-compartmentalization of the GI immune system.

Primary immunization with one dose of OVA delivered to the upper SI with L30D with 1.0 mg OVA encapsulated within (L30D/1.0 mg OVA) resulted in the production of anti-OVA serum antibodies (Table 2). This response differed between different types of mice. BDF₁ mice generated a primary antibody response of the IgG₁ isotype, while BALB/c produced primarily IgM. Secondary immunizations with L30D/1.0 mg OVA produced detectable levels

of anti-OVA IgG₁ in BALB/c mice and boosted the levels in BDF₁ mice (Table 2). It would seem surprising that BDF₁ mice produced anti-OVA antibodies of the IgG₁ isotype in the primary response against one dose of L30D/1.0 mg OVA and BALB/c did not, because BALB/c mice have a greater propensity to produce Th₂ responses; however, IgG₁ production requires genes responsible for GC formation as well as IL 4 [18,25]. BDF₁ mice produce greater Th₁ responses which are involved in GC formation [26,43,44].

Anti-OVA IgG_{2a} was not detected in the secondary response to L30D/1.0 mg OVA in either type of mice when OVA was delivered once in each immunization for a total of two doses. Multiple doses delivered to the upper SI of BDF₁ mice with three daily doses of L30D/1.0 mg OVA for primary and secondary immunizations did induce IgG_{2a}.

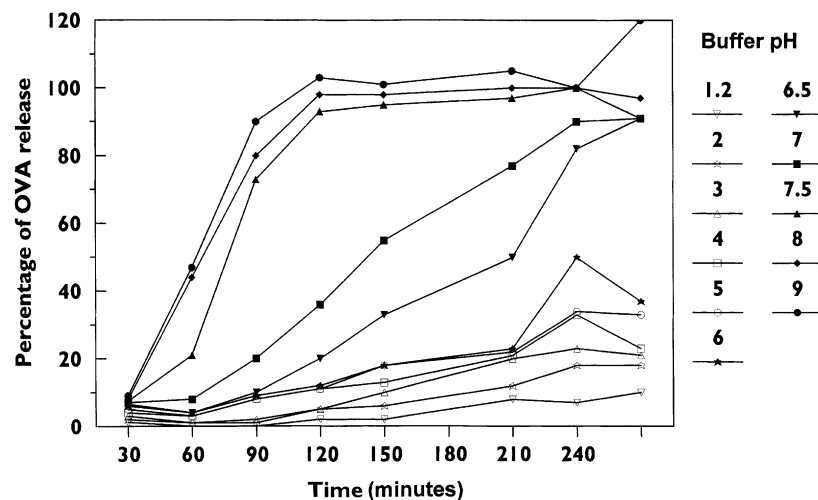


Fig. 2. Dissolution of FS30D/OVA in buffers of different pH. Fifty milligrams of FS30D/OVA was rocked in PBS of different pH values. Protein concentration in the supernatant was determined at the times indicated as described in Section 2. The results are expressed as the mean of triplicate measurements of the proportion of the protein released compared to an equivalent sample mechanically disrupted.

Table 1
Dissolution of L30D/OVA and FS30D/OVA microbeads in vivo

Coat copolymer	Time (min)	Position of beads in the GI tract ^a	Condition of the microbeads in the GI tract
L30D	0	–	–
	30	Stomach	White intact beads
	45	SI, 2–9 cm from stomach	Intact beads or damaged or fragile white beads or yellow chyme bolus
	60	SI, 15 cm from stomach	No intact beads, yellow chyme bolus
	90	–	No intact beads
	120	–	No intact beads
FS30D	0	–	White intact beads
	30	Stomach	White intact beads
	60	SI, 18 cm from stomach	White intact beads in chyme
	120	SI, 23 cm from stomach	White intact beads in chyme
	240	SI, 31 cm from stomach	No intact beads, yellow chyme bolus

^a BALB/c mice were fasted for 4 h prior to being given L30D/OVA or FS30D/OVA, p.o. They were then returned to their cages without food. After the indicated times of incubation, the mice were sacrificed, and their GI tracts dissected to determine the positions and conditions of the microbeads. The results represent the summary of three separate experiments. The distances to the cecum and rectum are approximately 33 and 43 cm from the murine stomach.

This demonstrates that IgG_{2a} induction is difficult in this region, but that this can be overcome (Table 2). These results may reflect the requirement for GCs as well as T_H1 cytokines in production of the IgG_{2a} and the small number of follicles present in the upper SI [1,18,25]. It is much easier to induce IgG_{2a} in the lower SI where a greater number of follicles exist as well as DCs that promote T_H1 responses [1,45,46]. In fact, five times less antigen delivered to the lower SI (FS30D/0.2 mg OVA) versus the upper SI (L30D/1.0 mg OVA) stimulated the same level of production of IgG_{2a} (Table 2). Furthermore, the upper SI appears to be more biased toward the T_H2 phenotype than the lower SI (Tables 2–4). This is by shown by the production of IgG₁ as well as the induction of T_H2 memory cells and/or memory B cells with isotypes induced by T_H2 cytokines that migrate to the lower SI (Table 4). Immunization of BDF₁ mice with multiple doses in the primary and secondary immune responses followed by immunization of the lower SI

with FS30D/0.2 mg OVA actually produced lower levels of IgG_{2a} than immunization of the upper SI with only one dose per immunization (Table 4). These results are supported by those obtained by for BALB/c mice. Only one of six BALB/c mice that were chronically immunized with L30D/0.5 mg OVA every other day for 2 weeks demonstrated detectable IgG_{2a} (Table 3). Two of six BALB/c mice that were immunized with L30D/0.5 mg OVA every other day for 2 weeks did not respond or responded very poorly. Yet eight of eight BALB/c mice which were immunized with FS30D/0.5 mg OVA responded well and four of the eight produced IgG_{2a} (Table 3).

GCs are involved in most types of immune memory and are required for normal control of commensal bacteria in the SI [24,29]. Since GC induction requires T_H1 cytokines, a sequential segregated immunization protocol in the SI may select for T_H1 memory responses because memory cells migrate [30–32]. TGF- β may also be involved in the migration

Table 2
Titers of OVA-specific antibody isotypes in sera pools of BALB/c and BDF₁ mice induced by immunization of the two different regions of the GI tract

Mice	Copolymer utilized for each immunization ^a		Titer ^b				
	Primary	Secondary	IgM	IgG ₁	IgG _{2a}	IgA	IgE
BDF ₁	None ^c		<90	<90	<90	<90	<90
	L30D/1.0 mg OVA		<90	2,430	<90	<90	<90
	L30D/1.0 mg OVA	L30D/1.0 mg OVA	<90	7,290	<90	270	270
	L30D/1.0 mg OVA 3×	L30D/1.0 mg OVA 3×	90	>7,290	810	2,430	270
	FS30D/0.2 mg OVA	–	<90	<90	<90	<90	<90
	FS30D/0.2 mg OVA	FS30D/0.2 mg OVA	90	810	<90	<90	270
	FS30D/0.2 mg OVA 3×	–	<90	810	<90	<90	<90
	FS30D/0.2 mg OVA 3×	FS30D/0.2 mg OVA 3×	<90	7,290	810	810	90
BALB/c	None ^c		90	<90	<90	<90	nd
	L30D/1.0 mg OVA		270	<90	<90	<90	nd
	L30D/1.0 mg OVA	L30D/1.0 mg OVA	270	810	<90	<90	nd

^a Mice were immunized with 20 mg of the microbeads, L30D/5% OVA (1 mg OVA) or FS30D/1% OVA (0.2 mg OVA) p.o. one time (1×) or three times over three consecutive days (3×). They received the second immunization 4 weeks later.

^b The titer of the pool of sera was determined as the reciprocal of the last dilution with an optical density of ≥ 0.2 of the control immune serum as described in Section 2. All results represent the means of sera pools analyzed in triplicate, $P < 0.05$ Mann–Whitney U -test.

^c Methacrylic copolymer beads that do not contain OVA do not induce OVA-specific antibodies.

Table 3

Titers of OVA-specific antibody isotypes in individual BALB/c mice after chronic “primary” immunization of the GI tract

Mouse number	Immunization ^a	Titer ^b			
		IgM	IgG ₁	IgG _{2a}	IgA
1	Soluble OVA 0.5 mg dosed by gavage every other day for a total of 6 doses	90	810	<90	<90
2		90	7,290	<90	<90
3		90	2,430	<90	<90
4		90	810	<90	<90
5		90	810	<90	<90
1	L30D/OVA 0.5 mg OVA encapsulated with L30D was administered every other day for a total of 6 doses	90	<90	<90	<90
2		90	90	<90	<90
3		270	7,290	<90	90
4		270	7,290	810	90
5		270	7,290	<90	<90
6		270	2,430	<90	<90
1	FS30D/OVA 0.5 mg OVA encapsulated with FS30D was administered every other day for a total of 6 doses	270	7,290	<90	<90
2		270	7,290	90	<90
3		270	2,430	<90	<90
4		270	7,290	270	90
5		270	810	<90	90
6		270	7,290	810	90
7		270	2,430	<90	<90
8		270	21,870	90	<90
1	FS30D/OVA 0.5 mg OVA encapsulated with FS30D was administered on 3 consecutive days	270	2,430	810	90
2		270	810	<90	<90
3		270	2,430	270	90
4		270	2,430	<90	<90
5		270	2,430	270	<90

^a Mice were immunized with 10 mg of the microbeads, p.o., every other day for a total of six doses or three times over three consecutive days.^b See Table 2 legend and Section 2.

of these memory cells to the ileum. TGF- β is chemotactic for memory T cells and a higher production of TGF- β in the lower SI would favor chemotaxis to that location [47]. Little TGF- β is produced in the jejunal crypts [48]. In fact, significant levels of TGF- β are not induced upon feeding of OVA, unless anti-IL 12 is supplied to inhibit IFN- γ induction [49]. Immunization of the upper SI followed by immunization of the lower SI of BDF₁ mice, does induce IgG_{2a}, as well as high levels of IgG₁ (Table 4). This was more apparent when BDF₁ mice were given primary and secondary

immunizations with only one dose of L30D/1.0 mg OVA, and then a tertiary immunization with FS30D/OVA. Animals immunized with this protocol produced significantly higher levels of IgG_{2a}, yet low levels of IgE. A higher dose of OVA, FS30D/0.5 mg OVA, produced even higher levels of IgG_{2a}. The response of BDF₁ mice to a sequential segregated immunization protocol is likely the result of the specific stimulation of memory cells that have migrated to the lower SI as well as the environment of the lower SI of BDF₁ mice. The same sequential segregated immunization protocol yielded

Table 4

Titers of OVA-specific antibody isotypes in sera pools of BDF₁ mice induced by sequential segregated immunization of the two regions of the GI tract

Strain	Immunizations protocols ^a			Titer ^b				
	1st	2nd	3rd	IgM	IgG ₁	IgG _{2a}	IgA	IgE
BDF ₁	L30D, 1 $\times$ 1.0 mg	FS30D, 1 $\times$ 0.2 mg	–	<90	7,290	90	<90	90
	FS30D, 1 $\times$ 0.2 mg	L30D, 1 $\times$ 1.0 mg	–	90	2,430	<90	<90	270
	L30D, 1 $\times$ 1.0 mg	FS30D, 1 $\times$ 0.2 mg	FS30D, 3 $\times$ 0.1 mg	90	>7,290	810	810	810
	L30D, 1 $\times$ 1.0 mg	L30D, 1 $\times$ 1.0 mg	FS30D, 3 $\times$ 0.1 mg	90	>7,290	810	810	270
	L30D, 1 $\times$ 1.0 mg	L30D, 1 $\times$ 1.0 mg	FS30D, 3 $\times$ 0.5 mg	270	21,870	2,430	7,290	270 ^c
	L30D, 3 $\times$ 1.0 mg	L30D, 3 $\times$ 1.0 mg	FS30D, 3 $\times$ 0.5 mg	90	21,870	270	810	270
BALB/c	L30D, 1 $\times$ 1.0 mg	FS30D, 1 $\times$ 0.2 mg		270	2,430	<90	270	nd

^a Mice were immunized with microbeads containing the amounts of OVA indicated p.o., one time (1 $\times$) or three times over three consecutive days (3 $\times$).^b See Table 2 legend and Section 2.^c Cannot be accurately measured with OVA-reactive IgM present (see Section 2).

different results for BALB/c mice. BALB/c did not produce detectable levels of IgG_{2a} when immunized in this manner. The migrating memory cells appeared to be a mixture of T_h1, T_h2, and/or T_h3 cells in BDF₁ mice, while only T_h2 and/or T_h3 phenotypes were detected in BALB/c mice (Table 4).

The anti-OVA IgA responses of the different regions of the GI tract differed significantly between the types of mice. No detectable anti-OVA IgA was produced in the secondary response of the upper SI of BALB/c mice, while it was produced in BDF₁ mice immunized in this manner (Table 2). However, anti-OVA IgA was more easily induced in the lower SI of BALB/c than BDF₁ mice. Immunization of the upper SI with L30D/1.0 mg OVA did prime BDF₁ mice for a secondary antibody response 1 month later in the ileum to FS30D/0.2 mg OVA; however, this secondary anti-OVA response produced anti-OVA IgG₁ and IgG_{2a}, but not anti-OVA IgA antibodies. Yet this same dosing regime induced OVA-binding IgA in BALB/c mice, but not IgG_{2a}. The influence of the genetic differences between these mice on IgA induction in the ileum can be overcome by increasing the dose of antigen. BDF₁ mice did not produce significant levels of anti-OVA IgA antibody when given single primary and secondary doses of FS30D/0.2 mg OVA, but three daily doses of FS30D/0.2 mg OVA for each immunization did result in the production of anti-OVA IgA (Table 2).

There are probably several mechanisms responsible for the differences in IgA induction in the different regions of the SI. First, differences in the amounts of TGF- β produced in the upper and lower SI may also be responsible for some of the differences in IgA induction between the upper and lower SI. Second, the relative difficulty inducing IgA in the lower SI of BDF₁ mice is likely due to the inhibition of T_h3 differentiation by the higher production of T_h1 cytokines by these mice [15,43,44,49]. One possible explanation for the difference in the responses of these two types of mice to OVA in the upper SI is that the response of B1 B cells differs between these mice. IgA is produced by B1 B cells against commensal bacteria, including membrane proteins [12]. Although OVA has typically been classified as a B2 antigen, it is a glycoprotein. It is therefore likely an antigen for B1 B cells [50–53]. B1 B cells stimulated by immunization of the upper SI of BDF₁ mice with OVA may produce predominantly IgA, while those of the BALB/c mice IgM.

This would be supported by the differences in the levels of T_h1 cytokines in these mice [43,44,49]. Furthermore, a low level of TGF β in the upper SI would not likely influence IgA production by B1 B cells, since TGF- β does not enhance their production of IgA [54,55].

There is one other mechanism that could also be responsible for the differences in IgA induction between the two different types of mice. Antigen-reactive IgM or IgA may aid in the transport of antigen into the PP via Fc μ / α receptors (Fc μ / α Rs) [1,56]. Since IgM is induced in the primary response of BALB/c mice in the upper SI, it may aid in the uptake of OVA when it is delivered to the lower SI with FS30D. However, increasing the number of doses in the primary and secondary immunizations of the upper SI of BDF₁ mice did not increase the level of IgA obtained after a tertiary immunization of the lower SI with FS30D, even though higher levels of IgA were induced in the secondary response to L30D/1.0 mg OVA (Table 4). Furthermore, antigen uptake via the Fc α / μ Rs could not be the only mechanism involved in the differences in IgA induction between these types of mice, because BDF₁ mice did produce IgG_{2a} before they produced IgA upon immunization of the ileum (Tables 3 and 4).

3.4. Chronic immunization of the SI follicles increases the T_h2-influenced immunoglobulin responses, including IgE

The lower SI of BDF₁ mice initially demonstrated greater T_h1 responses than the upper SI; however, repeated dosing of the lower SI eventually greatly increased the levels of IgE, as well as IgG₁ and IgA (Table 5). This indicates an eventual shift of OVA-reactive T cells to the T_h2 phenotype in the lower SI, even in BDF₁ mice. As a consequence of the larger number of follicles in the lower SI there are more GCs induced and this allows for vigorous T_h2 and T_h3 responses after repeated immunization [14,15]. IgG_{2a} was probably not detected in BALB/c mice with a sequential segregated immunization protocol because fewer T_h1 cells are produced in the upper SI and their GCs are quickly converted to a T_h2/T_h3 environment [43,44]. Chronic immunization of lower SI of BALB/c did induce detectable levels of IgG_{2a} in a “primary” response, presumably because a large enough number of GCs were induced in that

Table 5

Titers of OVA-specific antibody isotypes in sera pools of BDF₁ mice induced by long term immunization of different regions of the GI tract

Immunization method ^a					Titer ^b				
1st	2nd	3rd	4th	5th	IgM	IgG ₁	IgG _{2a}	IgA	IgE
FS30D, 1 × 0.2 mg	FS30D, 1 × 0.2 mg	FS30D, 3 × 0.1 mg			<90	2,430	270	270	270
FS30D, 3 × 0.2 mg	FS30D, 3 × 0.2 mg	FS30D, 1 × 0.5 mg			90	21,870	810	810	270
FS30D, 1 × 0.2 mg	FS30D, 1 × 0.2 mg	FS30D, 3 × 0.1 mg	FS30D, 1 × 0.5 mg		90	21,870	810	810	270
FS30D, 3 × 0.2 mg	FS30D, 3 × 0.2 mg	FS30D, 1 × 0.5 mg	FS30D, 3 × 0.5 mg		90	21,870	810	810	270
FS30D, 1 × 0.2 mg	FS30D, 1 × 0.2 mg	FS30D, 3 × 0.1 mg	FS30D, 1 × 0.5 mg	FS30D, 3 × 0.5 mg	270	21,870	270	810	2,430

^a Mice were immunized with the amounts of OVA indicated with microbeads, p.o., one time (1×) or three times over three consecutive days (3×).

^b See Table 2 legend and Section 2.

Table 6

Titers of OVA-specific antibody isotypes in sera pools induced by pervasive immunization of the SI of BDF₁ mice

Microbead preparations used for immunization ^a				Titer ^b				
1	2	3	4	IgM	IgG ₁	IgG _{2a}	IgA	IgE
Mix				90	90	<90	<90	<90
Mix	Mix			90	7,290	<90	90	90 ^c
Mix	Mix	FS30D		90	7,290	<90	270	810
Mix	Mix	FS30D	MIX	90	21,870	810	810	2,430

^a Mice were immunized with the microbeads, p.o. one time (1×) or three times over three consecutive days (3×). The following doses were each given 4 weeks apart. Mix, 20 mg of a 1:1 mix of L30D/5% OVA and FS30D/1% OVA; FS30D 1×, 20 mg of FS30D/5% OVA; MIX, 10 mg of a 1:1 mix of L30D/5% OVA and FS30D/5% OVA.

^b See Table 2 legend and Section 2.

^c Cannot be accurately measured with OVA-reactive IgM present (see Section 2).

manner before that genetic environment, which favors T_h2 cytokines, fully converted their OVA-reactive lymphocytes to a T_h2/T_h3 response (Table 3).

3.5. Pervasive immunization resembles chronic immunization of the SI

Pervasive immunization could induce a larger number of GCs which might increase T_h1 responses and IgG_{2a} production. L30D/OVA and FS30D/OVA were mixed and used to simultaneously immunize the upper and lower SI of BDF₁ mice (Table 6). Significant levels of IgG_{2a} were induced by this method; however, multiple pervasive immunizations also induced very high titers of IgE. The reason for the exacerbation of the IgE response by pervasive immunization probably involves the same mechanisms that are involved in the IgE induced by chronic immunization of the lower SI, together with the bias of the upper SI toward T_h2 responses. Although IgE production requires IL 4 and CD40 stimulation, it does not require GCs [18,25,27]; Therefore, IgE could be induced in many areas of the SI, not just in GCs. Memory cells of the T_h2 phenotype that migrate from the upper SI to the lower SI might also allow for greater IgE induction because less IFN-γ may be produced there by the resident IELs than in the upper SI [57,58]. Furthermore, since only 20 mg of microbeads can be fed to a mouse at one time, only half of each normal dose was delivered to each region. Therefore, the increase in IgE could in part be due to a decreased dose to each region. However, the presence of

increased levels of IgG_{2a} in this response demonstrates that a dose high enough to induce GCs was achieved by this dose (Table 6). Furthermore, IgE is produced even when high doses of OVA are delivered only to the ileum after priming both regions (Table 6). Therefore, it is the pervasive immunization procedure in the SI that appears to induce high levels of IgE.

3.6. Compartmentalization of the systemic and the GI immune responses to OVA

To study communication between the systemic and mucosal immune systems, sera of BDF₁ mice which had been immunized i.p. with soluble OVA only, or following immunization with OVA p.o., were analyzed. Analysis of this data clearly indicated that a good T_h1 response, as determined by IgG_{2a} levels, was induced with soluble OVA in the primary response without any adjuvant (Table 7). IgA was induced in the secondary and tertiary i.p. immunizations. The data do not suggest a substantial boost of the mucosal immune response by parenteral immunization (Table 7). Boosting mice, i.p. that had been previously immunized with OVA p.o. resulted in drastically reduced levels of anti-OVA IgA and IgG₁ as compared to the p.o. immunizations (Table 7). Although IgA was induced after secondary immunization with OVA i.p., and with secondary immunization with OVA p.o., little IgA was found in mice that had been immunized twice p.o. with L30D/OVA and then once with OVA i.p. (Table 7). Whereas a tertiary immunization of a

Table 7

Titers of OVA-specific antibody isotypes induced in BDF₁ mice after i.p. immunization with OVA in saline or p.o. immunization with pH-sensitive microbeads

Primary immunization ^a	Secondary immunization	Tertiary immunization	Titer ^b				
			IgM	IgG ₁	IgG _{2a}	IgA	IgE
Soluble OVA, 50 mg, i.p.	–	–	270	270	90	<90	nd
Soluble OVA, 50 mg, i.p.	Soluble OVA, 50 mg, i.p.	–	270	7,290	810	270	nd
Soluble OVA, 50 mg, i.p.	Soluble OVA 50 mg, i.p.	Soluble OVA, 50 mg, i.p.	270	7,290	21,870	810	nd
L30D/OVA, 3 × 0.2 mg	L30D/OVA, 3 × 0.2 mg	Soluble OVA, 50 mg, i.p.	810	810	270	<90	nd

^a For oral immunization, mice were immunized with the indicated amount of grade II OVA in saline, i.p., or grade V OVA in the pH sensitive microbeads, p.o., one time (1×) or three times on three consecutive days (3×). The immunizations were given 4 weeks apart.

^b See Table 2 legend and Section 2.

similar group of animals with FS30D/OVA induced significant amounts anti-OVA IgA (Table 4). Therefore, help for IgA production exists in the mucosa after mucosal immunization, but not the systemic immune system. The two systems appeared to be completely isolated for IgA responses. The slight increase in IgM, IgG₁, and IgG_{2a} responses as compared to i.p. injection alone may reflect the presence of a population of memory cells in the splenic marginal zone which were generated at the mucosa [59].

The low level of IgG₁, as well as the production of significant amounts of OVA-reactive IgM and IgG_{2a} in response to soluble OVA, i.p., also suggests the participation of B1 B cells of the peritoneum or the “B1 like” MZ B cells of the spleen in this response [22,50,52,53,59–61]. Although most reports for i.p. injection of soluble OVA or OVA/alum have described T_h2 responses, some reports have noted significant production of isotypes influenced by T_h1 cytokines [21,22,62–64]. The most likely explanation for the production of T_h1 cytokine dependent isotypes in this response, as well as the discrepancies in the literature, is due to differences in antigenic form. The i.p. injections of OVA consisted of crude egg albumin extract or “grade II” OVA. Grade II OVA in these i.p. injections is quite aggregated, since a good portion of OVA in solution is aggregated (unpublished observations). Natural anti-carbohydrate antibody or OVA-binding B1 B cells may then allow enough complement fixation to cause degranulation of the CTMCs and the release of IFN- γ , even in the absence of OVA-reactive IgE [7]. It has been shown that the aggregation of OVA is important for T_h1 responses to OVA; however a role of contaminants cannot be completely ruled out [63].

The effective compartmentalization of these immune responses also has significant implications for allergy treatment. It suggests that a combination of an optimal GI tract immunization may be complemented by systemic immunization therapies, such as subcutaneous immunization therapy (S.I.T.). At the least, it suggests that optimal GI tract immunization will not negatively affect S.I.T.

Acknowledgements

The Eudragit coated OVA microbeads used in this study were kindly provided at no charge by Allerganics Inc., 395 Oyster Point Blvd, Suite 113, South San Francisco, CA 94080. This work was supported by Senmed Medical Ventures, 4445 Lake Forest Drive, Suite 600, Cincinnati, OH 45242, USA.

References

- [1] Neutra MR, Mantis NJ, Kraehenbuhl JP. Collaboration of epithelial cells with organized mucosal lymphoid tissue. *Nat Immunol* 2001;2(11):1004–9.
- [2] Quiding-Järbrink M, Ahlstedt I, Lindholm C, Johansson EL, Lönnroth H. Homing commitment of lymphocytes activated in the human gastric and intestinal mucosa. *Gut* 2001;49(4):519–25.
- [3] Lee HO, Miller SD, Hurst SD, Tan LJ, Cooper CJ, Barrett TA. Interferon gamma induction during oral tolerance reduces T cell migration to sites of inflammation. *Gastroenterology* 2000;119(1):129–38.
- [4] Suzuki H, Jeong KI, Okutani T, Doi K. Regional variations in the number and subsets of intraepithelial lymphocytes in the mouse small intestine. *Comp Med* 2000;50(2):39–42.
- [5] van den Brink GR, van den Boogard DEM, van Deventer SJH, Peppelenbosch MP. Feed a cold, starve a fever. *Clin Diag Lab Immunol* 2002;9(1):182–3.
- [6] Costa JJ, Galli SJ. Mast cells and Basophils: basic biology and roles in gastrointestinal diseases. In: Frieri M, Kettelhut B, editors. *Food hypersensitivity and adverse reactions: a practical guide for diagnosis and management*. New York: Marcel Dekker; 1999. p. 1–38.
- [7] Wasserman SI. Mast cell biology. *J Allergy Clin Immunol* 1990;86(4 pt 2):590–3.
- [8] Smith TJ, Ducharme LA, Weis JH. Preferential expression of interleukin-12 or interleukin-4 by murine bone marrow mast cells derived in mast cell growth factor or interleukin 3. *Eur J Immunol* 1994;24(4):822–6.
- [9] Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* 2001;2(4):361–7.
- [10] Penney L, Kilshaw PJ, MacDonald TT. Regional variation in the proliferative rate and lifespan of alpha beta TCR+ and gamma delta TCR+ intraepithelial lymphocytes in the murine small intestine. *Immunology* 1995;86(2):212–8.
- [11] Kararli TT. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm Drug Dispos* 1995;16(5):351–80.
- [12] MacPherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 2000;288(5474):2222–6.
- [13] Tegoshi T, Okada M, Nishida M, Arizono N. Early increase of gut intraepithelial mast cell precursors following *Strongyloides venezuelensis* infection in mice. *Parasitology* 1997;114(pt 2):181–7.
- [14] Alpan O, Rudomen G, Matzinger P. The role of dendritic cells, B cells, and M cells in gut-oriented immune responses. *J Immunol* 2001;166(8):4843–52.
- [15] Weiner HL. Induction and mechanism of action of transforming growth factor- β -secreting T_h3 regulatory cells. *Immun Rev* 2001;182:207–14.
- [16] Astwood JD, Leach JN, Fuchs RL. Stability of food allergens to digestion in vitro. *Nat Biotechnol* 1996;14(10):1269–73.
- [17] Schödel F. Oral vaccination using recombinant bacteria. *Semin Immunol* 1990;2:341–9.
- [18] Snapper CM, Mond JJ. Towards a comprehensive view of immunoglobulin class switching. *Immunol Tod* 1993;14(1):15–7.
- [19] Whitacre CC. New insights into oral tolerance. *Gastroenterology* 2000;119(1):260–3.
- [20] Lanzavecchia A, Sallusto F. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* 2000;290(5489):92–7.
- [21] Barone KS, Reilly MR, Flanagan MP, Michael JG. Abrogation of oral tolerance by feeding encapsulated antigen. *Cell Immunol* 2000;199(2):65–72.
- [22] Flanagan MP, Battisti G, Michael JG. Oral administration of *Escherichia coli* in enteric coated microparticles induces serum antibodies against lipopolysaccharide antigens. *J Endotoxin Res* 1996;3(6):481–9.
- [23] Thatte J, Rath S, Bal V. Immunization with live versus killed *Salmonella typhimurium* leads to the generation of an IFN- γ -dominant versus an IL-4-dominant immune response. *Int Immunol* 1993;5(11):1431–6.

- [24] Cottier H, Keiser G, Odartchenko N, Hess M, Stoner RD. De novo formation and rapid growth of germinal centers during secondary antibody responses to tetanus toxoid in mice. In: Cottier H, Keiser G, Odartchenko N, Hess M, Stoner RD, editors. Germinal centers in immune responses. New York: Springer-Verlag; 1967. p. 271.
- [25] Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by bcl 6. *Science* 1997;276(5312):589–92.
- [26] Matsumoto M, Mariathasan S, Nahm MH, Baranyay F, Peschon JJ, Chaplin DD. Role of lymphotoxin in the formation of germinal centers. *Science* 1996;271(5253):1289–91.
- [27] Maliszewski CR, Kennedy MK. CD40 ligand knockout mice. In: Durum SK, Muegge K, editors. Cytokine knockouts. Totowa, NJ: Humana Press; 1998. p. 53–67.
- [28] Ozkaki M, Spolski R, Feng CG, Qi CF, Cheng J, Sher A, et al. A critical role for IL21 in regulating immunoglobulin production. *Science* 2002;298(5598):1630–4.
- [29] Fagarasaon S, Muramatsu M, Suzuki K, Nagaoka H, Hiai H, Honjo T. Critical roles of activation-induced cytidine deaminase in the homeostasis of Gut Flora. *Science* 2002;298:1424–7.
- [30] Holtmeier W, Hennemann A, Caspary WF. IgA and IgM V_H repertoires in human colon: evidence for clonally expanded B cells that are widely disseminated. *Gastroenterology* 2000;119(5):1253–66.
- [31] Sprent J, Tough DF. T cell death and memory. *Science* 2001;293(5528):245–8.
- [32] MacKay CR. Homing of naïve, memory and effector lymphocytes. *Curr Opin Immunol* 1993;5(3):423–7.
- [33] Fargeas C, Wu CY, Nakajia T, Cox D, Nutman T, Delespesse G. Differential effect of transforming growth factor beta on the synthesis of T_H1 and T_H2 like lymphokines by human T lymphocytes. *Eur J Immunol* 1992;22(8):2173–6.
- [34] Galibert L, Burdin N, de Saint-Vis B, Garrone P, Van Kooten C, Bancereau J, et al. CD40 and B cell antigen receptor dual triggering of resting B lymphocytes turns on a partial germinal center phenotype. *J Exp Med* 1996;183(1):77–85.
- [35] Litwin A, Flanagan M, Michael JG. Microencapsulation: the future of oral immunotherapy. *BioDrugs* 1998;9(4):261–70.
- [36] Andus T, Gross V, Caesar I, Schulz HJ, Sochs H, Strom WD, et al. Replacement of conventional glucocorticoids by oral pH-modified release budesonide in active and inactive Crohn's disease: results of an open prospective, multicenter trial. *Dig Dis Sci* 2003;48:373–8.
- [37] Barone KS, Jain SL, Michael JG. Effect of In Vivo Depletion of CD4+ and CD8+ Cells on the Induction and Maintenance of Oral Tolerance. *Cell Immunol* 1995;163(1):19–29.
- [38] Zondlo M. Final report on the safety assessment of acrylates copolymer and 33 related cosmetic ingredients. *Int J Toxicol* 2002;21(Suppl 3):1–50.
- [39] Jain SL, Barone S, Flanagan MP, Michael JG. Activation patterns of murine B cells after oral administration of an encapsulated soluble antigen. *Vaccine* 1996;14:1291–7.
- [40] Flanagan MF, Wang C, Michael JG. Characterization of the immune response following the oral administration of modified antigen in an adjuvant-free system. *J Immunol* 1993;150:35A.
- [41] Manohar M, Baumann DO, Bos NA, Cebra JJ. Gut colonization of mice with actA-negative mutant of *Listeria monocytogenes* can stimulate a humoral mucosal immune response. *Infect Immun* 2001;69:3542–9.
- [42] Schäfer F, Kewenig S, Stolter N, Stahl-Hennig CS, Stallmach A, Kaup F-J, et al. Lack of simian immunodeficiency virus SIV specific IgA response in the intestine of SIV infected rhesus macaques. *Gut* 2002;50:608–14.
- [43] Murphy EA, Sathiyaseelan J, Parent MA, Zou B, Baldwin CL. Interferon-gamma is crucial for surviving a *Brucella abortus* infection in both resistant C57BL/6 and susceptible BALB/c mice. *Immunology* 2001;103(4):511–8.
- [44] Gieni RS, Fang Y, Trinchieri G, Umetsu DT, DeKruyff RH. Differential production of IL-12 in BALB/c and DBA/2 mice controls IL-4 versus IFN-gamma synthesis in primed CD4 lymphocytes. *Int Immunol* 1996;8(10):1511–20.
- [45] Becker C, Wirtz S, Blessing M, Pirhonen J, Strand D, Bechthold O. Constitutive p40 promoter activation and IL 23 production in the terminal ileum mediated by dendritic cells. *J Clin Invest* 2003;112:693–706.
- [46] Allan RS, Smith CM, Beiz GT, van Lint AL, Wakim LM, Heath WR. Epidermal viral immunity induced by CD8 α ⁺ dendritic cells but not by langerhans cells. *Science* 2003;301:1925–8.
- [47] Wahl SM. Transforming growth factor beta: the good, the bad, and the ugly. *J Exp Med* 1994;180(5):1587–90.
- [48] Coffey RJ, Russel WE, Barnard JA. Pharmacokinetics of TGF- β with emphasis on effects in liver and gut. *Ann NY Acad Sci* 1990;593:285–91.
- [49] Strober W, Kelsall B, Fuss I, Marth T, Ludviksson G, Ehrhardt R, et al. Reciprocal IFN- γ and TGF- β responses regulate the occurrence of mucosal inflammation. *Immunol Tod* 1997;18(1):61–4.
- [50] Bao S, Beagley KW, Murray AM, Caristo V, Matthaehi KI, Young IG, et al. Intestinal IgA plasma cells of the B1 lineage are IL-5 dependent. *Immunology* 1998;94(2):181–8.
- [51] McReynolds L, O'Malley BW, Nisbet AD, Fothergill JE, Givol D, Fields S, et al. Sequence of chicken ovalbumin mRNA. *Nature* 1978;273(5665):723–8.
- [52] Fagarasan S, Watanabe N, Honjo T. Generation, expansion, migration and activation of mouse B1 cells. *Immunol Rev* 2000;176:205–15.
- [53] Martin F, Kearney JF. B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a natural immune memory. *Immunol Rev* 2000;175:70–9.
- [54] Chen SS, Li Q. Transforming growth factor- β 1 (TGF- β 1) is a bifunctional immune regulator for mucosal IgA responses. *Cell Immunol* 1990;128(2):353–61.
- [55] Cazac BB, Roes J. TGF- β receptor controls B cell responsiveness and induction of IgA in vivo. *Immunity* 2000;13(4):443–51.
- [56] Sakamoto N, Shibuya K, Shimizu Y, Yotsumoto K, Miyabayashi T, Sakano S, et al. A novel Fc receptor for IgA and IgM is expressed on both hematopoietic and non-hematopoietic tissues. *Eur J Immunol* 2001;31(5):1310–6.
- [57] McGhee JR, Beagley KW, Fujihashi K, Taguchi T, Xu J, Aicher WK, et al. Regulatory mechanisms in mucosal immunity: roles for T helper cell subsets and derived cytokines in the induction of IgA responses. In: Griffin PD, Johnson PM, editors. Local immunity in reproduction. New York: Oxford University Press; 1993. p. 17–51.
- [58] Wilson AD, Bailey M, Williams NA, Stokes CR. The in vitro production of cytokines by mucosal lymphocytes immunized by oral administration of keyhole limpet hemocyanin using cholera toxin as an adjuvant 1991;21(10):2333–9.
- [59] Dunn-Walters DK, Isaacson PG, Spencer J. Analysis of mutations in immunoglobulin heavy chain variable region genes of microdissected marginal zone (MGZ) B cells suggest that the MGZ of the human spleen is a reservoir of memory B cells. *J Exp Med* 1995;182(2):559–66.
- [60] Tarlinton DM, McLean M, Nossal GJ. B1 and B2 cells differ in their potential to switch immunoglobulin isotype. *Eur J Immunol* 1995;25(12):3388–93.
- [61] Oliver AM, Martin F, Garland GL, Carter RH, Kearney JF. Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. *Eur J Immunol* 1997;27(9):2366–74.
- [62] Hogan SP, Mishra A, Brandt EB, Royalty MP, Pope SM, Zimmermann N, et al. Pathological function for eotaxin and eosinophils in eosinophilic gastrointestinal inflammation. *Nat Immunol* 2001;2(4):353–60.
- [63] Hayglass KT, Wang M, Gieni RS, Ellison C, Gartner J. In vivo direction of CD4 T cells to T_H1 and T_H2-like patterns of cytokine synthesis. *Adv Exp Med Biol* 1996;409:309–16.
- [64] Wu X-M, Nakashima M, Watanabe T. Selective suppression of antigen-specific T_H2 cells by continuous micro-dose oral tolerance. *Eur J Immunol* 1998;28(1):134–42.