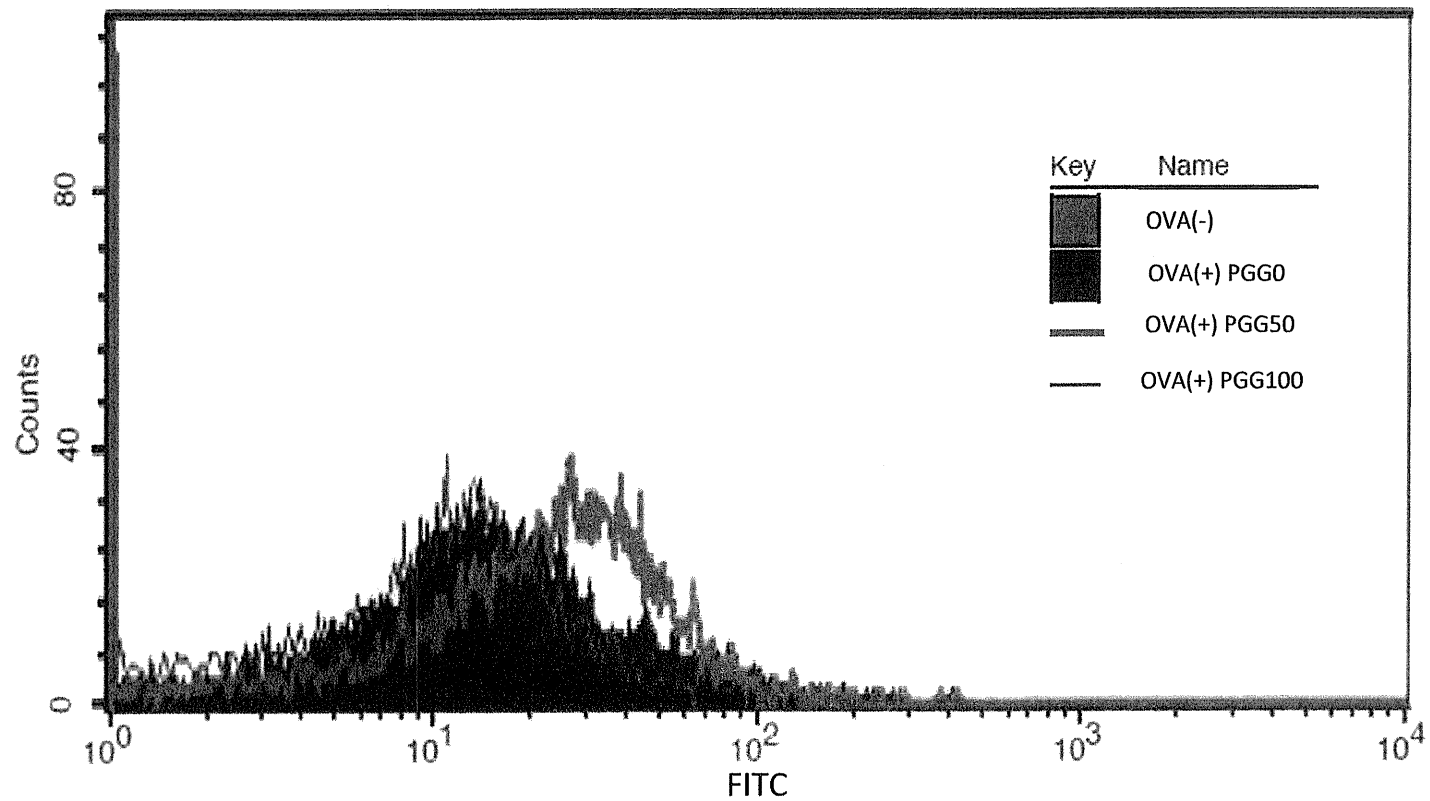
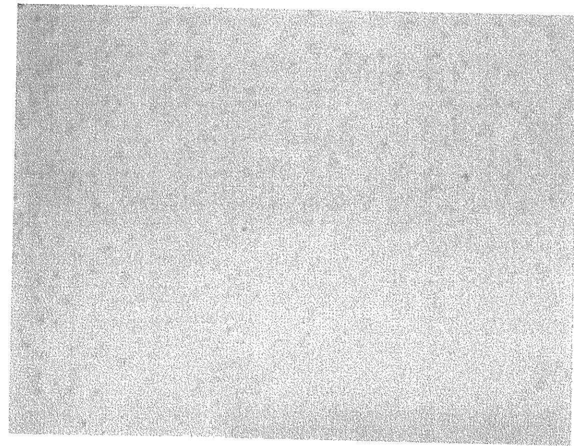
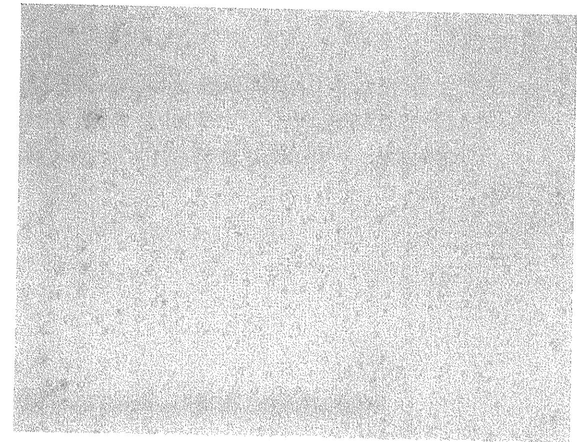
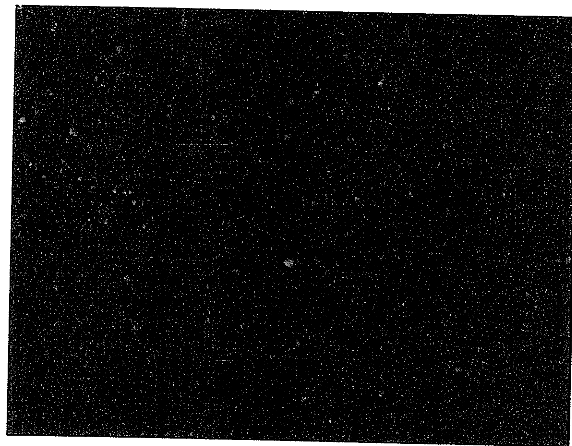


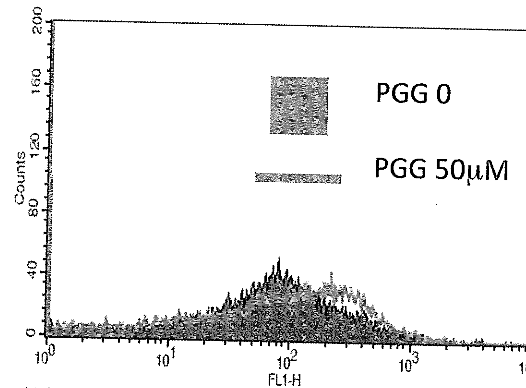
Fig.5　PGGによる樹状細胞の抗原取り込みの増加（1）
DC2.4細胞を、3.5cm dish に4×10^5 cells/ 500μlで播種。30分後、200μMに調整したPGGを500μlずつ加え(終濃度50μM)、1時間後、OVA-FITC/LPF溶液を1ml/ dishで添加（最終濃度OVA50μg/ml : LPF 20 μg/ml）。20時間培養後、細胞を回収し、FACS解析を行った。
■はOVA-FITCを加えていない、■はOVA-FITCを加えPGGを加えていない、ーはOVA-FITCを加えPGGを50μMで作用させたとき、ーはOVA-FITCを加えPGGを100μMで作用させたときの蛍光強度を示す。

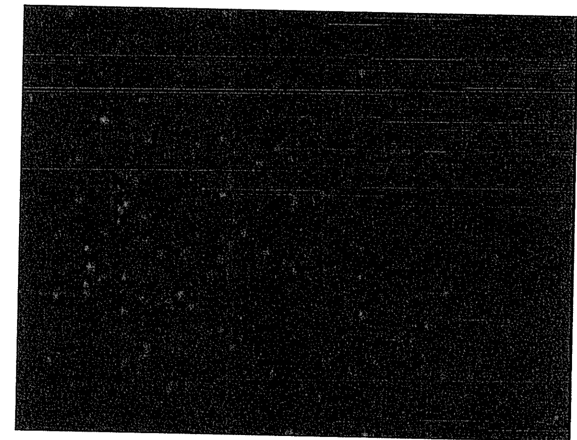






PGG 0





PGG 50μM

Fig.6　PGGによる樹状細胞の抗原取り込みの増加（2）
DC2.4細胞を、3.5cm dish に4×10^5 cells/ 500μlで播種。30分後、200μMに調整したPGGを500μlずつ加え(終濃度50μM)、1時間後、OVA-FITC/LPF溶液を1ml/ dishで添加（最終濃度OVA50μg/ml : LPF 20 μg/ml）。20時間培養後、PBSで洗浄、蛍光顕微鏡で細胞を観察した。左図はPGGを作用させないときで、上図は明視野写真、下図は蛍光写真。右図はPGGを50μMで作用させたときで、上図は明視野写真、下図は蛍光写真。
中央のヒストグラフは同時に行ったFACSの結果。■はPGGを作用させないとき、━はPGGを50μMで作用させたときの、細胞の蛍光強度を示す。

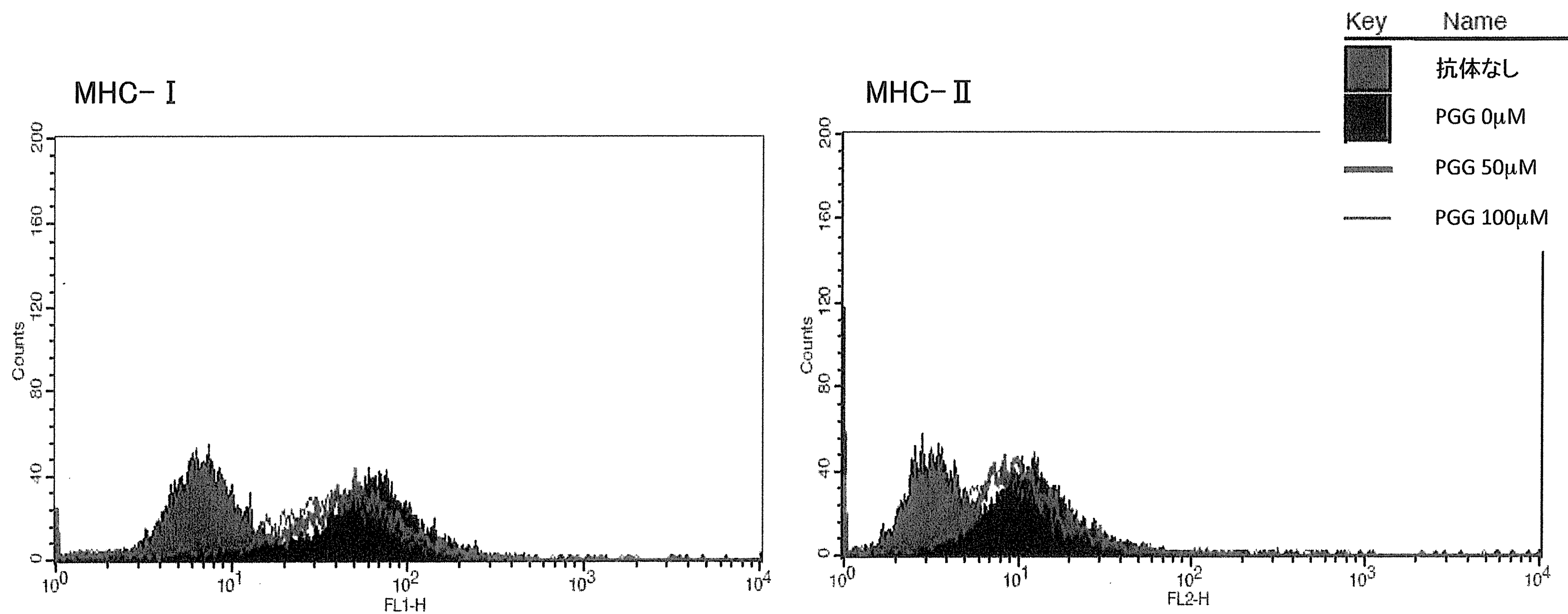


Fig.7　PGGによる樹状細胞のMHCの発現の低下
DC2.4細胞を、3.5cm dish に4×10⁵ cells/ 500μlで播種し、30分後、200μMに調整したPGGを500μlずつ加えた(終濃度50μM)。1時間後、OVA/LPF溶液を1ml/ dishで添加（最終濃度OVA100μg/ml：LPF 20 μg/ml）し、20時間培養し抗原を取り込ませた後、細胞を回収し、FACS 解析を行った。
■は各抗体を加えていないとき、■はPGGを作用させていない細胞に各抗体を作用させたとき、ーはPGGを50μMで作用させた細胞に各抗体を作用させたとき、ーはPGGを100μMで作用させた細胞に各抗体を作用させたときの蛍光強度を示す。

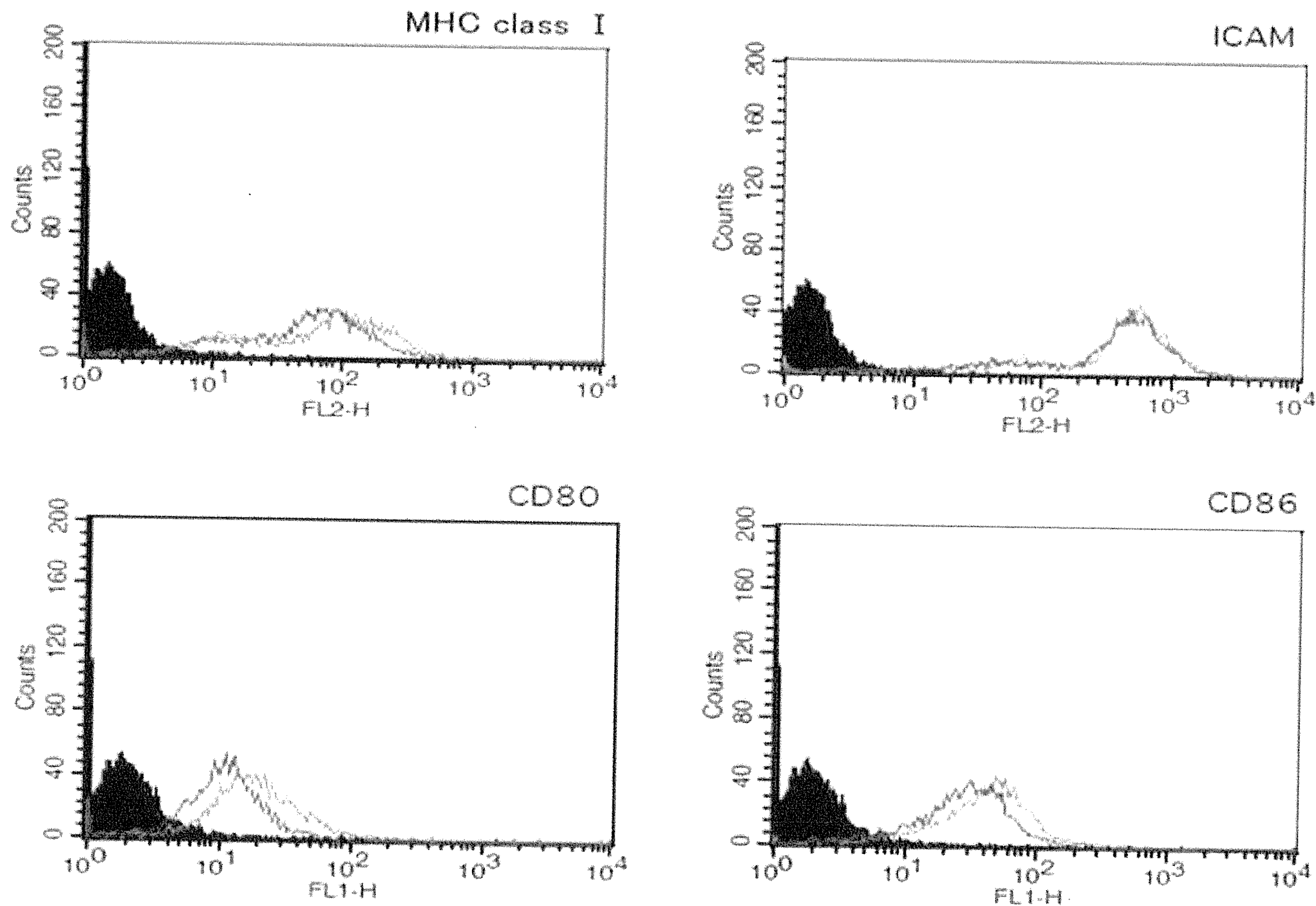
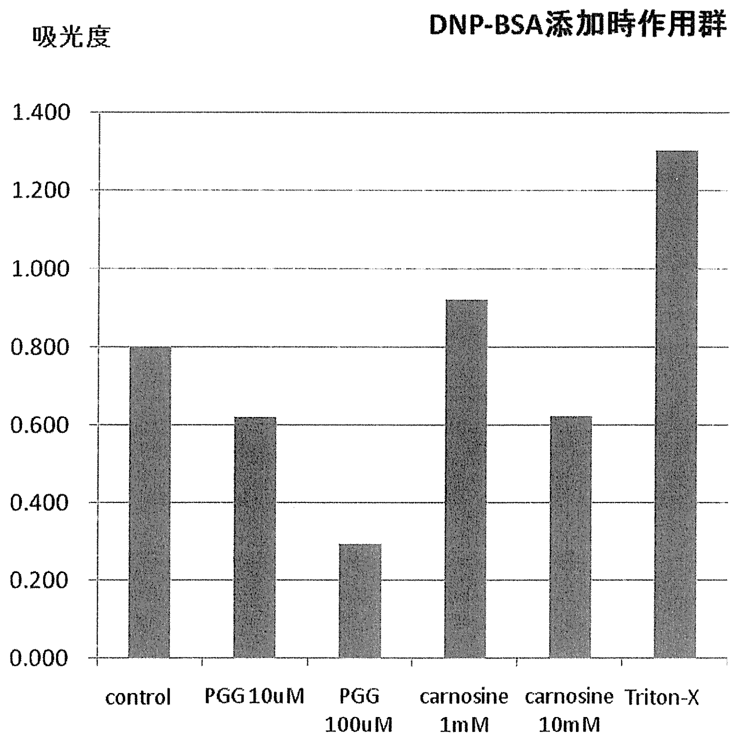


Fig.8　PGG作用時の樹状細胞の細胞表面マーカーの発現
DC2.4細胞を、3.5cm dish に4×10^5 cells/ 500μlで播種し、30分後、200μMに調整したPGGを500μlずつ加えた(終濃度50μM)。1時間後、OVA/LPF溶液を1ml/ dishで添加（最終濃度OVA100μg/ml : LPF 20 μg/ml）し、20時間培養し抗原を取り込ませた後、細胞を回収し、FACS 解析を行った。
■は各抗体を加えていないとき、━はPGGを作用させない細胞に各抗体を作用させたとき、━はPGGを50μMで作用させた細胞に各抗体を作用させたときの蛍光強度を示す。

Key	Name
■	Control
━	PGG0μM
━	PGG50μM



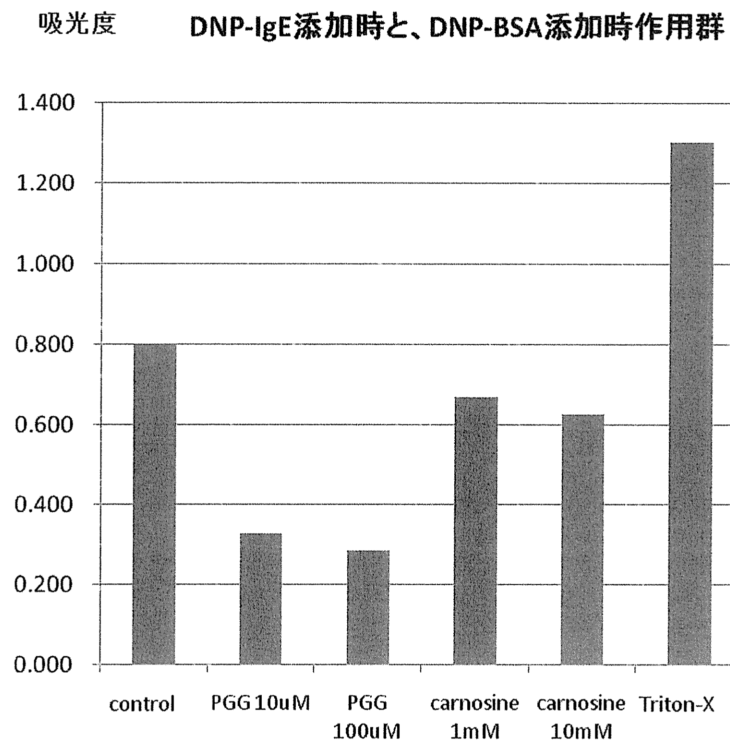


Fig.9　PGGによる脱顆粒反応抑制効果
RBH-2H3細胞を、48 well plateに1×10^5 cells/ 300μlで播種し、10～12時間後、上清を除去し、新たにEMEMあるいは、PGGまたはcarnosineを加えたEMEMを100μlずつ加える。10分後、DNP-IgE溶液を100μlずつ加え（終濃度；100ng/ml）6時間感作。siraganian bufferで2回洗浄し、siraganian buffer contain 5.6mM glucose,1mM CaCl2,0.1% BSAを80ml加え10分間予備加温後、この溶液、あるいはこの溶液で希釈したPGGまたはcarnosineを100μl加えた。10分後、DNP-BSA溶液（10μg/ml）を20μlずつ各wellに加え（最終濃度1μg/ml）、30分間反応させ、氷上で10分間冷却し反応を停止。1000gで5分間遠心し、上清を回収。総酵素量の測定は、細胞をDNP-BSAで反応させる時点で、0.1%tween in PBS を加え、細胞溶解液を回収。
回収した溶液25μlを96well plateに入れ、各wellに基質溶液を50μl加え30分間インキュベート。グリシン緩衝液100μlを加え反応を停止させ、405nmの吸光度を測定。
左図は、DNP-BSA添加時のみPGGまたはcarnosineを作用させたとき、右図は、DNP-IgE感作時と、DNP-BSA添加時にPGGまたは、carnosineを作用させたときの結果を示す。縦軸に405nmの吸光度を取り、横軸に、作用させた成分と、その濃度を示す。

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分担報告書

漢方薬ならびにその有効成分による粘膜免疫強化機序の解明

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研究要旨

古くより多くの漢方薬は免疫系に作用することが知られている。ここ最近の精力的な研究から、脾臓などの全身系の免疫に対する作用メカニズムは徐々に明らかとなっている。一方で、漢方薬の多くが内服処方なのにも関わらず、最も影響を受けると考えられる腸管の免疫システムへの影響についてはほとんど未解明である。本事業においては、研究分担者の國澤がこれまで得てきた腸管免疫に関する知的・技術基盤をもとに、漢方薬の腸管免疫に対する作用を明らかにする為の研究を推進している。本事業の2年度にあたる23年度は、初年度に得られた知見をもとに、腸管IgA反応を増強する補中益気湯に焦点を当て、その作用機序の解明を試みた。その結果、補中益気湯による IgA 産生増強効果は経口ワクチンと同時投与した時に認められることを見いだした。これらの結果は補中益気湯が腸管 IgA 産生の誘導相において作用していることを示唆する結果であると考えられる。

A.研究目的

古くから免疫調整作用があることが知られている漢方薬であるが、その作用メカニズムの多くは不明である。特に漢方薬の多くが内服処方なのにも関わらず、直接的な影響を受けると予想される腸管免疫に対する作用はほとんど未解明であると言っても過言ではない。研究分担者の國澤はこれまでに冬虫夏草をリード化合物とするFTY720を用いた研究から、生体防御に関わるIgAの産生やT細胞の遊走制御を始めとする腸管免疫制御機構に関する研究を進めてきた。本事業においてはこれらの研究から得た知的・技術基盤をもとに、漢方薬の有する腸管免疫に対する制御機能、ならびに経口ワクチンに用いるアジュバントとしての作用を中心にその作用メカニズムを明らかにする為の研究を遂行している。本事業の初年度にあたる昨年度に行った研究から、免疫調節機能があることが報告されている漢方薬のうち、補中益気湯が経口ワクチン投与時における抗原特異的腸管IgA産生を増強することを見いだした。そこで本年度は補中益気湯に焦点を当て、IgA 産生増強の作用機序の解明を目的とした研究を遂行した。

B.研究方法

1. 経口投与した抗原に対する特異的な免疫応答を検討する目的で、モデル抗原であるニワトリ卵白アルブミン（OVA, 1 mg/time/head）を粘膜アジュバントであるコレラトキシン(10 μg/time/head)と共に経口投与した。投与は1週間に1回の頻度で行い、計3回投与した。

2. 補中益気湯の投与は週5回の頻度とし、一回当たりマウスに 40 mg 経口投与した。作用機序を解明する目的で、免疫スケジュールの異なるタイミングで補中益気湯を投与する群を用意した。
3. 免疫後の各タイミングで糞便、ならびに単核球を回収し、それぞれ ELISA 法と ELISPOT 法により OVA 特異的抗体反応を測定した。また同マウスの IgA 産生形質細胞を IgA と CD138 をマーカーに用いたフローサイトメトリー法にて測定した。

（倫理面への配慮）
動物実験は東京大学医科学研究所のガイドラインに則り行った。

C. 研究結果
1. 経口ワクチンの投与を週一回の頻度で計3回行った。補中益気湯の作用を検討する目的で以下の2グループを用意した。
グループ 1. 経口免疫を行っている期間のみ、補中益気湯を投与
グループ 2. 経口免疫を行っている期間は補中益気湯を投与せず、経口免疫後より投与開始
グループ1においては、昨年度の結果と同様、補中益気湯を投与した群で糞便中の抗原特異的 IgA の産生増強が認められた。またこの増強効果は約1ヶ月間持続した。
一方、グループ2において、経口ワクチン接種後から補中益気湯の投与を開始した群においては、1 ヶ月間補中益気湯を投与してもIgA の産生増強効果は認められなかった。
2. 1. で得られた結果をもとに、グループ1のマウスの腸管組織から単核球を回収し、OVA 特異的抗体産生細胞数を ELISPOT 法にて測定した。その結果、1 の結果と相関し、補中益気湯投与群において腸管の OVA 特異的抗体産生細胞数の増加が認められた。一方で、フローサイトメトリーを用いて CD138 陽性 IgA 産生形質細胞を測定したところ、IgA 産生形質細胞の全体数には変化が認められなかった。

D. 考察
E. 結論
昨年度の検討から腸管 IgA 産生の増強効果があることが示された補中益気湯に焦点を当て、その作用メカニズムを検証する実験を行った。その結果、補中益気湯による経口ワクチンに対する IgA 産生増強強化は、ワクチン接種時に投与することで発揮されること、一方でワクチン接種後の投与では効果が得られないことが判明した。このことは補中益気湯が IgA 産生応答の中で特に誘導相において作用していることを強く示唆する結果であると考えられる。この考えを示唆する結果として、補中益気湯は抗原特異的 IgA 産生細胞の数は増やしているものの、IgA 産生細胞の総量には影響を与えていない、すなわち IgA 産生細胞の数を非特異的に増加させているのではないと推測される結果が得られた。
腸管組織において IgA 産生の主要誘導組織として機能しているのがパイエル板である。パイエル板を覆う上皮細胞層には M 細胞と呼ばれる抗原取り込みに特化した細胞が存在し、経口的に投与された抗原をパイエル板内に輸送する。M 細胞の直下においては樹状細胞が存在し、M 細胞を介して取り込んだ抗原を補足し、T 細胞、ならびに B 細胞を介した抗原特異的免疫応答を惹起する。これら一連の反応において樹状細胞が免疫誘導の鍵となる細胞の一つである。本事業の研究代表者である小泉がこれまで行ってきた検討から、補中益気湯は樹状細胞による抗原取り込みを増強することが判明していることを考慮すると、補中益気湯による IgA 産生増強効果は、IgA 誘導の場で

あるパイエル板における樹状細胞の抗原取り込みの促進が作用機序の一つの可能性として考えられる。
今後はこれら作用メカニズムの詳細を検討していくと共に、実際に感染モデルを用いて生体防御反応をより詳細に検討していく予定である。さらに補中益気湯の活性を担う漢方成分を同定し、そのアジュバント作用を検討していく。漢方の分野において「中」とは腹部を指し「補中」は「腹部を補う」という意味であるが、本事業での研究をさらに発展させることで、免疫学的観点からも腹部（消化管）の免疫機能を増強させる補中益気湯の有効性を検証していくことが重要であると考える。

F. 健康危険情報
総括研究報告書を参照

G. 研究発表
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H. 知的財産権の出願・登録状況

1. 特許出願

なし

2. 実用新案登録

なし

3. その他

なし

研究成果の刊行に関する一覧表

書籍
なし

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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J. Kunisawa, Y. Kurashima, and H. Kiyono	Gut-associated lymphoid tissues for the development of oral vaccine	Adv Drug Deliv Rev	64	4253-4262	2011
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J. Kunisawa and H. Kiyono	Commensal bacteria habituated in the gut-associated lymphoid tissues regulates the intestinal IgA responses	Front in Immunol	3	1-5	2012

Cell PRESS

Peaceful Mutualism in the Gut: Revealing Key Commensal Bacteria for the Creation and Maintenance of Immunological Homeostasis

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Quantitative and qualitative aspects of commensal bacteria determine the active and quiescent status of host immunity. In a recent *Science* paper, **Atarashi et al. (2011)** identify *Clostridium* clusters IV and XIVa as indigenous commensal bacteria that induce regulatory T cells for the creation and maintenance of immunological homeostasis.

The intestinal tract of mammals is home to 10^{13} to 10^{14} commensal bacteria composed of hundreds of species that benefit the host by supplying nutrients, metabolizing otherwise indigestible food, and preventing colonization by pathogens. Additionally, immune system development requires interactions with commensal bacteria (Hill and Artis, 2010). Because commensal bacteria commonly produce ligands of innate immunity, it was thought that unspecified commensal bacteria indiscriminately induced immune system development. However, accumulating evidence has indicated that individual species of commensal bacteria play specific roles in determining the immunological balance in the mucosal and systemic compartments. In a recent issue of *Science*, Honda and colleagues identified a cluster of indigenous commensal bacteria that are key to maintaining quiescent immunity (Atarashi et al., 2011).

Recent advances in genetic analyses of the composition of commensal bacteria led to the discovery that changes in microbial composition accompany alterations in the quality of host immunity and occasionally underlie immune diseases such as inflammatory bowel diseases (IBD) (Hill and Artis, 2010). These findings straightforwardly led to works addressing the puzzling question of how specific species of commensal bacteria regulate particular immune responses. One example of recent success in this area is the identification of segmented filamentous bacteria (SFB) as inducers of active immunity. Several groups, including Honda's, showed that SFB efficiently induce effector T cells, especially Th17 cells observed predominantly in the gut, where they provide protective immunity against intestinal infection (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009).

In addition to immunosurveillance against harmful pathogens, the gut immune system mediates quiescent immunity (or tolerance/unresponsiveness) against harmless and beneficial nonself materials such as dietary antigens and commensal bacteria. Among multiple immunoregulatory pathways, regulatory T (Treg) cells play pivotal roles in achieving quiescent immunity. Like Th17 cells, Treg cells are abundantly present in the gut, which is explained at least partly by the function of the vitamin A metabolite retinoic acid that is produced by gut-associated dendritic cells (Mucida et al., 2009). Although probiotic strains could also induce Treg cells in the gut (Kwon et al., 2010), whether and how indigenous commensal bacteria induce Treg cells remained unclear.

In their recent *Science* paper, Honda's group extends their studies and identifies *Clostridium* clusters IV and XIVa (also known as the *Clostridium leptum* and *coccoides groups*) as among the indigenous commensal bacteria inducing colonic Treg cells. Atarashi et al. (2011) demonstrated that only a few Treg cells were present in the colon of germ-free mice but increased to normal levels in specific pathogen-free (SPF) mice by colonization with commensal bacteria originating from SPF mice. By eliminating bacteria using antibiotics and chemical reagents, together with information about prominent commensal bacteria in the colon, they identified gram-positive and spore-forming *Clostridia* as candidate commensal bacteria that induce colonic Treg cells. Direct evidence was obtained from gnotobiotic mice that were generated by colonization with *Clostridium* clusters IV and XIVa. Intriguingly, the induction of Treg cells by commensal bacteria was observed specifically in the colon, whereas Treg cells in the small intestine were normally present in germ-free mice (Atarashi et al., 2011). The physiological functions of the small and large intestines differ substantially, and the small intestine is specialized to digest and absorb dietary materials. Treg cells in the small intestine increase after weaning (Atarashi et al., 2011), raising the possibility that materials in the diet and/or breast milk may regulate the induction of Treg cells in the small intestine.

Atarashi et al. also showed that an artificial increase in *Clostridium* in neonatal SPF mice resulted in the attenuation of intestinal inflammation in adulthood, which is potentially related to the lower levels of *Clostridium* clusters IV and XIVa in IBD patients (Frank et al., 2007). These regulatory effects were mediated by the preferential induction of Treg cells that produced IL-10 and expressed high levels of cytotoxic T-lymphocyte antigen

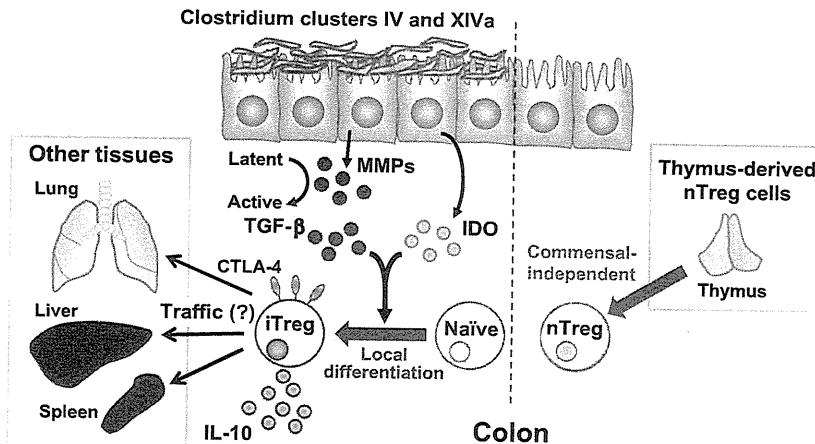

Figure 1. Induction of IL-10-Producing-Induced Treg (iTreg) Cells through the Interaction between Indigenous *Clostridium* Species and Epithelial Cells
After weaning, *Clostridium* clusters IV and XIVa become prominent in the colon, where they form a thick layer on the epithelium. *Clostridium* clusters IV and XIVa promote the production of matrix metalloproteinases (MMPs) from epithelial cells to convert TGF-β from the latent to the active form. Together with indoleamine 2,3-dioxygenase (IDO) produced by epithelial cells, the active form of TGF-β converts non-Treg cells into induced Treg (iTreg) cells that produce IL-10 and express high levels of CTLA-4. The locally differentiated iTreg cells prevent inflammatory and allergic responses in the gut and presumably other remote tissues. In contrast, thymus-derived naturally occurring Treg (nTreg) cells do not require stimulation by commensal bacteria.

4 (CTLA-4) (Figure 1). Interestingly, colonization with *Clostridium* resulted in the specific increase of IL-10-producing Treg cells at distant tissues, such as the spleen and lung, and inhibited allergic responses. These data suggest that T cells educated by commensal bacteria may move from the gut to remote tissues, where they determine the T cell-mediated immunological balance. This idea is plausible based on recent findings that Th17 cells induced by gut-resident SFB have pathogenic roles in the development of arthritis (Wu et al., 2010) and that probiotic-induced Treg cells accumulate at inflammatory sites of various tissues (Kwon et al., 2010).

Investigating the mechanisms of *Clostridium*-mediated induction of Treg cells, Atarashi et al. showed that *Clostridium* formed a thick colonizing layer on the epithelium where it enhanced the release of the active form of TGF-β and indoleamine 2,3-dioxygenase (IDO) from epithelial cells (Atarashi et al., 2011) (Figure 1). The TGF-β pathway was mediated by increasing the gene transcription of matrix metalloproteinases that converted latent TGF-β into the active form. Therefore, colonization with *Clostridium* preferentially converts non-Treg cells into Helios-negative induced Treg cells with little effect on Helios-positive thymus-derived naturally occurring Treg cells. A recent study demonstrated that a mixture of probiotic strains, including *Lactobacillus* and *Bifidobacterium*, enhanced the production of TGF-β and IDO from dendritic cells and consequently induced Treg cells (Kwon et al., 2010), similar to the effects of *Clostridium* on epithelial cells. Interestingly, Atarashi et al. (2011) demonstrated that colonization with a mixture of three *Lactobacillus* strains was not sufficient to induce colonic Treg cells, suggesting that the generation of a bacterial community in which bacteria respond to each other's metabolic products and establish a niche among commensals is important to create an environment that facilitates the induction of Treg cells. Another major unresolved question is the function of *Clostridium* in the induction of colonic Treg cells. Atarashi et al. mention that pattern-recognition receptors were not involved in this pathway, in contrast to the Toll-like receptor 2-dependent conversion of Treg cells induced by polysaccharide A by the human commensal *Bacteroides fragilis* (Round and Mazmanian, 2010). Collectively, these findings suggest that there are versatile pathways in the commensal bacteria-mediated induction of Treg cells, and thus it is important to examine not only bacteria-host interactions but also the role of the bacterial community in the establishment of immunological mutualism. The role of dietary materials (e.g., fatty acids, vitamins, and carbohydrates) in the three-way communications with the host and commensal bacteria is an additional fascinating subject (Maslowski and Mackay, 2011). These future studies will facilitate our understanding of how our immune system mutually evolves with commensal bacteria to achieve the protective but still homeostatic immunity in the intricate environment of the gut, and will also lead to novel strategies to prevent and treat inflammatory, allergic, and infectious diseases.

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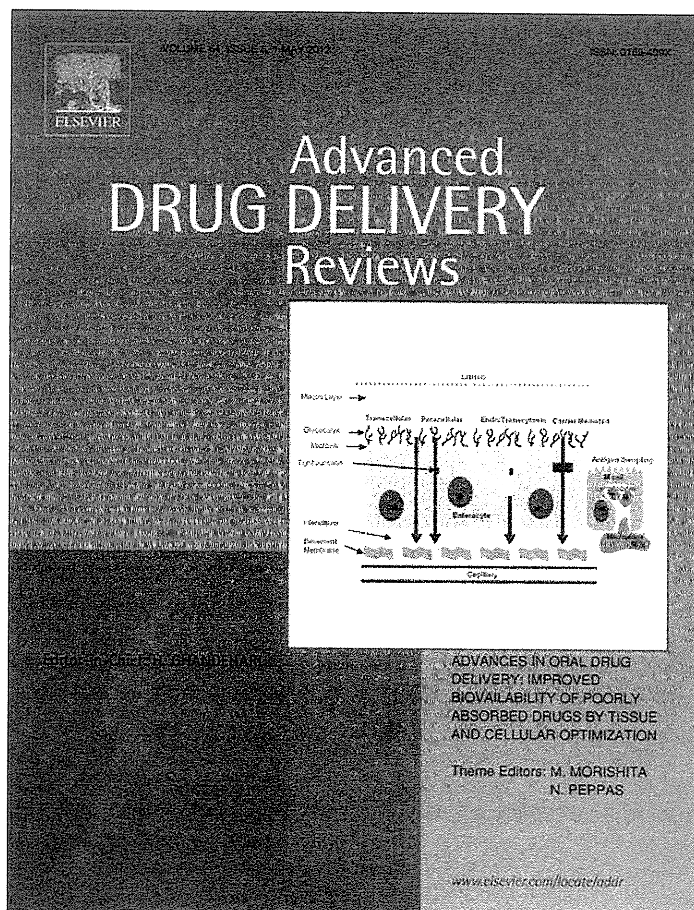

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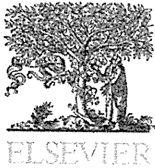

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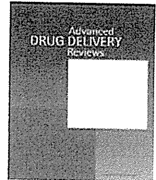

Gut-associated lymphoid tissues for the development of oral vaccines☆

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ABSTRACT

Oral vaccine has been considered to be a prospective vaccine against many pathogens especially invading across gastrointestinal tracts. One key element of oral vaccine is targeting efficient delivery of antigen to gut-associated lymphoid tissue (GALT), the inductive site in the intestine where antigen-specific immune responses are initiated. Various chemical and biological antigen delivery systems have been developed and some are in clinical trials. In this review, we describe the immunological features of GALT and the current status of antigen delivery system candidates for successful oral vaccine.

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1. Introduction

Despite physical and biological barriers, the gastrointestinal tract is a major route of entry for numerous pathogens. Barriers include epithelial cells (EC) joined firmly by tight junction proteins, brush-border microvilli, and a dense layer of mucin [1]. Antimicrobial peptides, such as defensins produced by ECs and Paneth cells, are additional barrier to provide further protection [2].

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In addition to these barriers, the gastrointestinal tract includes immunological defense system, in particular secretory-immunoglobulin A (IgA) [3], which is predominantly produced at intestinal mucosa by the harmonious interaction between ECs and mucosal lymphocytes and blocks microbial infections by inhibiting adherence of mucosal pathogens at the intestinal lumen to host ECs. Secretory IgA (SIgA) can also neutralize toxins produced by gut pathogens by binding to biologically active sites of toxins.

The immunological characteristics of the gastrointestinal tract have focused attention on the development of effective oral vaccines. Oral vaccination offers several advantages over parenteral vaccination, including needle-free delivery, easy and comfortable administration, and the possibility of self-delivery. Most importantly, oral vaccination can induce both mucosal and systemic immunity, leading to the double layers of protective immune responses [4]. In contrast, parenteral immunization primarily yields a systemic immune response. Therefore, effective oral vaccination could establish a first line of immunological defense in the intestinal tract, a major site of pathogen entry, as well as promote immune surveillance perhaps at other mucosal and systemic sites. One of the major strategies of oral vaccine has been induction of pathogen- or toxin-specific SIgA.

The hostile environment of the gastrointestinal tract (low pH, presence of digestive enzymes, and the detergent activity of bile salts) often makes it difficult to induce protective immune responses by oral vaccination with antigen alone. Additionally, effective oral delivery of antigen to the induction site of the mucosal immune system (e.g., gut-associated lymphoid tissues :GALT) is made difficult by the significant dilution and dispersion of antigen that occurs in the lumen since a total interior area of the intestinal wall is thought to be equivalent to over one tennis coat surface. Further, physical barriers, such as mucus and the tight junctions between the ECs prevent the effective delivery of vaccine antigen. To overcome these obstacles, effort has focused on development of effective antigen delivery systems. In this review, we describe the immunological features of gut-associated lymphoid tissue as the most obvious target site of antigen delivery in the development of oral vaccines. We also describe the current strategies being used to develop versatile antigen delivery systems for efficient oral vaccination.

2. Immunological features of GALT

GALTs comprise several different organized lymphoid structures [5]. Among them, Peyer's patches (PPs) are well characterized as sites for the initiation of intestinal IgA responses. Isolated lymphoid tissue (ILF) is another GALT structure, which is also important in the induction of intestinal IgA responses.

2.1. Peyer's patches (PPs)

PPs are considered to be one of the largest organized lymphoid tissues in the gastrointestinal immune system. There are generally 8 to 10 PPs in the small intestine of mice and hundreds in humans [6]. Each PP is composed of several B cell-rich follicles surrounded by a mesh-like structure consisting of T cells known as interfollicular region (IFR) (Fig. 1).

Although PPs share some common immunological and micro-architectural features with peripheral secondary lymphoid organs, they are harboring unique features as the mucosa-associated lymphoid tissue [6]. For example, PPs contain efferent but not afferent lymphatics. To compensate, PPs are covered with a specialized epithelial region, termed follicle-associated epithelium (FAE), containing specialized antigen-sampling microfold or membranous cells (M cells). The M cells are characterized by short microvilli, a thin mucus layer, small cytoplasmic vesicles, and efficient transcytosis activity, allowing the selective and efficient transfer of antigens from the intestinal lumen into PPs (Fig. 2) [7]. Thus, M cells are considered to be a professional antigen sampling and gateway cells for the mucosal immune system.

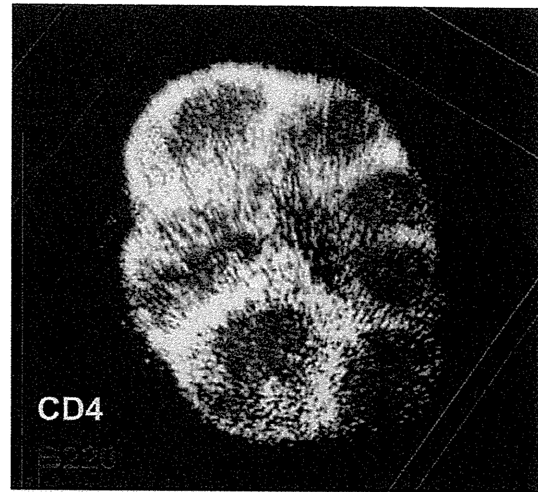

Fig. 1. Microarchitecture of murine Peyer's patches. Purified T cells (green) and B cells (red) were chemically labeled with carboxyfluorescein succinimidyl ester and arboxy SNARF-1, respectively, and adoptively transferred into mice. Fifteen hours after the transfer, cell distribution in the Peyer's patches was observed at the whole tissue level by using macro-confocal microscopy.

Dendritic cells (DCs) are abundant in the subepithelial dome region (SED) under the FAE, which thus can immediately take up orally encountered antigens from M cells and process and present antigenic peptides to mucosal T and B cells for the initiation of antigen-specific immune responses (Fig. 3). DCs are also found in the IFR. They are composed of at least three distinct subsets: $CD11c^+$ DCs in the SED, $CD8\alpha^+$ DCs in the T cell-rich IFRs, and double-negative DCs in both the SED and IFRs [8]. In addition to antigen presentation, DCs in the intestinal tissues express retinal dehydrogenase, an enzyme that converts vitamin A into retinoic acid. Retinoic acid promotes the preferential homing of activated antigen-specific T and B cells into the intestinal lamina propria by inducing the expression of gut imprinting molecules, such as $\alpha4\beta7$ integrin and CCR9 [9,10].

B cells, a major component of PP cells (~75%), are preferentially located in the follicle region (Figs. 1 and 3). Unlike other lymphoid organs, formation of germinal centers (GC) occurs in the PPs even under homeostatic conditions by the continuous stimulation from commensal bacteria, in which leads to the creation of molecular and

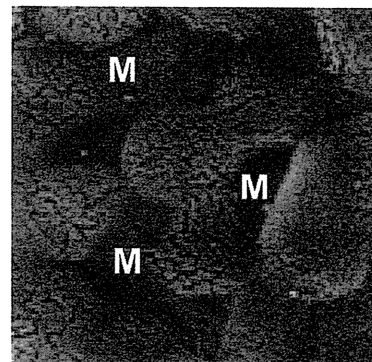

Fig. 2. Scanning electron micrograph of M cells in the Peyer's patches. Scanning electronic microscopy demonstrates that the M cells (indicated as "M") in the Peyer's patches are distinguished from surrounding ECs by their depressed position relative to the ECs, dark brush border, and short microvilli.

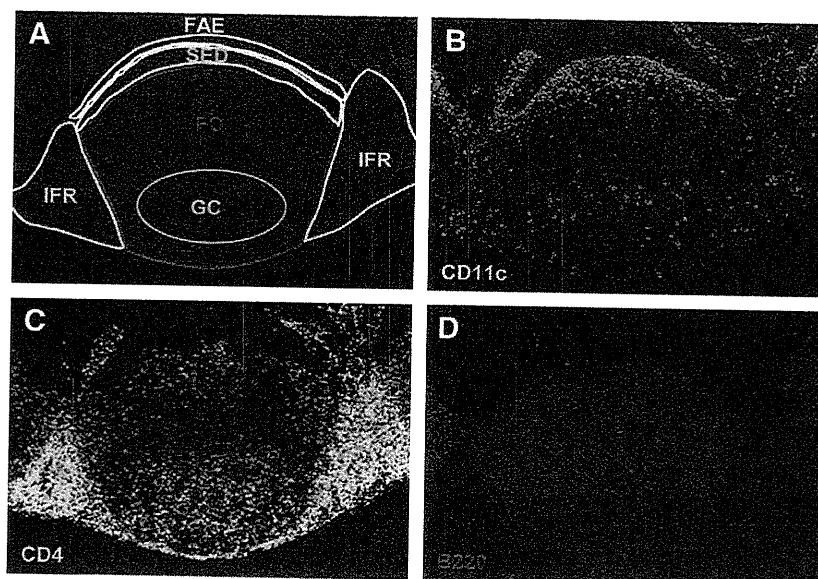

Fig. 3. Distinct cell distribution in the Peyer's patches. Immunohistochemical data on Peyer's patches is shown. (A) Each cell was identified with 4′,6-diamidino-2-phenylindole staining. PP compartments are outlined and labeled as follows: FO, follicle; FAE, follicle-associated epithelium; GC, germinal center; IFR, intrafollicular region; SED subepithelial dome. (B–D) Immunohistochemical staining of PPs for: dendritic cells (anti-CD11c; B), T cells (anti-CD4; C), B cells (anti-B220; D).

cellular environment for class switching of B cells from IgM to IgA (Fig. 3). Thus, PPs contain B cells at several differentiation and maturation stages: IgM^+B220^+ (~70%), $IgM^+IgA^+B220^+$ (~1%), IgA^+B220^+ (~3%), and IgA^+B220^- (~0.5%).

Approximately 20% of PP cells are T cells. Some portions of T cells are found in the IFRs of the PPs, which contain mainly naive T cells (Figs. 1 and 3) [11]. In addition to naïve T cells, other T cells exhibit active phenotype, including IFN-γ-producing Th1, IL-4-producing Th2, and IL-10-producing $Foxp3^+$ regulatory T cells [12]. A recent study demonstrated that at least some portions of $Foxp3^+$ regulatory T cells differentiated into follicular helper T cells which facilitate the B cell class switching to IgA^+ B cells in the GC [13].

Organogenesis of PPs is initiated in the embryonic stage. In mice, clustering of mesenchymal-lineage $VCAM\text{-}1^+ICAM\text{-}1^+$ PP organizer (PPo) cells starts at the site of tissue anlagen at embryonic days 14–16 [14]. PP inducer (PPi) cell are also key cells that initiate PP organogenesis. PPi cells are a component of lymphoid tissue inducer (LTi) cells that express key transcription factors, Id2 and RORγt, as well as a unique pattern of cell surface markers (IL-7 receptor $[IL\text{-}7R]^+$, $CD3^-$, $CD4^+$, $CD45^+$, lymphotoxin [LT] $\alpha 1\beta 2$). The interaction between PPi and PPo cells through the IL-7R and LTβ receptors (LTβR) with corresponding cytokines results in production of lymphoid chemokines such as CXCL13 and CCL19/CCL21 from PPo cells. These chemokines recruit lymphocytes and DCs to form the PP micro-lymphoid structure. Several lines of evidence have demonstrated that the loss of any part of the organogenesis pathways results in the disruption or impairment of PP development [14]. Of note, disruption of the PP organogenesis pathway by blockade of IL-7R and/or LTβR signaling during a limited time period leads to the selective loss of PPs without affecting other lymphoid tissue organogenesis [14]. Experiments with PP-deficient mice showed that they failed to develop antigen-specific immune responses against orally administered particle-form antigens but retained their ability to respond to soluble forms of antigens [15,16], suggesting that PPs play an important role in the induction of antigen-specific immune responses against particulate antigen. The finding may provide a clue for the creation of mucosal antigen delivery vehicle which effectively distributes vaccine to appropriate intestinal inductive lymphoid tissues (e.g., GALT or PPs) covered by FAE containing M cells.

2.2. Isolated lymphoid follicles

Mice selectively deficient in PPs retain certain levels of intestinal IgA responses [15,16]; this finding demonstrates the presence of alternative induction pathways for intestinal IgA production that are independent of PPs. In fact, ILFs were identified as an additional inductive tissue for IgA production. ILFs are located throughout the small intestine as clusters of 100–200 lymphocytes [17]. As for PPs, the formation of ILFs is mediated by the crosstalk between LTi cells and organizer cells. Thus, ILF formation was impaired in RORγt-deficient mice, which lack both PPs and ILFs. When RORγt-deficient mice were reconstituted with $ROR\gamma t^+$ LTi, naturally produced intestinal IgA responses were recovered with the newly formed ILFs [18].

ILFs are composed of a single follicle that contains predominantly B cells and some DCs and are covered with a FAE, which contains M cells [17]. In contrast to PPs, ILFs lack T cell-rich IFRs. In agreement with this finding, a recent report indicated that ILFs are a site for T cell-independent IgA production. Indeed, in contrast to PPs, which lack the IgA^+ cells in T cell-deficient mice, many IgA^+ B cells were still noted in the ILFs of TCR-deficient mice [18]. For the delivery of vaccine antigen to the gut mucosal immune system, an interesting strategy might be the selective delivery of T cell-dependent and -independent antigens to PPs and ILFs, respectively.

3. Antigen-sampling system in the gut

3.1. M cells in the GALT are specialized for antigen sampling

As mentioned above, FAE in the PPs contains M cells that act as a portal for uptake of antigen from the intestinal lumen and transfer into the PPs [19]. Approximately 10% (mouse) and 5% (human) of cells in FAE are M cells [19]. In both mouse and humans, M cells have been shown to harboring some biological and immunological uniqueness that distinguishes them from surrounding ECs. For example, M cells are characterized by short microvilli, a thin glycocalyx, and reduced activity of intracellular lysosomes [19]. In addition, M cells exhibit an intra-pocket structure at basal sites, where lymphocytes and/or antigen-presenting cells including DCs locate. These features allow the M cells to easily take particle-form antigens including microorganisms from the

lumen and transport them into the PPs without digestion and processing [19]. M cells also show a unique glycosylation pattern. Thus, ulex europaeus (UEA-1) lectin binds $\alpha(1,2)$ fucose residues that are specifically expressed on mouse M cells and Goblet cells [20]. Similarly, sialyl Lewis A antigen recognized by specific antibody (LM112) is a potential candidate for an M cell marker in humans [21]. We recently developed a murine M cell-specific antibody (NKM 16-2-4) [22]. Intriguingly, the antibody also recognized $\alpha(1,2)$ fucose like UEA-1, but did not bind to Goblet cells that are recognized by UEA-1[20], indicating that additional unique glycosylation pattern exists in M cells. Thus, one interesting and novel approach would be continuous search and characterization of glycoprotein modification patterns of FAE cells for the development of glycosylation targeted vaccine delivery system.

In addition to physiological and morphological features, several receptors important for invasion of pathogens and/or uptake of luminal antigens have been identified on M cells. For example, $\beta1$ integrin, identified as a receptor for invasin-mediated infection by *Yersinia*, is expressed on M cells [23]. *Salmonella typhimurium* encodes the specific adhesion molecule, long polar fimbria, which targets M cells [24]. Reovirus derived protein $\sigma1$ binds to M cells [25]. Recently, glycoprotein 2 (gp2) was found to be expressed specifically on both human and murine M cells; it recognizes FimH, a component of type I pili on bacterial outer membranes, and thus gp2 acts as a receptor for FimH-expressing bacteria such as *Escherichia coli* and *S. Typhimurium* [26,27].

Several key pathways important in the development of M cells were also recently identified [28]. At the cellular level, studies in B cell-deficient mice suggest that B cells play an important role in the M cell development in PPs. B cell-deficient mice had a decreased number of M cells in PPs and adoptive transfer of B cells reversed this phenotype [29]. At the molecular level, the TNF superfamily plays a critical role in the development of M cells. A recent study demonstrated that CD137 (also known as 4-1BB and induced by lymphocyte activation [ILA]) is required for the functionality of M cells. CD137 deficiency thus resulted in a defect in particle transcytosis by M cells [30]. The fact that the ligand of CD137, 4-1BBL, is expressed on B cells and myeloid lineage cells may explain why M cell development is impaired in B cell-deficient mice. In addition to CD137, another TNF receptor superfamily member, receptor activator of nuclear factor κ-B ligand (RANKL), is reported to be involved in M cell differentiation. The number of M cells in FAE of PPs is reduced in mice lacking RANKL or treated with RANKL-specific neutralizing antibody [31]. These findings will likely yield novel strategies to enhance the M cell development and function, resulting in more efficient antigen delivery in the GALT. Thus, M cell development and function regulating molecules may become new generation of mucosal adjuvants for supporting and enhancing antigen-specific immune responses to orally administered vaccine.

3.2. Epithelial cells and villous M cells

Intestinal ECs not only act as a physiological barrier, but also take part in the immunological function of the intestine by the formation of secretary form of immunoglobulin leading to the secretion of IgA and IgM into the intestinal lumen [1]. Reciprocally, IgG, which is involved in the antigen transport system, is transported from the intestinal lumen via the neonatal Fc receptor (FcRn) expressed on the apical surface of ECs [32]. In addition, ECs release exosomes containing antigen bound to MHC class II. The released MHC-bound antigen is thought to induce tolerance, not activation, of antigen-specific T cell responses [33]. This system might be important aspect of the gut immune system for the creation of immunologically quiescence condition at the harsh environment of intestine.

Among ECs in the villous epithelium, we identified M cells sharing similar characteristic with the M cells originally found in the FAE of PPs (or PP M cells) and termed them villous M cells [34]. Villous M cells are thus morphologically similar to M cells in the PPs and are recognized by UEA-1 lectin and M cell-specific NKM16-2-4 antibody, a marker of murine M cells. The specificity for UAE-1 and NKM 16-2-4 antibody suggests that villous M cells most likely harbor identical $\alpha(1,2)$ fucose based glycosylation molecules. Like M cells, villous M cells were capable of taking up *Salmonella*, *Yersinia*, and *Escherichia coli* expressing invasin. In addition, they are found in villous epithelium in PP-deficient mice, which allow them to still induce antigen-specific IgA responses [15,16]. Thus, villous M cells are an alternative antigen-sampling site and can be consider as the additional targeting site for oral vaccine delivery.

We recently reported that M cell-like $\alpha(1,2)$ fucose based glycosylation can be induced on intestinal ECs by environmental stimuli such as colonization with commensal biota, treatment with cholera toxin, or treatment with dextran sodium sulfate and termed these cells as fucosylated ECs (F-ECs) [35]. Although a functional role of F-ECs in the induction of immune responses against intestinal antigens needs further investigation, these findings suggest additional possible strategies to induce F-ECs for the enrichment of antigen-sampling system at the intestinal epithelium to vaccine administered via oral route.

3.3. Intraepithelial DCs

It is also known that the gut immune system is full of antigen-presenting cells including different subsets of DCs [8]. Some DCs are observed in the epithelium of the terminal ileum, where they extend their dendrites into the lumen and thus capable of taking-up intestinal microorganisms. Among the several subsets of DCs, epithelial DCs uniquely express CX3CR1. They penetrate the epithelial layer without disrupting the epithelial barrier connected with highly sophisticated tight junction molecules such as occludin, claudin 1 and zonula occludens 1, and capture luminal bacteria [36,37]. Because of their unique histological positioning at intestinal epithelium, these DCs can be called as "intraepithelial DCs". Unlike other DCs, CX3CR1$^+$ intraepithelial DCs are a non-migratory and gut-resident population, suggesting that the CX3CR1$^+$ population might play a critical role in the initiation or modulation of local immune responses in the intestinal epithelium or lamina propria regions [38]. Thus, these CX3CR1$^+$ DCs resided in the intestinal epithelium could also be useful targeted cell population for oral vaccine delivery.

4. Induction and regulation of IgA-mediated immune responses in the gut

4.1. GALT-mediated induction of IgA responses

A highly integrated sequence of processes of cellular and molecular interaction occurs in the PPs that lead to the initiation of antigen-specific immune responses (Fig. 4). Antigen transport from intestinal lumen by M cells at the FAE of PPs is an initial step for the induction of antigen-specific immune responses after oral immunization. Antigen is then taken up by DCs that are localized in the pocket of M cells or underneath M cells. Resultant up-regulation of CCR7 chemokine receptor expression on the DCs, allows them to move to the T cell region via locally produced corresponding chemokines (CCL19 and CCL21) in the PP or mesenteric lymph nodes and then present the processed peptide antigen for the generation of antigen-specific T cells [39].

Antigen-primed T cells support IgA class switching and somatic hyper mutation of B cells in the GC through antigen-specific interactions, CD40/CD40 ligand interaction, and cytokine expression (e.g., TGF-β, IL-4, and IL-21) [5]. Simultaneously, retinoic acid derived from PP DCs induces the expression on primed T and B cells of the gut-imprinting molecules $\alpha4\beta7$ integrin and CCR9 [9,10]. B cells also alter their expression of receptors for other chemokines (e.g., CXCR5 and CCR10) and sphingosine 1-phosphate, thus determining whether they

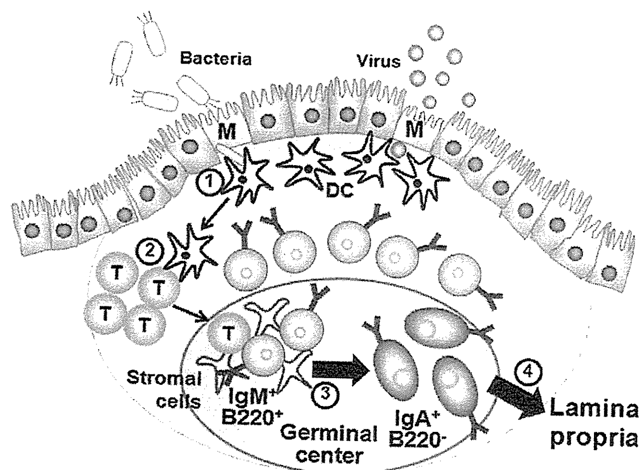

Fig. 4. Sequential processes for initiation of antigen-specific immune responses in Peyer's patches. (1) After transport of antigen by M cells, dendritic cells (DC) take up antigen, and (2) migrate to the T cell region. There, the DCs prime antigen-specific T cells by presenting antigen on MHC molecules and providing co-stimulatory signals. (3) Some of the antigen-primed T cells migrate to the germinal center, where, in coordination with stromal cells and follicular DCs, they induce immunoglobulin class switching and further differentiation of IgM$^+$ B220$^+$ B cells into IgA$^+$ B220$^-$ plasmablasts. These germinal center events are dependent on the interaction of CD40 with CD40 ligand, and cytokine activity (in particular TGF-β, IL-4, and IL-21). (4) IgA$^+$ B220$^-$ plasmablasts modulate their expression of integrins (such as α4β7 integrin) and receptors for chemokines (such as CCR9 and CXCR5) and sphingosine 1-phospahte. These changes promote their emigration from the PPs and trafficking to the intestinal lamina propria where differentiation occurs into plasma cells producing polymeric IgA.

stay in the GC or emigrate from the PPs for the migration to distant effector region (e.g., intestinal lamina propria) [40,41].

After emigration from the PPs, expression of gut-homing molecules (e.g., α4β7 integrin and CCR9/CCR10) on IgA$^+$ plasmablasts allows them to home to intestinal lamina propria, where IL-5, IL-6, and IL-10 induce terminal differentiation into plasma cells that produce dimeric or polymeric IgA. Polymeric IgA binds polymeric-immunoglobulin receptors expressed on the basal membrane of ECs and is transported to the intestinal lumen as the form of SIgA.

In contrast to events in the PPs, T cell help is not required for the IgA production in the ILFs. As described above, ILFs contain few T cells [17]. A previous study showed that stromal cells could be activated by LTβR-mediated interaction with RORγt$^+$ LTi and bacterial stimulation through toll-like receptors. This activation resulted in recruitment of DCs and B cells for the subsequent formation of ILFs [18]. Another study demonstrated that simultaneous stimulation of stromal cells with bacteria and retinoic acid induced production of CXCL13, TGF-β, and BAFF and led to preferential generation of IgA$^+$ B cells [42]. These events occurred in the absence of T cell help [42]. T cell-independent antigens, such as polysaccharides, have been thus considered for use as vaccine antigens [43]. Thus, induction of T cell-independent IgA responses via ILFs could be a novel strategy for the development of oral vaccines.

4.2. GALT-independent IgA production pathway

In addition to conventional B cells (named B-2 cells) which generally located in the organized lymphoid tissues (e.g., PPs), the peritoneal cavity contains large numbers of B-1 cells, another major source of intestinal IgA, especially against T cell-independent antigens [44]. A site for IgA class switching of peritoneal B cells has been elusive, but several lines of evidence indicate the involvement of DCs in the intestinal lamina propria for the creation of class switching molecular and cellular niche. Among the several types of DCs, those that express TNFα and inducible nitric oxide synthase, Tip-DCs, and TLR5$^+$ DCs, induce IgA$^+$ B cells by producing key molecules, such as APRIL, BAFF, IL-6, and retinoic acid without the involvement of organized lymphoid structure such as PPs [45,46]. However, it was previously reported that lamina propria DCs are capable of initiating systemic IgG responses, whereas antigen transport by M cells into the PPs is required for the initiation of intestinal IgA production pathway [47], which was consistent with another finding that DCs in the PPs are responsible for the intestinal IgA synthesis system [48]. Therefore, although it is generally accepted that lamina propria DCs act as antigen-presenting cells for intestinal antigens and are capable of inducing antibody responses, it is still obscure how lamina propria DCs regulate the induction of intestinal IgA and systemic IgG responses.

As ILF-mediate initiated IgA responses, GALT-independent IgA responses are involved in the immune responses against T cell-independent antigens, such as polysaccharides and phosphoryl choline [49]. Since these T cell-independent antigens have been considered as vaccine antigens such as *Streptococcus pneumonia*[43], the use of GALT-independent IgA induction pathway could be an additional strategy for the development of oral vaccines.

5. Application of drug delivery systems to the development of oral vaccines

Antigen delivery is central and key to the development of effective and successful oral vaccines. Particulate antigens appear to be more effective than soluble ones. This phenomenon is at least partially due to protection of the antigen from the harsh conditions of the gastrointestinal environment of digestive tract, such as low pH, detergent effects of bile salts, and extensive proteolytic enzyme activity. In addition, particulate antigens are preferentially taken up in the GALTs, especially by M cells serving as a gateway of the mucosal immune system, thus enhancing their antigenic activity. Several systems have been developed for targeting vaccine antigen selectively to the M cells in the FAE of GALTs.

5.1. Passive transport system

A variety of biodegradable antigen delivery systems have been developed for oral vaccines. These include incorporation of antigens into polymer-based particles (e.g., poly-lactide-co-glycolide-micro-particles) [50], liposomes [51], ISCOM [52], and chitosan particles [53]. Their utility as oral delivery vehicles is enhanced by the fact that they are biodegradable and can be formulated for controlled drug release. The effect of particle size on passive targeting to M cells has been evaluated. M cells preferentially take up particles with diameters less than 10 μm whereas a few micrometer- or nanometer-sized particles are taken up by ECs as well as M cells [54]. For example, small poly-lactide microparticles (e.g., 4 μm) in diameter enhanced only plasma IgG responses without IgA responses in the intestine. In contrast, 8–10 μm poly-lactide microparticles enhanced IgA responses in the intestine [55]. These findings suggest that the former size of particles is effectively transported antigen to the systemic immune system (or peripheral lymph nodes) via ECs for the initiation of IgG responses, while the latter sizes are successfully taken up by M cells for the initiation of mucosal IgA antibody responses via PPs. The combination of optimal sizing of capsule is important consideration for the development of oral vaccine which can induce simultaneously both mucosal and systemic protective immunity.

In addition to particle size, modifications to chemical features have been exploited to enhance antigen delivery. For instance, enterocoated-type particles were employed to protect the encapsulated antigen from the acidic environment of the upper part of intestine and to allow rapid release of antigen in the small intestine [56]. An additional example is the use of chemical mucoadhesive molecules (e.g., carboxy vinyl polymer) to elongate particles containing protein antigens, thereby prolonging antigen persistence in the intestine [57]. Liposomes can also

be made more stable in acid by constructing them with dipalmitoyl phosphatidylserine, dipalmitoyl-phosphatidylcholine, and cholesterol [58,59].

5.2. Use of M cell-specific ligands

In addition to passive one, active delivery of particles to GALT fascinates the induction efficacy of oral vaccines. In this issue, several mucosal antigen delivery systems have been explored that deliver antigen selectively to M cells (Table 1). Lectins have been widely exploited in vaccines to gain or to enhance access of antigen to M cells. The unique reactivity of UEA-1 to M cells allowed the selective and effective delivery of orally administered microparticles or liposomes to murine M cells [60,61]. A similar approach can be taken by using M cell-specific antibodies. NKM16-2-4 recognizing α1,2-fucose-containing carbohydrates. The NKM16-2-4 antibody can be conjugated to vaccine antigen for efficient delivery of antigen to M cells [22]. Thus the targeting to M cells resulted in the induction of antigen-specific IgA antibody responses by the use of low amount of vaccine antigen when compared with the non-targeting form of oral vaccine. Additional studies identified GP2, a receptor for some bacteria expressing Fim(H) [27], as a specific marker of M cells [27] [26]. Because anti-GP2 antibodies have been shown to bind to both murine and human M cells [27], they may be useful for oral antigen delivery in both systems.

The use of organic molecules or peptides that mimic the functional activity of UEA-1 has also been explored to promote efficient delivery of antigen to M cells (Table 1). In these studies, molecules that bound UEA-1 ligands were identified in mixture-based positional scanning synthetic combinatorial libraries or in phage peptide libraries. The former approach revealed that a digalloyl D-Lysine amide construct and a tetragalloyl D-Lysine amide construct bound effectively to M cells; coating of polystyrene particles with these compounds resulted in the selective and efficient delivery of the particles to M cells [62]. The latter approach yielded peptide sequence (YQCSYTMPHPPV) that selectively bound to the M cell-rich SED region of the PP and enhanced the delivery of polystyrene microparticles to M cells [63]. These accumulative evidences suggest that a combination of intestinal friendly characteristics of chemically modified particle and M cell targeting molecule could be a logical strategy for the development of oral vaccine.

5.3. Applying microbial invasion systems to M cell targeting

Another logical approach has been to use components of microbial invasion systems to deliver synthetic particles to M cells (Table 1). Enhanced antigen uptake was achieved by coating polystyrene nanoparticles with *Yersinia*-derived invasin, a ligand for β1 integrins that is expressed on the apical side of M cells [64]. Similarly, mucosal immune responses were significantly increased by mucosal immunization with an antigen coupled to σ1, a protein derived from reoviruses, which are known to be an invading molecule for the virus to enter the M cells [65]. Long polar fimbria (LPF) mediates the binding of *Salmonella* and adherent-invasive *E. coli* to M cells [24,66], but additional pathways appear to exist, as long polar fimbria-deficient *Salmonella* still invade through M cells [67]. In this issue, FimH, the adhesin portion of long polar fimbria, was found to be involved in the binding of FimH(+) *E. coli* and *Salmonella* to M cells [27]. FimH binds to glycoproteins in a mannose-dependent manner and mediates binding to GP2 expressed on M cells [27,68]. Thus, just as for GP2-specific antibodies, FimH is a candidate targeting bacterial molecule for specific delivery of antigen to M cells.

Recently, we employed genetic analyses to identify indigenous commensal bacteria that specifically localized inside of PPs. *Alcaligenes* species, for example, were observed predominantly inside of PPs, in contrast to their absence on the surface as well as other tissues [69]. It has been suggested that at least some component of *Alcaligenes* was taken up by DCs, which induced IL-6 and BAFF expression for the enhancement of IgA production [69]. These findings suggested an interesting possibility that *Alcaligenes* species can be used as a new form of commensal flora based vaccine antigen-delivery micro-vehicle specifically transport vaccine to PPs.

In related to our new observation for the intra-tissue co-habitation of commensal flora, mucosal IgA antibodies have been suggested to play a critical role for guiding and colonizing *Alcaligenes* in PPs since immunoglobulin-deficient mice showed a significant reduction of *Alcaligenes* in the PPs [69]. It is thus possible that antibody-mediated pathway appears to be involved in the uptake of *Alcaligenes* into the PPs [69]. It was previously revealed that immunogloblins preferentially adhere to M cells [70,71], implicating that *Alcaligenes* was taken up by M cells into the PPs via immunoglobulin-mediated pathway. In addition, it was demonstrated that secretory IgA was recognized by DC-SIGN on DCs [72], implicating that M cells and DCs cooperatively use IgA antibody to efficiently enhance the gut immune responses. In line with this, it was previously reported that coating particles with immonoglobulins would target oral vaccines to M cells and consequently enhanced antigen-specific immune responses [73,74].

Table 1
Tools for M cell targeting.

Ligand	Receptor	Reference
UEA-1 lectin	α1,2 fucose	20, 58, 59
Antibody (LM112)	Sialyl Lewis A	21
Antibody (NKM-16-2-4)	α1,2 fucose-containing carbohydrate	20
Antibody (3G7-H9, 2F11-C3)	Glycoprotein 2	26, 27
Digalloyl D-lysine amide	Unknown	60
Tetragalloyl D-lysine amide	Unknown	60
Peptides (YQCSYTMPHPPV)	Unknown	61
σ1 protein (reovirus)	α2,3 sialic acid	25, 63
Invasin (*Yersinia*)	β1 integrin	23, 62
Long Polar fimbriae (*E. coli*, *Salmonella*)	Unknown	24, 64
FimH (*E. coli*, *Salmonella*)	Glycoprotein 2	27
IgA	Immunoglobin receptors	71, 72

6. Conclusion

It is generally accepted that mucosal vaccines are an attractive strategy for protecting against many infectious diseases. Recent advances in biomaterial technologies have allowed the development of versatile antigen delivery systems. In addition, significant progress in our understanding of mucosal immunology and M cell biology has enhanced the possibility of targeting mucosal vaccines to the mucosal antigen-sampling and presenting system including M cells, DCs and ECs. Furthermore, because immunological environment in the intestinal tract is dominantly quiescent by several lines of regulatory/suppressor system to maintain the immunological homeostasis in order to deal with the harsh environment of intestine, we also have to consider the development of mucosal adjuvant/modulator to temporary break the immunological suppression for the initiation of antigen-specific positive responses. Thus, integration of the all knowledge gained in the biomaterial, immunological, and cellular biological fields should facilitate the development of a new generation of mucosal vaccines.

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