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粘膜型マスト細胞特異的抗体ライブラリを用いた

アレルギー･炎症性疾患の早期診断･治療法の開発

平成２３年度　総括研究報告書

研究代表者　國澤　純

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I.　総括研究報告書

厚生労働科学研究費補助金（医療機器開発推進研究事業）
総括研究報告書

粘膜型マスト細胞特異的抗体ライブラリを用いた免疫疾患の早期診断・治療法の開発

研究代表者：國澤　純（東京大学医科学研究所　講師）

研究要旨：近年患者数が爆発的に増加しているアレルギー炎症疾患においてマスト細胞が主要エフェクター細胞の一つとして知られている。一方で最近の研究から、マスト細胞は様々なサブセットに分画され、それぞれが生体防御応答を含む異なる免疫反応に関わっていることが判明してきた。その中でアレルギー炎症疾患の発症には粘膜型マスト細胞が強く関わっていることが示唆されつつあるが、粘膜型マスト細胞の特異性に立脚したアレルギー炎症疾患に対する診断・予防・治療法は開発されていないのが現状である。本研究課題においては、研究代表者がこれまで行ってきた粘膜組織におけるアレルギー炎症疾患に関する知的・技術基盤と世界に先駆け独自に樹立した粘膜型マスト細胞特異的抗体ライブラリを駆使し、アレルギー炎症疾患における早期診断技術と治療法の開発を進める。本事業の初年度にあたる23年度の研究においては、マウス免疫疾患モデルを用いた解析から、clone 4E8とclone 1F11がそれぞれ食物アレルギーと炎症性腸疾患に対して有効であること、clone 5A9はマスト細胞の活性化（脱顆粒）を検出するプローブとして有効であることを見いだした。またこれら3種類の抗体の認識分子を同定することに成功した。さらに細胞外核酸(ATP)受容体の一つであるP2X7受容体を認識するclone 1F11は、細胞外ATPによるマスト細胞からのサイトカンや脂質メディエーターの産生を抑制出来ることが判明した。これらの結果は、腸管免疫疾患における早期診断・予防・治療技術としての粘膜型マスト細胞特異的抗体の有効性を強く示唆するものであると考えられる。

本研究課題においては分担研究者を配していない。

A. 研究目的

近年患者数が爆発的に増加しているアレルギー炎症疾患においてマスト細胞が主要エフェクター細胞の一つとして知られている。本研究では、アレルギー炎症疾患の主要発症部位である粘膜組織に存在するマスト細胞を標的とした新規予防・診断・治療戦略の確立を目指した研究を遂行している。

一般にマスト細胞は結合組織型と粘膜型の２種類に大別され、粘膜型マスト細胞がアレルギー・炎症疾患に主に関わると考えられているが、その特異的分子や分化経路についてはほとんど解明されていない。このような背景のもと、これまでに研究代表者は粘膜型マスト細胞を特異的に識別できる抗体の樹立を試み、現在までに６種類の特異的抗体を

樹立した。本事業においては研究代表者が世界に先駆け独自に樹立した粘膜型マスト細胞特異的抗体の認識分子の同定を進めていくと同時に、食物アレルギーや炎症性腸疾患モデルを用い治療や予防、診断への応用を進める。これにより、粘膜型マスト細胞の特異性に立脚した早期診断技術と粘膜型マスト細胞を標的とした抗体療法の確立を目指す。

B. 研究方法

1. 食物アレルギーに対する効果の検討

鶏卵白アルブミン(OVA)に対するアレルギー性の下痢を発症するマウス食物アレルギーモデルを用い、アレルギー治療法としての樹立抗体の有効性を検証した。本食物アレルギーモデルでは、OVA をフロイント完全アジュバントと共に背部皮下に免疫することで全身感作を行い、その1週間後より、週3回の頻度で OVA を経口投与することでアレルギー性下痢を誘導する。アレルギー性下痢を呈するマウスに樹立抗体を尾静脈ならびに腹腔へ投与した後、再度 OVA を経口投与し、アレルギー性下痢の発症を観察した。効果が得られた抗体については、腸管組織よりマスト細胞を回収し、樹立抗体に対する反応性をフローサイトメトリー法により解析した。

2. 炎症性腸疾患に対する効果の検討

トリニトロベンゼンスルホン酸(TNBS)の直腸投与による炎症性腸疾患モデルマウスを用い、直腸投与前後に樹立抗体の尾静脈および腹腔投与を行った。その後、マウスの体重変化を測定した。効果が得られた抗体については、腸管組織よりマスト細胞を回収し、樹立抗体に対する反応性をフローサイトメトリー法により解析した。

3. 樹立抗体の抗原同定

樹立抗体およびマスト細胞溶解液を用いた免疫沈降法と SDS-PAGE により認識分子を精製した。PAGE ゲルより切り出したサンプルに含まれる候補分子の同定を質量分析法により行った。得られた候補分子は DNA クローニングし、CHO 細胞へトランスフェクションした後、樹立抗体との反応性を検証することで認識分子を同定した。

4. in vitro におけるマスト細胞の活性化

IL-3 と stem cell factor を用いて骨髄細胞より誘導したマスト細胞に抗 DNP-IgE 抗体＋DNP-BSA、もしくは ATP 単独を加え、30 分間反応させることでマスト細胞の活性化を誘導した。活性化誘導後のマスト細胞に対する反応性について FITC 標識した樹立抗体を用い検討した。

(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

各種マウス疾患モデルを用いた解析から、樹立した 6 種類の抗体のうち、clone 4E8 は食物アレルギーモデルにおけるアレルギー性下痢の発症を抑制することが示された。一方、clone 1F11 は炎症性腸疾患モデルにおいて体重減少など炎症性腸疾患に伴う病態を抑制できることが判明した。さらに上記の疾患を発症したマウスにおいては大腸組織においてマスト細胞の増加が認められたが、これらのマスト細胞は両 clone に反応することが確認された。さらには病態の形成に伴い clone 5A9 に認識されるマスト細胞の割合が、腸管粘膜内で増加していた。これらのことから、clone 4E8 と 1F11 はそれぞれ食物アレルギーと腸炎に対して有効であること、clone 5A9 はマスト細胞の活性化マーカーの指標として有効であることが示唆された。

各抗体による免疫沈降物を用いた質量分析により、各 clone の認識分子の同定を試み、いくつかの候補分子を見いだした。これら候補分子を DNA クローニングした後、CHO 細胞に発現させ樹立抗体に対する反応性を検証した結果、clone 4E8 は IgE を、clone 1F11 は細胞外核酸(ATP)受容体の一つである P2X7、clone 5A9 は CD63 を認識していることが判明した。

炎症性腸疾患モデルに対し有効である clone 1F11 が、細胞外核酸(ATP)受容体である P2X7 を認識しているという上記知見から、ATP によるマスト細胞の活性化とそれらに対する clone 1F11 の効果を検討した。In vitro の培養系において、ATP をマスト細胞に添加すると炎症性サイトカイン(TNF、IL-6)や脂質メディエーター(ロイコトリエン B4、C4)の産生が確認されたが、これらの炎症性物質の産生は培養系に clone 1F11 を添加することにより抑制された。

D. 考察

本事業の初年度である 23 年度は、clone 4E8 と 1F11 はそれぞれ食物アレルギーと腸炎に対して有効であること、clone 5A9 はマスト細胞の活性化マーカーの指標として有効であることを明らかにした。clone 4E8 は IgE を認識することから、IgE 依存的アレルギーモデルである食物アレルギーにおいて有効であるという予想され得る結果となった。一方、炎症性腸疾患に対して有効であることが示された clone 1F11 は細胞外核酸(ATP)の受容体の一つである P2X7 を認識していた。これまでに P2X7 受容体に関しては、神経細胞や樹状細胞、T 細胞といったマスト細胞以外の細胞にも発現していることが報告されているが、マスト細胞培養系を用いた解析から、ATP による直接的作用により促進されるマスト細胞の炎症性サイトカインや脂質メディエーターの産生が clone 1F11 の添加により抑制されたことから、少なくとも clone 1F11 はマスト細胞を標的の一つとしていることが示唆された。これを支持する予備的検討結果として、マスト細胞欠損マウスに P2X7 欠損マスト細胞を再構築したマウスにおいては炎症性腸疾患の発症が認められないことから、

ATP/P2X7 を介したマスト細胞の活性化が炎症性腸疾患の発症において重要であることが強く示唆される。*In vivo* ならびに *in vitro* の解析から、clone 5A9 は活性化したマスト細胞のみを認識していることが判明した。このことから、clone 5A9 はマスト細胞の活性のプローブとして活用することが可能である。また、clone 5A9 の認識分子である CD63 は通常ライソゾーム膜に存在しており、脱顆粒に伴って細胞膜表面に露出し、それを clone 5A9 が認識していると考えられる。

本研究においてマウス免疫疾患モデルを用いて得られた知見のヒト疾患との関連は今後の重要な検討課題だと思われる。さらに、樹立抗体が疾患マーカーの測定に用いることができるかの検討を行うため、血清サンプルなどを用いた ELISA 法などの確立を目指したいと考えている。

E．結論

これまでに樹立した抗体のうち、食物アレルギーと炎症性腸疾患に有効な抗体を 2 クローン見いだし、その認識抗原を同定した。また同様に活性化（脱顆粒）したマスト細胞を識別できる clone 5A9 の認識抗原を同定した。今後さらに免疫疾患モデル、ヒト患者サンプルを用いた検討を進めることで、腸管免疫疾患における早期診断・予防・治療技術としての粘膜型マスト細胞特異的抗体の有効性が提示できると期待される。

F. 健康危機情報

なし

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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得
   該当事項なし

2. 実用新案登録
   該当事項なし

3. その他
   特記事項なし

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

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III.研究成果の刊行物・別冊
（主要なもの）

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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

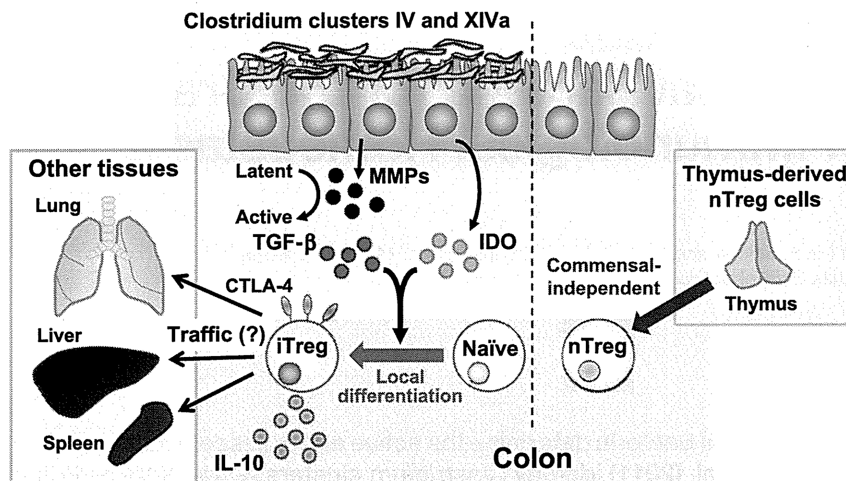

Figure 1. Induction of IL-10-Producing-Induced Treg (iTreg) Cells through the Interaction between Indigenous *Clostridium* Species and Epithelial Cells
After weaning, *Clostridium* clusters IV and XIVa become prominent in the colon, where they form a thick layer on the epithelium. *Clostridium* clusters 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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The Airway Antigen Sampling System: Respiratory M Cells as an Alternative Gateway for Inhaled Antigens

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In this study, we demonstrated a new airway Ag sampling site by analyzing tissue sections of the murine nasal passages. We revealed the presence of respiratory M cells, which had the ability to take up OVA and recombinant *Salmonella typhimurium* expressing GFP, in the turbinates covered with single-layer epithelium. These M cells were also capable of taking up respiratory pathogen group A *Streptococcus* after nasal challenge. Inhibitor of DNA binding/differentiation 2 (Id2)-deficient mice, which are deficient in lymphoid tissues, including nasopharynx-associated lymphoid tissue, had a similar frequency of M cell clusters in their nasal epithelia to that of their littermates, $Id2^{+/-}$ mice. The titers of Ag-specific Abs were as high in $Id2^{-/-}$ mice as in $Id2^{+/-}$ mice after nasal immunization with recombinant *Salmonella*-ToxC or group A *Streptococcus*, indicating that respiratory M cells were capable of sampling inhaled bacterial Ag to initiate an Ag-specific immune response. Taken together, these findings suggest that respiratory M cells act as a nasopharynx-associated lymphoid tissue-independent alternative gateway for Ag sampling and subsequent induction of Ag-specific immune responses in the upper respiratory tract. ***The Journal of Immunology*, 2011, 186: 4253–4262.**

The initiation of Ag-specific immune responses occurs at special gateways, M cells, which are located in the epithelium overlying MALT follicles such as nasopharynx-associated lymphoid tissue (NALT) and Peyer's patches (1). Peyer's patches contain all of the immunocompetent cells that are required for the generation of an immune response and are the key inductive tissues for the mucosal immune system. Peyer's patches are interconnected with effector tissues (e.g., the lamina propria of the intestine) for the induction of IgA immune responses specific to ingested Ags (2). NALT also contains all of the necessary lymphoid cells, including T cells, B cells, and APCs, for the induction and regulation of inhaled Ag-specific mucosal immune responses (1, 3). This tissue is rich in Th0-type $CD4^+$ T cells, which can become either Th1- or Th2-type cells (4). NALT is also equipped with the molecular and cellular environments for class-switch recombination of μ to α genes for the generation of IgA-committed B cells and the induction of memory B cells (5, 6). It is thus widely accepted that NALT M cells are key players in the uptake of nasally delivered Ags for the subsequent induction of Ag-specific IgA immune responses (1). As a result, NALT is considered a potent target for mucosal vaccines (1).

A recent study identified NALT-like structures of lymphocyte aggregates with follicle formation in the human nasal mucosa, especially in the middle turbinate of children <2 y old (7). Another recent study showed that, postinfection of mice with influenza via the upper respiratory tract, the levels of Ag-specific Ig observed in the serum and in nasal mucosal secretions after surgical removal of NALT were comparable to those in tissue-intact mice (8). Other studies have demonstrated that Ag-specific immune responses are induced in lymphotoxin-$\alpha^{-/-}$ and $CXCL13^{-/-}$ mice, in which the NALT exhibits structural and functional defects (9, 10). Thus, despite the central role of NALT in the generation of Ag-specific Th cells and IgA-committed B cells against inhaled Ags, these tissues do not appear essential for the induction of Ag-specific immune responses, suggesting that additional inductive sites and/or M cells are present in the upper respiratory tract.

The major goal of our study was to search for an NALT-independent M cell-operated gateway by examining and characterizing the entire nasal mucosa. We were able to identify M cells developed in the murine nasal passage epithelium as an alternative and NALT-independent gateway for the sampling of respiratory Ags and the subsequent induction of Ag-specific immune

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; dLN, draining lymph node; GAS, group A *Streptococcus*; GFP-*Salmonella*, GFP-expressing *Salmonella*; Id2, inhibitor of DNA binding/differentiation 2; NALT, nasopharynx-associated lymphoid tissue; *Salmonella*-GFP, *Salmonella typhimurium* expressing GFP; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TT, tetanus toxoid; UEA-1, *Ulex europaeus* agglutinin-1; WGA, wheat germ agglutinin.

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responses. Characterization of respiratory M cells should accelerate our understanding of the Ag sampling system at work in the upper respiratory tract.

Materials and Methods

Mice

BALB/c mice were purchased from SLC (Shizuoka, Japan). Inhibitor of DNA binding/differentiation 2 $(Id2)^{-/-}$ mice (129/Sv), generated as previously described (11), were maintained together with their littermate $Id2^{+/-}$ mice in a specific pathogen-free environment at the experimental animal facility of the Institute of Medical Science, University of Tokyo. All experiments were carried out according to the guidelines provided by the Animal Care and Use Committees of the University of Tokyo.

M cell staining

For the preparation of nasal cavity samples for confocal microscopy, we decapitated euthanized mice and then, with their heads immobilized, removed the lower jaw together with the tongue. Using the hard palate as a guide, we then used a large scalpel to remove the snout with a transverse cut behind the back molars. After removing the skin and any excess soft tissue, we flushed the external nares with PBS to wash out any blood within the nasal cavity before freezing the nasal passage tissue in Tissue-Tek OCT embedding medium (Miles, Elkhart, IN) in a Tissue-Tek Cryomold. For immunofluorescence staining, we prepared 5-μm–thick frozen sections by using a CryoJane Tape-Transfer System (Instrumedics, St. Louis, MO), allowed the sections to air dry, and then fixed them in acetone at 4°C. We then rehydrated the sections in PBS and incubated them for a further 30 min in Fc blocking solution. For M cell staining, sections were incubated overnight with rhodamine-labeled *Ulex europaeus* agglutinin-1 (UEA-1; Vector Laboratories, Burlingame, CA) at a concentration of 20 μg/ml and FITC-labeled M cell-specific mAb NKM 16-2-4 (12) at 5 μg/ml or FITC-labeled wheat germ agglutinin (WGA; Vector Laboratories, Burlingame, CA) at 10 μg/ml and counterstained with DAPI (Molecular Probes, Eugene, OR) at 0.2 μg/ml in PBS (13).

Electron microscopic analysis of respiratory M cells

For electron microscopic analysis, the nasal cavity sample was prepared and vigorously washed as described above, and then fixed on ice for 1 h in a solution containing 0.5% glutaraldehyde, 4% paraformaldehyde, and 0.1 M sodium phosphate buffer (pH 7.6). After being washed with 4% sucrose in 0.1 M phosphate buffer, the tissues were incubated in an HRP-conjugated UEA-1 solution (20 μg/ml) for 1 h at room temperature. The peroxidase reaction was developed by incubating the tissues for 10 min at room temperature with 0.02% 3,3′-diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl (pH 8) containing 0.01% H_2O_2. After being washed with the same buffer, the tissues were fixed again with 2.5% glutaraldehyde in 0.1 M phosphate buffer overnight. The nasal passage tissue was decalcified with 2.5% EDTA solution for 5 d. After being washed three times with the same buffer, samples were fixed with 2% osmium tetraoxide on ice for 1 h before being dehydrated with a series of ethanol gradients. For scanning electron microscopy (SEM), dehydrated tissues were freeze-embedded in *t*-butyl alcohol and freeze-dried, then coated with osmium and observed with a Hitachi S-4200 scanning electron microscope (Hitachi, Tokyo, Japan). For transmission electron microscopy (TEM) analysis, the samples were embedded in Epon 812 Resin mixture (TAAB Laboratories Equipment, Berks, U.K.), and ultrathin (70-nm) sections were cut with a Reichert Ultracut N Ultramicrotome (Leica Microsystems, Heidelberg, Germany). Ultrathin sections were stained with 2% uranyl acetate in 70% ethanol for 5 min at room temperature and then in Reynolds lead citrate for 5 min at room temperature. Sections were examined with a Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Elucidation of M cell numbers

To examine the numbers of respiratory and NALT M cells, mononuclear cells (including M cells, epithelial cells, and lymphocytes) were isolated from the nasal passages and NALT as previously described, with some modifications (4). In brief, the palatine plate containing NALT was removed, and then NALT was dissected out. Nasal passage tissues without NALT were also extracted from the nasal cavity, and mononuclear cells from individual tissues were isolated by gentle teasing using needles through 40-μm nylon mesh. The total numbers of cells isolated from the two preparations were counted. These single-cell preparations were then labeled with PE–UEA-1 (Biogenesis, Poole, England), and the percentages of UEA-1–positive epithelial cells in the nasal passages and NALT were determined with a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ). The numbers of M cells and goblet cells in the nasal passages and NALT were counted by confocal microscopic analysis according to the patterns of staining with UEA-1 and WGA. That is, the frequencies of M cells ($UEA\text{-}1^{+}WGA^{-}$) and goblet cells ($UEA\text{-}1^{+}WGA^{+}$) were determined by the enumeration of each type in 100 $UEA\text{-}1^{+}$ cells. The formula used to estimate the number of M cells was: [(total number of mononuclear cells × percentage of $UEA\text{-}1^{+}$ epithelial cells) × M cells/ $UEA\text{-}1^{+}$ epithelial cells]. The number of respiratory M cells in $Id2^{-/-}$ mice was calculated in the same manner.

Ag uptake in situ

DQ OVA was purchased from Molecular Probes. *Salmonella typhimurium* PhoPc strain transformed with the pKKGFP plasmid was kindly provided by F. Niedergang (14, 15). Group A *Streptococcus* (GAS; *Streptococcus pyogenes* ATCC BAA-1064) was obtained from the American Type Culture Collection (Manassas, VA), and immunofluorescence staining with FITC-conjugated goat anti-*Streptococcus* A Ab (Cortex Biochem, San Leandro, CA) was used to detect GAS uptake. DQ OVA (0.5 mg), GFP-expressing *Salmonella* (GFP-*Salmonella*) (5×10^8 CFU), or GAS (5×10^8 CFU) was intranasally administered and incubated in situ. Thirty minutes after the intranasal administration, the nasal passages were removed as described above and extensively washed with cold PBS with antibiotic solution to remove weakly adherent and/or extracellular DQ OVA or bacteria, as described (13).

The airway fluorescence-labeled Ag-treated nasal passages were processed for confocal microscopy as described above or for FACSCalibur flow cytometric analysis as follows. Mononuclear cells (including M cells, epithelial cells, and lymphocytes) were physically isolated from the nasal passages and NALT as described above, fixed in 4% paraformaldehyde, and labeled with PE–UEA-1 (Biogenesis, Poole, England). The percentage of green fluorescence (BODYPI FL or GFP)/UEA-1 double-positive nasal passage epithelial cells was determined by using an FACSCalibur (BD Biosciences).

To clarify the uptake of the bacteria by M cells, $UEA\text{-}1^{+}GFP^{+}$ cells, which were sorted from the nasal passages of mice intranasally infected with GFP-*Salmonella* by using an FACSAria cell sorter (BD Biosciences) were analyzed under three-dimensional confocal microscopy (Leica Microsystems).

To demonstrate the presence of dendritic cells (DCs) in the submucosa of the nasal passages, especially underneath respiratory M cells, after intranasal instillation of GAS, we used FITC- or allophycocyanin-conjugated anti-mouse CD11c (BD Pharmingen, San Jose, CA) Abs for subsequent confocal microscopic analysis.

Immunization

The recombinant *S. typhimurium* BRD 847 strain used in this study was a double *aroA aroD* mutant that expressed the nontoxic, immunogenic 50-kDa ToxC fragment of tetanus toxin from the plasmid pTET*nir*15 under the control of the anaerobically inducible *nirB* promoter (recombinant *Salmonella*-ToxC) (16). As a control, recombinant *Salmonella* that did not express ToxC was used. The recombinant *Salmonella* organisms were resuspended in PBS to a concentration of 2.5×10^{10} CFU/ml. Bacterial suspensions were intranasally administered by pipette (10 μl/mouse) three times at weekly intervals. To eliminate any possible GALT-associated induction of Ag-specific immune responses from the swallowing of bacterial solutions after intranasal immunization, mice were given drinking water containing gentamicin from 1 wk before the immunization to the end of the experiment and were also subjected to intragastric lavage with 500 μl gentamicin solution before and after intranasal immunization. This protocol successfully eliminated the possibility of the intranasally delivered bacteria becoming deposition in the intestine (Supplemental Fig. 1). The titers of tetanus toxoid (TT)-specific serum IgG and mucosal IgA Abs were determined by end-point ELISA, as described previously (17).

To measure GAS-specific immune responses, GAS was suspended in PBS to a concentration of 2×10^{10} CFU/ml. Ten microliters bacterial suspension was intranasally administered once using a pipette. Six weeks after the administration, serum and nasal washes were prepared, and the titers of GAS-specific Ab were measured by ELISA using a previously described protocol (18).

Statistical analysis

Data are expressed as means ± SD, and the difference between groups was assessed by the Mann–Whitney *U* test. The *p* values <0.05 were considered to be statistically significant.

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Results

Respiratory M cells in single-layer epithelium of the nasal passage

The nasal respiratory epithelium of the mouse is composed mainly of pseudostratified ciliated columnar epithelium (19). However, when H&E-stained sections of the whole nasal cavity were examined, a single-layer epithelium was found to cover some regions of the nasal cavity, especially the lateral surfaces of the nasal turbinates (Fig. 1*A–C*). Frozen sections of nasal passages from naive BALB/c mice were prepared and stained with FITC-WGA (green) and rhodamine–UEA-1 (red), and then counterstained with DAPI (blue). Clusters of UEA-1^{+}WGA^{-} cells that shared M cell characteristics were found exclusively in the single-layer epithelium of the nasal passage covered by ciliated columnar epithelial cells (Fig. 1*D*, 1*E*). Some respiratory M cells were also occasionally found on the transitional area between the single-layer and stratified epithelium. Notably, respiratory M cells also reacted with our previously developed M cell-specific mAb NKM 16-2-4 (12), demonstrating colocalization of the signals of UEA-1 and NKM 16-2-4 (Fig. 1*F*, 1*G*).

Electron microscopic analysis of respiratory M cells

SEM of the respiratory M cells revealed the characteristic features of M cells: a depressed surface with short and irregular microvilli (Fig. 2*A*, 2*B*). TEM analysis revealed that the respiratory M cell was covered by shorter and more irregular microvilli (with definite UEA-1^{+} signals; Fig. 2*C*, 2*D*) than were found in neighboring ciliated columnar respiratory epithelial cells (Fig. 2*E*). However, no pocket formation (or pocket lymphocytes) was seen in the basal membranes of respiratory M cells, unlike in NALT M cells (Fig. 2*F*, 2*G*). These findings indicated that the newly identified respiratory M cells had most of the unique morphological characteristics of classical M cells.

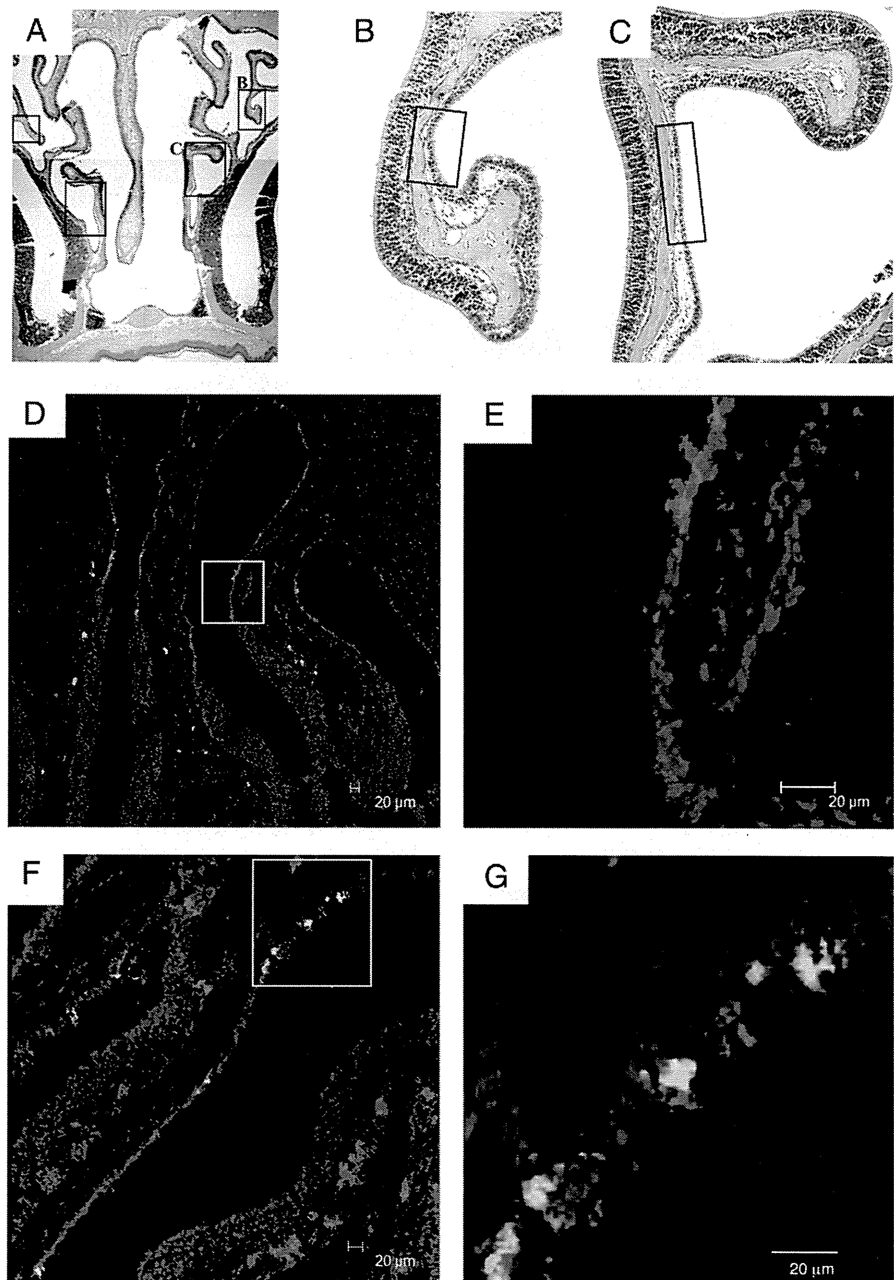

FIGURE 1. Clusters of UEA-1^{+}WGA^{-} respiratory M cells are found selectively in the single-layer epithelium of the nasal passage. *A–C*, H&E staining reveals the anatomy and general histology of the murine nasal passage (*A*, original magnification ×40). The nasal respiratory epithelium of the mouse is covered with a pseudostratified ciliated columnar epithelium. However, a single-layer epithelium was found on the lateral surfaces of the nasal turbinates (*B*, *C*). Original magnification ×100. Rectangles indicate areas covered with the single-layer epithelium. The results are representative of three independent experiments. *D–G*, Confocal views of UEA-1^{+} cells in the nasal epithelium of turbinates. Frozen sections were prepared and stained with FITC-WGA (green) and rhodamine–UEA-1 (red), and then counterstained with DAPI (blue) (*D*, *E*). Scale bars, 20 μm. The merged image is shown in *D*. An enlargement of the area in the rectangle in *D* is shown in *E*. UEA-1^{+}WGA^{-} cells are clustered on the single-layer nasal epithelium of the turbinate. *F* and *G*, UEA-1^{+} cells also reacted with our previously developed M cell-specific mAb NKM 16-2-4, demonstrating colocalization of signals of rhodamine–UEA-1 (red) and FITC-NKM 16-2-4 (green). The merged image is shown in *F*. An enlargement of an area from the rectangle in *F* is shown in *G*. The results are representative of five independent experiments.

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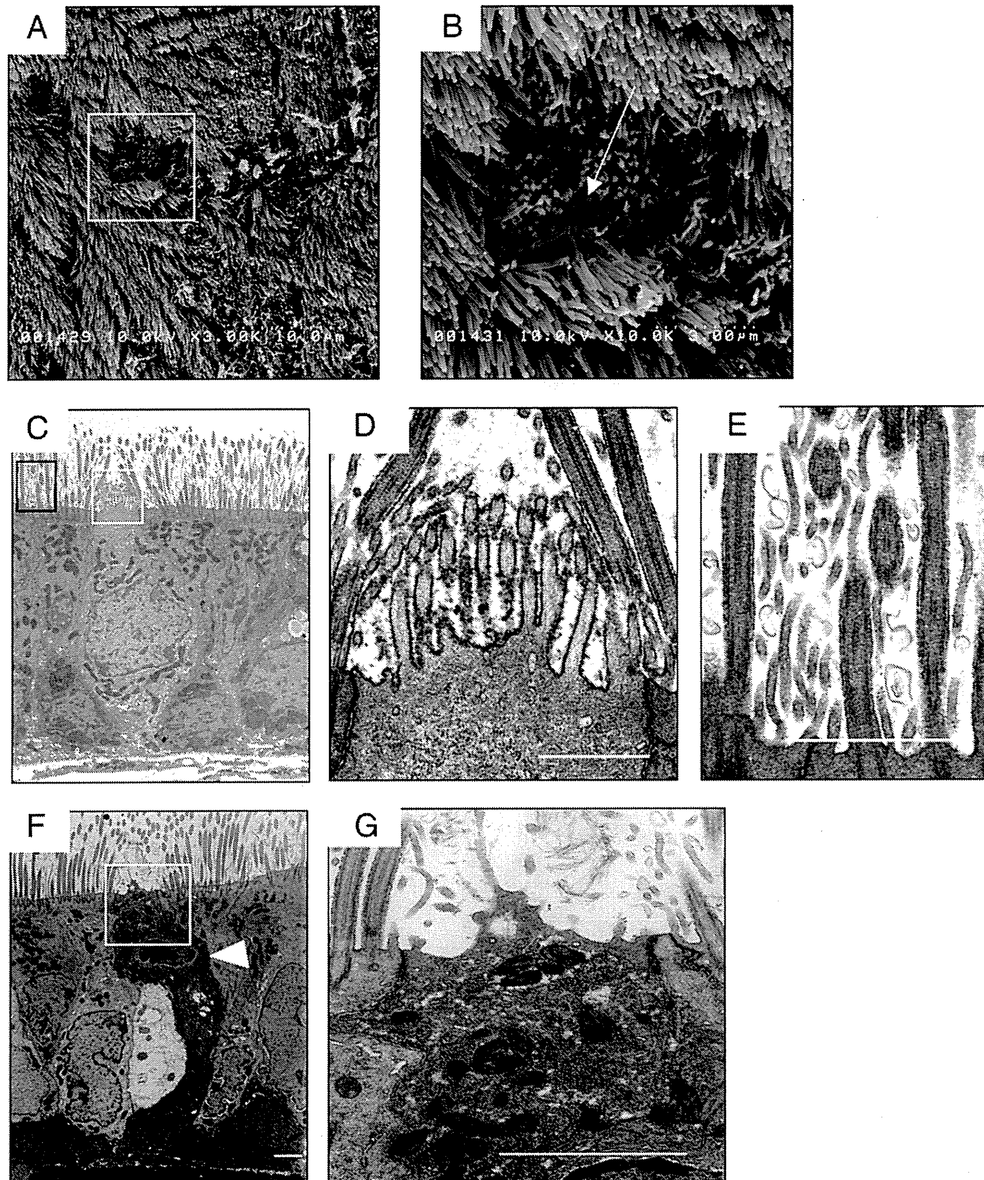

FIGURE 2. Electron microscopic analysis of respiratory M cells. *A* and *B*, SEM analysis shows that the M cells (*B*, arrow) in the nasal passage epithelium are distinguishable from adjacent respiratory epithelial cells by their relatively depressed and dark brush borders. An enlargement of the area in the rectangle in *A* is shown in *B*. As indicated in the *Materials and Methods*, the tissue specimen was incubated with HRP-conjugated UEA-1 before TEM analysis. *C–E*, TEM analysis of respiratory M cells reveals shorter and more irregular microvilli with definite UEA-1^+ signals (*D*), unlike the cilia of neighboring respiratory epithelial cells (*E*). *F* and *G*, TEM analysis of NALT M cells. A readily apparent intraepithelial pocket with mononuclear cells (*F*, arrowhead) and short microvilli on the apical surfaces of NALT M cells are seen. The white squares in *C* and *F* indicate UEA-1^+ respiratory and NALT M cells, respectively, and are magnified in *D* and *G*, respectively. The black rectangle in *C* indicates an adjacent respiratory epithelial cell and is magnified in *E*. *C–G*, Scale bars, 0.5 μm. Results are representative of four independent experiments.

Protein and bacterial Ag uptake by respiratory M cells

Because M cells were frequently found in the single layer of nasal passage epithelium (Fig. 1*D–G*), we next examined the ability of respiratory M cells to take up various forms of Ag from the lumen of the nasal cavity. DQ OVA or recombinant *Salmonella typhimurium* expressing GFP (*Salmonella*-GFP) was instilled into the nasal cavities of BALB/c mice via the nares. Thirty minutes after the intranasal instillation, immunohistological analyses revealed that the M cells located on the lateral surfaces of the nasal turbinates in the single layer of nasal epithelium had taken up DQ OVA (Fig. 3*A*, 3*B*), as had the M cells located in the NALT epithelium (Fig. 3*C*). Recombinant *Salmonella*-GFP was also observed in M cells in the single layer of nasal epithelium after intranasal administration (Fig. 4*A*, 4*B*). These findings demonstrate that, like NALT M cells (Figs. 3*C*, 4*C*), respiratory M cells were capable of taking up both soluble protein and bacterial Ags.

To further demonstrate the biological significance of respiratory M cells, the numbers of these M cells per mouse were examined and compared with those of NALT M cells (Fig. 3*D*). The number of respiratory M cells was significantly higher than that of NALT M cells. Next, we examined the efficiency of Ag uptake per respiratory M cell and NALT M cell (Figs. 3*E–J*, 4*D–I*). Nasal passage and NALT epithelial cells isolated from BALB/c mice 30 min after intranasal instillation of DQ OVA or recombinant *Salmonella*-GFP were counterstained with PE–UEA-1 for flow cytometric analysis. The UEA-1^+ fraction showed a significantly greater efficiency of uptake of DQ OVA Ag and recombinant *Salmonella*-GFP than did UEA-1^- cells isolated from the re-

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