厚生労働科学研究費補助金

食品の安全確保推進研究事業

肥満・脂質代謝を標的にした機能性健康食品の免疫学的機能・安全性評価

平成２３年度　総括研究報告書

研究代表者　國澤　純

平成２４（２０１２）年５月

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I. 総括研究報告書

厚生労働科学研究費補助金（食品の安全確保推進研究事業）

総括研究報告書

肥満・脂質代謝を標的にした機能性健康食品の免疫学的機能・安全性評価

研究代表者：　國澤　純（東京大学医科学研究所　講師）

研究要旨：　現在、脂質代謝を標的にし、“やせる、太らない”を謳った多くの機能性健康食品が開発されている。脂質は免疫制御や各種免疫疾患の発症において重要な役割を担っていることを考慮すると、脂質代謝を標的とした機能性健康食品が特に油の吸収部位である腸管組織における免疫機能に影響を与え、その結果、各種免疫疾患につながる危険性が考えられる。本事業においては脂質代謝を標的とした機能性健康食品、特に機能性食用油の免疫学的機能・安全性を評価することを目的とする。本事業の２年目である２３年度は、機能性食用油のうち飽和脂肪酸を多く含むヤシ科食用油を基本組成とする食用油に焦点をあて、これらの食用油を含む餌で飼育したマウスに食物アレルギーモデルを適用した際のアレルギー・免疫応答について解析した。本事業の結果から、飽和脂肪酸であっても炭素鎖の長さの違いによりアレルギー発症度合いが異なることが示され、太らないと謳われている中鎖飽和脂肪酸を多く含むヤシ油は食物アレルギーの発症にほとんど影響を示さないことが示された。

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

現在、“やせる、太らない”を謳った機能性健康食品が開発されているが、その多くは脂質代謝を標的にしたものである。一方、脂質の代謝や吸収を司る腸管には多くの免疫担当細胞が存在し、生体の免疫学的防御と恒常性維持において重要な役割を担っている。研究代表者の研究を含む最近の研究結果から、食餌性脂質の量と質が腸管免疫の制御に深く関わっていること、ならびに腸管免疫を介した恒常性維持機構の破綻が各種炎症・アレルギー性疾患などの免疫疾患の発症につながることが示されている。これらを考え合わせると脂質代謝を標的とした機能性健康食品が腸管免疫に影響を与え、その結果、各種免疫疾患につながる危険性が考えられる。しかしながら、食の安全性が危惧されている現在においても、脂質代謝を標的とした機能性健康食品と各種免疫疾患との関連に関する実験的検証はほとんど行われていないのが現状である。本研究においては、研究代表者がこれまで行ってきた脂質を介した腸管免疫の制御と各種免疫疾患との関連に関する研究から得ら

れた知的・技術基盤を発展させ、脂質代謝を標的にした機能性健康食品、特に機能性食用油の免疫学的機能・安全性を評価することを目的とする。本事業の２年目である２３年度は、機能性食用油のうち飽和脂肪酸を多く含むヤシ科食用油を基本組成とする食用油に焦点をあてた免疫学的研究を遂行した。

ヤシ科の食用油のうちヤシ油は炭素鎖が12以下の中鎖脂肪酸が80%以上を占めているという特徴を示す。これら中鎖脂肪酸は体内での分解が早いという性質を示すことから、体に油のつきにくい機能性食用油として市販されている。また近年、健康への影響が危惧されている飽和脂肪酸を多く含むことも特徴としており、炭素鎖14以上の長鎖脂肪酸も含めるとヤシ油は90%以上が飽和脂肪酸となっている。また同じくヤシ科の食用油であるパーム油は、長鎖飽和脂肪酸であるパルミチン酸を多く含むという特徴を有し、酸化されにくい油として日本国内においても爆発的に使用量が増加している。本年度はこれらの食用油を含む餌で飼育したマウスに食物アレルギーモデルを適用した際のアレルギー・免疫応答について解析した。

B. 研究方法

通常のマウス用食餌に用いられる大豆油、もしくは脂肪酸組成の異なるヤシ科食用油を重量比で 4%になるように加えた飼料を作製し、Balb/c マウスに２ヶ月間与えたマウスを実験に供した。

これらのマウスに、フロイント完全アジュバントと混合した OVA を背部皮下に免疫することで全身感作を行った。全身感作の一週間後から週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し、その症状を観察した。さらにOVAの経口投与7もしくは8回後に血清中のOVA特異的IgE 産生を ELISA 法で定量すると共に、フローサイトメーター法を用いた大腸組織へのマスト細胞の浸潤と活性化を検証した。

（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

ヤシ科の食用油であるパーム油を 4%含む餌で飼育したマウスに食物アレルギーモデルを適用したところ、アレルギー性下痢の増悪化が認められた。パーム油は長鎖飽和脂肪酸であるパルミチン酸を多く含むことを特徴としている。最近、飽和脂肪酸はデンマークを中心とした欧州で健康への影響が危惧されていることから飽和脂肪酸が影響を与えた可能性が考えられた。

そこで次に同じくヤシ科の食用油であるヤシ油に着目した。ヤシ油は 90%以上が飽和脂肪酸となっているが、パーム油と異なり80%以上が炭素鎖12以下の中鎖脂肪酸である。これまでに報告されているように中鎖脂肪酸は速やかに分解されるため、ヤシ油群では体重の増加抑制が認められた。これらのマウスに食物アレルギーモデルを適

用したところ、アレルギー性下痢の発症はコントロール群である大豆油と同程度であった。これらの結果と相関しアレルギー発症の誘導因子であるアレルゲン特異的 IgE の産生に変化は認められなかった。さらに、アレルギー発症のエフェクター因子であるマスト細胞にも変化は認められなかった。

D. 考察

本研究事業の２年目である２３年度においては、ヤシ科食用油に注目した研究を中心に行った。ヤシ科食用油の一つであるパーム油は近年東南アジアを中心に輸入が増加している油であり、日本国民においても使用量が増えており、菜種油、大豆油に並び日本国内で使用されている油であり、近年増加している免疫疾患との関連も含め検討が必要であると考える。

一方、パーム油と同じヤシ科に属するヤシ油はパーム油とは異なり、カプリル酸、カプリン酸、ラウリン酸、ミリスチン酸などの中鎖脂肪酸を多く含む。これは日本国内において頻用されている菜種油や大豆油には全く含まれない脂肪酸であるため、その免疫学的作用が注目されたが、少なくとも 2 ヶ月間摂取したマウスに食物アレルギーモデを適用した際にはコントロール群として比較して違いは認められなかった。

パーム油に多く含まれるパルミチン酸は Toll 様受容体 4 などの自然免疫受容体のリガンドとなることで炎症を誘導することが最近報告されている。これらは炎症性疾患だけではなく、各種代謝性疾患にも関与することが示唆されている。これらのことから最近、デンマークにおいて飽和脂肪酸を含む食品に対し課税されるようになった。一方、同じヤシ科油であるヤシ油は飽和脂肪酸を 90%以上含むことから、アレルギー発症への影響が危惧されたが、今回の結果から食物アレルギーの発症に大きな影響を与えないことが示された。これらを考えると同じ飽和脂肪酸でも炭素鎖の長さの違いにより免疫学な効果は大きく異なることが示された。

これら本年度に得られた知見は、“脂質と腸管免疫”といった新視点から機能性健康食品の安全性に関する情報提供となる。これは国民の健康維持に直結する食品安全行政にとって重要な知見となると共に、これらの研究から得られる知見を応用することで、各種免疫疾患のリスク低減による国民の保健医療、ならびに免疫疾患等の患者数減少による医療経済の改善が予想され、今後さらに検討を進めることで、厚生労働行政に貢献できると期待される。

E. 結論

本研究から、健康への影響が危惧されている飽和脂肪酸であるが、その効果は炭素鎖の長さの違いにより異なり、機能性食用油と指定しているヤシ油に多く含まれる中鎖脂肪酸は、アレルギー発症に対してはあまり影響を与えないことが示された。

F. 健康危機情報

なし

G. 研究発表

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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

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ
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Kim DY, Sato A, Fukuyama S, Sagara H, Nagatake T, Kong IG, Goda K, Nochi T, Kunisawa J, Sato S, Yokota Y, Lee CH, Kiyono H	The airway antigen sampling system: respiratory M cells as an alternative gateway for inhaled antigens.	*J Immunol*	186	4253-62	2011
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國澤　純	マクロ共焦点顕微鏡を用いた粘膜組織における細胞動態	*Drug Delivery System*	30	409-412	2011
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III. 研究成果の刊行物・別冊
（主要なもの）

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

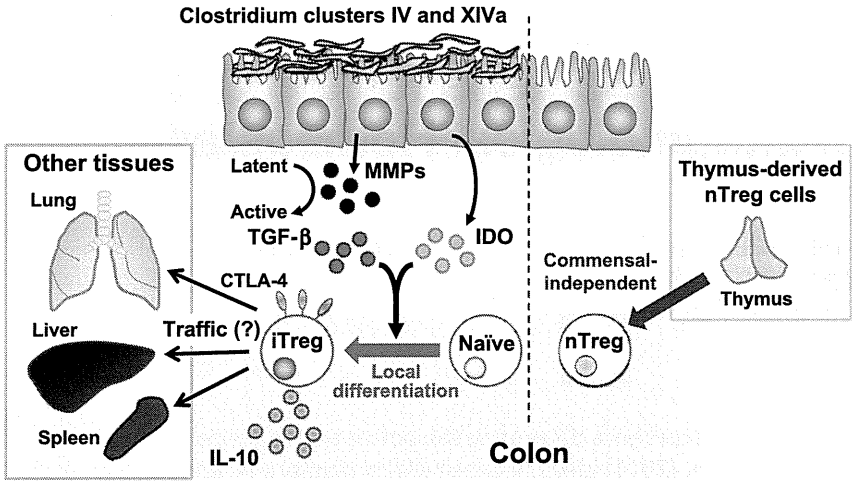

Figure 1. Induction of IL-10-Producing-Induced Treg (iTreg) Cells through the Interaction between Indigenous *Clostridium* Species and Epithelial Cells
After weaning, *Clostridium* clusters IV and XIVa become prominent in the colon, where they form a thick layer on the epithelium. *Clostridium* clusters 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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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-1$^+$WGA$^-$) and goblet cells (UEA-1$^+$WGA$^+$) were determined by the enumeration of each type in 100 UEA-1$^+$ cells. The formula used to estimate the number of M cells was: [(total number of mononuclear cells × percentage of UEA-1$^+$ epithelial cells) × M cells/UEA-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-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.

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.

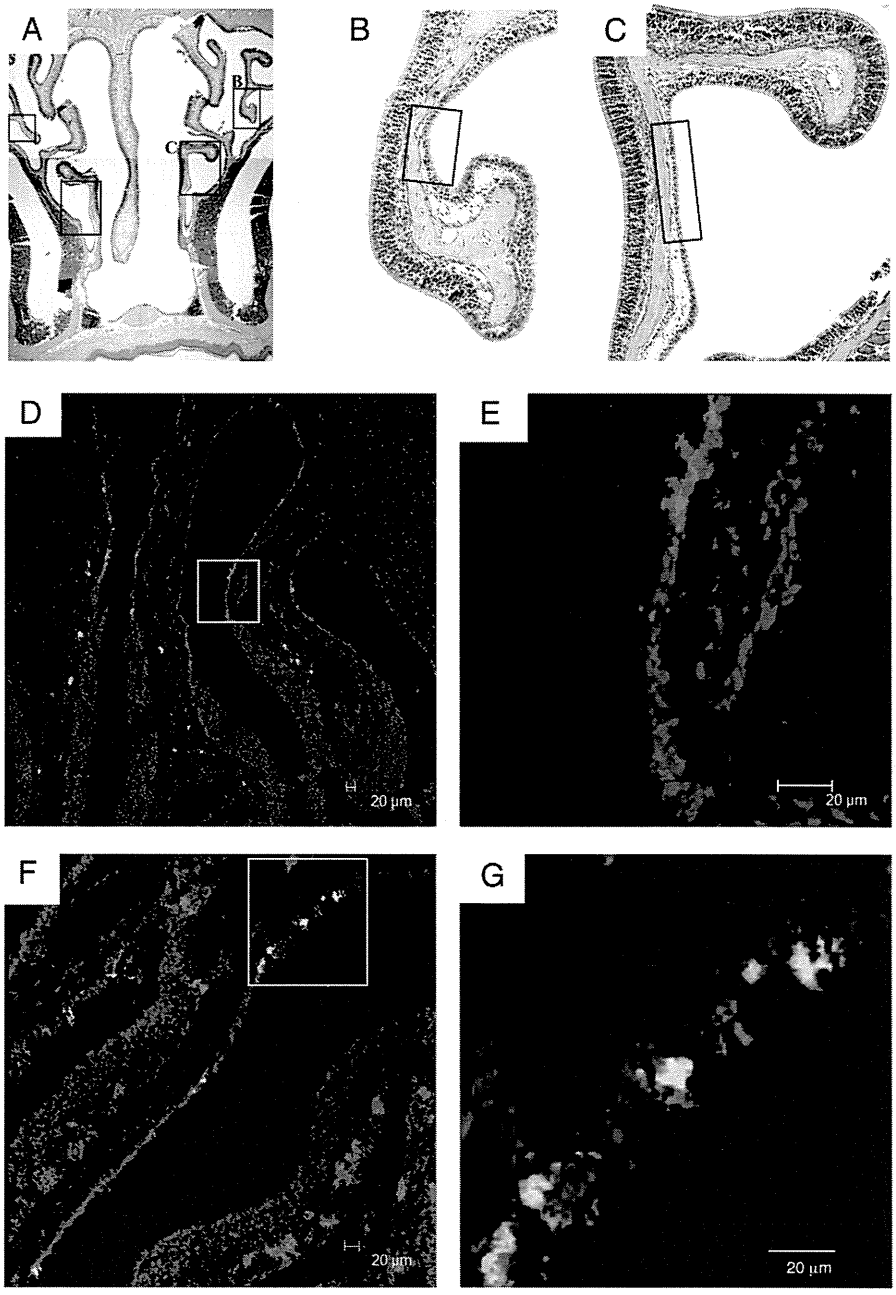

FIGURE 1. Clusters of UEA-1$^+$WGA$^-$ respiratory M cells are found selectively in the single-layer epithelium of the nasal passage. *A–C*, H&E staining reveals the anatomy and general histology of the murine nasal passage (*A*, original magnification ×40). The nasal respiratory epithelium of the mouse 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.

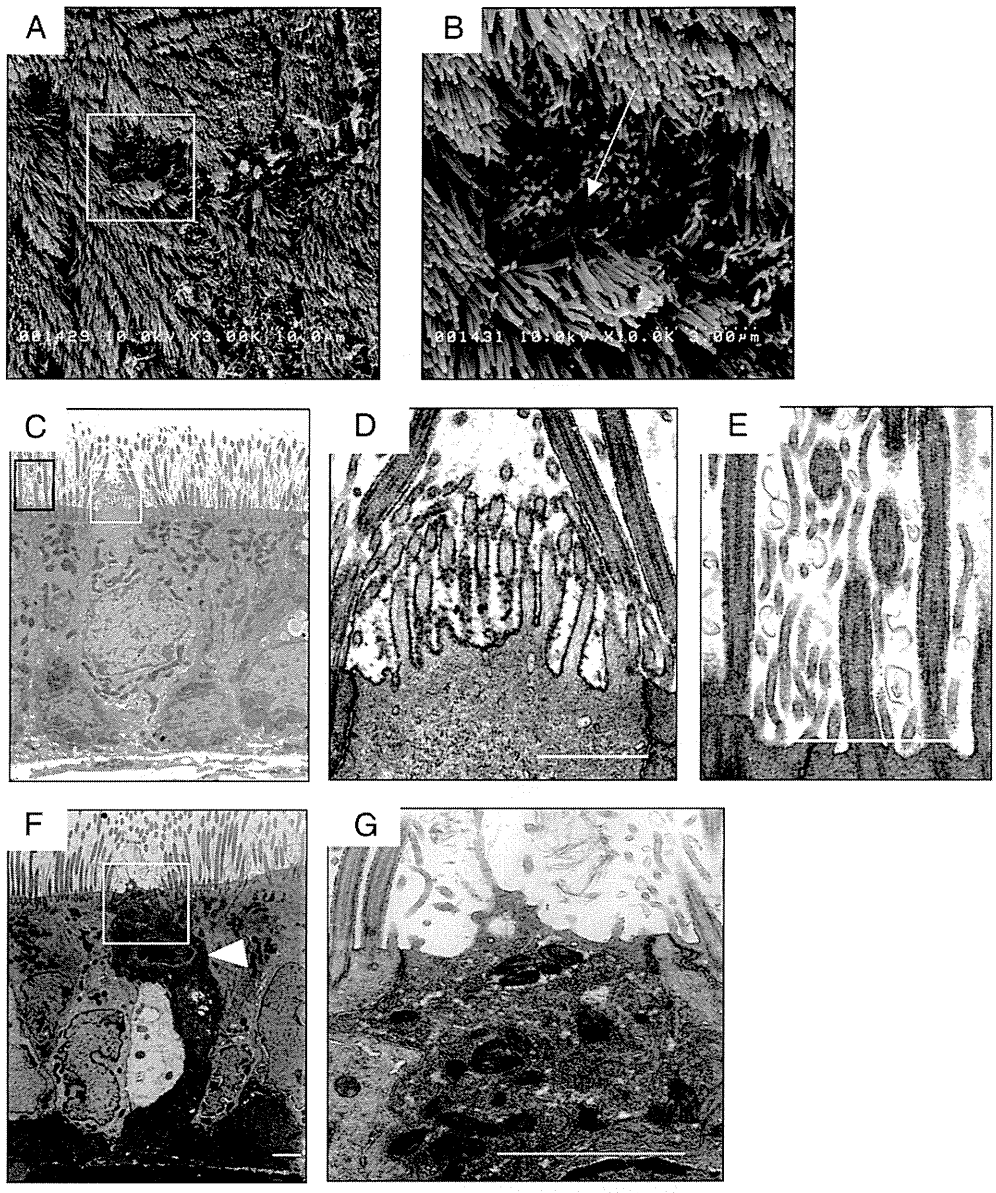

FIGURE 2. Electron microscopic analysis of respiratory M cells. *A* and *B*, SEM analysis shows that the M cells (*B*, arrow) in the nasal passage epithelium are distinguishable from adjacent respiratory epithelial cells by their relatively depressed and dark brush borders. An enlargement of the area 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-

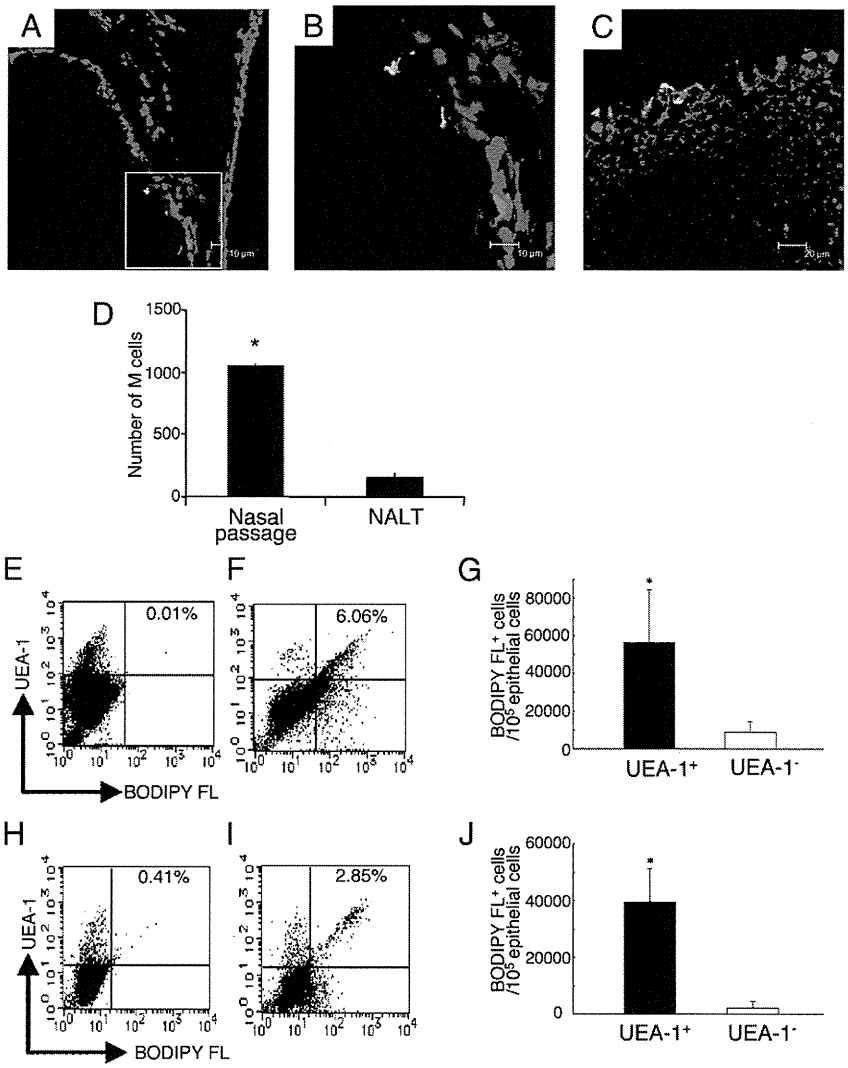

FIGURE 3. Respiratory M cells can take up DQ OVA. *A* and *B*, Immunofluorescence staining of nasal passages in BALB/c mice 30 min after DQ OVA (0.5 mg, green) instillation. Frozen sections of nasal passage were stained with rhodamine–UEA-1 (red) and DAPI (blue). Scale bars, 10 μm. The merged image is shown in *A*. An enlargement of the area in the rectangle in *A* is shown in *B*. These pictures demonstrate DQ OVA uptake by UEA-1^+ respiratory M cells. *C*, UEA-1^+ (red) NALT M cells in BALB/c mice also show an ability to take up DQ OVA (green). Scale bar, 20 μm. The results are representative of seven independent experiments. *D*, The numbers of UEA-1^+WGA$^-$ cells in nasal passages and NALT were quantified. The results are representative of four independent experiments. Flow cytometric analysis of DQ OVA uptake by UEA-1^+ respiratory (*E–G*) and NALT (*H–J*) M cells 30 min after intranasal instillation of PBS (*E*, *H*; control) or DQ OVA (*F*, *I*). *G* and *J*, UEA-1^+ cells showed significantly higher uptake of DQ OVA than did UEA-1^- cells in the nasal passages and NALT. The results are representative of four independent experiments. $*p < 0.05$.

spiratory epithelium of the nasal passage (Figs. 3*E–G*, 4*D–F*) and NALT (Figs. 3*H–J*, 4*G–I*).

Three-dimensional confocal microscopic analysis demonstrated that UEA-1^+ GFP$^+$ cells, which were sorted from the nasal passages of the mice intranasally infected with GFP-*Salmonella*, had captured and taken up the bacteria (Fig. 4*J*, Supplemental Video 1).

Cluster formation by respiratory M cells and DCs in response to inhaled respiratory pathogens

Because respiratory M cells are capable of capturing bacterial Ag, we considered it important to assess these cells as potential new entry sites for respiratory pathogens such as GAS. Confocal microscopic analysis demonstrated that, after its intranasal instillation, GAS stained with FITC–anti-*Streptococcus* A Ab was taken up by UEA-1^+ respiratory M cells (Fig. 5*B–E*). SEM analysis also revealed the presence of GAS-like microorganisms on the membranes of respiratory M cells after nasal challenge with GAS (Supplemental Fig. 2*A*). As one might expect, GAS were found in UEA-1^+ NALT M cells (Supplemental Fig. 2*B*) as well, confirming a previously reported result (20). Our findings suggest that respiratory M cells act as alternative entry sites for respiratory pathogens.

When we examined the site of invasion by GAS, we noted the presence of CD11c$^+$ DCs underneath the respiratory M cells (Fig. 5). Confocal microscopic analysis of the nasal passage epithelium after intranasal instillation of GAS revealed evidence of the recruitment of DCs, some having contact with the GAS, to the area underneath the respiratory M cells (Fig. 5*B–E*). A few DCs were also observed in the nasal passages of naive mice (Fig. 5*A*); these nasal DCs might preferentially migrate to the area underneath the respiratory M cells to receive Ags from these cells for the initiation of Ag-specific immune responses.

Presence of respiratory M cells in NALT-deficient mice

When we examined the numbers of respiratory M cells in the lymphoid structure-deficient Id$2^{-/-}$ mice (including NALT, NALT-null), the frequency of occurrence of respiratory M cells was comparable to that found in their littermate Id$2^{+/-}$ mice (Fig. 6*A*). This finding suggested that development of respiratory M cells occurred normally under NALT-null or Id2-deficient conditions. Frozen tissue samples were next prepared from NALT-null mice that had received fluorescence-labeled bacteria by intranasal instillation. Immunohistological analysis of these samples revealed the presence of recombinant *Salmonella*-GFP in UEA-1^+ cells from the nasal epithelium of Id$2^{-/-}$ mice. GFP-positive bacteria were also located in the subepithelial region of the nasal passages, suggesting that, in the NALT-null mice, some of the nasally deposited bacteria were taken up by respiratory M cells (Fig. 6*B*, 6*C*). Flow cytometric analysis confirmed the uptake of recombinant *Salmonella*-GFP by UEA-1^+ M cells, with UEA-1^+ cells in the nasal passages of Id$2^{-/-}$ mice showing a significantly higher uptake than UEA-1^- cells (Fig. 6*D–F*).