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免疫アレルギー疾患等予防・治療研究事業

食餌性脂質を中心とした生理活性脂質による粘膜免疫制御ならびにアレルギー疾患との関連解明

平成２１年度　総括研究報告書

研究代表者　國澤　純

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目　次

I.　統括研究報告
食餌性脂質を中心とした生理活性脂質による粘膜免疫制御ならびにアレルギー疾患との関連解明
國澤　純　--------------1

II.　研究成果に関する一覧表　--------------7

III.　研究成果の刊行物・別刷（主要なもの）　--------------9

I.　総括研究報告書

厚生労働科学研究費補助金（免疫アレルギー疾患等予防・治療研究事業）
総括研究報告書

食餌性脂質を中心とした生理活性脂質による粘膜免疫制御ならびにアレルギー疾患との関連解明

研究代表者： 國澤　純（東京大学医科学研究所　講師）

研究要旨： 本研究においてはこれまでの我々の研究から食物アレルギーに関わることを示している脂質メディエーターの一つであるスフィンゴシン１リン酸（S1P）に関する研究を起点に、食餌性脂肪酸を介した腸管免疫制御と食物アレルギーとの関連を検討した。その結果、我々が日常摂取している食用油であってもその脂肪酸組成の違いにより腸管免疫の活性化や抑制が起こることが示された。さらに腸管免疫の活性化を主要食餌性脂肪酸の一つであるパルミチン酸が担っていることを明らかにした。また食餌性脂肪酸の間に免疫活性と抑制における階層が存在していることを示し、その制御を行うことで生体防御を増強させつつ、副作用となるアレルギー反応を抑制できる可能性を示唆した。

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

食の欧米化に伴いアレルギー疾患の患者数が増加していることから、食餌性成分を介した免疫制御がアレルギー発症に関与していると考えられている。これまでの国内外のグループによる研究から、アレルギー発症に関わる食餌性成分の一つとして油の関与を示唆する結果が報告されている。これらの研究においては主に炎症性脂質メディエーターであるプロスタグランジンやロイコトリエンの前駆体となるリノール酸や、抗炎症作用が知られているリノレン酸に着目された検討がなされてきた。

一方、申請者はこれまでに脂質メディエーターの一つであるスフィンゴシン１リン酸（S1P）が分泌型 IgA の産生や上皮細胞間 T リンパ球を介した腸管免疫制御を行うのと同時に、食物アレルギーの発症に関与する活性型 T 細胞やマスト細胞の遊走を制御していることを示してきた。S1P は生体内において細胞膜の主成分であるスフィンゴミエリンやセラミドを前駆体とし産生されると言われている。しかし食餌性脂質の代謝・吸収部位となっている腸管においては食餌性脂質もその前駆体になり得ると予想されるが、脂質の代謝経路を考えてみると、S1P は従来言われているリノール酸やリノレン酸から産生されるとは考えにくい。本研究においてはこれまでの研究結果を基盤に、これまで提唱されてきたリノール酸や

リノレン酸ではない食餌性脂肪酸にも腸管免疫制御活性があるという仮説をたて、アレルギー発症との関連も含め実証を行っている。

3 年計画の 2 年度である本年度は脂肪酸組成の異なる食用油を用いた特殊餌をマウスに与えた際の腸管免疫への影響を調べることを第一の目的とした。また腸管免疫の機能に違いが見られた食用油においては、その食用油に特徴的な脂肪酸に焦点を当て、その腸管免疫機能と食物アレルギーとの関連における脂肪酸の関与について検討した。

B. 研究方法

1) 異なる食餌性脂質を含む餌を摂取した際の腸管免疫応答の変化

通常のマウス用食餌に用いられる大豆油の代わりに、脂肪酸組成の異なる食用油、もしくは精製脂肪酸を各食用油に混合したものを重量比で 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、糞便中の IgA 産生を ELISA 法にて測定した。糞便中 IgA 産生に変化が見られた群においては、フローサイトメトリー等の免疫学的手法を用い免疫学的機能変化について検討した。また抗原特異的免疫応答を検討するために、ニワトリ卵白アルブミン (OVA) を実験的粘膜アジュバントであるコレラトキシンと共に経口免疫し、産生される OVA 特異的糞便中 IgA を ELISA 法にて測定した。

2) 異なる食餌性脂質を含む餌を摂取した際の食物アレルギーの発症

異なる食餌性脂質を 4%含む餌を 2 ヶ月間摂取させた同マウスに、フロイント完全アジュバントを用い OVA 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。

(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) 異なる食餌性脂質を含む餌を摂取した際の腸管免疫応答の変化

食用油として用いられている植物油の脂肪酸組成に関する情報をもとにユニークな脂肪酸組成を示す食用油を選択し、重量比で 4%含む特殊飼料を作製した。これらの特殊飼料を 7 週齢のマウスに 2 ヶ月間与え、糞便中の IgA を ELISA 法により測定した。その結果、亜麻仁油を用いて調整した飼料を摂取したマウスではコントロール群である大豆油を用いた飼料で飼育した場合に比べ糞便中 IgA の量が約 3 分の 1 に減少していた。一方、パーム油を用いた飼料により飼育した場合、糞便中 IgA の量が約 2 倍に増加した。

パーム油の脂肪酸組成をその他の食用油と比較したところ、パルミチン酸を非常に多く含有していることが判明した。そこでパルミチン酸の腸管免疫に与える影響を調べる目的で、大豆油にパーム油と同程度となるような量のパルミチン酸を加えた飼料

を作製し、その飼料を用いて飼育したマウスの糞便中 IgA を測定した。その結果、大豆油にパルミチン酸を加えた油を含む飼料で飼育したマウスにおいても、パーム油を用いた場合と同様、糞便中 IgA の産生増強が確認された。また IgA 産生の減少が観察された亜麻仁油においてもパルミチン酸を加えることで亜麻仁油飼料に対して約 10 倍、大豆油飼料に対して約 3 倍の IgA 産生増強効果が観察された。

次にパルミチン酸に焦点を絞り、その機能について解析を行った。まず始めに飼育開始時の週齢の影響について検討したところ、パルミチン酸による腸管 IgA の増強は 6 週齢のマウスを用いた際に最も強く認められ、週齢が増す毎にその効果は減少し 34 週齢以上のリタイアマウスでは逆に IgA は減少していた。IgA の産生増強が認められたマウスを用い、フローサイトメトリーにて免疫学的解析を行ったところ、主に大腸における CD138 陽性の IgA 産生形質細胞の増加が観察された。またこれらの IgA 産生増強効果は抗原特異的免疫応答においても観察され、経口免疫したタンパク質抗原に対する特異的 IgA の産生も増加していた。

2) 異なる食餌性脂質を含む餌を摂取した際の食物アレルギーの発症

上記の研究よりパルミチン酸を多く含む餌を用いて飼育した場合、主に大腸において免疫の活性化が観察されたことから、次に大腸において症状が観察されるマウス食物アレルギーモデルを用い、その発症状態を検討した。その結果、IgA の産生増強と同様、大豆油にパルミチン酸を加えた油で飼育した群やパーム油を含む飼料で飼育した群で食物アレルギーの増悪化が観察された。一方、IgA 産生の減少が観察された亜麻仁油を用いた飼料で飼育した場合、アレルギーの発症抑制が観察された。興味深いことに亜麻仁油にパルミチン酸を加えることで IgA の産生は約 10 倍に増加したのにも関わらず、アレルギーの発症は抑制されたままであった。

D. 考察

本研究においては、新たな腸管免疫活性型の脂肪酸としてパルミチン酸を同定した。大豆油にパルミチン酸を加えた食用油やパーム油を用いた飼料で飼育した場合、生体防御に関わる IgA の産生増強だけではなく、生体にとって有害な免疫応答であるアレルギー反応の増悪化も同時に観察された。これは S1P などの多くの生理活性脂質が生体防御に働く免疫経路とアレルギーに働く免疫経路の両者に対して促進的に働くという結果により説明される現象であると考えられる。また同様の腸管免疫活性化がパルミチン酸を多く含有するパーム油でも観察されたことから、パルミチン酸を人為的に加えた特異的なケースだけではなく、我々の日常においても同様の現象が観察されることが示唆される結果であると考えられる。

一方、抗炎症作用があるリノレン酸を多く含む亜麻仁油を用いて飼育した場合、IgA の産生抑制が観察されたが、大豆油の場合

と同様、パルミチン酸の添加により IgA の産生が増強した。亜麻仁油は現在、健康食品として注目されているが、今回の結果から、亜麻仁油だけを大量に摂取した場合、IgA を中心とする生体防御免疫機能が低下し、感染症のリスクが高まる危険性があること、これらを改善するためにはパルミチン酸などの免疫活性化機能を有する脂肪酸を摂取することが重要であることが示唆された。また特筆すべきことは、亜麻仁油にパルミチン酸を加えた場合、IgA の産生は著しく増加するのにも関わらず、アレルギーの発症の抑制は維持されたままであるという結果である。これは各脂肪酸間である種の階層が存在しており、IgA の産生とアレルギーの発症を異なる作用点により制御していることを示唆する結果であると考えられる。今後はパルミチン酸による免疫活性化機構と亜麻仁油（or リノレン酸）によるアレルギー抑制の分子機構の解明を行っていくと共に、ヒトにおける影響についても検討する予定である。

また一般食生活との関連を考えると、パーム油は多くの飲食関連業界で使用されるようになってきており、またその脂肪酸組成は牛脂に類似している。一方、亜麻仁油の脂肪酸は魚油に類似している。今回得られた知見は、食の欧米化に伴いパルミチン酸を多く含む食事（パーム油、牛脂）の摂取量が増加したことがアレルギー疾患増加の一因である可能性、ならびに肉と魚をバランス良く摂取することが生体の免疫バランス制御において重要であるという知見に関する一つの分子機序を提唱するものであると考えられる。

E. 結論

本研究から、日常的に摂取している食用油であっても脂肪酸組成の違いにより腸管免疫の活性化や抑制が起こることが示された。また活性化の一端をパルミチン酸が担うこと、さらに脂肪酸間でその活性制御における階層が存在し、その制御により生体防御を増強させつつ、アレルギー反応を抑制できることが示唆された。

F. 健康危機情報

なし

G. 研究発表

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1. 特許取得

（出願番号）特願 2009-169720；(発明者）國澤　純、清野　宏；(発明の名称）免疫増強剤；(出願人）株式会社東京大学 TLO；(出願日）平成 21 年 7 月 21 日

2. 実用新案登録
   特記事項なし。

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
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ARTICLE

Id2-, RORγt-, and LTβR-independent initiation of lymphoid organogenesis in ocular immunity

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Abbreviations used: AID, activation-induced cytidine deaminase; CALT, conjunctiva-associated lymphoid tissue; CT, cholera toxin; FAE, follicle-associated epithelium; FDC, follicular DC; GC, germinal center; HE, hematoxylin and eosin; HEV, high endothelial venule; Id2, inhibitor of DNA binding/differentiation 2; ILF, isolated lymphoid follicle; LT, lymphotoxin; LTi, lymphoid tissue inducer; MAdCAM-1, mucosal addressin cell adhesion molecule 1; MALT, mucosa-associated lymphoid tissue; NALT, nasopharynx-associated lymphoid tissue; NIK, NF-κB-inducing kinase; NP, nasal passage; pLN, peripheral LN; PNA, peanut agglutinin; PNAd, pLN addressin; PP, Peyer's patch; ROR, retinoic acid-related orphan receptor; TALT, tear duct-associated lymphoid tissue; TLR, Toll-like receptor; TRAF, TNF receptor-associated factor; TRANCE, TNF-related activation-induced cytokine; UEA, *Ulex europaeus* agglutinin; VCAM-1, vascular cell adhesion molecule 1.

The eye is protected by the ocular immunosurveillance system. We show that tear duct-associated lymphoid tissue (TALT) is located in the mouse lacrimal sac and shares immunological characteristics with mucosa-associated lymphoid tissues (MALTs), including the presence of M cells and immunocompetent cells for antigen uptake and subsequent generation of mucosal immune responses against ocularly encountered antigens and bacteria such as *Pseudomonas aeruginosa*. Initiation of TALT genesis began postnatally; it occurred even in germ-free conditions and was independent of signaling through organogenesis regulators, including inhibitor of DNA binding/differentiation 2, retinoic acid-related orphan receptor γt, lymphotoxin (LT) $\alpha 1\beta 2$-LTβR, and lymphoid chemokines (CCL19, CCL21, and CXCL13). Thus, TALT shares immunological features with MALT but has a distinct tissue genesis mechanism and plays a key role in ocular immunity.

Mucosa-associated lymphoid tissues (MALTs), including nasopharynx-associated lymphoid tissue (NALT) and Peyer's patches (PPs), are gateways for the uptake of inhaled and ingested antigens from the lumen of the aerodigestive tract, and are considered to be the sites of induction of mucosal immune responses (Mestecky et al., 2003; Kiyono and Fukuyama, 2004). The ocular surface leading to the lacrimal sac and nasolacrimal duct also forms an interface with the outside environment. In fact, it has been proposed that conjunctiva-associated lymphoid tissue (CALT), together with tear duct–associated lymphoid tissue (TALT), organizes eye-associated lymphoid tissue to create mucosal surveillance and a barrier in the eye

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region of humans (Knop and Knop, 2000, 2001). Past investigations have focused on the identification and characterization of CALT (Gomes et al., 1997; Chodosh et al., 1998; Knop and Knop, 2000, 2005; Giuliano et al., 2002; Cain and Phillips, 2008). Mice and rats do not possess CALT, whereas other mammals (e.g., cats, dogs, and humans) do develop CALT (Chodosh et al., 1998). Rat conjunctivae, lacrimal glands, and harderian glands contain immunocompetent cells (e.g., $CD4^+$ and $CD8^+$ cells; Gomes et al., 1997); however, the immunocompetent cells do not form any organized microarchitecture at the conjunctiva and are thus diffusely located. Tears contain cytokines (e.g., IL-1, IL-4, IL-6, and TGF-β), antimicrobial peptides (e.g., lactoferrin and defensin), and secretory IgA; these secretions are an important arm of mucosal innate and acquired immunity, and respond to antigens that contact and invade the eye (Allansmith et al., 1985; Kijlstra, 1990; Gupta et al., 1996; Haynes et al., 1998; Nakamura et al., 1998; Uchio et al., 2000). Tear flow does not just provide mucosal protection at the ocular surface; it also connects the ocular surface with the nasal cavity via the tear duct, suggesting that tear flow is integral to regulating the homeostasis of the oculonasal mucosal barrier. On the other hand, the eye is considered to be an immune-privileged site, because the microenvironment of the eye is regulated by several complex aspects of the immune system (Stein-Streilein and Taylor, 2007). However, little information is currently available about the immunological nature of the eye-associated lymphoid tissue system—particularly the regulation of the tissue genesis of TALT and its immunological functions—despite the fact that TALT develops in humans (Knop and Knop, 2000, 2001).

Organogenesis of secondary lymphoid tissues, such as PPs and peripheral LNs (pLNs), is dependent on inflammatory cytokines, the release of which is mediated by lymphotoxin (LT) β receptor (LTβR) signals during the embryonic period (Mebius, 2003). The basis of LT-mediated lymphoid organ development at a molecular level was first shown in genetically manipulated $Lt\alpha^{-/-}$ mice, which lack PPs and pLNs (De Togni et al., 1994). Injection of an agonistic LTβR antibody into $Lt\alpha^{-/-}$ mice during a limited period in embryogenesis regenerates pLNs (Rennert et al., 1998). The physiological ligand of LTβR is a membrane-bound form of $LT\alpha1\beta2$ produced by $CD3^-CD4^+CD45^+$ lymphoid tissue inducer (LTi) cells expressing IL-7Rα (Mebius et al., 1997). IL-7Rα–mediated signals trigger LTi cells to produce $LT\alpha1\beta2$, and $Il\text{-}7r\alpha^{-/-}$ mice do not form PPs (Adachi et al., 1998b; Honda et al., 2001). In addition, deficiency of either inhibitor of DNA binding/differentiation 2 (Id2) or the retinoic acid–related orphan receptor (ROR) γt gene results in a lack of PPs and pLNs because the differentiation of $CD3^-CD4^+CD45^+$ LTi cells is impaired (Yokota et al., 1999; Sun et al., 2000; Eberl et al., 2004). These facts indicate the importance of inflammation-related cytokines, as well as Id2- and RORγt-subordinated $CD3^-CD4^+CD45^+$ LTi cells, in the organogenesis of lymphoid tissues, including PPs and pLNs (Kiyono and Fukuyama, 2004). However, NALT does not follow the general biological rule of the dependence of embryonic genesis on inflammatory cytokines (Fukuyama et al., 2002; Harmsen et al., 2002). NALT organogenesis, which occurs postnatally, is independent of LTβR-mediated signals and RORγt but does require Id2 (Fukuyama et al., 2002; Harmsen et al., 2002).

In this study, we provide evidence that TALT develops independently of organogenesis regulators, a finding that distinguishes TALT genesis from that of other lymphoid organs. In addition, we found that TALT plays a central role in the induction of antigen-specific immune responses against ocularly encountered antigens.

RESULTS

Identification of TALT in mice

TALT develops in the human tear duct (Knop and Knop, 2000, 2001; Paulsen et al., 2000, 2003), but to our knowledge no information is currently available on TALT in mice. To identify organized lymphoid tissue in the mouse tear duct and to elucidate its immunological and developmental features, we first examined the anatomy of this duct in mice. To visualize the position of the tear duct, we administered hematoxylin to mice in eye drops. We clearly observed the location of the tear duct where it connects the ocular surface to the nasal cavity (Fig. 1 A). In coronal and horizontal views, we were able to identify TALT in both the left and right side of the lacrimal sac in C57BL/6 WT mice (Fig. 1, B and C).

Postnatal development of TALT

The genesis of each type of lymphoid tissue occurs within a given time window: for example, PPs develop during late embryogenesis and NALT develops postnatally (Fukuyama et al., 2002; Mebius, 2003; Kiyono and Fukuyama, 2004). The initiation of intestinal isolated lymphoid follicles (ILFs) also occurs after birth, and the genetic background (e.g., whether the mouse is of the C57BL/6 or BALB/c strain) influences the postnatal time of initiation of tissue genesis (Hamada et al., 2002). To determine when TALT genesis is initiated and to evaluate the influence of genetic background on tissue genesis, we took tissue samples from both C57BL/6 and BALB/c mice at various pre- and postnatal stages for histological analysis. No sign of mononuclear cell accumulation was observed at embryonic day (E) 18 or postnatal day (D) 5 in C57BL/6 or BALB/c mice (Fig. 1 D and Fig. S1). In contrast, we detected accumulation of mononuclear cells at D10 in both C57BL/6 and BALB/c mice, indicating that the TALT development was initiated between D5 and D10, and that the different genetics of the two strains did not influence TALT organogenesis (Fig. 1 D and Fig. S1). To support the data, the initial appearance of mononuclear cells was noted at D7 in both C57BL/6 and BALB/c mice (Fig. 1 D and Fig. S1). Furthermore, pLN addressin (PNAd)–positive high endothelial venules (HEVs) developed at D10 but not at D5 and D7 (Fig. 1 E). These findings suggest that TALT develops postnatally, as does NALT. Unlike in the genesis

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of other lymphoid tissues (Mebius, 2003), expression of vascular cell adhesion molecule 1 (VCAM-1) was not observed at the TALT anlage (unpublished data).

To determine which cell population initially migrates to the TALT anlage, we analyzed the tissue genesis site at D5, D7, and D10 by confocal microscopy. The D5 anlagen did not

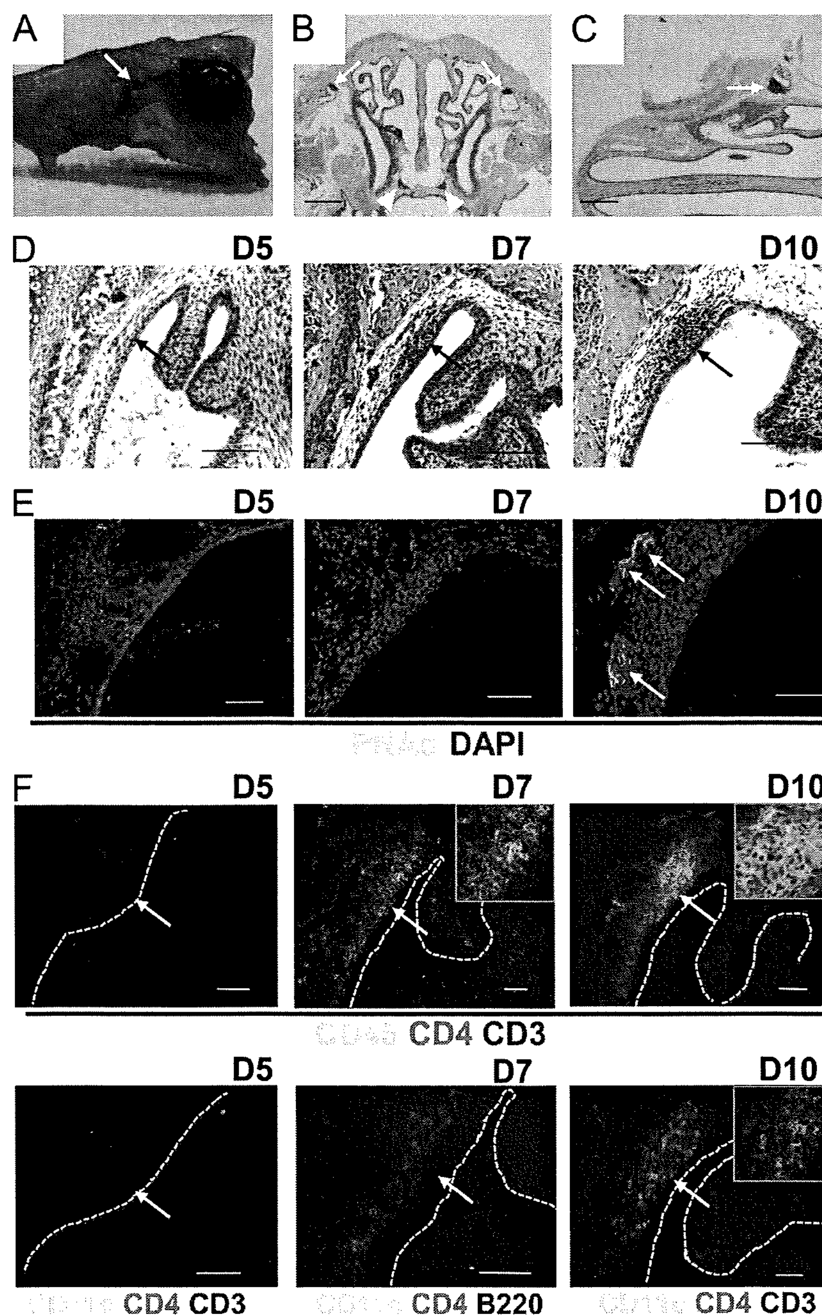

Figure 1. Postnatal development of TALT. (A–C) 10 µl of hematoxylin solution was added to the ocular surface of 8-wk-old C57BL/6 mice to visualize the tear duct (A, arrow). Coronal (B) and horizontal (C) paraffinized sections of the head were stained with HE. Arrows and arrowheads indicate TALT and NALT, respectively (n = 3 mice/group). Bars, 1 mm. (D) Paraffinized tissues of heads from D5, D7, and D10 C57BL/6 mice were examined by HE staining. Arrows indicate the site of TALT genesis (n = 5 mice/group). Bars, 100 µm. (E and F) Head tissue of C57BL/6 mice was examined by confocal microscopy with the indicated antibodies at D5, D7, and D10. Arrows indicate PNAd^{+} HEVs (E) and the site of TALT genesis (F). Some magnified pictures are shown in F (insets). Dashed lines indicate the edge between the TALT epithelium and tear duct lumen. These data are representative of at least three independent experiments (n = 5 mice/group). Bars, 50 µm.

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contain any $CD45^+$ cells, whereas the D7 TALT anlage possessed $CD45^+$ cells (Fig. 1 F). $CD3^-CD4^+CD45^+$ cells have been shown to be LTi cells (Mebius, 2003). Among the $CD45^+$ cells in the D7 TALT anlage, we identified $CD3^-CD4^+CD45^+$ cells and $B220^+$ B cells (Fig. 1 F, D7). $CD11c^+$ DCs were not found at D5 and D7. At D10, $CD11c^+$ DCs and increased numbers of $CD3^-CD4^+CD45^+$ cells were found in the TALT (Fig. 1 F). Because $B220^+$ B cells were among the first cells to migrate at the TALT anlage (Fig. 1 F, D7), we examined B cell–deficient $Igh6^{-/-}$ mice for the development of TALT. The TALT genesis occurred normally, even in the B cell–deficient condition (Fig. S2 A). Further, TALT also developed in T cell–deficient $Tcr\beta^{-/-}$, $Tcr\delta^{-/-}$ mice (Fig. S2 B). These findings show that B and T lymphocytes and DCs are dispensable for the initiation of TALT development.

Organogenesis of TALT does not require microbial stimulation

TALT has been identified in only 30–40% of humans examined (Paulsen et al., 2000, 2003; Knop and Knop, 2001), raising the possibility that environmental conditions, including microbial infections and allergic responses, are involved in the initiation of TALT development. In addition, our finding that TALT develops postnatally also points to the possible involvement of microbial stimulation in TALT genesis. However, we detected TALT in mice deficient in Toll-like receptor (TLR) signals, including $Tlr2^{-/-}$, $Tlr4^{-/-}$, and $MyD88^{-/-}$ mice (Fig. S2, C–E). Further, we found that germ-free mice developed TALT (Fig. 2 A). Thus, TALT organogenesis is most likely independent of microbial stimulation.

TALT development is independent of organogenesis regulators

We next determined the molecular requirements for TALT development. PPs and pLNs are not present in alymphoplasia (*aly/aly*) mice, which carry a null mutation of NF-κB–inducing kinase (NIK), resulting in a failure to transmit LTβR-mediated signals (Shinkura et al., 1999). However, our histological analysis showed that the TALT structure was preserved in *aly/aly* and $Lt\alpha^{-/-}$ mice, although it was smaller than in WT TALT (Fig. 2, A and B; and Fig. S3). In addition, TALT was even observed in $Il\text{-}7r\alpha^{-/-}$ mice, although the size was again small (Fig. 2 B and Fig. S3). Thus, the initiation of TALT development mediated by inducer cells was independent of the IL-7R and LTα1β2–LTβR–NIK pathway signaling cascades, whereas the maturation process of accumulating lymphocytes required the cytokine signaling cascade, as is the case with other lymphoid organs (Mebius, 2003; Kiyono and Fukuyama, 2004).

Lymphoid chemokines, including CXCL13, CCL19, and CCL21, play important roles in the migration of LTi cells to the sites of tissue genesis (Honda et al., 2001; Luther et al., 2003; Fukuyama et al., 2006). We therefore examined the involvement of these lymphoid chemokines in TALT genesis. The TALT structure was preserved in $Cxcl13^{-/-}$ mice, although they lacked some pLNs and had reduced numbers of PPs (Fig. 2 B; Ansel et al., 2000). TALT also developed well in *plt/plt* mice (Fig. 2 B), which carry null mutations of both the *Ccl19* and *Ccl21* genes (Nakano et al., 1998). Furthermore, the initiation of TALT formation was maintained in triple mutant ($Cxcl13^{-/-}$ *plt/plt*) mice (Fig. 2 B), confirming that these lymphoid chemokines are not required for the initiation of TALT organogenesis, although the TALT was smaller in the lymphoid chemokine–null condition than in WT (Fig. S3). These results supported the observation that an NIK-mediated pathway is required to recruit large numbers of lymphocytes to TALT.

We then addressed the involvement of Id2 and RORγt, key transcriptional regulators in the induction of lymphoid organogenesis by $CD3^-CD4^+CD45^+$ LTi cells (Yokota et al., 1999; Sun et al., 2000; Eberl et al., 2004). Surprisingly, TALT formation was preserved in $Id2^{-/-}$ and $Ror\gamma t^{-/-}$ mice (Fig. 2 B). Consistent with this finding, FACS and confocal microscopy analyses detected $CD3^-CD4^+$ LTi cells in the TALT anlage of $Id2^{-/-}$ and $Ror\gamma t^{-/-}$ mice as well as in WT mice (Fig. 3, A and B). Furthermore, when we isolated these $CD3^-CD4^+CD45^+$ cells from WT mice and examined the gene expression of the tissue genesis–associated transcription factors by RT-PCR, we found that $CD3^-CD4^+CD45^+$ cells isolated from the TALT anlagen did not express either *Id2* or *Rorγt*, whereas $CD3^-CD4^+CD45^+$ cells isolated from the embryonic intestine, i.e., PP inducer cells, expressed both *Id2* and *Rorγt* (Fig. 3 C). Thus, TALT organogenesis proceeds independently of Id2, RORγt, and LT. Therefore, TALT genesis is quite different from the genesis of other secondary lymphoid tissues, including PPs, pLNs, and NALT (Mebius, 2003; Kiyono and Fukuyama, 2004).

Microarchitecture of TALT

The structure of the MALT epithelium is characterized by the presence of follicle-associated epithelium (FAE; Kiyono and Fukuyama, 2004). Indeed, we were able to divide the epithelial layer of the lacrimal sac into two populations on the basis of its morphological structure (Fig. 4 A): TALT-FAE was characterized by a thin layer of squamous epithelium (Fig. 4 B), whereas the lacrimal sac epithelium had a multilayered and squamous morphology (Fig. 4 C). Interestingly, TALT-FAE lacked mucus-producing goblet cells and cilia (Fig. 4 B), a fact that distinguished this tissue from NALT-FAE, which has some goblet cells (Fig. 4 D). Instead, mucus-producing gland tissue was frequently observed on the conjunctiva (unpublished data). Confocal microscopic analysis of TALT revealed large numbers of $B220^+$ B cells (Fig. 4 E) and $CD11c^+$ DCs in the subepithelial dome region of the FAE (Fig. 4 F), as well as $CD3^+CD4^+$ T helper cells distributed around the B cell follicles (Fig. 4, G and H). These data indicate that TALT is composed of a highly compartmentalized and organized lymphoid structure. In accordance with the finding that neonatal TALT developed $PNAd^+$ HEVs (Fig. 1 E), adult TALT HEVs expressed PNAd but not mucosal addressin cell adhesion molecule 1 (MAdCAM-1; Fig. 4 I and Fig. S4 A); this is similar to the case with NALT HEVs

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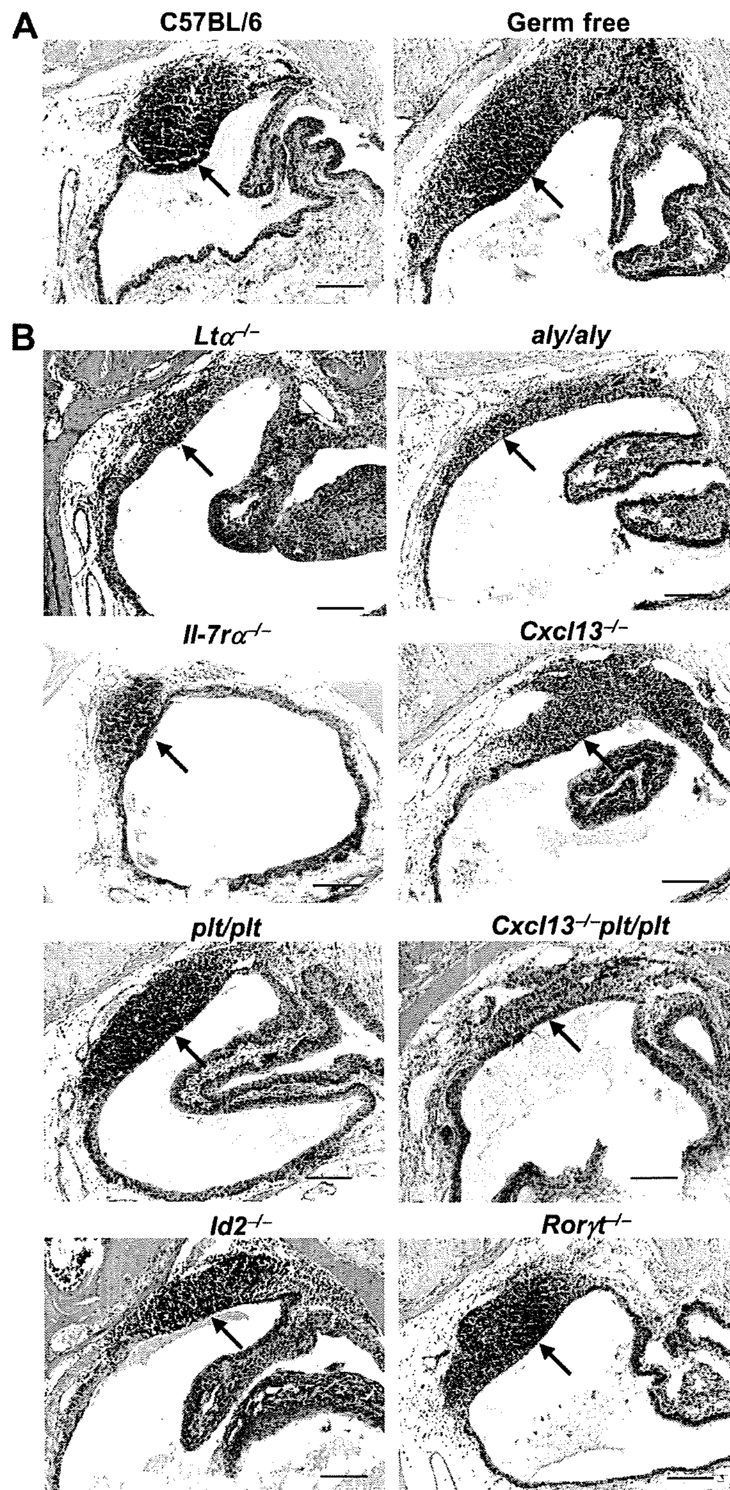

Figure 2. TALT genesis is independent of microbial stimulation and organogenesis-associated molecules. Paraffin-embedded tissue sections were analyzed by HE staining for TALT development. (A) The presence of TALT in germ-free mice, as well as C57BL/6 WT mice, shows that TALT develops independently of microbial stimulation (n = 3 mice/group). (B) Development of TALT in 8-wk-old $Lt\alpha^{-/-}$, *aly/aly*, $Il\text{-}7r\alpha^{-/-}$, $Cxcl13^{-/-}$, *plt/plt*, $Cxcl13^{-/-}plt/plt$, $Id2^{-/-}$, and $Ror\gamma t^{-/-}$ mice shows that the initiation of TALT development occurs independently of organogenesis-associated molecules. Arrows indicate the presence of TALT. These data are representative of at least three independent experiments per group (n = 5 mice/group). Bars, 100 µm.

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(Fig. 4 J and Fig. S4 B). These observations suggest that cellular trafficking to TALT and NALT is regulated via an L-selectin–PNAd interaction and is distinguishable from gut-trafficking mechanisms, which are dependent on α4β7 integrin/MAdCAM-1 (Kiyono and Fukuyama, 2004). These results indicate that TALT possesses many of the characteristic traits of organized MALT but is distinguished by the lack of goblet cells in its FAE region.

TALT is a site of immunological induction

To investigate the physiological function of TALT, we examined whether TALT takes up ocularly administered antigens. M cells, characterized by the M cell–specific mAb NKM16-2-4$^+$ (Nochi et al., 2007), *Ulex europaeus* agglutinin (UEA) 1$^+$, and wheat germ agglutinin$^-$, were found in the FAE of TALT (Fig. 5 A). Electron microscopic analysis showed that TALT-FAE contained cells bearing the hallmarks of M cells: microvilli and a unique pocket formation with lymphocytes (Fig. 5, B and C). When mice were ocularly dosed with GFP-expressing *Salmonella*, we observed the uptake of *Salmonella* by UEA-1$^+$ M cells (Fig. 5 D) as well as by CD11c$^+$ DCs (Fig. 5 E). Moreover, when mice were ocularly challenged with *Pseudomonas aeruginosa* PAO1, large amounts of *P. aeruginosa* PAO1 were located within the TALT (Fig. 5 F). *P. aeruginosa* PAO1 was not detected in naive mice (Fig. 5 G). Mice ocularly challenged with *P. aeruginosa* PAO1 formed germinal centers (GCs; Fig. 5 H); this is an important immunological event for the initiation of hypersomatic mutations and Ig class switching, which enable the production of memory B cells with high-affinity B cell receptors (Kelsoe, 1996; Shapiro-Shelef and Calame, 2005). In contrast, GC formation was not present in the TALT of naive mice (Fig. 5 I; and Fig. 6, A and B). These data suggest that TALT is a preferential site for the uptake of ocularly encountered antigens/pathogens and for the subsequent induction of antigen-specific B cell responses.

Supporting this notion, immunization of the eyes with eye drops containing cholera toxin (CT), a well-known mucosal immunogen, resulted in the formation of GCs with a follicular DC (FDC) network in the TALT (Fig. 6, C and D). Interestingly, ocular immunization also induced GC formation in NALT (Fig. 6, E and F), suggesting that the anatomical

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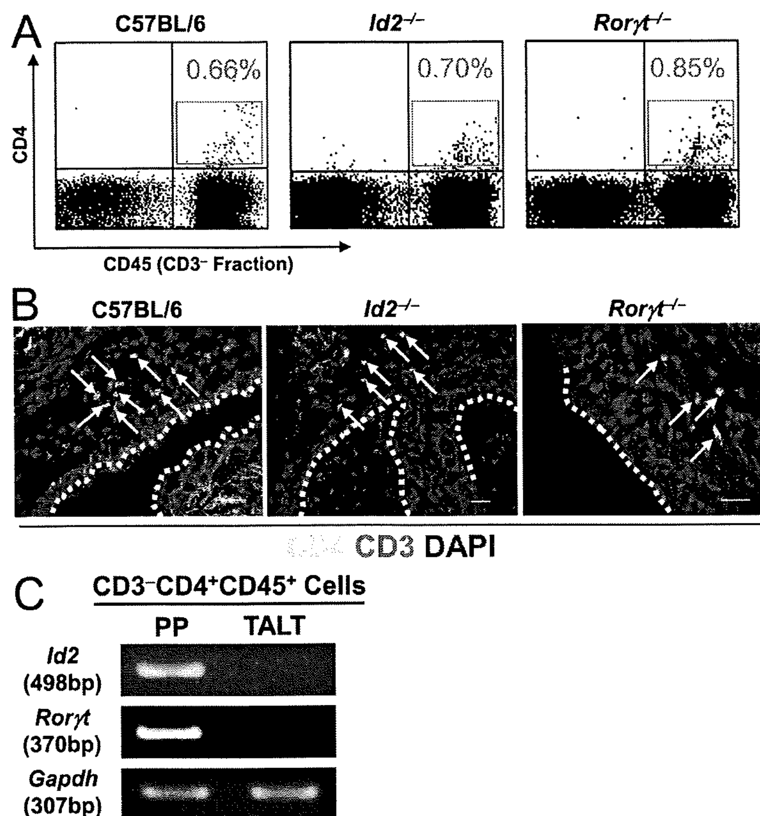

Figure 3. Presence of CD3$^-$CD4$^+$CD45$^+$ cells in the TALT anlagen. (A) Because substantial numbers of CD3$^-$CD4$^+$CD45$^+$ cells were noted in the TALT anlagen of D10 mice, we analyzed mononuclear cells from D10 tear ducts by FACS. Percentages of CD3$^-$CD4$^+$CD45$^+$ cells are shown in red (n = 6 mice/group). (B) Confocal microscopic analysis of the site of TALT genesis at D10. Frozen tissue samples were stained with the antibodies indicated. Arrows point to CD3$^-$CD4$^+$ cells (n = 6 mice/group). Dotted lines indicate the edge between the TALT epithelium and tear duct lumen. Bars, 50 µm. (C) CD3$^-$CD4$^+$CD45$^+$ cells from PP and TALT were isolated from an E17 intestine and D10 tear duct, respectively. Gene expression of *Id2* and *Rorγt* were analyzed by RT-PCR. The expression of *Gapdh* is shown as an internal control. These data are representative of at least three independent experiments (n = 18–20 mice/group).

connection through the tear duct lays the groundwork for a cooperative immunological network between TALT and NALT that responds to ocularly encountered antigens. Activation-induced cytidine deaminase (AID), an essential Ig class switch–related molecule, is expressed in organized lymphoid tissues, including NALT, PPs, and intestinal ILFs (Shikina et al., 2004). Parallel to our findings of GC formation after ocular immunization, confocal microscopy revealed the presence

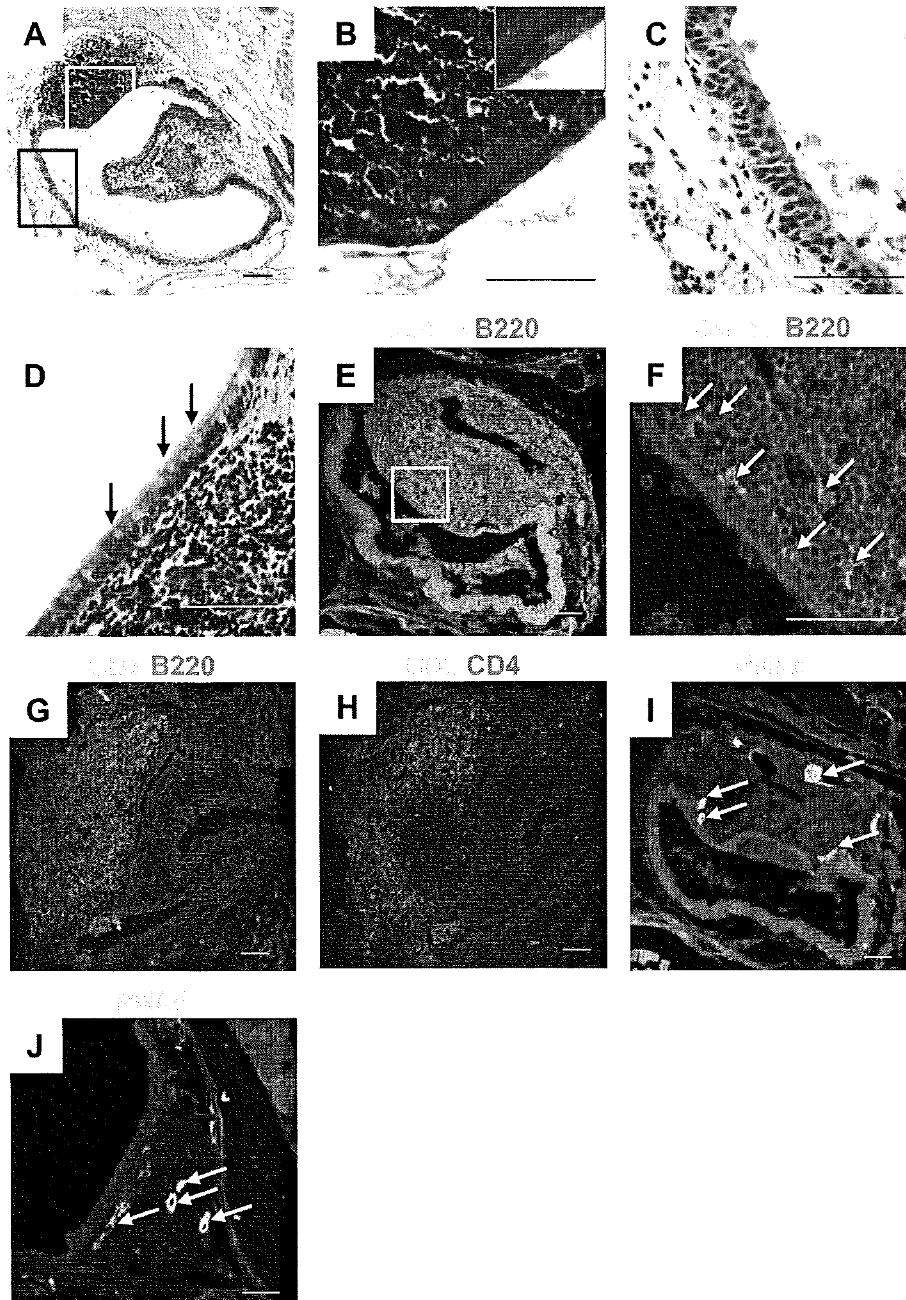

Figure 4. Microarchitecture of TALT. (A–C) The epithelial region of TALT in WT mice was analyzed by HE staining. B and C are magnifications, respectively, of the white and black boxes in A, and show TALT-FAE (B) and the lacrimal sac epithelium (C). The inset in B shows single-layered squamous epithelium in the TALT FAE (n = 3 mice/group). (D) The presence of goblet cells in NALT-FAE is indicated by arrows (n = 3 mice). (E–J) Confocal microscopic analysis of TALT (E–I) and NALT (J) in C57BL/6 WT mice. Tissue samples were stained with the antibodies indicated. F is a magnified view of the box in E; arrows point to the presence of CD11c$^+$ DCs. Arrows in I and J indicate PNAd$^+$ HEVs. These data are representative of at least three independent experiments (n = 3 mice/group). Bars, 50 µm.

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