本研究では、主に実験用動物ならびに分離した細胞、細胞株を使用して実験を行った。実験動物使用にあたっては、独立行政法人国立大学実験動物施設協議会指針に基づき、島根大学医学部動物実験施設の管理下で実験を行った。

C. 研究結果

1. アレルギー性鼻炎に対する抗原特異的免疫療法としての舌下免疫療法

OVA の舌下免疫療法モデルにおいて、舌下免疫をアレルギーの誘導相の前（感作前）もしくは誘導相と反応相の間（感作後）に行った系では、PBS のみを舌下投与したコントロール群と比較してそれぞれ血清中の抗原特異的 IgE 値の有意な減少が認められたが、舌下免疫を反応相の後（発症後）に行った系では、OVA 投与群とコントロール群との間に IgE 値に有意な差は認められなかった。感作前 OVA 舌下投与群において、脾臓および頚部リンパ節由来のリンパ球からの Th2 サイトカイン産生がコントロール群と比較して有意に抑制された。感作前 OVA 舌下投与群の頚部リンパ節において、CD4 陽性 CD25 陽性制御性 T 細胞の数や頻度にはコントロール群と比較して有意な差を認めなかったが、Foxp3 や IL-10 の mRNA の有意な発現上昇が認められた。これらの結果から、アレルギー性鼻炎の舌下免疫療法において、頚部リンパ節における制御性 T 細胞や抑制性サイトカインがアレルギー反応の抑制に関与している可能性が示唆された。

2. マウスアレルギー性鼻炎モデルにおける IL-15 の役割

IL-15 は粘膜免疫に関与する細胞群の増殖維持因子として重要な役割を果たしている。IL-15 あるいは IL-15Rα の遺伝子欠損マウスでは、腸管上皮間・・型 T 細胞、NK、NKT 細胞及びメモリー$CD8^+$T 細胞が減少しており、さらに IL-15 遺伝子導入マウスでは、メモリー$CD8^+$T 細胞の増加、Tc1 反応を介しての気道アレルギー性炎症の抑制が報告されている。さらに、IL-15 は肥満細胞の増殖活性化因子として知られており、本研究では、粘膜面でのアレルギー反応における IL-15 の役割を調べるため、IL-15 ノックアウト（KO）マウスと野生型マウスのマウスアレルギー性鼻炎について比較検討した。

その結果、OVA 感作後の IL-15KO マウスにおける OVA 特異的 IgE 量及び脾臓における Th1/Th2 応答は、野生型マウスと比較して有意差はなかった。感作マウスにおける OVA 点鼻後の症状は、IL-15KO マウスで増悪しており、鼻粘膜への好酸球浸潤も亢進していた。野生型マウス骨髄由来肥満細胞（BMMC）と IL-15KO マウス由来 BMMC では、FcεR 及び CD117 の発現に差は認められなかったが、IL-15KO マウス由来 BMMC で脱顆粒率が高く、リコンビナント IL-15 を添加することで野生型マウスおよび IL-15KO 由来いずれの BMMC でも脱顆粒が抑制された。

更に OVA で感作した野生型マウスに OVA と共にリコンビナント IL-15 を点鼻投与したところ、症状および鼻粘膜への好酸球浸潤が抑制された結果より、IL-15 は鼻粘膜局所の実効相における Th2 反応を抑制することにより、アレルギー反応を制御しているものと考えられた。さらに、IL-15 は肥満細胞の脱顆粒を抑制することにより、鼻アレルギー症状を制御している可能性も示唆された。

D. 考　　察

1．マウスを用いてアレルギー性鼻炎に対する舌下免疫療法の動物モデルを作製した。OVA をアレルギー性鼻炎の発症前（感作前もしくは感作後）に舌下投与することでアレルギー反応が抑制され、舌下免疫療法の動物モデルとして有用であ

ることが示された。今後、ヒトでのアレルギー性鼻炎（特に花粉症）の病態により近付けた舌下免疫療法モデルの作製を目指し、抗原の種類や投与プロトコールなどにさらなる検討が必要である。

また、アレルギー性鼻炎の舌下免疫療法のメカニズムとして、頚部リンパ節における制御性T細胞や抑制性サイトカインがアレルギー反応の抑制に関与している可能性が示唆された。アレルギー性鼻炎の舌下免疫療法における制御性T細胞の役割、作用メカニズムについて更なる詳細な解析を進めるとともに、ヒトの検体を用いてその裏付けを行う必要があると考えられる。

2. IL-15は、鼻粘膜局所の実効相におけるTh2反応を抑制することにより、アレルギー反応を制御しているものと考えられた。更にIL-15は肥満細胞の脱顆粒を抑制することにより、鼻アレルギー症状を制御しているものと考えられた。rIL-15の点鼻投与は、アレルギー性鼻炎の治療のひとつとなりうるものと考えられた。

E. 結　論

アレルギー性鼻炎に対する舌下免疫療法の有用な動物モデルを作製した。アレルギー性鼻炎の舌下免疫療法において、頚部リンパ節における制御性T細胞や抑制性サイトカインがアレルギー反応の抑制に関与している可能性が示唆された。また、サイトカイン療法のひとつとして、rIL-15の点鼻投与は、アレルギー性鼻炎の治療のひとつとなりうるものと考えられた。

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
   なし
2. 実用新案登録
   なし
3. その他
   なし

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

摂取食餌量が気道炎症に及ぼす影響に関する研究

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研究要旨
食餌量の制限がアレルギー発症に及ぼす影響を調べるために、自由摂取群のマウスと食餌量制限マウスに気道炎症を誘導した。その結果、摂取食餌量を 60%に制限すると肺胞洗浄液に浸潤する好酸球が有意に減少し血清 IgE の上昇が抑えられた。食餌量制限によるアレルギー発症の抑制は 7 週齢、18 週齢のいずれのマウスでもみられ、末梢血中のアレルゲン特異的 IL-4 産生 T 細胞の頻度が食餌量制限により有意に低下した。

A. 研究目的

アレルギー反応は、特定の外来抗原に対する IgE 抗体の増加や、外来抗原を認識して T 細胞が産生するサイトカイン産生バランスの異常により引き起こされる。T 細胞のサイトカイン産生応答は、健常児では出生直後は Th2 側にシフトしているが、生後腸内フローラの定着に伴って Th1 側へシフトする。一方、アレルギー児では Th2 サイトカイン産生応答が優位な状態にあり、アレルギー児と健常児の腸内フローラに違いが見られることから、腸内フローラの異常が Th1 側へのシフトを遅らせてアレルギー発症リスクを高める可能性が提示されている。

これまでに、動物モデルで食餌量の制限が癌や自己免疫疾患などの発症を抑えることが知られている。本研究では、食餌量の制限によるアレルギー発症予防の可能性を追究するために、マウスモデルを用いて摂取食餌量の制限が免疫機能や気道炎症の誘導に及ぼす影響を検討した。

B. 研究方法

BALB/c マウス（雄、7 週齢および 18 週齢）に、合成飼料（AIN-76）を自由に、または自由摂取量の 60%、80%に制限して与え、4 週間飼育した。常法に従い、卵白アルブミン（OVA）を用いて気道炎症反応を誘発した。炎症反応を評価するために、肺胞気管洗浄液（BALF）および血清を回収し、アレルギー関連パラメーター（好酸球の集積、IL-4 レベル、OVA 特異的 IgE レベル）を測定した。免疫応答バランスへの影響を解析するために、末梢血単核細胞を調製し、抗 CD3 抗体刺激による T 細胞の IFN-γ/IL-4 産生応答および OVA 特異的 IL-4 産生 T 細胞の頻度を解析した。細胞性免疫応答への影響を解析するために脾臓細胞の NK 活性を測定した。食餌量制限下で 4 週間飼育後糞便を回収し、培養法で腸内フローラの解析を行った。

(倫理面への配慮)
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C. 研究結果

食餌量を自由摂取量の60%に制限することにより、末梢血単核細胞に占める B 細胞の割合が減少し T 細胞の割合が増加した。また、T 細胞を抗 CD3 抗体で刺激

したところ、食餌量制限により IFN-γ産生が減少し IL-4 産生が増加した。

次に、食餌量の制限がアレルギー発症に及ぼす影響を調べるために、7 週齢のマウスを OVA で免疫後再度 OVA を経鼻投与して気道炎症を誘発した。その結果、食餌量を 80%および 60%に制限すると、制限の程度に依存して OVA の経鼻投与で誘発される気道炎症反応が改善し（BALF に回収される好酸球の減少、血清中 IgE レベルの低下）、BALF 中の IL-4 レベルの低下が観察された。

食餌量制限によるアレルギー発症の抑制に及ぼす加齢の影響を探るために、同様な実験を 18 週齢のマウスで行った。その結果、摂取食餌量を60%に制限すると、18週齢のマウスでも同様にアレルギー症状の抑制が認められた。

アレルギー抑制の機序を調べるために、自由摂取マウスおよび食餌量制限マウスの末梢血から T 細胞を精製し、抗原提示細胞の存在下で、OVA で刺激した。その結果、食餌量制限により OVA 特異的 IL-4 産生が低下し、OVA 特異的 IL-4 産生 T 細胞の頻度が食餌量制限により減少することがわかった。

食餌量制限の副作用を検討するために脾臓の NK 活性に及ぼす影響を調べた。その結果、7 週齢のマウスでは食餌量制限により脾臓 NK 活性が有意に低下したが、18 週齢のマウスでは NK 活性の低下は見られなかった。また、7 週齢のマウスでは食餌量制限により一過性に血便が観察されたが、18 週齢のマウスでは血便は見られなかった。

最後に、食餌量制限が腸内フローラに及ぼす影響を調べたところ、7 週齢のマウスでは食餌量を60%に制限することにより *Lactobacillus* 属の低下が見られたが、18 週齢のマウスではこれらの変化は見られなかった。

D. 考　察

マウスモデルを用いて、食餌量の制限が気道炎症を軽減することを示した。近年、臨床試験で摂取カロリーの制限がアレルギー症状を改善することが報告されており、本研究の結果はそれらの結果を支持している。しかしながら、若齢期の食餌量制限は腸内フローラを不安定化し、腸管バリアーを脆弱化するリスクがある。今後は、食餌量の制御と腸内フローラを安定化する手法を組み合わせることにより、安全で有効なアレルギー予防法を検討する必要がある。

E. 結　論

食餌量の制限がアレルギー発症を予防する可能性が示された。作用機序の一つとして、食餌量の制限がアレルゲン特異的 Th2 細胞クローンの過剰な増殖を抑え、アレルゲンによる IL-4 産生誘導を抑えることが考えられた。

F. 健康危機情報

特記事項なし。

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

1)インターロイキン 12 産生促進剤（特願 2007-331913、平成 19 年 12 月 25 日出願）

2. 実用新案登録

特記事項なし。

3. その他

特記事項なし。

Ⅳ. 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 平成20年度(2008)

〈雑誌〉

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Ⅴ．研究成果の刊行物・別冊
（主なもの）

Colonic Patches Direct the Cross-Talk Between Systemic Compartments and Large Intestine Independently of Innate Immunity[1]

Sun-Young Chang,* Hye-Ran Cha,* Satoshi Uematsu,† Shizuo Akira,† Osamu Igarashi,‡ Hiroshi Kiyono,‡ and Mi-Na Kweon[2]*

Although the mucosal and the systemic immune compartments are structurally and functionally independent, they engage in cross-talk under specific conditions. To investigate this cross-talk, we vaccinated mice with tetanus toxoid together with cholera toxin with s.c. priming followed by intrarectal (IR) boosting. Interestingly, higher numbers of Ag-specific IgA and IgG Ab-secreting cells (ASCs) were detected in the lamina propria of the large intestine of mice vaccinated s.c.-IR. Ag-specific ASCs from the colon migrated to SDF-1α/CXCL12 and mucosae-associated epithelial chemokine/CCL28, suggesting that $CXCR4^+$ and/or $CCR10^+$ IgA ASCs found in the large intestine after s.c.-IR are of systemic origin. In the colonic patches-null mice, IgA ASCs in the large intestine were completely depleted. Furthermore, the accumulation of IgA ASCs in the colonic patches by inhibition of their migration with FTY720 revealed that colonic patches are the IgA class-switching site after s.c.-IR. Most interestingly, s.c.-IR induced numbers of Ag-specific IgA ASCs in the large intestine of $TLR2^{-/-}$, $TLR4^{-/-}$, $MyD88^{-/-}$, and $TRIF^{-/-}$ mice that were comparable with those of wild-type mice. Taken together, our results suggest the possibility that cross-talk could occur between the large intestine and the systemic immune compartments via the colonic patches without the assistance of innate immunity. *The Journal of Immunology,* **2008, 180: 1609–1618.**

The possibility of developing safe and effective mucosal vaccines against a variety of microbial pathogens has aroused great interest because such vaccines would provide two layers of host protection—at mucosal surfaces and in systemic compartments (1). To realize the potential benefits of mucosal vaccines, a number of novel vaccination routes and adjuvants have been proposed but many of these have been challenged (2). Parenteral immunization is generally thought not to induce significant immune responses at mucosal surfaces. For instance, systemic vaccination with vaccinia induces cellular and humoral immune responses in systemic sites but not in mucosal sites. However, vaccination via mucosal routes can elicit both systemic and mucosal immunity (3). Mucosal vaccination has sometimes been used to overcome the barrier to recombinant viral vector immunization caused by preexisting same viral immunity (4). Therefore, an effective mucosal vaccine must be able to overcome preexisting immunity in systemic tissues that can provoke immune tolerance and/or hypersensitivity.

Sensitized T and B cells leave the mucosal inductive sites (e.g., Peyer's patches (PPs)[3] and nasopharynx-associated lymphoid tissue) transit through the thoracic duct and enter the circulation before settling down in selected mucosal tissues where they differentiate into memory/effector or plasma cells (5, 6). Recent studies (7) demonstrate that murine dendritic cells (DCs) isolated from PPs, but not from spleen and peripheral lymph nodes (LNs), increase the expression of mucosal homing receptors (e.g., $\alpha4\beta7$ and CCR9). Furthermore, retinoic acid, specifically secreted by mucosal DCs, regulates the imprinting of mucosal DCs including DCs from PPs and mesenteric LNs (MLNs) for gut T (8) and B cell homing (9). Thus, these results may explain how the "common mucosal immune system" is compartmentalized and restricted within mucosal immune tissues.

However, results obtained by our own group and others have revealed the possibility of cross-talk between systemic compartments and some mucosal tissues. For instance, the combined systemic prime/mucosal boost strategy for the induction of both systemic and mucosal immune responses has been used to develop an efficient vaccination regimen (10–12). In addition, intrarectal (IR) immunization following systemic priming results in increases in both systemic and mucosal CTL responses, although systemic immunization alone could induce systemic but not mucosal CTL responses (13). In the murine allergic diarrhea model, systemically primed Ag-specific allergic responses selectively occur in the large but not the small intestine of BALB/c mice (14). Adoptive transfer of Ag-primed splenic $CD4^+$ cells obtained from GFP transgenic donor mice preferentially migrate into the large intestine. In addition, systemically primed Ag-specific $CD4^+$ T cells containing both $CD45RB^{high}$ and $CD45RB^{low}$ populations act as a pathogenic subset, in turn, leading to selective inflammatory responses in the large intestine (15). These results together with those of other studies suggest that a unique and important immunological cross-talk system exists between systemic compartments such as the spleen and the

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[3] Abbreviations used in this paper: PP, Peyer's patch; IR, intrarectal; CT, cholera toxin; TT, tetanus toxoid; ASC, Ab-secreting cells; CP, colonic patch; DC, dendritic cell; MNC, mononuclear cell; LN, lymph node; MLN, mesenteric lymph node; CLN, cutaneous LN; ILN, iliac lymph node; pIgR, polyimmunoglobulin receptor; TCI, transcutaneous; CSR, class switching recombination; AID, activation-induced cytidine deaminase; ILN, iliac LN; MEC, mucosae-associated epithelial chemokine.

large intestine. We have previously offered several hypotheses to explain the phenomenon as, for example, the differences in the immune environments and microenvironments and/or existence of a specific cross-talk immune pathway. However, the exact underlying mechanism has yet to be elucidated.

To clarify the cross-talk interaction between mucosal and systemic compartments, we used a simple regimen of s.c. immunization followed by an IR boost. Interestingly, this regimen dramatically increased the number of Ag-specific IgA Ab-secreting cells (ASC) expressing functional CXCR4 and CCR10, thought to be systemically derived, in the large intestine. In contrast, two IR immunizations induced IgA ASC expressing CCR10 alone. These responses depended on colonic patches (CP), one of the major inductive sites in the large intestine, and seemed to be independent of innate immunity. Based on these results, we concluded that s.c. priming could modulate immune responses if followed by IR immunization, suggesting that the mucosal compartment, especially the large intestine, long thought to be separate from the systemic immunological compartment, is instead in close communication with it.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Orient). Timed pregnant C57BL/6 mice were purchased from the Daehan Biolink. To generate both PP- and CP-null mice or PP-null but CP-intact mice, pregnant C57BL/6 or BALB/c mice were injected i.v. with 600 μg of anti-IL-7Rα mAb on gestational day 14 (16). The structure of CP is circular and its center forms a protruding configuration giving it the appearance of a dome (17). Naive C57BL/6 mice possess 3 or 4 CP in the large intestine and are completely depleted by in utero treatment with anti-IL-7R mAb (provided by Dr. Masafumi Yamamoto, Nihon University at Matsudo, Chiba, Japan). The polyimmunoglobulin receptor (pIgR)$^{-/-}$ mice (18) was provided by Dr. Masanobu Nanno (Yakult Central Institute for Microbiological Research, Tokyo, Japan) and the OVA epitope (323–339)-specific TCR transgenic (DO11.10) mice was provided by Dr. Kazuhiko Yamamoto (University of Tokyo, Tokyo, Japan). All mice were maintained under pathogen-free conditions in the experimental facility at the International Vaccine Institute (Seoul, Korea), where they received sterilized food and water ad libitum.

Immunization

Mice were injected s.c. into the shoulder region and by i.p. routes with 10 μg of tetanus toxoid (TT) plus 1 μg of cholera toxin (CT; List Biological Laboratories; Campbell, CA) or by the transcutaneous (TCI) route with 100 μg of TT plus 50 μg of CT. For IR immunization, we anesthetized mice with a ketamine-xylazine mixture before administration of a 40-μl vaccine solution containing 10 μg of TT plus 2 μg of CT using disposable polystyrene tubing for oral feeding. For direct injection into the lumen of the small intestine, mouse abdomens were surgically opened, and the same volume and amount of TT plus CT as used in IR immunization was injected above the upper jejunum lumen with a fine needle. TT was provided by Dr. Yasushi Higashi (Biken Foundation, Osaka University, Osaka, Japan).

ELISA and ELISPOT for assessment of Ag-specific ASCs

We used serum and fecal extracts to determine Ag-specific Ab titers by ELISA as described elsewhere (19). Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution giving an OD at 450 nm of 0.1 greater than background. Mononuclear cells (MNCs) were obtained from the spleen, LN, and intestinal lamina propria as previously described (20). MNCs from the lamina propria of the small and large intestine were dissociated by digestion using a collagenase/DNase I enzyme solution after removal of PPs. Cells were then enriched by a discontinuous density gradient containing 40 and 75% Percoll (Amersham Biosciences). The number of total or Ag-specific ASCs was measured by an ELISPOT assay in accord with an established protocol (21). ASCs were counted with the aid of a stereomicroscope (SZ2-ILST; Olympus, Tokyo, Japan).

Chemotaxis assay

To evaluate the expression of chemokine receptors on Ag-specific ASCs, a chemotaxis assay and ELISPOT were combined. MNCs were isolated from the lamina propria of the large intestine and subjected to a chemotaxis assay (22). In brief, 5-μm Transwell inserts (Corning Costar) containing 1×10^6 MNCs were placed in the 24-well plate so as to make contact with 600 μl of the medium alone (basal) or with one of the following chemokines as well: 100 nM of SDF-1α/CXCL12 (R&D Systems), 100 nM of MIP-3α/CCL20 (R&D Systems), 100 nM of SLC/CCL21 (PeproTech), 300 nM of TECK/CCL25 (R&D Systems), 100 nM of CTACK/CCL27 (PeproTech), or 250 nM of mucosae-associated epithelial chemokine/CCL28 (R & D Systems). We did a preliminary experiment to determine the optimal concentration of different chemokines using MNCs from splenocytes and lamina propria of the small intestine. Two hours later, inserts were removed and the population that had migrated to the bottom wells was added into the wells of ELISPOT plates to measure the number of Ag-specific ASCs.

The proliferation of OVA-specific TCR transgenic CD4$^+$ T cells

We isolated MNCs from the spleen and LNs of DO11.10 mice, labeled the CD4$^+$KJ1.26$^+$ T cells with 10 μM of CFSE (Molecular Probes) for 15 min in RPMI at 37°C and washed the cells several times in PBS. In all 5×10^6 CD4$^+$KJ1.26$^+$ T cells were transferred into sex-matched naive or s.c.-primed BALB/c mice via the tail vein. One day after adoptive transfer when transferred CD4$^+$ T cells were circulated and distributed throughout the body, naive or s.c.-primed recipient mice were vaccinated IR with a mixture of 200 μg of OVA and 2 μg of CT or s.c. with a mixture of 20 μg of OVA and 1 μg of CT. The CFSE dilution in the MNCs of each tissue was analyzed after staining with anti-KJ1.26-PE (BD Pharmingen) (53-6.7) mAbs specific to DO11.10 clonotypic TCR in accord with the manufacturer's instructions.

CT-I-A^b tetramer staining

To detect CT-specific TCR-expressing CD4$^+$ T cells, CT-I-A^b tetramers were formed by incubation of I-A^b monomers and streptavidin-PE (Molecular Probes) with a molecular ratio of 5:1 for 2 h at 37°C. CT-I-A^b tetramers were incubated with cells for 2.5 h at 37°C in a CO_2 incubator. Then, the cells were stained with anti-CD4-APC. The data were obtained using FACSCalibur (BD Biosciences) with CellQuest (BD Biosciences), and the profiles were analyzed using FlowJo software (Tree Star).

RT-PCR

MNCs isolated from the colon lamina propria were sorted as B220$^-$IgA$^+$ and B220$^+$IgA$^+$ cells by FACSAria Cell Sorter (BD Bioscience). Total RNA was extracted from mouse tissues using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized by Superscript II reverse transcriptase (Invitrogen) with oligo(dT) primer (Invitrogen). Activation-induced cytidine deaminase (AID), αCT, Iμ-Cα, and β-actin were amplified as described previously (23, 24). To check CCR10 mRNA expression, murine CCR10-specific primer (forward 5'-AGAGCTCTGTTACAAGGCTGATGTC-3' and reverse 5'-CAGGTGG TACTTCCTAGATTCCAGC-3') was used.

FTY 720 treatment

To induce lymphocyte retention in secondary lymphoid organs, we administered mice i.p. with FTY 720 (1 mg/kg body weight; Cayman Chemicals) every other day (25). The effect of FTY 720 treatment was monitored by regular analysis of peripheral blood lymphocyte and tissue lymphocyte counts.

Statistics

Data are expressed as the mean $\pm$ SD. Statistical comparison between experimental groups was performed using ANOVA or Student's t test.

Results

Dramatic increase in Ag-specific IgA and IgG ASCs in the large intestine after s.c.-IR immunization

As the first step in investigating the relation between systemic and mucosal immune systems, C57BL/6 mice were immunized by the IR route two weeks after s.c. immunization (s.c.-IR) with TT and CT as adjuvant. As expected, mice vaccinated by s.c.-s.c. (Fig. 1*a*) and s.c. or IR (data not shown) did not have significant numbers of Ag-specific ASCs in the small and large intestines; however, Ag-specific IgG ASCs were detected in the spleen of s.c.-immunized mice although in low levels because IgG ASCs migrate to the effective site or bone marrow rather than reside in spleen once they switch class from IgM to IgG (26) (Fig. 1*a*). Interestingly, however, brisk numbers of TT-specific IgA and IgG ASCs were detected in the lamina propria of the large intestine after s.c.-IR at levels similar to those induced by IR-IR immunization (Fig. 1*a*). Dramatic increases in the induction of IgA

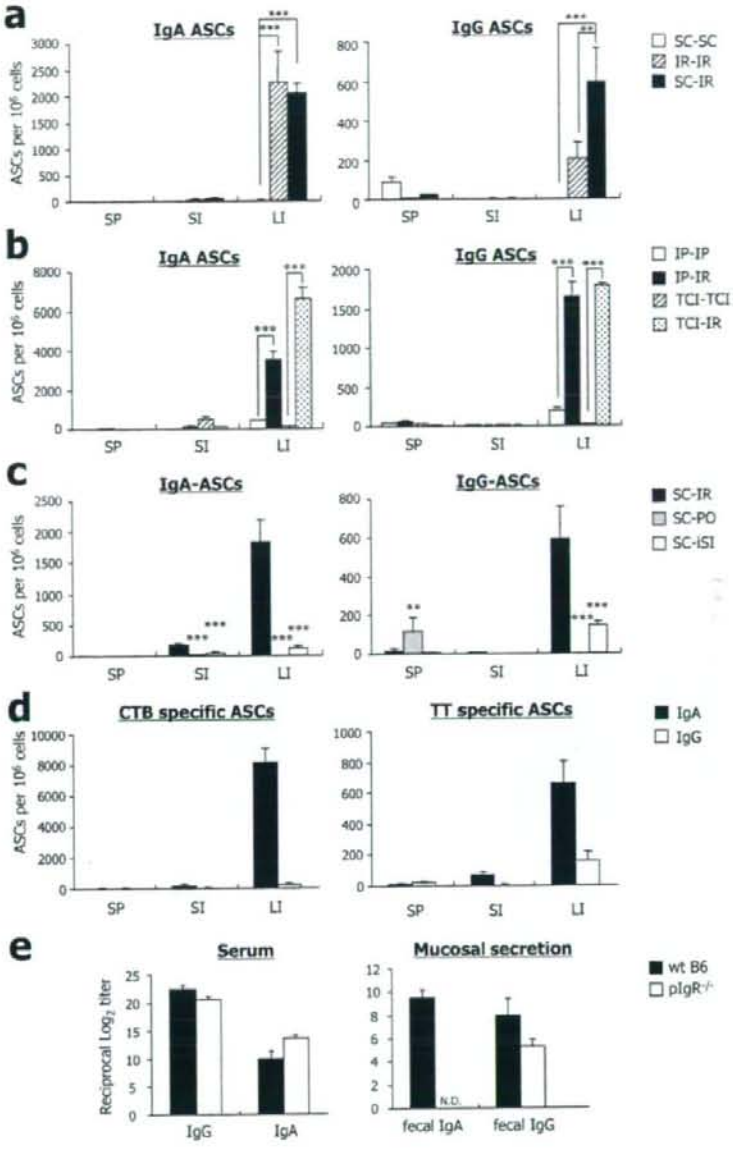

FIGURE 1. High numbers of Ag-specific IgA and IgG ASCs in the mouse large intestine are induced by s.c.-IR immunization. Mice received IR boosting following s.c. (*a*) or i.p. or TCI priming (*b*) with TT plus CT. Seven days after boosting, MNCs isolated from the spleen (SP) and lamina propria of the small (SI) and large intestine (LI) of the immunized mice were prepared. TT-specific ASCs per 10^6 cells were determined by ELISPOT. ***, $p < 0.001$ by ANOVA test (*a*) or Student's *t* test (*b*). *c*, Mice received IR or oral boosting (PO) following s.c. priming with TT plus CT. For another group of s.c. primed mice, the same dose of TT plus CT as used in the IR immunization was injected into the upper jejunum lumen of the small intestine (s.c.-iSI) to exclude the chance of inactivation of protein Ag by the acidic conditions and enzyme digestion of the stomach. **, $p < 0.01$ and ***, $p < 0.001$ by ANOVA test. *d*, Mice received IR boosting 100 days following s.c. priming to check the mucosal boosting effects on the long-term memory phase of systemic immunity. CTB- and TT-specific ASCs per 10^6 cells were determined using ELISPOT. *e*, Wild-type C57BL/6 and $pIgR^{-/-}$ mice receiving s.c.-IR immunization were used to determine the secretion of Ag-specific polymeric secretory IgA Abs into the gut lumen. TT-specific IgG or IgA Ab responses were determined in the sera and fecal extracts by ELISA.

and IgG ASCs in the large intestine against the B subunit of CT (CT-B) used as an adjuvant were also noted (data not shown). In addition, these highly enhanced responses were obtained regardless of mouse strain (data not shown). Ag-specific IgA Ab was also greatly increased in vaginal and nasal wash samples and in saliva and fecal extracts after s.c.-IR vaccination with TT plus CT (data not shown). These results strongly suggest that s.c.-IR combine effects not only in the colon but also in other mucosal compartments. To determine whether similar responses could be obtained by other routes of systemic priming combined with IR boosting, mice were boosted with IR immunization following i.p. or TCI immunization (i.p.-IR, TCI-IR) (Fig. 1*b*). The i.p.-IR and TCI-IR regimens induced large numbers of Ag-specific IgA and IgG ASCs in the large intestine, suggesting systemic priming was not limited to the s.c. route. However, when s.c.-primed animals were boosted orally (s.c.-PO), few TT-specific IgA and IgG ASCs were induced in the gut, demonstrating that the large but not the small intestine is the mucosal site interconnected with the systemic immune system (Fig. 1*c*). To confirm definitively the low responsiveness of small intestine-targeting immunization following systemic priming by bypassing the digestion and acidic conditions of the stomach, s.c.-primed mice were injected in the upper jejunum lumen of the small intestine (s.c.-iSI) with the same dose of TT plus CT used for IR immunization. This direct injection into the lumen of the small intestine induced a small amount of Ag-specific IgA and IgG ASCs in the small intestine and induced few Ag-specific IgA and IgG ASCs in the large

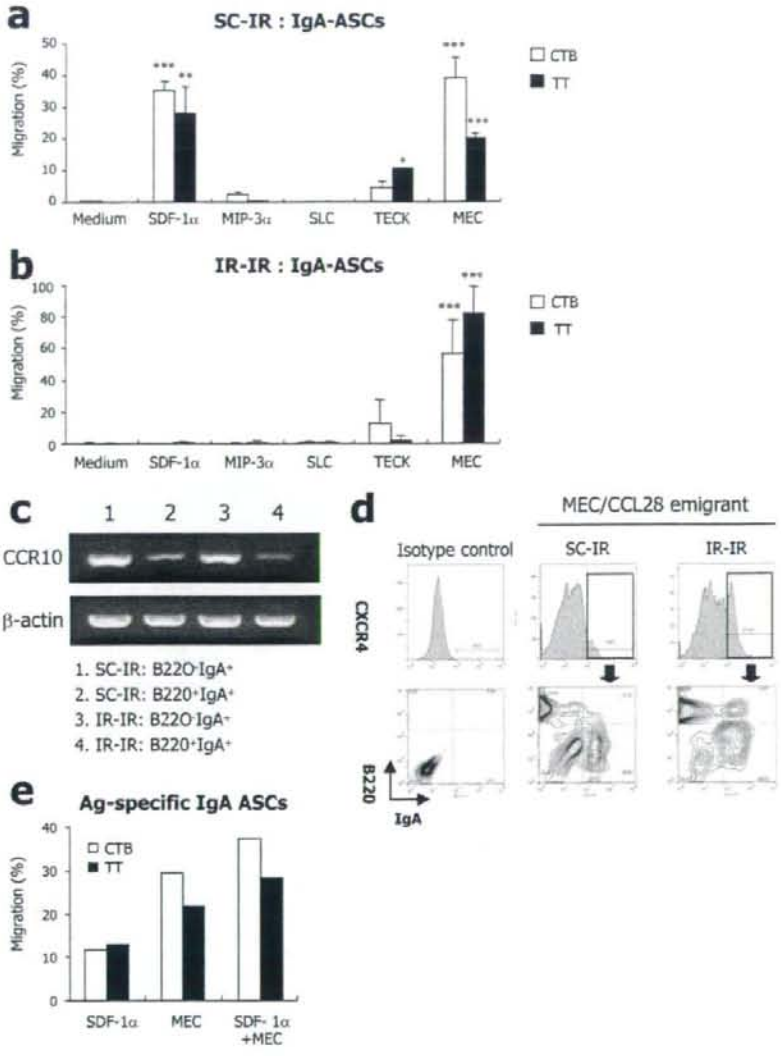

FIGURE 2. SDF-1α and MEC attracted Ag-specific IgA ASCs of the large intestine after s.c.-IR immunization. Each chemokine was added to the lower chamber and MNCs isolated from the lamina propria of the large intestine following s.c.-IR (*a*) or IR-IR immunization (*b*) were applied to the upper chamber well. CTB-specific and TT-specific IgA ASCs were determined using an ELISPOT assay to analyze the cells that had migrated into the lower chamber. The data represent the percentage of Ag-specific IgA ASCs that migrated into each chemokine relative to the total Ag-specific IgA ASCs. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ by ANOVA test. *c*, MNCs isolated from the colon lamina propria at day 7 after s.c.-IR or IR-IR were sorted as two population of $B220^-IgA^+$ and $B220^+IgA^+$ cells. CCR10 mRNA expression was determined by RT-PCR using CCR10-specific primer. β-actin was used as a control. *d*, MNCs from colon lamina propria after s.c.-IR or IR-IR were used for chemotaxis assay with a Transwell system. Cells that migrated to MEC/CCL28 were stained with anti-IgA, anti-B220, and anti-CXCR4 Ab to evaluate the CXCR4 expression on $CCR10^+$ IgA ASCs. *e*, MNCs isolated from colon lamina propria after s.c.-IR were applied to chemotaxis assay by a mixture of SDF-α and MEC and then CTB-specific and TT-specific IgA ASCs were determined using an ELISPOT assay to analyze the cells that had migrated into the lower chamber.

intestine, but not enough to be statistically significant (Fig. 1*c*). To assess the stress of surgery, we did sham surgery with PBS in s.c.-IR immunized mice. As expected, there were no significant changes in the numbers of Ag-specific IgA and IgG ASCs in the large intestine after sham surgery when compared with s.c.-IR mice that did not have surgery (data not shown).

To evaluate whether IR boosting affects the memory phase of immunity after systemic priming and induces a dramatic increase in Ag-specific IgA ASCs in the large intestine, mice received IR boosting 100 days after s.c. priming. Brisk numbers of CTB- and TT-specific IgA and IgG ASCs in the lamina propria of the large intestine were induced by IR boosting of the memory phase of systemic immunization (Fig. 1*d*). To further determine whether the IgA ASCs in the large intestine after s.c.-IR could secrete the dimeric form of IgA Abs associated with the secretory component into the gut lumen, IgA Ab responses after s.c.-IR were analyzed in $pIgR^{-/-}$ mice lacking this IgA secretion pathway (18). No IgA Abs were found in the fecal extracts of these mice, showing that Ag-specific IgA ASCs in the large intestine after s.c.-IR are able to secrete polymeric IgA into the lumen of the colon (Fig. 1*e*). As expected, no impairment in the Ag-specific IgG Ab responses was noted in the fecal extract of $pIgR^{-/-}$ mice, because the secretion of IgG Abs was regulated by the neonatal Fc receptor but not by pIgR (27). All of these data suggest that IR boosting during the effector and memory phases of systemic priming could induce a dramatic increase in Ag-specific IgA and IgG ASCs, which secrete soluble Abs into the lumen in the lamina propria of the large intestine.

CXCR4- and CCR10-expressing IgA ASCs were detected in the large intestine after s.c.-IR immunization

Because IR-IR immunization can induce numerous Ag-specific IgA ASCs in the colon (Fig. 1*a*), we wondered whether Ag-specific IgA ASCs induced by s.c.-IR differed from those induced by IR-IR. If so, IgA ASCs induced by s.c.-IR might originate from or

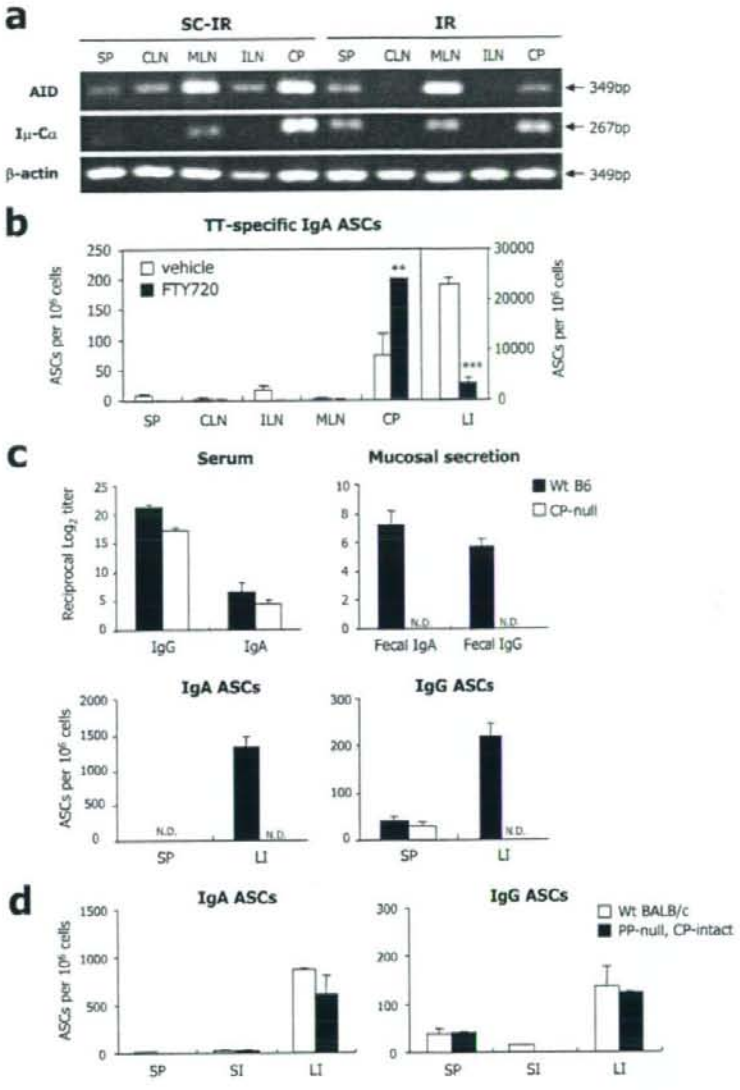

FIGURE 3. CPs are essential for IgA class switching after s.c.-IR immunization. *a*, Expression of a series of IgA isotype CSR-associated mRNA including AID and Iμ-Cα was determined in the organized lymphoid tissues of spleen, CLN, MLN, and ILN, and CP at day 7 after s.c.-IR or IR immunization alone using RT-PCR. β-actin was used as a cDNA control. *b*, Mice were treated with FTY 720 every other day beginning 1 day before IR immunization. The numbers of TT-specific IgA ASCs in the spleen, CLN, ILN, MLN, CP, and lamina propria of the large intestine were measured at day 7 after s.c.-IR immunization. **, $p < 0.01$, ***, $p < 0.001$ compared with the vehicle group (Student's *t* test). *c*, TT-specific Ab responses in the serum and fecal extract, and TT-specific IgA and IgG ASCs in the MNCs isolated from spleen and large intestine were evaluated in the CP-null C57BL/6 progeny treated with anti-IL-7Rα mAb in utero at day 7 following s.c.-IR immunization. N.D.; Not detected. *d*, TT-specific IgA and IgG ASCs were evaluated in the PP-null and CP-intact BALB/c progeny treated with anti-IL-7Rα mAb in utero following s.c.-IR.

be affected by systemic derived cells. We evaluated the migratory characteristics of Ag-specific IgA ASCs induced by s.c.-IR or IR-IR immunization by testing the chemokine responsiveness of these cells using a Transwell chemotaxis assay. As expected on the basis of earlier findings that MEC expression predominates in the colon (28), we found that Ag-specific IgA ASCs elicited by an IR-IR regime migrated principally toward MEC/CCL28 (~70–80%) and, to a much lesser degree, toward TECK/CCL25 (5–10%) (Fig. 2*b*). Surprisingly, similar numbers of Ag-specific IgA ASCs (30–40%) induced by an s.c.-IR regime migrated toward SDF-1α/CXCL12 and toward MEC (Fig. 2*a*). Similar results were obtained from both TT-specific and CTB-specific IgA ASCs. In addition, we checked expression levels of CCR10 mRNA on polyclonal B cells such as $B220^-IgA^+$ and $B220^+IgA^+$ cells after s.c.-IR or IR-IR immunization (Fig. 2*c*). CCR10 mRNA was highly expressed on IgA^+ cells in the colon LP after s.c.-IR or IR-IR. The level of CCR10 mRNA was higher in the $B220^-IgA^+$ cells than in $B220^+IgA^+$ cells. To investigate whether Ag specific IgA ASCs induced by s.c.-IR will coexpress CXCR4 and CCR10, we stained colon MNCs with anti-IgA, anti-B220, or anti-CXCR4 Ab following migration toward MEC. The $CCR10^+$ IgA ASCs after both s.c.-IR and IR-IR immunization partially coexpressed CXCR4 (Fig. 2*d*). Furthermore, we performed the chemotaxis assay to check whether the number of IgA ASCs that migrate toward SDF-1α plus MEC will be additive or not. Of interest, more Ag-specific IgA ASCs migrated toward the mixture of SDF-α and MEC than did each chemokine (Fig. 2*e*). These results also suggest that Ag-specific IgA ASCs partially coexpressed CCR10 and CXCR4. In contrast, Ag-specific IgG ASCs elicited by s.c.-IR or IR-IR immunization did not migrate toward any chemokine we tested (data not shown), suggesting that they do not use these chemokine-chemokine receptor interactions for migration into the colon. The use