感染性結核患者の放置→蔓延などがある．

医療従事者の健康管理として，機関の管理者は労働安全衛生法や結核予防法[20]などに基づき，健康診断を実施し，徹底する．管轄保健所長や医療機関管理者は，医療機関において結核患者が発生した場合，協力して定期外健康診断を実施する．

結核の主要な感染源は，排菌量の多い喀痰塗抹陽性肺結核や喉頭結核患者(塗抹陰性や肺外結核の感染性は低い)であり，塗抹陽性結核患者の喀痰は10,000結核菌/mL以上を含む．結核菌の感染経路は飛沫核(空気)感染様式であり，結核菌が直径1～5 μmの落下しがたい，浮遊性飛沫核に含まれ，吸入することで感染する[21]．感染は結核菌の被曝露(吸入)者の約30%に成立する[22]．

結核のみならず感染症の蔓延防止は，感染源，感染経路および感受性宿主対策を基本としている．施設内結核感染防止対策は，① 管理対策(administrative measures)，② 環境や設備整備計画(engineering or environmental programs)，および，③ 個人防御対策(personal respiratory protection)から構成される[23](表6)．

未発病感染者の化学予防を実施する際，日本ではBCG(bacille Calmette-Guérin)接種が普及しているため，新規結核菌感染者をツベルクリン皮内反応(TST)で選定することは困難である．すなわち，① 現行TSTはBCGと結核菌に双方に共通な蛋白質抗原を用いているため，BCG接種と結核菌感染による反応の区別が不可能，② TST反応は経時的に減弱すること，さらに，③ 減弱した反応は再度のTSTによって，回復する(免疫学的ブースター効果)などが判定を困難にしている．

院内結核感染防止対策として，職員の新規採用に際し，39歳以下の場合，TST二段階法(two-step testing)を行い，その成績を発赤長径ミリ数，副反応の種類・有無を含めて記録しておくことが望まれる(なお，米国など諸外国はTST結果を硬結径で表現し，日本とは異なる)．第2回目の成績をTST基礎値として，その後の定期的TST，または結核患者発生時の接触者検診で実施するTSTに際して，結核菌感染の判定に有力な参考となる．実際的には，第1回目のTST強陽性者を除き，全員に第2回目のTSTを1～3週間後に行う方式が勧められる．

表6　施設内結核感染防止対策

管理対策
● 施設内感染防止委員会や感染制御部隊(infection control team)の設置
● 院内結核発生動向調査
● 医療従事者へ結核に関する啓発
● 医療従事者のツベルクリン皮内反応
● 持続性(2週間以上)咳嗽患者の優先診療
● 喀痰塗抹陽性結核患者の早期発見や迅速な個室収容/隔離，有効な抗結核化学療法
環境や設備整備計画
● 陰圧個室の整備
● 換気(7回以上/時間)
● 紫外線照射やHEPAフィルター装備
個人防御対策
● 飛沫核を除去できるマスク(例：N95)
● 咳嗽を誘発する医療行為(例：気管支内視鏡検査や気管内挿管)における細心の注意
● 未発病感染者の化学予防(INH服用など)

定期外検診(特に，塗抹陽性結核患者発生)におけるTSTは発生約2カ月後に施行し，結核菌感染判定基準として，① 発赤≧30 mmや，② TST基礎値に比し，10 mm以上の増強などが一般的である[23]．この基準に従い，未発病感染者(潜在性結核菌感染者)を選定し，化学予防を実施している．結核菌の潜伏感染者や感染した未発病医療従事者に対する発病予防戦略は，抗結核化学療法薬の6カ月間投与が一般的である．INHによる化学予防の場合，発病予防効果は50～70%，効果持続は約10年間である．ただし，感染源の結核菌がINH感受性であることを確認する．INH耐性の場合，RIFによる化学予防を考慮する．

潜在性結核菌感染の診断と対策

結核菌とBCGは結核菌群に属し，相同性がきわめて高く，BCGは乳幼児結核の発病予防ワクチンとして繁用されている．ツベルクリンは結核菌培養濾液から精製された蛋白抗原であり，

表7 内因性再燃における発病相対危険度

状況	発病相対危険度
● HIV 感染者	9.4～9.9
● 陳旧性結核	5.2
● 慢性腎不全	2.4
● 抗サイトカイン療法(IL-1 拮抗薬, TNF 抗体や可溶性受容体)	2.0
● 糖尿病(管理不良)	1.7
● 珪肺症	1.2～1.7
● 低体重(基準値の 10%以上のるいそう)	1.6
● 胃切除	1.4

TST が臨床応用されている．TST 陽性は結核菌感染，非結核性抗酸菌感染(*Mycobacterium avium* complex や *M. kansasii* など)や BCG 接種を示唆し，TST は結核菌感染，非結核性抗酸菌感染や BCG 接種を区別することは不可能である．結核菌感染者の治療(抗結核薬の発病予防内服)に際し，結核菌感染者の特定をするうえで，TST は支障をきたしている．

結核菌と BCG のゲノム情報から，BCG で欠失している遺伝子領域(region of deletion 1：RD1)産物(early secreted antigen target 6：ESAT-6 や，culture filtrate protein 10：CFP-10)は結核菌特異的蛋白であり，TST では識別できなかった結核菌感染，非結核性抗酸菌感染や BCG 接種による TST 陽性を ESAT-6 や CFP-10 抗原による特異免疫反応(末梢血に RD1 抗原を添加培養し，上清に産生される interferon γ を定量)で結核菌感染のみを検出することが可能となった[24～26]．RD1 領域を利用した結核菌特異的診断法は，潜在性感染の発見，集団感染の拡がりの把握や化学予防対象者の選定に有用である．

結核の発病様式は，① 潜在性結核菌感染(約 20 億人，人類の 1/3)を起源とした内因性再燃と，② 外来性再感染に大別される．結核菌感染後の発病率は 5～10%であるが，その多くは“内因性再燃”機序である．したがって，潜在性結核菌感染者を科学・効率的に発見し，発病高危険群(表7)や濃厚接触者などの潜在性感染者に治療(発病予防)介入することは結核制圧に新戦略を提供するであろう[27]．

BCG の有効性

現行の結核発病予防ワクチンである BCG の有効性に関し，根拠に基づく医療(evidence-based medicine：EBM)の観点から，見直しが進められた．その結果，乳幼児結核(結核性髄膜炎など播種性結核)の有効性は認められたが，成人肺結核に対する BCG の有効性は実証されていないこと，さらに，BCG 再接種による TST の陽転化が結核菌感染の診断を妨げること[28]などの事由により，結核予防法を改正し，BCG は乳幼児期(原則として，生後 6 カ月までに TST を省略した BCG 接種)の初回接種のみに限定し，BCG 再接種および TST(小学 1 年生および中学 1 年生時)は 2003 年 4 月から廃止された[29]．

BCG を凌駕する安全，かつ，有効な新規ワクチンの研究・開発は大きな課題である[22]．

移植医療と抗酸菌感染症

結核など抗酸菌感染に対する宿主防御は細胞性免疫に依存しており，細胞性免疫の低下した易感染性宿主(compromised hosts)における日和見感染症(opportunistic infections)でもある．臓器移植(造血幹細胞や固形臓器)の普及に伴い，被移植者における抗酸菌感染症が増加している．その理由として，① 被移植者の増加，② 移植臓器拒絶反応を回避するための強力な免疫抑制療法，③ 被移植者の寿命延長，および，④ 診断技術の向上が考えられる．

一般人口の抗酸菌感染症の罹患率と比較した場合，被移植者における結核は 40～70 倍，非結核性抗酸菌感染症は 50～600 倍のきわめて高い罹患率が報告されている．抗酸菌感染症は移植 3 カ月以降に発症することが多い．病型では，① 限局型，② 肺外型，③ 播種型があるが，②および③が 60%以上を占め，非定型的であることが多い[30]．

おわりに

最近，世界を震撼させたSARS-コロナウイルスによる重症急性呼吸器症候群の集団発生事例からも明らかなように，感染症は現在でも，人類の健康に甚大な健康被害を提供し，さらに，新興・再興感染症や薬剤耐性病原体感染症は世界的に増加しており，油断できない状況にある[31]．代表的再興感染症である結核は単一病原体感染症として，人類の健康に最大の脅威であり，一般国民や医療従事者への啓発・教育，感染源，感染経路および感受性宿主対策などの結核対策は，将来も重要な課題である．結核制圧目標は喀痰塗抹陽性肺結核罹患率：0.1/10万人以下であるが，日本の現状は9.4であり，制圧目標には程遠く，中進国である．加えて，結核が呼吸器のみならず，全身性感染症であることを考慮すると，結核を念頭において日常の診療に従事することが肝要である．

今後の結核対策における重点項目として，① DOTSの普及，② 潜在性結核菌感染対策，③ HIV感染/AIDSと結核の重複感染に対する効果的な戦略，④ 多剤耐性結核に対する抗結核薬，⑤ 迅速・簡便な診断法，⑥ 有効なワクチンの開発，⑦ 集団や院内感染対策，が推進され，結核が制圧されることを期待している．

本稿は，厚生労働省 厚生労働科学研究費補助金(新興・再興感染症研究事業)，文部科学省 科学研究費補助金，文部科学省 科学研究費補助金，日米医学協力研究会結核・ハンセン病専門部会および大阪市立大学都市問題研究費により支援された．

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The Role of IL-5 for Mature B-1 Cells in Homeostatic Proliferation, Cell Survival, and Ig Production[1]

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B-1 cells, distinguishable from conventional B-2 cells by their cell surface marker, anatomical location, and self-replenishing activity, play an important role in innate immune responses. *B-1 cells constitutively* express the IL-5R α-chain (IL-5Rα) and give rise to Ab-producing cells in response to various stimuli, including IL-5 and LPS. Here we report that the IL-5/IL-5R system plays an important role in maintaining the number and the cell size as well as the functions of mature B-1 cells. The administration of anti-IL-5 mAb into wild-type mice, T cell-depleted mice, or mast cell-depleted mice resulted in reduction in the total number and cell size of B-1 cells to an extent similar to that of IL-5Rα-deficient (IL-5R$\alpha^{-/-}$) mice. Cell transfer experiments have demonstrated that B-1 cell survival in wild-type mice and homeostatic proliferation in recombination-activating gene 2-deficient mice are impaired in the absence of IL-5Rα. IL-5 stimulation of wild-type B-1 cells, but not IL-5R$\alpha^{-/-}$ B-1 cells, enhances CD40 expression and augments IgM and IgG production after stimulation with anti-CD40 mAb. Enhanced IgA production in feces induced by the oral administration of LPS was not observed in IL-5R$\alpha^{-/-}$ mice. Our results illuminate the role of IL-5 in the homeostatic proliferation and survival of mature B-1 cells and in IgA production in the mucosal tissues. *The Journal of Immunology*, 2004, 172: 6020–6029.

B-1 cells differ from conventional B-2 cells in their surface phenotype, anatomical localization, self-replenishing activity, and V_H usage of IgM (1, 2). B-1 cells constitutively express three different markers, namely *Mac-1* (CD11b/CD18), FcεR (CD23), and the IL-5R α-chain (IL-5Rα).[3] Mac-1 is present in peritoneal and pleural cavity B-1 cells but is not expressed on B-2 cells, whereas FcεR is preferentially expressed on B-2 cells in the peritoneal cavity and in the spleen (3, 4). IL-5Rα is constitutively expressed on all B-1 cells, but is expressed on a small proportion (2–4%) of resting B-2 cells in the spleen (5).

The progenitors of B-1 cells are abundant in the fetal omentum and liver but are missing in the bone morrow of adult animals (6, 7). In contrast with B-2 cells, which are supplied from progenitors in the bone marrow throughout life, B-1 cells maintain their number in adult animals by their self-replenishing capacity (3, 7). In the adult, these self-replenishing B-1 cells are clearly enriched in the peritoneal and pleural cavities, and a low frequency is seen in the spleen, but B-1 cells are virtually absent from the lymph nodes, Peyer's patches (PP), and peripheral blood, where most conventional B-2 cells are localized (3). B-1 cells are categorized into B-1a cells that express CD5 and B-1b cells that express cell surface markers similar to those of B-1a cells, except for the low expression, if any, of CD5 (2, 3).

B-1 cells are believed to be the primary source of natural IgM Ab, although they can become Ig-producing cells for all isotypes. Consistent with a major role of B-1 cells in natural IgM production, a number of specificities of natural IgM Ab have been identified in the B-1 repertoire. These include specificities for LPS, phosphorylcholine, undefined determinants on *Escherichia coli* and *Salmonella* spp., phosphatidylcholine, and complement-binding Abs (3). Furthermore, B-1 cells in the peritoneal cavity serve as an important source of IgA-producing plasma cells at mucosal sites. These findings are largely supported by transfer experiments of peritoneal B-1 cells in irradiated mice or otherwise B cell-depleted mice and by analysis of genetically altered immune-deficient mice (8). The role of B-1 cells in IgA production in the gut is further supported by evidence that mice with a selective B-1 cell reduction in number showed decreased frequencies of IgA-producing cells in the lamina propria (LP) (9). The helper T cell dependency of B-1 cells on gut-associated IgA production is still controversial (9, 10).

Studies of gene-targeted and transgenic mice have revealed that B cell receptor (BCR) signaling is critical for B-1 cell development or maintenance. Mutant mice that lack Bruton's tyrosine kinase protein kinase C β, CD19, the p85α subunit of phosphatidylinositol-3 kinase, $p95^{vav}$, CD21/CD35, and CD81, which are strongly associated with BCR signaling, have substantial depletion of B-1 cells but largely spare B-2 cells (11–18). Conversely, mutation or overexpression of Src homology protein-1, CD22, or CD72 that induces enhanced BCR signaling results in an expanded B-1 cell compartment (19–21).

IL-5, mainly produced by activated Th2 cells and mast cells, acts on B-1 and B-2 cells to induce proliferation and differentiation into Ig-producing cells (22–25). IL-5 also controls the production and functions of eosinophils and basophils. The IL-5R consists of two distinct membrane proteins, IL-5Rα and βc, each of which is a member of the cytokine receptor superfamily (5). The binding of IL-5 occurs through the IL-5Rα, and the βc forms a high-affinity IL-5R in combination with the IL-5Rα, transducing signals into nuclei. Although the molecular mechanisms for IL-5 signal

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Received for publication October 30, 2003. Accepted for publication March 3, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by a research grant from the Human Frontier Science Program (to K.T.), by Special Coordination Funds for Promoting Science and Technology (to K.T.), by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture (Japan), and by an international postdoctoral fellowship from the Japan Society for the Promotion of Science (to B.M.).

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[3] Abbreviations used in this paper: IL-5Rα, IL-5R α-chain; PP, Peyer's patch; LP, lamina propria; BCR, B cell receptor; RAG, recombination-activating gene; PEC, peritoneal exudate cell; MLN, mesenteric lymph node; s, surface; TLR, Toll-like receptor 4; HPRT, hypoxanthine phosphoribosyltransferase; m, murine; CD40L, CD40 ligand.

0022-1767/04/$02.00

transduction are not fully characterized, the activation of Bruton's tyrosine kinase and Janus kinase 2 kinases, rapid tyrosine phosphorylation of βc and Src homology 2/Src homology 3-containing cellular proteins, and the induction of the transcription of several nuclear proto-oncogenes are essential for signal transduction (26–32).

Transgenic mice expressing the *IL-5* gene exhibit elevated levels of serum IgM, IgA, and IgE, and an increase in the number of B-1 cells and autoantibody production and show persistent eosinophilia (33, 34). IL-5Rα$^{-/-}$ mice and IL-5$^{-/-}$ mice show a decrease in B-1 cells in the peritoneal cavity and in B-1 cell-derived surface (s)IgA^{+} cells in the LP (35–39). Although these results suggest that IL-5 is an important cytokine for B-1 cell development, maintenance, or triggering, the role of IL-5 in mature B-1 cell maintenance and activation in vivo remains to be properly evaluated.

This study examines whether the IL-5/IL-5R system plays an important role in the homeostatic proliferation and survival of mature B-1 cells. We show that IL-5 regulates the cell number and cell size of B-1 cells in the absence of T cells or mast cells. We also demonstrate the role of IL-5 in gut-associated B-1 cell response to CD40 and LPS.

Materials and Methods

Mice

C57BL/6J (IL-5Rα$^{+/+}$) and W/W^{V} mice were purchased from Japan SLC (Hamamatsu, Japan). Recombination-activating gene (RAG)2-deficient (RAG-2$^{-/-}$) mice and TCRβ δ double null mutant (TCRβ$^{-/-}$ δ$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The IL-5Rα null mutant (IL-5Rα$^{-/-}$) mice (35) used in this study were backcrossed with C57BL/6J mice for >10 generations. IL-5 null mutant (IL-5$^{-/-}$) mice on a C57BL/6 background (38) were donated by M. Kopf (University of Freiburg, Germany). All of the mice were bred and maintained in animal facilities under specific pathogen-free conditions using ventilated microisolater cages in the experimental animal facility at the Institute of Medical Science, University of Tokyo. All experiments were conducted according to our institution's guidelines for the care and treatment of experimental animals.

Administration of anti-IL-5 mAb and LPS

A single i.p. administration of anti-IL-5 mAb (clone NC17) or isotype-matched control IgG into 6- to 8-wk-old mice (1 mg in a volume of 250 μl per mouse) was performed (40, 41). Six days after treatment, peritoneal washouts were obtained and analyzed. LPS (*E. coli* serotype O55: B5; Sigma-Aldrich, St. Louis, MO) dissolved in PBS was orally administered (0.1 mg in a volume of 200 μl per mouse per week) for 3 wk into the gut of 8-wk-old mice through a 1-mm diameter polyethylene tube. Seven days after the last administration, the mice were anesthetized with ether, sacrificed, and analyzed.

Cell preparation

Single cell suspensions were prepared from the lymphoid organs of 6- to 8-wk-old mice. A standard procedure was used to prepare single cell suspensions from the peritoneal exudate cells (PECs), mesenteric lymph nodes (MLNs), PP, SP, lung, and the LP of the small intestine. Briefly, PECs were obtained by washing the peritoneal cavity with HBSS (Life Technologies, Grand Island, NY) containing 3% FCS. Mononuclear cells from MLNs or PP were isolated by a mechanical method using a stainless steel screen. Mononuclear cells from the lung and LP were isolated by a procedure of shaking in an RPMI 1640 medium (Life Technologies) containing 5 mM EDTA and by enzymatic dissociation procedures with collagenase type VIII (Sigma-Aldrich) (37).

Purification of B-1 cells

PECs were collected from >10 mice and were mixed together. After washing twice with PBS containing 1% BSA, the cells were incubated with anti-FcγR (2.4G2; American Type Culture Collection, Rockville, MD) to prevent the nonspecific binding of the labeled Abs. After another washing, macrophages, B-2 cells, and T cells were depleted from the cells using a MACS system (Miltenyi Biotec, Cologne, Germany) after incubation with a mixture of biotinylated Abs (anti-F4/80, anti-CD23, and anti-CD3) and streptavidine-coupled microbeads (Miltenyi Biotec). In B-1 cell transfer experiments, we took another purification step of B-1 cells using a FACSVantage (BD Biosciences, San Jose, CA) to obtain B-1 cells with a higher degree of purity. In addition to using the MACS system, the resulting F4/80^{-}/CD23^{-}/CD3^{-} cells were stained with FITC-labeled anti-CD23 and PE-labeled F(ab')$_2$ of anti-IgM, and the CD23^{-}sIgM^{+} cells were sorted using a FACSVantage.

Flow cytometry

The cells (1–10 × 10^5) were stained with predetermined optimal concentrations of the respective Abs together with 2.4G2 (10 μg/ml). After washing, the cells were analyzed on FACScan or a FACSCaliber instrument (BD Biosciences). The following mAbs were used: biotinylated anti-IL-5Rα (T21) (42); FITC-labeled, PE-labeled, or biotinylated anti-CD23 (B3B4), PE-labeled or biotinylated anti-CD5 (53-7.3) and biotinylated anti-CD3 (145-2C11) (all purchased from BD PharMingen, San Diego, CA); FITC-labeled, PE-labeled, or biotinylated anti-B220 (RA3-6B2), PE-labeled F(ab')$_2$ of anti-mouse IgM, and FITC-labeled or biotinylated anti-Mac-1 (M1/70) (all obtained from Caltag Laboratories, Burlingame, CA); PE-labeled anti-Toll-like receptor 4 (TLR4)/MD2 (MTS 510) (43) and PE-labeled anti-RP105 (RP/14) (44); biotinylated anti-CD40 (1C10; R&D Systems, Minneapolis, MN), biotinylated anti-F4/80 (A3-1; Serotec, Oxford, U.K.); and biotinylated anti-IgA (Southern Biotechnology, Birmingham, AL). PE-labeled streptavidin (Ancell, Bayport, MN) or allophycocyanin-conjugated streptavidine (BD PharMingen) were also used. In some stainings, 2 μg/ml 7-amino-actinomycin D (Sigma-Aldrich) was used to gate out dead cells.

Cell transfer and homeostatic proliferation assay

PECs obtained from IL-5Rα$^{+/+}$ or IL-5Rα$^{-/-}$ mice were washed with PBS and suspended in PBS at 1 × 10^7 cells/ml. CFSE (Molecular Probes, Eugene, OR) was then added to the cell suspensions at a final concentration of 1 μM. The cell suspensions were incubated at 37°C for 10 min and washed three times with cold sterile PBS. The resulting CFSE-labeled cells (1 × 10^6) were injected i.p. into IL-5Rα$^{+/+}$ or RAG-2$^{-/-}$ mice. In some experiments, sorted B-1 cells (1 × 10^5) were injected i.p. into RAG-2$^{-/-}$ mice. The PECs of recipient mice were recovered on days 2, 30, and 60 after the cell transfer, and their cellularities in the B-1 cell compartment were analyzed.

RNA isolation and semiquantitative RT-PCR

Total RNA was isolated from various mouse tissues using the SV Total RNA Isolation System (Promega, Madison, WI), according to the manufacturer's instructions, and first strand cDNA templates synthesized by Superscript II reverse transcriptase (Life Technologies) using random primers (TaKaRa, Kyoto, Japan). Serial dilutions of cDNA templates were subjected to PCR amplification by using primer sets encompassing several introns for IL-5 (forward primer, 5′-ATGGAGATTCCCATGAGCAC; reverse primer, 5′-GCACAGTTTTGTGGGGTTTT) or hypoxanthine phosphoribosyltransferase (HPRT; forward primer, 5′-TGCTCGAGATGTCATGAAGG; reverse primer, 5′-TTGCGCTCATCTTAGGCTTT). The cycling parameters were 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for 35 cycles to detect *IL-5* mRNA or 27 cycles for HPRT. The PCR products were separated through 1.0% agarose gel and were stained with ethidium bromide.

Assay for B-1 cell proliferation and differentiation

MACS-sorted PECs were cultured in an RPMI 1640 medium supplemented with 8% heat-inactivated FCS, 2 mM glutamine, 5 μM 2-ME, penicillin (100 U/ml), and streptomycin (100 μg/ml) in 96-well flat-bottom microtiter plates (1 × 10^5/well in 200 μl of medium) with or without stimulants. Anti-CD40 mAb (1C10; R&D Systems; 1 μg/ml), LPS (40 μg/ml), IL-4 (1000 U/ml), or a selected combination of these agents was added at the onset of cell culture. For the proliferation assay, cells were pulse-labeled with [^{3}H]thymidine (0.2 μCi per well) during the last 8 h of the 72-h culture period, and the incorporated [^{3}H]thymidine was measured using a MATRIX 96 Direct Beta Counter (Packard, Meriden, CT). The results were expressed as the mean cpm and the SD of the duplicate cultures. For determining IgM, IgG1, and IgG3 secretion, cells (1 × 10^5 in a 200-μl culture) were cultured for 7 days. The cultured supernatants were used for ELISA to determine the amounts of IgM, IgG1, and IgG3. Each experiment was repeated at least three times.

Enumeration of Ig-producing cells using ELISPOT

An ELISPOT assay was conducted according to the procedures previously described (37). The 96-well filtration plates with a nitrocellulose base (Millipore, Bedford, MA) were coated with 5 μg/ml anti-Ig (Southern Biotechnology) overnight and were blocked with a culture medium. The mononuclear cells suspended in the culture medium were added at various concentrations and were incubated for 6 h. After washing, 1 μg/ml HRP-conjugated anti-IgM, anti-IgG, or anti-IgA Ab (all obtained from Southern Biotechnology) was added, and the plates were incubated for 10 h at 4°C.

After the incubation, the spots were developed with 2-amino-9-ethylcarbazole containing hydrogen peroxide (Polysciences, Warrington, PA). Reddish-brown-colored spots were counted as Ab-forming cells using the KS ELISPOT compact system (Carl Zeiss, Jena, Germany).

ELISA

Freshly collected fecal samples were weighed, dissolved in PBS (0.1 g/ml), and centrifuged at 15,000 rpm for 5 min. The supernatants were used as fecal extract. The amount of each Ig isotype in sera and in fecal extract was measured by sandwich ELISA with Abs specific for each murine (m)Ig isotype according to the procedures previously described (36). In brief, 96-well trays (Greiner, Frickenhausen, Germany) were coated with 10 μg/ml isotype-specific goat anti-mIg polyclonal Abs for total Igs. Samples were added to the wells and the trays were incubated for 2 h. After washing with PBS containing 0.05% Tween 20 (washing buffer), biotinylated isotype-specific goat anti-mIg polyclonal Abs were added to each well. After washing, HRP-streptavidine was added to each well, and the incubation continued for 1 h. Finally, the trays were washed with the buffer, and 100-μl aliquots of substrate, *o*-phenylene-diamine (final 0.4 mg/ml), and hydrogen peroxide (final 0.015%), dissolved in 0.1 M citrate buffer (pH 5.0), were added to each well. Enzyme reaction was terminated by adding 2 M sulfuric acid, and OD at 495 nm was measured with a V-max kinetic Micro Plate Reader (Molecular Devices, Sunnyvale, CA). Using myeloma proteins (BD PharMingen), standard curves were generated for each isotype and the concentration of mIg was determined.

Results

Decrease in cell number and cell size of mature B-1 cells by administration of anti-IL-5 mAb in vivo

As we reported previously, IL-5Rα$^{-/-}$ mice have a significant reduction in cell number and cell size of IgM$^+$ CD5$^+$ B-1a cells in the PECs (36). When we analyzed the entire IgM$^+$ CD23$^-$ B-1 cell populations, a significant reduction in percentage and smaller cell size of B-1 cells was also observed (Fig. 1*A*). These results were confirmed by the analysis of IgM$^+$ CD5$^+$ B-1 cell populations. We also found that the total number and size of B-1 cells in the PECs decreased in IL-5$^{-/-}$ mice (data not shown). In contrast with B-1 cells, the total percentage and size of IgM$^+$CD23$^+$ (or IgM^{low}CD5$^-$) B-2 cells in IL-5Rα$^{-/-}$ mice were similar to those of wild-type mice (Fig. 1*A*). The total numbers of B-1 cells in IL-5Rα$^{+/+}$ and IL-5Rα$^{-/-}$ mice were 4.2×10^5 and 3.1×10^5 on average, respectively, (statistically significant, $p < 0.05$), whereas those of B-2 cells were 4.0×10^5 and 4.3×10^5, respectively (Table I).

Our first question is whether the abnormalities observed in B-1 cells from IL-5Rα$^{-/-}$ and IL-5$^{-/-}$ mice originated in the developmental process or in fully developed mature B-1 cells. We administered anti-IL-5 mAb i.p. into a group of 8-wk-old wild-type mice, which developed mature B-1 cells. As a control, isotype-matched rat IgG was injected in another group of mice. A smaller B-1 cell size was observed in anti-IL-5-treated mice 3 days after treatment (data not shown). Six days after anti-IL-5 treatment, not only cell size but also the total percentage of B-1 cells significantly decreased (Fig. 1*B*). The total numbers of B-1 cells on average in the control and anti-IL-5-treated mice were 4×10^5 and 3.2×10^5, respectively (statistically significant, $p < 0.05$), whereas those of B-2 cells were 3.8×10^5 and 3.7×10^5, respectively (Table I). The levels of reduction in B-1 cell number and size in anti-IL-5-treated mice were similar to those of B-1 cells in IL-5Rα$^{-/-}$ mice. Anti-IL-5 treatment did not cause significant changes in total number or cell size in the IgM$^+$CD23$^+$ (or IgM^{low}CD5$^-$) B-2 cell compartment (Fig. 1*B*). We infer from these results that the abnormality of B-1 cells observed in IL-5Rα$^{-/-}$ mice is reproduced in mature B-1 cells in wild-type mice by blocking IL-5 signals.

Impaired survival and homeostatic proliferation of B-1 cells in IL-5Rα$^{-/-}$ mice

To examine the role of IL-5 in maintaining the mature B-1 cell compartment in more detail, PECs from IL-5Rα$^{+/+}$ or IL-5Rα$^{-/-}$ mice were labeled with CFSE and transferred into the peritoneal cavity of unirradiated IL-5Rα$^{+/+}$ mice, where the normal number

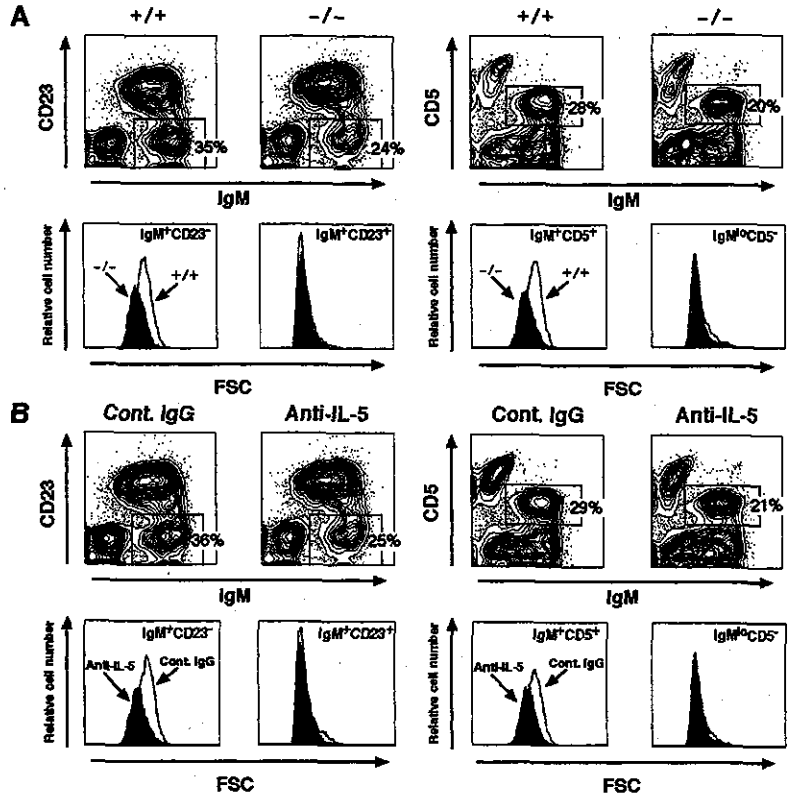

FIGURE 1. Decreased cell number and size of B-1 cells by blocking IL-5 and IL-5R interaction. Representative two-color contour plots show the expression of IgM/CD23 and IgM/CD5 on PECs from 8-wk-old IL-5Rα$^{+/+}$ or IL-5Rα$^{-/-}$ mice (*A*) and from control IgG- or anti-IL-5-treated IL-5Rα$^{+/+}$ mice (*B*). A single i.p. administration of anti-IL-5 mAbs or control IgG (1 mg/250 μl) into IL-5Rα$^{+/+}$ mice was performed. PECs were obtained and analyzed on day 6 after treatment (*B*). The percentages represent the fractions of the lymphocyte-gated live cells that fall into the indicated boxes. Representative histograms depict the relative cell number and sizes of B-1 cells (IgM$^+$CD23$^-$), B-1a cells (IgM$^+$CD5$^+$), and B-2 cells (IgM$^+$CD23$^+$ or IgM^{low}CD5$^-$). The representative results of three independent experiments are shown.

Table I. *Reduced absolute cell number of B-1 cells by blocking IL-5 and IL-5R interaction*[a]

	IL-5Rα$^{+/+}$	IL-5Rα$^{-/-}$	Control IgG	Anti-IL-5
B-1 (IgM$^+$CD23$^-$)	4.2 ± 0.36	3.1 ± 0.34*	4.4 ± 0.32	3.2 ± 0.17*
B-1a (IgM$^+$CD5$^+$)	3.8 ± 0.28	2.8 ± 0.39*	4.0 ± 0.41	3.0 ± 0.23*
B-2 (IgM$^+$CD23$^+$)	4.0 ± 0.42	4.3 ± 0.52	3.8 ± 0.21	3.7 ± 0.33

[a] The results indicate the mean cell numbers ± SEM (× 10^5) of indicated groups of five mice.
*, $p < 0.05$ compared with IL-5Rα$^{+/+}$ or control IgG-administered mice.

of B-1 cells resides. The CFSE$^+$ B-1 and CFSE$^+$ B-2 cells in the PECs of the recipient mice were examined by FACS analysis on day 2 or on day 30. As shown in Fig. 2*A* (*left panel*), the proportion of CFSE$^+$IL-5Rα$^{+/+}$ B-1 cells on day 30 (94%) was close to that on day 2, suggesting the long-term survival of CFSE-labeled B-1 cells in the recipient. In contrast, the proportion of CFSE$^+$ IL-5Rα$^{-/-}$ B-1 cells was reduced to 39% on day 30 compared with that on day 2. The intensity of CFSE labeling showed a broad distribution in both IL-5Rα$^{+/+}$ B-1 and IL-5Rα$^{-/-}$ B-1 cells on day 30 (Fig. 2*B*), indicating that cells have divided slightly. However, we could not estimate how many times the B-1 cells divided, because of faint intensities of CFSE labeling. CFSE$^+$ IL-5Rα$^{+/+}$ B-2 cells in the recipient on day 30 were reduced to 55%, which was comparable with CFSE$^+$IL-5Rα$^{-/-}$ B-2 cells (52%) (Fig. 2*A*, *right panel*). We examined the distribution of CFSE-positive B cells from IL-5Rα$^{+/+}$ or IL-5Rα$^{-/-}$ mice in the LP, PP, and MLNs of recipient mice 30 days after cell transfer. CFSE-positive B cells were rarely detected in the LP (data not shown). We observed some CFSE-positive B cells (0.02–0.03% of the total cells) in MLNs and PP (data not shown). However, there was no significant difference between recipient mice transferred IL-5Rα$^{+/+}$B-1 and IL-5Rα$^{-/-}$B-1 cells. Thus, the survival of mature B-1 cells, but not B-2 cells, in the peritoneal cavity was severely impaired in the absence of IL-5Rα.

Next, the role of IL-5 in the self-replenishing activity of B-1 cells was examined. Peritoneal exudate cells from IL-5Rα$^{+/+}$ or IL-5Rα$^{-/-}$ mice were CFSE-labeled and transferred into RAG-2$^{-/-}$ mice. On day 30, the proportion of CFSE$^+$ IL-5Rα$^{+/+}$ B-1 cells in the recipient RAG-2$^{-/-}$ mice increased ~3-fold (276%) compared with that on day 2, whereas the proportion of CFSE$^+$ IL-5Rα$^{-/-}$ B-1 cells did not increase (70%) (Fig. 3*A*, *upper panel*). The CFSE intensity of IL-5Rα$^{-/-}$ B-1 cells somewhat decreased, but maintained higher intensities than did IL-5Rα$^{+/+}$ B-1 cells (Fig. 3*B*, *lower left panel*). The difference in B-2 cell number on day 30 in the RAG-2$^{-/-}$ recipient mice between IL-5Rα$^{+/+}$ B-2 cells and IL-5Rα$^{-/-}$ B-2 cells (62% and 48%, respectively) was not obvious compared with that of B-1 cells (Fig. 3*A*, *lower panel*). The CFSE intensity of IL-5Rα$^{-/-}$ B-2 cells was comparable with that of IL-5Rα$^{+/+}$ B-2 cells (Fig. 3*B*, *lower right panel*). In the LP 30 days after cell transfer, the proportion of CFSE$^+$B220$^-$sIgA$^+$ cells in RAG-2$^{-/-}$ mice transferred with IL-5Rα$^{+/+}$ cells was significantly higher than that transferred with IL-5Rα$^{-/-}$ cells (Fig. 3*C*, *left panels*). We examined the levels of CFSE in sIgA$^+$ cells in the LP in recipients transferred IL-5Rα$^{+/+}$ PECs or IL-5Rα$^{-/-}$ PECs and found that the levels of CFSE of sIgA$^+$ cells were very low and were similar between the two experimental groups (Fig. 3*C*, *right panels*). Intriguingly, ~3-fold higher levels of IgM, IgG1, IgG3, and IgA in serum were observed in RAG-2$^{-/-}$ mice transferred IL-5Rα$^{+/+}$ cells compared with those transferred with IL-5Rα$^{-/-}$ cells, whereas the serum levels of IgG2a and IgG2b were comparable between the two groups (Fig. 3*D*, *left panel*). The amount of IgA in fecal extracts was also ~3-fold higher in the recipients of IL-5Rα$^{+/+}$ cells compared with that in IL-5Rα$^{-/-}$ cells (Fig. 3*D*, *right panel*). These results imply that IL-5 plays a critical role in the homeostatic proliferation of mature B-1 cells and leads to the maintenance of optimal levels of Ig production, although these processes may occur inefficiently even in the absence of IL-5.

Homeostatic proliferation and Ig production of B-1 cells in the absence of T cells

We examined the effect of T cell dependency in the IL-5-mediated homeostatic proliferation of mature B-1 cells. IgM$^+$CD23$^-$ B-1 cells were purified (>98% purity) from the PECs of IL-5Rα$^{+/+}$ or IL-5Rα$^{-/-}$ mice by cell sorting and were transferred into RAG-2$^{-/-}$ mice. As shown in Fig. 4*A*, the proportion of IL-5Rα$^{+/+}$ B-1 cells in PECs increased ~2-fold (198%) on day 30 and ~3-fold (276%) on day 60 in the RAG-2$^{-/-}$ recipient mice. An increase in both the IgM$^+$CD23$^-$CD5$^+$B-1a and IgM$^+$CD23$^-$CD5$^-$B-1b cell populations was also observed (Fig. 4*A*, *center* and *right*

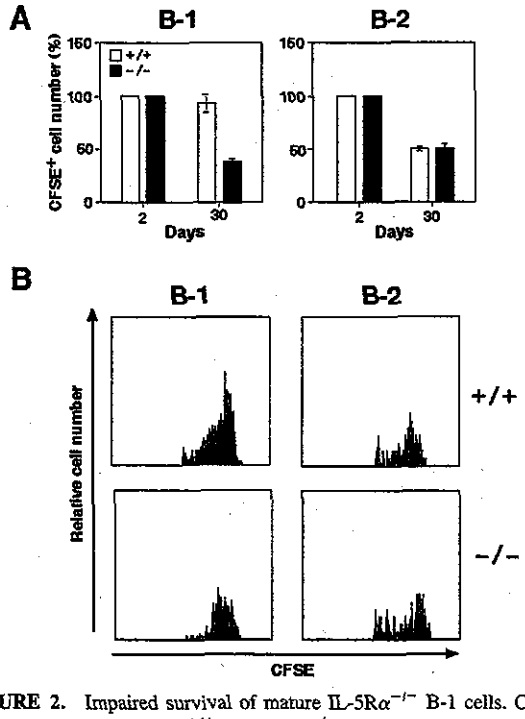

FIGURE 2. Impaired survival of mature IL-5Rα$^{-/-}$ B-1 cells. CFSE-labeled PECs from IL-5Rα$^{+/+}$ or IL-5Rα$^{-/-}$ mice were transferred (1 × 10^6 cells per head) into the peritoneal cavity of IL-5Rα$^{+/+}$ mice. On days 2 and 30 after cell transfer, the recipient mice were killed and the numbers of CFSE$^+$ B-1 (IgM$^+$CD23$^-$) and B-2 (IgM$^+$CD23$^+$) cells in PECs were analyzed by flow cytometry. Two and three recipient mice in each group were analyzed and the mean ± SEM is shown (*A*). The mean cell number of CFSE$^+$ peritoneal cells from two mice on day 2 was set as 100% (*A*). Representative histograms show the intensity of CFSE in B-1 cells (CFSE$^+$IgM$^+$CD23$^-$) and B-2 cells (CFSE$^+$IgM$^+$CD23$^+$) in the peritoneal cavity of the recipients (*B*). The data shown are representative results from three independent experiments.

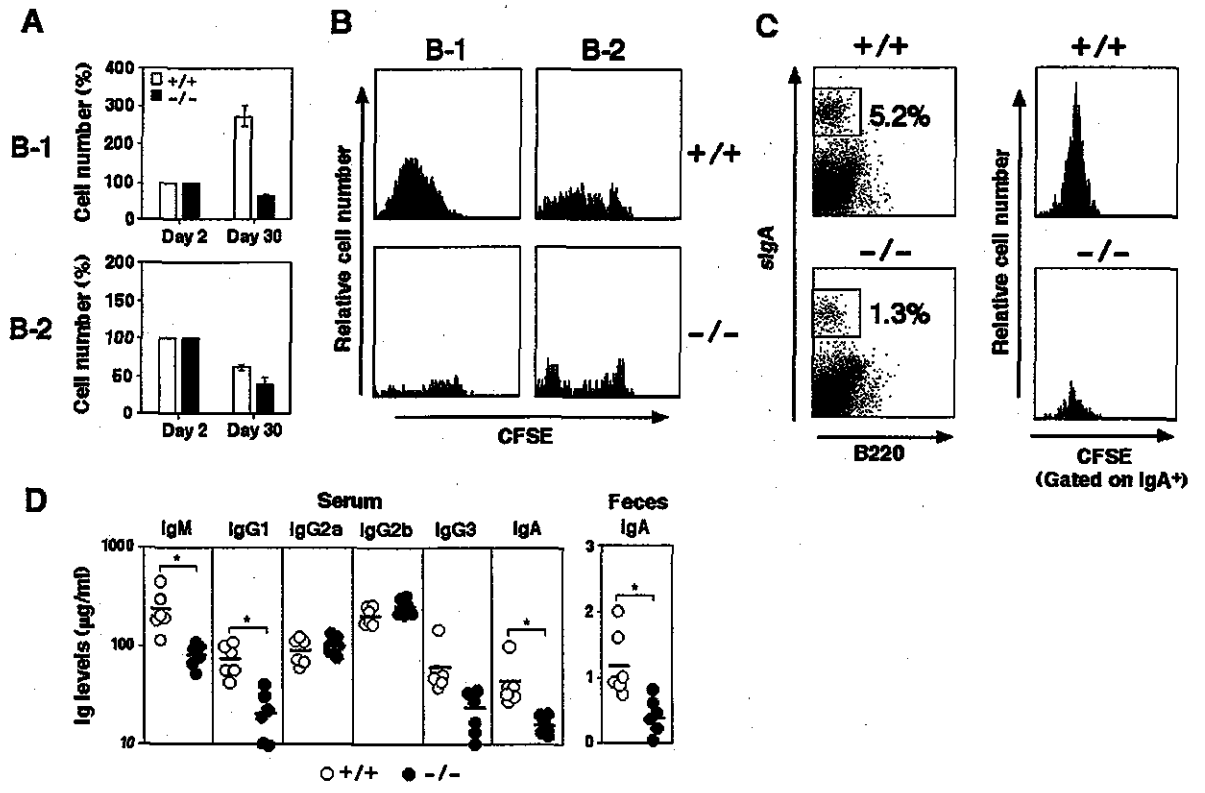

FIGURE 3. Impaired homeostatic B-1 cell proliferation and Ig production from IL-5Rα$^{-/-}$ mice. *A*, IL-5Rα$^{-/-}$ B-1 cells have a defect in homeostatic proliferation. CFSE-labeled peritoneal cells were injected i.p. into RAG-2$^{-/-}$ mice. The recipient mice of each group were analyzed as described in Fig. 2. *B*, IL-5Rα$^{-/-}$ B-1 cells maintain high CFSE intensity. Representative histograms depict CFSE intensity in B-1 cells and B-2 cells in PECs of RAG-2$^{-/-}$ recipient mice. *C*, IL-5Rα$^{-/-}$ B-1 cells migrate to the LP, but the number of sIgA$^+$ cells is decreased. Cells in the LP were purified as in the method described and were stained with anti-B220 and anti-IgA. Representative two-color fluorescence plots show B220$^+$ and sIgA$^+$ cells in the LP of the recipient mice on day 30. Representative histograms depict CFSE intensity in sIgA$^+$ cells in LP of RAG-2$^{-/-}$ recipient mice. The percentages represent the fractions of the lymphocyte gated live cells that fall into the indicated box. Representative results of three independent experiments are shown (*A–C*). *D*, IL-5Rα$^{-/-}$ peritoneal cells transferred into RAG-2$^{-/-}$ mice produce low levels of Igs. The concentration of Ig subclasses in serum or in fecal extracts in the RAG-2$^{-/-}$ recipient mice on day 30 was determined by isotype-specific ELISA. The mean values of Igs in the indicated groups of recipient mice are represented as a bar. *, $p < 0.05$ by Student's *t* test.

panels), whereas CD5^{high} T cells or CD23$^+$ B-2 cells were not detected even 60 days after cell transfer (data not shown). In contrast, IL-5Rα$^{-/-}$ B-1 cells did not show a significant increase on day 30 (114%) or on day 60 (103%) compared with that 2 days after cell transfer (Fig. 4*A*, *left panel*). IL-5Rα$^{-/-}$ B-1a cells decreased to ~71% on day 30 and on day 60, whereas IL-5Rα$^{-/-}$ B-1b cells showed a small but significant increase up to 129% on day 30 (Fig. 4*A*, *center* and *right panels*). These results indicate that mature B-1 cells undergo homeostatic proliferation in T cell-deficient conditions. The serum levels of IgM, IgG3, and IgA were elevated to ~3- to 5-fold in the recipient transferred IL-5Rα$^{+/+}$ B-1 cells compared with those of IL-5Rα$^{-/-}$ B-1 cells (Fig. 4*B*). The Ig levels of IgG1 and IgG2 were virtually undetectable in both groups of recipient mice on day 30 (Fig. 4*B* and data not shown).

Production of IL-5 in T cell- and mast cell-deficient mice

IL-5 is produced by T cells, mast cells, and eosinophils once they are activated (22). In particular, not only αβT cells in the peritoneal cavity and intestinal intraepithelial lymphocytes (8, 45), but also freshly isolated γδT cells in the intraepithelial lymphocytes are capable of producing IL-5 (45). We injected anti-IL-5 mAb into TCRβ$^{-/-}$δ$^{-/-}$ mice and examined B-1 cell survival in T cell-deficient conditions. Anti-IL-5-treated TCRβ$^{-/-}$δ$^{-/-}$ mice showed a decrease in B-1 cell number and cell size 6 days after treatment, compared with the control group of mice (Fig. 5*A*, *left panel*). Anti-IL-5 injection into W/W^V mice also caused a decrease in B-1 cell size (Fig. 5*A*, *right panel*), although the total B-1 cell number did not change significantly. The total number and size of B-2 cells did not change in either the TCRβ$^{-/-}$δ$^{-/-}$ mice or the W/W^V mice as a result of anti-IL-5 treatment (data not shown).

To evaluate *IL-5* mRNA expression in tissues, total RNA was isolated from the various tissues of RAG-2$^{-/-}$, TCRβ$^{-/-}$δ$^{-/-}$, and W/W^V mice and was used for *IL-5* mRNA expression analysis. As controls, wild-type mice and IL-5$^{-/-}$ mice were also used. RT-PCR analysis revealed significant *IL-5* mRNA expression in the lungs, spleen, small intestine, and stomach of wild-type mice, RAG-2$^{-/-}$ mice, TCRβ$^{-/-}$δ$^{-/-}$, and W/W^V mice (Fig. 5*B*). A lesser extent of *IL-5* mRNA expression was observed in PECs. We did not detect any *IL-5* mRNA expression in the tissues of the IL-5$^{-/-}$ mice. *IL-5* mRNA expression was not observed in the liver. To examine *IL-5* mRNA expression in cells other than T cells, mast cells, and eosinophils, we purified c-*kit*$^-$IL-5Rα$^-$ cells by sorting (>99% purity) from the tissues of RAG-2$^{-/-}$ mice. The RNA from these cells was isolated and used for RT-PCR analysis. As shown in Fig. 5*C*, high levels of *IL-5* mRNA expression were observed in c-*kit*$^-$IL-5Rα$^-$ cells in the lungs and small intestine of RAG-2$^{-/-}$ mice. The c-*kit*$^-$IL-5Rα$^-$ PECs also expressed *IL-5* mRNA, although the expression levels were low. These results

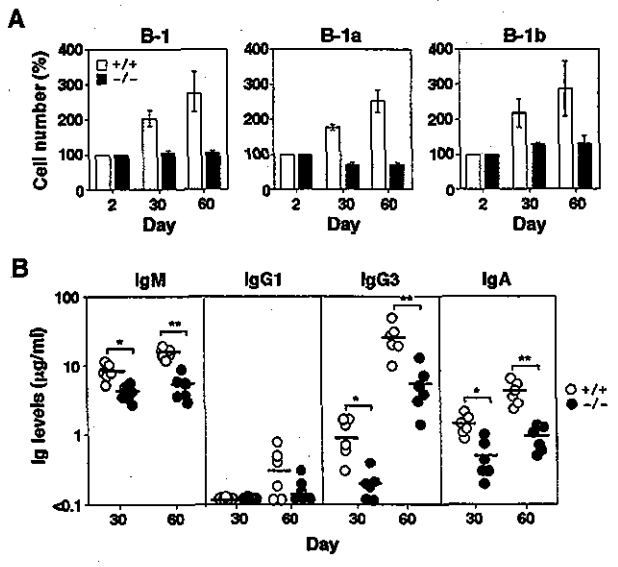

FIGURE 4. T cell-independent homeostatic proliferation of B-1 cells. *A*, B-1 cells from IL-5Rα$^{+/+}$ mice but not from IL-5Rα$^{-/-}$ mice show homeostatic proliferation even in T cell-deficient conditions. Purified B-1 cells were transferred (1×10^5 per head) i.p. into RAG-2$^{-/-}$ mice. The proportion of B-1 (IgM$^+$CD23$^-$), B-1a (IgM$^+$CD23$^-$CD5$^+$), or B-1b (IgM$^+$CD23$^-$CD5$^-$) cells in the PECs of RAG-2$^{-/-}$ mice was analyzed by flow cytometry. The recipient mice of each group were analyzed as described in Fig. 2 on days 2, 30, and 60. The data shown are representative results from two independent experiments. *B*, RAG-2$^{-/-}$ mice that have received IL-5Rα$^{-/-}$ B-1 cell transfer show low levels of Igs. On days 30 and 60, the concentrations of Igs in the serum of the recipient mice were determined by isotype-specific ELISA. The mean values of the indicated groups of mice are represented as a bar. **, $p < 0.01$; *, $p < 0.05$; by Student's *t* test.

suggest that IL-5 is also produced by non-T/non-mast/non-eosinophil cells and may support the maintenance and Ab production of mature B-1 cells in vivo.

Defective responses of IL-5Rα$^{-/-}$ B-1 cells to anti-CD40 mAb and LPS

B-1 cells that were smaller in size in IL-5Rα$^{-/-}$ mice and anti-IL-5-treated mice led us to address the possibility that these small cells might show an impaired response to various activation signals. We purified B-1 cells in PECs from IL-5Rα$^{+/+}$ and IL-5Rα$^{-/-}$ mice and stimulated them with anti-CD40, LPS, IL-4, or combinations of these. Interestingly, IL-5Rα$^{-/-}$ B-1 cells showed lower proliferation than did IL-5Rα$^{+/+}$ B-1 cells in response to anti-CD40 (58% of IL-5Rα$^{+/+}$ cells) and LPS (49% of IL-5Rα$^{+/+}$ cells) (Fig. 6*A*). Similar results were obtained when the cells were stimulated with anti-CD40 plus IL-4 and LPS plus IL-4. IL-5Rα$^{-/-}$ B-1 cells secreted significantly lower levels of IgM when they were cultured with anti-CD40, LPS, anti-CD40 plus IL-4, or LPS plus IL-4 than they did IgM secreted from IL-5Rα$^{+/+}$ B-1 cells (Fig. 6*B*, *upper panel*). Although IL-5Rα$^{-/-}$ B-1 cells were capable of producing IgG1 upon stimulation with anti-CD40 plus IL-4 or LPS plus IL-4, the amount of IgG1 produced by these cells was significantly lower (~55% and ~70%, respectively) than the amount of IgG1 produced by IL-5Rα$^{+/+}$ B-1 cells (Fig. 6*B*, *middle panel*). IgG3 production induced by LPS was also impaired in IL-5Rα$^{-/-}$ B-1 cells (Fig. 6*B*, *lower panel*). In contrast with B-1 cells, IL-5Rα$^{+/+}$ and IL-5Rα$^{-/-}$ B-2 cells in the spleen responded comparably upon anti-CD40 or LPS stimulation (data not shown).

Regulation of CD40 expression in B-1 cells by IL-5

One possible reason for the impaired response of IL-5Rα$^{-/-}$ B-1 cells to anti-CD40 may be the impaired expression of CD40. We compared the expression levels of CD40 on IL-5Rα$^{-/-}$ B-1 cells with those on IL-5Rα$^{+/+}$ B-1 cells and found that IL-5Rα$^{-/-}$ B-1 cells showed a significantly lower expression of CD40 than did IL-5Rα$^{+/+}$ B-1 cells (Fig. 7*A*). In contrast with B-1 cells, IL-5Rα$^{-/-}$ B-2 cells showed CD40 expression comparable with that of IL-5Rα$^{+/+}$ B-2 cells. CD40 expression on IL-5$^{-/-}$ B-1 cells was also lower than on wild-type B-1 cells (data not shown). B-1 cells from the anti-IL-5-treated mice showed reduced CD40 expression 6 days after treatment, whereas CD40 expression on B-2 cells was not affected (Fig. 7*B*). Conversely, IL-5 stimulation of IL-5$^{-/-}$ B-1 cells enhanced CD40 expression, whereas CD40 expression on B-2 cells was unaltered (Fig. 7*C*). These results strongly suggest that the IL-5 signal is important for CD40 expression and CD40-related activation in B-1 cells.

Defective IgA production in LPS-administered IL-5Rα$^{-/-}$ mice

As described previously, IL-5Rα$^{-/-}$ B-1 cells respond poorly to LPS stimulation (Fig. 6). Because TLR4/MD2 and RP105 expressed on B cells play an essential role in LPS-mediated B cell activation (43, 44), we examined TLR4/MD2 and RP105 expression on B-1 cells. IL-5Rα$^{+/+}$ B-1 cells showed very low levels of TLR4/MD2 expression and significant levels of RP105 expression. The expression levels of TLR4/MD2 and RP105 on B-1 cells were comparable between IL-5Rα$^{+/+}$ B-1 and IL-5Rα$^{-/-}$ B-1 cells (Fig. 8*A*). IL-5 might not be involved in the regulation of TLR4/MD2 or RP105 expression, but rather might participate in modulating LPS-induced intracellular signaling.

LPS is capable of inducing differentiation of B-1 cells in gut-associated lymphoid tissue (46). We orally administered LPS to IL-5Rα$^{+/+}$ and IL-5Rα$^{-/-}$ mice and examined Ig levels in serum and fecal extracts by ELISA. IgA levels in serum and fecal extracts in LPS-administered IL-5Rα$^{+/+}$ mice were elevated ~40% and ~400%, respectively, compared with those in the PBS-treated control group of mice. The levels of other Ig isotypes were comparable with those in the PBS-treated control mice (Fig. 8*B*). In contrast with the IL-5Rα$^{+/+}$ mice, the IgA levels in serum and fecal extracts in the IL-5Rα$^{-/-}$ mice did not increase as a result of oral LPS administration (Fig. 8*B*). To determine whether impaired IgA production due to LPS administration in IL-5Rα$^{-/-}$ mice was because of a decrease in IgA-producing cells, mononuclear cells were isolated from different tissues in LPS-administered mice and isotype-specific ELISPOT assays were performed. As shown in Fig. 8*C*, we detected a significant number of Ig-producing cells in the LP and PP in both IL-5Rα$^{+/+}$ and IL-5Rα$^{-/-}$ mice. Importantly, the number of IgA-producing cells in the LP and PP was significantly lower in LPS-administered IL-5Rα$^{-/-}$ mice than in LPS-administered IL-5Rα$^{+/+}$ mice. The number of IgM-producing cells in PECs in IL-5Rα$^{-/-}$ mice was also lower than in IL-5Rα$^{+/+}$ mice, whereas their splenocytes showed a number of IgM-producing cells comparable with that in IL-5Rα$^{+/+}$ mice. These results indicate that IL-5 signals are required for cells in the LP and PP to induce the optimal LPS response for terminal differentiation into Ab-producing cells in vivo.

Discussion

IL-5 and B-1 cell maintenance

A significant reduction in B-1 cells has been shown in IL-5Rα$^{-/-}$ and IL-5$^{-/-}$ mice (35, 38) and in 129 mice whose B cells show impaired response to IL-5 (47). These results imply that IL-5 is a

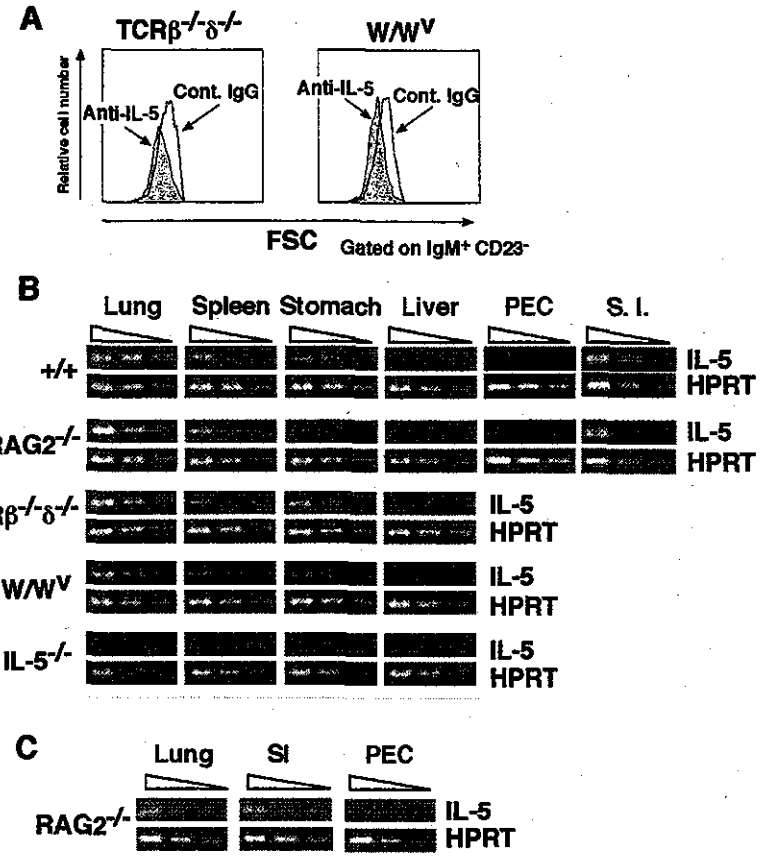

FIGURE 5. IL-5 production by cells other than T cells, mast cells, and eosinophils. *A*, Anti-IL-5 Ab treatment affects B-1 cell maintenance in T cell- or mast cell-deficient mice. A single i.p. administration of anti-IL-5 mAbs or control IgG (1 mg/250 μl) into TCR$\beta^{-/-}\delta^{-/-}$ or W/W^{V} mice was performed. On day 6 after treatment, peritoneal cells were obtained and analyzed. Representative histograms depict the relative cell number and size of the B-1 cells. Representative results of three independent experiments are shown. *B*, Tissues from T cell- or mast cell-deficient mice show *IL-5* mRNA expression. Various tissues were freshly isolated from IL-5R$\alpha^{+/+}$, RAG-$2^{-/-}$, TCR$\beta^{-/-}\delta^{-/-}$, W/W^{V}, and IL-$5^{-/-}$ mice. *C*, Non-T/non-mast/non-eosinophil cells express *IL-5* mRNA. Single cell suspensions were prepared from the lungs, small intestine (SI), and PECs of RAG-$2^{-/-}$ mice and c-*kit*$^{-}$ and IL-5Rα^{-} cells purified by negative sorting using MACS (>99% purity). Serial dilutions (4-fold) of cDNA templates were prepared and subjected to RT-PCR analysis using primer sets designed to amplify *IL-5* or HPRT cDNA fragments (*B* and *C*).

crucial cytokine for B-1 cell development or maintenance. Supporting this notion, we showed B-1 cells that were fully restored in number and function in *IL-5*R$\alpha^{-/-}$ mice due to the enforced expression of IL-5Rα by crossing with IL-5Rα transgenic mice (36). It should be noted that the decrease in B-1 cell proportion and number is more obvious in young IL-5R$\alpha^{-/-}$ mice than in older ones. This suggests that the IL-5 signal is at least required to facilitate B-1 cell development. However, it is not clear whether IL-5 is required for the maintenance of mature B-1 cells. Thus, the key question is to what extent IL-5 is involved in mature B-1 cell survival and homeostatic proliferation.

This study demonstrates the marked impairment of the maintenance of mature B-1 cell survival and its homeostatic proliferation by blocking IL-5 signals. Intriguingly, the administration of anti-IL-5 mAb into IL-5R$\alpha^{+/+}$ mice could induce a rapid reduction in the total number and size of B-1 cells within 6 days to a degree comparable with that observed in IL-5R$\alpha^{-/-}$ B-1 cells (Fig. 1*B*). Cell transfer experiments of CFSE-labeled B-1 cells to wild-type mice revealed that CFSE^{+} B-1 cells from wild-type mice survived longer in the peritoneal cavity than did those from IL-5R$\alpha^{-/-}$ B-1 cells (Fig. 2). This may not be due to the impairment of the migratory activity of IL-5R$\alpha^{-/-}$ B-1 cells, because the distribution pattern of CFSE-positive IL-5R$\alpha^{-/-}$ B cells in the LP, PP, and MLNs in the recipient mice 30 days after cell transfer was similar to that of CFSE-positive IL-5R$\alpha^{+/+}$ B cells (data not shown). We were surprised to see CFSE-positive B-2 cells (>50% more than starting cells) in the recipients 30 days after cell transfer, because B-2 cells are thought to be recirculating cells that do not reside in the peritoneal cavity. A proportion of B-2 cells in the peritoneal cavity tend to reside in or to migrate to the peritoneal cavity.

CFSE-labeled B-1 cells of wild-type mice expanded in the peritoneal cavity on day 30 of cell transfer in the RAG-$2^{-/-}$ mice, whereas IL-5R$\alpha^{-/-}$ B-1 cells did not (Figs. 3, *A* and *B*, and 4*A*). This again may not be due to the enhanced migration of IL-5R$\alpha^{-/-}$ B-1 cells in RAG-$2^{-/-}$ mice to the B cell compartment other than in the peritoneal cavity, because *IL-5*R$\alpha^{-/-}$sIgA^{+} B cells resided in the LP to lesser extent than did IL-5R$\alpha^{+/+}$sIgA^{+} B cells (Fig. 3*C*). The CFSE-labeled B-2 cells in RAG-$2^{-/-}$ mice expressed a wide range of CFSE labeling intensities (Fig. 3*B*). It is likely that cotransferred T cells may expand in the peritoneal cavity of RAG-$2^{-/-}$ recipient mice because of their ability to homeostatically proliferate, during which they may produce cytokines that induce the proliferation of B-2 cells. Alternatively, the B-2 cells that we detected may have been contaminated B-1 cell progenitors with long-lived and self-replenishing activity.

We were surprised to observe that both IL-5R$\alpha^{+/+}$sIgA^{+} B cells and IL-5R$\alpha^{-/-}$sIgA^{+} B cells showed relatively low CFSE labeling (Fig. 3*C*), suggesting extensive cell divisions before differentiation to sIgA^{+} B cells. Although CFSE intensities of B cells were similar, a reduced proportion of sIgA^{+} B cells in the LP of RAG-$2^{-/-}$ mice transferred with IL-5R$\alpha^{-/-}$ B cells was observed compared with mice transferred with IL-5R$\alpha^{+/+}$ B cells. This may be due to the impairment of cell survival and expansion of IL-5R$\alpha^{-/-}$ B-1 cells, although a small proportion of IL-5R$\alpha^{-/-}$ B-1 cells may be sufficient for proliferation and differentiation to sIgA^{+} B cells. Our results imply that the IL-5/IL-5R system plays an important role in maintaining mature B-1 cell survival and homeostatic proliferation in our short-term cell transfer assay.

IL-5-dependent B-1 cell maintenance in T cell- and mast cell-deficient mice

Although it is well known that T cells are a major IL-5 producer, we observed *IL-5*-mediated homeostatic proliferation of purified B-1 cells in recipient RAG-$2^{-/-}$ mice (Fig. 4). It was possible that

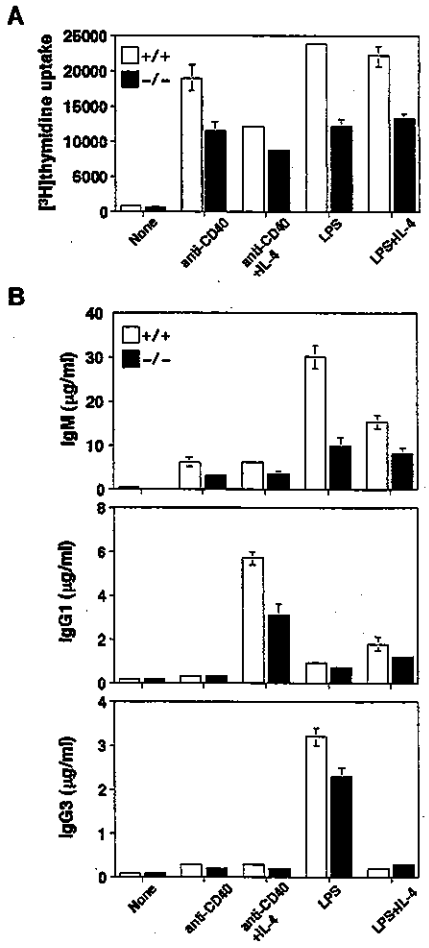

FIGURE 6. Defective activation of IL-5R$\alpha^{-/-}$ B-1 cells to anti-CD40 mAb or LPS. *A*, IL-5R$\alpha^{-/-}$ B-1 cells have defective proliferative responses to anti-CD40 mAb or LPS. B-1 cells purified by the MACS system were cultured (1×10^5 cells in a 200-μl culture) for 3 days with anti-CD40 mAb (1 μg/ml), LPS (40 μg/ml), IL-4 (1000 U/ml), or a selected combination of these agents. The cells were pulse-labeled with [^{3}H]thymidine (0.2 μCi/well) for the last 8 h of the culture. The results represent the mean cpm ± SD of the duplicate determinations. *B*, IL-5R$\alpha^{-/-}$ B-1 cells produce a small amount of Igs in response to anti-CD40 mAb or LPS. Purified B-1 cells were cultured (1×10^5 cells in a 200-μl culture) for 7 days with each stimulant as described in *A*. The IgM, IgG1, and IgG3 concentrations in the cultured supernatants were determined by ELISA. The values represent the mean and SD of the duplicate wells. The data shown are representative results from three independent experiments (*A* and *B*).

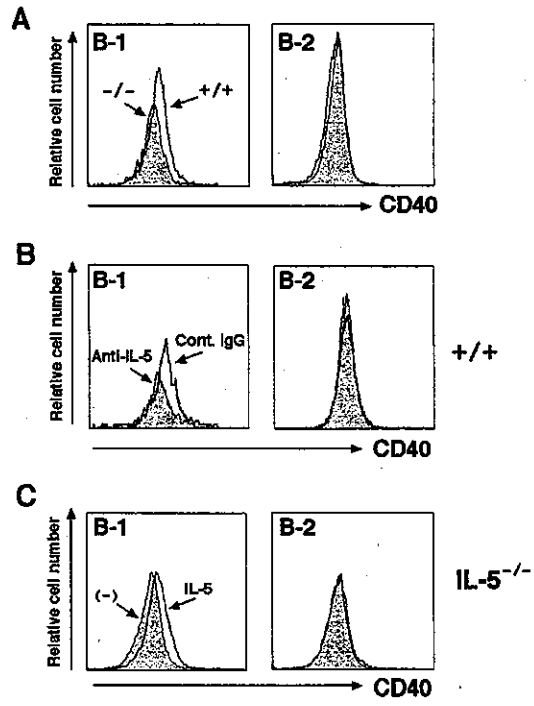

FIGURE 7. IL-5-dependent CD40 expression on B-1 cells. *A*, IL-5R$\alpha^{-/-}$ B-1 cells express low levels of CD40. *B*, Decreased CD40 expression on B-1 cells from anti-IL-5-treated mice is reduced. *C*, CD40 expression on IL-5$^{-/-}$ B-1 cells is recovered by IL-5 stimulation. Peritoneal cells from IL-5$^{-/-}$ mice were cultured (1×10^6 cells in a 2-ml culture) for 2 days with or without IL-5 (500 U/ml). The cultured cells were stained and analyzed by flow cytometry. Representative histograms show the CD40 expression of B-1 or B-2 cells from IL-5$^{-/-}$ mice, which were cultured for 2 days. Representative results from three different experimental sets are shown.

extremely low numbers of T cells in RAG-2$^{-/-}$ mice may provide T cell help, as described by Kushnir et al. (48), but we found no significant T cell population when we examined the PECs from RAG-2$^{-/-}$ mice 30 days after purified B-1 cell transfer (data not shown). Moreover, the results in which anti-IL-5-treated T cell-deficient mice show a reduction in number and cell size of B-1 cells in the peritoneal cavity also support our conclusion that non-T cells produce the IL-5 that supports maintenance and Ab production by B-1 cells (Fig. 5*A*). In fact, cells in various tissues including the lungs, stomach, and spleen of RAG-2$^{-/-}$ mice or TCR$\beta^{-/-}\delta^{-/-}$ mice showed *IL-5* mRNA expression (Fig. 5*B*). Moreover, small intestine and peritoneal washouts also expressed *IL-5* mRNA. Fort et al. (49) demonstrated using RAG-2$^{-/-}$ splenocytes that non-T/non-B cells produce IL-5 in response to IL-25 and that cells responding to IL-25 are accessory cells, which belong to the MHC class II^{high}, CD11c^{dull}, F4/80^{low}, CD8α^-, and CD4$^-$ populations. In vitro stimulation of mouse mast cells by FcϵRI cross-linking induces increased levels of mRNA expression or secretion of various inflammatory cytokines including IL-5 (50). W/W^V mice showed *IL-5* mRNA expression and IL-5-dependent B-1 cell maintenance (Fig. 5, *A* and *B*). In addition to T cells and mast cells, eosinophils and NK cells have also been shown to possess IL-5-producing ability (51, 52). This study shows that c-*kit*$^-$IL-5Rα^- cells purified from the lungs and small intestine of RAG-2$^{-/-}$ mice expressed *IL-5* mRNA (Fig. 5*C*). Our results support the notion that IL-5 can be produced even in T cell-, mast cell-, and eosinophil-deficient conditions, possibly by nonhemopoietic cells, leading to the support of B-1 cell maintenance.

IL-5 and CD40-related response of B-1 cells

T cell-dependent activation of B cells requires CD40-CD40 ligand (CD40L) interaction and a defined set of cytokines. Although B-1 cells are classified as B cells responding to T cell-independent Ags, T cells can influence other aspects of B-1 cell activation and differentiation. In fact, B-1 cells exhibit a strong proliferation and IgG1 production when cocultured with activated T cells plus IL-4 or with recombinant CD40L plus IL-4 (53). T cells enhance Ig production by B-1 cells and induce switching from IgM to IgG1 in B-1 cell-transferred SCID mice (10). Moreover, Erickson et al. (53) have demonstrated that B-1 cells require IL-5 in conjunction with CD40-CD40L interaction for maximal T cell-dependent responses. We showed that IL-5 regulates CD40 expression solely

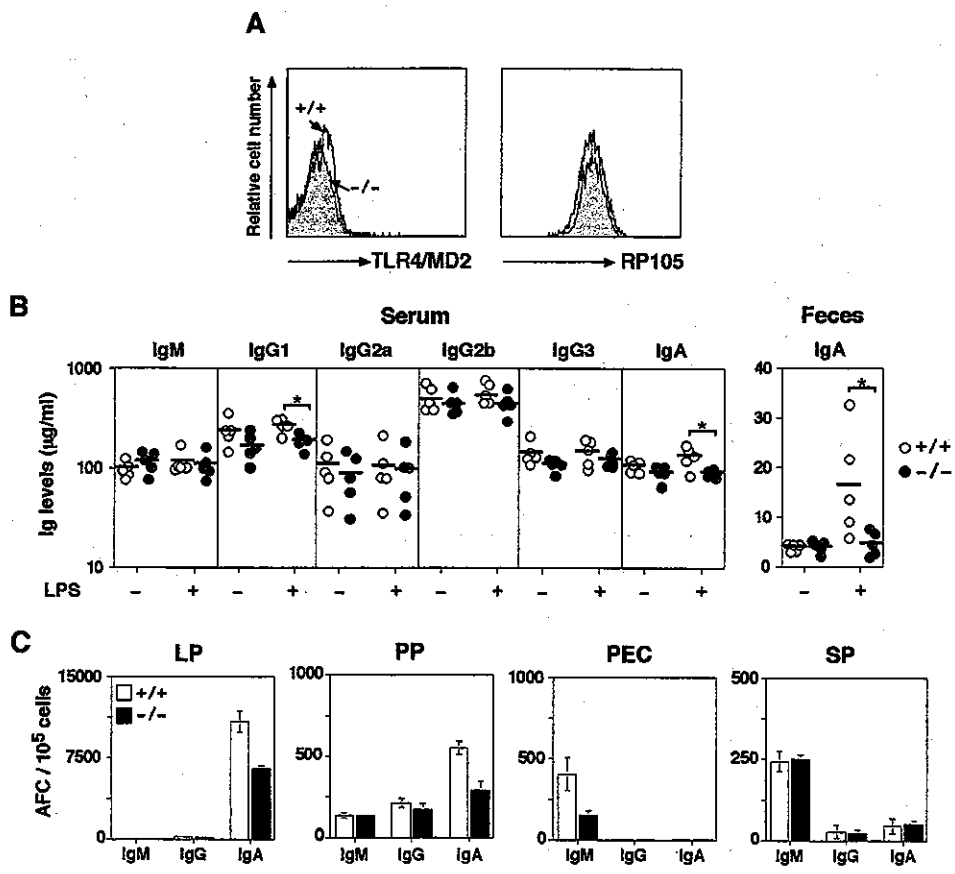

FIGURE 8. Ab production in IL-5R$\alpha^{-/-}$ mice that were administered LPS orally. *A*, IL-5R$\alpha^{-/-}$ B-1 cells express normal levels of TLR4/MD2 and RP105. Representative histograms show TLR/MD2 or RP105 expression on B-1 cells from IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice. *B*, IgA and IgG1 levels are not elevated in IL-5R$\alpha^{-/-}$ mice by oral injection of LPS. LPS (0.1 mg/200 μl/week) was injected orally into the gut of IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice for 3 wk. On day 7 after the last injection, the serum and fecal Ig levels of the mice were analyzed by isotype-specific ELISA. The mean Ig levels of the indicated group of mice are represented as a bar. *, $p < 0.05$. *C*, The numbers of Ab-producing cells are reduced in LPS-injected IL-5R$\alpha^{-/-}$ mice. IgM-, IgG-, or IgA-producing cells were examined in the LP, PP, peritoneal cavity, and spleen (SP) from LPS-injected mice by isotype-specific ELISPOT assay. The results represent the mean ± SD of the duplicate wells. Representative results of three independent experiments are shown (*A* and *C*).

on B-1 cells, not on B-2 cells (Fig. 7). Moreover, IL-5R$\alpha^{-/-}$ B-1 cells showed a defective response to anti-CD40 or anti-CD40 plus IL-4 (Fig. 6*A*). Taking these results together, we propose that constitutive stimulation by IL-5 is important for the full activation of B-1 cells in T cell-dependent response as well as LPS-dependent response in mucosal tissues as described below.

IL-5 and B-1 cell-derived IgA

IL-5 is an important cytokine for the mucosal immune system, which distinguishes it from the systemic immune compartment (54). IL-5 is postulated to be a major cytokine that induces sIgA$^+$ B-2 cells to differentiate into IgA-producing plasma cells in PP and to a lesser extent in the spleen (25, 54). Approximately one-half of IgA plasma cells in the LP of the intestine appear to be derived from B-1 cells in the peritoneal cavity, and B-1 cell-derived IgA is specific for commensal bacteria (55). Hiroi et al. (37) have reported the critical role of IL-5 in IgA secretion in mucosal tissues using IL-5R$\alpha^{-/-}$ mice. In IL-5R$\alpha^{-/-}$, the number of sIgA$^+$ B-1 cells from the effector site are significantly reduced, and IgA levels in mucosal secretions are reduced (37). Interestingly, there were significant differences in serum and fecal IgA levels in LPS-treated IL-5R$\alpha^{+/+}$ and IL-5R$\alpha^{-/-}$ mice (Fig. 8*B*). Although the B-1 cells of IL-5R$\alpha^{-/-}$ mice showed defective proliferation and Ig production upon LPS stimulation in vitro (Fig. 6), the expression levels of TLR4/MD2 and RP105 and the sensor of LPS signals on IL-5R$\alpha^{+/+}$ B-1 cells were comparable with those on IL-5R$\alpha^{-/-}$ B-1 cells (Fig. 8*A*). The IL-5-mediated signaling pathway may couple or cross-talk with the LPS-induced signaling pathway. Because LPS, CD40, and BCR triggering of B cells results in the activation of NF-κB factors (56), NF-κB activation induced by LPS or CD40 may be influenced by IL-5 in B-1 cells.

In summary, the present study provides new insight for an understanding of the important role of IL-5 in homeostatic proliferation and survival of mature B-1 cells. Furthermore, constant IL-5 stimulation may be required for optimal B-1 cell activation in response to CD40 or LPS requirements.

Acknowledgments

We are grateful to A. Kariyone, C. Kubo-Akashi, Y. Tezuka, and R. Shiraishi for their technical assistance. We thank T. Hiroi, H. Kaku, K. Horikawa, Y. Oe-Kikuchi, and T. Tamura for helpful discussion and our colleagues for critical reading of the manuscript.

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International Immunology, Vol. 16, No. 12, pp. 1691–1699
doi:10.1093/intimm/dxh170 Advance Access publication 11 October 2004

The role of antigenic peptide in CD4$^+$ T helper phenotype development in a T cell receptor transgenic model

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Keywords: altered peptide ligand, IFN-γ, Th1, Th2, Th1-inducing peptide, transgenic mouse

Abstract

CD4$^+$ Th1 cells play a critical role in the induction of cell-mediated immune responses that are important for the eradication of intracellular pathogens. Peptide-25 is the major Th1 epitope for Ag85B of *Mycobacterium tuberculosis* and is immunogenic in I-A^b mice. To elucidate the role of the TCR and IFN-γ/IL-12 signals in Th1 induction, we generated TCR transgenic mice (P25 TCR-Tg) expressing TCR α- and β-chains of Peptide-25-reactive cloned T cells and analyzed Th1 development of CD4$^+$ T cells from P25 TCR-Tg. Naive CD4$^+$ T cells from P25 TCR-Tg differentiate into both Th1 and Th2 cells upon stimulation with anti-CD3. Naive CD4$^+$ T cells from P25 TCR-Tg preferentially develop Th1 cells upon Peptide-25 stimulation in the presence of I-A^b splenic antigen-presenting cells under neutral conditions. In contrast, a mutant of Peptide-25 can induce solely Th2 differentiation. Peptide-25-induced Th1 differentiation is observed even in the presence of anti-IFN-γ and anti-IL-12. Furthermore, naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg also differentiate into Th1 cells upon Peptide-25 stimulation. Moreover, Peptide-25-loaded I-A^b-transfected Chinese hamster ovary cells induce Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg in the absence of IFN-γ or IL-12. These results imply that interaction between Peptide-25/I-A^b and TCR may primarily influence determination of the fate of naive CD4$^+$ T cells in their differentiation towards the Th1 subset.

Introduction

Naive CD4$^+$ Th cells recognize an antigenic peptide through their TCR in the context of MHC class II molecules on antigen-presenting cells (APC) and undergo differentiation to effector cells that can produce cytokines and chemokines. During this process, naive CD4$^+$ T cells can differentiate to at least two functionally distinct subsets of cells, represented by Th1 and Th2 (1). Th1 cells produce IFN-γ and lymphotoxin (TNF-β) in addition to IL-2 and are responsible for directing cell-mediated immune responses leading to the eradication of intracellular pathogens such as *Mycobacterium*, viruses and parasites (1–4). Th1 cells also regulate IgG2a and IgG3 antibody production via IFN-γ production, which is involved in the opsonization and phagocytosis of particulate microbes. Th2 cells secrete IL-4, IL-5 and IL-13 as effector cytokines and are responsible for humoral immune responses for the eradication of helminths. Th2 cells also cause inflammatory damage during allergic diseases, such as asthma and atopic dermatitis. The process by which an uncommitted Th cell develops into a mature Th1 or Th2 subset is a matter of fact for regulating the immune response to various antigens.

Considerable progress has been made in identifying the factors that govern the progression of cell differentiation during the generation of Th subsets (2–4). Using T cells stimulated with polyclonal activators or T cells from mice expressing transgenic antigen receptors of known specificities, it has become clear that Th1 and Th2 subsets develop

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Transmitting editor: K. Sugamura

Received 10 August 2004, *accepted* 13 September 2004

from the same T cell precursor (5–7), which is a naive $CD4^+$ T cell. There is a body of evidence to indicate that the cytokines IL-12 and IL-4 are key determinants of the Th1 and Th2 response, respectively (4). For example, IL-12 directs Th1 development from antigen-stimulated naive $CD4^+$ T cells and activates STAT4 in Th1 cells (8,9). In terminally differentiated Th1 cells, successive IFN-γ production can occur through TCR ligation or IL-12 and IL-18 stimulation. Using mice deficient in either cytokines or STAT, it has been shown that activation of the IFN-γR/STAT1 is also important for the differentiation of $CD4^+$ T cells into Th1 cells (10,11). The IL-4R/STAT6 signaling pathway plays a central role in the differentiation of naive $CD4^+$ T cells into Th2 cells (12–14). The balance of IFN-γ and IL-4 levels present during T cell activation is considered to be the major influence on Th1 versus Th2 differentiation. Although the strength of the interaction mediated through TCR and MHC/peptide complex is suspected to affect the lineage commitment of Th cells to Th1 cells and clonal expansion (15–17), it remains unclear whether Th1 cells can develop from naive $CD4^+$ T cells upon antigenic peptide stimulation in the presence of APC under neutral conditions.

Ag85B (also known as α antigen or MPT59) is the most potent antigen species yet purified for both humans and mice (18). Ag85B can elicit strong Th1 response *in vitro* from PPD^+ asymptomatic individuals (19–21). We have shown that *in vitro* stimulation of lymph node cells from *Mycobacterium tuberculosis*-primed C57BL/6 mice with Ag85B induces the production of IFN-γ and IL-2 and expansion of $CD4^+$ T cells expressing Vβ11 of TCR (TCRVβ11) in an $I\text{-}A^b$-restricted manner (22,23). We identified the 15-mer peptide (Peptide-25), covering amino acids residues 240–254 (FQDAYNAAGGHNAVF) of Ag85B, as the major epitope for Ag85B-specific $TCRV\beta11^+$ T cells (22). Using Peptide-25-reactive $V\beta11^+$ T cell clones (BP1, BP4, BM5, BM7 and BM12) and substituted Peptide-25 mutants, we determined which amino acid residues within Peptide-25 were critical for TCR recognition (23,24). Peptide-25 contains the motif that is conserved for $I\text{-}A^b$ binding and requires processing by APC to trigger Ag85B-specific $TCRV\beta11^+$ T cells (22). Active immunization of C57BL/6 mice with Peptide-25 can induce the differentiation of $CD4^+$ $TCRV\beta11^+$ Th1 that produce IFN-γ and TNF-α and protect against subsequent infection with live *M. tuberculosis* H37Rv (23).

Here we generate transgenic mice (P25 TCR-Tg) expressing functional TCR that interacts with Peptide-25 in conjunction with $I\text{-}A^b$. We report that naive $CD4^+$ T cells in the spleen of P25 TCR-Tg mice respond specifically to Peptide-25 in the presence of APC from $I\text{-}A^b$ mice and differentiate to Th1 cells in the absence of IFN-γ or IL-12 under neutral conditions.

Methods

Mice

C57BL/6 mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). STAT1 deficient mice were kindly provided by Dr R. D. Schreiber, Center for Immunology, Washington University School of Medicine. These mice were maintained under specific pathogen-free conditions in our animal facility according to our Institute's guidelines, and used at 8–15 weeks of age.

Cell lines

Five different Peptide-25-reactive $CD4^+$ Th1 clones (BP1, BP4, BM5, BM7 and BM12) were established *in vitro* by culturing lymph node cells from C57BL/6 mice immunized with heat-killed *M. tuberculosis* H37Rv as described (23). TG40 is a variant T cell hybridoma cell line lacking the expression of surface TCR-α and -β chains that has been used a recipient cells for TCR (25). PLAT-E is a packaging cell line that produces retroviruses (26). Chinese hamster ovary cells expressing $I\text{-}A^b$ ($I\text{-}A^b$-CHO) (27) were kindly provided by Dr Y. Fukui (Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan).

Reagents and antibodies

All peptides including Peptide-25 and its substituted mutants were synthesized by Funakoshi Co. Ltd (Tokyo, Japan). Anti-IFN-γ–FITC (XMG1.2), anti-IL-4–allophycocyaninn (11B11), anti-Vβ11–PE (RR3-15), anti-CD4–FITC or -PE (GK1.5), anti-CD8–PE (53.6.72), anti-CD25–FITC (7D4), anti-CD28–FITC (37.51), anti-CD69–FITC (H1.2F3), anti-CD44–FITC (IM7), anti-CD45RB–PE (16A) and anti-LFA1–FITC (2D7) were purchased from BD Biosciences PharMingen (San Diego, CA). Purified anti-CD3 ε (2C11), anti-IFN-γ (R4-6A2) and anti-IL-12 (C17.8) were purchased from BD Biosciences PharMingen.

Subcloning of TCR

Total cellular RNA was isolated from BP1 by using acid guanidinium–phenol–chloroform method. cDNA was synthesized with random hexamer primers and superscript II cDNA kit (GIBCO BRL, Grand Island, NY). 5′-Rapid amplification of cDNA end (5′-RACE) was performed using the 5′-RACE System Ver.2.0 (Life Technologies, Rockville, MD) according to the manufacturer's instructions. The first strand of cDNA was synthesized with gene-specific primer 1 (5′-ATCCATAGCTTTCATGTCCA for TCR α-chain and 5′-GCCATTCACCCACCAGCTCA for TCR β-chain). The first PCR amplification was carried out by using gene-specific primer 2 (5′-GCGAATTCTGAGACCGAGGATCTTTTAACTGGTAC for TCR α-chain and 5′-GCGTCGACTCTGCTTTTGATGGCTCAAAC for TCR β-chain). The second PCR amplification was carried out with nested gene-specific primer (5′-GCGTCGACACAGCAGGTTCTGGGTTCTGGAT for TCR α-chain and 5′-GCGTCGACAAGGAGACCTTGGGTGGAGTCAC for TCR β-chain). The PCR fragment was subcloned in Bluescript SK^+ and sequences by automatic DNA sequencer (ABI PRISM 3700 DNA analyzer, Applied Biosystems, Foster City, CA).

Retrovirus-mediated gene transfer

Full length cDNAs genes encoding the TCR α- and β-chains of BP1 were inserted into a retroviral vector pMX-IRES-GFP vector, pMX-BP1-α and pMX-BP1-β, respectively, and were transfected into a retroviral packaging cell line, PLAT-E with LipofectAMINE Plus Reagent (GIBCO BRL) (28) The cultured supernatant of PLAT-E after 24 h culture was collected, and added to TG40 cells together with DOTAP Liposomal Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) (29). Transfection was monitored by the cell surface expression of TCR by FACS analysis. TG40 cells were transfected with each of plasmids or in their combinations

and selected TG40 cells expressing TCR-αβ (TG40-BP1). TG40-BP1 cell line for expression of CD4 (TG40-BP1/CD4) was established by electroporation of the expressible constructs of full length CD4 cDNA into TG40-BP1 cells by Gene Pulser (Bio-Rad laboratories, Hercules, CA).

Establishment of transgenic mice

The transgenic TCR-α and -β genes were isolated from BP-1 as described in the previous session. The DNA sequences of the PCR products revealed that BP1-TCR-α was composed of Vα5, Jα15 and Cα1, and the TCR-β chain of Vβ11, Jβ2.3 and Cβ2. The pHSE3′ plasmid contains the H-2K^b promoter (provided by H. Pircher), a poly(A) signal from β-globin and the immunoglobulin heavy chain enhancer (30). The full-length BP1 TCR α and β cDNAs were subcloned into the *Sal*I and *Bam*HI sites of the expression vector pHSE3′ plasmid under control of the H-2K^b promoter. The constructs were excised from these plasmids by *Xho*I cleavage for TCR-α chain and *Apa*LI cleavage for TCR-β, and purified by using QIAEX II gel extraction system (Qiagen Inc., Valencia, CA). The purified expression constructs for TCR α and β cDNAs were co-injected into fertilized eggs of C57BL/6 mice. We finally obtained a TCR-Tg line of mice expressing TCR-Vα5-Vβ11 (P25 TCR-Tg). P25 TCR-Tg mice were bred to STAT1 deficient mice (STAT1 deficient P25 TCR-Tg) on a C57BL/6 background in our animal faculty under specific pathogen-free conditions.

Preparation of naive CD4$^+$ T cells and APC

Splenic T cells from either P25 TCR-Tg or littermate C57BL/6 mice were enriched by passing splenocytes through a nylon wool column. To further purify primary CD4$^+$ T cells, the splenic T cells were incubated with a mixture of Microbead-bound monoclonal antibodies that were specific for CD8 (53-6.72), CD49b (DX5), B220 (RA3-6B2) and I-A^b (M5/114.15.2) (Miltenyi Biotec, Bergisch Gladbach, Germany). MEL-14^{high} T cells were purified from splenic CD4$^+$ T cells by positive sorting using MACS after treatment with anti-CD62L (MEL-14)-Microbeads (Miltenyi Biotec) and were used as naive CD4$^+$ T cells. The purity of CD4$^+$ naive T cells was >98%. Splenocytes from wild-type (WT) C57BL/6 mice were incubated with a mixture of anti-Thy1 (30-H12)-Microbeads and anti-CD49b-Microbeads (Miltenyi Biotec) to deplete T cells and NK cells. Cells were then recovered by passage through a MACS column according to the manufacturer's instructions. Recovered cells were irradiated with a total of 3500 Rad, and used as APC. I-A^b-CHO was incubated with 10 μg/ml Peptide-25 for 12 h and extensively washed and incubated with 50 μg/ml mitomycin C for 15 min in 37°C and used as APC in some experiments.

Cell culture

Stably transfected TG40-BP1 or TG40-BP1/CD4 cells (1 × 10^4/culture) were stimulated with various concentrations of peptide in the presence of irradiated spleen cells (5 × 10^5/culture) from various strains of mouse in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark). The cultured supernatants were collected and subjected to ELISA.

To examine Th differentiation *in vitro*, two-step cultures were employed. For the first culture, purified splenic naive CD4$^+$ T cells (5 × 10^5/culture) were activated for 6 days with 10 μg/ml of anti-CD3 or 10 μg/ml Peptide-25 or its substituted mutant in the presence of T- and NK cell-depleted C57BL/6 splenic APC (2.5 × 10^6/culture) in a 48-well plate. In some experiments, we used Peptide-25 loaded I-A^b-CHO (2.5 × 10^5/culture) as APC. For the second culture, the cells collected from the first culture were extensively washed and dead cells were removed by centrifugation through Ficol–Hypaque gradients. The viable primed CD4$^+$ T cells were re-stimulated with 10 μg/ml of anti-CD3 or 10 μg/ml of Peptide-25 in the presence of splenic APC or 1 μg/well of immobilized anti-CD3.

Intracellular cytokine staining and FACS analysis

We identified cytokine-producing cells by cytoplasmic staining with anti-cytokine antibody as previously described (24). First, 2 μM of Monensin (BD Biosciences PharMingen) was added to the secondary culture for the last 4 h of each stimulation. The cells were harvested at 24 h of the secondary culture and stained with 7-amino-actinomycin D and with anti-Vβ11–PE or anti-CD4–PE. The cells were fixed with 4% formaldehyde after washing with 0.05% azide–1% FCS–PBS, permeabilized with 0.1% saponin, and stained with both anti-IFN-γ–FITC and anti-IL-4–allophycocyanin. Isotype-matched control antibodies were also used. The cells stained were gated on live Vβ11- or CD4-positive cells and analyzed on a FACSCalibur instrument (Becton Dickinson, Mountain View, CA).

ELISA

Amounts of IL-2, IL-4 and IFN-γ in the culture supernatant were measured by ELISA. All monoclonal antibodies specific for mouse IL-2, IL-4 and IFN-γ used for capture and detection of cytokines were purchased from BD Biosciences PharMingen. ELISA was performed following the instruction of BD Biosciences PharMingen.

ELISPOT assay

Cytokine producing cells were identified by ELISPOT assay, using the IFN-γ and IL-4 ELISPOT assay kits (R&D Systems, Minneapolis, MN). After naive CD4$^+$ T cells from P25 TCR-Tg mice were cultured with Peptide-25-loaded I-A^b-CHO for 20 h in a 96-well plate coated with capture antibodies, ELISPOT assay was performed following the manufacturer's instructions. Spots were analyzed by KS ELISPOT compact (Carl Zeiss, Oberkochen, Germany).

Results

Analysis of Peptide-25 recognition by reconstituted TCR-αβ pairs

To investigate the functional TCR able to bind a Peptide-25/MHC complex at the clonal level, we first determined the usage of TCR-α and -β chains of Peptide-25-reactive Vβ11$^+$ Th1 clone (BP1) that was of C57BL/6 (I-A^b) mouse origin (23) with the use of 5′-RACE. BP1-TCR α-chain was found to be composed of Vα5 and Jα15 and Cα (Accession No.: AB183189). BP1-TCR β-chain was also identified to be Vβ11, Jβ2.3 and Cβ2 (Accession No.: AB183190).

In order to analyze Peptide-25-recognition by TCR dimers composed of the TCR α- and β-chains of BP1, TCR α- and

β-chain were subcloned into a retrovirus vector and then transfected by retrovirus-mediated gene transfer into a TCR-αβ- and CD4-deficient recipient T cell hybridoma cell line, TG40 (28), and the reconstruction and functional specificity of the TCR was assessed by measuring IL-2 production (Fig. 1). TG40-BP1 produced substantial amounts of IL-2 in response to Peptide-25 plus APC in a dose dependent manner. Enforced expression of CD4 molecules on TG40-BP1 (TG40-BP1/CD4) augmented IL-2 production even upon a lower dose of Peptide-25 stimulation (0.3 μg/ml). TG40-BP1/CD4 produced much more IL-2 than TG40-BP1 when stimulated with higher concentrations of Peptide-25 (10 μg/ml) (Fig. 1A). TG40 transfectants of TCR-α alone or TCR-β alone did not respond to Peptide-25 in the presence of splenic APC (data not shown). These results indicate that recombinant TCR α- and β-chains can reconstruct functional TCR and recognize Peptide-25/I-A^b complex to become IL-2-producing cells.

The specificity of BP1 TCR for Peptide-25 and splenic APC from C57BL/6 mice was examined by culturing TG40-BP1/CD4 with various I-A^b-binding peptides in the presence of APC from different strains of mice. Although we do not show data here, among the various peptides only Peptide-25 could induce IL-2 production by TG40-BP1/CD4 in the presence of splenic APC from C57BL/6 (I-A^b) mice. The 11-mer from Peptide-25 was stimulatory while the 8-mer from Peptide-25 was ineffective. We then stimulated TG40-BP1/CD4 cells with a mutant of Peptide-25 as an altered peptide ligand (APL). The APL preserves those amino acid residues within Peptide-25 essential for I-A^b binding, while one of TCR-binding amino acid residues, glutamic acid at position 248 of Peptide-25, was substituted to alanine, G248A. The APL stimulation at 10 μg/ml of TG40-BP1/CD4 induced marginal IL-2 production, and the stimulatory activity was much lower than with Peptide-25 (Fig. 1B).

We then determined the amino acid sequences for the TCR-α and -β chains of four other Peptide-25-reactive Th1 clones (BP4, BM5, BM7 and BM12). All these Th1 clones responded to Peptide-25 for proliferation and IFN-γ production (23). Analysis of the TCR-α and -β chain amino acid sequences for each clone revealed no obvious differences from BP1 except in the sequence and in the length of CDR3 regions of TCR α- and β-chain (Supplementary table 1, available at *International Immunology* Online). Taking all these results together, the TCR-α5 and -β11 can reconstitute a functional TCR complex that is able to recognize and respond to Peptide-25 when presented in the context of I-A^b. As BP1 is the best Peptide-25-reactive Th1 clone with respect to IFN-γ production in response to Peptide-25, we chose BP1 TCR cDNAs for generating P25 TCR-Tg mice.

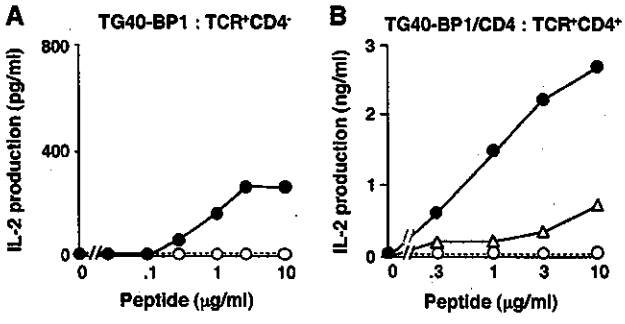

Fig. 1. IL-2 production of TG40 transfectants upon stimulation with Peptide-25. TG40 transfectants retrovirally introduced TCR-αβ of BP1, TG40-BP1 (TCR^+ $CD4^-$) and TG40-BP1 transfectants of CD4, TG40-BP1/CD4 (TCR^+ $CD4^+$) (1×10^4 cells/culture) were stimulated with various concentrations of Peptide-25 in the presence (closed circles) or absence (open circles) of irradiated C57BL/6 spleen cells (5×10^5 cells/culture) as APC in 96-well microplates. We also stimulated TG40-BP1/CD4 with APL (triangles) in the presence of C57BL/6 spleen cells (5×10^5 cells/culture) as APC. After incubation for 24 h, IL-2 in the cultured supernatants were titrated by ELISA.

Generation of Peptide-25-reactive TCR-Tg mice

We then analyzed the clonal basis of preferential Th1 development by single TCR-Tg mice line expressing TCR-α5 and -β11. We constructed transgenes for TCR α5- and β11-chains under the control of the H-$2K^b$ promoter, the poly(A) signal from human β-globin gene and the immunoglobulin heavy chain enhancer. The transgenes were excised from the vector sequences and co-microinjected into fertilized eggs from C57BL/6 mice. Transgenic mice were screened by Southern blot analysis of tail DNA and by staining peripheral blood T cells with anti-Vβ11, followed by FACS analysis. We obtained founder mice expressing $Vα5^+$-$Vβ11^-$, $Vα5^-$-$Vβ11^+$ and $Vα5^+$-$Vβ11^+$ T cells. In the present study, we have mainly analyzed TCR transgenic (P25 TCR-Tg) mice expressing both TCR-Vα5 and -Vβ11.

FACS analysis revealed that >85% of splenic $CD4^+$ T cells from the P25 TCR-Tg mice expressed TCR β11-chain, while 5–7% of splenic $CD4^+$ T cells were $Vβ11^+$ in WT mice (22). Over 98% of splenic $CD4^+$ T cells from the RAG-2 deficient P25 TCR-Tg mice expressed TCR Vβ11-chain. Similar results were obtained by staining splenic $CD4^+$ T cells from P25 TCR-Tg mice with anti-idiotypic antibody (KN7) for the recombinant TCR αβ (A.K. and K.T., unpublished observation). We did not observe any significant $KN7^+$ lymph node cells from transgenic mice expressing TCR α-chain alone or β-chain alone. We compared the expression patterns of LFA-1, CD25, CD28, CD44, CD45RB and CD69 on splenic $CD4^+$ T cells from P25 TCR-Tg mice with those from WT mice. There were no significant differences in the expression pattern or mean fluorescence intensity of these cell surface molecules between the two groups. RT-PCR analysis revealed that T-bet and IFN-γ mRNA expressions were not detected in freshly prepared splenic $CD4^+$ cells of P25 TCR-Tg mice. Taking these results together, $CD4^+$ T cells from P25 TCR-Tg mice are not pre-activated *in vivo*.

Naive $CD4^+$ T cells from P25 TCR-Tg mice are able to differentiate into both Th1 and Th2

Naive $CD4^+$ T cells from P25 TCR-Tg and WT mice were purified from the spleen and stimulated *in vitro* with anti-CD3 in the presence of exhaustively T- and NK cell-depleted irradiated C57BL/6 splenocytes as APC. After 6 days in culture, the proliferated cells were harvested and re-stimulated for another day with anti-CD3 in the presence of APC. After culturing, IFN-γ- and IL-4-producing cells were analyzed by intracellular staining. The cultured supernatants were subjected to ELISA assay for cytokine titration. The results revealed that *in vitro* stimulation of naive $CD4^+$ T cells from

P25 TCR-Tg mice with anti-CD3 induced the propagation of both IFN-γ- and IL-4-producing cells to a similar extent as from WT mice (Fig. 2A). The IFN-γ and IL-4 production were confirmed by ELISA (Fig. 2B). It is also evident from Fig. 2 that P25 TCR-Tg T cells has a higher proportion of IFN-γ-producing cells and IFN-γ production upon anti-CD3 stimulation compared with T cells from WT mice. These results indicate that naive CD4$^+$ T cells from P25 TCR-Tg mice can differentiate into both Th1 and Th2 upon TCR cross-linking.

Induction of naive CD4$^+$ T cells from P25 TCR-Tg mice to Th1 differentiation upon Peptide-25 stimulation

To examine the differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon *in vitro* Peptide-25 stimulation, naive CD4$^+$ splenic T cells were purified from P25 TCR-Tg mice and stimulated *in vitro* for 6 days with Peptide-25 in the presence of T and NK cell-depleted irradiated C57BL/6 splenocytes as APC. The activated cells produced IL-2 and proliferated upon Peptide-25 stimulation in a dose dependent manner in the presence of APC, but they did not produce IL-2 in the absence of Peptide-25 or in the presence of APC from strains of mice other than C57BL/6 mice (data not shown).

In another set of cultures, we stimulated naive CD4$^+$ T cells from P25 TCR-Tg mice *in vitro* with Peptide-25. After 6 days in culture, the proliferated cells were re-stimulated for another day with immobilized anti-CD3. After culturing, IFN-γ- and IL-4-producing cells were analyzed by cytoplasmic staining, followed by FACS analysis. The cultured supernatants were subjected to ELISA for titration of cytokine levels. As a control, we also cultured the cells with APL or medium alone. Naive CD4$^+$ T cells stimulated with Peptide-25 in the presence of splenic APC became solely IFN-γ-producing cells under neutral conditions (Fig. 3A). IFN-γ production was detected on the first day of culture and increased for the rest of the culture period at day 5 (data not shown). IL-4 secretion was not detected even after 5 days of culture. Importantly, stimulation of the cells with APL, in place of Peptide-25, solely induced IL-4-producing cells (Fig. 3B). When we cultured naive CD4$^+$ T cells and splenic APC in the absence of Peptide-25 or APL in the primary culture, cells did not proliferate well (data not shown). These results indicate that naive CD4$^+$ T cells from P25 TCR-Tg mice can be activated leading to proliferation and differentiate solely into Th1 cells upon stimulation with Peptide-25 under neutral conditions.

Roles of IFN-γ/STAT1 and IL-12 signaling in the Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice

It is well known that in addition to the TCR signals IFN-γ and IL-12 play an important role in the Th1 development. To examine whether IFN-γ and IL-12 are required for Th1 development, we

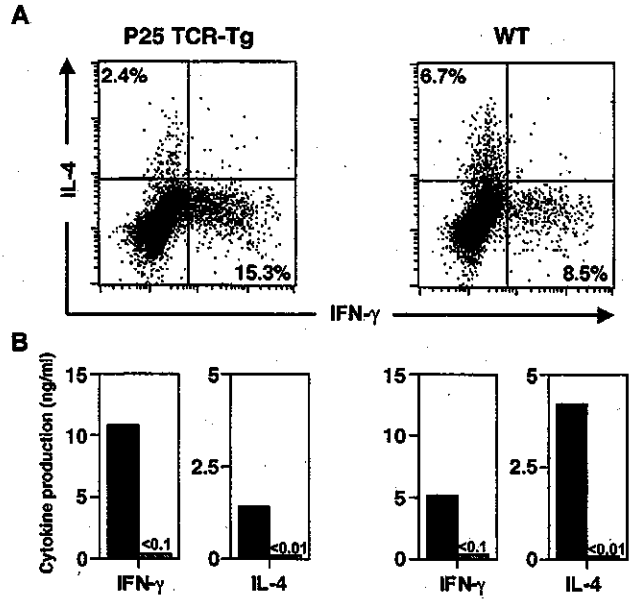

Fig. 2. Induction of Th1 and Th2 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with anti-CD3. Naive CD4$^+$ T cells from P25 TCR-Tg and WT mice were purified and cultured with 10 μg of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for 6 days. (A) After the culture, the cells were washed extensively and re-stimulated with 10 μg/ml of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for another day. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively. (B) After the culture, the cells were washed extensively and re-stimulated with (black bar) or without (hatched bar) 10 μg/ml of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for another day. IFN-γ and IL-4 in the cultured supernatants were titrated by ELISA.

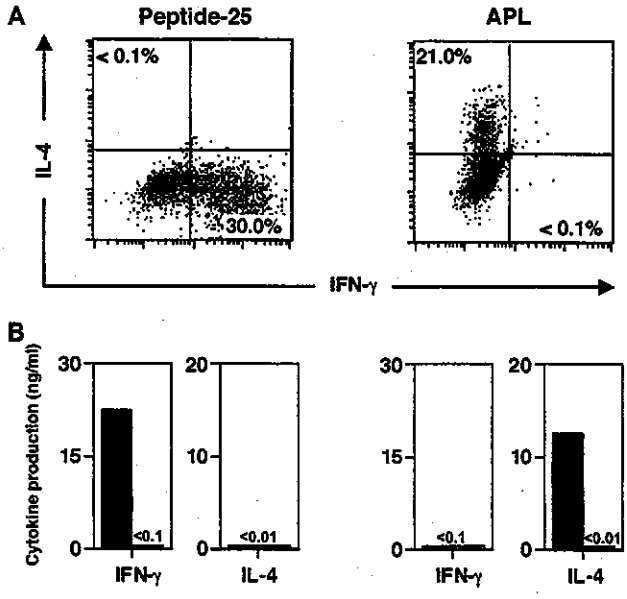

Fig. 3. Induction of Th1 and Th2 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25 and APL, respectively. Naive CD4$^+$ T cells from P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 or APL for 6 days. (A) On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for another day. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively. (B) On day 6, the cells were washed and re-stimulated with (black bar) or without (hatched bar) 1 μg/well of immobilized anti-CD3 for another day. IFN-γ and IL-4 in the cultured supernatants were titrated by ELISA.

cultured naive CD4$^+$ T cells from P25 TCR-Tg mice with Peptide-25 and splenic APC in the presence of anti-IFN-γ, anti-IL-12 or anti-IFN-γ and anti-IL-12 for 6 days. Results revealed that IFN-γ-producing cells were predominantly observed even when cultured in the presence of anti-IFN-γ and anti-IL-12 (Fig. 4). It was also evident that addition of anti-IL-12 partially reduced the proportion of IFN-γ-producing cells without enhancing IL-4-producing cells, while the addition of anti-IFN-γ treatment slightly increased the frequencies of both IFN-γ- and IL-4-producing cells. These results imply that IFN-γ and IL-12 are not essential for Th1 development of CD4$^+$ T cells from P25 TCR-Tg mice in response to Peptide-25. To evaluate further the role of IFN-γ in the Th1 development, we examined the differentiation fate of naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg mice upon Peptide-25 stimulation. This result revealed that Peptide-25-stimulated naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg mice became solely IFN-γ-producing cells after 6 days of culture under neutral conditions (Fig. 5).

Induction of IFN-γ-producing cells upon stimulation of naive CD4$^+$ T cells from P25 TCR-Tg mice with Peptide-25-loaded I-A^b-CHO

To elucidate the mechanism that ensures Th1 differentiation upon TCR stimulation with peptide/MHC, naive CD4$^+$ T cells were stimulated *in vitro* with Peptide-25-loaded I-A^b-CHO for 20 h and assayed for IFN-γ and IL-4 production by ELISPOT assay. IFN-γ-producing cells were induced upon treatment with Peptide-25-loaded I-A^b-CHO stimulation in a dose-dependent manner; however, IL-4-producing spots were not detected. Neither IFN-γ nor IL-4 spots were detected when naive CD4$^+$ T cells from P25 TCR-Tg mice were cultured *in vitro* without Peptide-25-loaded I-A^b-CHO for 20 h. These results indicate that activated CD4$^+$ T cells stimulated with Peptide-25/I-A^b produced IFN-γ in primary culture within 24 h.

To evaluate the role of IFN-γ and IL-12 in Th1 development, naive CD4$^+$ T cells from P25 TCR-Tg mice were stimulated for 6 days *in vitro* with Peptide-25-loaded I-A^b-CHO in the presence of anti-IFN-γ and anti-IL-12. At 24 h after the re-stimulation with immobilized anti-CD3, the frequency of IFN-γ producing cells was 14.5% for the live CD4$^+$ T cells (13% for the live TCRVβ11$^+$ T cells) (Fig. 6), indicating that naive CD4$^+$ T cells can differentiate into Th1 by TCR activation with Peptide-25/I-A^b stimulation even in the absence of IFN-γ and IL-12. In separate experiments, we confirmed IFN-γ-producing cells when CD4$^+$ naive T cells from RAG-2$^{-/-}$ P25 TCR-Tg mice were cultured with Peptide-25-loaded I-A^b-CHO even in the presence of anti-IFN-γ or anti-IL-12. Therefore, direct interaction between Peptide-25/I-A^b and TCR may determine the fate of naive CD4$^+$ T cells for differentiating into Th1 subsets.

Discussion

Peptide-25 is the major antigenic epitope for Ag85B of *M. tuberculosis*, is immunogenic in C57BL/6 (I-A^b) mice, and preferentially induces Vβ11$^+$ Th1 cells. It remains unclear why Peptide-25 can preferentially induce Th1 immune responses in C57BL/6 mice. We approached this question by analyzing naive CD4$^+$ T cells from transgenic mice, whose T cells express functional TCR capable of recognizing Peptide-25 in the context with I-A^b molecules. In the present study we generated TCR-Tg mice for the Th1-inducing peptide, Peptide-25, to elucidate the role of TCR signals in the decision of CD4$^+$ T cells to development into either a Th1 or Th2 cell. Our data support the notion that TCR signals may play a role in the determination of Th1 development under neutral conditions in the absence of IFN-γ or IL-12.

We determined usage of TCR α-chain in five different Peptide-25-reactive Vβ11$^+$ Th1 clones. All Peptide-25-reactive Vβ11$^+$ Th1 clones expressed Vα5, while each clone showed slightly different amino acid sequences in CDR3 regions of both Vα5 and Vβ11 chains (Supplementary table 1). Although each Th1 clone responds to Peptide-25 to a similar extent with regard to proliferation and IFN-γ production, it responds differently to a mutant of Peptide-25 where an amino acid required for TCR-binding had been substituted to alanine (data not shown). However, this may be due to the heterogeneity of the CDR3 regions of both Vα5 and Vβ11 chain. TG40 transfectants (TG40-BP1) expressing α and β chains from the BP1 clone constructed functional TCRs that recognize Peptide-25 in the context of I-A^b on APC resulting in IL-2 production even in the absence of CD4 expression (Fig. 1A). Enforced expression of CD4 in TG40-BP1 enhanced IL-2 production along with a low dose of Peptide-25 stimulation (Fig. 1B), suggesting that the avidity of the TCR and Peptide-25/I-A^b complex is potent enough to trigger TG40-BP1

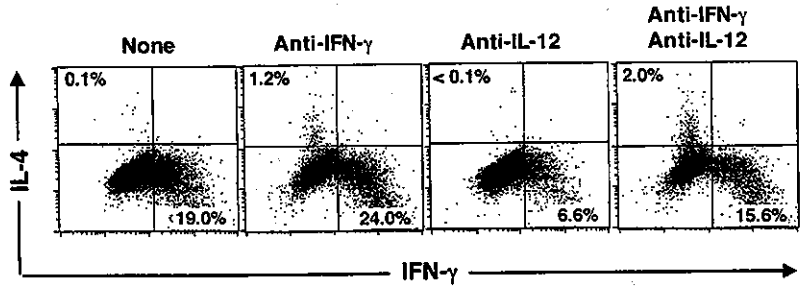

Fig. 4. Effect of anti-IFN-γ and anti-IL-12 on Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25. Naive CD4$^+$ T cells in the spleen of P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 for 6 days. Anti-IFN-γ (10 μg/ml), anti-IL-12 (10 μg/ml) or anti-IFN-γ (10 μg/ml) plus anti-IL-12 (10 μg/ml) were added at the onset of culture. On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for another day in the absence of antibodies. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.