CD19 陽性細胞上の CD40 と CD80 は投与後速やかに発現分子数が減少し、半年後も減弱が維持された。興味深いことに、治療 4 週後も $CD4^+C45RO^+$メモリー T 細胞数は変化がなかったが、CD4 陽性細胞上の CD69, CD40L と ICOS の発現が低下した。

一方、DNA マイクロアレイによる末梢血 mRNA 発現の短期的変動解析では、p38MAPK などのシグナル関連分子の低下を認めた。

さらに、治療抵抗性 SLE 患者では、多剤耐性遺伝子 MDR-1 の産物である P-糖蛋白質の発現が亢進していたが、リツキシマブ治療により $CD19^+$B 細胞、$CD4^+$T 細胞の双方にて発現量、発現率が低下した。実際、患者リンパ球に In vitro で放射線標識デキサメタゾンを添加すると、リツキシマブにより細胞内のデキサメタゾン残留が回復した。

D. 考察

リツキシマブの作用機序としては、メモリーB 細胞の再出現を制御してナイーブ B 細胞の再構築を生じ、免疫複合体が関与する腎障害などが改善したと考えられてきた(液性免疫の制御)。しかし、中枢神経症状の速やかな改善については、共刺激分子を発現するメモリーB 細胞を優先的に除去して B-T 細胞間相互作用を抑制し、リンパ球の活性化の制御を介して血管障害などを改善した(細胞性免疫の制御)可能性も考えられる。これらの結果は、SLE の病態形成において液性免疫のみならず、細胞性免疫も介在することを示唆する。また、DNA アレイでもシグナル関連分子の発現低下を認め、リンパ球の活性化の制御が示唆された。

さらに、多剤耐性遺伝子 MDR-1 の産物である P-糖蛋白質の発現亢進が、リツキシマブ投与によるリンパ球活性化制御の結果、P-糖蛋白質を介する薬剤ポンプ機能の抑制と共に、細胞内のデキサメタゾン残留の回復が示唆され、リンパ球の活性化制御を裏付けると共に、リツキシマブの薬剤耐性回復という治療意義も示された。

しかし、抗 CD20 抗体をはじめ SLE に対して生物学的製剤を用いた治験は、疾患の多様性、評価方法、PML などの有害事象の問題があり、捗々しい結果が得られていない。今後、寛解導入に伴い減弱する遺伝子に関する DNA マイクロアレイ解析とも併せて、より効率的な疾患制御の可能性を探求する。さらに、CD20 に加えて CD22 や TACI 等の B 細胞特異的表面抗原、および、B 細胞受容体やサイトカイン等のシグナルを伝達する細胞内蛋白質(JAK3 や Syk など)の重要な治療標的に対する疾患制御を目指した細胞やモデルマウスレベルでの研究を遂行し、臨床応用の基礎成績を確立する予定である。

E. 結論

治療抵抗性 SLE に対して抗 CD20 抗体リツキシマブ療法は早期効果を示した。その機序としては、単なる B 細胞除去によるものに加えて、共刺激分子を発現するメモリーB 細胞を優先的に除去して B-T 細胞間相互作用を抑制した(細胞性免疫の制御)可能性も考えられた。

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) Fas 抗原発現増強剤（特許出願番号： 特開 2003-171282）
2) Akt シグナル経路の活性化阻害を目的として使用するレフルノミド（特願 2005-81972）

2. 実用新案登録

なし

3. その他

なし

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研究分担報告書

# ポリクローナル抗原刺激下における、ステロイド・免疫抑制剤の制御性T細胞の誘導に関する研究

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研究要旨　SLE などの自己免疫疾患ではステロイド・免疫抑制剤が寛解導入及び維持療法に用いられている。薬剤の治療効果や病態把握の目的で末梢血中の Foxp3+制御性 T 細胞(Treg)の定量が行われている。しかし、Treg はヒトでは少数分画であるため in vivo での定量的比較では正確な解釈が困難な場合も少なくない。本研究では、CD3/CD28-Ab ビーズ(Bs)のポリクロナール抗原で PBMC を刺激して Treg 発現誘導を行い、臨床的な血中濃度を与える薬剤量でのステロイド・免疫抑制剤の Treg の発現誘導に及ぼす影響をサイトカイン産生・ヒストンのアセチル化の観点ら比較検討した。その結果、1)タクロリムス(TAC)とラパマイシン(RAP)はエフェクターT 細胞(Teff)増殖を強く抑制し、それと同時に Treg の発現誘導も抑制した、,2)デキサメタゾ(DXA)とミゾリビン(MZR)での Teff の増殖抑制作用は TAC,RAP での抑制作用に比べて弱かったが、経口内服量相当の薬剤濃度で Treg 誘導抑制効果が無かった、3)MZR と DXA による IL-6 の産生抑制は極めて弱かったが、これらは Bs 刺激によるヒストンのアセチル化を促進させた。以上から、DXA や MZR などは TAC に比較して免疫抑制効果が少ないため寛解導入効果は少ないと思われるが、一方で経口内服量相当の薬剤濃度で Treg 誘導抑制効果が無いため内因性の免疫制御機序の保持に有利であると考えられた。優れた寛解導入効果を有する TAC は Treg の発現誘導も抑制するため、長期間の寛解維持においては内因性の免疫制御機序の保持に配慮することが肝要と思われた。

## A. 研究目的

SLE を初めとする自己免疫疾患では自己抗原が様々な免疫応答を惹起する。自己抗原の刺激によりエフェクターT 細胞(Teff)の活性化と増殖が誘導されるが、それと同時に過剰な免疫応答を抑制する目的で制御性T細胞(Treg)も生み出すと考えられる。本研究ではこの内因性の免疫制御機序、adaptive Treg の誘導をステロイドや免疫抑制剤がどのように修飾するかを検討した。すなわち、各種薬剤での Treg 誘導を比較し、それらの modality の違いを検討することにステロイド・免疫抑制剤を用いた治療戦略の新たな指針を与える基礎的データの収集を目的とした。

## B. 研究方法

I. Bs 刺激による PBMC 中の Treg 誘導の検討。
健常者 PBMC を各種濃度のステロイド・免疫抑制剤:デキサメタゾン(DXA:1~100ng/ml),タクロリムス(TAC:1~100ng/ml),ラパマイシン(RAP:0.1~10nM), ミゾリビン(MZR:0.1~10 μg/ml)を加えた培養液中で CD3/CD28-Ab ビーズ,expansion beads,(Bs)を用いて7日間刺激ながら培養した。刺激後の生細胞数をトリパンブルー染色を用いて定量し、各種薬剤による細胞増殖抑制効果を検討した。
II:7日間刺激後、フローサイトメーターで表面マーカーCD4、転写因子の Fox3 の発現を検討した。
III. 上記の I.及び II.のデータから Teff, Treg の実数(個/$mm^3$)を計算して各種薬剤による Treg 誘導効果を検討した。
IV. 上記の培養上清中のサイトカインレベル(IL-2,IL-6,IL-17)を ELISA で検討した。
V.刺激後の PBMC のヒストンのアセチル化を FACS で検討した。この実験ではヒストンアセチル化の positive control に HDACI(ヒストン脱アセチル化酵素阻害剤)である TSA(Trichostatin-A)を用いた。
(倫理面への配慮)
対象者にはあらかじめ本研究の目的と方法を十分に説明し同意を得た。

## C. 研究結果

I.-1:Bs 単独で7日間刺激後、PBMC 細胞数は刺激前に比較して約 3 倍に増加した。一方、各種薬物存在下では、MZR(2.5 倍),DXA(2.2 倍),RAP(1.8 倍)、TAC(0.6 倍)だった。Bs 刺激なしでは細胞数は 0.7 倍に減少した。TAC が最も細胞増殖抑制効果が強く、

MZRが最も弱く、DXAはその中間だった。これらの変化は統計的に有意だった($p<0.05$)。

II-1：刺激後の CD4+Foxp3+(Treg)のパーセンテージ(%)は,3,3%(刺激前),30%(Bs 単独刺激),10%(TAC:1ng/ml), 20%(RAP:0.1nM), 40%(MZR:1 μg/ml), 40%(DXA:10ng/ml)だった。Bs 単独刺激に比較して、TAC では Treg の発現%が著明に低下し、MZR と DXA では増加した。これらの変化は統計的に有意だった($p<0.05$)。

III-1. Treg の実数(個/$mm^3$)は Bs 単独刺激より Bs+MZR が多く、Bs+TAC は Bs+MZR の約 10%と低値だった。Teff の増殖抑制は TAC が最も強く、MZR は弱かった。Teff 実数は Bs+TAC は Bs+MZR の約 20%と低値だった。これらの変化は統計的に有意だった($p<0.05$)。

IV：MZR, DXA の IL-6,IL-17 産生抑制は弱く、TAC,RAP は IL-6,IL-17 を強く抑制した。IL-2 は全てのサンプルで検出感度以下だった。これらの変化は統計的に有意だった($p<0.05$)。

V:Bs 刺激により PBMC のヒストンアセチル化は亢進したが、HDACI である TSA および MZR,DXA を加えつつ Bs 刺激を行なうと、ヒストンアセチル化が Bs 刺激単独よりさらに亢進した。一方、TAC,RAP を加えつつ Bs 刺激を行なうと Bs 刺激単独よりにヒストンのアセチル化が減少した。これらの変化は統計的に有意だった($P<0.05$)。

D. 考察

今回の研究で、CD3/CD28-Ab(Bs)のポリクローナルな刺激下では、i)TAC の免疫抑制効果は Teff を強力に抑制することにより発揮されるが、これは同時に Treg の誘導にも抑制的に作用していること、ii)一方 DXA や MZR は TAC と比較すると Teff の抑制作用が弱いが、経口内服量相当の薬剤濃度では Treg に対する抑制作用が無いことが明らかとなった。これらの事実は疾患の速やかな寛解導入と長期の寛解維持を考える上で重要なファクターである。TAC はカルシニューリン阻害薬(CNI)として転写因子レベルで IL-2 産生を抑制するが、これ以外にもヒストンのアセチル化を阻害して Foxp3 の転写を阻害している可能性が考えられる(実験結果 V)。

一般に、ヒストンのアセチル化については以下の事実が知られている。I)ヒストンのアセチル化はクロマチンの高次構造を変化させる。この結果、転写因子が遺伝子のプロモーター領域に結合できるようになり転写が開始される。II)Foxp3 の発現調節もこのようなクロマチン・リモデリングの支配を受けている。III)CD3/CD28 抗体による抗原刺激もヒストンをアセチル化して Foxp3 の発現を誘導する。IV)抗原刺激により誘導される Foxp3 には functional なものと non-functional なものが存在するが、non-functional な Foxp3 の発現は Bs 刺激後 3 日をピークに減少して7日後にはほぼ全数が functional な Foxp3 となる。今回使用した HDACI の TSA (Trichostain-A)はヒストン脱アセチル化酵素の作用を阻害してヒストンのアセチル化を促進するが、Bs 刺激を加えつつ TSA を作用させた PBMC では Foxp3 の発現が Bs 単独刺激に比して有意に増加したことから抗原刺激によるアセチル化と TSA によるアセチル化は相加的に作用すると思われる。また、DXA や MZR も TSA と同様の傾向を示したことからこれらの薬剤も抗原刺激の際は相加的にヒストンをアセチル化して Foxp3 の発現を上昇させると思われる。一方、TAC は DXA や MZR とは対照的に抗原刺激時のヒストンのアセチル化を抑制したので、TAC の強い T 細胞増殖抑制効果には CNI の作用だけでなくクロマチン・リモデリング阻害作用も関与する可能性がある。このような TAC の強力な Teff 抑制作用は SLE の寛解導入に優れた効果を発揮するが、同時に Treg の誘導にも抑制的に作用している。注目すべきは、経口内服量相当の薬剤濃度では DXA や MZR は Treg 誘導の抑制作用の無いことである。従って、日和見感染症のリスクを回避しつつ長機関の寛解維持を得るためには、薬剤のモダリティーの異なる TAC と DXA あるいは MZR を適切に組み合わせる必要があると思われる。

E. 結論

TAC と MZR の免疫抑制機序は異なっており、TAC は主に Teff を抑制することで、一方 DXA や MZR は主に弱い Teff 抑制作用に加えて Treg を増加させることで免疫抑制作用を示すことが示唆された。これらの事実は疾患の速やかな寛解導入と長期の寛解維持を考える上で重要なファクターと考えられた。

F. 健康危機情報

なし

G. 研究発表

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H. 知的財産権の出願·登録状況(予定を含む)

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

# Ⅳ.　研究成果の刊行に関する一覧表

雑誌

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# V.　研究成果の刊行物・別刷

# $CD4^+CD25^-LAG3^+$ regulatory T cells controlled by the transcription factor Egr-2

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**Regulatory T cells (Tregs) are engaged in the maintenance of immunological self-tolerance and immune homeostasis. IL-10 has an important role in maintaining the normal immune state. Here, we show that IL-10-secreting Tregs can be delineated in normal mice as $CD4^+CD25^-Foxp3^-$ T cells that express lymphocyte activation gene 3 (LAG-3), an MHC-class-II-binding CD4 homolog. Although ≈2% of the $CD4^+CD25^-$ T cell population consisted of $CD4^+CD25^-LAG3^+$ T cells in the spleen, $CD4^+CD25^-LAG3^+$ T cells are enriched to ≈8% in the Peyer's patch. They are hypoproliferative upon in vitro antigenic stimulation and suppress in vivo development of colitis. Gene expression analysis reveals that $CD4^+CD25^-LAG3^+$ Tregs characteristically express early growth response gene 2 (Egr-2), a key molecule for anergy induction. Retroviral gene transfer of Egr-2 converts naïve $CD4^+$ T cells into the IL-10-secreting and LAG-3-expressing phenotype, and Egr-2-transduced $CD4^+$ T cells exhibit antigen-specific immunosuppressive capacity in vivo. Unlike $Foxp3^+$ natural Tregs, high-affinity interactions with selecting peptide/MHC ligands expressed in the thymus do not induce the development of $CD4^+CD25^-LAG3^+$ Tregs. In contrast, the number of $CD4^+CD25^-LAG3^+$ Tregs is influenced by the presence of environmental microbiota. Thus, IL-10-secreting $Egr\text{-}2^+LAG3^+CD4^+$ Tregs can be exploited for the control of peripheral immunity.**

anergy | Blimp-1 | inflammatory bowel disease | IL-10 | type 1 regulatory T cells

Thymic T cell development efficiently regulates tolerance to self antigens (1, 2). However, in the last decade, rapid progress revealed the key role of peripheral tolerance in the maintenance of immunological homeostasis (3–5). In view of the recent reports, T cell subsets in the periphery are quite diverse. Naïve $CD4^+$ T helper cells may develop into different committed helper cell subsets characterized by distinct cytokine profiles, such as IFN-γ-secreting Th1, IL-4-secreting Th2, and IL-17-secreting Th17 cells (6–8). The versatile nature of T cells is found most strikingly in $Foxp3^+$ regulatory T cells (Tregs) (9). Therefore, identifying new subsets of effector and regulatory T cells is possible.

Naturally occurring $CD4^+CD25^+$ Tregs, which characteristically express the transcription factor Foxp3 (9), have been studied intensively, because their deficiency abrogates self-tolerance and causes autoimmune diseases (10). Mice with a null mutation of Foxp3, scurfy mice, have massive lymphoproliferation and severe inflammatory infiltration of the skin and liver (11). However, Aire is a gene responsible for autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy, which influences on the central induction of tolerance by regulating the clonal deletion of self-reactive thymocytes (12). Aire regulates the ectopic expression of a battery of peripheral-tissue antigens in the thymus [e.g., insulin, fatty-acid-binding protein, and salivary protein 1 (13)]. By an additional defect in central tolerance induction in scurfy mice, generated by crossing in a null mutation of the Aire gene, the range of affected sites was not noticeably extended, and many organs remained unaffected (3). This result suggests that additional important mechanisms other than central tolerance and the Foxp3 system are required to enforce immunological self-tolerance in the periphery.

Indeed, there are T cell populations with regulatory activity other than $CD4^+CD25^+Foxp3^+$ Tregs. The IL-10-secreting $Foxp3^-CD4^+$ T cells (4, 14) also have been a focus of active investigation, because, in contrast to $Foxp3^+$ natural Tregs, antigen-specific IL-10-secreting T cells can be adaptively induced in vitro and in vivo (15, 16). Because IL-10-secreting T cells also appear to be capable of controlling tissue inflammation under various disease conditions (14), IL-10-secreting regulatory T cells may be a tolerogenic machinery complementing $CD4^+CD25^+Foxp3^+$ Tregs. However, assessing the in vivo physiological function of IL-10-secreting regulatory T cells is difficult, because of the lack of specific markers that can reliably differentiate them from the other T cells (17).

Known regulatory T cells are closely related to anergy. Anergy is a tolerance mechanism in that T cells are functionally inactivated following an antigen encounter but remain alive for an extended period in the hyporesponsive state (18). A set of functional limitations characterizes the anergic state, including cell division, cell differentiation, and cytokine production. The E3 ligases c-Cbl, Cbl-b, GRAIL, Itch, and Nedd4 have been linked to the promotion of T cell anergy (19, 20). The RING-type E3 ubiquitin ligase Cbl-b promotes ubiquitination and degradation of signaling components, such as phospholipase C-γ and PKC-θ. Recently, early growth response gene 2 (Egr-2) and Egr-3 were reported to be transcription factors for the T cell receptor (TCR)-induced negative regulatory program controlling Cbl-b expression (21). Egr-2 is a C2H2-type zinc finger transcription factor that plays an essential role in hindbrain development and myelination of the peripheral nervous system (22), and Egr-2 null mutation resulted in perinatal or neonatal death. However, the role of Egr-2 in the regulatory function of T cells has not been described extensively.

We here report the identification of a Treg population that expresses Egr-2 and lymphocyte activation gene 3 (LAG-3). LAG-3, which negatively controls T cell proliferation (23, 24), was reported to be required for maximal regulatory functioning of murine $CD4^+CD25^+$ T cells. Ectopic expression of LAG-3 conferred regulatory activity to naïve T cells (25). Interestingly, LAG-3 protein was hardly detected on the cell surface of $CD4^+CD25^+$ T cells but was expressed by a sizable population of $CD4^+CD25^-$ T cells (26). We have found that IL-10-secreting $CD4^+CD25^-LAG3^+$ T cells show a significant regulatory activ-

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The authors declare no conflict of interest.

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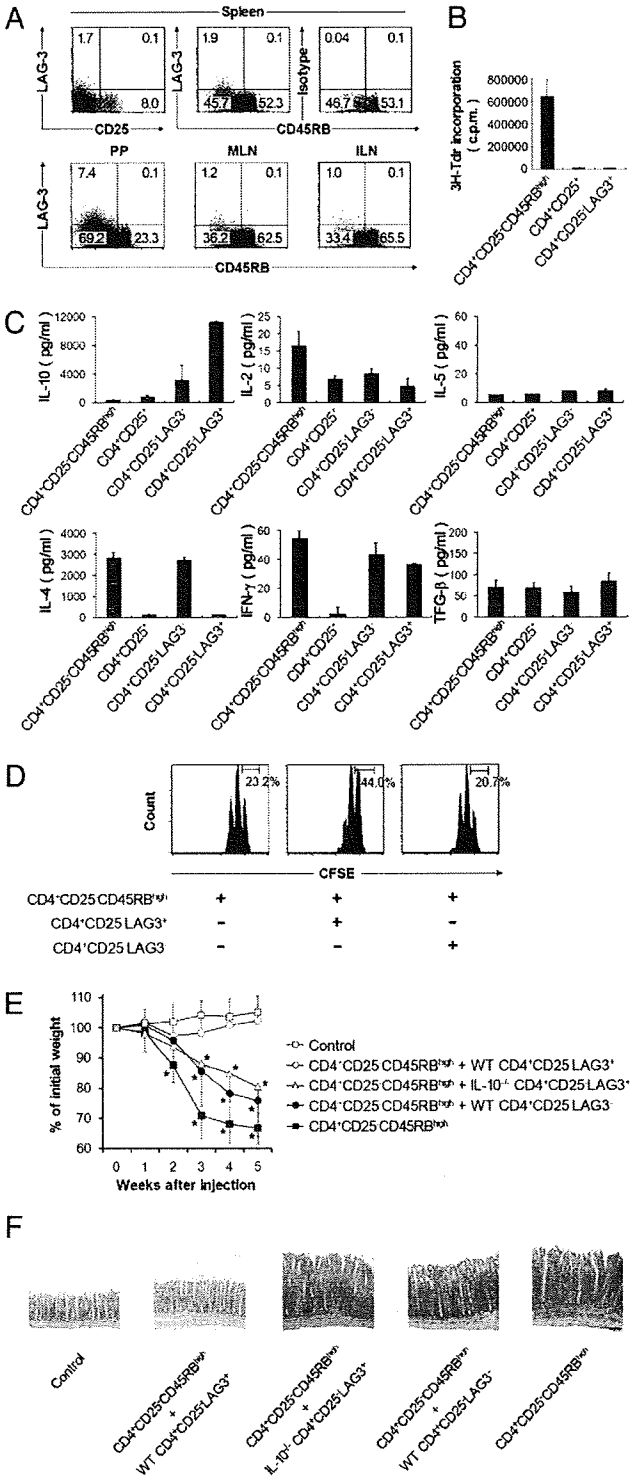


**Fig. 1.** Identification of $CD4^+CD25^-LAG3^+$ regulatory T cells. (*A*) LAG-3 expression in the spleen, Peyer's patch (PP), mesenteric lymph node (MLN), and inguinal lymph node (ILN). (*Top*) LAG-3 and CD25 or CD45RB expression in splenocytes from C57BL/6 is shown for the gated on $CD4^+$ (*Upper Left*) or $CD4^+CD25^-$ (*Upper Center* and *Upper Right*) T cells, respectively. (*Lower*) LAG-3 and CD45RB expression of PP, MLN, and ILN gated on $CD4^+CD25^-$ T cells. Representative FACS dot plots from at least three independent experiments are shown. (*B*) Proliferation of $CD4^+CD25^-CD45RB^{high}$, $CD4^+CD25^-$, and $CD4^+CD25^-LAG3^+$ splenocytes after 72 h stimulation with anti-CD3/anti-CD28. The results are the means of three independent experiments. (*C*) Cytokines in the culture supernatants of $CD4^+CD25^-CD45RB^{high}$, $CD4^+CD25^-$, and $CD4^+CD25^-LAG3^+$ T cells stimulated for 5 days with anti-CD3 mAb. Representative data from at least three independent experiments are shown. (*D*) Suppressive function of $CD4^+CD25^-LAG3^+$ T cells. Naïve $CD4^+CD25^-CD45RB^{high}$ $Thy1.1^+$ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cocultured with the indicated $Thy1.2^+$ T cells and irradiated whole splenocytes plus anti-CD3 mAb. Representative data from three independent experiments are shown. (*E*) Suppression of $CD4^+CD25^-CD45RB^{high}$ T-cell-mediated colitis in RAG-$1^{-/-}$ mice by $CD4^+CD25^-LAG3^+$ T cells. Data represent body weight as a percentage of the initial weight of individual mice; $n = 6$ per group. (*F*) Representative photomicrographs of the colons stained with hematoxylin and eosin after transfer of the indicated cell populations. All error bars represent ±SD. (Scale bar, 100 μm.)

ity in vivo and characteristically express Egr-2. Conversion of Egr-2 transduced naïve $CD4^+$ T cells to IL-10-secreting and LAG-3-expressing Tregs suggested that Egr-2 is a key transcription factor for $CD4^+CD25^-LAG3^+$ T cells.

## Results

**IL-10-Secreting $CD4^+CD25^-CD45RB^{low}LAG3^+$ T Cells Exert Regulatory Activity.** In agreement with previous results (26), flow cytometric analysis revealed that >90% of LAG-3-expressing cells belonged to the $CD4^+CD25^-CD45RB^{low}$ population (hereafter called $CD4^+CD25^-LAG3^+$ cells) (Fig. 1*A*). These $CD4^+CD25^-LAG3^+$ cells showed staining profiles of conventional $CD4^+TCR\alpha\beta^+$ T cells that did not express CD8, $TCR\gamma\delta$, and NK1.1 antigens (Fig. S1). The frequencies of $LAG3^+$ T cells in the $CD4^+CD25^-$ population were relatively low in the spleen (1.8 ± 0.18%), mesenteric lymph node (1.1 ± 0.09%), and inguinal lymph node (1.0 ± 0.07%) but characteristically high in Peyer's patch (PP) (7.7 ± 0.87%). These cells were hypoproliferative upon in vitro stimulation in a manner similar to $CD4^+CD25^+$ Tregs (Fig. 1*B*). They exclusively produced large amounts of IL-10 and low amounts of IL-2 and IL-4 (Fig. 1*C*). There were no significant differences in IL-5 and TGF-β production among the populations compared in the experiment. In anti-CD3-stimulated cocultures of $LAG3^+$ or $LAG3^-CD4^-CD25^-$ T cells with $CD4^+CD25^-CD45RB^{high}$ T cells, $CD4^+CD25^-LAG3^+$ T cells exhibited weak suppressive activity (Fig. 1*D*). In contrast, $CD4^+CD25^-LAG3^+$ T cells effectively inhibited colitis induced in RAG-1-deficient (RAG-$1^{-/-}$) recipients by the transfer of $CD4^+CD25^-CD45RB^{high}$ T cells (Fig. 1 *E* and *F* and Fig. S2). The in vivo suppressive activity was IL-10-dependent, because the transfer of $CD4^+CD25^-LAG3^+$ T cells from congenic IL-10-deficient (IL-$10^{-/-}$) mice failed to suppress colitis.

Cytofluorometric analysis revealed that $CD4^+CD25^-LAG3^+$ T cells did not express Foxp3 protein (Fig. S3 and Fig. S4). In addition, the number of $CD4^+CD25^-LAG3^+$ T cells was significantly increased in scurfy mice that lack functional Foxp3 protein (11). These cells expressed LAG-3 and IL-10 mRNA equivalently and exhibited distinct in vitro suppressive activity (Fig. S5). $CD4^+CD25^-LAG3^+$ T cells hardly expressed CD103 and latency-associated peptide (LAP) on the cell surface (Fig. S1 and Fig. S6), indicating that they were different from $CD103^+$ regulatory T cells and $CD4^+CD25^-LAP^+$ regulatory T cells, respectively (27, 28). These findings collectively indicate that $CD4^+CD25^-LAG3^+$ T cells exert regulatory activity in an IL-10-dependent and Foxp3-independent manner.

**$CD4^+CD25^-CD45RB^{low}LAG3^+$ T Cells Exhibit a Distinct Transcriptional Profile.** To further characterize $CD4^+CD25^-LAG3^+$ T cells, the mRNA expression profiles of four $CD4^+$ subsets ($CD4^+CD25^-LAG3^+$, $CD4^+CD25^-LAG3^-$, $CD4^+CD25^+$, and $CD4^+CD25^-CD45RB^{high}$) were examined. Gene expression profiling revealed six clusters of differentially expressed genes

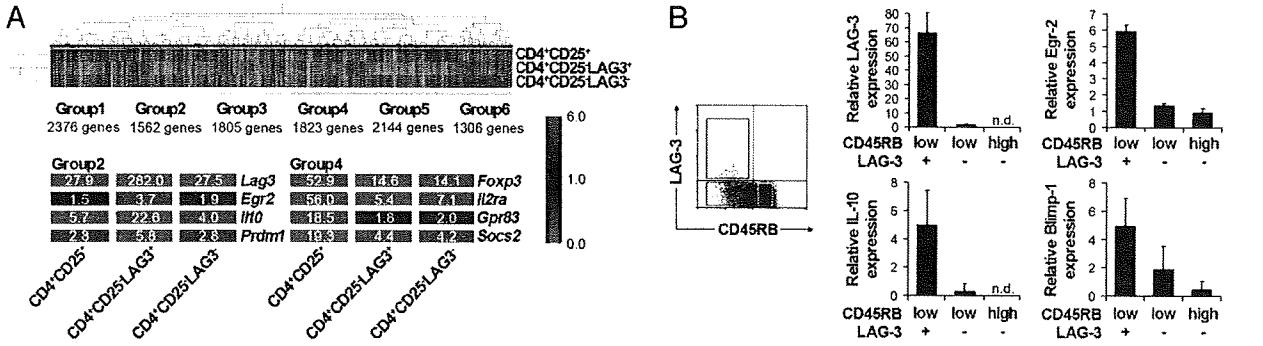


Fig. 2. Expression of Egr-2 in CD4+CD25−LAG3+ T cells confers regulatory function. (*A*) Microarray comparisons of the gene expression profiles among CD4+CD25+, CD4+CD25−LAG3+, and CD4+CD25−LAG3− cells. Normalized expression values from naïve CD4+CD25− CD45RB$^{high}$ T cells are depicted according to the color scale shown. The expression profiles for each gene were classified into six groups. (*B*) LAG-3 and CD45RB expression of splenocytes from C57BL/6 mice gated-on CD4+CD25− T cells (*Left*). Quantitative PCR for LAG-3, Egr-2, IL-10, and Blimp-1 in the indicated T cell subsets (*Right*). The results are the means of three independent experiments. All error bars represent ±SD.

(Fig. 2*A*). Signature genes for CD4+CD25+ Tregs, such as *Foxp3*, *Il2ra* (CD25), *Gpr83*, and *Socs2*, were preferentially expressed in one group (group 4). In contrast, the supposed signature genes for CD4+CD25−LAG3−, such as *Lag3*, *Il10*, and *Prdm1* [B-lymphocyte-induced maturation protein 1 (Blimp-1)], were preferentially expressed in another group (group 2). Because CD4+CD25−LAG3+ T cells were anergic in response to TCR stimulation (Fig. 1*B*), that the expression of the anergy-associated Egr2 gene was significantly increased in group 2 is particularly notable. Egr-2 was reported recently as a key negative regulator of T cell activation and was required for full induction of clonal anergy (21, 29). In accordance with the microarray analysis, quantitative real-time PCR confirmed the high expression levels of Egr2, LAG3, IL-10, and Blimp-1 genes in CD4+CD25−LAG3+ T cells (Fig. 2*B*).

**Retroviral Transduction of Egr-2 Converts Naïve CD4+ T Cells into IL-10-Secreting and LAG-3-Expressing Tregs.** Next, we examined whether forced expression of Egr-2 in naïve CD4+ T cells could convert them to the CD4+CD25−LAG3+ phenotype using retrovirus vectors that coexpressed GFP and Egr-2 (pMIG-Egr2) (Fig. 3*A*). The TCR-stimulated pMIG-Egr2-transduced GFP+ cells showed significant up-regulation of Egr2, LAG3, IL-10, and Blimp-1 genes (Fig. 3*B*). In addition, pMIG-Egr2-transduced GFP+ cells produced significantly higher amounts of IL-10 and lower amounts of IL-2, IL-4, and IL-5 proteins (Fig. 3*C*).

Despite the expression of LAG-3 and IL-10 proteins, the present study was not able to confirm sufficient suppressive activity of pMIG-Egr2-transduced GFP+ cells in in vitro coculture with freshly isolated CD4+CD25−CD45RB$^{high}$ responder T cells stimulated with anti-CD3 mAb (Fig. 3*D*). To examine the in vivo suppressive activity of Egr-2, we next performed the delayed-type hypersensitivity (DTH) reaction of BALB/c mice against chicken ovalbumin (OVA) by using T cells transduced with the Egr2 gene. The in vivo functions of T cells transduced with regulatory genes have been verified (30, 31). In this experiment, CD4+ T cells from BALB/c mice were transduced with pMIG or pMIG-Egr2. FACS-sorted retrovirus-infected CD4+GFP+ cells were injected intravenously 6 days after immunization with OVA, and OVA was rechallenged 2 days after the cell transfer. Notably, BALB/c CD4+ T cells transduced with pMIG-Egr2 significantly suppressed DTH responses compared with BALB/c CD4+ T cells transduced with pMIG (Fig. 3*E*). To explore the influence of antigen specificity, CD4+ T cells from OVA-specific DO11.10 TCR transgenic mice also were transduced with pMIG or pMIG-Egr2, and mice transferred with these CD4+GFP− cells were simultaneously analyzed for DTH. DO11.10 CD4+ T cells transduced with pMIG-Egr2 significantly suppressed DTH responses compared with BALB/c CD4+ T cells transduced with pMIG. Moreover, DO11.10 CD4+ T cells transduced with pMIG-Egr2 suppressed DTH responses more efficiently than BALB/c CD4+ T cells transduced with pMIG-Egr2, indicating a contribution of the antigen specificity to the enhancement of suppressive activity in Egr2-transduced cells. Thus, Egr-2 can confer in vivo suppressive activity on naïve T cells.

**Development of CD4+CD25−LAG3+ T Cells.** We then explored whether CD4+CD25−LAG3+ T cells could develop through the thymic selection process in a similar manner to Foxp3+ Tregs, which require a high-affinity agonistic interaction with self-peptide/MHCs expressed by thymic stromal cells (32). RIP-mOVA/OT-II double-transgenic mice express a membrane-bound form of OVA in the pancreatic islets and the thymus together with a transgenic TCR (Vα2 and Vβ5.1) that recognizes the $OVA_{323-339}$ peptide in the context of I-A$^b$. The frequency of CD4+CD25−LAG3+ T cells was not increased in the thymus and spleen of RIP-mOVA/OT-II mice, in contrast with an increase in the frequency of CD4+CD25+ Tregs in these organs as reported in ref. 32 (Fig. 4*A*). Thus, unlike Foxp3+ natural Tregs, the development of CD4+CD25−LAG3+ T cells does not appear to require high-affinity interactions with selecting peptide/MHC ligands expressed in the thymus.

Next, the influence of the environmental microbiota was studied for the development of CD4+CD25−LAG3+ T cells with germfree (GF) mice. Although GF mice are exposed to self antigens, to food-derived antigens, and to microbial particles from dead microorganisms in the sterilized food or bedding, the absence of viable microbiota affects the immune homeostasis (33, 34). As shown in Fig. 4*B*, GF mice contained fewer CD4+CD25−LAG3+ T cells than specific-pathogen-free mice in the spleen and PP. This result suggested that the exposure to viable microbiota affects the development of CD4+CD25−LAG3+ T cells.

## Discussion

We have shown the natural presence of Egr-2-dependent CD4+CD25− Tregs in the normal immune system and characterized their function and development. CD4+CD25−LAG3+ Tregs are clearly different from CD4+CD25+ Tregs in Foxp3 independency and development. T-cell-mediated colitis driven by enteric bacteria develops in lymphopenic mice after the transfer of CD4+CD45RB$^{high}$ T cells (35). The development of colitis can be prevented by cotransfer of the reciprocal