厚生労働科学研究費補助金 難治性疾患克服研究事業

小腸生検検体大規模解析による特定難治性疾患病態理解に関する研究

平成21年度 総括・分担研究報告書

研究代表者　渡辺　守

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小腸はこれまで検査法がほとんど無く、暗黒大陸と称されるほど状態を把握することは困難であった。さらに小腸を主座とするクローン病などの難治性腸疾患だけでなく、免疫異常や神経異常を背景とした特定難治性疾患の多くも小腸に病変を併発することが知られ、出血、潰瘍、吸収不良、狭窄などの症状を認めるも、その病態把握、効果的な治療がなく患者のQOL低下を助長している。本邦で開発されたダブルバルーン内視鏡は全小腸の観察を可能し、病状の把握、治療に非常に有効な検査法となる一方で、予想以上に小腸病変が描出され新たな病変に対する対策が急務となっている。

そこで本研究では内視鏡を利用した小腸生検検体の大規模・網羅的解析がこれまで不明であった全小腸の構造、病態解明に繋がると考え、さらに新規治療法の開発にまで発展させることを目指して、平成21年4月研究班がスタートした。研究班では　これまで到達できなかった小腸内部をライブ環境で観察しその状態での小腸粘膜の収集、解析が可能となった。また検査、検体収集、保存、解析までの大規模解析に向けたバンクシステムの構築を行うことができた。欧米ではカプセル内視鏡が主な小腸検査となっており、本邦で開発された内視鏡による小腸生検検体は本邦独自の解析手法であることからも国際的な評価は高く、小腸はヒトで最大の器官であり、多機能である組織であることからも基本構造を理解し病態を解明するという本研究班の社会的意義は非常に大きいと考えられた。

1年間の限られた期間内ではあったものの、各々のプロジェクトの順調な遂行により、予想を超える大きな成果を得ることができた。本研究班の成果を基礎とし、さらなる研究の発展を図ることにより、多数の検体収集、保存、解析が期待でき、小腸難治性疾患病態の解明に繋がると期待される。

この1年間、実りある研究成果を挙げていただいた研究協力者の諸先生に深謝致したい。

平成22年3月

研究代表者　　渡　辺　　守

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Ⅰ．総括研究報告

厚生労働科学研究費補助金難治性疾患克服研究事業
小腸生検検体大規模解析による特定難治性疾患
病態理解に関する研究
総括研究報告書

小腸生検検体大規模解析による特定難治性疾患病態理解に関する研究

研究代表者　　渡辺　守　東京医科歯科大学大学院消化器病態学分野　教授

研究要旨：本研究は小腸というこれまで暗黒大陸であった器官に対して、ダブルバルーン内視鏡という本邦で開発された内視鏡により全小腸が観察可能になったことを機に、生検検体を用いて小腸の構造解析、難治性疾患の病態理解を目的とすることである。まず正常小腸の全小腸のマッピング生検にて今回長軸方向の細胞構成制御を明らかとし、腸管分化に必須の転写因子群が空腸から回腸まで局在を変化させることで各細胞種への分化を制御することが判明した。さらに各細胞が分泌する蛋白群を解析することによって小腸の各部位での機能差異を明確にすることができた。以上より生検検体を用いた解析が小腸の機能制御理解に有用であり、さらには難治疾患の病態解明に繋がると考え、大規模生検検体収集を行っている。これは内視鏡を多施設で多数の患者に対して行う本邦の特徴を生かした計画であり、既に 300 を超える生検検体の収集に成功した。また難治疾患の腸病変に関しても収集を開始し、正常人との比較においてクローン病では非病変部を含む全小腸において抗菌物質の産生が低下していることを発見している。以上より収集された生検検体が難治疾患病態理解、新規治療法の開発のためには非常に貴重な試料であると思われ、今後バンク化されることが期待される。

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１．研究目的

小腸は全長 6m、表面積はテニスコートと同じ大きさとなり体内で最大の器官であること、器官内の組織は均一でなく部位により細胞構成、機能が異なること、常に異なる食餌抗原、腸内細菌により暴露されていること、パイエル板などの免疫器官も有し体内で最大の免疫装置で有るという非常に複雑な多機能器官である。さらに小腸は「暗黒大陸」と表現されるほど検査法がないため基本的な小腸構造・機能・病態の理解が全くされず、小腸の経時的かつ空間的な機能制御を理解することはこれまで不可能であった。

しかし本邦で開発されたダブルバルーン小腸内視鏡により全小腸の観察、生検、内視鏡的治療等で小腸病変の経時的、空間的評価が可能となりクローン病などの小腸を主座にする疾患に関して診断、治療などに有用されている。その一方で、内視鏡観察にて予想以上に小腸に病変が多く、その背景疾患に関してクローン病以外にもベーチェット病、アミロイドーシス、高安病、SLE、GVHD などの特定難治性疾患に小腸病変が付随し腹痛、下血、吸収不良など患者の QOL に直結する症状を呈する場合が多いことが指摘されている。さらに原因不明の小腸潰瘍、びらんが多数発見され、病変は同定できるも確定診断ができず苦慮する場面が多く見られる。以上の現況から小腸の構造・機能制御の理解が必要であり病変における制御破綻機構を解明することが急務である。

そこで申請者は小腸疾患患者の全小腸粘膜をマッピングすることで小腸機能を空間的、経時的に解析することが可能であると考え、本研究計画で

は１）小腸上皮細胞構成成分解析、２）粘膜内リンパ球解析、３）遺伝子発現解析を大規模に網羅的に施行することで小腸疾患の病態理解、新規診断法・治療法開発の基盤を創成し、小腸組織バンク設立の素地作成を目的とする。具体的には小腸内視鏡拠点病院において小腸粘膜を病変部だけでなく全腸のマッピング生検を施行し、採取した検体は部位別に病理学解析、免疫組織学的解析、小腸発現遺伝子発現解析を行うことで、小腸上皮細胞分化、機能制御機構、免疫制御機構を空間的に理解する。

本研究は海外ではカプセル内視鏡が主であり生検できず小腸組織バンクは存在しないこと、本邦で開発された内視鏡を用いて本邦では施行数が多く、生検検体の集積が期待されることから小腸組織バンク設立により得られる成果は独創的であり世界的に評価されると考えられる。

２．研究方法

研究環境として本研究は東京医科歯科大学施設内の消化器病態学研究室、医学部附属病院、疾患モデルセンターで施行され、研究計画を遂行するための設備は全て整っている。

本研究は研究協力者を組織し遂行する。

１）生検検体採取および内視鏡所見の集約
　当施設光学医療診療部および全国１０大学のダブルバルーン内視鏡拠点病院
２）病理学的解析　　当大学　人体病理学講座
３）遺伝子発現解析　当大学　消化器病態学講座
４）免疫制御解析　　当大学　消化管先端治療学
５）免疫組織学的解析　当大学消化管先端治療学
の分担により各スタッフおよび大学院生が実行する。

具体的な施行方法として内視鏡所見上小腸を５区域（空腸２区域、回腸３区域）に分けて、それぞれダブルバルーン内視鏡にて生検を施行する。病理組織用、免疫染色用、遺伝子発現用に一カ所ごとに３個生検し、それぞれパラフィン固定、ホルマリン固定後 OCT 包埋、RNA 精製試薬処理を行い、室温または冷凍庫に保存する。

目標症例として正常人５０例、２５０検体、クローン病患者２０例、１００検体、その他の小腸疾患患者（ベーチェット病、アミロイドーシス、高安病、SLE、GVHD）合計２０例、１００検体とする。

当該年度は正常小腸検体解析による正常小腸構造、機能制御を理解する。次年度以降は各疾患の検体を解析し正常と比較することで異常点を抽出する。

解析方法としては以下に記載のごとく行う。

１）ヒト小腸粘膜分化・機能機構解析

A)正常小腸粘膜生検検体による細胞種、機能タンパク発現解析

各部位において下記のごとく発現解析を行う。

○粘膜形態（HE 染色）細胞増殖解析（Ki-67 染色）
○分化マスター遺伝子（Hath1 染色）
○細胞種類解析杯細胞、内分泌細胞、パネート細胞、吸収上皮
○細胞の特異蛋白の染色

Wnt シグナル解析(Wnt 染色、β-catenin 染色、c-myc 染色)、Notch シグナル解析(Notch 細胞内ドメイン染色、HES1 染色、Musashi-1 染色)
として評価し、部位別特徴の有無を検討し分化機構、発現タンパクの差異から機能制御機構を理解する。

さらに各検体の RNA を用い、上記遺伝子の発現変化を RT-PCR により確認すると共に、マイクロアレイ解析を施行し網羅的に遺伝子発現を解析し部位別特徴を把握する。

B)小腸疾患における腸管機能評価

クローン病を始めとした小腸病変を有する特定難治疾患症例に関して、症状、病変部位、重症度などの臨床情報を検討し、内視鏡所見と併せ生検検体を採取し上記項目に置いて同様に解析を行い、正常との差異を解明する。またマイクロアレイ解析を用いて、正常人と同部位における網羅的に遺伝子の差異を明らかとする。さらに治療後内視鏡にて再検査し、生検検体を同様に解析し、治療法、治療抵抗性と併せて考慮することで予後予測因子を探索する。

２）小腸間質及び上皮内リンパ球組成解析

A)正常小腸組織におけるリンパ球性状解析

上記小腸検体の固定標本を用い、粘膜下及び上皮内リンパ球の組成を下記の染色にて解析する。

T細胞(CD4, CD8, FoxP3)、 B細胞(IgG, IgM, IgA, IgE)

マクロファージ(CD11c)、 サイトカイン(IL-7, IL-6, IL-8)についてそれぞれ解析し評価する。

さらにパイエル板を生検しリンパ球性状解析を行う。

また小腸生検検体をコラゲナーゼ処理にてリンパ球を分離し、FACSにて表面マーカー解析を行う。Foxp3陽性の抑制性リンパ球を分離し抑制効果を in vitroで確認し、抑制リンパ球の機能制御を解析する。

B)小腸疾患における腸管機能評価

小腸疾患患者において同様の解析を行い、正常と比較してリンパ球の組成の差異、機能異常を抽出する。また潰瘍の病変部位の違いにおける性状の差異を明らかとする。

（倫理面への配慮）

申請者らがヒトの細胞および組織を用いた研究に

あたっては、厚生科学審議会の「遺伝子解析研究に付随する倫理問題等に対応するための指針」などに準じて、人権及び利益の確保を下記のように行うよう配慮する。検体の提供に関しては「小腸びらん・潰瘍病変に関する研究」として申請し、当学の倫理審査委員会で、研究の適否などを議論・審査し既に承認を得ている。意義と必要性を説明しその自由意志に基づき同意を得られた場合にのみ検体提供を受ける。代諾による同意は今回の研究では無効とする。検体提供の有無により、治療など不利益を被ることはない。個人のプライバシーの保護を厳密に行う。希望に応じ検体提供者やその保護者への研究結果の説明を行う。研究目的でのみ検体を使用し、その他の目的では使用しない。

３．研究結果及び考察

１）小腸粘膜の検体採取

申請者の施設において小腸出血疑いなど、ダブルバルーン内視鏡の適応のある患者に対し十分なインフォームドコンセントを行い(「小腸びらん・潰瘍病変に関する研究」(承認番号 No.314))、ほとんどの患者から同意を得ることができた。約６０例のダブルバルーン内視鏡を施行し、３００検体以上の小腸粘膜生検検体を採取した。
そのうち特定難治性疾患においてはクローン病５例、ベーチェット病１例、GVHD２例、高安病１例の計４５の生検検体の採取を行った。

２）検体保存方法の検討

まずバンクシステムが機能するかを当施設にて試みた。小腸一カ所から生検検体を３個採取し、パラフィンブロック保存、凍結切片にてOCT包埋にて保存、RNA試薬にて保存をそれぞれ行った。パラフィンブロックは常温、凍結切片及びRNA検体は-80℃冷凍庫にて保存した。

パラフィンブロックにて小腸粘膜のHE染色を行ったところ、病理学的評価可能な状態であった。また免疫染色にてもTFF3, CgAなどの染色も可能であった。

凍結切片にても分化マーカーであるHath1, Hes1, Klf4などの免疫染色が可能であり、局在解析による分化評価が可能と判断した。

RNA用の検体ではRNAを抽出し、逆転写反応を行いcDNAを作成した。各種遺伝子の発現量をPCR法にて解析可能であった。
以上から生検検体の処理および保存は当研究計画に十分耐えうる品質を維持することを明らかとした。

３）バンクシステムの構築

上記検体保存にて有用な情報が得られることが判明したため、バンクシステムの構築を開始した。

厚生労働省　難易疾患克服事業　「難治性疾患克服のための難病研究資源バンク開発研究」の代表者である独立行政法人医薬基盤研究所　亀岡洋祐氏と当施設において会議を行い、保存方法、輸送方法について具体的に協議を行ったところ、基盤研において収集検体の保存が可能であるとの結論であり、現在さらに実現にむけて調整中である。

４）小腸部位別の構造解析

結果的に基礎疾患がなく、内視鏡観察にて正常所見であった患者を健常人とし、それら１０例、計５０検体を用いて部位別構造解析を行った。

A)絨毛形態解析　パラフィンブロックからのHE染色標本ではそれぞれ絨毛の形態及び杯細胞、パネート細胞の構成の評価が可能であった。絨毛の形態は全小腸に渡って絨毛長および幅に変化を認めなかった。

B)細胞構成解析　HE染色にて杯細胞、パネート細胞の細胞数を各部位で計測した。またCgA染色にて内分泌細胞を描出し絨毛あたりの数を計測した。その３種類以外の細胞を吸収上皮細胞としてカウントした。

空腸側では吸収上皮が多く、杯細胞が少ないが、回腸側では逆に吸収上皮は減少し、杯細胞は増加していた。パネート細胞、内分泌細胞は部位別にて数の変化を認めなかった。

C)形質遺伝子発現解析

部位別の生検検体からのRNAを用いてPCRを行い、各種形質発現検討を行った。杯細胞の形質発現マーカーであるMucin2の発現は杯細胞数と同様に回腸側で増加していた。吸収上皮形質であるラクターゼは細胞構成と同様に回腸側で発現低下を認めた。内分泌細胞の形質であるCgAおよびパネート細胞の形質であるディフェンシン５の発現は部位による変化を認めなかった。

D)分化遺伝子発現解析

回腸側における杯細胞増加機構解析のため、杯細胞への分化遺伝子であるHath1及びKlf4について発現解析を行った。PCRでの遺伝子発現は両者ともに回腸側での発現増加を認めた。免疫染色における局在解析では、興味深いことにHath1は空腸側では絨毛の基底側に発現し回腸になるにつれて管腔側まで徐々に発現細胞の増加を認めた。一方、Klf4は空腸側では管腔側のみに発現していたが、回腸になるにつれて基底側まで発現細胞が増加し結果として回腸側ではHath1とKlf4が共発現することで、杯細胞が回腸側で増加することを明らかとした。

E)Notchシグナル制御

また吸収上皮細胞への分化はNotchシグナルが関わるため、HES1の遺伝子発現、局在を解析したが、全小腸で変化を認めなかった。

以上より同一人物の小腸内では空腸側と回腸側で細胞構成が異なり、それぞれの分化規定遺伝子の発現、局在により長軸方向の細胞構成が制御されていることを解明し得た。

５)クローン病での病態解析

クローン病患者の検体の一部を解析し、健常人との差異を解析した。驚いたことに、クローン病患者の病変部ではない空腸側の生検検体において病理学的解析では絨毛形態、間質は異常を認めないが、遺伝子発現解析においてディフェンシン５の発現が健常人と比較して１０００倍減少していることを明らかとした。これまでの病理学的検索では発見し得ないことであり、健常人との同部位を比較検討できる本計画でのみ描出可能な結果を得ることができた。

４．評価

１）達成度について

本研究の目的に沿って研究計画をほぼ遂行することができた。ダブルバルーン内視鏡の施行数が多い本邦では期待通りの検体数を採取することが可能であった。その結果健常人における小腸の長軸方向の構造解析を可能とし、分子生物学的な厳密な制御により小腸構造が制御されていることを世界で初めて明らかとできた。また難治疾患における小腸病変に関する解析も開始しており初年度としての達成度としては十分と思われる。

２）研究成果の学術的・国際的・社会的意義について

これまでライブ環境における同一人物の全長小腸を解析できた例はなく、本結果が初めて小腸内環境を解析することができたことから学術的意義は非常に高い。また欧米ではカプセル内視鏡が主な小腸検査となっており、本邦で開発された内視鏡による小腸生検検体は本邦独自の解析手法であることからも国際的な評価は高いと考える。小腸はヒトで最大の器官であり、多機能である組織であることからも基本構造を理解し病態を解明することは社会的意義も多い。

３）今後の展望について

健常人における基本構造解析は、分子生物学的解析の進歩により多大な進歩が期待される。その一つとしては次世代シークエンスの登場であり、小腸生検検体内のすべての発現している遺伝子をすべて網羅的に同定することが可能となる。
小腸生検検体内には①上皮に付着する腸内細菌、②上皮細胞③上皮内リンパ球、④間質細胞が含まれておりそれらをすべて解析することが可能であり、一部解析をスタートさせていることからも大きなブレークスルーとなる機構を解明できると期待される。

また病態に関しても難治疾患の小腸検体の収集は進行中であり、本計画の継続により多施設共同研究を含めたさらなる検体の収集が期待できる。

４）研究内容の効率性について

当初たてた目標を着実に遂行できており、施行件数の多さ、患者へのインフォームドコンセント、同意率の高さなどから検体収集の効率は非常に高い。また解析についてもそれぞれ得られた検体の品質の高さから十分な成果が挙げられた。

５．結論

これまで到達できなかった小腸内部をライブ環境で観察しその状態での小腸粘膜の収集、解析が可能となった。また検査、検体収集、保存、解析までの大規模解析に向けたシステムの構築を行うことができた。以上から今後本計画の継続により、多数の検体収集、保存、解析が期待でき、小腸病態の解明に繋がると考える。

Ⅱ．　研究成果の刊行に関する一覧

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Ⅳ.　研究成果の別刷

RANK-RANKL Signaling Pathway Is Critically Involved in the Function of $CD4^+CD25^+$ Regulatory T Cells in Chronic Colitis[1]

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It is now clear that functional $CD4^+CD25^+$ regulatory T (T_R) cells exist as part of the normal immune population and prevent the development of intestinal inflammation. We have recently shown that $CD4^+CD25^+$ T_R cells reside in the intestine and control intestinal homeostasis in humans and mice. In this study, we demonstrate that the TNF family molecule RANKL and its receptor RANK are critically involved in controlling the function of $CD4^+CD25^+$ T_R cells in the intestine. We first found that RANKL was preferentially expressed on both $CD4^+CD25^+$ T_R cells and colitogenic $CD4^+$ T cells, whereas RANK was expressed on dendritic cells. Although neutralizing anti-RANKL mAb did not affect T_R activity of $CD4^+CD25^+$ T_R cells to suppress the proliferation of $CD4^+$ responder cells in vitro, in vivo administration of anti-RANKL mAb abrogated $CD4^+CD25^+$ T_R cell-mediated suppression of colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID mice. Interestingly, an adoptive transfer experiment using $Ly5.1^+CD4^+CD45RB^{high}$ cells and $Ly5.2^+CD4^+CD25^+$ T_R cells revealed that the ratio of $CD4^+CD25^+$ T_R cells in total $CD4^+$ T cells in inflamed mucosa was significantly decreased by anti-RANKL mAb treatment. Consistent with this, the expression of RANK on lamina propria $CD11c^+$ cells from colitic mice was significantly increased as compared with that from normal mice, and in vitro treatment with anti-RANKL mAb suppressed the expansion of $CD4^+Foxp3^+$ T_R cells in culture with colitic lamina propria $CD11c^+$ cells. Together, these results suggest that the RANK-RANKL signaling pathway is critically involved in regulating the function of $CD4^+CD25^+$ T_R cells in colitis. *The Journal of Immunology,* **2009, 182: 6079–6087.**

Intestinal mucosal surfaces are exposed to a large number of dietary and bacterial Ags (1–5). However, the gut-associated immune system fences off harmful Ags from systemic circulation and induces systemic tolerance against luminal Ags. In contrast, inflammatory bowel diseases (IBD)[4] and animal models of T cell-mediated chronic colitis are associated with the activation of intestinal and systemic immune responses (2, 3). In this regard, $CD4^+CD25^+$ regulatory T (T_R) cells play a central role in the maintenance of immunological homeostasis (6, 7). $CD4^+CD25^+$ T_R cells have been detected mainly in lymphoid sites, including thymus, lymph nodes, and spleen. Because numerous studies have demonstrated the capacity of $CD4^+CD25^+$ T_R cells to prevent the induction of immune responses and this suppression requires direct cell-cell contact with responder T cells or APCs, it is conceivable that $CD4^+CD25^+$ T_R cells act as a central regulator within lymphoid tissues (6–8).

The GALT can be divided into effector sites, which consist of lymphocytes scattered throughout the lamina propria (LP) of the intestinal mucosa and organized lymphoid tissues (inductive sites) such as mesenteric lymph nodes (MLNs) and Peyer's patches, which are responsible for the induction phase of immune responses (2, 9). It is thought that presentation of Ags to naive, effector, and memory T cells is concentrated at these inductive sites of organized mucosal lymphoid follicles, and thus APCs finely tune the balance between intestinal immune tolerance and inflammation.

In addition to the inductive sites, however, it remains unclear where $CD4^+CD25^+$ T_R cells suppress the development of colitis. Although it is reasonable to hypothesize that mechanisms for the induction, maintenance, and suppression of colitis would be centrally controlled in the inductive sites by $CD4^+CD25^+$ T_R cells, two-thirds of which constitutively express the lymph node-homing receptor CD62L (10), we previously demonstrated that human intestinal LP $CD4^+CD25^{bright}$ T cells obtained from normal individuals possess T_R activity in vitro and therefore questioned whether these inductive sites alone were involved in the induction and suppression of intestinal inflammation (11). We also reported that peripheral $CD4^+CD25^+$ T_R cells actually migrated to the intestine and suppressed the development of colitis in the $CD4^+CD45RB^{high}$ cell transfer model of colitis without the involvement of lymph nodes in lymph node-null $LT\alpha^{-/-} \times RAG\text{-}2^{-/-}$ recipient mice (12). Consistent with our previous reports, it has recently been reported that $CD4^+CD25^+$ T_R cells were detected in peripheral tissues and at sites of ongoing immune responses, such as synovial fluid from rheumatoid arthritis patients (13), tumors (14), transplants (15), skin lesions in mice infected

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[4] Abbreviations used in this paper: IBD, inflammatory bowel disease; LP, lamina propria; MLN, mesenteric lymph node; RANK, receptor activator of NF-κB; SP, spleen; RANKL, receptor activator of NF-κB ligand; T_R, regulatory T; IEL, intraepithelial lymphocyte; HPF, high-power field; Fwd, forward; Rev, reverse; MMC, mitomycin C; DC, dendritic cell; RA, retinoic acid.

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with *Leishmania major* (16), lungs from mice infected with *Pneumocystis carinii* (17), and diseased lesions in delayed-type hypersensitivity models (18), as well as in inflamed mucosa of colitic mice (8, 19).

However, it remains largely unknown which molecular mechanisms actually control the function of $CD4^+CD25^+$ T_R cells in the intestine to suppress intestinal inflammation. In the present study, we show that both $CD4^+CD25^+$ T_R cells and colitogenic $CD4^+$ T cells preferentially express a TNF family member, receptor activator of NF-κB ligand (RANKL), and that blockade of the signaling pathway via RANKL and its receptor activator of NF-κB (RANK) (20) by administering neutralizing anti-RANKL mAb abrogates the $CD4^+CD25^+$ T_R cell-mediated suppression of colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID mice, indicating a critical role for the RANKL/RANK signaling pathway in the function of intestinal $CD4^+CD25^+$ T_R cells in attenuating colitis.

Materials and Methods

Animals

Female BALB/c, CB-17 SCID, and C57BL/6-Ly5.2 mice were purchased from Japan Clea. C57BL/6-Ly5.1 mice and C57BL/6-Ly5.2 RAG-2-deficient ($RAG\text{-}2^{-/-}$) mice were obtained from Taconic Farms and Central Laboratories for Experimental Animals. Mice were maintained under specific pathogen-free conditions in the animal care facility of Tokyo Medical and Dental University. Mice were used at 7–12 wk of age. All experiments were approved by the regional animal study committees.

Antibodies

The following mAbs except anti-CCR9 mAb (R&D Systems) and reagents were purchased from BD Pharmingen: RM4-5-, PE-, or PerCP-conjugated anti-mouse CD4 (rat IgG2a); 7D4, FITC-conjugated anti-mouse CD25 (rat IgM); PC61, PE-conjugated anti-mouse CD25 (rat IgG1); H1.3F3, FITC-conjugated anti-CD69 (Ham IgG1); FJK-16s, allophycocyanin-conjugated anti-mouse Foxp3 (rat IgG2a); DATK32, PE-conjugated anti-integrin $\alpha_4\beta_7$ (rat IgG2a); M290, PE-conjugated anti-integrin $\alpha_E\beta_7$ (rat IgG2a); 242503, PE-conjugated anti-CCR9 (rat IgG2b); isotype control Abs, biotin-conjugated rat IgG2, FITC-conjugated rat IgM, PE-conjugated rat IgG2a, and PE-conjugated mouse IgG2a; PE-conjugated streptavidin; and CyChrome-conjugated streptavidin. The neutralizing anti-mouse RANKL mAb (IK22-5, rat IgG2a), and anti-mouse RANK mAb (12-31, rat IgG2a) were prepared as described previously (21).

Purification of T cell subsets

$CD4^+$ T cells were isolated from spleen cells of BALB/c mice using the anti-CD4 (L3T4) MACS system (Miltenyi Biotec) according to the manufacturer's instructions. Enriched $CD4^+$ T cells (96–97% pure, as estimated by FACSCalibur; BD Biosciences) were then labeled with PE-conjugated anti-mouse CD4 (RM4-5), FITC-conjugated anti-CD45RB (16A), FITC-conjugated anti-CD25 (7D4), and streptavidin-PE. Subpopulations of $CD4^+$ cells were isolated by two-color sorting on a FACSVantage (BD Biosciences). All populations were >98.0% pure on reanalysis.

In vivo adoptive transfer experiments

A series of in vivo experiments was conducted to investigate the role of the RANK/RANKL pathway in the expansion and function of $CD4^+CD25^+$ T_R cells in the suppression of murine chronic colitis. In experiment 1, chronic colitis was induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID mice (22). CB-17 SCID mice were injected i.p. with one or two subpopulations of sorted $CD4^+$ T cells in PBS and then administered 250 μg of anti-RANKL mAb or control rat IgG in 250 μl of PBS three times per week for 6 wk as follows: 1) $CD4^+CD45RB^{high}$ alone (3×10^5/mouse) plus control IgG ($n = 8$), 2) $CD4^+CD45RB^{high}$ alone (3×10^5) plus anti-RANKL mAb ($n = 8$), 3) $CD4^+CD45RB^{high}$ (3×10^5) plus $CD4^+CD25^+$ (1×10^5) plus control IgG ($n = 8$), or 4) $CD4^+CD45RB^{high}$ (3×10^5) plus $CD4^+CD25^+$ (1×10^5) plus anti-RANKL mAb ($n = 8$). Mice were sacrificed at 6 wk after T cell transfer. For experiment 2, to further assess the localization of effector T cells and T_R cells in the recipients, we used $Ly5.1^+CD4^+CD45RB^{high}$ cells and $Ly5.2^+CD4^+CD25^+$ cells as donors and C57BL/6 $RAG\text{-}2^{-/-}$ mice as recipients in the same treatment setting as experiment 1 (12). In experiment 3, to more properly assess the effect of anti-RANKL mAb on the trafficking of T_R cells to inflamed mucosa of colitic mice, we conducted another in vivo setting. First, $RAG\text{-}2^{-/-}$ mice were transferred with $CD4^+CD45RB^{high}$ T cells and, 4 wk after transfer, these colitic mice were treated with 250 μg of anti-RANKL mAb or control IgG two times within 1 day. Then they were retransferred with splenic $Ly5.1^+CD4^+$ T cells from normal mice, and we evaluated the cell number of $Ly5.1^+CD4^+Foxp3^+$ and $Foxp3^-$ cells recovered from LP, spleen (SP), and MLNs at 24 h after the retransfer.

Disease monitoring and clinical scoring

The recipient SCID mice after T cell transfer were weighed initially, then three times per week thereafter. They were observed for clinical signs of illness: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. Mice were sacrificed and assessed for a clinical score as the sum of four parameters: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; and an additional point was added if gross blood was noted) (12).

Histological examination

Tissue samples were fixed in PBS containing 6% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. Three tissue samples from the proximal, middle, and distal parts of the colon were prepared. The sections were analyzed without prior knowledge of the type of T cell reconstitution or treatment. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated using a modification of a previously described scoring system (12), as follows: mucosal damage, 0; normal, 1; 3–10 intraepithelial lymphocytes (IEL)/high-power field (HPF) and focal damage, 2; >10 IEL/HPF and rare crypt abscesses, 3; and >10 IEL/HPF, multiple crypt abscesses and erosion/ulceration, submucosal damage, 0; normal or widely scattered leukocytes, 1; focal aggregates of leukocytes, 2; diffuse leukocyte infiltration with expansion of submucosa, 3; diffuse leukocyte infiltration, muscularis damage, 0; normal or widely scattered leukocytes, 1; widely scattered leukocyte aggregates between muscle layers, 2; leukocyte infiltration with focal effacement of the muscularis, and 3; extensive leukocyte infiltration with transmural effacement of the muscularis.

Preparation of mucosal LP mononuclear cells

Colonic LP mononuclear cells were isolated using a method described previously (22). In brief, the entire length of intestine was opened longitudinally, washed with PBS, and cut into small (~5-mm) pieces. To remove epithelium including IEL, the dissected mucosa was incubated two times with $Ca^{2+}Mg^{2+}$-free HBSS containing 1 mM DTT (Sigma-Aldrich) for 30 min and then serially incubated two times in medium containing 0.75 mM EDTA (Sigma-Aldrich) for 60 min at 37°C under gentle shaking. The supernatants from these incubations, which included the epithelium and IEL, were deserted, and the residual fragments were pooled and treated with 2 mg/ml collagenase A (Worthington Biomedical) and 0.01% DNase (Worthington Biochemical) in 5% CO_2 humidified air at 37°C for 2 h. The cells were then pelleted two times through a 40% isotonic Percoll solution and further purified by Ficoll-Hypaque (Pharmacia) density gradient centrifugation (40%/75%). Enriched $CD4^+$ LP T cells were obtained by positive selection using an anti-CD4 (L3T4) MACS magnetic separation system. The resultant cells when analyzed by FACSCalibur contained >96% $CD4^+$ cells.

RT-PCR

Total cellular RNA was extracted from 7×10^5 cells using a RNeasy Mini Kit (Qiagen). Five micrograms of total RNA was reverse-transcribed using the Superscript II Reverse Transcriptase (Invitrogen). RANK and RANKL levels were measured with a QuantiTect SYBER green PCR kit using Applied Biosytems I7500 real-time PCR system and 7500 system SDS software with the following primers: RANK: forward (Fwd), 5′-GGT CTG CAG CTC TTC CAT GAC-3′ and reverse (Rev) 5′-TGA GAC TGG GCA GGT AAG CC-3′; RANKL: Fwd, 5′-TTG CAC ACC TCA CCA TCA ATG-3′ and Rev, 5′-TTA GAG ATC TTG GCC CAG CCT-3′; and G3PDH: Fwd, 5′-CTA CTG GCG CTG CCA AGG CAG T-3′ and Rev, 5′-GCC ATG AGG TCC ACC ACC CTG-3′. PCR cycling conditions consisted of 95°C for 15 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 40 s. Data are expressed as the relative amount of indicated mRNA as normalized against G3PDH.

Flow cytometry

To detect the surface expression of various molecules, isolated splenocytes or LP mononuclear cells were preincubated with a FcγR-blocking mAb

(CD16/32, 2.4G2; BD Pharmingen) for 15 min, then incubated with specific FITC-, PE-, or biotin-labeled Abs for 20 min on ice. Biotinylated Abs were detected with PE- or CyChrome-streptavidin. Intracellular Foxp3 staining was performed with the allophycocyanin-anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer's instructions. Standard two- or three-color flow cytometric analyses were obtained using the FACSCalibur with CellQuest software. Background fluorescence was assessed by staining with control isotype-matched mAbs.

Cytokine ELISA

To measure cytokine production, 1×10^5 LP $CD4^+$ T cells were cultured in 200 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar) precoated with 5 μg/ml hamster anti-mouse CD3ε mAb (145-2C11; BD Pharmingen) and 2 μg/ml hamster anti-mouse CD28 mAb (37.51; BD Pharmingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA per the manufacturer's recommendation (R&D Systems).

In vitro regulatory function of $CD4^+CD25^+$ T cells

Spleen cells from BALB/c mice were separated into unfractionated whole $CD4^+$ T cells and $CD4^+CD25^+$ T cells using the anti-CD4 (L3T4) MACS magnetic separation system and/or FACSVantage as described above. Responder $CD4^+$ cells (7×10^4) and mitomycin C (MMC)-treated $CD4^-$ cells (5×10^4) as APCs, with or without $CD4^+CD25^+$ cells (1×10^4), were cultured in the presence or absence of neutralizing anti-RANKL mAb or anti-RANK mAb (1, 3, or 10 μg/ml) for 72 h in round-bottom 96-well plates in RPMI 1640 medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-ME. Incorporation of [^{3}H]thymidine (1μCi/well) by proliferating cells was measured during the last 9 h of culture. In some experiments, a CFSE dilution assay was performed. To this end, splenic $CD4^+$ T cells were negatively obtained from whole spleen cells of normal C57BL/6 mice using a CD4 T Cell Isolation Kit (Miltenyi Biotec). $CD4^+CD25^-$ responder T and $CD4^+CD25^+$ T_R cells were separately isolated using anti-CD25 MACS beads (Miltenyi Biotec) from the $CD4^+$ T cells. $CD11c^+$ dendritic cells (DC) were isolated from SP and LP of normal C57BL/6 mice and colitic C57BL/6 background RAG-$2^{-/-}$ mice previously transferred with $CD4^+CD45RB^{high}$ T cells. All populations were >92% pure on reanalysis. Then, $CD4^+CD25^-$ responder T cells were labeled with 1 μM CFSE (Molecular Probes). CFSE-labeled $CD4^+$ $CD25^-$ responder T cells (1×10^5) were cocultured with unlabeled $Ly5.2^+CD4^+CD25^+$ T_R cells (0.33×10^5) with $CD11c^+$ DC (2×10^4) and anti-CD3 (1 μg/ml) in the presence of anti-RANKL mAb (1 μg/ml) or control IgG (1 μg/ml) in 96-well round-bottom plates for 120 h in triplicates. After incubation, cells were collected and analyzed by FACS. Propidium iodide was added to exclude dead cells. Proliferation analysis was based on division times of responder $CFSE^+CD4^+$ T cells at the condition that can discriminate the unlabeled $CD4^+CD25^+$ T_R cells and $CD11c^+$ DC cells.

In vitro induction of gut-homing receptors and $CD4^+Foxp3^+$ T_R cells

For the isolation of DC from LP, MLN, and SP of colitic mice, total cells obtained by digestion with collagenase were passed through a 40-μm cell strainer. $CD11c^+$ DC were further purified using an anti-CD11c MACS magnetic separation system, resulting in >80% purity. In vitro assay for the induction of gut-homing receptors was performed by the modified protocol established by others (23). In short, 2×10^5 $CD4^+$ T cells purified from normal BALB/c mice were cultured with 2×10^3 purified LP, MLN, or splenic $CD11c^+$ DC from colitic mice in addition to a soluble 1 μg/ml anti-CD3 mAb (BD Biosciences), human 10 ng/ml rTGF-β (PeproTech), all-*trans* retinoic acid (RA), and 5 ng/ml human rIL-2 (Shionogi Pharm) with or without 1 μg/ml anti-RANKL mAb. On day 4, cells were stained with PE-conjugated anti-CD4 mAb and FITC-conjugated anti-integrin $\alpha_4\beta_7$, FITC-conjugated anti-integrin $\alpha_E\beta_7$, or FITC-conjugated anti-CCR9 mAb. In some experiments, 2×10^5 $CD4^+$ T cells purified from normal BALB/c mice were cultured with 2×10^3 purified LP, MLN, or SP $CD11c^+$ DC from colitic mice in addition to soluble 1 μg/ml anti-CD3 mAb (BD Biosciences) with or without 1 μg/ml anti-RANKL mAb. On day 4, intracellular Foxp3 staining was performed with the allophycocyanin-anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer's instructions.

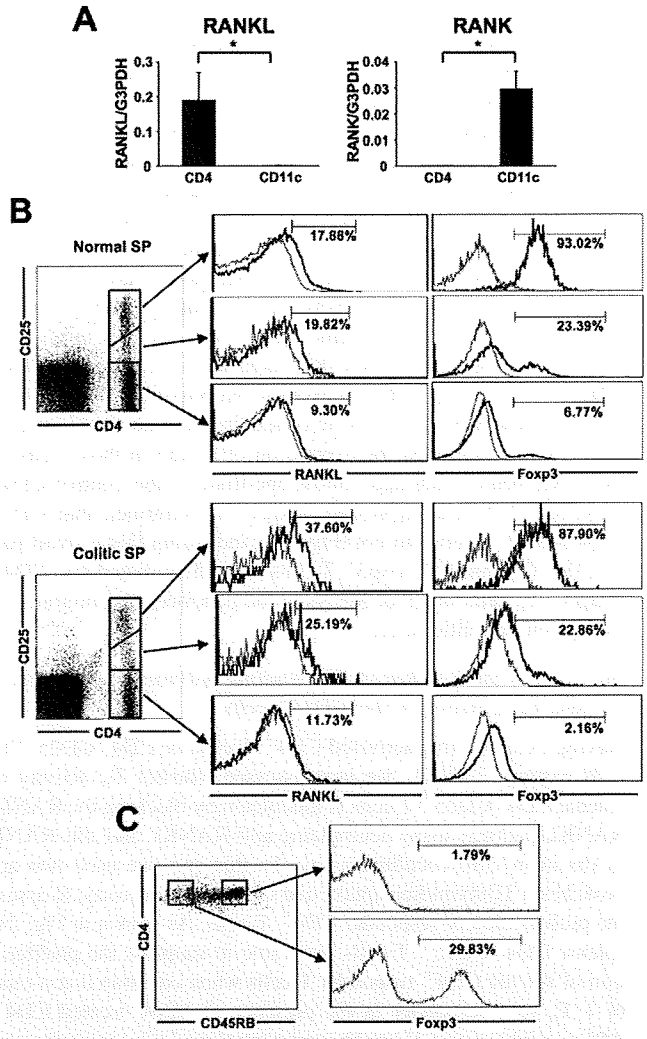

FIGURE 1. Expression of RANK/RANKL in spleen cells from normal and colitic mice. *A*, Expression of RANK, RANKL, and G3PDH mRNA was determined by RT-PCR using five SP samples each from normal and colitic mice and is shown as relative amount of the indicated mRNA normalized to G3PDH. Data are represented as the mean ± SEM of five samples. *, $p < 0.005$. *B*, Phenotypic characterization of splenic $CD4^+$ T cells expressing CD25 and Foxp3 by FACS. Correlation of CD25/Foxp3 and RANKL expression on/in splenic $CD4^+$ T cells obtained from normal or colitic mice. Representatives of four separate samples in each group. *C*, Expression of Foxp3 in a population of $CD4^+CD45RB^{high}$ or $CD45RB^{low}$ T cells purified from spleen of normal BALB/c mice.

Statistical analysis

The results are expressed as the mean ± SEM. Groups of data were compared using the Mann-Whitney *U* test. Differences were considered to be statistically significant when $p < 0.05$.

Results

Murine splenic $CD4^+CD25^+$ cells constitutively express RANKL

To assess the role of the RANK/RANKL axis in the pathogenesis of chronic colitis in terms of stimulatory and inhibitory effects, we first assessed the expression of RANK and RANKL molecules in normal splenic $CD4^+$ T cells and $CD11c^+$ DC by RT-PCR. As shown in Fig. 1*A*, $CD4^+$ T cells preferentially expressed RANKL, but not RANK mRNA, while $CD11c^+$ cells preferentially expressed RANK, but not RANKL mRNA. We further assessed the expression of RANKL on $CD4^+$ T cells obtained from colitic SCID mice induced by adoptive transfer of $CD4^+CD45RB^{high}$ T

cells and age-matched normal BALB/c mice at a protein level by flow cytometry. As shown in Fig. 1*B*, splenic $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells in normal mice slightly but substantially expressed RANKL, while $CD4^+CD25^-$ cells did not. We confirmed that almost all $CD4^+CD25^{high}$ T cells expressed Foxp3, while $CD4^+CD25^{low}$ T cells did not, indicating that $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells are T_R and previously activated effector T cells, respectively (Fig. 1*B*). Interestingly, although colitic SCID mice were reconstituted with a splenic $CD4^+CD45RB^{high}$ T cell population that lacks $CD4^+CD25^+$ T_R cells, inducible $CD4^+CD25^{high}Foxp3^+$ T cells as well as activated $CD4^+CD25^{low}Foxp3^-$ T cells were found in the spleen and these cells expressed the RANKL molecule, in contrast to $CD4^+CD25^-$ T cells (Fig. 1*B*). To exclude the possibility that isolated $CD4^+CD45RB^{high}$ T cells were contaminated with $CD4^+Foxp3^+$ T_R cells, we examined the expression of Foxp3 in those cells by flow cytometry. Although almost one-third of the control $CD4^+CD45RB^{low}$ T cells expressed Foxp3, we confirmed that $CD4^+CD45RB^{high}$ T cells did not (Fig. 1*C*), indicating that a small part of $CD4^+CD45RB^{high}Foxp3^-$ T cells had differentiated into $CD4^+Foxp3^+$ T_R cells in LP or MLNs in the periphery and migrated to the spleen in colitic mice.

Blockade of the RANK/RANKL signaling pathway did not affect in vitro T_R activity of $CD4^+CD25^+$ cells

Having evidence that activated $CD4^+$ T cells or $CD4^+CD25^+$ T_R cells express RANKL, we next assessed whether T_R activity of splenic $CD4^+CD25^+$ T cells is modulated by blocking the RANK/RANKL pathway using neutralizing anti-RANKL and anti-RANK mAbs in in vitro coculture assay. To this end, we used two approaches, [^{3}H]thymidine uptake and CFSE dilution assay, to assess the proliferation of responder $CD4^+$ T cells. As shown in Fig. 2*A*, splenic $CD4^+CD25^+$ T cells were able to suppress the proliferation of splenic $CD4^+$ responder T cells when cocultured at a ratio of 1 T_R to 1 responder in the presence of MMC-treated $CD4^-$ APCs, soluble anti-CD3 mAb, and control rat IgG. Similarly, neither anti-RANKL nor anti-RANK mAb at concentrations of 1–10 μg/ml affected the T_R activity of $CD4^+CD25^+$ T_R cells (Fig. 2*A*). To further precisely assess the role of the RANK/RANKL pathway in the suppressive activity of $CD4^+CD25^+$ T_R cells to block the proliferation of $CD4^+CD25^-$ responder T cells at the same ratio of the following in vivo adoptive transfer experiment, we next used $CD4^+CD25^-$ T cells as responder T cells and SP or LP $CD11c^+$ DC from normal or colitic mice and adopted the CFSE dilution assay to assess the proliferation of responder $CD4^+CD25^-$ T cells without the impact of the proliferation of cocultured $CD4^+CD25^+$ T_R cells. As shown in Fig. 2*B*, the proliferation of $CD4^+CD25^-$ responder T cells is seen in the right part of the histogram, and they extensively proliferated in response to anti-CD3 mAb in the absence of $CD4^+CD25^+$ T_R cells with any $CD11c^+$ DC regardless of SP or LP and normal or colitic DC. In contrast, the addition of $CD4^+CD25^+$ T_R cells prevented the proliferation of $CD4^+CD25^-$ responder T cells in the presence of anti-RANKL mAb to a similar extent in the presence of control IgG, indicating that the blockade of the RANK/RANKL pathway does not affect the suppressive activity of $CD4^+CD25^+$ T_R cells at least in vitro.

Blockade of the RANK/RANKL signaling pathway abolished $CD4^+CD25^+$ T_R cell-mediated suppression of colitis in vivo

Although blockade of the RANK/RANKL signaling pathway did not affect the T_R function of $CD4^+CD25^+$ cells in the coculture assay (Fig. 2), in vitro assays do not always reflect function in vivo. Thus, we next administered neutralizing anti-RANKL mAb to SCID mice transferred with $CD4^+CD45RB^{high}$ cells alone or a

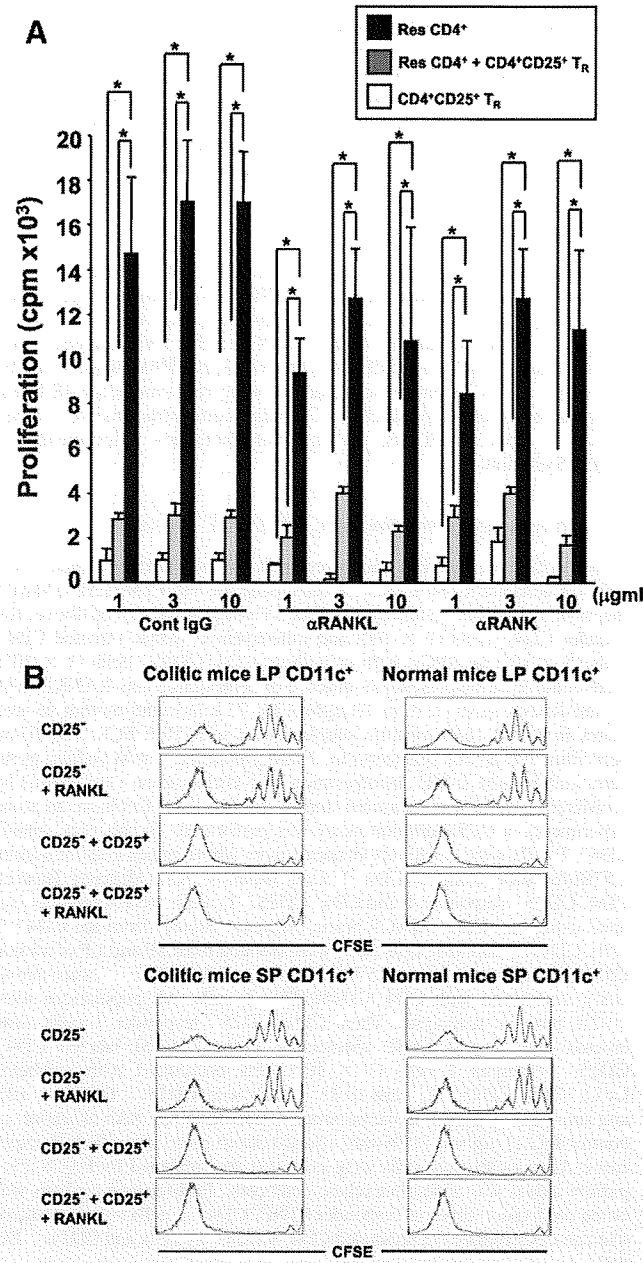

FIGURE 2. Blockade of the RANK/RANKL signaling pathway does not affect T_R activity in in vitro coculture assay. *A*, Splenic $CD4^+CD25^+$ T cells suppress the proliferation of responder (Res) $CD4^+$ T cells in vitro in the presence of neutralizing anti-RANKL or anti-RANK mAb. Responder $CD4^+$ T cells and sorted $CD4^+CD25^+$ T cells were cocultured for 72 h with anti-CD3 mAb at a 1:1 ratio in the presence of MMC-treated APCs and in the presence of various concentrations (1, 3, or 10 μg/ml) of control IgG, anti-RANKL, or anti-RANK mAb. [^{3}H]Thymidine ([^{3}H]TdR) uptake was determined for the last 9 h. Data are represented as the mean $\pm$ SEM of triplicate samples. *, $p < 0.05$. *B*, Splenic $CD4^+CD25^+$ T_R cells suppress the proliferation of $CD4^+CD25^-$ responder T cells in vitro in the presence of anti-RANKL mAb. CFSE-labeled $CD4^+CD25^-$ responder T cells (1×10^5) and $CD4^+CD25^+$ T_R cells (0.33×10^5) were cocultured for 120 h with $CD11c^+$ DC obtained from SP or LP of normal or colitic mice (2×10^4) in the presence of anti-RANK mAb (1 μg/ml) or control IgG (1 μg/ml) in addition to anti-CD3 mAb (1 μg/ml). Proliferation of the $CD4^+CD25^-$ responder T cells was measured by the dilution of CFSE on $CD4^+CD25^-$ responder T cells that is shown at the *right part* of the histogram at 120 h after culture. The unlabeled cells including $CD4^+CD25^+$ T_R cells and contaminated DC are seen at the *left part* of the histogram. Data are represented from four independent experiments.

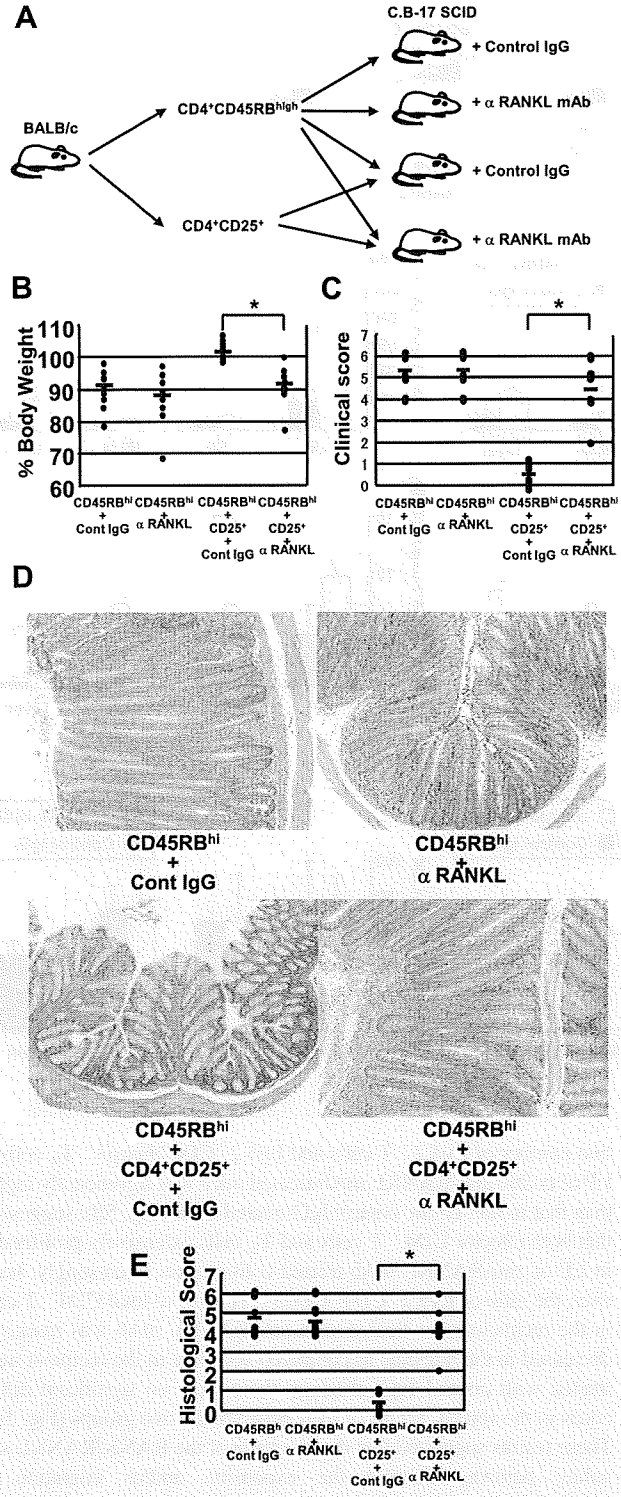

FIGURE 3. Anti-RANKL mAb abrogates $CD4^+CD25^+$ T_R cell-mediated suppression of colitis in vivo. *A*, C.B-17 SCID mice were injected i.p. with $CD4^+CD45RB^{high}$ (3×10^5/mouse) alone or $CD4^+CD45RB^{high}$ (3×10^5) + $CD4^+CD25^+$ T cells (1×10^5) and treated with control IgG or anti-RANKL mAb by i.p. injection at a dose of 250 μg three times per week over 6 wk starting at the time of T cell transfer. *B*, Change in body weight over time is expressed as percentage of the original weight. Data are represented as the mean ± SEM of seven mice in each group. *, $p < 0.05$. *C*, Clinical scores were determined at 6 wk after transfer as described in *Materials and Methods*. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.05$. *D*, Histological examination of the colon at 6 wk after transfer. Original magnification, ×100. *E*, Histological scores were determined at 6 wk after transfer as described in *Materials and Methods*. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.05$.

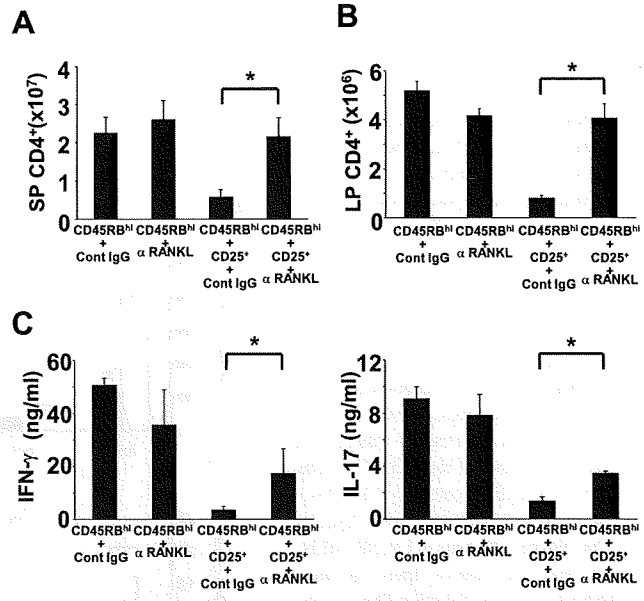

FIGURE 4. Anti-RANKL mAb abrogates $CD4^+CD25^+$ T_R cell-mediated suppression of Th1 and Th17 cells in vivo. *A*, SP and LP $CD4^+$ T (*B*) cells were isolated from the colon at 6 wk after adoptive T cell transfer and treated as described in Fig. 3, and the number of $CD4^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.01$. *C*, Cytokine production by $CD4^+$ lamina propria lymphocyte. LP $CD4^+$ cells were stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. IFN-γ and IL-17 concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean ± SEM of six mice in each group. *, $p < 0.01$.

mixture of $CD4^+CD45RB^{high}$ cells and $CD4^+CD25^+$ T cells (Fig. 3*A*). As a positive control, mice transferred with $CD4^+CD45RB^{high}$ T cells alone and treated with control rat IgG developed wasting disease, which became evident 3–5 wk after transfer (Fig. 3*B*), and clinical (Fig. 3*C*) and histological evidence of severe chronic colitis as estimated at 6 wk after transfer (Fig. 3, *D* and *E*). As a negative control, mice transferred with both $CD4^+CD45RB^{high}$ and $CD4^+CD25^+$ T_R cell populations and administered with the control rat IgG did not develop wasting disease or colitis (Fig. 3, *A–E*). Although we showed that activated $CD4^+CD25^{low}Foxp3^-$ effector T cells express RANKL (Fig. 1), the administration of anti-RANKL mAb to mice transferred with $CD4^+CD45RB^{high}$ cells alone did not affect the severity of colitis as compared with the control IgG-treated mice transferred with $CD4^+CD45RB^{high}$ cells alone (Fig. 3, *A–E*), suggesting that the RANK/RANKL pathway between RANKL-expressing activated effector $CD4^+$ T cells and RANK-expressing APCs is not critically involved in the development of colitis. Surprisingly, however, the administration of anti-RANKL mAb did induce severe colitis in mice transferred with $CD4^+CD45RB^{high}$ cells and $CD4^+CD25^+$ T_R cell populations, indicating that the anti-RANKL mAb treatment abolished the $CD4^+CD25^+$ T_R cell-mediated in vivo suppression of colitis induced by adoptive transfer of $CD4^+$ $CD45RB^{high}$ T cells.

A further quantitative evaluation of $CD4^+$ T cell infiltration was made by isolating spleen cells and LP cells from the resected bowels. Significantly higher numbers of $CD4^+$ T cells were recovered from the spleen (Fig. 4*A*) and LP (Fig. 4*B*) of the mice transferred with both $CD4^+CD45RB^{high}$ and $CD4^+CD25^+$ T_R cell populations and administered with anti-RANKL mAb as compared with control IgG, being comparable to those from mice transferred with $CD4^+$ $CD45RB^{high}$ T cells alone and treated with anti-RANKL mAb or the control rat IgG. We also examined the cytokine production by $CD4^+$