付き添いとなる場合が多く，患児のみならずドナーでもある母親の術後経過にも注意を払う必要がある。実際に，生体ドナーの12.4%が胆汁瘻などの術後合併症を患うことも報じられており，対症療法として用いられるステロイド剤の情報提供は必須と考えられる[7]。

(2) 成人症例

小児症例のドナーはほとんどが両親であるが，成人患者のドナーは配偶者の場合が多い。この場合，血液型不適合症例が増える傾向にあり，血液型一致/適合症例に比して術前からの十分な準備が必要となる。すなわち，血漿交換による抗A抗体，抗B抗体，IgMの物理的な除去に加えて，リツキシマブを用いたB細胞の排除が実施される。したがって，多様な免疫抑制薬それぞれについて，簡単にまとめた情報提供シートをもとに十分に説明することが求められる。場合によっては術中に脾臓を摘出するため，術後の感染対策について十分に情報提供を行うことが求められている。

2. 医師，看護師など医療従事者

肝臓移植患者およびその家族は，移植術後の集中治療室（ICU）管理を経て病棟にて加療，社会復帰を目指すためさまざまな職種が関わることになる。移植病棟担当の薬剤師は，患者およびその家族に対する薬物治療の継続的な説明と理解を促し，医師・看護師への同効薬や薬剤選択に関わる情報提供，血中濃度モニタリングが必要な薬物については採血タイミングのコンサルテーションなどを行うことが求められる。特に，退院後のかかりつけ医や薬局に対する情報提供は，移植コーディネーターを介して行われる場合が多いため，彼らに対する薬物の理解を促すという側面も重要である。同時に，当該施設において実施されてきた過去の症例分析に加えて，論文などで公表されている多施設の情報を考えあわせて，常に最も合理的な薬物治療を選択できる体制を維持することが肝要である。

タクロリムス，シクロスポリンの血中濃度管理に関わる情報提供

1. カルシニューリン阻害薬の特徴

いずれもT細胞の脱リン酸化酵素であるカルシニューリンに対する特異的な阻害薬として，nuclear factor activated T cell（NFATC）の脱リン酸化を抑制し，T細胞の増殖を促すサイトカインであるインターロイキン(IL)-2の産生抑制を薬効としている。シクロスポリンは，細胞内受容体であるシクロフィリンと結合し，カルシニューリン阻害活性を示す。一方，タクロリムスは白血球内のFK506 binding protein 12kd（FKBP1A）と結合し，シクロスポリンと同様にカルシニューリン阻害活性を示す。いずれも化学構造上まったく異なるにもかかわらず，薬理効果の作用機序や薬物動態の制御因子は類似すると考えられてきた。また，いずれの薬物も副作用として腎毒性や中枢毒性を示す。

2. 薬物動態

いずれもバイオアベイラビリティに大きな個人差を示し，タクロリムスのそれは15～80%とされる。タクロリムスの体内動態には大きな個人差があるものの，トラフ(C_0)レベルの血中濃度がAUCと良好な相関関係を示すことから，TDMにおいてトラフモニタリングが推奨されている[8]。一方，マイクロエマルション製剤が開発され，シクロスポリンの吸収特性は飛躍的に向上した。Mahalatiらは，従来のトラフモニタリングでは，シクロスポリンのAUCにおける個人差を反映できないことから，吸収相（$AUC_{0\sim4}$）を根拠に投与設計をすることが拒絶反応の抑制，副作用の予防に有効であると発表している[9]。実地診療において複数の採血は，患者と医療従事者の双方に大きな負担を強いることから，最小の採血ポイントを見いだす試みが続けられ，$AUC_{0\sim4}$と最も相関性の高い服用2時間後の血中濃度値（C_2）のモニタリングが有用であると発表された。われわれは，周術期肝臓移植患者におけるシクロスポリンの1日1回投与法の有用性を発表しているが，そのなかでトラフレベルの継続的な高値は高確率で腎障害を引き起こすことを見いだしている[10]。したがって，薬効の指標としてC_2モニタリング，副作用予防の指標としてトラフモニタリングを考慮するのが望ましいと考え，日常診療で活用している。しかしながら，最高血中濃度を示す経口投与後の時間（T_{max}）の個人差も見いだされており，すべての患者でC_2値をモニタリングすることの妥当性については，一定

の考慮を要すると考えられる。

タクロリムスやシクロスポリンは，消化管粘膜における初回通過効果を経て，門脈を通じて引き続き肝臓における初回通過効果を受ける。これらの薬物は肝臓において，主として薬物代謝酵素チトクロムP4503A4（CYP3A4）によって代謝されること，代謝物および未変化体薬物はmultidrug resistance 1（MDR1）遺伝子の産物であるP糖蛋白質（Pgp）を介して胆汁中に排泄されることが知られている。また，CYP3A4やPgpは小腸粘膜にも発現しており，経口投与されたこれら薬物の吸収過程における代謝・排泄を媒介することによって，薬物の吸収障壁として協働的に機能することから，経口投与された免疫抑制薬の血中濃度を支配する重要な生体因子として位置づけられている[10),11)]。したがって，小腸のPgpやCYP3A4の同一患者における発現変動や個人差に関する情報は，これら免疫抑制薬の個別投与設計を行ううえで有用な指標になると考えられる。最近では，CYP3A4の類縁体であるCYP3A5がタクロリムスの代謝に重要な役割を担うことも明らかにされ，これらの代謝酵素や薬物トランスポーターを中心とした薬物相互作用などが注目されている。

3. ファーマコゲノミクス

(1) 薬物動態制御因子の発現レベルと免疫抑制薬の体内動態における個人差の関係

生体肝移植手術では，胆道系の疾患を有する患者において，患者の小腸を一部用いて移植肝から流れ出る胆汁を通すための胆管再建を行う場合がある。われわれは，このときに切除される小腸組織片の一部を用いて，小腸上皮細胞に発現するPgpおよびCYP3A4の発現量を定量し，術後のタクロリムス体内動態との相関解析を行った。その結果，術後早期におけるタクロリムスの血中濃度/投与量（C/D）比は，小腸Pgpの発現レベルと良好な逆相関を示した[11)]。一方，CYP3A4発現レベルとの相関関係は認められなかった。さらに，MDR1高発現群における平均C/D比はMDR1低発現群の約50%であり，一定のタクロリムス血中濃度を得るために，MDR1高発現群では，MDR1低発現群と比較して約2倍のタクロリムス投与量を必要とすることが明らかとなった。これらの結果から，小腸Pgp発現量は，タクロリムス体内動態に大きく影響することが判明した。生体肝移植治療に用いられる移植肝は部分肝であり，そのサイズは移植後経日的に回復する。タクロリムスの体内動態についても経日的な全身クリアランスの増大を認めるが[12)]，術後初期における有用な投与量設定のための指標が不明であった。これらの結果から，術後初期においては移植された肝組織の機能レベルが低値であり，小腸Pgpの発現量が，特に術後初期段階における投与量設定マーカー，すなわちタクロリムス治療の際の個体間変動要因となると考えられる。

現在われわれは，小腸を用いた胆管再建の対象となる患者に対し，術時小腸組織を用いたMDR1 mRNA定量数値化とそれに基づくタクロリムスの初期投与量設定を継続しており，一定の成果を上げつつある。一方，良好な小腸Pgp発現量とタクロリムスのC/D比の逆相関関係は，術後10日以降乖離しはじめる。このことは，術後の経時的な肝機能の回復とそれに伴う肝クリアランスの増大に起因すると考えられ[13)]，術後2週間以降はPPKパラメータとベイジアン解析を中心に[12)]，タクロリムスの個別投与設計を行うことで補えるであろう。

小腸Pgp発現レベルは，生体肝移植直後におけるタクロリムス吸収特性の個人差を予測するうえで有用であるが，個体内における変動については不明であった。われわれは，原発性硬化性胆管炎により生体肝移植治療を受けた後，慢性拒絶反応による再移植を受けた患者において小腸Pgp発現量が初回移植術時に比して3.5倍上昇していることを見いだした。さらに，それによると考えられるシクロスポリンの著しい吸収不良と考えられる事象について報告した[14)]。本症例では，慢性拒絶反応に加え慢性的な胆管炎も合併しており，さまざまな臨床症状が小腸のPgp発現量を変化させること，肝機能が低下している場合には小腸Pgpを介した免疫抑制薬の小腸管腔中への排出活性の影響が，薬物の肝代謝よりも大きくなることが示唆された。

(2) *MDR1*遺伝子多型の影響

これまで述べてきたように，肝臓移植患者に対する小腸Pgp発現レベルは，術後のタクロリムスまたはシクロスポリンを中心とした免疫抑制療法を円滑に行ううえで

有用であると考えられた。近年，ジゴキシンなどPgpの基質について，その薬物動態が*MDR1*遺伝子多型によって影響を受けることが報告されている[15]。すでに述べたように，生体肝移植患者における術後初期のタクロリムス体内動態の個人差は，小腸Pgpの発現レベルと良好な負の相関を示した。したがって，小腸Pgpの発現量に影響を与える遺伝子多型を見いだすことができれば，患者一人ひとりの小腸組織を採取・解析するまでもなく，初期投与量を設定することが可能になると考えられる。そこで，*MDR1*遺伝子多型のなかから主な10カ所について遺伝子多型解析を行った。そのなかで，小腸Pgpの発現量や機能に関係があると考えられているMDR1 mRNAの3435番目のCからTに変異する多型について解析を進めた。その結果，小腸Pgpの発現量や術後初期のタクロリムスのC/D比に対してC3435T多型は，有意な影響を示さなかった[16]。生体肝移植患者において*MDR1*遺伝子のC3435T多型が，小腸CYP3A4発現量や薬物体内動態とどのような関係にあるかについては，今後さらに詳細な解析を実施する必要があると考える。

(3) *CYP3A5*遺伝子多型の影響

CYP3A4のアイソフォームであるCYP3A5の多型性について，その発現はCYP3A5ゲノム上のイントロン3における一塩基多型（SNP）が引き起こすCYP3A5 mRNAのスプライシング異常とそれに引き続く機能蛋白質の欠失に左右される。この遺伝子変異は欧米系コーカサス人種においては高い頻度で認められ，その結果，60～90%のコーカサス人種はCYP3A5機能を欠失する。一方，少なくとも50%以上のアフリカ系人種は野生型である*CYP3A5**1遺伝子多型を有し，機能蛋白質を発現するとされている。そして日本人を含むアジア系人種では，*CYP3A5**1遺伝子多型の頻度は27.6%であり，コーカサス人種とアフリカ系人種の中間と理解できる[17),18)]。生体肝移植術を受けた患者については，小腸は患者自身の遺伝子型を反映するものの肝臓はドナー由来であり，両者の遺伝子型が異なることが想定される。したがって，肝臓移植患者においてはCYP3A5活性が肝臓のみ，あるいは小腸のみに認められる症例が存在する。そこで，移植術後の経過日数に伴うタクロリムス体内動態の個体内変動と*CYP3A5*遺伝子多型との関係について調べた結果，術直後では小腸における多型が，術後2週以降では移植肝の多型が強く影響を及ぼすことが明らかになっている。これらの検討により，移植肝/患者体重比（%），術時の小腸MDR1 mRNA発現量，患者小腸および移植肝*CYP3A5*遺伝子多型情報をタクロリムス動態影響因子として日常診療への応用を進めている[19]。

薬物相互作用に関わる情報

タクロリムスやシクロスポリンは，PgpやCYP3A4/5の基質であるためさまざまな併用薬による薬物動態学上の影響を受ける。個々の事例については膨大な蓄積があると考えられるが，ここでは筆者らの経験を交えて若干紹介するにとどめる。

(1) グレープフルーツジュース

果皮，果肉に含まれるフラノクマリン誘導体などが相互作用の原因物質とされており，経口投与された薬物の小腸粘膜における薬物代謝能を撹乱することによってシクロスポリンの血中濃度上昇を引き起こすことが知られている。われわれは，タクロリムスの血中濃度コントロールに難渋していた生体肝移植後の症例に対して，グレープフルーツジュースを適用した。患者自身の訴えによりグレープフルーツジュースの飲用を中止したが，遅れてタクロリムスの血中濃度上昇が観察され，飲用中止後も注意が必要であると考えられた[20]。一方，グレープフルーツジュースの飲用は免疫抑制薬の服用量の削減に加えて，血中濃度コントロールに難渋する際の一手法として考慮している。

(2) プロトンポンプ阻害薬

高度な侵襲を伴う肝移植術を受けた患者の多くは，ストレス性の潰瘍性消化管出血の予防を目的に，術直後からプロトンポンプ阻害薬が投与される。プロトンポンプ阻害薬として用いられるオメプラゾールの解毒機構は，80%をCYP2C19，20%をCYP3A4が担うとされる。一方，ランソプラゾールのそれはCYP2C19とCYP3A4がほぼ同程度の寄与を示すことが知られている[21]。欧米系コーカサス人種に比して日本人を含むアジア系人種では，CYP2C19の機能欠損型を示す割合が約20%と高いこと，肝臓移植患者においては小腸と肝臓の遺伝子型が異なる

症例がしばしば存在することから，タクロリムスとの相互作用には遺伝子型が影響を及ぼすことが想定される。われわれは，タクロリムスとプロトンポンプ阻害薬の相互作用に，肝移植患者小腸およびドナー肝の*CYP2C19*と*CYP3A5*遺伝子多型の組み合わせが影響を及ぼすと想定し，89症例を対象に検討を行った。その結果，*CYP2C19*および*CYP3A5*遺伝子多型が患者小腸およびドナー肝において同時に機能欠損型である症例において，オメプラゾール併用によってタクロリムスのC/D比が約3倍上昇することが判明した。一方，ランソプラゾールを併用した患者では*CYP2C19*遺伝子多型の影響はほとんどみられず，むしろ*CYP3A5*遺伝子多型の影響を強く受けることが示された。生体における消失経路のほとんどが代謝酵素非依存性であるラベプラゾールについては，遺伝子型によらずタクロリムスのC/D比は変動しないことも見いだされた[21]。

(3) アゾール系抗真菌薬

アスペルギルス感染症の予防を目的として，β-Dグルカン値の上昇ならびにアスペルギルス抗原陽性を呈した患者に対して，イトラコナゾールまたはボリコナゾールが用いられる。イトラコナゾールは強力なCYP3A4阻害作用を有しており，併用開始とともに必然的にタクロリムスやシクロスポリンの血中濃度上昇を引き起こす[22]。また，ボリコナゾールはその主要代謝酵素がCYP2C19とCYP3A4とされる[23]。すなわち，オメプラゾールの場合と同様に，ボリコナゾール併用によるタクロリムスやシクロスポリンの血中濃度上昇には，一部薬物代謝酵素の多型性も関わる可能性が考えられる。またボリコナゾールは，イトラコナゾールと同様に強くCYP3A4阻害作用を示すことから，薬物相互作用において広く注意を払う必要がある。

退院時および外来通院時における注意点

生体肝移植治療を受けた患者の多くは，退院後にかかりつけ医やかかりつけ薬局にフォローを受ける場合が多い。したがって，入院中に見いだされたさまざまなイベントを適切にまとめ，情報交換を通じて円滑な術後管理の継続を進める必要がある。特に，免疫抑制薬の血中濃度管理については，どのタイミングで測定したのか，測定方法はどの方法なのかについても確認する必要がある。シクロスポリンおよびタクロリムスの血中濃度測定については，CLIA法（chemiluminescence enzyme immunoassay）であるアボット社のARCHITECTシステム，ACMIA法（affinity column-mediated immunoassay）であるシーメンス社のディメンションシステムで，用いる抗薬物抗体の特性の差異から，同一検体においても異なるデータが得られる場合が報じられている[24),25]。免疫抑制薬の測定法など施設間における差異を明確にしたうえで，かかりつけ医との意思疎通を円滑にすることが望ましい。

おわりに

生体肝移植術後の免疫抑制療法は，過去15年以上にわたる経験を積み重ね，免疫抑制薬の体内動態制御機構の解明や薬物相互作用の分子メカニズム解明などを経て，ようやく落ち着きをみせてきたと考えられる。生体ドナーによる移植医療は，術前の詳細な検査などを通じて十分な準備のもと実施される。一方，最近の脳死ドナー由来の肝臓移植件数の増加は，臨機応変な対応が薬剤師にも求められる。すなわち，当該移植患者の情報を移植コーディネーターからいち早く入手し，術後の薬物治療全般にわたる計画について医師・看護師に適切にコンサルトを実施することが求められる。これまで生体肝移植症例の積み重ねによって蓄えられてきた経験が，今後増加することが想定される脳死肝移植治療にも活かされることを期待する。

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OPINION

透析療法の選択についてひとこと
——腹膜透析か血液透析か

深津　敦司*

わが国においては，諸外国に比べて，血液透析（HD）に対して腹膜透析（PD）がきわめて少ない（3〜4％）ことは周知の事実である．その理由についてはさまざまな意見がある．PDに懐疑的な医師からは，PDは長期にできない，被嚢性腹膜硬化症への懸念，患者の理解力が必要など否定的な意見が出る．一方，PD推進派からはPD firstが残存機能の保護に良い，家庭でできる，循環系に負荷が少ないなどの意見が出される．しかし現状ではPDは増えていない．私見であるが最大の要因は，HDを主体的に行っている施設が大半であるが，そこではPDはしていないか，片手間で施行していることが多く，少数のPDを主体としている施設ではPDが優先的に行われており，両者のどちらでも患者の希望に従って自由に選択でき，同等に実施できるという本来あるべき施設が多くないことにあると考えている．またわれわれも経験したようにPDを始めて，ある程度患者数が増えると厄介な合併症が一時期集中的に起こり，それで懲りてしまうというのも考えられる．

われわれの施設ではPDを積極的に開始して約10年，現在脱落と導入で維持患者が約20数名で横ばい安定という状態である．途中，腹膜炎や横隔膜交通症，カテーテルトラブルなどが集中して起こり，正直PD導入を減らそうかと考えた時期もあった．初期にはPDでの導入を比較的積極的に勧めていたが（当時PDが一般的ではなかった），

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スタッフの経験が蓄積されてきた現在，われわれの施設ではPD firstという考え方は強調しないようになった．「PD firstの予後が良い」という外国での成績は，導入時期やHD方法がわが国と同じでなく，そのまま受け入れられない．HDの成績がきわめてよいわが国で，本当に導入時からの残存腎機能の保護，日常生活動作（ADL；activities of daily living），予後にPDとHDで差があるかの臨床的データが存在しないからである．以前からHD，PDがどちらも同等に選択できる施設で，導入時からの残存腎機能とADLについてのHDとPD間の比較臨床研究を呼びかけているが，なかなか協力施設がない．

このような状況で，現在のわれわれの透析導入時のスタンスは以下のようになった．適応が限定される例（PDが不可能な例や心機能が悪く，PDが推奨される例など）を除いて，少なくとも開始後3〜5年間はHDとPDは原則同等に確立された方法でどちらも選択可能というのが基本である．実際の手技や器具を見せ，できるだけバイアスは排除して，十分その利点欠点を説明し，納得したうえで患者と家族の意思で自分に合ったほうを選択してもらう．さらにPDを選択した患者には2社のデバイスから，実際に操作して自分に合った（好みの）ほうを選択してもらっている．また変更も可能としている．

このような方針で導入時PDを選択する患者は約2割となり，これはほぼ国際的平均であると思っている．

Immunosuppressive Effects of Tacrolimus on Macrophages Ameliorate Experimental Colitis

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Background: Tacrolimus is a novel immunomodulator for inflammatory bowel diseases. Immunosuppressive effects of tacrolimus on T cells are well known; however, the effects of tacrolimus on macrophages remain unclear. The aim of this study was to investigate the effects of tacrolimus on activated macrophages and to examine its efficacy in murine colitis models.

Methods: Proinflammatory cytokine production from lipopolysaccharide (LPS)–stimulated peritoneal macrophages of IL-10-knockout (KO) mice with and without tacrolimus was measured. We investigated the effects of tacrolimus on nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), and caspase activation in macrophages and the induction of apoptosis in macrophages in vitro and examined the in vivo apoptotic effect of tacrolimus on colonic macrophages in IL-10-KO mice. We evaluated the effect of the rectal administration of tacrolimus on colonic inflammation in IL-10-KO mice and dextran sulfate sodium (DSS)–induced colitis in CB.17/SCID mice.

Results: Proinflammatory cytokine production from tacrolimus-treated macrophages was significantly lower than that from untreated cells. Tacrolimus suppressed LPS-induced activation of both NF-κB and MAPK in macrophages and induced apoptosis of macrophages via activation of caspases 3 and 9. Rectal administration of tacrolimus evoked apoptosis of colonic macrophages in IL-10-KO mice. Moreover, the rectal administration of tacrolimus ameliorated colitis in IL-10-KO mice and DSS-induced colitis in CB.17/SCID mice. Gene expression of inflammatory cytokines in colonic mucosa was significantly lower in tacrolimus-treated mice than in untreated mice.

Conclusions: Tacrolimus suppresses the function of activated macrophages and promotes their apoptosis, which may lead to the amelioration of colonic inflammation.

(Inflamm Bowel Dis 2010;16:2022–2033)

Key Words: inflammatory bowel disease, tacrolimus, macrophage, NF-κB, MAPK, apoptosis

Inflammatory bowel disease (IBD) is the chronic disease characterized by recurrent and severe inflammation of the gastrointestinal tract.[1,2] Despite recent advances in understanding the pathophysiology of IBD, therapeutic options are still limited and the management of refractory IBD is particularly challenging.[3–5]

Recent genomewide studies have shown that dysregulation of both innate and adaptive immunity are important risk factors for the development of IBD.[6] In particular, abnormalities of the genes involved in innate immunity by recognizing and/or processing bacterial components, such as *NOD2/CARD15*, *IRGM*, and *ATG16L1*, have been revealed to play critical roles in the development of IBD.[7–9] These data suggest that the control of abnormal innate-immune responses of antigen-presenting cells to commensal bacteria is important in the treatment of IBD.

Macrophages, which recognize bacteria and incorporate bacteria or their components, play a pivotal role in innate immunity.[10] Recent studies have suggested that macrophages in the intestinal mucosa are deeply involved in maintaining intestinal homeostasis and negatively regulate excess immune responses evoked by commensal bacteria.[11] Moreover, it was also reported that abnormal responses of intestinal macrophages to commensal bacteria result in chronic intestinal inflammation.[12] In agreement with these data, recent studies have suggested that macrophage-

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targeting treatment ameliorates colonic inflammation in experimental colitis models.[13,14] Thus, the regulation of abnormal responses of macrophages appears to be a promising therapeutic approach for the treatment of IBD.

Tacrolimus is a potent immunomodulator that was isolated from *Streptomyces tsukubaensis*.[15] Tacrolimus binds the tacrolimus-binding protein, and the complex inhibits the Ca^{2+}-dependent phosphatase calcineurin, thereby preventing calcineurin-dependent interleukin (IL)–2 transcription and T-cell proliferation in a manner similar to that of cyclosporine A (CyA).[16,17] Moreover, tacrolimus has been shown to down-regulate the nuclear factor-κB (NF-κB) pathway and induce apoptosis of activated T cells by activating caspase 3.[18] Because it has such potent immunosuppressive effects on T cells, tacrolimus is now widely used for prophylaxis against not only organ rejection and autoimmune diseases but also IBD,[19,20] and it is generally accepted that the therapeutic effects of tacrolimus are a result of its inhibitory effects on T-cell functions.[15,17] However, it remains unclear whether tacrolimus also has immunoregulatory effects on macrophages.

In the present study, therefore, we examined whether tacrolimus influences the function of activated macrophages and, if so, whether this effect of tacrolimus on macrophages may have beneficial roles in the treatment of IBD.

MATERIALS AND METHODS

Animals

Female IL-10-KO mice, CB.17/SCID mice, and C57BL/6 mice (Charles River Japan, Inc., Kanagawa, Japan) 8–10 weeks old were used for the experiments. They were fed standard laboratory chow and supplied drinking water ad libitum. In our animal facility, IL-10-KO mice developed colonic inflammation at approximately 10 weeks.

Reagents

The calcineurin inhibitor tacrolimus (FR900506) was provided by Astellas Pharmaceutical Inc. (Tokyo, Japan). Lipopolysaccharide (LPS, *Escherichia coli* 0127:B8) and antibody to β-actin were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to I-κBα, p65, p38, JNK, phospho-p65, phospho-p38, phospho-JNK, caspase 3, caspase 8, and caspase 9 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to rat antimouse CD11b and rat IgG_{2b} were purchased from BD Bioscience Pharmingen (San Jose, CA).

Peritoneal Macrophages Isolation and Stimulation

Peritoneal macrophages of IL-10-KO mice were elicited by intraperitoneal injection of 2 mL of 4% thioglycolate (Eiken Chemical Co., Ltd.) in distilled water. After 4 days, elicited macrophages were collected by peritoneal lavage with phosphate buffered saline (PBS), and the cells were suspended in RPMI1640 medium (Invitrogen Corp, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (GIBCO, Invitrogen, Grand Island, NY). A total of 1×10^5 cells were plated in a 96-well plate and incubated for 2 hours in 5% CO_2 at 37°C. After incubation, nonadherent cells were removed by washing with PBS. Macrophages were stimulated with LPS (1 μg/mL) alone or with LPS (1 μg/mL) plus tacrolimus (0.01, 0.1 mg/mL) for 24 hours in 5% CO_2 at 37°C, and the cytokine secretion in the supernatant was measured by enzyme-linked immunosorbent assay (ELISA). For measurement of IL-12/IL-23p40, tumor necrosis factor (TNF)–α and IL-6, sandwich ELISA was performed according to the manufacturer's instructions (eBioscience, San Diego, CA).

Western Blotting

For the analysis of the effects of tacrolimus on NF-κB signaling and mitogen-activated protein kinase (MAPK) in macrophages, 1×10^6 peritoneal macrophages from IL-10-KO mice were stimulated with LPS (1 μg/mL) with or without pretreatment with tacrolimus (0.1 mg/mL) for 2 hours in 5% CO_2 at 37 °C. Then, 0, 1, 5, 10, 15, 30, and 60 minutes after stimulation, the cells were washed with PBS, and nuclear and cytoplasmic protein extractions from isolated cells were performed with an NE-PER kit (PIERCE Biotechnology, Rockford, IL) according to the manufacturer's instructions. For the analysis of the effect of tacrolimus on caspase signaling in macrophages, we used 1×10^6 RAW264.7 cells, a murine macrophage cell line, with or without tacrolimus stimulation (0.1 mg/mL) for 0 and 12 hours in 5% CO_2 at 37°C. The cells were then washed with PBS, and protein extractions from isolated cells were performed. Protein content was measured by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membrane was blocked with Tris-buffered saline with 0.1% Tween-20 (TBS-T) and 5% skim milk for 1 hour at room temperature. Blots were incubated overnight with the primary antibody at a dilution of 1:1000 at 4°C. After incubation, the membrane was washed 3 times for 5 minutes with TBS-T and incubated in TBS-T with 5% skim milk containing antirabbit IgG antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, England) at a 1:2000 dilution for 1 hour at room temperature. Immunoreactive bands were visualized using the Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA).

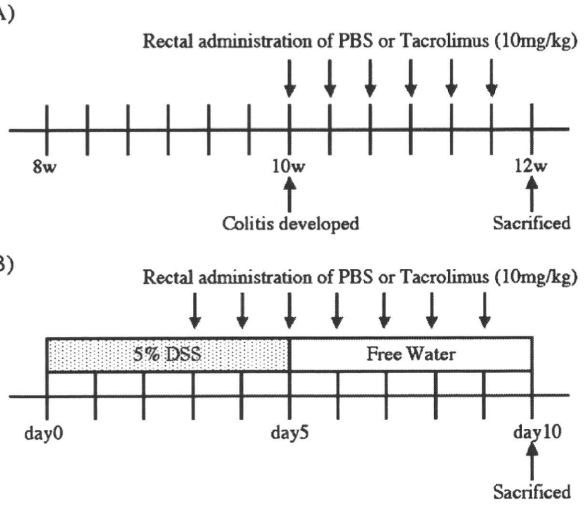

FIGURE 1. Experimental protocols of in vivo study. A: Therapeutic study using IL-10-KO mice. B: Therapeutic study using CB.17/SCID mice with DSS-induced colitis.

Evaluation of Apoptosis

To evaluate the apoptotic effect of tacrolimus on macrophages in vitro and in vivo, an Annexin V assay and a terminal deoxyribonucleotodyl tranferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay were performed with an Annexin V-FITC Apoptosis Detection Kit (Bio Vision, Mountain View, CA) and an In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturers' instructions.

To evaluate the apoptotic effect of tacrolimus on macrophages, 5×10^5 RAW264.7 cells were collected 0, 1, 6, 12, and 24 hours after stimulation with tacrolimus, resuspended in 500 μL of binding buffer, and stained with Annexin V-FITC and propidium iodide at room temperature for 5 minutes in the dark. The percentage of apoptotic cells was analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ).

Apoptosis in colonic tissue of mice treated with rectal administration of tacrolimus or PBS was detected by the TUNEL assay. IL-10-KO mice who were 12 weeks old were treated with rectal administration of tacrolimus (10 mg/kg) or PBS. Then 0, 12, and 24 hours after rectal administration of tacrolimus or PBS, mice were sacrificed, the distal colons were collected, and TUNEL staining and immunohistochemistry for CD11b were performed.

Experimental Designs of In Vivo Studies

The therapeutic studies with tacrolimus included 2 protocols, as described in Figure 1. In the first protocol, the therapeutic effect of rectal administration of tacrolimus was studied using an immune-mediated colitis model. IL-10-KO mice who were 10 weeks old were treated with rectal administration of tacrolimus (10 mg/kg) or PBS every other day 3 times a week for 2 weeks. Mice were sacrificed on day 12. As a control, 12-week-old C57BL/6 mice were also sacrificed on day 12.

In the second protocol, the therapeutic effect of the rectal administration of tacrolimus was studied in CB.17/SCID mice with dextran sulfate sodium (DSS)–induced colitis. To induce colitis in CB.17/SCID mice, 5% DSS (molecular weight 36,000–50,000 MP Biomedicals, Inc., Aurora, OH) was dissolved in water and given to CB.17/SCID mice.[21] DSS water was provided ad libitum up to 5 days. On day 5, they were switched to normal drinking water. Treatment with rectal administration of PBS or tacrolimus was performed from day 3 to day 9. Mice were sacrificed on day 10.

Evaluation of Colitis

After sacrifice, the colons of IL-10-KO and C57BL/6 mice were divided into 4 segments to represent the rectum, cecum, proximal colon, and middle colon. The histology of each segment of the colon was evaluated with hematoxylin and eosin staining. The severity of inflammation of each section was scored using a histological index ranging from 0 to 4, as previously described.[22] This index was based on the degree of epithelial layer erosion, goblet cell depletion, and inflammatory cell infiltrate [0, normal; 1, small number of inflammatory cells; 2, more extensive by number and involvement; 3, significant evidence of inflammatory infiltrate with goblet cell depletion; 4, significant evidence of inflammatory infiltrate (ulcers and crypt abscess)].

We evaluated the severity of DSS-induced colitis in CB.17/SCID mice using disease activity index (DAI) scores as described in Table 1.[23] After sacrifice, colon length was also evaluated, and the severity of inflammation of colon was scored using a histological index, as described previously.[24] Three independent parameters were measured: severity of inflammation (0–3: none, slight, moderate, and severe, respectively), depth of injury (0–3: none, mucosal, mucosal and submucosal, and transmural, respectively), and crypt damage (0–4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, and entire crypt and epithelium lost, respectively). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement (×1, 0%–25%; ×2, 26%–50%; ×3, 51%–75%; ×4, 76%–100%), and all numbers were added. The maximum possible score was 40.

Semiquantitative Analysis of Gene Expression of Proinflammatory Cytokines

Samples of colonic tissues for mRNA isolation were removed from the distal third of the colon. Total RNA was extracted using TRIzol RNA isolation reagent (Invitrogen

TABLE 1. Disease Activity Index Score

Score	Weight loss (%)	Stool consistency	Occult/gross bleeding
0	None	Normal	Normal
1	1–5		
2	6–10	Loose stool	Occult bleeding
3	11–20		
4	> 20	Diarrhea	Gross bleeding

The disease activity index (DAI) is a mean of individual scores of weight loss, stool consistency and bleeding. Normal stool = formed pellets, loose stool = pasty and semiformed stool, diarrhea = liquid stool.

Corp, Carlsbad, CA) according to the manufacturer's instructions. RNA (1 μg) was reverse-transcribed with Superscript II (Invitrogen), and the resulting complementary DNA was analyzed for IL-12/IL-23p40, TNF-α, IFN-γ, IL-17A, IL-6, and β-actin mRNA expression by semiquantitative polymerase chain reaction (PCR) using a Dyad Thermal Cycler (MJ Research, Waltham, MA). The primer sets used are described in Table 2. A 5-μL aliquot PCR product was electrophoresed on 2% agarose gel containing ethidium bromide, and the bands were examined using an image autoanalyzing system (AE-6911CX, ATTO Corp, Tokyo, Japan). The densities of bands on the gels were measured by image analysis software (CS analyzer, ATTO Corp.). Semiquantitative level of each product was corrected for the β-actin density of each sample.

Statistical Analysis

The Student t test and the Mann-Whitney U test were used where appropriate for statistical analysis. Data are expressed as means ± SDs. A P value < 0.05 was considered statistically significant.

RESULTS

Tacrolimus Inhibits Proinflammatory Cytokine Production in LPS-Stimulated Peritoneal Macrophages from IL-10-KO Mice

Macrophages are deeply involved in the onset and development of IBD.[25] Therefore, we focused on the effect of tacrolimus on macrophage function. To examine whether tacrolimus suppresses activated macrophages, we evaluated the effect of tacrolimus on proinflammatory cytokine production in LPS-stimulated peritoneal macrophages from IL-10-KO mice. As shown in Figure 2, the increases in IL-12/IL-23p40, TNF-α, and IL-6 production from peritoneal macrophages of IL-10-KO mice were all significantly inhibited by tacrolimus in a dose-dependent manner ($P < 0.001$). Thus, tacrolimus strongly suppresses LPS-stimulated inflammatory cytokine production from activated macrophages.

Tacrolimus Inhibits NF-κB and MAPK Activation Pathways

To elucidate the mechanism of the inhibitory effect of tacrolimus on proinflammatory cytokine production in LPS-stimulated macrophages, we investigated the effect of tacrolimus on the intracellular signaling pathway of peritoneal macrophages. Tacrolimus inhibited the LPS-induced degradation of I-κBα and the phosphorylation of NF-κB p65 in peritoneal macrophages from IL-10-KO mice (Fig. 3A). Furthermore, tacrolimus also inhibited LPS-induced phosphorylation of p38 and JNK in peritoneal macrophages from IL-10-KO mice (Fig. 3B). These results demonstrated that tacrolimus inhibits both the NF-κB and MAPK activation pathways induced by LPS in peritoneal macrophages from IL-10-KO mice.

TABLE 2. Primer Sets for Semiquantitative PCR

IL-12/IL-23p40	forward:	CGGTCATCTGCCGCAAA
	reverse:	TGCCCATTCGCTCCAAGA
TNF-α	forward:	TTCTGTCTACTGAACTTCGGGGTGATCGGTCC
	reverse:	GTATGAGATAGCAAATCGGCTGACGGTGTGGG
IFN-γ	forward:	TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC
	reverse:	TGGACCTGTCGGGTTGTTGACCTCAAACTTGGC
IL-17A	forward:	TCTCTGATGCTGTTGCTGCT
	reverse:	CGTGGAACGGTTGAGGTAGT
IL-6	forward:	ATGAAGTTCCTCTCTGCAAGAGACT
	reverse:	CACTAGGTTTGCCGAGTAGATCTC
β-Actin	forward:	GTGGGCCGCCCTAGGCACCAG
	reverse:	CTCTTTGATGTCACGCACGATTTC

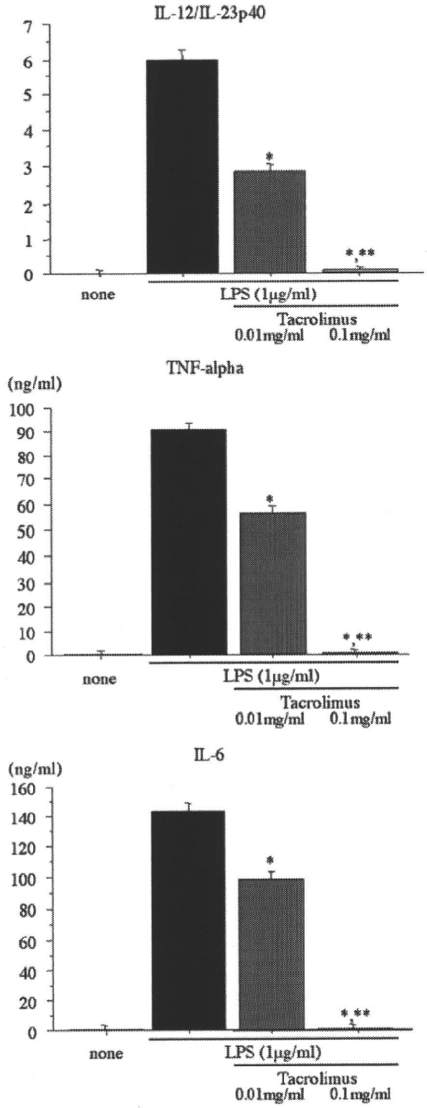

FIGURE 2. Dose-dependent effect of tacrolimus on proinflammatory cytokine production of LPS-stimulated peritoneal macrophages from IL-10-KO mice. Peritoneal macrophages from IL-10-KO mice were stimulated with medium alone or LPS (1 μg/mL) with or without tacrolimus (0.01, 0.1 mg/mL) for 24 hours, and proinflammatory cytokine secretion in the supernatant was measured by ELISA. Data are expressed as the mean ± SD ($n = 5$ in each group), and similar results were obtained in 3 independent experiments; $^{*}P < 0.001$ compared with untreated cells (LPS alone), $^{**}P < 0.001$ between tacrolimus (0.01 mg/mL) group and tacrolimus (0.1 mg/mL) group.

Tacrolimus Induces Apoptosis of Macrophages Both In Vitro and In Vivo

Induction of apoptosis in immune cells is considered one of the therapeutic strategies for IBD.[6] Therefore, we evaluated the apoptotic effects of tacrolimus on macrophages both in vitro and in vivo. As shown in Figure 4A, the percentage of annexin V–positive cells was significantly higher in RAW264.7 cells treated with tacrolimus than in those treated with PBS alone. This effect of tacrolimus was both dose- and time dependent. We also evaluated the effect of tacrolimus on caspase signaling. Tacrolimus induced activation of caspase 3 and 9, whereas it did not activate caspase 8 (Fig. 4B). These data suggested that tacrolimus-induced apoptosis of macrophages is a result of activation of the mitochondrial apoptotic pathway.

Moreover, we evaluated the in vivo apoptotic effect of rectal administration of tacrolimus on colonic macrophages in IL-10-KO mice. As shown in Figure 4C, apoptosis of colonic CD11b-positive cells in IL-10-KO mice was observed 12 and 24 hours after rectal administration of tacrolimus, whereas it was not observed in PBS-treated IL-10-KO mice. We also evaluated the in vivo apoptotic effect of tacrolimus on colonic T cells. In agreement with previous reports, tacrolimus induced apoptosis of CD4-positive T cells after 12 and 24 hours (data not shown). These in vivo data revealed that tacrolimus induces apoptosis of not only T cells but also macrophages in inflammatory colonic mucosa.

Tacrolimus Ameliorates Immune-Mediated Colitis of IL-10-KO Mice

To evaluate the therapeutic effect of tacrolimus on immune-mediated colitis, we performed the therapeutic study in IL-10-KO mice with colitis at 10 weeks. In PBS-treated IL-10-KO mice, body weight gradually decreased and did not recover during the experiment. In contrast, the body weight of tacrolimus-treated IL-10-KO mice was significantly recovered compared with that of PBS-treated IL-10-KO mice ($P < 0.05$; Fig. 5A), although there was no difference in colon length between mice with and without tacrolimus treatment (Fig. 5B). Histological findings revealed that the severe hyperplasia of colonic epithelial cells, infiltration of mononuclear cells in the colonic lamina propria, and loss of Goblet cells observed in IL-10-KO mice were significantly reduced by rectal administration of tacrolimus (Fig. 5C). Histological scores of tacrolimus-treated IL-10-KO mice were significantly lower than those of PBS-treated IL-10-KO mice ($P < 0.05$; Fig. 5D). We confirmed that indigo carmine reached the cecum after rectal administration of PBS with indigo carmine (data not shown), indicating that rectal administration of tacrolimus could be delivered throughout the colon.

Additionally, immunohistochemistry revealed that CD11b-positive cells were significantly decreased in tacrolimus-treated IL-10-KO mice compared with PBS-treated IL-10-KO mice (Fig. 6A,B). These data suggested that amelioration of colonic inflammation observed in tacrolimus-treated IL-10-KO mice involves the depletion of colonic macrophages.

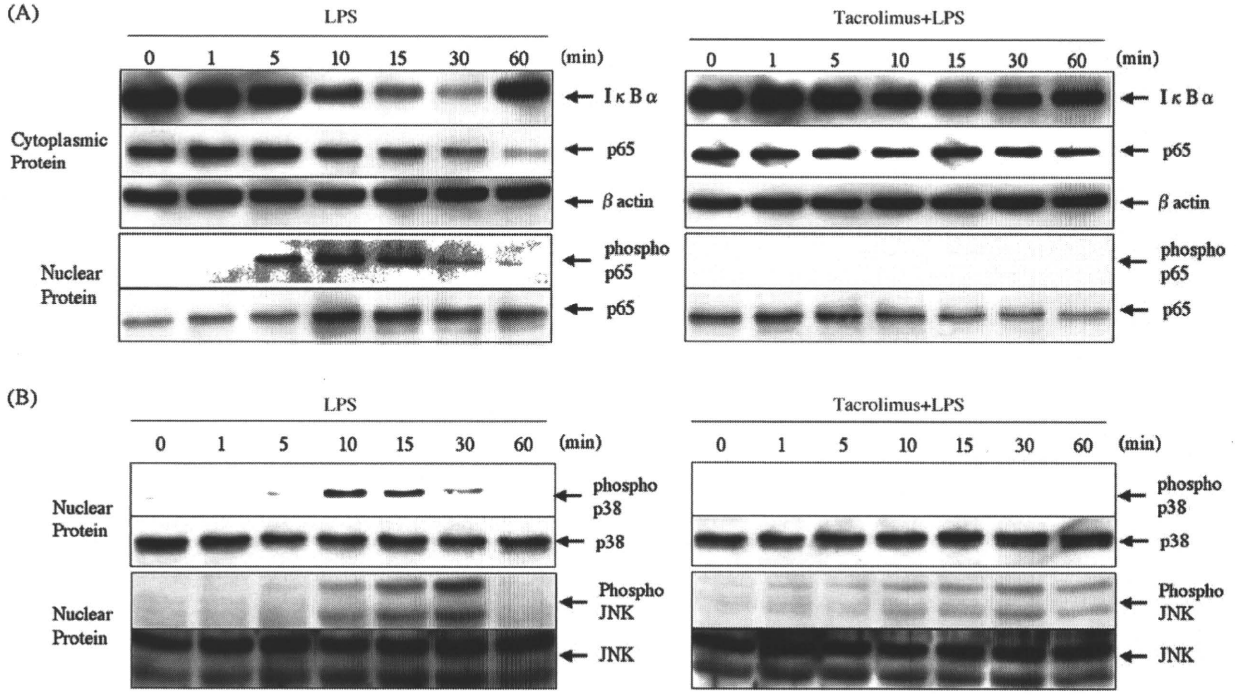

FIGURE 3. Effects of tacrolimus on NF-κB and MAPK activation in LPS-stimulated peritoneal macrophages from IL-10-KO mice. Peritoneal macrophages were stimulated by LPS (1 μg/mL) for the indicated times with (right) or without (left) pretreatment by tacrolimus (0.1 mg/mL) for 2 hours, and cell lysates were prepared and subjected to SDS-PAGE, followed by Western blot with indicated antibodies. A: Effect of tacrolimus on NF-κB activation pathway. B: Effect of tacrolimus on the MAPK activation pathway.

Tacrolimus Inhibits Gene Expression of Proinflammatory Cytokines In Vivo

To evaluate the effect of rectal administration of tacrolimus on the gene expression of proinflammatory cytokines in colonic tissues, RNA was extracted from colonic specimens of tacrolimus-treated IL-10-KO mice. As shown in Figure 7, significant increases in IL-12/IL-23p40, TNF-α, IFN-γ, IL-17A, and IL-6 transcripts were observed in the colonic tissue of IL-10-KO mice compared with those in the control mice. However, these transcripts were significantly lower in the colonic tissues of tacrolimus-treated IL-10-KO mice than in those of PBS-treated IL-10-KO mice ($P < 0.05$). These results suggested that rectal administration of tacrolimus reduces colonic inflammation of IL-10-KO mice at least in part by decreasing proinflammatory cytokines, which may be related to the depletion of colonic macrophages.

Tacrolimus Also Ameliorates DSS-Induced Colitis in CB.17/SCID Mice

To confirm whether tacrolimus-induced inhibition of macrophage functions and apoptosis contribute to the amelioration of colitis, we investigated the effect of rectal administration of tacrolimus on DSS-induced colitis in CB.17/SCID mice, which lack lymphocytes. Tacrolimus treatment significantly reduced body weight loss in CB.17/SCID mice with DSS-induced colitis (Fig. 8A). The DAI score of tacrolimus-treated CB.17/SCID mice with DSS-induced colitis was significantly lower than that of PBS-treated CB.17/SCID mice on day 10 (Fig. 8B). Macroscopically, colon length was significantly longer in tacrolimus-treated mice than in PBS-treated mice (Fig. 8C). Pathologically, tacrolimus-treated CB.17/SCID mice with DSS-induced colitis showed little cellular infiltration and regeneration of epithelial cells characterized by the presence of surface epithelium and crypt formation. In contrast, PBS-treated CB.17/SCID mice with DSS colitis showed severe cellular infiltration, loss of goblet cells, crypt damage, and mucosal ulceration (Fig. 8D). Histological scores of tacrolimus-treated CB.17/SCID mice with DSS-induced colitis were significantly lower than those of PBS-treated CB.17/SCID mice (Fig. 8E). These data suggested that apoptosis and suppression of cytokine production from macrophages induced by rectal administration of tacrolimus contribute to the amelioration of DSS-induced colitis in CB.17/SCID mice.

DISCUSSION

In this study, we examined whether tacrolimus has inhibitory effects on macrophages in addition to T cells

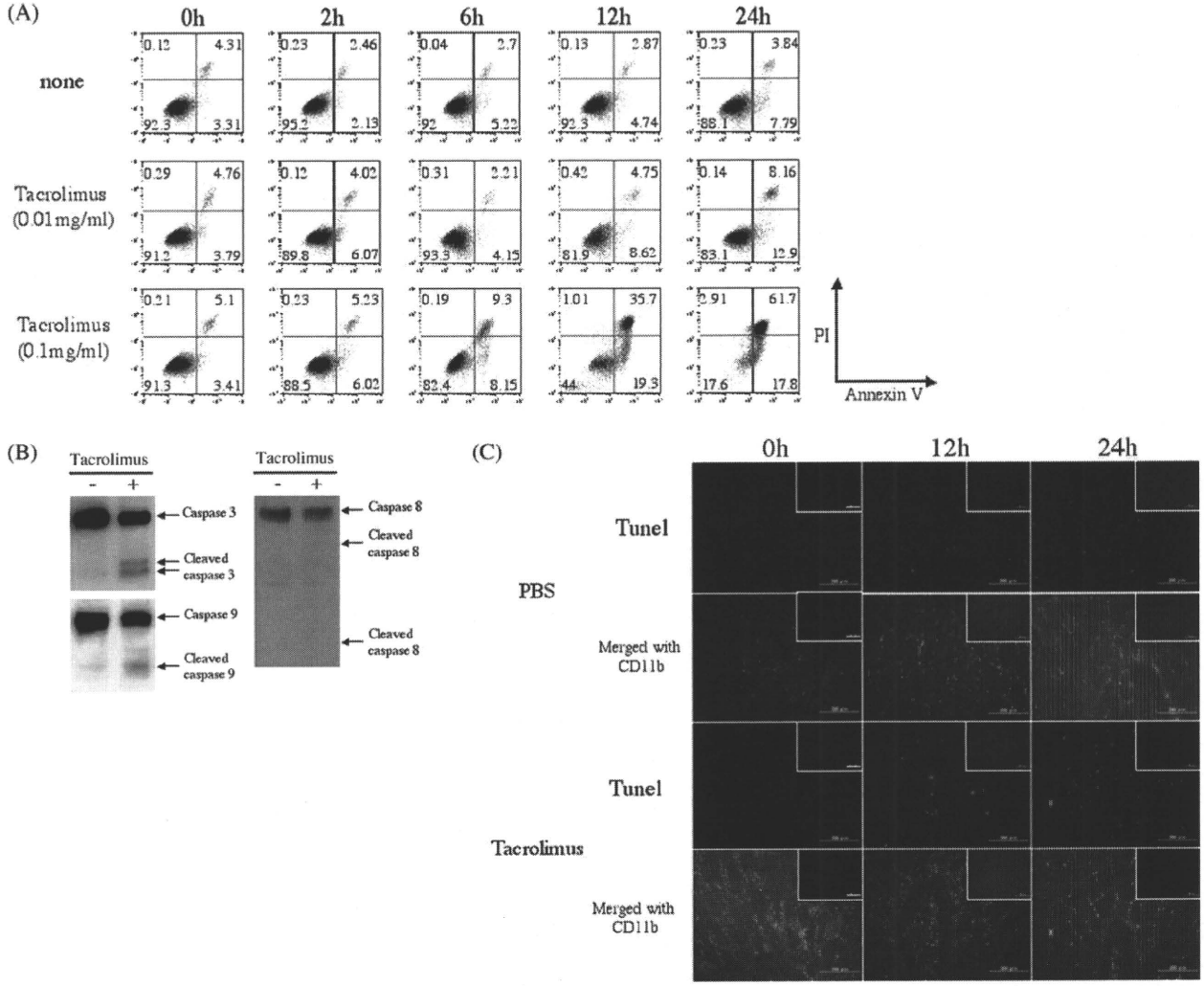

FIGURE 4. Apoptotic effects of tacrolimus on macrophages. A: Apoptotic effect of tacrolimus on a murine macrophage cell line RAW264.7. RAW264.7 cells were stimulated with or without tacrolimus (0.01 or 0.1 mg/mL) for 0, 1, 6, 12, and 24 hours. The percentage of apoptotic cells (shown at the right lower part of each panel) was analyzed by flow cytometry. Similar results were obtained in 3 independent experiments. B: Effect of tacrolimus on caspase signals. RAW264.7 cells were stimulated with or without tacrolimus (0.1 mg/mL) for 12 hours, and cell lysates were prepared and subjected to SDS-PAGE, followed by Western blot with indicated antibodies. C: In situ TUNEL assay in colonic tissue of IL-10-KO mice treated with PBS or tacrolimus. Zero, 12, and 24 hours after treatment with rectal administration of tacrolimus or PBS, TUNEL staining (TMR red labeled), and immunohistochemistry for CD11b (green) were performed on frozen sections of distal colon. Immunohistochemistry of the negative control is shown in the right upper corner (original magnification 200×). Apoptosis cells merged with CD11b were colored yellow.

and found by in vitro study that tacrolimus directly inhibited LPS-stimulated proinflammatory cytokine production in macrophages and induced apoptosis. Furthermore, we showed in vivo that rectal administration of tacrolimus attenuated colonic inflammation in both IL-10-KO mice and CB.17/SCID mice with DSS-induced colitis, which lack lymphocytes. Thus, our study demonstrated that tacrolimus directly inhibits macrophage functions and that these inhibitory effects of tacrolimus on macrophages may contribute to the therapeutic action of tacrolimus in IBD.

To evaluate the direct effects of tacrolimus on macrophages, we first investigated the effect of tacrolimus on proinflammatory cytokine production in peritoneal macrophages of IL-10-KO mice in vitro. Our results clearly demonstrated that tacrolimus directly and dose-dependently reduced LPS-induced production of IL-12/IL-23p40, TNF-

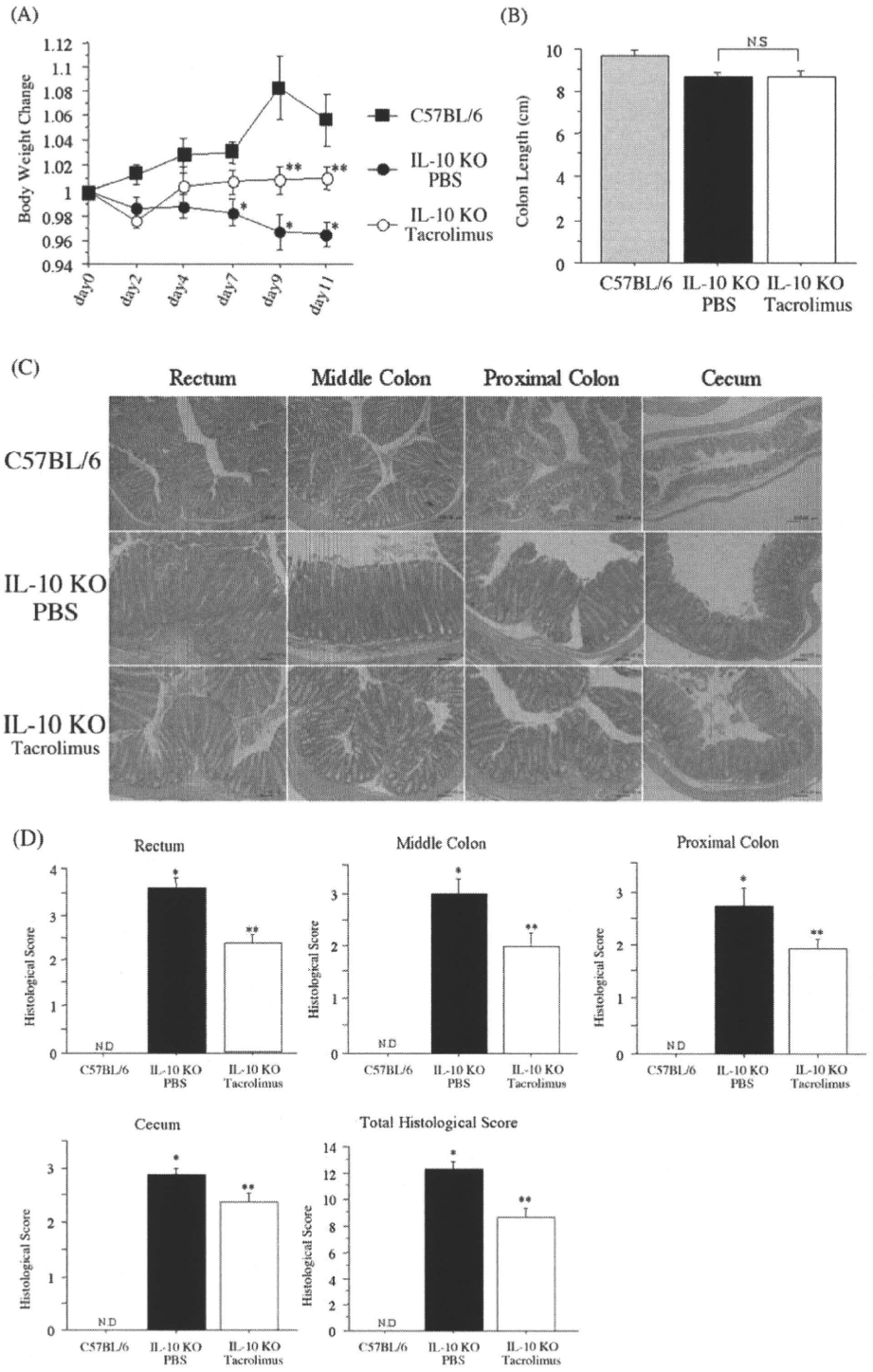

FIGURE 5. Therapeutic effects of rectal administration of tacrolimus on colitis in IL-10-KO mice. A: Effect of rectal administration of tacrolimus on body weight changes in the therapeutic study. Serial changes of body weight were measured. Data are expressed as the mean ± SD (C57BL/6 mice, $n = 3$; IL-10-KO mice, $n = 7$ in each group); $^{*}P < 0.05$ compared with C57BL/6 mice, $^{**}P < 0.05$ compared with IL-10-KO mice treated without tacrolimus (PBS alone). B: Effect of rectal administration of tacrolimus on colon length of mice. Colon length was measured from the ileocecal junction to the anal verge. Data are expressed as the mean ± SD (C57BL/6 mice, $n = 3$; IL-10-KO mice, $n = 7$ in each group). C: Representative histological findings of mice treated with rectal administration of tacrolimus or PBS (original magnification 100×). D: Histological scores of colonic tissues in mice treated with rectal administration of tacrolimus or PBS. Data are expressed as the mean ± SD (C57BL/6 mice, $n = 3$; IL-10-KO mice, $n = 7$ in each group); $^{*}P < 0.05$ compared with IL-10-KO mice treated without tacrolimus (PBS alone), $^{**}P < 0.05$ compared with C57BL/6 mice.

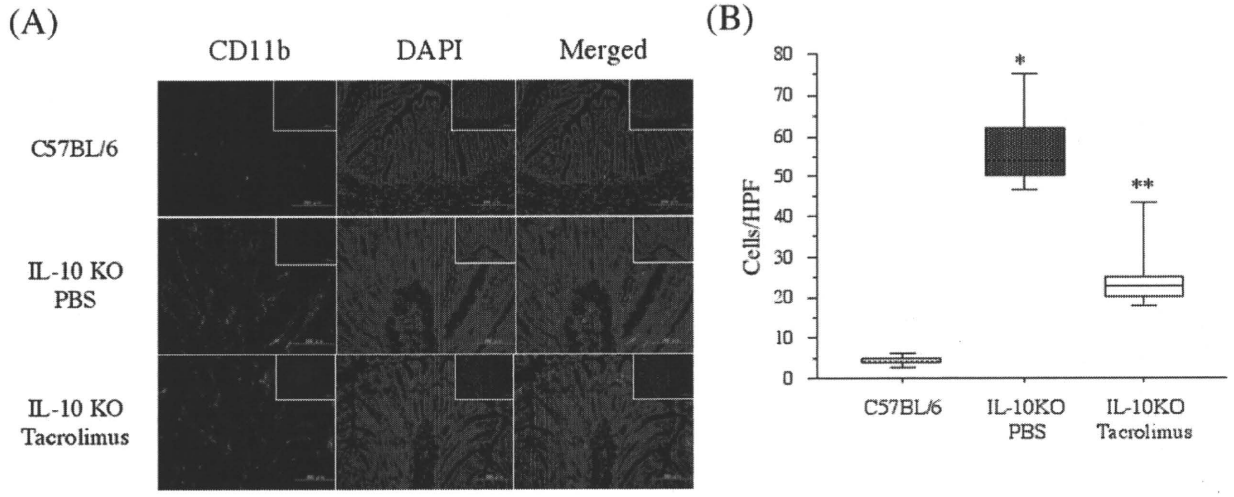

FIGURE 6. Apoptotic effects of rectal administration of tacrolimus on colonic macrophages in IL-10-KO mice. A: Immunohistochemistry for CD11b (green) and DAPI (blue) was performed on frozen sections of distal colon isolated from C57BL/6 mice and IL-10-KO mice treated with tacrolimus or PBS. Immunohistochemistry of the negative control is shown in the right upper corner (original magnification 200×). B: Cell count of CD11b-positive cells on frozen sections of distal colon isolated from C57BL/6 mice and IL-10-KO mice treated with PBS or tacrolimus. Data are expressed as the mean ± SD cell count per high-power field (HPF); $^{*}P < 0.001$ compared with C57BL/6 mice, $^{**}P < 0.05$ compared with IL-10-KO mice treated with rectal administration of PBS.

α, and IL-6 in peritoneal macrophages of IL-10-KO mice. Our data are the first to show the direct inhibitory effect of tacrolimus on the function of macrophages. Recent studies have revealed that IL-12, IL-23, TNF-α, and IL-6 play important roles in the pathophysiology of IBD.[26,27] Among them, IL-12 and IL-23 are mainly produced by activated myeloid cells such as macrophages and dendritic cells.[28] IL-12 promotes IFN-γ-producing T-cell (Th1) polarization,[29] whereas IL-23 promotes expansion of IL-17-producing T cells (Th17),[30] both of which are deeply involved in the development of IBD.[31,32] Thus, it appears reasonable that the direct inhibitory effects of tacrolimus on the production of various cytokines in macrophages as observed in this study, explain the therapeutic effect of tacrolimus on the colonic inflammation of IBD.

Tacrolimus has been reported to inhibit NF-κB activation in human $CD4^+$ T cells and keratinocytes[33] and MAPK activation in human endothelial cells.[34] Therefore, we examined the effects of tacrolimus on both NF-κB and MAPK activation in peritoneal macrophages and found that tacrolimus directly and significantly inhibited both NF-κB and MAPK activation. It is well established that activation of TLR signals by microbial components is important for macrophages to produce proinflammatory cytokines and that both the NF-κB and MAPK pathways play pivotal roles in TLR-stimulated cytokine production.[35] Indeed, Neurath et al and Zhang et al reported that the NF-κB pathway regulates LPS-induced expression of IL-6, IL-12, and TNF-α,[36,37] and Saklatvala et al reported that MAPK activation is associated with enhanced production of TNF-α.[38] Taken together with our data, it is suggested that inhibition of both NF-κB and MAPK activation by tacrolimus leads to inhibition of the production of various cytokines by macrophages.

Another important finding in this study is that, in addition to inhibition of proinflammatory cytokine production, tacrolimus directly induced apoptosis of macrophages in vitro, and this in vitro effect was confirmed in our in vivo study, as shown by the increase of apoptotic macrophages in the colonic mucosa of IL-10-KO mice by rectal administration of tacrolimus. It is well known that tacrolimus strongly induces apoptosis of $CD4^+$ T cells.[39] In this study, we observed apoptosis of not only T cells but also macrophages in IL-10-KO mice. Thus, tacrolimus appears to induce apoptosis in both macrophages and T cells. We also observed in this study that the apoptotic effect of tacrolimus on macrophages is associated with caspase 3 and 9 activation. This result is consistent with previous data that tacrolimus enhances T-cell apoptosis through the activation of caspase 3.[39,40] In addition to caspase signaling, our data demonstrated that tacrolimus directly inhibits NF-κB activation in macrophages. Several reports have suggested that NF-κB activation is involved in the inhibition of apoptosis.[41,42] Thus, inhibition of NF-κB activation by tacrolimus may have roles not only in the suppression of proinflammatory cytokine production, but also in the induction of apoptosis in macrophages.

Finally, to confirm that tacrolimus ameliorates colonic inflammation through the inhibition of macrophages, we performed an in vivo study. We first evaluated the effects of tacrolimus in immune-mediated colitis of IL-10-KO mice, which mimic well the immune-mediated chronic

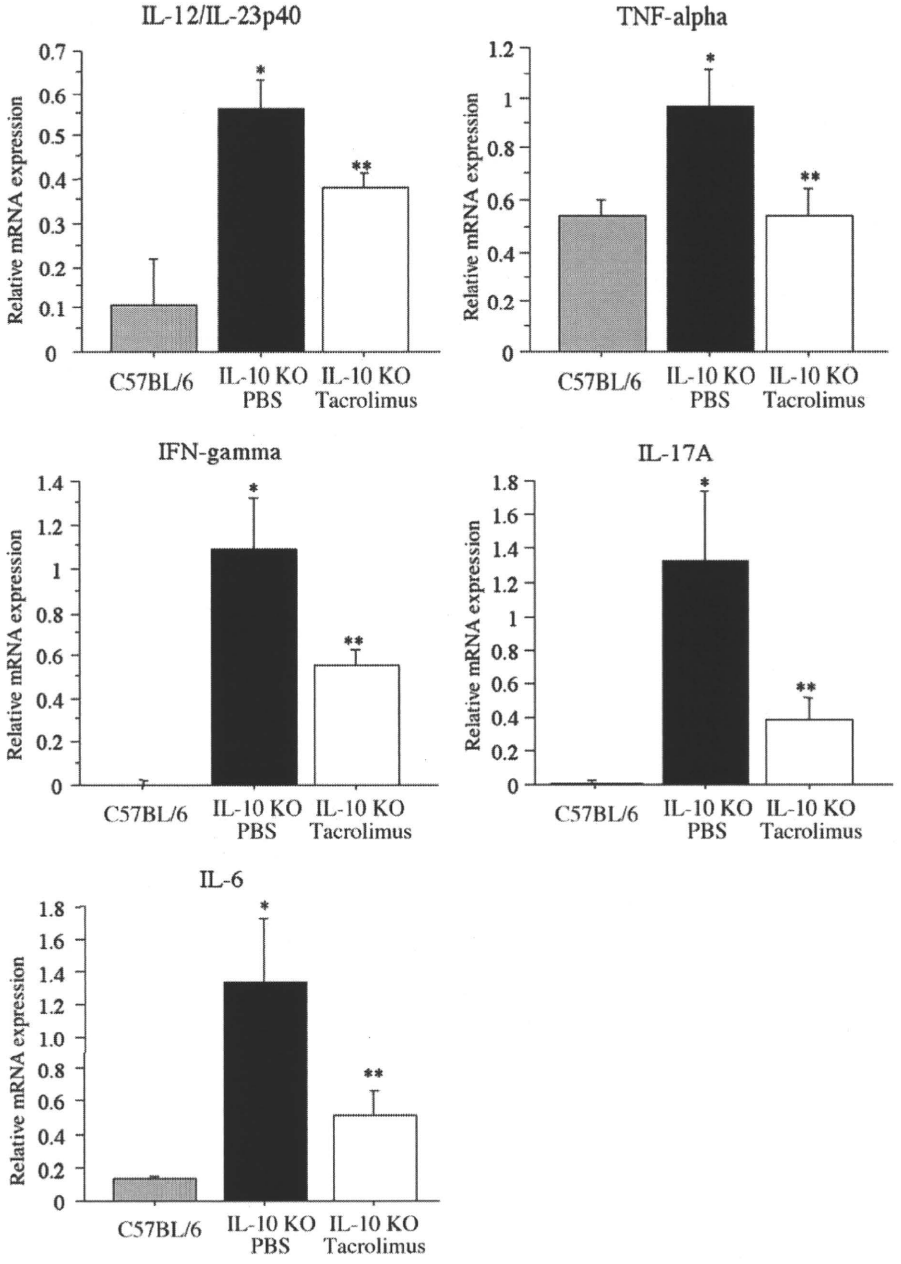

FIGURE 7. Effects of rectal administration of tacrolimus on transcript levels of IL-12/IL-23p40, TNF-α, IFN-γ, IL-17A, and IL-6 in colonic tissues of IL-10-KO mice in the therapeutic study. The gene expression of each target molecule was determined by semiquantitative PCR and was standardized against β-actin. Data are expressed as the mean ± SD (C57BL/6 mice, $n = 3$; IL-10-KO mice, $n = 7$ in each group); $^{*}P < 0.05$ compared with C57BL/6 mice, $^{**}P < 0.05$ compared with IL-10-KO mice without rectal administration of tacrolimus (PBS alone).

colonic inflammation observed in human IBD.[43] Our data clearly demonstrated that tacrolimus significantly improves colonic inflammation in IL-10-KO mice, and this is associated with apoptosis of macrophages in the colonic mucosa. Furthermore, we examined the effects of tacrolimus on DSS-induced colitis in CB.17/SCID mice. Because CB.17/SCID mice lack lymphocytes, T-cell-immune responses are not involved in the development of DSS-induced colitis in

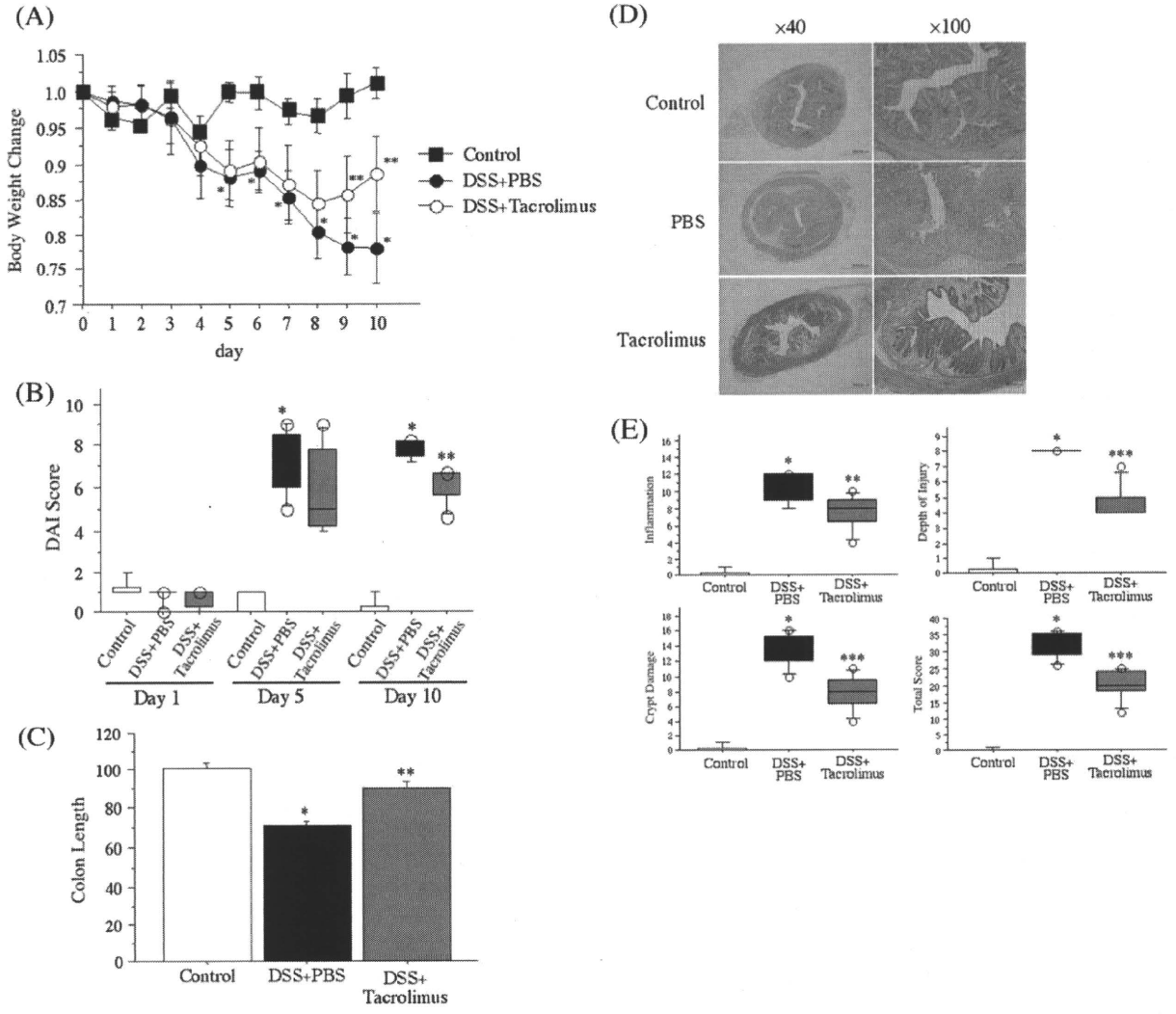

FIGURE 8. Therapeutic effects of tacrolimus on DSS-induced colitis in CB.17/SCID mice. A: Effects of rectal administration of tacrolimus on body weight changes. Serial changes in body weight were measured. Data are expressed as the mean ± SD (control group, $n = 5$; PBS group, $n = 8$; tacrolimus group, $n = 8$); $^{*}P < 0.05$ compared with control group (without DSS), $^{**}P < 0.05$ compared with PBS group (without tacrolimus). B: DAI score of DSS-induced colitis in CB.17/SCID mice treated with tacrolimus or PBS. Data are expressed as the mean ± SD (control group, $n = 5$; PBS group, $n = 8$; tacrolimus group, $n = 8$); $^{*}P < 0.001$ compared with control group (without DSS), $^{**}P < 0.05$ compared with PBS group (without tacrolimus). C: Effect of rectal administration of tacrolimus on colon length. Colon length was measured from the ileocecal junction to the anal verge. Data are expressed as the mean ± SD (control group, $n = 5$; PBS group, $n = 8$; tacrolimus group, $n = 8$); $^{*}P < 0.001$ compared with control group (without DSS), $^{**}P < 0.001$ compared with PBS group (without tacrolimus). D: Representative histological findings of mice treated with rectal administration of tacrolimus or PBS (original magnification 40× and 100×). E: Histological scores of colonic tissues in mice treated with rectal administration of tacrolimus or PBS. Data are expressed as the mean ± SD (control group, $n = 5$; PBS group, $n = 8$; tacrolimus group, $n = 8$); $^{*}P < 0.001$ compared with control group (without DSS), $^{**}P < 0.05$ and $^{***}P < 0.001$ compared with PBS group (without tacrolimus).

these mice. Interestingly, we found that tacrolimus ameliorated DSS-induced colitis in CB.17/SCID mice. These data strongly suggested that inhibitory effects of tacrolimus on macrophages contribute to amelioration of colonic inflammation.

In conclusion, our results confirmed the potential immunosuppressive effects of tacrolimus on activated macrophages. These inhibitory effects of tacrolimus on macrophages appear to play important roles in the treatment of colonic inflammation in patients with IBD.

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Identification and Comparative Functional Characterization of a New Human Riboflavin Transporter hRFT3 Expressed in the Brain[1–3]

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Abstract

We isolated cDNA coding a new human riboflavin transporter (hRFT)3, which exhibits 86.7 and 44.1% amino acid identity with hRFT1 and hRFT2, respectively. It was predicted to have 10 putative membrane-spanning domains. The functional characteristics of hRFT3 were examined and compared with those of its isoforms, hRFT1 and hRFT2. Real-time PCR revealed that hRFT3 mRNA was strongly expressed in the brain and salivary gland. hRFT1 mRNA was strongly expressed in the placenta and small intestine, whereas hRFT2 mRNA was most abundantly expressed in the testis and strongly in the small intestine and prostate. hRFT-mediated uptake of [^{3}H]riboflavin was evaluated using human embryonic kidney 293 cells transiently transfected with the cDNA coding each hRFT. The apparent Michaelis-Menten constants of hRFT1, hRFT2, and hRFT3 for riboflavin were 1.38, 0.98, and 0.33 μmol/L, respectively. The hRFT-mediated [^{3}H]riboflavin uptake was independent of extracellular Na^+ and Cl^-. Specific uptake of [^{3}H]riboflavin by hRFT2, but not hRFT1 and hRFT3, decreased as extracellular pH was changed from 5.4 to 8.4. The substrate specificities of the hRFT family were similar. hRFT-mediated uptake of [^{3}H]riboflavin was inhibited by some riboflavin analogs, but not D-ribose, organic ions, or other vitamins. The newly isolated hRFT3 may play an important role in brain riboflavin homeostasis. Its amino acid sequence and functional characteristics are similar to those of hRFT1, but not hRFT2. J. Nutr. 140: 1220–1226, 2010.

Introduction

The water-soluble vitamin riboflavin is essential for normal cellular functions. It is converted to the coenzyme forms flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) by flavokinases and FAD synthases. These flavins participate in cellular metabolic reactions as intermediaries in biochemical oxidation-reduction reactions, including carbohydrate, lipid, and amino acid metabolism (1). Humans are unable to synthesize riboflavin and therefore must obtain it via intestinal absorption. Over the last 5 decades, many studies using intestinal specimens, intestinal membrane vesicles, and cell lines have indicated that absorption of riboflavin in the intestine is mediated by transporter(s) (2).

Recently, we identified novel human riboflavin transporter (hRFT)1[4] and rat riboflavin transporter (rRFT)1 using our rat kidney mRNA expression database (3). hRFT1 was originally annotated as G protein-coupled receptor (GPR) 172B (GenBank accession no. NM_017986.2), but its molecular function had yet to be determined. It was predicted to have 10 putative membrane-spanning domains by the SOSUI program (4) and, thus, this protein was postulated to be a transporter rather than a receptor. Screening for its substrate(s) was carried out using the small interfering RNA approach, because it was endogenously expressed in various cultured cells. We found that riboflavin is a specifically transported substrate for it and renamed it hRFT1. It exhibits no similarity to a bacterial riboflavin transporter RibU or impX (5,6), a yeast riboflavin transporter Mch5p (7), or any other mammalian transporters. Riboflavin transporter (RFT)1 is

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[3] Supplemental Figures 1 and 2 are available with the online posting of this paper at jn.nutrition.org.
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[4] Abbreviations used: EGFP, enhanced green fluorescent protein; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GPR, G protein-coupled receptor; HEK, human embryonic kidney; hRFT, human riboflavin transporter; K_m, Michaelis-Menten constant; RFT, riboflavin transporter; rRFT, rat riboflavin transporter; V_{max}, maximal transport rate.

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