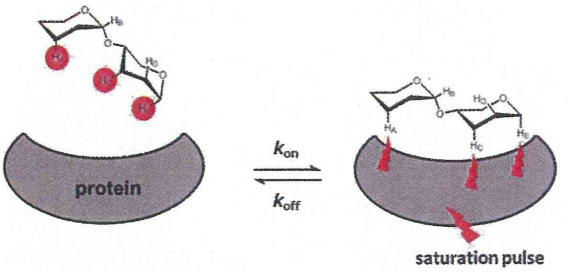

図９，　STD-NMR法の概念図

hCD22-Fcの溶液にシアル酸誘導体（阻害剤Y）を加えて、STD-NMRスペクトルを測定したところ、阻害剤の芳香族部分がタンパク質との相互作用に大きく寄与していることが判明した（図10）。

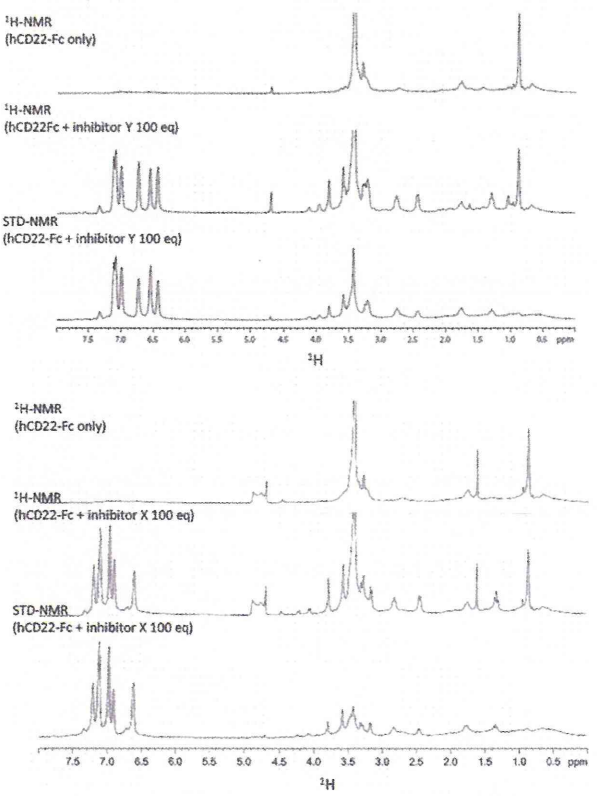

図 10，　STD-NMR 法による hCD22 と阻害剤 Y（上段）および阻害剤 X（下段）の相互作用解析

阻害剤 X と hCD22-Fc との相互作用解析も同様に STD-NMR 解析を行ったところ、阻害剤 X も阻害剤 Y と同様の面を使って hCD22-Fc と結合していることが判明した。

一方で mCD22-Fc と阻害剤 Y との相互作用を STD-NMR 法で解析したところ、大きな STD シグナルを与えなかった。その結果は、阻害剤 Y と mCD22-Fc の結合が弱い、もしくはリガンドの結合解離の速度が遅いことを意味する。阻害剤 X と mCD22-Fc の相互作用も同様であった。

CD22-Fc と阻害剤との相互作用解析：TR-NOE

TR-NOE 法はタンパク質に結合した状態のリガンドのコンフォメーションに関する情報をもたらす。

NOE は ^{1}H-^{1}H 間の距離が近い時に観測される（通常 約 5 Å 以内）（図 11）。

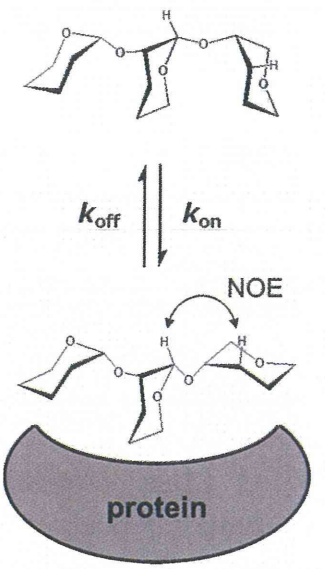

図11，　TR-NOE法の概念図

その結果、大変興味深いことに、化学構造上離れている ^{1}H 同士で TR-NOE シグナルが観測された（図 12)。このことは阻害剤 Y が hCD22-Fc に結合しているときは両末端が空間的に近接した状態になっていることが明らかになった。また、阻害剤 X と阻害剤 Y はほぼ同様のコンフォメーションで hCD22-Fc と結合していることも判明した。

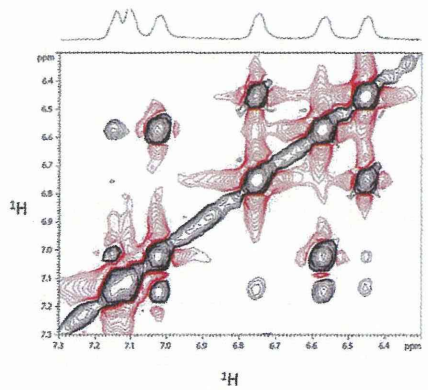

図 12,　hCD22-Fc 存在下における阻害剤 Y の TR-NOE スペクトル（芳香族領域）

D. 考　察

GSC-718 の合成展開と活性評価

シアル酸誘導体 GSC718 は CD22 に高親和性（IC50~0.1 μ M）で結合し、B 細胞の活性化を増強する。我々は、GSC718 のシアル酸の２位と５位を中心に合成展開して得た化合物１６個を得た。これらの化合物について CD22 への親和性を測定し、うち２つ(MF6 および MF13)で CD22 への親和性の上昇を認めた。次いで、化合物の B 細胞の活性化・増殖への作用を調べたところ、CD22 への親和性が 1 μ M 程度以上になると B 細胞の活性化・増殖の亢進は認められなかったが、GSC718 より親和性の高い化合物でも B 細胞の活性化・増殖は GSC718 と同程度であった。また、予備的に MF6 および MF13 を抗原とともにマウスに投与したが、GSC718 に比べてアジュバント活性の増強を認めなかった。この点についてはさらに追試が必要であるものの、親和性の増強のみではアジュバント活性の増強は難しい可能性が示唆された。

そこで、我々は代謝安定性をめざした GSC718 の誘導体 W の評価をおこなったところ、CD22 への親和性や in vitro での B 細胞活性化・増殖増強作用は GSC718 と遜色なかった。この化合物を抗原とともにマウスに投与したところ、GSC718 よりも多量の特異抗体の産生を認め、アジュバント活性が増強していることが明らかとなった。したがって、優れたアジュバント活性を得るのに代謝安定性の増強が重要であることが示唆された。

本研究では、マクロファージや好中球などの炎症細胞で CD22 の発現がないことを示した。また、樹状細胞では CD22 の発現があるものの、GSC718 で刺激しても活性化はおこらない。これらの知見から GSC718 はマクロファージ、樹状細胞、好中球といった先天免疫細胞を活性化できないことが明らかである。この知見は、脾細胞を GSC718 で刺激しても炎症性サイトカインの産生がおこらないという我々の知見（未発表）とよく合致し、GSC718 が抗体産生を増強するが炎症をおこさない画期的な化合物であることを強く示唆している。

シアル酸誘導体による B 細胞活性化のメカニズム

本研究では、GSC718 が CD22 に作用することにより BCR 架橋の際の BCR シグナル伝達が減弱することを示した。この結果は、GSC718 が CD22 の抑制機能を増強することを示すものである。B 細胞は CD22 のリガンドである α2,6 シアル酸を細胞表面の糖脂質や糖タンパク上に多量に発現し、CD22 は同じ B 細胞上の α2,6 シアル酸と構成的に結合していることが知られている。このような同じ細胞上のリガンドはシスリガンドと呼ばれる。GSC718 は CD22 とシスリガンドの結合を阻害し、シスリガンドによる CD22 の機能制御をキャンセルするものと考えられるので、GSC718 が CD22 のシグナル抑制機能を増強するという我々の結果は、シスリガンドが CD22 の機能を抑制することを示唆し、α2,6 シアル酸産生に必須のシアリルトランスフェラーゼ ST6GalI 欠損マウス B 細胞での UCSD の Marth 博士やスクリプス研究所の Paulson 博士ら結果とよく合致する。一方、B 細胞を in vitro で抗 CD40 抗体や CpG オリゴとともに培養した場合、正常 B 細胞では GSC718 の添加により B 細胞の活性化マーカーの発現や B 細胞の増殖は顕著に増強するが、このような B 細胞の活性化増強は CD22 欠損 B 細胞でも認められる。CD22 の機能を増強して BCR シグナル伝達を抑制する GSC718 がどのようにして、CD22 欠損 B 細胞と同様の B 細胞活性化増強を誘導するのかが問題である。

我々は、GSC718 が BCR 架橋による BCR シグナル伝達を抑制するが、BCR 架橋非存在下での緊張性シグナルを増強することを明らかにした。シスリガンドによる制御が BCR 架橋によるシグナル伝達と緊張性シグナルで異なるために、GSC718 が BCR 架橋によるシグナル伝達を抑制し、緊張性シグナルを増強するという相反する作用がおこるのであろう。また、GSC718 による B 細胞活性化増強は、おそらく緊張性シグナル伝達の増強によっておこるのであろう。B 細胞での緊張性シグナルは B 細胞の生存維持に必須であり、その分子メカニズムの解明が進んでいる。我々の結果は、緊張性シグナルが B 細胞の生存のみならず、B 細胞の活性化でも重要な役割を果たすことを示唆している。

組み換え CD22 タンパクの大量発現と結晶化

CD22 について，N 末端側のドメイン 1 から 3 までを対象とし，大量発現系の構築と結晶化用標品の精製を行った．発現させるドメインの長さを変化させ，さらに点変異の導入，folding や可溶性を改善させるタグタンパク質の融合，バクテリアとヒト培養細胞での発現系の検討など，多くの条件を検討したが，総じて CD22 が凝集しやすいという特性が，効率の良い発現と精製を困難にしていると考えられた．発現させるドメインの長さが増えるごとに発現量が減少し，また凝集性は上昇する傾向がみられ，ドメイン 1 から 3 までを発現させると，バクテリア，ヒト培養細胞いずれの系でも少量の可溶性の凝集体が得られるのみであった．

ドメイン1のみ，あるいはドメイン1と2を発現させた場合，いくつかの条件で結晶化実験を行うに足る精製度と収量を達成することが出来た．ジスルフィド結合形成促進タンパク質を融合させて発現させる系では，このタグタンパク質が融合した状態でのCD22ドメイン1と2，またタグタンパク質を除去したCD22ドメイン1について最終標品を得たが，これらはいずれもリガンド結合活性が低いことがわかり，正しいfoldingがなされていないか，あるいはタグタンパク質部分がリガンド結合部位を覆い隠している可能性が考えられた．また，結晶化を行ったタグタンパク質融合CD22では現在までに結晶が得られていないが，今回使用したジスルフィド結合形成促進タンパク質は結晶化が難しいとされるタンパク質であるため，やはりタグタンパク質を除去することが望ましいと考えられる．この場合，タグタンパク質を除去しても十分な収量を確保できるよう改善が必要である．

高可溶性タグタンパク質を融合させて発現させる系では，タグタンパク質融合CD22ドメイン1について精製度，収量ともに結晶化実験に十分な水準を達成し，結晶化実験を行った．本高可溶性タグタンパク質はシステイン残基がないために，タグタンパク質を介した多量体化が基本的に生じることがなく，またタグタンパク質単体では結晶化が容易であることなどから，CD22融合タンパク質についても結晶化には有利であると考えられた．しかしながら結晶化条件の探索において，多くの条件でアモルファス状の凝集体がえられており，これはCD22部位の一部がfoldしていないことが原因として考えられる．結晶構造解析が可能な良質の単結晶を得るためには，CD22部位が強固に一定のfoldを形成するよう，さらなる点変異の導入，タグタンパク質の変更，追加，あるいはタグタンパク質とCD22部分を繋ぐリンカー配列の改善などが必要である．

CD22とシアル酸誘導体の相互作用

シアル酸誘導体の両末端が空間的に近接したコンフォメーションでhCD22-Fcと結合することは、これまでのmouse siglec-1と阻害剤との複合体の結晶構造からも支持される。mouse siglec-1とシアル酸の9位にビフェニルが結合した阻害剤の複合体の結晶構造、およびシアル酸の2位にベンジル基が結合した阻害剤の複合体との結晶構造が過去に報告されているが（図13)、両構造においてシアル酸ユニットは同じ部位に結合しており、両構造を重ねると9位側のビフェニル基と2位のベンジル基が空間的に近接することがわかる。

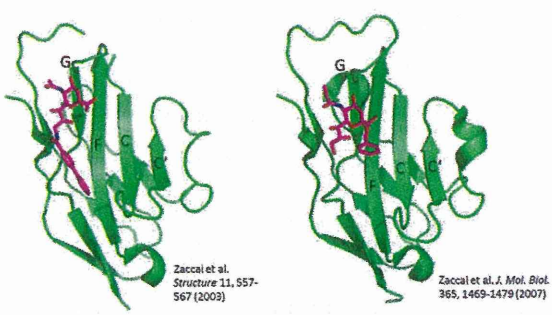

図13，mouse siglec-1の糖鎖結合ドメインとシアル酸誘導体の結晶構造

また STD-NMR から特定の芳香族領域がよりタンパク質に近接していることが明らかになった。この実験結果から反対側に位置する部分は親和性向上のために改変の余地が残されていると考えられる。実際、mouse siglec-1 とシアル酸の２位にベンジル基が結合した阻害剤の複合体との結晶構造では、ベンジル基は直接タンパク質と相互作用しておらず、結晶中において隣接する分子のベンジル基とスタックしている。

E. 結　論

我々は、GSC718 より系統的に合成展開した化合物の活性評価を行い、GSC718 よりも CD22 に高親和性に結合する化合物２つを得た。これまでのところ、これらの化合物が GSC718 よりも高い抗体産生増強作用を示すという証拠は得られていない。一方、我々は代謝安定性をめざした GSC718 の誘導体 Z が GSC718 よりも抗体産生増強作用が有意に高いことを示し、GSC718 よりも抗体産生増強作用の強い化合物の合成に成功した。また、GSC718 が B 細胞活性化を増強するメカニズムの解明を行い、GSC718 による B 細胞緊張性シグナルの増強が B 細胞活性化に重要であることを明らかにした。CD22 の立体構造情報および阻害剤との相互作用に関する情報を得るために、CD22 の大量発現系の構築を行い、N 末端ドメイン１のみ，あるいは N 末端ドメイン１と２を発現範囲とした条件で，結晶化可能な純度と収量で精製標品を得た。また、シアル酸誘導体のタンパク質結合部位やコンフォメーションに関する情報を得ることができた。これらの知見はより活性の高い CD22 阻害剤のデザインに貢献する。

F．健康危険情報

特記すべきことはなかった。

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2. 実用新案登録
該当なし。

3. その他
該当なし。

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

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Constitutively CD40–Activated B Cells Regulate CD8 T Cell Inflammatory Response by IL-10 Induction

Pandelakis A. Koni,[*,†] Anna Bolduc,[†] Mayuko Takezaki,[†] Yutetsu Ametani,[†] Lei Huang,[†,‡] Jeffrey R. Lee,[†,§,¶] Stephen L. Nutt,[‖] Masahito Kamanaka,[#] Richard A. Flavell,[#] Andrew L. Mellor,[*,†] Takeshi Tsubata,[**] and Michiko Shimoda[†,§]

B cells are exposed to high levels of CD40 ligand (CD40L, CD154) in chronic inflammatory diseases. In addition, B cells expressing both CD40 and CD40L have been identified in human diseases such as autoimmune diseases and lymphoma. However, how such constitutively CD40–activated B cells under inflammation may impact on T cell response remains unknown. Using a mouse model in which B cells express a CD40L transgene (CD40LTg) and receive autocrine CD40/CD40L signaling, we show that CD40LTg B cells stimulated memory-like CD4 and CD8 T cells to express IL-10. This IL-10 expression by CD8 T cells was dependent on IFN-I and programmed cell death protein 1, and was critical for CD8 T cells to counterregulate their overactivation. Furthermore, adoptive transfer of naive CD8 T cells in RAG-1$^{-/-}$ mice normally induces colitis in association with IL-17 and IFN-γ cytokine production. Using this model, we show that adoptive cotransfer of CD40LTg B cells, but not wild-type B cells, significantly reduced IL-17 response and regulated colitis in association with IL-10 induction in CD8 T cells. Thus, B cells expressing CD40L can be a therapeutic goal to regulate inflammatory CD8 T cell response by IL-10 induction. *The Journal of Immunology*, **2013, 190: 3189–3196.**

CD40-CD154 (CD40 ligand [CD40L], CD40L) interaction delivers a critical costimulatory signal for B cell differentiation and function (1). CD40L is highly expressed by activated T cells as well as by platelets and various other cell types under chronic inflammatory diseases such as autoimmune diseases (2). CD40L derived from platelets has been shown to modulate adaptive immune response (3). In multiple sclerosis patients, B cells had a trait of CD40-activated B cells and stimulated CD8 T cells in vitro via IL-15 (4). Moreover, CD40L is functionally expressed on some B cells in patients with EBV infection (5), autoimmune diseases (6–8), and lymphoma (9–11). In B cell lymphoma, this autonomous CD40/CD40L interaction has been shown to increase their survival through constitutive NF-κB and NFAT activation (12, 13). These findings support the hypothesis that the heightened B cell CD40/CD40L signaling due to elevated CD40L expression during chronic inflammatory diseases changes B cell functions and has an impact on ongoing immune response through altered B cell reactivity.

In this study, we employed CD40L transgenic (CD40LTg) mice that express CD40L under the *IgVH* promoter specifically on B cells (14). Thus, CD40LTg mice serve as a model for human diseases in which B cells abnormally express CD40L and are exposed to excessive CD40/CD40L signaling under chronic inflammation. Based on their phenotype, B cells in CD40LBTg mice are not constitutively activated (14, 15). However, binding of CD40L or anti-CD40 Ab breaks up the CD40 and CD40L complex formed on the cell surface of B cells and triggers *cis* activation of B cells (16), as evidenced by robust NF-κB-1 activation (15), without triggering *trans*-activation of dendritic cells (DCs) (16). This augmented B cell–specific CD40/CD40L signaling enhanced the magnitude of primary Ag-specific humoral response as a result of premature termination of ongoing germinal center response (15, 16). Moreover, aged CD40LBTg mice have been shown to develop B cell–mediated lupus-like disease and colitis with autoantibody production (14, 17).

In this study, we show that CD40LTg B cells stimulated memory-like CD4 and CD8 T cells to express IL-10. Furthermore, in a RAG-1$^{-/-}$ colitis model, adoptive cotransfer of CD40LTg B cells could suppress inflammatory CD8 T cell response by inducing IL-10 expression and regulated CD8 T cell–mediated colitis.

Materials and Methods

Mice, immunization, and inflammatory challenge

RAG-1$^{-/-}$, C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I), and C57BL/6-Tg (TcraTcrb)425Cbn/J (OT-II) mice were all on a C57BL6/J background and were purchased from The Jackson Laboratory, as were C57BL6/J mice. All other mice used were on a C57BL6/J background ($n > 10$) and were bred in our facility under specific pathogen-free conditions. CD40LTg mice (14), IL-10-GFP reporter mice (18), JH$^{-/-}$ mice (19), and Blimp-1-GFP reporter mice (20) were described before. IFN-α/βR$^{-/-}$ mice and programmed cell death protein 1 (PD-1)$^{-/-}$ mice were the gifts of D. Moskofidis (Georgia Regents University) and T. Honjo (Kyoto University), respectively. Typical experiments used mice at 6–12 wk of age. For immunization and inflammatory challenge, mice 6–10 wk of age

*Department of Medicine, Georgia Regents University, Augusta, GA 30912; †Georgia Regents University Cancer Center, Georgia Regents University, Augusta, GA 30912; ‡Department of Radiology, Georgia Regents University, Augusta, GA 30912; §Department of Pathology, Georgia Regents University, Augusta, GA 30912; ¶Charlie Norwood Veterans Affairs Medical Center, Augusta, GA 30904; ‖Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia; #Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520; and **Department of Immunology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113, Japan

Received for publication December 7, 2012. Accepted for publication January 28, 2013.

This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR052470 (to M.S.), National Institute of Allergy and Infectious Diseases Grant AI064752 (to M.S.), and Georgia Regents University Pilot Study Research Program grant (to M.S.).

Address correspondence and reprint requests to Dr. Michiko Shimoda, Georgia Regents University, CN4155, 1120 15th Street, Augusta, GA 30912. E-mail address: mshimoda@gru.edu

Abbreviations used in this article: CD40L, CD40 ligand; DC, dendritic cell; MLN, mesenteric lymph node; MZ, marginal zone; PD-1, programmed cell death protein 1; PD-L1, programmed cell death protein ligand 1; PD-L2, programmed cell death protein ligand 2; T_{EM}, effector memory T; Tg, transgenic.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1203364

were given an i.p. challenge with 100 μl PBS containing 2 mg alum (Sigma-Aldrich) with or without 200 μg OVA. All studies were reviewed and approved by the institutional animal care and use committee.

Abs and reagents

Abs used in this study were against I-A^b (AF6-120.1), CD4 (RM4-5), CD11c (HL3), CD8α (53-6.7), CD90.1 (OX-7), TCRβ (H57-597), CD19 (1D3), CD21 (7G6), CD23 (B3B4), CD44 (IM7), CD62L (MEL-14), CD103 (M290), CD122 (TMB1), CD127 (A7R34), IFN-γ (XMG1.2), B7H-1 (programmed cell death protein ligand 1 [PD-L1]), B7-DC (PD-L1, programmed cell death protein ligand 2 [PD-L2]), CD80, CXCR5-biotin, CD16/CD32 (2.4G2), and H-2kd (SF1-1.1) from BD Biosciences. Streptavidin-eFluor780 and Abs against Foxp3 (FJK-16a), CD8β (H35-17.2), MHC-II (M5/114.15.2), CD4 (GK5.1), and PD-1 (J43) were from eBioscience. Peanut agglutinin FITC was from Vector Laboratories (Southfield, MI). Mouse rIL-2 and rIL-27 were from R&D Systems (Minneapolis, MN).

Flow cytometry and cell sorting

For analysis of DCs, spleens were diced and incubated in RPMI 1640/1% FCS containing 0.5 mg/ml collagenase type IV (Sigma-Aldrich) for 30 min at 37°C; single-cell suspensions were prepared; and RBCs were depleted with ACK lysing buffer (BioWhittaker). Spleen cells were then washed twice with PBS and filtered through nylon mesh in RPMI 1640/1% FCS. For general analysis, cells were pretreated with anti-CD16/CD32 Ab on ice for 15 min and then incubated at 4°C for 30 min with specific Abs. For intracellular staining for IFN-γ and granzyme B, cells were incubated with anti-CD3 Ab (100 ng/ml) for 3 h in the presence of brefeldin A (eBioscience), followed by fixation/permeabilization and staining with anti–IFN-γ (BD Bioscience) and anti-granzyme B Ab (Invitrogen). For IL-10-GFP and granzyme B staining, cells were treated with 0.01% paraformaldehyde/PBS for 5 min and 0.05% Tween 20/PBS for 5 min, followed by anti-granzyme B Ab.

Cells were analyzed on a FACS Canto (BD Biosciences) with FlowJo software (Ashland, OR). Singlet lymphocyte gates were set based on forward scatter and side scatter channels, and autofluorescent cells were gated out using a dump channel. Positive gates were set using fluorescently labeled isotype-control nonrelevant Abs, or internal nonrelevant cellular populations. IL-10-GFP^+ gates were set using non–IL-10-GFP reporter cells as a negative control. B cells were sorted from spleen cells using B cell isolation kit (Miltenyi Biotec) by depletion of $CD43^+$ B cells and non-B cells. CD4 T cells, CD8 T cells, and CD11c DCs were sorted from spleen cells using magnetic beads (Miltenyi Biotec), whereas $CD62L^{low}$ CD8 or CD4 T cells were purified using an Aria cell sorter (BD Biosciences).

Cell culture

For CFSE-dilution assays, sorted CD4 and CD8 T cells were labeled with 5 nM CFSE (Invitrogen) for 10 min at 37°C, followed by washing with cold PBS. Purified B cells (1×10^6) and DCs (2×10^4) were cultured with 10^5 CFSE-labeled OT-I, OT-II cells, and CD8 T cells, or FACS Aria-sorted $CD62L^{low}$ CD8 T cells and $CD62L^{low}$ CD4 T cells in 250 μl RPMI 1640/10% FCS containing various concentrations of OVA (Sigma-Aldrich) or 100 ng/ml anti-CD3 Ab for 2–3 d. CFSE dilution was analyzed on a FACS Canto (BD Biosciences) with FlowJo software. IL-10 in the culture supernatant was measured by IL-10 ELISA kit (R&D Systems).

In vivo cytotoxicity

Spleen cells or B cells purified were pulsed with 2 μM $OVA_{257–264}$ peptide for 2 h at 37°C. $OVA_{257–264}$ peptide-pulsed cells and control, nonpulsed cells were labeled with 3 and 0.3 μM CFSE, respectively, for 3 min at 37°C. After washing, these two were mixed 1:1 and injected i.v. (10^7) into day 10–14 immunized mice. The ratios of $CFSE^{high}$ versus $CFSE^{low}$ B cells in the host spleen were measured after 18 h.

Quantitative RT-PCR

RNA was prepared using a Qiagen mini kit, according to the manufacturer's protocol. cDNA was prepared using a cDNA synthesis kit from SABioscience (Qiagen). Quantitative RT-PCR analyses were performed in triplicate using primers and SYBR Green master mix from SABioscience with an iQ5 cycler (Bio-Rad). Gene expression levels in each sample were normalized against β-actin expression and statistically analyzed with software from SABioscience.

Adoptive transfer

Six-week-old sex-matched RAG-$1^{-/-}$ recipient mice received an i.v. injection in 100 μl PBS of either wild-type or CD40LTg B cells (5×10^6), plus one or other combination of IL-10–deficient or sufficient (IL-10-GFP reporter) CD4 T cells (10^6) and CD8 T cells (10^6). Likewise, RAG-$1^{-/-}$ recipient mice received CD40LTg B cells with IL-10-GFP reporter CD4 T cells (10^6) and CD8 T cells (10^6) from either PD-$1^{-/-}$ or IFN-α/β$R^{-/-}$ mice. Two to three weeks after transfer, spleen cells were analyzed by FACS.

Colitis model

Purified naive $CD62L^+CD44^-$CD8 T cells (10^6) by Aria sort from IL-10-GFP wild-type or IL-$10^{-/-}$ mice were i.v. transferred into RAG-$1^{-/-}$ mice (6-wk-old male) with or without B cells (5×10^6) from wild-type or CD40LTg mice. The recipients were weighed weekly for clinical signs of disease. After 5 wk of transfer, mice were sacrificed for tissue harvest. Each colon was graded by an experienced pathologist blinded to the treatment group using a 0–3 scoring system to evaluate acute and chronic inflammation, crypt damage, and regeneration. The highest injury score could be as high as 12, and the lowest without injury 1 (physiological inflammation).

Statistics

The p values were determined by applying the two-tailed, two-sample equal variance Student t test or Mann–Whitney U test.

Results

CD40LTg B cells activate CD8 T cells and induce augmented Ag-specific cytotoxic response

Previous studies have shown that in vitro CD40-activated B cells become potent APCs for CD8 T cells and generate augmented Ag-specific cytotoxic response (4, 21, 22). To test whether CD40LTg B cells can activate T cells, CD40LTg and wild-type B cells were

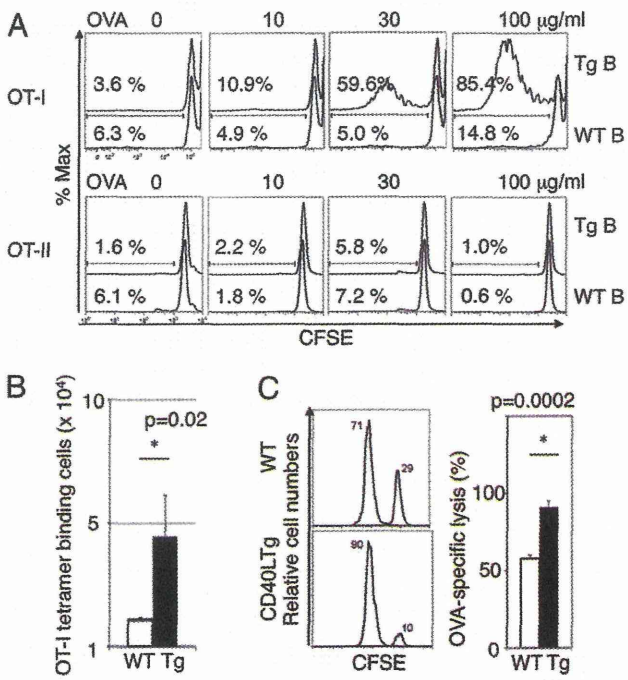

FIGURE 1. CD40LTg B cells prime CD8 T cells in vitro and generate augmented in vivo Ag-specific cytotoxicity. (**A**) CFSE-labeled OT-I or OT-II cells were cultured with wild-type (WT) or CD40LTg (Tg) B cells and with various concentrations of OVA protein for 3 d. Representative histogram overlays with percentages of $CFSE^{low}$ OT-I and OT-II cells cocultured with B cells from WT (black) or Tg (red) mice are shown. (**B**) Averages with SDs of total OT-I tetramer-binding cell numbers in spleen of WT (n = 3) or Tg mice (n = 3) after day 10 immunization with OVA-alum are shown. *p = 0.02. (**C**) Representative histograms with percentages of $CFSE^{low}$ control (black) and $CFSE^{high}$ $OVA_{257–264}$ peptide-loaded (red) $B220^+$ donor cells in Tg or WT mice (n = 3/group) preimmunized with OVA plus alum. Averages with SDs of $OVA_{257–264}$-specific lysis (%), determined as ($CFSE^{low}$[%] − $CFSE^{high}$[%])/$CFSE^{low}$[%] × 100. *p = 0.0002. Experiments in (A)–(C) were repeated twice.

cultured with CFSE-labeled OVA-specific CD8 (OT-I), or CD4 (OT-II), TCR Tg T cells in the presence of various amounts of soluble OVA. We found that CD40LTg, but not wild-type, B cells induced proliferation of OT-I, but not OT-II, cells, as evidenced by significantly higher percentage of $CFSE^{low}$ population in the OT-I cell culture, in a manner dependent on the amounts of OVA (Fig. 1A). Also, at 2 wk after immunization with OVA plus alum, CD40LTg mice generated significantly greater numbers of OVA-specific tetramer-binding CD8 T cells (Fig. 1B) with augmented OVA-specific in vivo cytotoxicity compared with wild-type control mice (Fig. 1C). It should be noted that DCs in Tg mice were not functionally activated (16) and, in fact, upregulated PD-L1 expression and suppressed CD8 T cell activation (P.A. Koni, A. Bolduc, M. Takezaki, Y. Ametani, L. Huang, S.L. Nutt, M. Kamanaka, R.A. Flavell, A.L. Mellor, T. Tsubata, and M. Shimoda, manuscript in preparation). These results indicate that like CD40-activated B cells, CD40LTg B cells are potent APCs for CD8 T cells.

Adjuvant induces activation of natural memory-like CD8 T cells in CD40LBTg mice

During the immunization experiment described above, we found that, unlike wild-type control mice, CD40LTg mice exhibited spontaneous global activation of CD8 T cells in their spleen with accumulation of $CD62L^{low}CD44^{high/int}$CD8 T cells. This was substantially augmented after i.p. alum (2 mg) injection even without Ag (Fig. 2A), although the accumulation of $CD62L^{low}$ $CD44^{high/int}$CD4 T cells was not so obvious (data not shown). At 2 wk after alum injection, significantly higher levels of CD4 and CD8 T cells in CD40LTg mice were still in cell cycle, as judged by 24-h BrdU labeling compared with those in wild-type mice (Fig. 2B). The proliferating $BrdU^+$ CD8 T cells were $CXCR5^+$ $CD62L^{low}CD44^{high/int}$ with effector memory T (T_{EM}) phenotype (Fig. 2B), which are similar to those found in the B cell mantle zone of human tonsils (23). This accumulation of $CD62L^{low}$ $CD44^{high/int}$CD8 T_{EM} cells after alum injection did not occur in CD40LTg mice on a B cell–deficient $Jh^{-/-}$ background (data not shown).

Total numbers of CD4 and CD8 T cells and the frequency of $Foxp3^+$ regulatory CD4 and CD8 T cells were not significantly different between wild-type and CD40LTg mice 2 wk after alum injection, although $Foxp3^+$ CD4 T cells from CD40LTg mice had a more activated phenotype with downregulated CD62L expression compared with those from wild-type mice (data not shown).

Activated natural effector memory CD8 T cells express IL-10

The $CD62L^{low}CD44^{high/int}$CD8 T cells in CD40LTg did not express IFN-γ by intracellular staining. By gene expression analysis, we found that CD8 T cells in CD40LTg mice expressed significantly higher levels (67.4-fold) of IL-10 compared with those in wild-type mice. To better track IL-10 differentiation in CD8 T cells, IL-10-GFP reporter mice (18) and Blimp-1-GFP reporter mice (20, 24) were crossed onto a CD40LTg background. As shown in Fig. 2C, the $CD62L^{low}CD44^{int/low}$ population has a phenotype consistent with effector/effector memory CD8 T cells defined by Blimp-1-GFP reporter expression (20, 24). This population in CD40LTg/IL-10-GFP mice also expressed elevated PD-1 and IL-10-GFP reporter expression. This population barely contained Foxp3-GFP^+ cells (data not shown).

In IL-10-GFP reporter mice, the level of GFP expression correlates with the amount of IL-10 mRNA (18). Because the mean fluorescent intensity of IL-10-GFP reporter expression in GFP^+ CD8 T cells was consistently lower than that in GFP^+ CD4 T cells (data not shown), we tested whether IL-10-GFP^+ CD8 T cells in

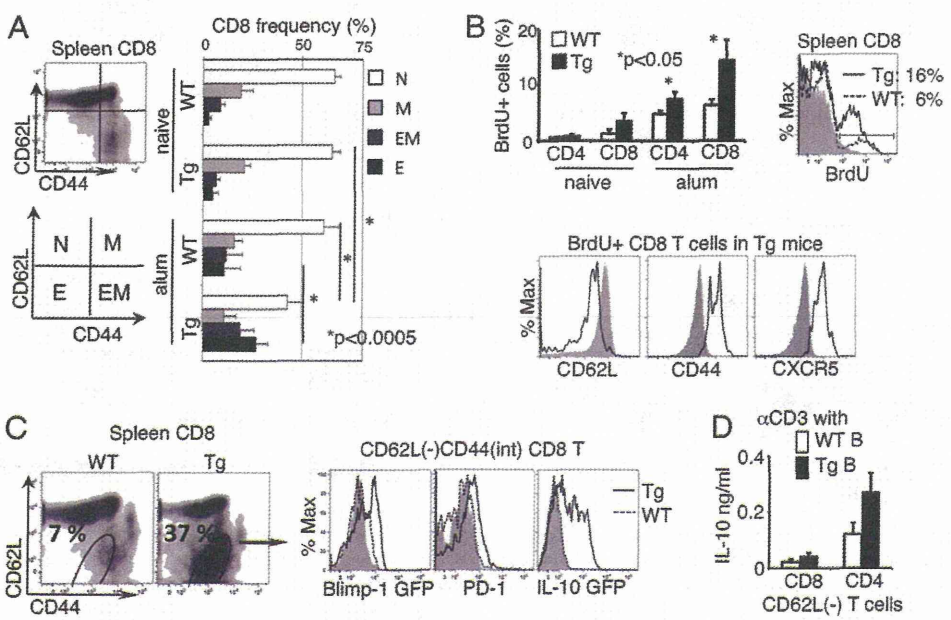

FIGURE 2. CD40LTg mice trigger activation of CD8 T_{EM} cells during alum-mediated inflammatory response. Wild-type (WT) and CD40LTg (Tg) mice were given alum, and their spleen cells were analyzed 2 wk later. (**A**) Average frequencies with SDs of naive (N), memory (M), effector memory (EM), and effector (E) CD8 T cells (as gated on the *left*) in WT and Tg mice with (alum) ($n = 9$/group) or without (naive) ($n = 6$/group) alum injection are shown. $*p < 0.0005$. (**B**) Average percentages with SDs of $BrdU^+$ CD4 and CD8 T cells in naive and alum-treated wild-type (white) and Tg (black) mice are shown ($n = 3$/group). Representative histograms for BrdU staining of CD8 T cells in Tg (solid) and WT mice (dash) with isotype control (shaded) and for CD62L, CD44, and CXCR5 expression of $BrdU^+$ cells are shown. $*p < 0.05$. (**C**) Representative CD62L versus CD44 FACS profiles of CD8 T cells in wild-type or Tg Blimp-1-GFP reporter mice and gating strategy of $CD62L^{low}CD44^{high/int}$CD8 T_{EM} cell population with percentages are shown (*left*). Representative histograms of Blimp-1-GFP, PD-1, and IL-10-GFP expression in $CD62L^{low}CD44^{high/int}$CD8 T_{EM} cell population in Tg (solid) and WT mice (dash) with isotype control (shaded) are shown. (**D**) ELISA IL-10 concentration in triplicate 4-d culture supernatants for anti-CD3–stimulated $CD62L^{low}$ CD8 and CD4 T cells of Tg mice with B cells from WT or Tg mice.

CD40LTg mice produce IL-10. Thus, CD62L^{low} CD8 and CD4 T cells enriched with IL-10-GFP^{+} cells were purified from CD40LTg and restimulated with anti-CD3 Ab in the presence of wild-type or CD40LTg B cells. As expected and shown in Fig. 2D, the amount of IL-10 produced by CD62L^{low}CD8 T cells was significantly lower than that produced by CD4 T cells from these mice.

CD40LTg B cells induce IL-10-GFP expression in T cells during lymphopenic proliferation

Based on the CD8 T cell IL-10 expression in CD40LTg mice during alum-induced inflammatory response, we hypothesized that CD40LTg B cells stimulate memory-like CD8 T cells to express IL-10. To test the hypothesis, we performed adoptive transfer experiments in RAG-1$^{-/-}$ mice. CD4 and CD8 T cells transferred into the lymphopenic environment of RAG-1$^{-/-}$ mice undergo spontaneous proliferation and generate natural memory/effector memory T cells (25). When RAG-1$^{-/-}$ mice were reconstituted with CD4 and CD8 T cells (1×10^6 each) from IL-10-GFP reporter mice and B cells (5×10^6) either from wild-type or CD40LBTg mice, the recipients of CD40LTg B cells had significantly greater numbers of CD8 T cells (8.7-fold) with a higher frequency of CD62L^{low}CD44^{low} effectors (2.8-fold) compared with the recipients of wild-type B cells at 2 wk posttransfer (Fig.

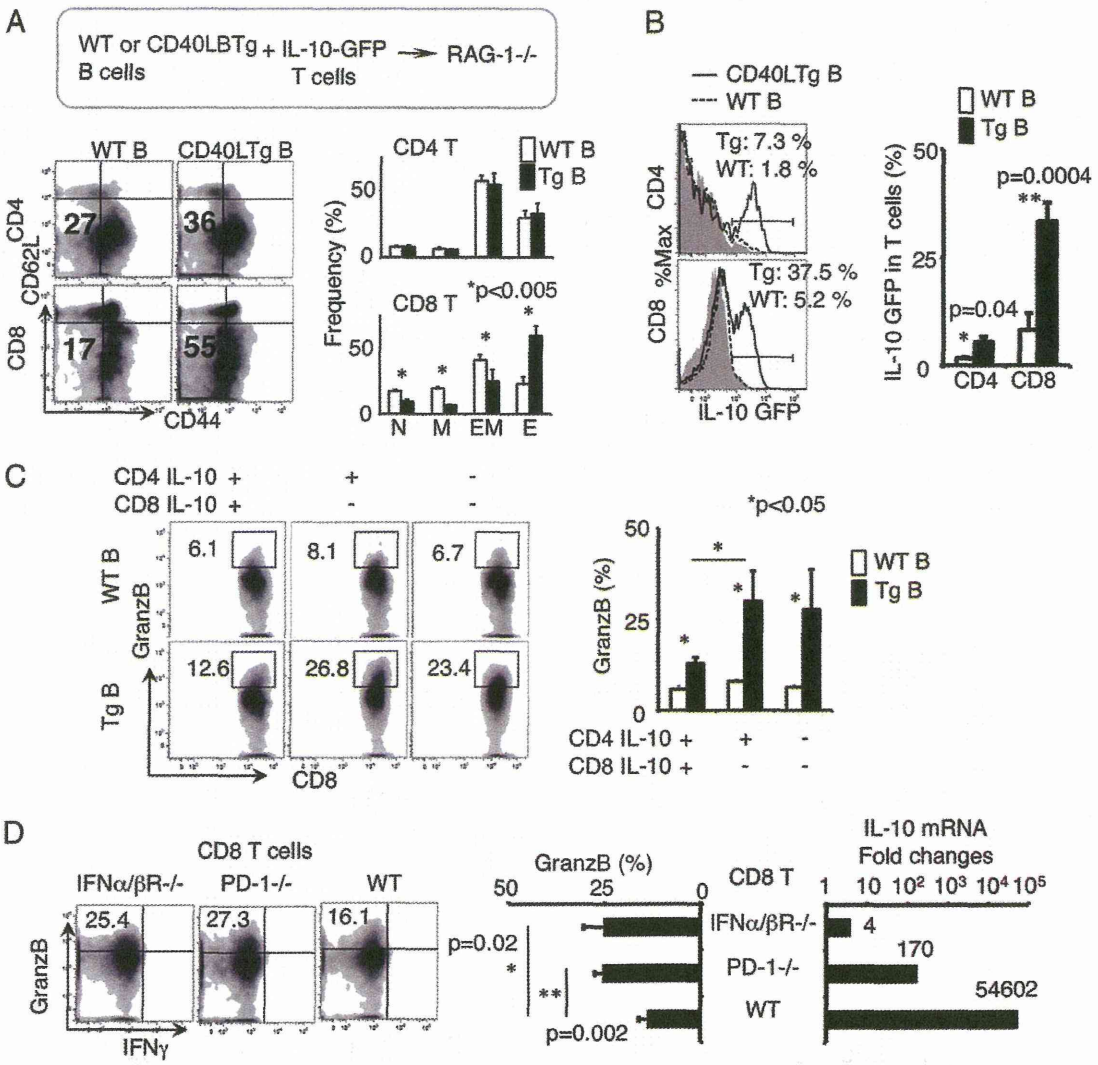

FIGURE 3. CD40LTg B cells induce IL-10 expression from CD8 T cells during lymphopenic response in a manner dependent on IFN-I and PD-1 to counterregulate overactivation. Wild-type (WT) or CD40LTg (Tg) B cells and T cells from IL-10-GFP or IL-10$^{-/-}$ mice were transferred into RAG-1$^{-/-}$ mice. Their spleen cells were analyzed after 2 wk. (**A**) Representative CD62L versus CD44 FACS profiles for CD4 and CD8 T cells of RAG-1$^{-/-}$ recipients of WT or Tg B cells. Average frequencies with SDs of naive (N), memory (M), effector memory (EM), and effector (E) cells among CD8 T cells are shown (n = 3/group). *$p < 0.005$. Experiments in (A) were repeated twice. (**B**) Representative histograms for IL-10-GFP expression of CD4 and CD8 T cells in RAG-1$^{-/-}$ recipients of WT (dash) or Tg (solid) B cells. Average percentages with SDs of IL-10-GFP^{+} cells are shown (n = 7/group). *$p = 0.04$, **p = 0.0004. Experiments in (B) were repeated twice. (**C**) RAG-1$^{-/-}$ mice received B cells from WT (n = 3) or CD40LBTg (n = 5) mice together with CD4 and CD8 T cells (IL-10$^{-/-}$ or IL Please add a p value to the figure legend, otherwise explain the asterisks in Fig. 1, or provide a revised figure without the asterisks.-10$^{+/+}$). Representative granzyme B expression FACS profiles of CD8 T cells in the recipients at 5 wk postcell transfer, with percentages for the gated granzyme B^{+} CD8 T cells. Average percentages with SDs of granzyme B^{+} CD8 T cells (n = 3/group) are shown. *$p < 0.05$ The experiment in (C) was repeated once. (**D**) RAG-1$^{-/-}$ mice received CD8 T cells from WT, PD-1$^{-/-}$, or IFN-α/βR$^{-/-}$ mice together with WT CD4 T cells and Tg B cells. Representative granzyme B versus IFN-γ FACS profiles of CD8 T cells in RAG-1$^{-/-}$ recipients, with granzyme B^{+} percentages. Average percentages with SDs of granzyme B^{+} CD8 T cells and fold changes of IL-10 mRNA level in CD8 T cells compared with that in control WT CD8 T cells are shown (n = 3/group). The experiment in (D) was repeated once.

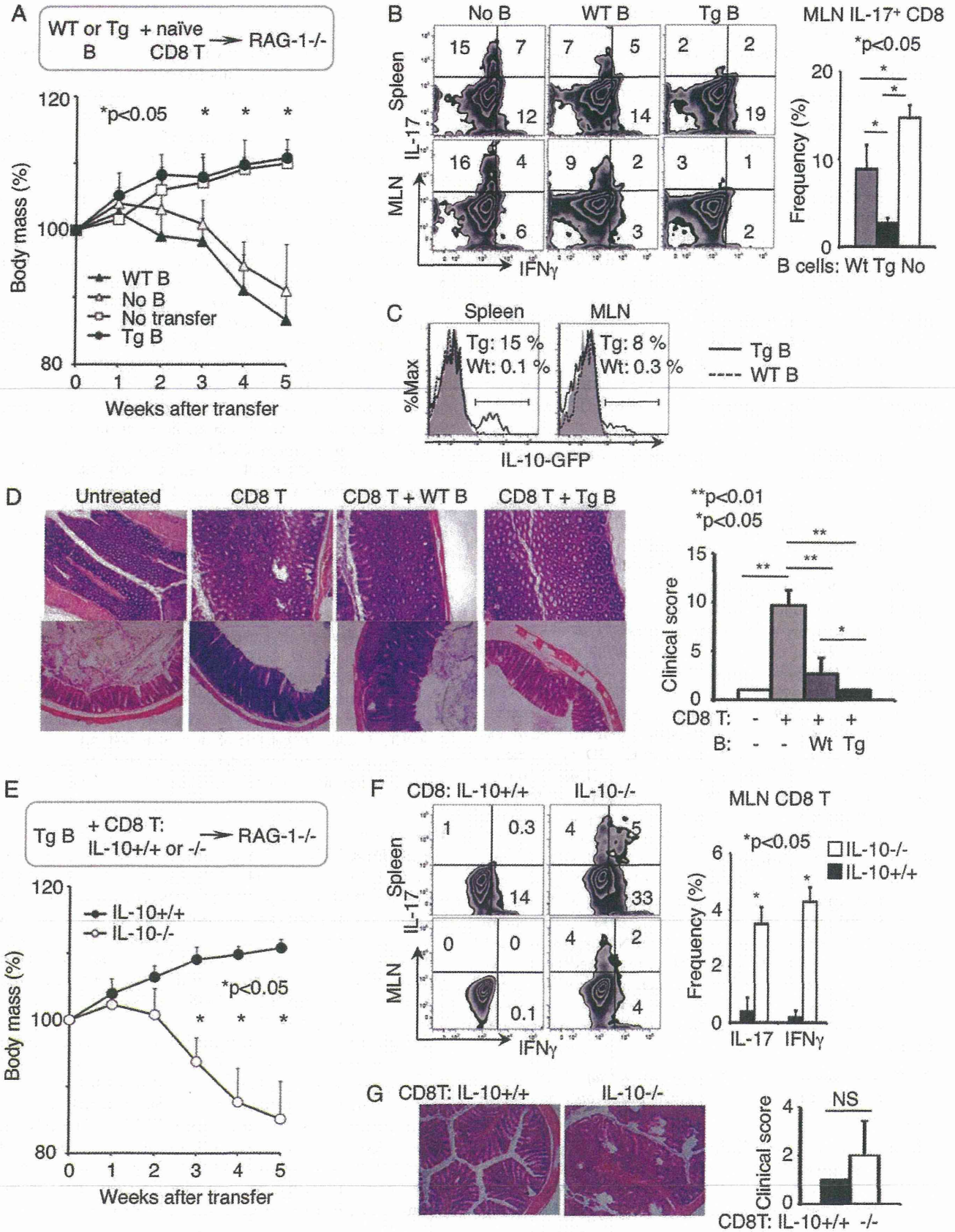

FIGURE 4. CD40LTg B cells regulate naive CD8 T cell–mediated inflammatory colitis by IL-10 induction. (**A–D**) RAG-$1^{-/-}$ mice received $CD62L^{+}CD44^{-}$CD8 T cells from IL-10-GFP reporter mice with B cells from wild-type or Tg mice ($n = 4$ each), without B cells ($n = 4$ each) or left untreated ($n = 3$ each). (**E–G**) RAG-$1^{-/-}$ mice received $CD62L^{+}CD44^{-}$CD8 T cells from IL-10-GFP reporter or IL-$10^{-/-}$ mice with Tg B cells ($n = 4$ each). (A and E) Average percentages with SDs of body mass changes (%) for each recipient group are shown. (B, F) Representative intracellular staining IL-17 versus IFN-γ FACS profiles for spleen or MLN CD8 T cells in the recipients with percentages for each quadrant. Average frequencies with (*Figure legend continues*)

3A). In addition, their CD4 and CD8 T cells had significantly higher frequencies of IL-10-GFP expression compared with those in the recipients of wild-type B cells (Fig. 3B). These results confirm that CD40LTg B cells stimulate CD4 and CD8 T cells during lymphopenic proliferation to express IL-10.

Autocrine IL-10 expression is essential for CD8 T cells to counterregulate their overactivation during lymphopenic proliferation

Using this adoptive transfer model, we further addressed the role of IL-10 expression in activated CD8 T cells by CD40LTg B cells. When RAG-$1^{-/-}$ mice were reconstituted with CD40LTg or wild-type B cells and different combinations of IL-10–deficient or sufficient CD4 and CD8 T cells, the frequency of $CD62L^{low}$ $CD44^{low}$ effector CD8 T cells (data not shown) and granzyme B^+ CD8 T cells significantly increased when CD8 T cells lacked IL-10, and this was not further increased when CD4 T cells were also IL-10 deficient (Fig. 3C). These results indicate that autonomous IL-10 expression in CD8 T cells is critical to counterregulate their overactivation and granzyme B expression when CD40LTg B cells stimulate CD8 T cells.

IFN-α/βR and PD-1 signaling is essential for effector memory CD8 T cells to express IL-10

Type I IFN signaling is critical for CD8 downregulation (26) and PD-1 up-regulation on CD8 T cells (27) upon TCR engagement. PD-1 engagement has been shown to induce IL-10 expression in T cells (28). As shown in Fig. 2C, $CD62L^{low}CD44^{high/int}$CD8 T cells in CD40LTg mice, enriched with IL-10-GFP expression, expressed elevated levels of PD-1. Therefore, we hypothesize that CD8 T cell IL-10 expression is triggered as a regulatory mechanism downstream of type I IFN and PD-1 pathway. To test the hypothesis, CD8 T cells from IFN-α/$βR^{-/-}$ or PD-$1^{-/-}$ mice were transferred into RAG-$1^{-/-}$ along with CD40LTg B cells. At 2 wk after transfer, CD8 T cells were recovered from the recipient mice, and their granzyme B expression and IL-10 mRNA expression level was examined. As shown in Fig. 3D, the frequency of granzyme B^+ CD8 T cells in the recipients of CD40LTg B cells significantly increased when CD8 T cells lacked IFN-α/βR or PD-1. Thus, signals from IFN-α/βR and PD-1 are important to block the activation of CD8 T cells. Furthermore, whereas IL-10 mRNA level in CD8 T cells increased by ~54,000-fold after transfer into RAG-$1^{-/-}$ recipients together with CD40LTg B cells, it increased only 4- and 170-fold when CD8 T cells lacked IFN-α/βR and PD-1, respectively. Thus, IFN-α/βR-deficient CD8 T cells almost completely failed to upregulate IL-10 mRNA expression upon stimulation with CD40LTg B cells. These results indicate that the signal from IFN-α/βR triggers IL-10 expression in CD8 T cells, and this IL-10 expression is further enhanced by PD-1 signal delivered by PD-1/PD-L1 and PD-L2 interaction during CD8 T cell activation.

CD40LTg B cells induce IL-10 expression and suppress intestinal inflammation caused by spontaneous proliferation of CD8 T cells

Adoptive transfer of naive $CD62L^+CD44^-$CD8 T cells into RAG-$1^{-/-}$ mice causes colitis in association with MHC/Ag-driven rapid spontaneous proliferation and differentiation of inflammatory effector CD8 T cells producing IL-17 and IFN-γ (29). Using this model, we tested whether cotransfer of CD40LTg B cells could suppress this CD8 T cell–mediated inflammatory response with induction of IL-10. As previously reported, RAG-$1^{-/-}$ recipients of naive CD8 T cells significantly lost body weight (Fig. 4A) in association with the differentiation of IL-17^+ and IFN-γ^+ effector CD8 T cells in their mesenteric lymph nodes (MLNs) and spleen (Fig. 4B). As shown in Fig. 4A, cotransfer of CD40LTg, but not wild-type, B cells could suppress inflammation and maintained the body weight of recipient RAG-$1^{-/-}$ mice during the period studied. Also, the frequency of IL-17^+, but not IFN-γ^+, CD8 T cells was significantly reduced in the spleen (data not shown) and MLNs (Fig. 4B) of the recipients of CD40LTg B cells compared with recipients of wild-type B cells or without B cell transfer. As expected, CD8 T cells of the recipients of CD40LTg B cells had a significantly higher frequency of IL-10-GFP expression compared with those in the recipients of wild-type B cells (Fig. 4C). By histology, colon pathology was significantly improved in the recipients of CD8 T cells with B cell transfer compared with the recipients of CD8 T cells alone, and this was even more significant in the recipients with CD40LTg B cell cotransfer, compared with wild-type B cell cotransfer, in association with reduced lymphocyte infiltration in the colon (Fig. 4D).

To test whether CD8 T cell IL-10 expression is essential for the regulation of inflammatory response by CD40LTg B cells, IL-$10^{+/+}$ or IL-$10^{-/-}$ CD8 T cells were transferred into RAG-$1^{-/-}$ mice along with CD40LTg B cells. As shown in Fig. 4E, the therapeutic effect of CD40LTg B cells was largely dependent on IL-10 expression by CD8 T cells because the recipients of IL-$10^{-/-}$ CD8 T cells with CD40LTg B cell cotransfer exhibited systemic inflammation, as evidenced by significant weight loss, along with significantly elevated frequency of IL-17^+ and IFN-γ^+ CD8 T cells in their spleens and MLNs (Fig. 4F). The recipients with IL-$10^{-/-}$ CD8 T cells also exhibited enhanced lymphocyte infiltration into the colon tissues, although the clinical scoring of this effect was not statistically significant (Fig. 4G). These results demonstrate that CD40LTg B cells are capable of suppressing systemic as well as intestinal inflammation in spontaneously proliferating CD8 T cells under lymphopenic conditions by inducing IL-10 expression.

Discussion

Using CD40LTg B cells as a model for B cells in human inflammatory diseases that abnormally express CD40L or constitutively receive CD40L, this study presents the possibility that such B cells induce suppressive mechanisms in memory-like T cells with IL-10 expression. Furthermore, this study demonstrates in a mouse model that adoptive transfer of CD40LTg B cells can regulate inflammatory CD8 T cell response under lymphopenia, indicating a potential therapeutic use of CD40L-expressing B cells in CD8 T cell–mediated inflammatory diseases.

Lymphopenia, a condition characterized by reduced numbers of lymphocytes, is a critical cofactor of autoimmunity (30–32). Under lymphopenic conditions, residual low numbers of CD8 T cells proliferate in response to cytokines and self- and commensal bacterial Ags presented by DCs (25, 29), and quickly form memory-like cells. Such memory-like cells are functionally indistinguishable from adaptive memory T cells to provide immediate protection (33), but may cause tissue damage. In the absence or failure of regulatory mechanisms, self- and microbial reactive

SDs of IL-17^+ (B, F) or IL-17^+ and IFN-γ^+ (F) CD8 T cells in the MLN are shown ($n = 4$/group). (C) Representative histograms for IL-10-GFP expression of spleen and MLN CD8 T cells in the recipients of wild-type (WT; dash) or Tg (solid) B cells, with percentages of IL-10-GFP^+ cells. (D, G) Representative H&E staining of colon histology are shown (×100). Average clinical scores with SDs are shown to the *right*. Results from one of two similar experiments are shown. $*p < 0.05$, $**p < 0.01$.

T cells are abnormally activated and differentiated into inflammatory IL-17 and IFN-γ effector cells to cause autoimmune diseases (31, 32). Lymphopenia-induced proliferation of autoreactive CD8 T cells closely correlates with the onset of diabetes in NOD mice (34). Furthermore, memory-like CD8 and CD4 T cells cooperate to break peripheral tolerance under lymphopenic conditions in an autoimmune diabetes model (35). These studies collectively support the notion that regulating effector differentiation of CD8 T cells during lymphopenic proliferation is a critical therapeutic target for autoimmune diseases.

In the autoimmune NOD mouse diabetes model, a nanoparticle vaccine coated with peptide–MHC complex could prevent and cure diabetes by selective expansion of low-affinity memory-like autoregulatory CD8 T cells, in an epitope-specific manner, to blunt the activation and recruitment of CD8 T cells with other specificities to the islets (36). In this context, CD40LTg B cells induced the expansion of effector memory-like CD8 T cells under lymphopenia (Fig. 3A) and substantially reduced recruitment of CD8 T cells to the colon compared with wild-type B cells (Fig. 4D). It is interesting to speculate that CD40L-expressing B cells have a capacity similar to the nanoparticle vaccine and may be used for an adoptive cell transfer therapy to treat CD8 T cell–mediated inflammatory autoimmune diseases.

The current study identified CD40LTg B cells to be potent APCs for CD8 T cells, which can be used for a therapeutic target. In fact, accumulating evidence indicates a potential physical interaction between memory-like CD8 T cells and B cells under physiological settings. In a human/SCID rheumatoid arthritis model, IFN-γ^+ CD40L$^+$CD8 T cells in the mantle zone were required for the maintenance of ectopic germinal centers (37). In tonsils, CXCR5$^+$ CD44^{high} memory-like CD8 T cells found in the mantle zone supported B cell Ab production (23). In a viral infection model, long-lived memory CD8 T cells were mainly found in B cell follicles (38). More recently, it was shown that MZ B cell numbers are determined by perforin-mediated CD8 T cell cytotoxicity (39). Further studies are needed to examine the role and significance of interactions between CD40-activated B cells and memory-like CD8 T cells in immune response.

In line with the mechanisms of CD8 T cell activation by B cells, previous studies showed that anti-CD40–activated B cells modulated T cell response via overproduction of the cytokines IL-6, IL-10, and IL-15 (4, 40, 41). Also, injection of agonistic anti-CD40 Abs into mice induced bystander proliferation of memory-like CD8 T cells in a manner dependent on CD40 expression on APCs (i.e., B cells and DCs) and IL-15 (42). In our model, naive CD40LTg B cells expressed relatively higher levels of IL-6, IL-10, and IL-15 mRNAs (2.5-, 2.0-, and 2.0-fold, respectively) compared with wild-type B cells. Also, CD21^{high} CD23^{low} MZ B cells from CD40LTg mice produced greater amounts of IL-6 and IL-10 in response to various stimuli compared with those from control mice (P.A. Koni, A. Bolduc, M. Takezaki, Y. Ametani, Kamanaka, R.A. Flavell, A.L. Mellor, T. Tsubata, and M. Shimoda, manuscript in preparation). Thus, overproduction of certain cytokines could also be a mechanism of augmented CD8 T cell response in CD40LTg mice. Another potential mechanism for memory CD8 T cell activation by CD40LTg B cells could be through CD40 expressed on memory CD8 T cells (43), although this was not the case in bystander proliferation of memory-like CD8 T cells induced by agonistic anti-CD40 Abs (42). Further studies are needed to understand the molecular mechanisms of CD8 T cell activation by CD40L-expressing B cells for therapeutic use.

IL-10 expression has been found in virus-specific exhausted CD8 T cells (44, 45) under chronic infection and was implicated as a regulatory mechanism to prevent overactivation. In this context, the current study demonstrates that the absence of IL-10 expression in CD8 T cells increased granzyme B expression (Fig. 3C), inflammatory cytokine production (Fig. 4E), and lymphocyte infiltration in the colon (Fig. 4F). Thus, autonomous IL-10 expression is a critical safeguard mechanism in activated memory CD8 T cells to counterregulate overactivation and block inflammatory response. In a viral infection model, IL-10 production by cytotoxic T cells was amplified by IL-2 derived from CD4$^+$ Th cells with innate-derived IL-27 through a Blimp-1–dependent mechanism (46). Like in this chronic viral infection model, Blimp-1 and PD-1 expression coincided with IL-10 reporter expression in CD8 T_{EM} cells in our model (Fig. 2C). Furthermore, based on the data presented in Fig. 3D, we propose IL-10 expression in CD8 T cells to proceed at least in two steps. First, the IL-10 expression is induced under IFN-I signaling as a part of IFN-I–mediated regulatory mechanisms such as downregulation of CD8α to desensitize TCR signaling (26). Next, the IL-10 expression is dramatically upregulated through PD-1 engagement. In CD8 T cells, IFN-I induces IFN regulatory factor 9 that directly enhances PD-1 expression at the transcriptional level (27). PD-1 and the PD-L1/PD-L2 interaction critically regulates self-reactive CD8 T cell activation in various autoimmune disease models (47) via induction of CD8 T cell anergy (unresponsive) by limiting their autonomous IL-2 production (48) as well as by causing exhaustion in fully differentiated effector CD8 T cells to block cytotoxicity in association with upregulation of various negative regulators (49, 50). In this context, we show that IL-10 expression also is one of the critical regulatory mechanisms mediated by PD-1 engagement.

Finally, our finding may be related to regulatory B cell functions, which are induced by anti-CD40 activation (51–53). Regulatory functions of B cells have been mainly associated with their own overproduction of IL-10 (41, 52, 54, 55). In addition, the current study suggests that the regulatory action of anti-CD40–activated B cells may, in fact, involve induction of IL-10 in the memory-like CD4 and CD8 T cell compartment. Whereas such a mechanism is certainly important to prevent excessive tissue damage in infections (45), colitis, and other autoimmune diseases, it might also play an adverse role in chronic infections and malignancies by creating an immune-suppressive environment that impairs overall T cell function. Thus, our findings may be helpful to treat patients with chronic inflammatory diseases.

Acknowledgments

We thank Dr. David Gray for critical comments; Domenica Powell, Denise Gamble, Taejin Lee, and Danielle O'Connell for technical assistance; Dr. Dimitris Moskophidis for FACS Canto; and Dr. Shuhua Han for Blimp-1 GFP mice. We also thank Drs. D. Moskophidis and T. Honjo for IFN-α/βR$^{-/-}$ and PD-1$^{-/-}$ mice, respectively.

Disclosures

The authors have no financial conflicts of interest.

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Cd72^c Is a Modifier Gene that Regulates *Fas*^{lpr}-Induced Autoimmune Disease

Miduo Xu,*,† Rong Hou,*,† Aya Sato-Hayashizaki,*,† Rongyong Man,*,† Chenghua Zhu,*,† Chisato Wakabayashi,*,† Sachiko Hirose,‡ Takahiro Adachi,*,† and Takeshi Tsubata*,†

Although modifier genes are extensively studied in various diseases, little is known about modifier genes that regulate autoimmune diseases. Autoimmune disease caused by the *Fas*^{lpr} mutation depends on the genetic background of mouse strains, suggesting a crucial role of modifier genes. MRL/MpJ-*Fas*^{lpr} (MRL/lpr) and AKR/lpr mice develop severe and mild lupus-like autoimmune disease, respectively, whereas this mutation does not cause disease on C57BL/6 (B6) or C3H background. Both MRL and AKR carry the same haplotype of the *Cd72* gene encoding an inhibitory BCR coreceptor (CD72^c), and CD72^c contains several amino acid substitutions and a deletion in the extracellular region compared with CD72^a and CD72^b. To address the role of *Cd72*^{c} locus in the regulation of *Fas*^{lpr}-induced autoimmune disease, we generated B6.CD72^c/lpr and MRL.CD72^b/lpr congenic mice. Introduction of the chromosomal interval containing *Cd72*^{c} did not cause disease in B6 mice by itself, but caused development of lupus-like disease in the presence of *Fas*^{lpr} on B6 background, clearly demonstrating that this interval contains the modifier gene that regulates *Fas*^{lpr}-induced autoimmune disease. Conversely, MRL.CD72^b/lpr congenic mice showed milder disease compared with MRL/lpr mice. We further demonstrated that *Cd72*^{c} is a hypofunctional allele in BCR signal inhibition and that CD72 deficiency induces severe autoimmune disease in the presence of *Fas*^{lpr}. These results strongly suggest that the *Cd72*^{c} is a crucial modifier gene that regulates *Fas*^{lpr}-induced autoimmune disease due to its reduced activity of B cell signal regulation. *The Journal of Immunology*, **2013, 190: 5436–5445.**

Modifier genes have been extensively studied in various diseases such as cancer, arrythmia, and cystic fibrosis, because penetrance and disease manifestations of the disease caused by disease-causing genes are extensively modified by modifier genes (1–3). In cystic fibrosis, contribution of modifier genes to the disease variability is almost equivalent to that of environmental factors. Mutation of the *Fas* gene causes autoimmune disease in both mice and human (4–7). Penetrance, severity, and manifestations of the disease induced by *Fas*^{lpr} mutation, a loss-of-function mutation of *Fas*, depend on the genetic background of mouse strains. MRL/MpJ-*Fas*^{lpr} (MRL/lpr) and AKR/lpr mice develop severe and mild lupus-like autoimmune disease, whereas *Fas*^{lpr} does not induce autoimmune disease in C57BL/6 and C3H mice (8, 9). Moreover, Fas-deficient BALB/c mice were recently shown to develop allergic inflammation (10). Thus, the disease caused by *Fas*^{lpr} or Fas deficiency is strongly regulated by modifier genes.

CD72 is a 45-kDa type II membrane protein expressed in B cells. CD72 contains a C-type lectin-like domain in the extracellular region and an immunoreceptor tyrosine-based inhibition motif (ITIM) in the cytoplasmic region (11–13). CD72 negatively regulates BCR signaling by recruiting SH2-containing tyrosine phosphatase-1 at the ITIM (12–16). In mice, four allelic forms of CD72 (i.e., CD72^a, CD72^b, CD72^c, and CD72^d) were serologically defined (17). CD72^a, CD72^b, and CD72^d are highly homologous (18, 19). In contrast, the extracellular region of CD72^c has a marked difference from the other alleles including a 7-aa deletion in the C-type lectin-like domain, although the amino acid sequence of the transmembrane and cytoplasmic regions of CD72^c is identical to that of the other alleles (18, 19). Interestingly, MRL and AKR, both of which develop autoimmune disease in the presence of *Fas*^{lpr}, carry CD72^c, whereas most of the other strains of mice, including BALB/c and C57BL/6 (B6), carry either CD72^a or CD72^b (18, 19). Moreover, studies using microsatellite markers revealed association of the loci containing *Cd72* to development of glomerulonephritis in MRL/lpr mice (20–22). Thus, *Cd72*^{c} is a candidate for a modifier gene that regulates *Fas*^{lpr}-induced autoimmune disease.

In this study, we addressed the role of the *Cd72*^{c} locus in the development of autoimmune disease by generating B6.CD72^c and MRL.CD72^b/lpr congenic mice. B cells from B6.CD72^c congenic mice showed augmented BCR signaling compared with B6 B cells, and B6.CD72^c/lpr developed severe autoimmune disease, whereas B6.CD72^c mice showed no disease. Conversely, MRL.CD72^b/lpr mice showed less severe autoimmune disease compared with MRL/lpr mice. These results suggest that *Cd72*^{c} is a functionally defective allele, and the *Cd72*^{c} locus does not cause any disease by itself but plays a role in development of severe autoimmune disease in MRL/lpr mice probably by augmenting BCR signaling. We further demonstrate that CD72 deficiency causes severe autoimmune disease in the presence of *Fas*^{lpr} by generating CD72-deficient mice. Thus, *Cd72*^{c} is a modifier gene that plays a crucial role in development of *Fas*^{lpr}-induced autoimmune disease probably through its defective regulatory function on BCR signaling.

*Laboratory of Immunology, Graduate School of Biomedical Sciences, Tokyo Medical and Dental University, Tokyo 113-8510, Japan; †Department of Immunology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan; and ‡Department of Pathology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

Received for publication January 2, 2013. Accepted for publication March 27, 2013.

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Japan Society for the Promotion of Science.

Address correspondence and reprint requests to Prof. Takeshi Tsubata, Department of Immunology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. E-mail address: tsubata.imm@mri.tmd.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this article: ALPS, autoimmune lymphoproliferative syndrome; B6, C57BL/6; BAC, bacterial artificial chromosome; BM, bone marrow; ES, embryonic stem; ITIM, immunoreceptor tyrosine-based inhibition motif; LN, lymph node; MRL/lpr, MRL/MpJ-*Fas*^{lpr}; NP, 4-hydroxy-3-nitrophenyl acetyl; PASH, periodic acid–Schiff and hematoxylin; PEC, peritoneal exudate cells.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1203576