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Dermatitis due to epiregulin deficiency and a critical role of epiregulin in immune-related responses of keratinocyte and macrophage

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Edited by Tadamitsu Kishimoto, Osaka University, Osaka, Japan, and approved August 15, 2004 (received for review June 14, 2004)

Epidermal growth factor (EGF) family members, including epiregulin (EP), play a fundamental role in epithelial tissues; however, their roles in immune responses and the physiological role of EP remain to be elucidated. The skin has a versatile system of immune surveillance. Biologically active IL-1α is released to extracellular space upon damage from keratinocytes and is a major player in skin inflammation. Here, we show that *EP* is expressed not only in keratinocytes but also in tissue-resident macrophages, and that *EP*-deficient (*EP*$^{-/-}$) mice develop chronic dermatitis. Wound healing in the skin in *EP*$^{-/-}$ mice was not impaired *in vivo*, nor was the growth rate of keratinocytes from *EP*$^{-/-}$ mice different from that of WT mice *in vitro*. Of interest is that in WT keratinocytes, both IL-1α and the secreted form of EP induced down-regulation of *IL-18* mRNA expression, which overexpression in the epidermis was reported to induce skin inflammation in mice, whereas the down-regulation of *IL-18* induced by IL-1α was impaired in *EP*$^{-/-}$ keratinocytes. Although bone marrow transfer experiments indicated that EP deficiency in non-bone-marrow-derived cells is essential for the development of dermatitis, production of proinflammatory cytokines by *EP*$^{-/-}$ macrophages in response to Toll-like receptor agonists was much lower, compared with WT macrophages, whose dysfunction in *EP*$^{-/-}$ macrophages was not compensated by the addition of the secreted form of EP. These findings, taken together, suggested that EP plays a critical role in immune/inflammatory-related responses of keratinocytes and macrophages at the barrier from the outside milieu and that the secreted and membrane-bound forms of EP have distinct functions.

The system of epidermal growth factor (EGF) superfamily (1) and EGF receptor (EGFR) family, including EGFR (ErbB1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4) (2), play a fundamental role in epithelial tissues. EGF family members, including EGF, transforming growth factor-α, amphiregulin, heparin-binding EGF (HB-EGF), epiregulin (EP), and other members, regulate these receptors by inducing their homo- and/or hetero-oligomerization (2, 3). EGF family members vary in their ability to activate distinct ErbB heterodimers, and this mechanism may, in part, account for the differences in their bioactivities (4–7).

The membrane-anchored precursor of the EGF family is enzymatically processed externally to release a mature soluble form that acts as autocrine and/or paracrine growth factor (8, 9), whereas some members of the EGF family act in the membrane-anchored form (8, 10). A bioactive transmembrane precursor, pro-HB-EGF, was suggested to induce growth inhibition or apoptosis rather than the proliferative response induced by soluble HB-EGF (10). EP (11–17) acts as an autocrine growth factor in normal human keratinocytes *in vitro* (15); however, its physiological role *in vivo* still remains to be elucidated.

Keratinocytes in the epidermis play critical roles in the cutaneous immune-related responses (18) and contain biologically active IL-1α (19), which is released to extracellular space upon damage and is a major player in skin inflammation (20). Overexpression of IL-1α in murine epidermis produces inflammatory skin lesions, indicating the critical role of IL-1α in skin inflammation (21). On the other hand, IL-18 is stored as a biologically inactive precursor form in keratinocytes, and overexpression of IL-18 in the mouse skin was reported to induce inflammatory skin lesions without inducing allergen-specific IgE production (22).

Macrophages normally reside in tissues and beneath mucosal surfaces and function at the front line of immune defense against incoming pathogens through ingestion of bacteria by phagocytosis, destruction of bacteria, and recruitment of inflammatory cells to the site of infection by using soluble mediators (23). Toll-like receptors (TLRs) (24, 25), which recognize molecular patterns that are common and shared by many microbial pathogens, are expressed on antigen-presenting cells (APCs), including macrophages and dendritic cells: TLR4 to lipopolysaccharide (LPS), TLR2 to peptidoglycan (PGN), and TLR9 to CpG DNA.

Here, we show the critical role of EP in immune/inflammatory-related responses of keratinocytes and macrophages through the establishment and analysis of *EP*$^{-/-}$ mice.

Methods

Generation of *EP*-Deficient Mice. We isolated the genomic DNA of the *EP* gene from a 129/Sv mouse genomic library (Stratagene) and constructed the targeting vector by replacing a 6.5-kb *SalI-XhoI* fragment containing exons 2–5 (90% of the coding region) of the *EP* gene, with a pGKneo cassette with opposite transcriptional orientation. The 5′ and 3′ arm of the targeting construct was composed of 2.0-kb and 11.0-kb genomic DNA, respectively. Diphtheria-toxin A fragment cassette (DT-A) flanked the 3′ genomic arm. The targeting vector was linearized with *SalI*. The mutant embryonic stem cells were microinjected into C57BL/6 blastocysts as described (26), and the resulting male chimeras were mated with C57BL/6 mice. Heterozygous offspring were intercrossed to obtain *EP*-deficient mice.

Histopathology. Tissues were fixed in 4% paraformaldehyde, embedded on paraffin, and stained with hematoxylin/eosin. Mast cells and eosinophils were detected by toluidine blue and Luna staining, respectively.

Quantification of Serum Ig Isotype Concentration. Blood samples were obtained from mouse eye vein. Serum Ig isotype concentration was determined by using an ELISA kit (Bethyl Laboratories, Montgomery, TX).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; HB-EGF, heparin-binding EGF; EP, epiregulin; rmEP, recombinant mouse EP; sEP, secreted form of EP; TLR, Toll-like receptor; BMDM, bone marrow-derived macrophage; LPS, lipopolysaccharide; PGN, peptidoglycan; TNF, tumor necrosis factor; APC, antigen-presenting cell.

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Preparation of Mice Macrophages and Keratinocytes. Peritoneal macrophages were isolated as described (27). Bone marrow-derived macrophages were generated as described (28). Primary epidermal keratinocytes were prepared from neonatal foreskins as described (29), then cultured on plates coated with 30 $\mu g \cdot ml^{-1}$ type I collagen (TOYOBO, Tokyo) in Keratinocytes-SFM (GIBCO/BRL) with 50 $ng \cdot ml^{-1}$ bovine pituitary extract (BPE), 10 $ng \cdot ml^{-1}$ recombinant murine EGF (Sigma), and 0.03 mM $CaCl_2$ (9). Keratinocytes (2×10^5 per well in a 24-well plate) were cultured without BPE and EGF for 24 h, then stimulated with recombinant mouse EP (rmEP) (R & D Systems) or recombinant mouse IL-1α (PeproTech), and mRNA expressions were determined by RT-PCR.

Quantification of Cytokine Concentration of the Culture Supernatants. Peritoneal macrophages (5×10^5 per well) or bone marrow-derived macrophages (5×10^5 per well) were cultured in 12-well plates for 24 h, then stimulated with rmEP, LPS, PGN, or CpG [LPS from *Salmonella typhimurium* (Sigma); PGN from *Staphylococcus aureus* (Fluka); CpG oligodeoxynucleotide (5′-TCCATGACGTTCCTGATGCT-3′) (Sigma)]. The culture supernatants were subjected to IL-6, IL-12p40, and tumor necrosis factor (TNF)-α by ELISA kit (BioSource International).

Isolation of RNA and RT-PCR. Epidermis was isolated from the ear. T, B, $CD11b^+$, and $CD11c^+$ cells were isolated by magnetic cell sorting (MACS) (Miltenyi Biotec, Auburn, CA). Total RNA from the tissues and sorted cells were isolated by ISOGEN (Nippon Gene, Toyama, Japan), and total RNA from primary epidermal keratinocytes and bone marrow-derived macrophage (BMDM) were isolated by using the RNeasy Mini kit (Qiagen, Valencia, CA). First-strand cDNA were synthesized from 1 μg of total RNA by Omniscript Reverse Transcriptase (Qiagen), and PCR was done by using LAtaq polymerase (Takara Shuzo, Kyoto) and gene-specific primers: for *EP*, 5′-TTGGGTCTTGACGCTGCTTTGT-3′ and 5′-TGAGGTCACTCTCATATTC-3′; for *IL-18*, 5′-ATGGCTGCCATGTCAGAAGACTCT-3′ and 5′-ACTCCATCTTGTTGTGTCCTGGAAC-3′; for *TLR2*, 5′-TCTCCTGTTGATCTTGCTCGTAGG-3′ and 5′-TTACCCAAAACACTTCCTGCTGGC-3′; for *TLR4* 5′-TCAGTCTTCTAACTTCCCTCCTGC-3′ and 5′-AGCTGTCCAATAGGGAAGCTTTCT-3′; for *NOD1*, 5′-CAGATAACTGATATCGGAGCCAGG-3′ and 5′-TTTGGCCTCCTCGGGCTTAATCAA-3′; for *NOD2*, 5′-AAGCAGAACTTCTTGTCCCTGAGG-3′ and 5′-TCACAACAAGAGTCTGGCGTCCCT-3′; for *MD2*, 5′-ATGTTGCCATTTATTCTCTTTTCGACG-3′ and 5′-ATTGACATCACGGCGGTGAATGATG-3′; *GAPDH* primers were from Clontech. The optimal cycle number for each gene was determined empirically under nonsaturating conditions.

Measurement of T Cell Responses and Growth Rate of Keratinocytes. $CD4^+$ T cells were isolated from spleens of mice immunized with 100 μg of ovalbumin (OVA) emulsified with complete Freund's adjuvant after 14 days. $CD4^+$ T cells (4×10^5 cells) were incubated with irradiated peritoneal macrophages (5×10^5 cells) in 96-well plates at the indicated concentration of OVA in RPMI medium 1640 containing 10% FCS for 72 h. They were pulsed with 1 μCi (1 Ci = 37 GBq) [^{3}H]thymidine for the final 12 h, and radioactivity was measured by liquid scintillation counting (30). For proliferation assay, primary epidermal keratinocytes (4×10^4 cells) were plated on a type I collagen-precoated 96-well plate and cultured in the presence of 10 $ng \cdot ml^{-1}$ EGF, then pulsed with 0.5 μCi of [^{3}H]thymidine for the final 8 h, and radioactivity was measured by using liquid scintillation counting (29).

Phagocytosis Assay. Mouse peritoneal macrophages (5×10^6 ml^{-1}) were incubated for 60 min at 37°C in the presence of 0.75 μM fluorescent beads (Polysciences). These cells were washed with ice-cold PBS twice and fixed with 2.5% formaldehyde in PBS for 20 min on ice. Phagocytosis was measured by the percentage of phagocytic cells per 100 cells by fluorescent microscopy, as described (31).

Cell Staining and Flow Cytometry. Single-cell suspensions were incubated at 2×10^5 cells per 100 μl on ice in staining buffer (PBS containing 2.5% FCS and 0.01% NaN_3) with the mAbs for 15 min (28). The mAbs used were anti-I-A^b, anti-CD80, anti-CD86, anti-CD40, and anti-CD11b (Pharmingen). Flow cytometry analysis was performed on a FACScan cytometer (Becton Dickinson).

Bone Marrow Transplantation. Bone marrow transplantation experiments were done as described (32). In brief, bone marrow cells were isolated from $EP^{-/-}$ and WT mice, and recipient mice were irradiated (11 Gy) 12 h before injection (i.v.) of 2×10^7 donor bone marrow cells.

Results

***EP* Expression in Epidermis and Tissue-Resident APCs.** Because human *EP* mRNA is expressed predominantly in peripheral blood lymphocytes (PBL) (12), mouse *EP* expression in immune-related tissues was examined by RT-PCR. Mouse *EP* expression was detected not only in the epidermis (15) but also in peritoneal macrophages, and in macrophages and $CD11c^+$ dendritic cells in Peyer's patches (Fig. 1*A*). On the other hand, *EP* was rarely detected in the immune system, including bone marrow, thymus, lymph nodes, and spleen (Fig. 1*A*). Furthermore, mouse *EP* expression in PBL was much lower, compared with that in tissue-resident APCs (Fig. 1*A*). These results suggested that EP may be involved in the functions of keratinocytes in the epidermis and tissue-resident APCs.

***EP* Deficiency Results in Dermatitis in Specific Pathogen-Free (SPF) Conditions.** To elucidate the function of EP *in vivo*, *EP*-deficient mice were generated, in which 90% of the coding region was deleted (Fig. 1 *B* and *C*). $EP^{-/-}$ mice were born from heterozygous matings and seemed to be indistinguishable from WT mice. However, $EP^{-/-}$ mice had chronic dermatitis at age 5 months at the earliest, even under SPF conditions. The rate of dermatitis observed in $EP^{-/-}$ mice was ≈20%, 50%, and 75% at the age of 9 months, 12 months, and 15 months, respectively, in the mixed genetic background of 129SV/J and C57BL6/J (Fig. 1*D*), suggesting that a genetic background and/or environmental factors affect the development of dermatitis in $EP^{-/-}$ mice.

Pathology of the Skin Legions in $EP^{-/-}$ Mice. Most of the skin lesions started as focal skin alterations in the ears or faces and gradually extended to the necks (Fig. 2*A*). The pathological findings of the skin legions in $EP^{-/-}$ mice were typical chronic dermatitis characterized by thickness of epithelial layers and fibrosis with infiltration of inflammatory cells (Fig. 2*B*), including mast cells (Fig. 2*C*) and eosinophils (Fig. 2*D*).

Serum Ig and Cytokine Concentrations in $EP^{-/-}$ Mice. Serum Ig isotype concentrations, including IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, and IgE, in $EP^{-/-}$ mice were not different from WT mice (Fig. 1*E*). Furthermore, the serum concentrations of cytokines, including IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, IL-18, TNF-α, and IFN-γ did not differ between WT and $EP^{-/-}$ mice without dermatitis (data not shown). All these results suggested that dermatitis in $EP^{-/-}$ mice is not IgE-dependent and systemic immunological disturbance might not exist in $EP^{-/-}$ mice.

Wound Healing Was Not Impaired in $EP^{-/-}$ Mice. Although EP was reported to be involved in tissue repairing and growth of keratinocytes, wound healing in $EP^{-/-}$ mice was not impaired *in vivo* (Fig. 3*A*), nor was the growth rate of keratinocytes from $EP^{-/-}$ mice different from that of WT mice *in vitro* (Fig. 3*B*). These findings

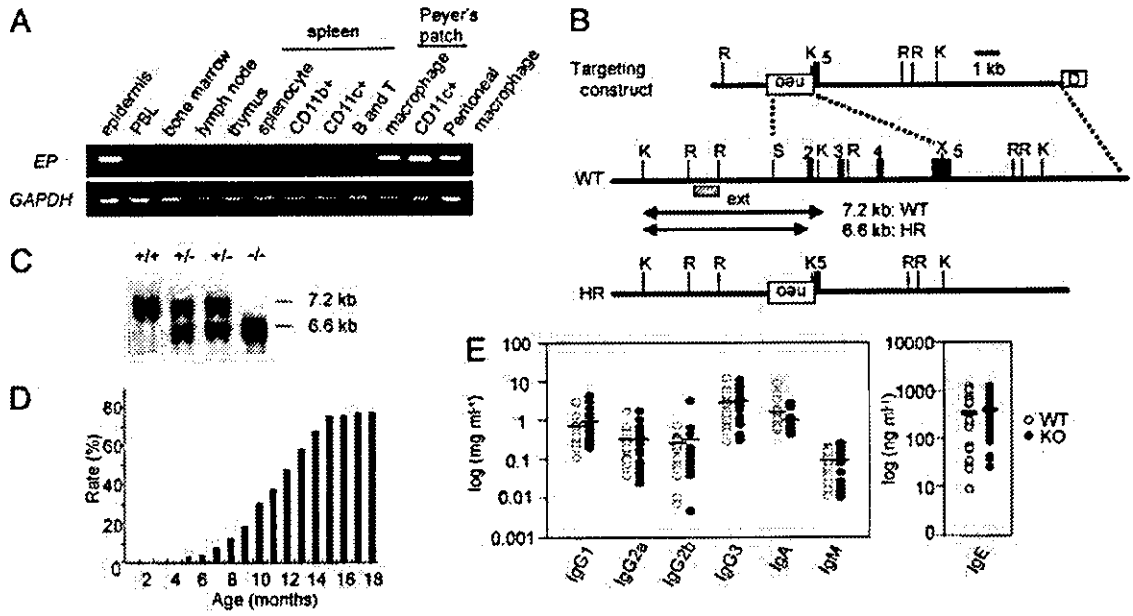

Fig. 1. *EP* expression in mouse immune-related tissues and generation of *EP*-deficient mice. (*A*) *EP* expression level was determined by using RT-PCR with *GAPDH* as control. PBL, peripheral blood lymphocytes. (*B*) Schematic representations of targeting construct, WT *EP* genomic DNA, and homologous recombinant (HR) allele. The numbers denote exons (filled boxes). External probe (ext) used in Southern blot to detect HR events is indicated by a dashed box. The germ-line and recombinant *KpnI* restriction fragments are denoted by lines with arrowheads. Restriction sites: K, *KpnI*; R, *EcoRI*; S, *SalI*; X, *XhoI*; neo, pGK-neo; D, diphtheria toxin A fragment. (*C*) Southern blot of *KpnI*-digested genomic DNA with the external probe. (*D*) The rate of chronic dermatitis observed in $EP^{-/-}$ mice. (*E*) Serum Ig isotype concentration. Serum Ig isotype concentration was determined by ELISA. WT mice (open circles; $n = 22$) and $EP^{-/-}$ mice without dermatitis (filled circles; $n = 20$) at the age of 4 months. Mean values are indicated by short horizontal lines.

taken, together, suggested that EP will not play a crucial role in the growth of keratinocytes *in vivo*, which will be possibly due to the functional redundancy of the EGF family.

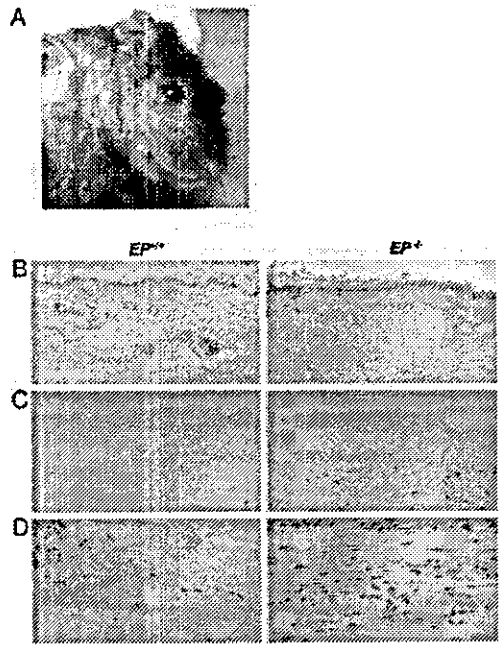

Fig. 2. Pathology of the skin legions in *EP*-deficient mice. (*A*) Chronic dermatitis around the face of $EP^{-/-}$ mice (8 months of age). (*B–D*) Chronic dermatitis in skin in $EP^{-/-}$ mice. Sections of skin were subjected to staining with hematoxylin/eosin (*B*), toluidine blue staining for mast cells (*C*), and Luna staining for eosinophils (*D*).

Induction of *EP* mRNA Expression and Down-Regulation of *IL-18* mRNA Expression in Keratinocytes by IL-1α. To elucidate the molecular mechanisms by which *EP* deficiency leads to dermatitis, we next analyzed the inflammatory responses of primary keratinocytes stimulated with IL-1α (21). IL-1α induced *EP* mRNA expression at 1 h after stimulation, and it was recovered to the basal level at 8 h after stimulation (Fig. 3*C*). Of note is that IL-1α evidently reduced the expression of *IL-18* mRNA at 4 h after stimulation in WT-derived keratinocytes and the reduced *IL-18* expression was still observed at 24 h after stimulation, whereas the decreased *IL-18* expression in $EP^{-/-}$ keratinocytes was not prominent, compared with the finding in WT keratinocytes (Fig. 3*C*). These results, together, suggested that IL-1α-induced down-regulation of *IL-18* expression is impaired in keratinocytes of $EP^{-/-}$ mice.

Down-Regulation of *IL-18* mRNA Expression Induced by the Secreted Form of EP. To elucidate whether the secreted form of EP (sEP) affects the down-regulation of *IL-18* expression, keratinocytes were stimulated with rmEP. rmEP reduced *IL-18* expression in WT- and $EP^{-/-}$-derived keratinocytes at 8 h after stimulation, and there was no obvious difference between WT and $EP^{-/-}$ (Fig. 3*D*). The expressions were still reduced at 24 h after stimulation in both keratinocytes, although their expressions were gradually increased at 24 h (Fig. 3*D*). These results, together, suggested that IL-1α-induced down-regulation of *IL-18* will be, in part, dependent on sEP. Furthermore, preexposure of keratinocytes with an EGFR kinase blocker (PD168393) (33) prevented rmEP-induced down-regulation of *IL-18* expression, whereas a platelet-derived growth factor receptor (PDGFR) blocker (AG1296) as a control did not do so (Fig. 3*E*). These findings, together, suggested that sEP down-regulates the *IL-18* expression through EGFR in keratinocytes and that loss of EP leads to the disturbance of tight regulation of *IL-18* expression in keratinocytes, which will, in part, cause the development of dermatitis in $EP^{-/-}$ mice.

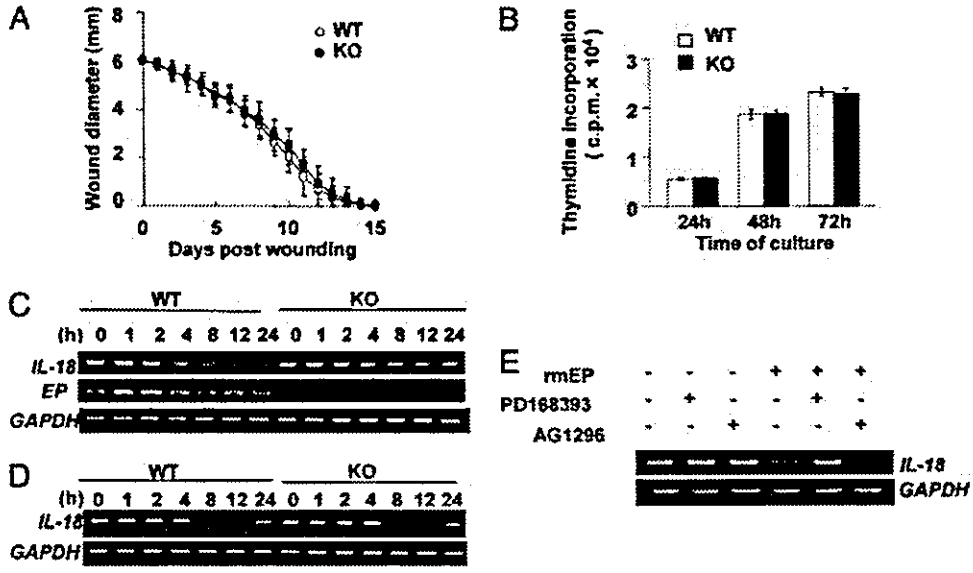

Fig. 3. Disturbance of IL-1α-induced down-regulation of *IL-18* mRNA expression in $EP^{-/-}$ mice-derived keratinocytes. (*A*) Wound healing in WT and $EP^{-/-}$ mice. A full-thickness skin excision (6 mm in diameter) was made on the back of WT (open circles; $n = 10$) and $EP^{-/-}$ (filled circles; $n = 10$) mice. Values indicate the mean diameter ± SD. (*B*) Proliferation of primary epidermal keratinocytes from WT (open bars) and $EP^{-/-}$ (filled bars) mice. Keratinocytes (4×10^4 per well in a 96-well plate) were cultured, and cell proliferation at the indicated times was determined by [^{3}H]thymidine incorporation. Data shown in triplicate are the mean ± SD. (*C* and *D*) Down-regulation of *IL-18* mRNA expression by IL-1α (*C*) and EP (*D*) in primary keratinocytes. Keratinocytes (2×10^5 per well in a 24-well plate) were stimulated with 50 ng·ml^{-1} recombinant mouse IL-1α (*C*) and 100 ng·ml^{-1} rmEP (*D*) for the indicated time, and the expressions were determined by using RT-PCR. Experiments were repeated five times with similar results. (*E*) Down-regulation of *IL-18* expression by EP depends on EGFR. Keratinocytes (2×10^5 per well in a 24-well plate) were preincubated with 2 μM of PD168393 (Calbiochem), 2 μM of AG1296 (Calbiochem), or vehicle for 30 min, followed by incubation with 100 ng·ml^{-1} rmEP in culture medium for 12 h. The expressions were determined by using RT-PCR.

Loss of EP in Non-Bone-Marrow-Derived Cells Is Essential for the Development of Dermatitis. To elucidate that *EP* expression in which cells, keratinocytes, or bone marrow-derived cells including macrophages is essential for the proper homeostasis of the skin region, bone marrow transfer experiments were done. The chimera mice, in which WT-derived bone marrow cells were transplanted into $EP^{-/-}$ mice, developed dermatitis, but not vice versa (Table 1), suggesting that loss of EP in non-bone-marrow-derived cells will be essential for the development of dermatitis and that EP expression in keratinocytes may be critical for the homeostasis of the skin.

Antigen Presentation and Phagocytosis of Peritoneal Macrophages. As *EP* was strongly expressed in tissue-resident macrophages (Fig. 1*A*), we focused on the functions of peritoneal macrophages. First, we examined the potential of antigen presentation of macrophages through T cell responses to the antigen by using immunized mice with ovalbumin. There was no significant difference in proliferation of T cells from the immunized WT and $EP^{-/-}$ mice, when stimulated by WT- or $EP^{-/-}$-derived peritoneal macrophages with ovalbumin (Fig. 4*A*), indicating that antigen presentation of macrophages and the T cell responses were not impaired in $EP^{-/-}$ mice. Furthermore, the potential of phagocytosis was not impaired in macrophages from $EP^{-/-}$ mice (Fig. 4*B*).

Table 1. Bone marrow transplantation

Recipient	Donor	Dermatitis*
WT	$EP^{-/-}$	0/7
$EP^{-/-}$	WT	8/10

The number of mice with dermatitis was determined 6 months after transplantation.
*The number of mice with dermatitis/the number of recipients.

Low Responsiveness to TLR Agonists in Proinflammatory Cytokine Production by $EP^{-/-}$ Peritoneal Macrophages. To elucidate a possibility that EP is involved in the innate immunity, we examined TLR signaling in macrophages. LPS induced surface expressions of CD80, CD86, and CD40, by peritoneal macrophages from WT and $EP^{-/-}$ mice, and differences were not observed between WT and $EP^{-/-}$ (data not shown). However, IL-6 and TNF-α productions were remarkably reduced in macrophages from $EP^{-/-}$ mice in response to LPS and PGN, compared with findings in WT mice (Fig. 4 *C* and *D*).

Membrane-Anchored EP, but Not Secreted EP, May Play Critical Roles in LPS- and PGN-Induced Proinflammatory Cytokine Production by Macrophages. To elucidate molecular mechanisms underlying the low responsiveness to TLR agonists, we analyzed BMDM. The three TLR agonists, including LPS, PGN, and CpG, induced the MHC class II molecule I-A^b and costimulatory molecules, including CD80, CD86, and CD40, by WT- and $EP^{-/-}$-derived BMDM, and there was no difference in the surface expressions of these molecules between WT- and $EP^{-/-}$-derived BMDM (Fig. 4*E* and data not shown). Obvious differences of the responses were observed in the IL-6, IL-12, and TNF-α secretion by BMDM between WT and $EP^{-/-}$ mice, when stimulated with LPS and PGN, but not CpG (Fig. 4 *F–H*). Expression levels of *TLR-2*, *TLR-4*, *nucleotide-binding oligomerization domain 1* (*NOD1*), *NOD2* (34), and *MD-2* (28) in $EP^{-/-}$ mice-derived BMDM were not different from findings in WT mice (Fig. 4*K*). These results, together, suggested that EP affects the TLR4- and TLR2-mediated signaling in macrophages.

Furthermore, the addition of rmEP to LPS stimulation did not show differences in cytokine production, compared with a single LPS stimulation (Fig. 4*I*), suggesting that sEP does not affect these responses, although LPS and PGN did induce rapid *EP* expression in macrophages (Fig. 4*L*). Preexposure of BMDM with high concentration of PD168393 before LPS stimulation

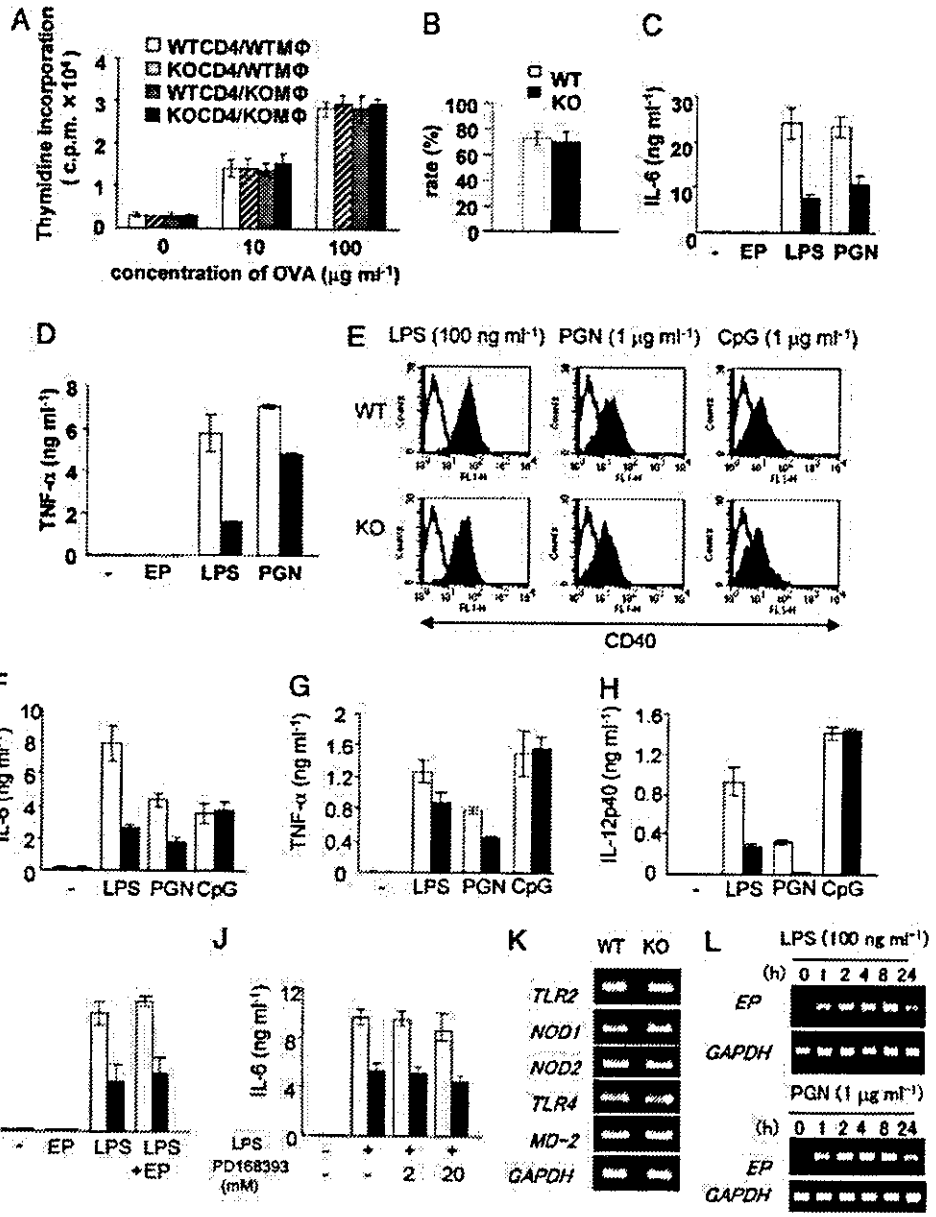

Fig. 4. TLR agonist-induced proinflammatory cytokine production in macrophages. (*A*) Shown are proliferative responses of T cells to the immunized antigen presented by macrophages. T cell proliferation to the irradiated peritoneal macrophages with the indicated concentration of ovalbumin was evaluated by [^{3}H]thymidine incorporation. Mϕ, macrophage. (*B*) Activity of phagocytosis of macrophages. Phagocytosis of fluorescent beads by macrophages was determined by using fluorescent microscopy. Solid bars, $EP^{-/-}$ mice; open bars, WT mice). The values are the means ± SD of three independent experiments. (*C* and *D*) Production of IL-6 (*C*) and TNF-α (*D*) by the peritoneal macrophages stimulated with LPS (100 ng·ml^{-1}), PGN (10 μg·ml^{-1}), and EP (100 ng·ml^{-1}). Cytokine concentration in the supernatant after 24 h was examined by using ELISA. Experiments were repeated five times in triplicate with similar results. Data shown in triplicate are the mean ± SD. Shown are WT (open bars) and $EP^{-/-}$ (filled bars). (*E*) Surface expression of CD40 on the BMDM stimulated with LPS (100 ng·ml^{-1}), PGN (1 μg·ml^{-1}), and CpG (1 μg·ml^{-1}). Expressions were determined at 24 h after stimulation by FACS. (*F–H*) Production of IL-6 (*F*), TNF-α (*G*), and IL-12p40 (*H*) by the BMDM stimulated with LPS, PGN, and CpG, respectively. (*I*) Production of IL-6 by the BMDM stimulated by rmEP (100 ng·ml^{-1}) in addition to LPS stimulation. (*J*) Production of IL-6 by the BMDM stimulated with LPS in the presence of EGFR blocker. BMDM (5×10^5 per well in 12-well plates) were preincubated with 2 μM or 20 μM of PD168393 for 30 min, followed by stimulation with LPS (100 ng·ml^{-1}). Cytokine concentration in the supernatant after 24 h was examined by using ELISA. Experiments were repeated five times in triplicate with similar results. Data shown in triplicate are the mean ± SD. Shown are WT (open bars) and $EP^{-/-}$ (filled bars). (*K*) Expression of *Toll-like receptor 2* (*TLR2*), *TLR4*, *nucleotide-binding oligomerization domain 1* (*NOD1*), *NOD2*, and *MD-2* in BMDM from WT and $EP^{-/-}$ mice. The expressions were determined by RT-PCR. (*L*) Induction of *EP* expression by LPS and PGN in peritoneal macrophages. Peritoneal macrophages were stimulated with 100 ng·ml^{-1} LPS and 1 μg·ml^{-1} PGN for the indicated time, and the expressions were determined by RT-PCR.

seemed to reduce the total amount of IL-6 production (35); however, the obvious difference in IL-6 secretion between WT and $EP^{-/-}$ BMDM still existed (Fig. 4*J*), indicating that the difference is not dependent on EGFR-mediated signaling. All these results suggested that the membrane-anchored EP will play critical roles in the cytokine production by macrophages and/or in macrophage differentiation (17, 36).

Discussion

Here, we demonstrate that *EP* deficiency results in chronic dermatitis in mice and that EP is physiologically a critical molecule for tight control of *IL-18* mRNA expression of keratinocytes and for proper production of proinflammatory cytokines by macrophages in response to LPS and PGN.

Wound healing in the skin in $EP^{-/-}$ mice and the growth of in $EP^{-/-}$ keratinocytes were not impaired (Fig. 3 *A* and *B*), suggesting that dermatitis in $EP^{-/-}$ mice will be caused rather by the dysregulation of immune-related responses of the skin, but not due to the dysfunction of the proliferative activities of keratinocytes. Skin is the place to be damaged frequently, and indigenous bacteria exist in the epidermis, the barrier from the outside milieu. It is intriguing that a member of the EGF family is critically involved in immune regulation of keratinocytes and macrophages. Elucidation of the functions of the EGF family on APCs and epithelial cells in immune regulation might lead to a better understanding of immune defense at the primary interface between the body and the environment and might provide insight into the pathogenesis of inflammatory epithelial disorders.

IL-1α rapidly induced *EP* expression in keratinocytes (Fig. 3*C*) and the skin lesions in $EP^{-/-}$ mice were mostly restricted to faces and ears, suggesting that IL-1α release by repeated grooming-induced injury may accumulate IL-18 in the skin of $EP^{-/-}$ mice, resulting in the initiation of development of dermatitis in $EP^{-/-}$ mice (22). IL-1α alone did not induce the active form of IL-18 by primary keratinocytes (data not shown), suggesting that some particular signals, in addition to IL-1α, may be necessary for the production of the active form of IL-18 by keratinocytes. On the other hand, dysregulation of *IL-18* expression in the skin legions in $EP^{-/-}$ mice *in vivo* was not constantly observed (data not shown), suggesting that dysregulation of IL-1α-induced *IL-18* down-regulation may not be involved in the promotion of dermatitis. Because IL-1α-deficient mice do not develop dermatitis (37), analysis of $EP^{-/-}$/IL-1$\alpha^{-/-}$ doubly mutant mice will show the relation between IL-1α and EP for the development of dermatitis in $EP^{-/-}$ mice.

Bone marrow transfer experiments supported the critical role of EP in keratinocytes for homeostasis of the skin; however, there are possibilities that EP by keratinocytes might affect the function and/or differentiation of tissue-resident macrophages and that loss of EP in macrophages might affect the progression of dermatitis.

Although *EP* expression in other epithelial tissues was not extensively examined in this study, there is a possibility that EP might be involved in immune/inflammatory responses in other epithelial tissues, including intestine and lung. Dendritic cells (DCs) initiate and regulate immune responses, linking innate and adaptive immunity (38). EP expression in Langerhans cells, the DCs in epidermis (39, 40), was not determined in this study, whereas EP expression in DCs in Peyer's patches was strongly observed. There might be a possibility that EP plays a crucial role not only in DCs in Peyer's patches but also in Langerhans cells.

The sEP is involved in the IL-1α-induced down-regulation of *IL-18* expression (Fig. 3*D*) and the mechanism by which sEP regulates *IL-18* expression depends on the EGFR (Fig. 3*E*). On the other hand, addition of sEP or EGFR blocker did not affect the proinflammatory cytokine production by macrophages (Fig. 4 *I* and *J*), suggesting that the membrane-anchored EP may be involved in the proinflammatory cytokine production by macrophages stimulated with LPS and PGN through non-EGFR-mediated signaling. A bioactive membrane precursor, pro-HB-EGF, functions as the diphtheria toxin receptor in primates (41) and distinctively signals growth inhibition or apoptosis rather than the proliferative response induced by soluble HB-EGF (10). Like HB-EGF, sEP and membrane-anchored EP will have distinct function and there might be a possibility that membrane-anchored EP transduces signals into the own cells as receptor (8).

In conclusion, we have demonstrated that $EP^{-/-}$ mice are susceptible for the development of dermatitis even in specific pathogen-free conditions and that sEP is critical for the proper regulation of *IL-18* in keratinocytes and membrane-anchored EP will be essential for the proper proinflammatory cytokine production in response to LPS and PGN by tissue resident macrophages. Further elucidation of EP function may shed light on epithelial inflammatory disorders.

We thank Dr. Kensuke Miyake for critical comments, Y. Yamada for the injections, Y. Fujimoto for technical assistance, M. Ohara for language assistance, and S. Ohkubo and Y. Hagishima for manuscript preparation. This work was supported in part by a grant from the Ministry of Education, Science, Technology, Sports, and Culture of Japan.

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Ectopic CD40 Ligand Expression on B Cells Triggers Intestinal Inflammation[1]

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Several studies indicate that CD4$^+$ T cells, macrophages, and dendritic cells initially mediate intestinal inflammation in murine models of human inflammatory bowel disease. However, the initial role of B cells in the development of intestinal inflammation remains unclear. In this study we present evidence that B cells can trigger intestinal inflammation using transgenic (Tg) mice expressing CD40 ligand (CD40L) ectopically on B cells (CD40L/B Tg). We demonstrated that CD40L/B Tg mice spontaneously developed severe transmural intestinal inflammation in both colon and ileum at 8–15 wk of age. In contrast, CD40L/B Tg×CD40$^{-/-}$ double-mutant mice did not develop colitis, indicating the direct involvement of CD40-CD40L interaction in the development of intestinal inflammation. The inflammatory infiltrates consisted predominantly of massive aggregated, IgM-positive B cells. These mice were also characterized by the presence of anti-colon autoantibodies and elevated IFN-γ production. Furthermore, although mice transferred with CD4$^+$ T cells alone or with both CD4$^+$ T and B220$^+$ B cells, but not B220$^+$ cells alone, from diseased CD40L/B Tg mice, develop colitis, mice transferred with B220$^+$ B cells from diseased CD40L/B Tg mice and CD4$^+$ T cells from wild-type mice also develop colitis, indicating that the Tg B cells should be a trigger for this colitis model, whereas T cells are involved as effectors. As it has been demonstrated that CD40L is ectopically expressed on B cells in some autoimmune diseases, the present study suggests the possible contribution of B cells in triggering intestinal inflammation in human inflammatory bowel disease. ***The Journal of Immunology,*** **2004, 172: 6388–6397.**

Crohn's disease (CD)[4] and ulcerative colitis (UC) are the two major forms of chronic inflammatory bowel disease (IBD). Although their etiopathology remains unknown, increasing evidence has shown that an immune mechanism plays an important role in their pathogenesis (1–3). These include increased T cell infiltrates into gut, the production of cytokines by lamina propria (LP) T cells, and remission after treatment with immunosuppressants or by targeting proinflammatory cytokines. Although many animal experimental models clinically mimicking human IBD have been established, all these models induce diseases characterized by the common features of T cell- or macrophage/dendritic cell-dependent immune responses (4, 5).

One particular molecule that may be a potent target for the treatment of IBD is CD40 ligand (CD40L; CD154). This is found predominantly on activated CD4$^+$ T cells and interacts with CD40, which is expressed on B cells, APCs, and endothelial cells (6–8). In patients with IBD, increased numbers of T cells expressing CD40L have been detected in the LP, and these contribute to induce proinflammatory cytokines, including IL-12, by macrophages/dendritic cells (9, 10). Furthermore, it is known that mice with overexpressed CD40L on T cells develop chronic colitis with the infiltration of CD40L$^+$ T cells and CD40$^+$ cells into disease tissues (11). In both 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced (12) and SCID-transferred colitis models (13, 14), neutralizing anti-CD40L Abs prevent colitis. These data suggest that CD40/CD40L interactions are probably relevant in the development of colitis in humans and mice.

In contrast, CD40-expressing B cells, which interact with CD40L-expressing T cells, are able to promote the proliferation and survival of B cells, Ig isotype switching, and germinal center reaction (7, 15, 16). B cells possess a variety of immune functions, including the production of Igs and various cytokines, the presentation of Ags, and the regulation of dendritic cell function. Interestingly, functionally distinct roles of B cells have been reported in autoimmune diseases such as systemic lupus erythematosus (SLE) (17). B cells drive the development of several autoimmune disorders through the production of pathogenic autoantibodies. Similarly, circulating autoantibodies to colon epithelial cells have been consistently identified in patients with UC or CD by many investigators in reports that span at least 2 decades (18–20). Interestingly, accumulating evidence suggests that CD40L is ectopically expressed on B cells in the same range of their T cells in patients with SLE, although the pathogenic role of CD40L on B cells in SLE is unknown (21). At present, however, to our knowledge,

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Received for publication June 25, 2003. Accepted for publication March 4, 2004.

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

[1] This work was supported in part by grants-in-aid for Scientific Research, Scientific Research on Priority Areas, Exploratory Research, and Creative Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science, and Technology; the Japanese Ministry of Health, Labor, and Welfare; the Japan Medical Association; Foundation for Advancement of International Science; Terumo Life Science Foundation; Ohyama Health Foundation; Yakult Bio-Science Foundation; and Research Fond of Mitsukoshi Health and Welfare Foundation.

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[4] Abbreviations used in this paper: CD, Crohn's disease; IBD, inflammatory bowel disease; LP, lamina propria; LPMC, lamina propria mononuclear cell; UC, ulcerative colitis; SLE, systemic lupus erythematosus; Tg, transgenic; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TRITC, tetramethylrhodamine; WT, wild type.

0022-1767/04/$02.00

there have been no reports describing the expression of CD40L on B cells in patients with IBD.

Based on the fact that CD40L can be inducible on B cells in some autoimmune disorders and also several reports that described the association between IBD and SLE (22, 23), we investigated the possibility that intestinal inflammation may be induced by the primarily dysregulated B cells. To this end, we used transgenic (Tg) mouse lines expressing CD40L ectopically on B cells (CD40L/B Tg) (24). At 8–12 wk of age, CD40L/B Tg mice show increased number of B cells in spleen and increased serum IgG and IgM concentrations by ~4- and ~5-fold, respectively. In addition, B cells in CD40L/B Tg mice are resistant to apoptosis induced in vitro, probably due to constitutive CD40 signaling in B cells via CD40 (our unpublished observations). Interestingly, these mice developed SLE-like autoimmune disease, probably due to the production of many kinds of autoantibodies, such as anti-DNA Abs (24).

Surprisingly, we found that CD40L/B Tg mice developed severe transmural intestinal inflammations. Remarkably, the inflammatory infiltrates consisted predominantly of B cells. Although T cells also infiltrated inflamed lesions, they did so to a much lesser degree than B cells, and the CD4/CD8 ratio of LP T cells was decreased compared with that in control mice. Furthermore, we demonstrated that mice transferred with $CD4^+$ T cells alone or with both $CD4^+$ T and $B220^+$ B cells, but not $B220^+$ cells alone from diseased CD40L/B Tg mice, develop colitis similar to the original disease, indicating that the final cause of colitis is dysregulated $CD4^+$ T cells. However, we demonstrated that mice transferred with $B220^+$ B cells from diseased CD40L/B Tg mice and $CD4^+$ T cells from wild-type (WT) mice also developed colitis, albeit to a lesser extent than those transferred with $CD4^+$ T and $B220^+$ B cells from diseased CD40L/B Tg mice, whereas mice reconstituted with both $B220^+$ B cells and $CD4^+$ T cells from WT mice did not. This indicates that Tg B cells should be involved as a trigger for this colitis model. These observations demonstrate that the CD40-CD40L interaction among B cells may introduce abnormal Ig isotype switching and consecutive B cell expansion and may contribute to the infiltration in gut. Thus, this experimental murine model seems to be sharply distinct from any established murine IBD models in which dysregulative function of T cells and macrophages/dendritic cells should be an essential pathogenic mechanism.

Materials and Methods

Mice

CD40L/B Tg mice expressing CD40L on B cells were previously established by injecting the DNA fragment containing the CD40L cDNA, a V_H promoter, the IgH intron enhancer, and the Igκ3′ enhancer into C57BL/6 fertilized eggs (24). $CD40^{-/-}$ mice were described previously (25). In the present study male, 4- to 20-wk-old CD40L/B Tg mice and WT littermate controls were used. CD40L/B Tg×$CD40^{-/-}$ double-mutant mice were also generated. The animals were housed under specific pathogen-free conditions. All experiments were approved by the regional animal study committees. Cross-breeding of these mice was performed, and the presence of each transgene was identified by tail DNA.

Histopathology and immunohistochemical analysis

Tissue specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections were stained with H&E. For the immunohistochemistry, sections were embedded in OCT compound (Tissue-Tek: Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −80°C. Six-micron sections were incubated with primary Abs. These included anti-murine CD4 (RM4-5, rat IgG1; BD PharMingen, San Diego, CA), anti-murine CD8 (53-6.7, rat IgG2a; BD PharMingen), anti-murine B220 (RA3-6B2, rat IgG2a; BD PharMingen), anti-murine CD11c (HL-3, hamster IgG; BD PharMingen), and anti-F4/80 (A3-1, rat IgG2b; Serotec, Oxford, U.K.). Isotype-matched control Abs (BD PharMingen) were also used. Biotinylated goat anti-hamster IgG or rat anti-mouse IgG (BD PharMingen) was chosen as second Abs. After three washes with PBS, positively stained cells were detected by streptavidin-biotinylated HRP complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA), and visualized by diaminobenzidine. Then the sections were counterstained with hematoxylin.

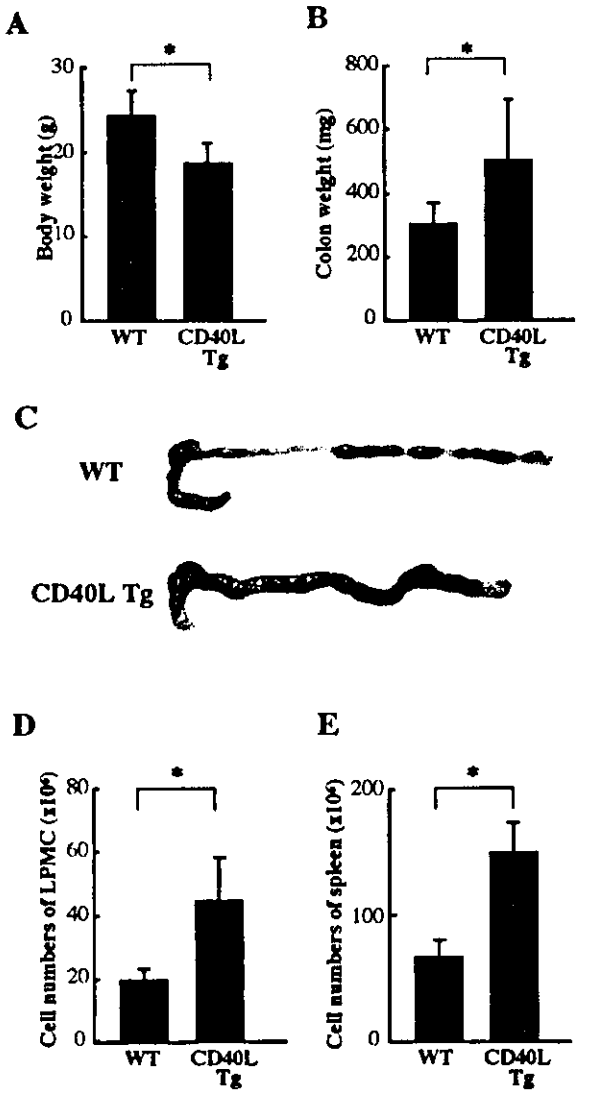

FIGURE 1. CD40L/B Tg mice developed the wasting disease with local and peripheral expansive cell growth. *A*, Approximately 90% of CD40L/B Tg mice (n = 29) had diarrhea and showed significant weight loss compared with WT littermates (n = 24) at 15 wk of age. *B*, The colonic weight of CD40L/B Tg mice was markedly larger than that of WT littermates at 15 wk of age. *C*, The macroscopic appearance of colon of CD40L/B Tg mice was enlarged, shortened, and had a greatly thickened wall compared with that of WT littermates. *D*, LPMCs were isolated from the colon of 15-wk-old CD40L/B Tg mice or their littermates, and the number of LPMCs was determined. Data are the mean ± SD of seven mice in each group. *E*, Splenocytes (Sp) were isolated from the colon of 15-wk-old CD40L/B Tg mice or their littermates, and the number of Sp was determined. *, $p < 0.05$.

Immunofluorescence analysis

In some experiments frozen sections of colon were prepared and fixed with cold acetone and then incubated with Block Ace (Dainippon-Pharmaceuticals, Tokyo, Japan). Sections were incubated with FITC-labeled goat anti-mouse IgM (magnification, ×500; Southern Biotechnology Associates, Birmingham, AL), tetramethylrhodamine (TRITC)-labeled goat anti-mouse IgG (magnification, ×500; Southern Biotechnology Associates), and biotin-labeled goat anti-mouse IgA (magnification, ×400; BD PharMingen), followed by incubation with aminocumarin-labeled streptavidin (magnification, ×500;

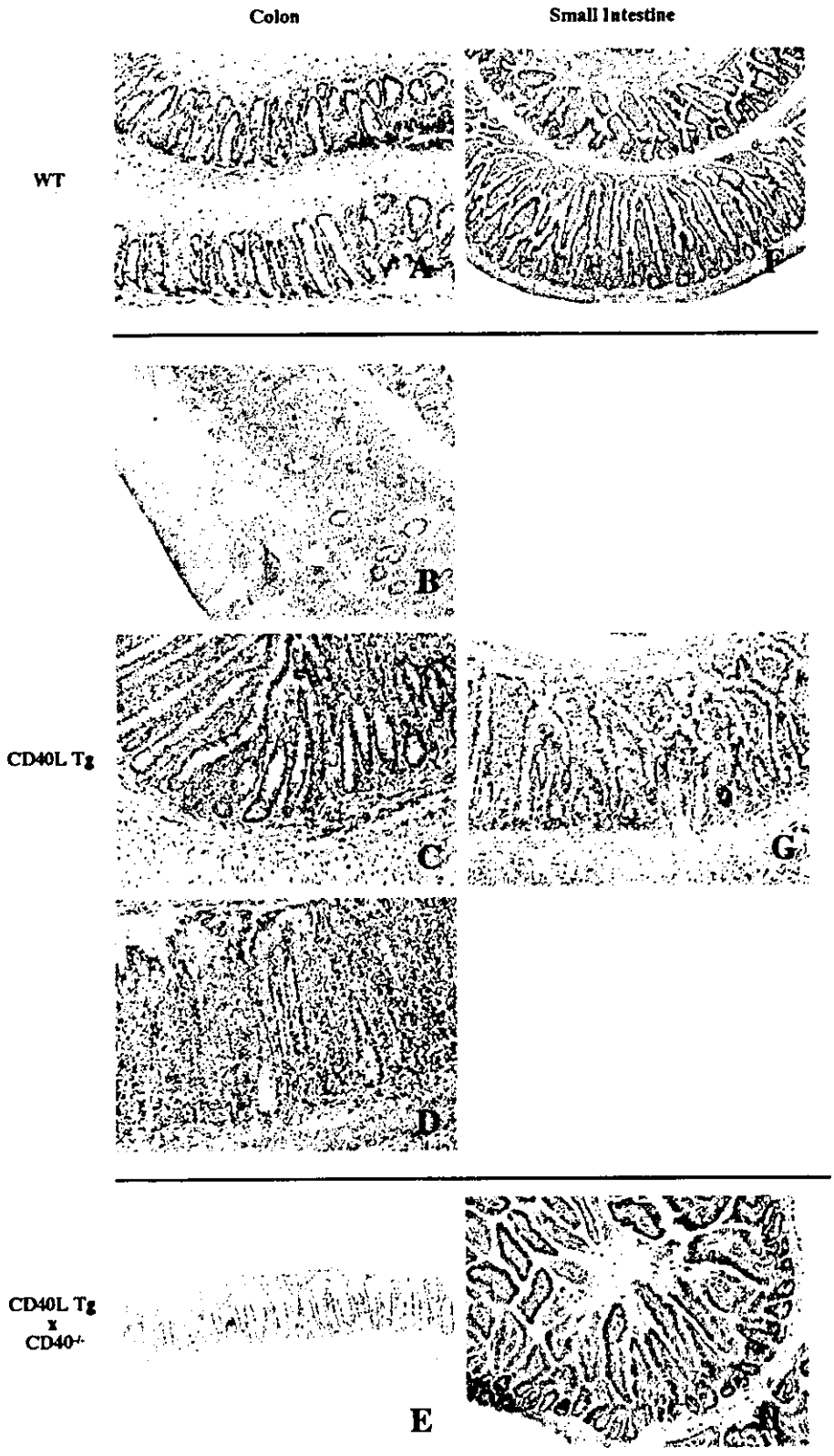

FIGURE 2. Histopathologic examination revealed the development of severe enterocolitis in CD40L/B Tg mice. Colons from WT littermates (*A*), CD40L/B Tg mice (*B–D*), and CD40L/B Tg×CD40$^{-/-}$ (*G*) mice and small intestines from WT littermates (*E*), CD40L/B Tg mice (*F*), and CD40L/B Tg×CD40$^{-/-}$ mice (*H*) were stained with H&E. Original magnification: *A*, *B*, *E–G*, and *H*, × 40; *C* and *D*, ×100.

Southern Biotechnology Associates) (26). Anti-colon autoantibodies in sera from 12- to 15-wk-old colitic CD40L/B Tg mice were detected by indirect immunofluorescence using colonic tissues from 6-wk-old nondiseased CD40L/B Tg mice. Sections were blocked with 10% goat serum in PBS for 30 min at room temperature and incubated with mouse serum diluted in 10% goat serum in PBS for 1 h, and then bound Ab was detected with TRITC-labeled anti-mouse IgG or FITC-labeled anti-mouse IgM. Sections were examined with a fluorescence microscope (BX50/BXFLA: Olympus, Tokyo, Japan) equipped with a charge-coupled device camera (Olympus) and an image capture system (Olympus). Combination images for multicolor staining were performed using Photoshop 4.0 (Adobe Systems, San Jose, CA).

Serum Ig

Total amounts of serum IgM, IgG, and IgA were measured by standard sandwich ELISA analysis using goat anti-IgM or IgG or IgA Abs (Southern Biotechnology Associates). Abs bound to the plates were detected using

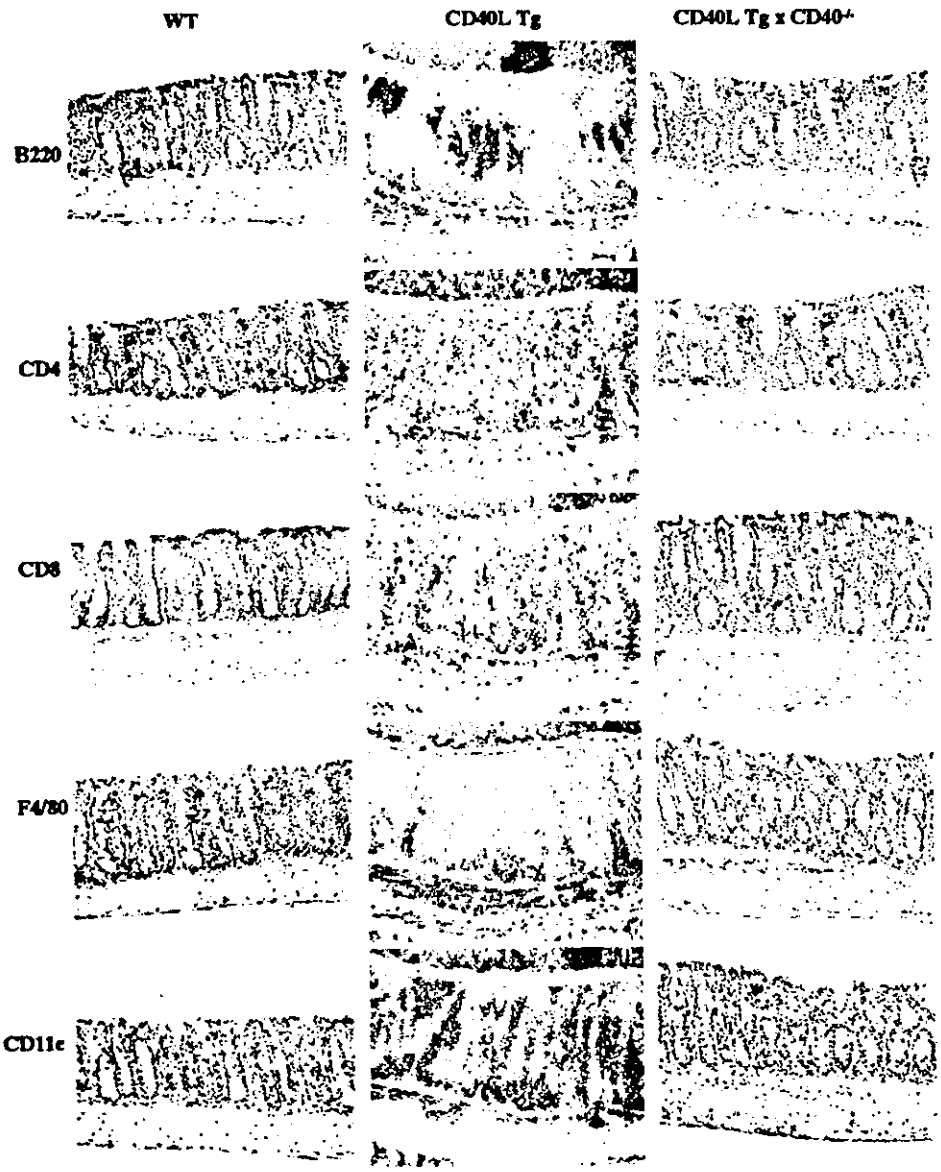

FIGURE 3. Immunohistochemical analysis showed the massive B220-positive cell infiltration to colonic tissue in CD40L/B Tg mice. A large number of aggregated $B220^+$ cells, which were structurally different from B cells in colonic patches, were infiltrated in the LP of diseased CD40L/B Tg mice. In addition, $CD4^+$, $CD8^+$, $F4/80^+$, and $CD11c^+$ cells were increased in the inflamed colonic mucosa of CD40L/B Tg mice compared with those of WT littermates or CD40L/B Tg×$CD40^{-/-}$ mice. Original magnification, ×100.

alkaline phosphatase-labeled anti-IgG, anti-IgM (BD PharMingen), and anti-IgA Abs (Southern Biotechnology Associates).

Cell preparation

LP mononuclear cells (LPMC) were isolated from the colon as described previously (27, 28). Briefly, the entire colon was opened longitudinally, washed with PBS, and cut into small pieces. The pieces were then incubated with Ca^{2+}- and Mg^{2+}-free HBSS containing 2.5% FBS and 1 mM DTT (Sigma-Aldrich, St. Louis, MO) for 30 min to remove mucus and then serially incubated twice in Ca^{2+}- and Mg^{2+}-free HBSS containing 2.5% FBS and 0.75 mM EDTA (Sigma-Aldrich) for 1 h each. The supernatants from these incubations were collected, pooled, and treated with 1 mg/ml collagenase (Worthington Biomedical, Freehold, NJ) and 0.01% DNase (Worthington Biomedical) in medium for 2 h. The cells were pelleted twice through a 40% isotonic Percoll solution and then further purified by Ficoll-Hypaque density gradient centrifugation (40/75%) at the interface.

Flow cytometry

The isolated LPMC were preincubated with an FcγR-blocking mAb (CD16/32; 2.4G2; BD PharMingen) for 20 min, followed by incubation with FITC-, PE-, or biotin-labeled mAb for 30 min on ice. Biotinylated Abs were detected with PE-streptavidin (BD Biosciences, Mountain View, CA). Two-color flow cytometric analysis was performed on a FACSCalibur (BD Biosciences) using CellQuest software. Background fluorescence was assessed by staining with isotype-matched control mAbs.

Cytokine analysis

Ninety-six-well culture plates were precoated with anti-CD3 mAb (5 μg/ml; 145-2C11; BD PharMingen) and anti-CD28 mAb (2 μg/ml; 37.51; BD PharMingen) in 100 μl of PBS at 37°C for 4 h and washed with PBS three times to remove unbound Abs. LP $CD4^+$ T cells (5×10^5/well) were then incubated at 37°C in 5% CO_2 humidified air for 48 h. Culture supernatants of littermate mice and Tg mice after onset of disease were harvested and assayed for IFN-γ and IL-4. Cytokine concentrations were determined by a specific ELISA kit for mouse IFN-γ and IL-4 (Endogen, Cambridge, MA). ODs were measured on an Intermet ELISA reader at a wavelength of 490 nm.

Adoptive transfer experiment

Adult female C57BL/6J SCID mice (6–8 wk old) were used as recipients in the following experiments. Diseased CD40L/B Tg and control WT littermates were euthanized at 18 wk of age. To obtain $CD4^+$ T or $B220^+$ B cells from the pooled splenocytes and mesenteric lymph node cells, $CD4^+$ T cells were purified using the anti-CD4 (L3T4) MACS beads (Miltenyi Biotec, Auburn, CA), and thereafter $B220^+$ cells were purified from the negatively selected $CD4^-$ cells using anti-B220 (RA3-6B2) MACS beads (Miltenyi Biotec). After the enriched $CD4^+$ T cells (96–97% pure, as estimated by FACSCalibur) and $B220^+$ B cells (95–96% pure) were labeled with PE-conjugated anti-mouse CD4 (RM4-5) and FITC-conjugated anti-B220 (RA3-6B2) mAbs, and the purified $CD4^+$ T cells and $B220^+$ B

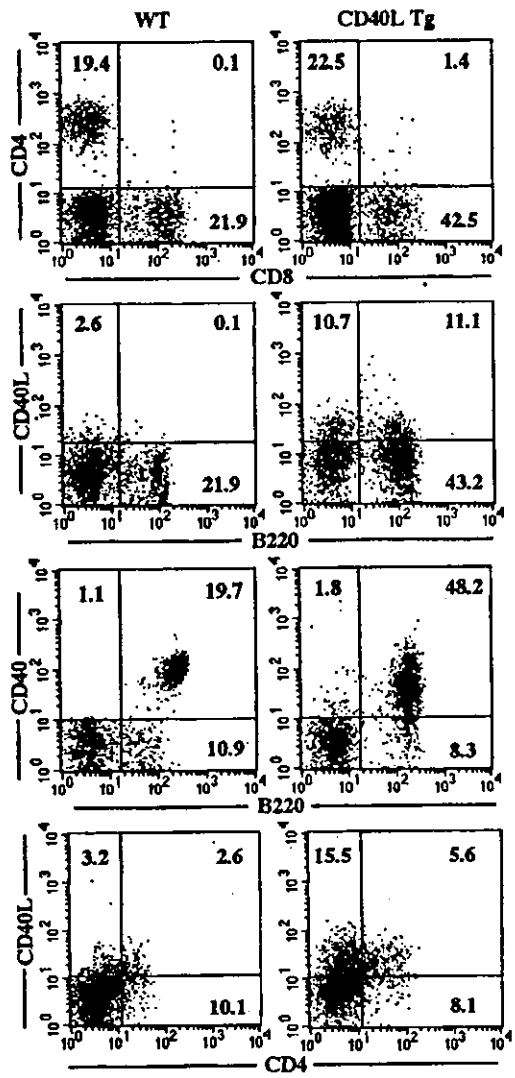

FIGURE 4. Flow cytometric analysis of LPMC from diseased CD40L/B Tg mice and WT littermates. The percentages of $CD8^+$ cells and the CD8/CD4 ratio were significantly increased in LPMC from diseased CD40L/B Tg mice compared with those from WT littermates. In CD40L Tg mice, the percentages of $B220^+$ LPMC were significantly increased and the expression of CD40L on B220-expressing LP B cells was also significantly up-regulated compared with those in WT littermates. In contrast, there were no differences in CD40 expression on LP B cells between CD40L/B Tg mice and littermates. Although we used the B cell-specific promoter and enhancer to establish CD40L/B Tg mice, CD40L/B on LP T cells in diseased mice was also slightly up-regulated compared with that in littermates. These data were representative of three experiments.

cells were then sorted by two-color sorting on a FACS Vantage (BD Biosciences). All populations were >99.0% pure on reanalysis. SCID mice were then injected i.p. with one or two subpopulations of the sorted $CD4^+$ T cell and/or $B220^+$ B cells in PBS: 1) CD40L/B Tg $B220^+$ (5×10^5/mouse), 2) CD40L/B Tg $CD4^+$ (5×10^5/mouse), 3) CD40L/B Tg $B220^+$ (5×10^5/mouse) and CD40L/B Tg $CD4^+$ (5×10^5/mouse), 4) CD40L/B Tg $B220^+$ (5×10^5/mouse) and WT $CD4^+$ (5×10^5/mouse), 5) WT $B220^+$ (5×10^5/mouse) and WT $CD4^+$ (5×10^5/mouse), 6) WT $B220^+$ (5×10^5/mouse), 7) WT $CD4^+$ (5×10^5/mouse), or 8) no transfer control. Mice were killed and analyzed 4 wk after transfer, and the colons were removed and evaluated histologically. For the histological score, the area of the proximal colon was scored on a scale of 0–3 in each of three criteria: cell infiltration, crypt elongation, and crypt abscesses. Histological grades were assigned in a blind fashion by one pathologist (R.I.).

Statistical analysis

Data were expressed as the mean and SD and are plotted in corresponding figures. The differences between experimental groups were statistically analyzed with the Mann-Whitney U test. A value of $p < 0.05$ was considered significant.

Results

CD40L/B Tg mice developed a wasting disease with severe intestinal inflammation

Clinical manifestation was monitored for up to 30 wk. IBD symptoms were first observed between 8 and 15 wk of age. Approximately 90% of CD40L/B Tg mice had diarrhea and developed significant weight loss until 20 wk of age ($p < 0.05$; Fig. 1*A*). In contrast, their WT littermates appeared healthy, with a gradual increase in body weight and absence of diarrhea during the period of observation (Fig. 1*A*). Macroscopic appearance showed enlarged colon with a greatly thickened wall in CD40L/B Tg mice (Fig. 1*C*), and the colonic weight of 15-wk-old CD40L/B Tg mice was significantly increased compared with that of littermates ($p < 0.005$; Fig. 1*B*). In addition, the spleen and mesenteric lymph node enlargement was also present in diseased CD40L/B Tg mice (data not shown). Consistent with these data, the numbers of mononuclear cells recovered from the colon and spleen of 15-wk-old CD40L Tg mice were significantly increased compared with those of their littermates (Fig. 1, *D* and *E*), indicating extensive mononuclear cell proliferation in the inflamed colon and spleen of CD40L/B Tg mice.

Histopathologic examination of colonic tissue revealed the development of severe colitis in 15-wk-old CD40L/B Tg mice (Fig. 2, *B–D*). Large numbers of mononuclear cells infiltrated transmurally in colonic mucosa (Fig. 2*B*). Prominent epithelial hyperplasia with glandular elongation (Fig. 2*C*) and goblet cell depletion (data not shown) were common in whole colon. Extensive leukocytic infiltrates were seen in the LP and submucosa and, to a lesser degree, in the muscularis, serosa, and mesentery (Fig. 2, *B* and *D*). The mucosal abnormalities, such as thickening of the intestinal wall, virous atrophy, crypt hyperplasia, and infiltrations of mononuclear cells were also seen in the ileum of CD40L/B Tg mice (Fig. 2*G*). Splenomegaly with large amounts of lymphocytes and markedly enlarged mesenteric lymph nodes were present in these colitic mice (data not shown). In contrast, colonic and ileac sections from WT littermates showed few lymphocytes and macrophage infiltration in LP (Fig. 2, *A* and *F*). To determine whether these abnormalities in CD40L/B Tg mice was really due to the direct CD40-CD40L interaction in vivo, we next generated CD40L/B Tg×$CD40^{-/-}$ double-mutant mice. These mice were monitored for up to 30 wk, but they appeared healthy with a gradual increase in body weight and the absence of diarrhea during the period of observation (data not shown). In addition, colonic and ileac sections from age-matched CD40L/B Tg×$CD40^{-/-}$ mice showed few lymphocytes and macrophage infiltration in the LP (Fig. 2, *E* and *H*).

To examine the phenotypical cell surface markers of infiltrated mononuclear cells, the sections of colon were immunohistochemically stained with Abs against B220, CD4, CD8, F4/80, and CD11c. The major population of cell infiltrates was $B220^+$ cell aggregates, which were structurally different from colonic patches. B cells were markedly infiltrated in the LP from diseased CD40L/B Tg mice as well as serosa and mesentery (Fig. 3). In addition, $CD4^+$, $CD8^+$, $F4/80^+$, and $CD11c^+$ cells in the inflamed mucosa from diseased CD40L/B Tg mice were increased in the inflamed colonic mucosa compared with those from WT littermates (Fig. 3). Unlike most reported animal models of IBD, $CD8^+$ T cells seemed to infiltrate to a greater extent in inflamed mucosa

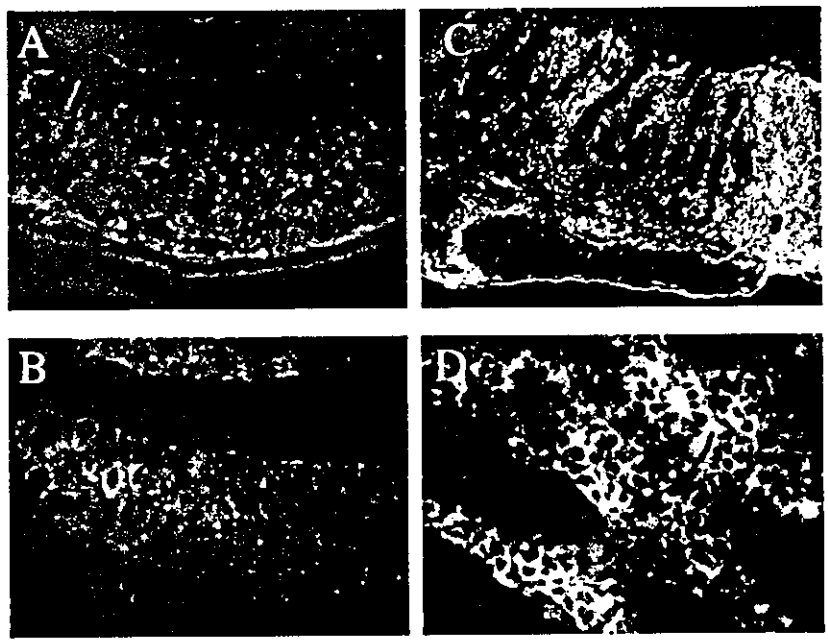

FIGURE 5. Colonic Ig-producing cells in CD40L/B Tg mice. Fifteen-week-old WT littermates (*A*), 6-wk-old nondiseased CD40L Tg mice (*B*), and 15-wk-old diseased CD40L/B Tg mice (*C* and *D*) were stained for IgG (red), IgM (green), and IgA (blue) by fluorescent immunohistochemistry. Diseased and nondiseased CD40L/B mice had almost similar numbers of IgA plasma cells in colon as WT littermates. In contrast, a markedly increased number of IgM^+ cells was observed in inflamed mucosa in diseased CD40L/B Tg mice. In addition, the number of IgG-producing cells in diseased CD40L/B Tg mice was slightly, but significantly, increased compared with those in nondiseased CD40L/B Tg mice and WT littermates. Original magnification: *A*–*C*, ×200; and *D*, ×1000. These data were representative of three experiments.

compared with $CD4^+$ T cells. In contrast, the littermates and age-matched CD40L/B Tg×$CD40^{-/-}$ mice showed no obvious infiltration of $B220^+$, $CD4^+$, $CD8^+$, $F4/80^+$, or $CD11c^+$ cells in the colon (Fig. 3).

Autoimmune diseases are often associated with the involvement of other organs, in particular the kidneys and sometimes the lungs, during disease progression. Therefore, cryostat sections of various tissues were analyzed for the presence of inflammation. Approximately half the CD40L/B Tg mice showed apparent glomerulonephritis and hyperplastic bronchus-associated lymphoid tissue in lungs (data not shown). However, no inflammation in other organs, such as skin, submaxillary gland, heart, liver, pancreas, and stomach, were observed (data not shown).

Increased expression of CD40L on B cells in diseased CD40L/B Tg mice

Flow cytometric analysis of LPMC from colitic mice showed that the percentages of $B220^+$ LPMC were significantly increased compared with those in WT littermates (Fig. 4). Consistent with immunohistochemical studies, unlike other T cell-mediated colitis models, the percentages of $CD8^+$ cells and the CD8:CD4 ratio were significantly increased compared with those in WT littermates (Fig. 4). In diseased CD40L/B Tg mice, the expression of CD40L on B220-expressing LP B cells was significantly up-regulated. The mean fluorescein intensity of $B220^+CD40L^+$ LP B cells in CD40L/B Tg mice was much higher than that in young nondiseased CD40L/B Tg mice (data not shown). In contrast, there were no differences in CD40 expression on B cells between CD40L/B Tg mice and littermates (Fig. 4). Although we used the B cell-specific promoter and enhancer to establish CD40L/B Tg mice, CD40L on LP T cells in diseased, but not in nondiseased, CD40L/B Tg mice was also up-regulated compared with that in littermates (Fig. 4). These data indicated that CD40L expression on T cells could be secondary to the response to inflammatory actions in colon.

To further investigate the role of CD40L-expressing B cells in the development of colitis in CD40L/B Tg mice, we assessed Ig-producing cells using confocal fluorescent immunohistochemistry. As shown in Fig. 5, diseased and nondiseased CD40L/B Tg mice had almost similar numbers of IgA plasma cells in colon compared with WT littermates. In contrast, it was of note that there was markedly increased number of surface IgM-positive cells were observed in inflamed mucosa in diseased CD40L/B Tg mice (Fig. 5). In addition, surface IgG-producing cells, which were rarely present in LP, were often detected in diseased CD40L/B Tg compared with nondiseased CD40L/B Tg mice (Fig. 5).

To next assess the systemic Ig production in CD40L/B Tg mice, we tested concentrations of serum IgG, IgM, and IgA using the specific sandwich ELISA. As shown in Fig. 6, concentrations of serum IgG and IgM were significantly increased by 4- and 5-fold, respectively, in diseased CD40L/B Tg mice compared with WT littermates (IgG; $p < 0.005$, IgM; $p < 0.05$). In some diseased CD40L/B Tg mice, serum IgA was increased, but not significantly different ($p = 0.29$).

Anti-colon autoantibodies

To assess the involvement of anti-colon autoantibodies in the pathogenesis of colitis, we tested whether sera obtained from colitic mice react with epithelial cells in the uninflamed colon of young nondiseased CD40L/B Tg mice. As shown in Fig. 7, both anti-colon IgG and IgM autoantibodies were detected in sera from colitic mice, IgM autoantibody reacted with crypt epithelium, and IgG autoantibody showed strong reaction with surface epithelium of the colon. These reactivities were seen with 1/125 diluted sera from diseased CD40L/B Tg mice. In contrast, staining of epithelial cells was not seen with sera from nondiseased mice and WT littermates at 1/25 or 1/125 dilution (Fig. 7).

B1/B2 differentiation in CD40L/B Tg mice

Based on the involvement of autoantibodies against intestinal epithelial cells in sera derived from diseased CD40L/B Tg mice, we further assessed B1 B cells in inflamed mucosa, because B1 cells appears to produce Abs in a T cell-independent manner (29). Flow cytometric analysis of LPMC from colitic mice showed no significant absolute increase in $CD5^+IgM^+$ B1 B cells on LPMC from diseased CD40L/B Tg mice (percentage of $CD5^+IgM^+$/lymphocyte gate: WT, 0.4 ± 0.2%; CD40L/B Tg, 0.9 ± 0.5%; $p = 0.18$), indicating that autoantibody production could require T cell-dependent help or the involvement of nonmucosal B1 B cells, which may be located in peritoneal and pleural cavities.

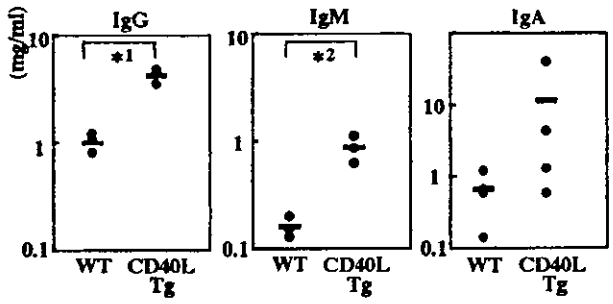

FIGURE 6. Serum concentrations of Igs from CD40L/B Tg mice (n = 4) and WT littermates (n = 4) at 18 wk of age. Serum IgG, IgM, and IgA were measured using the specific sandwich ELISA. The average concentrations of serum IgG and IgM were significantly increased by 4- and 5-fold, respectively, in diseased CD40L/B Tg mice compared with WT littermates. In some diseased CD40L/B Tg mice, serum IgA was markedly increased, but the difference was not significant ($p = 0.29$). *1, $p < 0.05$. *2, $p < 0.005$.

Th1-mediated immune responses in CD40L/B mice

As mentioned above (Fig. 4), $CD4^+$ and $CD8^+$ T cells were also increased in inflamed mucosa from diseased CD40L/B Tg mice. We then assessed the involvement of Th cells in the development of this colitis. Isolated LP $CD4^+$ T cells were cultured, and the supernatants were analyzed for concentrations of IFN-γ and IL-4 by specific ELISA. IFN-γ ($p < 0.05$), but not IL-4, production by anti-CD3/anti-CD28 mAbs-stimulated LP $CD4^+$ T cells was significantly increased in diseased CD40L/B Tg compared with WT littermates (IFN-γ: WT, 12.1 ± 3.0 ng/ml; CD40L/B Tg, 84.8 ± 12.1 ng/ml ($p = 0.001$); IL-4: WT, 575.2 ± 75.0 pg/ml; CD40L/B Tg, 469.0 ± 75.9 pg/ml ($p = 0.68$)), indicating that colitis induced in CD40L/B Tg mice could be mediated in secondary Th1-immune responses.

Adoptive transfer experiments

To establish which cell type mediates the inflammation in CD40L/B Tg mice, we next performed the adoptive transfer experiments using sorted B and T cells from diseased CD40L/B Tg and WT littermate mice in the indicated protocol (Fig. 8*A*). As shown in Fig. 8*B*, mice transferred with CD40L/B Tg $CD4^+$ T cells alone or with both CD40L/B Tg $CD4^+$ T and CD40L/B Tg $B220^+$ B cells, but not CD40L/B Tg $B220^+$ cells alone, develop colitis 4 wk after transfer, indicating that the final cause of colitis is the dysregulated $CD4^+$ T cells. However, mice reconstituted with CD40L/B Tg $B220^+$ B cells and WT $CD4^+$ T cells also developed colitis, whereas mice reconstituted with both WT $B220^+$ B cells and WT $CD4^+$ T cells did not. These differences were confirmed by histological scoring of multiple colon sections obtained from five mice in each group (Fig. 8*C*). This indicates that Tg B cells should be a trigger for this colitis model, and thereafter, dysregulated $CD4^+$ T cells are also needed as final effectors.

Discussion

This report describes a novel model of intestinal pathology induced by primarily B cell-triggered mechanism. To date, studies in animal models of IBD have mainly focused on $CD4^+$ T cells of unknown specificity and in part dendritic cells/macrophages (30) and $CD8^+$ T cells (31). In this study we show that abnormal B cells expressing CD40L can also trigger chronic colitis and may provide a link with autoimmune intestinal inflammation.

It is generally established that the interaction of CD40L on activated T cells with CD40 on APC leads to the secretion of IL-12 by APC, including dendritic cells and macrophages (32–37), and that such interactions are critical for IL-12 production in Ag-driven responses of animal models of colitis (12, 38, 39) and human IBD (9). In contrast, there were no reports that mentioned the involvement of CD40L-expressing B cells in IBD despite some evidence that CD40L is expressed on B cells as well as T cells in patients with SLE (21), and ectopic expression of CD40L on B cells has been observed in lupus-prone BXSB mice (40). The expression of CD40L is activation dependent; CD40L appears on the cell surface within 1–3 h after Ag recognition, and then immediately disappears by 24 h (7). In fact, it was difficult to detect CD40L on any type of cell, probably because CD40L expression in nondiseased young CD40L/B Tg mice was down-regulated in the presence of CD40 as described previously (25, 41). Indeed, we could detect surface expression of CD40L using CD40L/B Tg mice crossed with CD40-deficient mice in the spleen, lymph nodes, and bone marrow (Y. Aiba, details will be described elsewhere). Further study will be required to determine whether CD40L could be expressed on B cells in inflamed mucosa from patients with IBD as well as activated T cells, although it could be technically hard to determine this. Of importance, isolated LPMC from IBD mucosa have been reported to contain more B cells, particularly B cells producing IgG, compared with noninvolved IBD mucosa or control mucosa (42). Furthermore, circulating autoantibodies produced by abnormal B cells, such as colon epithelial cells and leukocyte nuclei, have been consistently identified in patients with UC or CD (43). The accumulating evidence led us to investigate the possible role of CD40L on B cells in the primary development of colitis using CD40L/B Tg mice expressing CD40L ectopically on B cells.

In the present study we demonstrate that CD40L/B Tg mice expressing CD40L on B cells develop chronic enterocolitis, which is first observed between 8 and 15 wk of age with massive infiltration of aggregated IgM-producing B cells in LP, and produce autoantibodies against colonic epithelial cells. First, we postulated that abnormal CD40-CD40L B-B interaction might be induced by ectopic expression of CD40L on B cells and may introduce 1) abnormal Ig isotype switching, 2) consecutive IgM-positive B cell proliferation, and 3) infiltration in the gastrointestinal tract. In contrast, T cells also infiltrate inflamed lesions, but the degree is much less than that of B cells. Interestingly, unlike most other animal models of colitis (1, 5), the CD4/CD8 ratio of LP T cells of inflamed colon was significantly decreased compared with that in WT littermates. These results indicated that CD40L/B Tg mice are sharply distinct from any established T cell/macrophages/dendritic cell-mediated murine models of intestinal inflammation.

Similarly, Clegg et al. (11) reported that CD40L Tg mice overexpressing CD40L on T cells (CD40L/T Tg) acquire a lethal chronic colitis marked by the infiltration of $CD40L^+$ T cells and $CD40^+$ cells into diseased tissues. Interestingly, they showed abnormal thymus development in CD40L/T Tg, such as a transgene copy-dependent decline in thymocyte number, loss of cortical epithelium, and expansion of $CD40^+$ medullary cells (11). In their model they postulated that CD40L/T Tg failed to develop regulatory T cells in thymus that suppress the development of colitis by abnormal T cell development. In addition to a defect in thymic negative selection in CD40L/T Tg, the chronic inflammation in colon might be caused by the continuation of transgene expression in peripheral T cells (11). In contrast, our CD40L/B Tg expressing CD40L on B cells showed no abnormalities in thymus in either flow cytometric or histological analysis (data not shown), indicating that not only could chronic intestinal inflammation in our CD40L/B Tg mice expressing CD40L on B cells be mediated in a thymus-independent manner, but also the CD40L transgene system

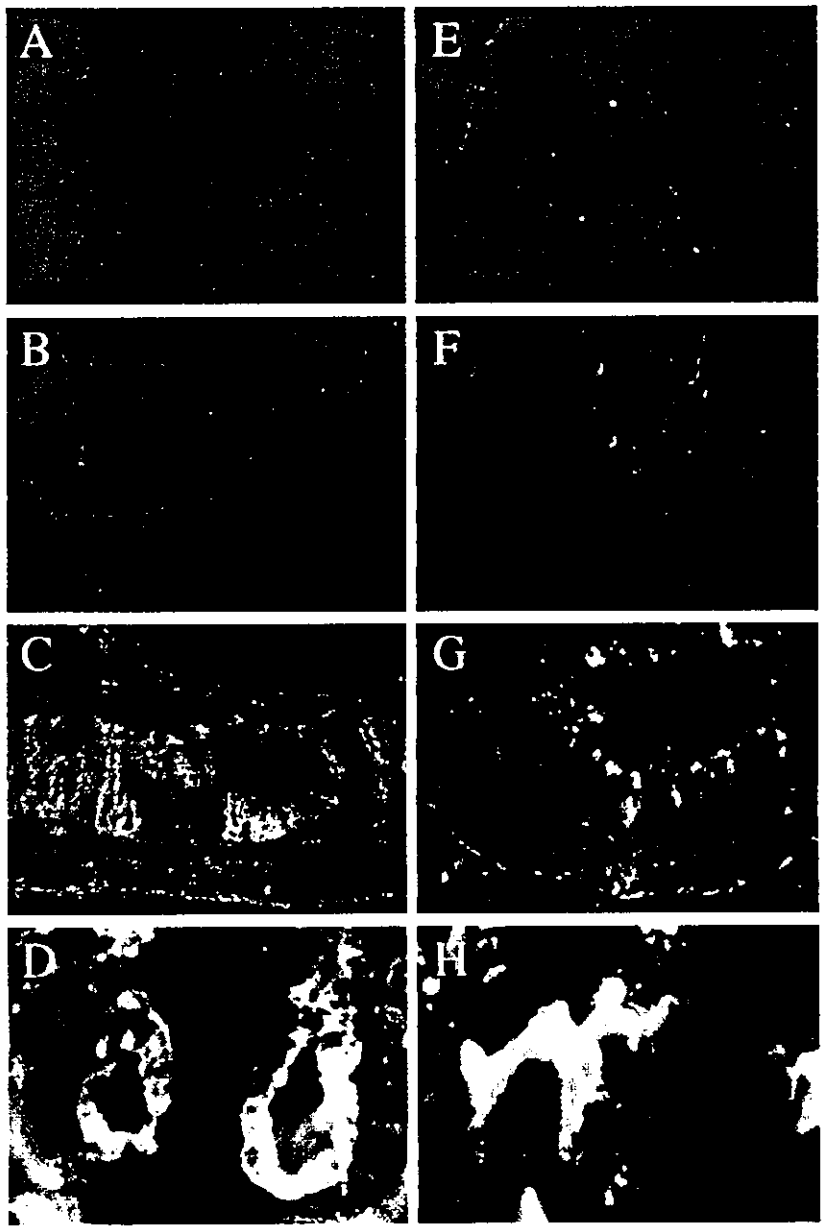

FIGURE 7. Anti-colon Abs were detected in sera from diseased CD40L/B Tg mice. Sera obtained from 12- to 15-wk-old colitic mice stained some surface epithelial cells in the uninflamed colon of young nondiseased CD40L/B Tg mice (*C* and *F*), but this was not observed when sera from WT littermate mice (*A* and D) and nondiseased young CD40L/B Tg mice (*B* and *E*) were used. For the detection of IgM (*A–D*) and IgG type (*E–H*) autoantibodies, FITC-labeled anti-mouse IgM and TRITC-labeled anti-mouse IgG, respectively, were used for the second antibodies. Original magnification: *A–C* and *E–G*, ×200; and *D* and *H*, ×1000. These data were representative of three experiments.

does not introduce CD40L expression into T cells because of the use of a B cell-specific promoter. Nevertheless, the fact that we detected CD40L protein on LP $CD4^+$ T cells from diseased CD40L/B Tg mice suggested that the expression of CD40L on LP $CD4^+$ T cells might be secondary in the reaction against the local intestinal inflammation. Indeed, like most CD40L-mediated animal models of IBD (9, 10, 12–14), LP $CD4^+$ T cells in inflamed mucosa from diseased CD40L/B Tg mice showed IFN-γ-dominant, rather than IL-4-dominant, cytokine production, indicating that our mice might be a good Th1-mediated chronic colitis model. It is also possible that CD40L-expressing LP B cells could directly stimulate CD40-expressing dendritic cells/macrophages to produce IL-12 and to introduce Th1-mediated immune responses. To determine the role of CD40L on B cells in the development of colitis in this model, we next performed an adoptive transfer of isolated T or B cells from diseased CD40L/B Tg and WT littermate mice into SCID mice. We demonstrated that mice transferred with $CD4^+$ T cells alone or with both $CD4^+$ T and $B220^+$ B cells, but not $B220^+$ cells alone, from diseased CD40L/B Tg mice develop colitis, indicating that the final cause of colitis is the dysregulated $CD4^+$ T cells. However, we also demonstrated that mice transferred with $B220^+$ B cells from diseased CD40L/B Tg mice and $CD4^+$ T cells from WT mice also developed colitis, whereas mice transferred with both $B220^+$ B cells and $CD4^+$ T cells from WT mice did not. This indicates that the Tg B cells should be involved in this colitis as a trigger for this colitis model. It should be noted that this transfer experiment also clearly excluded the possibility that the Tg CD40L was expressed on T cells in our Tg system.

In our CD40L/B Tg mice, serum IgM and IgG concentrations were 4- to 5-fold increased. Intestinal mucosal plasma cells producing IgG or IgM were apparently increased, but the number of IgA-producing cells was not significantly different compared with that in WT littermates. Comparison with the findings in human tissue from patients with IBD and in some experimental colitis models reveals some similarities. In human IBD, levels of IgG and IgM are increased along with disease progression. In contrast, it has been demonstrated the level of IgA is decreased in patients with chronic UC (44) and CD (45), but increased in the quiescent disease (46). Furthermore, we detected IgM- and IgG-type autoantibodies

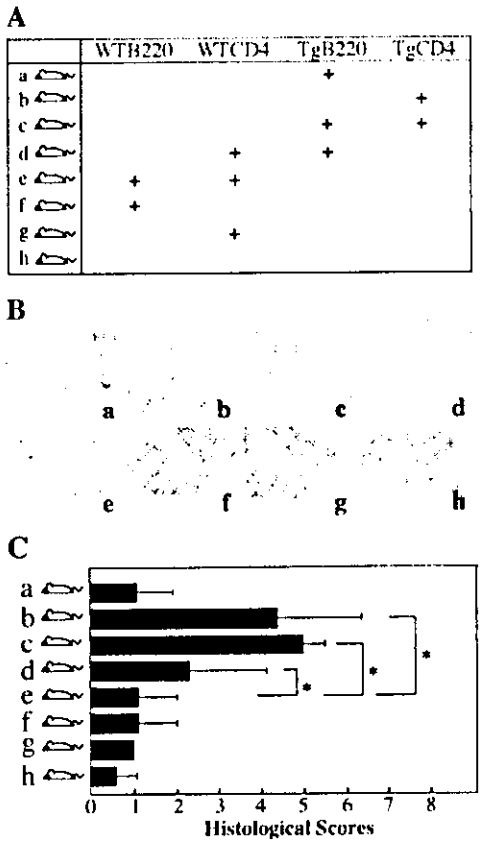

FIGURE 8. Adoptive transfer of sorted T and B cells obtained from spleens and mesenteric lymph nodes. *A*, Transfer protocol. C57BL 6 SCID mice were injected i.p. with one or two of the indicated subpopulations of sorted $CD4^+$ T cells and $B220^+$ B cells from diseased CD40L/B Tg mice (18 wk old) and WT littermates: a, CD40L/B Tg $B220^+$ (5×10^5 body); b, CD40L/B Tg $CD4^+$ (5×10^5 body); c, CD40L B Tg $B220^+$ (5×10^5/body) and CD40L/B Tg $CD4^+$ (5×10^5 body); d, CD40L B Tg $B220^+$ (5×10^5 body) and WT $CD4^+$ (5×10^5/body); e, WT $B220^+$ (5×10^5 body) and WT $CD4^+$ (5×10^5/body); f, WT $B220^+$ (5×10^5/body); g, WT $CD4^+$ (5×10^5 body); or h, no transfer control. Mice were euthanized 4 wk after transfer. *B*, Histopathology of distal colon from each mice. Original magnification, ×100. *C*, Histological scores were determined 4 wk after transfer as described in *Materials and Methods*. Data are presented as the mean ± SD (n = 5). *, $p < 0.05$.

against colonic epithelial cells. T cell-dependent immune responses generally involve conventional B2 B cells. In contrast, the other subset of B cells, B1 B cells, appear to produce Abs in a T-independent manner (27, 47, 48). B1 B cells, originally defined by the surface expression of CD5 and high levels of IgM, arise early in ontogeny, home predominantly to the peritoneal and pleural cavities, have a capacity for self-renewal, and display different receptor specificities. B1 cells appear to recognize self-Ag as well as common bacterial Ags. Production of autoantibodies by B1 cells is also supported by the fact that the neoplastic expansion of B1 cells, such as in chronic lymphocytic leukemia, is often associated with autoimmune symptoms (26, 47, 48). Based on the evidence, we assessed whether autoantibodies are produced via T cell-dependent (B1; $CD5^+IgM^+$) or T cell-independent (B2; $CD5^-IgM^+$) fashion. We showed no increase in $CD5^+IgM^+$ B cells in inflamed mucosa from these mice compared with those in WT littermates, indicating that autoantibodies in diseased CD40L/B Tg mice might be produced by T cell-dependent B2 cells. Alternatively, the autoantibodies in this model could be generated in other tissues, such as peritoneal cavity. In addition, it should be noted that Mizoguchi and colleagues (49, 50) recently demonstrated that highly CD1d-expressing B cells were significantly increased in mesenteric lymph nodes from colitic $TCR\alpha^{-/-}$ mice and had a unique ability to produce IL-10. They postulated that these B cells functioned as regulatory B cells to suppress the progression of intestinal inflammation by down-regulating inflammatory cascades associated with IL-1 up-regulation and STAT3 activation (49, 50). Although there might be a correlation between their regulatory B cells and our pathogenic B cells in the development of colitis at this point, it seems likely that our aggregated IgM-producing, activated B cells in inflamed mucosa from diseased CD40L/B Tg mice did not possess such a regulatory function, but differentiated to more pathogenic cells in the early stage of colitis development. Further study will be required to assess the features of CD40L-expressing B cells, such as cytokine production and APC function.

In conclusion, the present data suggested that under some conditions the CD40-CD40L interaction is constitutively generated on B cells by themselves, and IBD pathogenesis might be established by a primarily B cell-triggered mechanism without involving mainly T cells or macrophages/dendritic cells. Further investigation, such as establishing double-mutant mice crossed between CD40L/B Tg mice and nude mice, may be beneficial to elucidate the primary involvement of B cells in the pathogenesis of IBD.

Acknowledgments

We express special thanks to Dr. Morio Koike for critical comments, and to Saeko Matsumoto for manuscript preparation.

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American Journal of Pathology, Vol. 165, No. 4, October 2004

Gastrointestinal, Hepatobiliary and Pancreatic Pathology

$CD4^+CD45RB^{Hi}$ Interleukin-4 Defective T Cells Elicit Antral Gastritis and Duodenitis

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We have analyzed the gastrointestinal inflammation which develops following adoptive transfer of IL-4 gene knockout ($IL\text{-}4^{-/-}$) $CD4^+CD45RB^{Hi}$ (RB^{Hi}) T cells to severe combined immunodeficient (SCID) or to T cell-deficient, T cell receptor β and δ double knockout ($TCR^{-/-}$) mice. Transfer of $IL\text{-}4^{-/-}$ RB^{Hi} T cells induced a similar type of colitis to that seen in SCID or $TCR^{-/-}$ recipients of wild-type (wt) RB^{Hi} T cells as reported previously. Interestingly, transfer of both wt and $IL\text{-}4^{-/-}$ RB^{Hi} T cells to $TCR^{-/-}$ but not to SCID mice induced inflammation in the gastric mucosa. Notably, $TCR^{-/-}$ recipients of $IL\text{-}4^{-/-}$ RB^{Hi} T cells developed a more severe gastritis with erosion, apoptosis of the antral epithelium, and massive infiltration of macrophages. This gastritis was partially dependent on the indigenous microflora. Recipients of both wt and $IL\text{-}4^{-/-}$ RB^{Hi} T cells developed duodenitis with multinuclear giant cells, expansion of mucosal macrophages, and dendritic cells. Full B cell responses were reconstituted in $TCR^{-/-}$ recipients of RB^{Hi} T cells; however, anti-gastric autoantibodies were not detected. We have now developed and characterized a novel model of chronic gastroduodenitis in mice, which will help in our understanding of the mechanisms involved in chronic inflammation in the upper gastrointestinal tract of humans. ***(Am J Pathol 2004, 165:1257–1268)***

A number of murine models of colitis involve either aberrant T cell or cytokine expression. For example, spontaneous colitis develops in T cell receptor (TCR)-α gene knockout mice where aberrant T cells producing Th2-type cytokines actually mediate disease.[1–3] Perhaps the most useful murine model for both T cell and cytokine regulation of inflammatory bowel disease (IBD) has involved adoptive transfer of $CD45RB^{Hi}$ T cells from normal mice to either SCID[4–6] or to RAG 2 gene knockout ($RAG^{-/-}$) mice.[7,8] The transfer of RB^{Hi} T cells into SCID mice results in development of a severe mononuclear cell infiltration, epithelial cell hyperplasia, and tissue damage. Strong evidence has emerged provided that colitis results from enteric bacterial antigen (Ag)-driven Th1 cell induction,[9] since RB^{Hi} T cell transfer into SCID mice with reduced bacterial flora failed to develop large bowel disease.[10,11] Interestingly, T cell subsets producing TGF-β[12] and IL-10[13] in the RB^{low} T cell population, when co-delivered with RB^{Hi} T cells, actually suppressed colitis. The RB^{low} T cell population was enriched in $CD4^+CD25^+$ T regulatory (Tr) cells.[14] Despite this compelling evidence that RB^{Hi} T cells give rise to effector Th1 cells which mediate colitis, this model had not allowed study of either normal or abnormal B cell participation in the pathogenesis of $CD4^+$ T cells. Since the majority of antibody forming cells (AFCs) in humans and higher mammals reside in the gastrointestinal (GI) tract mucosa,[15,16] the impact of the presence of B cells and plasma cells in the mucosal pathology cannot be ignored. For example, we recently showed that dysregulated Th2 cells cause villus atrophy and goblet cell transformation in the small intestinal epithelium. Further, a wasting disease was seen and was mediated by excess IL-4 and the presence of B cells.[17]

Chronic GI tract inflammation is one of the most common types of inflammatory processes. For many years, gastric ulcers were thought to occur in susceptible individuals, especially those hypersecreting gastric hydrochloric acid.[18,19] However, it has become clear that gas-

Supported by U.S. Public Health Service National Institutes of Health Grants DK 44240, AI 18958, AI 43197, DE 12242, AI 35932, and DC 04976, and by grants and contracts from International Health Cooperation Research, the Ministry of Health, Labor, and Welfare, the Ministry of Education, Culture, Sports, Science, and Technology; the Japan Health Sciences Foundation and Organization; and The Organization for Pharmaceutical Safety and Research.

Accepted for publication June 21, 2004.

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tritis, peptic ulcer disease, and gastric cancer are the result of infection with the bacterium *Helicobacter pylori*.[19,20] Gastritis has been reproduced in experimental animals gastrically infected with either *H. pylori* or related bacteria.[21–24] Thus, gastritis and duodenitis are directly related to *H. pylori* infection; however, surprisingly little is known about what causes chronic inflammatory responses in the upper GI tract. Although many groups have shown that disease severity is related to certain virulence factors associated with *H. pylori*, only a minority of individuals infected with virulent strains of *H. pylori* develop severe disease.[25] In animal models, IL-10-deficient mice infected with *H. pylori* exhibited enhanced gastritis, lower bacterial loads, and higher serum IgG antibody (Ab) titers when compared with control mice.[26] These results have suggested the importance of the host immune system in perpetuating the gastritis and duodenitis. Indeed, a gastritis model in mice with *H. pylori* infection showed that $CD4^+$ T cells were both necessary and sufficient for gastritis, and IFN-γ contributed to this inflammation.[27] Further, oral immunization of mice with the *H. pylori* urease induced protection against *H. pylori* infection, but was often associated with corpus gastritis, which is now recognized as post-immunization gastritis.[28–30] In addition, *H. pylori*-infected SCID mice reconstituted with splenic T cells from *H. pylori*-infected, C57BL/6 mice developed severe gastritis, however, host colonization of *H. pylori* did not correlate to the severity of gastritis.[31] Thus, cell-mediated immunity appears necessary for gastritis development, and does not necessarily relate to the bacterial load. Although still controversial, there is significant evidence that $CD4^+$ Th1-type responses contribute to *H. pylori*-induced gastritis in humans.[32] For example, IFN-γ and TNF-α are the major cytokines produced by gastric T cells from *H. pylori*-infected subjects.[33,34]

In the murine colitis models induced by adoptive transfer of RB^{Hi} T cells, the microflora is necessary for disease; however, no priming of donor T cells with pathogens was required.[10,11] Furthermore, no specific colitogenic bacterial species has been identified to date. Nonetheless, regulation of colitis by Tr cells seems to occur in both innate and adaptive immunity.[35] These results indicate that T cells from normal mice have the potential to induce inflammation in response to the indigenous bacteria; however, the healthy host simultaneously develops Tr cells that attenuate the disease producing T cells. Similar mechanisms for upper GI tract inflammation may be present; however, there have been no reports of a gastritis model in the absence of infection or deliberate immunization, except for autoimmune gastritis induced by neonatal thymectomy[36,37] or ionizing radiation.[38]

In this study, we hypothesized that normal T cells prepared from pathogen-free mice would contain a subset, which potentially could cause inflammation in the upper GI tract, as well as colitis. We describe a new type of RB^{Hi} T cell transfer model in $TCR^{-/-}$ mice that allows assessment of RB^{Hi} T cells in the presence of responsive B cells. We discovered that transfer of RB^{Hi} T cells from IL-4 gene knockout ($IL\text{-}4^{-/-}$) mice resulted in gastroduodenitis in the absence of infection by pathogenic bacteria. Our results suggest the $CD4^+$ Th1-type T cells mediate both colitis and gastroduodenitis in $TCR^{-/-}$ mice reconstituted with RB^{Hi} T cells.

Materials and Methods

Mice

Normal wild-type (wt), $IL\text{-}4^{-/-}$, IFN-γ gene knockout ($IFN\text{-}\gamma^{-/-}$) TCR β and δ chain-defective ($TCR^{-/-}$) and SCID mice on the C57BL/6 background were originally obtained from the Jackson Laboratory (Bar Harbor, ME). This mouse colony was maintained under pathogen-free conditions in flexible Trexler isolators at the University of Alabama at Birmingham (UAB) Immunobiology Vaccine Center Mouse Facility. A separate colony was also maintained in the Immunocompromised Mouse Facility of the Research Institute, International Medical Center of Japan (IMCJ, Tokyo, Japan). We periodically and extensively performed health surveillance on these colonies. These tests were performed in laboratories with expertise in laboratory animal health care (Jackson Laboratories, Charles River Laboratories, Wilmington, MA and Central Laboratories for Experimental Animals, Kawasaki, Japan). This analysis included serological testing for viral infections, bacterial cultures of the nasopharynx, stomach and cecum, ecto- and endoparasitic examinations, and histology of all major organs and tissues. Feces and the stomach were also tested for *Helicobacter spp.* in these laboratories, including *H. hepaticus, H. bilis, H. muridarum, and "H. rappini"*, by PCR.[39–42] No lesions or pathogens including *Helicobacter spp.* and intestinal parasites have ever been detected in these two separate mouse colonies. We obtained essentially identical results in the mouse facilities of both UAB and the IMCJ, and these experiments are quite reproducible. Although original reports indicated that $TCR^{-/-}$ mice develop intestinal inflammation,[3] they did not develop histologically obvious gastritis, duodenitis, or colitis in our mouse facility until they were 24 weeks of age, which is well beyond the time frame of our experiments.

Purification of T Cells

Splenic T cells for adoptive transfer were purified as described previously.[17] In brief, following lysis of erythrocytes, splenic T cells were enriched by passage through a nylon wool column, and then stained with phycoerythrin (PE)-conjugated anti-CD45RB (23G2), FITC-labeled anti-B220, anti-CD11b, and anti-CD8 monoclonal antibodies (mAbs) (BD PharMingen, San Diego, CA). The $CD45RB^{Hi}$ T cell subset was separated by flow cytometry using a FACS Vantage (Becton-Dickinson Co., Sunnyvale, CA). Sorted, $CD45RB^{Hi}$ subsets were > 99% pure by the reanalysis using anti-CD4 Ab, anti-TCR $\gamma\delta$ Ab, anti-NK1.1 Ab, and anti-Gr-1 Ab.

Adoptive Transfer of T Cells

In these studies, purified populations of RB^{Hi} T cells were adoptively transferred to 6- to 8-week-old $TCR^{-/-}$ mice. We routinely transferred 1.0×10^6 T cells by the intrave-

nous (i.v.) route. Body weight was monitored weekly, and mice were taken for analysis when their weight became less than 75% of the initial weight, or at 12 weeks after adoptive transfer of RB^{Hi} T cells.

Histological Analysis

The stomach was removed by excising the esophagus and the anal side of the gastro-duodenal junction, opened along the greater curvature, washed and extended. Next the stomach was cut longitudinally in the middle first, and a 3- to 4-mm-wide strip was prepared. The lateral side of this strip included the fundus, antrum, and gastro-duodenal junction. This side was cut for preparation of sections for histological examination. At least one such section from each mouse prepared in this manner was examined. Duodenal tissues were obtained from 0.5 cm to 3 cm from the gastro-duodenal junction. Colonic tissue was taken from the middle and distal parts of the large intestine. The tissues were opened, fixed in 5% glacial acetic acid in ethanol, and paraffin-embedded. Tissue sections (4 μm) were prepared and stained with hematoxylin and eosin (H&E). In some experiments, 6 μm-frozen sections were prepared, dried and fixed with cold acetone for 10 minutes, and subjected to histological analysis. The immunohistochemical staining was performed using FITC- or biotin-labeled anti-CD3, anti-CD4, anti-B220, anti-CD11b, and anti-CD11c mAbs, respectively (all from BD PharMingen, San Diego, CA), followed by FITC- or TRITC-labeled streptavidin (BD PharMingen). For the detection of IgA^+, IgG^+ or IgM^+ plasma cells, sections were reacted with biotin-labeled anti-mouse IgA Ab, TRITC-labeled anti-mouse IgG Ab and FITC-labeled anti-mouse IgM Ab (BD PharMingen) followed by aminocoumarin-labeled streptavidin. Apoptotic cells were detected by TdT-mediated dUTP nick end labeling (TUNEL) using the Apoptosis *in Situ* Detection Kit *Wako* (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) on paraffin-embedded sections, according to the vender's protocol. To detect myeloperoxidase in granulocytes, sections were directly incubated with a substrate solution, 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in the presence of H_2O_2. Histological scores for gastritis were based on a summation of scores for cell infiltration (0, none; 1, moderate; 2, severe), epithelial hypertrophy (0, none; 1, moderate; or 2, severe), erosion (0, none; 1, focal; or 2, diffuse), giant cells (0, none; 1, less than 2; or 2, more than 3 per section), and deformity of pits (0, none; 1, moderate; or 2, severe). Histological scores for duodenitis and colitis were determined by severity of the inflammation (0, none; 1, mild; 2, moderate; or 3, severe). For histological examination, two observers performed all of the scoring. One was a pathologist who was not involved in this research, and for this examiner, the samples were provided in blinded fashion.

Purification of Lamina Propria Lymphocytes

Peyer's patches were excised from the intestinal wall, and small intestinal lamina propria lymphocytes (LPLs) were prepared as described previously.[43] Briefly, the intestinal tissue was treated with 1 mmol/L EDTA in phosphate-buffered saline (PBS) for 20 minutes to remove the epithelium. The tissue was then digested with type V collagenase (Sigma) for 20 minutes, and this step was repeated once more. The mononuclear cells were further purified by using a discontinuous Percoll gradient of 75% and 40%.

ELISPOT Assay

Enumeration of antibody forming cells (AFCs) was performed as described previously.[44] In brief, 96-well nitrocellulose plates (Millititer HA, Millipore, Bedford, MA) were coated with goat anti-mouse Ig (H+L) [Southern Biotechnology Associates (SBA), Birmingham, AL]. After blocking with 1% bovine serum albumin (BSA) in PBS, the LPLs were incubated for 4 hours at 37°C in moisturized atmosphere of 5% CO_2 in an incubator. After washing the plates, the captured Ig was visualized using peroxidase-labeled anti-mouse IgG, IgA or IgM (SBA). The spots in the individual wells were counted with the aid of a stereomicroscope.

Quantification of Plasma Immunoglobulin Levels

Plasma immunoglobulin (Ig) levels were determined by a sandwich ELISA using the combination of anti-mouse Ig (H+L) (SBA) and peroxidase-labeled anti-mouse IgG, IgA, or IgM (SBA) as described previously.[17]

RT-PCR for mRNA Analysis

Total RNA was prepared from gastric tissue or from separated cells using an RNA-Bee RNA isolation reagent (Tel-Test, Inc., Friendswood, TX). Complementary DNA was synthesized from RNA by reverse transcription (RT). The PCR primers for murine GAPDH used were 5'-AGCCAAACGGGTCATCATCTC and 5'-TGCCTGCTTCACCACCTTCTT; for TNF-α, 5'-TTCTGTCTACTGAACTTCGGGGTCATCGGTCC-3' and 5'-GTATGAGATAGCAAATCGGCTGACGTGTGCC. The step-cycle program was set for denaturing at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 45 seconds for a total of 40 cycles. Expression of mRNA was assessed by quantitative PCR using a SYBR Green PCR Master Mix (Applied Biosystems, Warrington, United Kingdom) and ABI PRISM 7700 Sequence Detector (Applied Biosystems). Quantification of mRNA for IFN-γ and IL-10 was performed using ABI Taqman probes (Applied Biosystems). The PCR primers for IFN-γ were 5'-TGATCCTTTGGACCCTCTGA and 5'-GCAAAGCCAGATGCAGTGT, for IL-10, 5'-GCTCTTGACTACCAAAGCCAC and 5'-CATGCCAGTCAGTAAGAGCAGG. The Taqman probes used for IFN-γ were 5'-CCTCCTGCGGCCTAGCTCTGAGAC and for IL-10, AAGAGAGCTCCATCATGCCTGGCTCA. Threshold cycle numbers (C_T) were determined with Sequence Detector Software (version 1.7; Applied Biosystems) and transformed using the $\Delta C_T/\Delta\Delta C_T$ method as described by the manufacturer, with GAPDH used as the calibrator gene.