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Antibodies to Complement Receptor 3 Treat Established Inflammation in Murine Models of Colitis and a Novel Model of Psoriasiform Dermatitis

Francisco Leon,† Nikhat Contractor,* Ivan Fuss,† Thomas Marth,* Edward Lahey,* Shoko Iwaki,‡ Andrea la Sala,* Victoria Hoffmann,§ Warren Strober,† and Brian L. Kelsall2*

Prior studies indicated the ability of Abs to complement receptor 3 (CR3, CD11b/CD18) to suppress the production of IL-12 from immune cells. Therefore, we tested the ability of an anti-CR3 Ab (clone M1/70) to treat established IL-12-dependent Th1-mediated inflammation in murine models. Systemic administration of anti-CR3 significantly ameliorated established intestinal inflammation following the intrarectal administration of trinitrobenzene sulfonic acid (TNBS-colitis), as well as colitis and skin inflammation in C57BL/10 RAG-2−/− mice reconstituted with CD4+CD45RBhigh T cells. The hyperproliferative skin inflammation in this novel murine model demonstrated many characteristics of human psoriasis, and was prevented by the adoptive transfer of CD45RBlow T cells. In vitro and in vivo studies suggest that anti-CR3 treatment may act, at least in part, by directly inhibiting IL-12 production by APCs. Administration of anti-CR3 may be a useful therapeutic approach to consider for the treatment of inflammatory bowel disease and psoriasis in humans. The Journal of Immunology, 2006, 177: 6974–6982.

Complement receptor 3 (CR3, αM/β2, CD11b/CD18, Mac-1) is a β₂ integrin expressed mainly on polymorphonuclear leukocytes, monocytes/macrophages, NK cells, and certain dendritic cell (DC) populations (1–3). It is a multi-functional glycoprotein that serves as an adhesion molecule, CR, and transmembrane signaling molecule (1–3). It has roles in cell trafficking via its ability to bind to ICAM-1 on activated endothelium, in phagocytosis of microorganisms, such as yeast and bacteria via direct or iC3b-dependent adhesion, and in pathogen killing via its ability to contribute to the release of reactive oxygen and nitrogen molecules (4–6).

CR3 is a heterodimer containing CD11b and CD18 and has two major extracellular binding regions on the CD11b chain. The α-domain mediates adhesion to iC3b, ICAM-1, and fibrinogen (7), and typical of many integrins, its binding affinity is increased dramatically following “activation” by “inside-out” and “outside-in” signaling by chemokines and CR3 ligands, respectively (8). More distal in the C terminus of CD11b is the lectin-binding domain, which recognizes polysaccharides such as LPS, yeast cell walls, and soluble β-glucan, and is important for the formation of membrane signaling complexes with specific GPI-anchored receptors, such as FcγRIIIB (CD16b) and urokinase-type plasminogen activator receptor, which do not signal on their own (9). Via these two binding domains, CR3 can function in innate immunity as a pattern recognition molecule that mediates the phagocytosis and killing of invading pathogens.

Signaling via CR3 may also regulate the production of cytokines such as IL-12 and IFN-α from phagocytes and DCs, which can influence innate, as well as adaptive immunity. In support of this possibility, exposure to CR3 ligands such as iC3b-opsonized Haemophilus influenzae and sheep RBC, as well as certain anti-CR3 Abs, led to down-regulation of IL-12/IL-23p40 and IL-12p70 production in vitro by human monocytes and mouse macrophages (10, 11). In addition, systemic administration of anti-CR3 suppressed the level of IL-12 in the sera of mice injected with LPS (10). Finally, human monocyte-derived DCs produce less IL-12 following exposure to fungi that are known to bind to CR3 (12). These studies demonstrate that signaling via CR3 acts to suppress IL-12 production by macrophages and possibly DCs. Under physiologic conditions, the suppression of IL-12 by CR3 may be important for preventing Th1-mediated inflammation during the scavenging of complement opsonized organisms and apoptotic cells and may help reinforce Th2 responses to extracellular pathogens that require Ab and complement for their clearance (13).

In view of these findings, we evaluated the capacity of an anti-CR3 Ab that suppresses IL-12 production to ameliorate Th1-mediated inflammation in two animal models: colitis induced by the intrarectal administration of trinitrobenzene sulfonic acid (TNBS) and a novel model of simultaneous skin and intestinal inflammation induced by the transfer of CD45RBhigh T cells to C57BL/10 mice. We found that anti-CR3 was effective in the treatment of established gut and skin inflammation in these models and that this was most likely mediated by effects on IL-12 production by macrophages and DCs.

Materials and Methods

Mice

Male SJL/J mice were purchased from The Jackson Laboratory, and female C57BL/10SgSnAi and C57BL/10SgSnAi-(KO) RAG2 (RAG-2−/−) mice.
were purchased from Taconic Farms. All animals used were housed under specific pathogen-free conditions at the National Institute of Allergy and Infectious Diseases. All housing and procedures were performed under an institutionally approved animal protocol.

**Antibodies**

Rat IgG2b anti-mouse CR3 eM chain (CD11b) Ab M1/70.15.11.5.HL (‘M1/70’; American Type Culture Collection) was obtained from hybridoma cultures performed in CELLine CL350 flasks (Integra Biosciences) and purified by protein G affinity chromatography. Rat-neutralizing anti-mouse IL-12/IL-23p40 was obtained from ascites fluid generated in male mice by hybridoma C17.8 (provided by Dr. G. Trinchieri, National Cancer Institute, National Institutes of Health, Bethesda, MD) using standard procedures. Abs were purified from the ascites fluid using ZE-SEP purification kits (Middlesex Sciences). The Abs were assessed to be >95% pure by SDS-PAGE and free of detectable endotoxin by Limulus amoebocyte lysate assay (QLC-1000; BioWhittaker). Ab concentration was determined by ELISA using paired anti-rat IgG2b Abs (BD Pharmingen). As a negative control, we used rat IgG (Chrom-Pure; Jackson ImmunoResearch Laboratories), which is >90% pure and has a low LPS content (<0.5 ng/mg).

Unmanipulated SJLJ mice given M1/70 or control IgG (1.6 mg/mouse, n = 4 for each Ab) experienced no symptoms or weight changes, and IgG-treated colitic mice had mortality and weight loss comparable to PBS-treated mice. Abs against CD3 (145-2C11), CD4 (LT34), CD8a (53-6.7), CD11b (M1/70), CD11c (HLC), CD16/32 (2.4.G2), CD28 (37.51), CD4 (3/23), Gr-1 (Ly6G/C; RB6-8C5), Ly6C (AL-21), CD71 (C2), CD122 (TM-β1), CD45RB (16A), and CD45R/B20 (RA3-6B2) were all purchased from BD Pharmingen. F4/80 Ab was obtained from Serotec. Isotype-matched control Abs used included rat IgG2a (R35-95), rat IgG2b (R35-38 or A95-1), and hamster IgG (G253-256; BD Pharmingen).

**Induction of TNBS colitis**

Colitis was induced in 6- to 7-wk-old, 19–22 g, male SJL/J mice by administration of 2.5% TNBS (picyrsulfonic acid (pH 1.5–2.0); Sigma-Aldrich) in 45% ethanol per rectum through a 3.5 French catheter (Sherwood Medical) as described previously (14). The catheter tip was inserted 4 cm proximal of the anal verge, 150 μl of fluid was slowly instilled into the colon, and the mouse was held in an inverted, vertical position for 30 s. Since weight loss has been shown to be associated with the degree of histological involvement (15), only those animals with a continuous weight loss for at least 4 days after TNBS administration were included in the study (n = 120 of 285 mice in four independent experiments).

**Induction of colitis and psoriasisform dermatitis (PD) by adoptive transfer of CD4⁺CD45RBhigh cells into C57BL/10 RAG-2⁻/⁻ mice**

CD4⁺ T cells were isolated from spleens of 5- to 8-wk-old female C57BL/10SgSnAi mice via negative selection using the MACS CD4 T cell isolation kit (Miltenyi Biotech) and the AutoMACS separation system (Miltenyi Biotech). Enriched cells were subsequently stained for CD4⁻ and CD45RB⁺ and sorted by flow cytometry into fractions containing the 35% of CD4⁺ cells with the brightest CD45RB-staining (CD4⁺ CD45RBhigh) and the 35% with the lowest CD45RB-staining (CD4⁺ CD45RBlow) using a FACSVantage cell sorter (BD Biosciences). Sorted cells were >95% pure. A total of 3 × 10⁵ CD45RBhigh cells or 3 × 10⁵ CD45RBlow plus 3 × 10⁵ CD45RBlow was transferred i.v. or i.p. to recipient 5- to 8-wk-old female RAG-2⁻/⁻ mice. Nontransferred RAG-2⁻/⁻ mice served as controls. Mice were followed for the development of weight loss, which correlates with the development of histological evidence of colitis (16) and for the presence of dermatitis, which only occurred in colitic mice. For therapeutic studies (see below), only mice transferred CD4⁺ CD45RBhigh with continuous weight loss for at least 1 wk, which generally took place 5–6 wk after transfer, were included in the study. Overall, we generated 127 colitic mice in six independent experiments and 32 (25%) of these mice developed skin inflammation.

**Treatment of inflammatory bowel disease and PD**

In the TNBS model of inflammatory bowel disease, the therapeutic capacity of anti-CR3 was assessed by treating mice between day 4 and 7 after administration of TNBS, after mice had reached a minimum of 15% weight loss (average 18%). In each experiment, ~30 colitic mice were randomized into treatment groups that were similar in terms of initial body weight, mean gain in weight and weight before treatment and weight after treatment with PBS alone (n = 25) or 75–100 mg/kg i.p. anti-CR3 (n = 40), rat IgG (n = 55), or anti-IL-12/IL-23p40 (n = 20) as a positive treatment control (14). Weights and clinical status were monitored, and where indicated, treated mice were sacrificed 48 h or 4–5 days after treatment (i.e., 9–11 days after administration of TNBS).

To determine the ability of anti-CR3 to treat colitis and prevent PD in the RAG-transfer model, colitic mice that had not yet developed dermatitis (weeks 6–8 after transfer) were administered control IgG (n = 14) or anti-CR3 (n = 17) at a dose of 50 mg/kg i.p. for three doses over a 2-wk period or left untreated (n = 20). At the time of Ab administration, colitic mice had lost a minimum of 5% body weight (average 8%; in this model, chronic wasting is not severe (15)). Mice were monitored for weight changes and the development of dermatitis for an additional 4–6 wk, at which time mice were euthanized and their colons collected for histology and cell isolation. To assess the capacity of anti-CR3 to treat active PD, 25 colitic mice that had clearly developed dermatitis (weeks 8–9 after transfer) were given PBS (n = 9), control IgG (n = 7), or anti-CR3 (n = 10) at a dose of 50 mg/kg for three doses over 2 wk. The evolution of the lesions was monitored daily and analyzed histologically 3 wk later. In each experiment, mice were randomized into treatment groups with similar initial body weight, percent weight loss, and degree of involvement with dermatitis.

**Histological assessment of inflammation**

Colonic tissue was separated into three pieces of tissue each containing representatively spaced fragments. For each animal, one set of colonic tissue fragments was rinsed in PBS for cell isolation, another was frozen in optimal cutting temperature solution (Sakura Finetek), and the third was fixed in 10% neutral-buffered formalin solution (Sigma-Aldrich). The fixed colonic specimens were embedded in paraffin, cut into 8-mm tissue sections and stained with H&E. Stained sections were examined for evidence of colitis using a previously reported scoring method (17) that uses as criteria the presence of lymphocyte infiltration, the elongation and/or distortion of crypts, the presence of frank ulceration, and the thickening of the bowel wall. Using these criteria, the degree of colonic inflammation was graded from 0 to 4 by two investigators (F. Leon and I. Fuss) in a blinded fashion. Skin fragments were also obtained from grossly involved or healed (in the case of Ab treatment) skin lesions and used for cell isolation, for frozen sections, or for formalin-fixed, embedded, sectioned, and H&E stained.

**In situ staining of tissues**

Myeloperoxidase (MPO) was detected in frozen tissue sections by exposure to Harker Yates’ reagent (Sigma-Aldrich) 1 mg/ml in 10 ml of 0.1 M Tris buffer (pH 7.6) containing 1 μMl ml 3% H₂O₂ for 30 min, followed by counterstaining with hematoxylin and examination by light microscopy (Axioplan; Zeiss). For BrDU labeling of cells in vivo, mice were injected i.p. with 1 mg of BrdU (BD Biosciences). After 6 h, skin specimens were collected and fixed in formalin. Paraffin sections were deparaffinized, subjected to Ag retrieval, and stained for DNA-incorporated BrdU with Abs to BrdU, according to the manufacturer’s instructions (BrdU In Situ Detection kit; BD Biosciences). For immunofluorescence staining, frozen sections were fixed in acetone, and nonspecific staining was prevented by treatment with blocking buffer (1% bovine serum albumin in PBS-Tris; pH 7.5) with blocking reagent; PerkinElmer), Peroxidase Blocking Solution (DakoCytomation), and excess avidin and biotin (Avidin-Biotin Blocking kit; Vector Laboratories). Sections were stained subsequently for CD4 using biotinylated anti-CD4 (clone GK 1.5; ebioscience) or the corresponding biotinylated rat IgG2b, k isotype control Ab, streptavidin-HRP, and Cy3-Tyramide (TSACyamine 3 System; PerkinElmer) and bis-benzimide for nuclear staining (Hoechst 33258; Sigma-Aldrich). Sections were analyzed by epifluorescence with a Zeiss Axioplan microscope. Images were visualized with Openlab 3.0 software (Improvision).

**Isolation of cells**

To obtain lamina propria mononuclear cells (LPCMs), freshly removed colonic specimens were washed in calcium- and magnesium-free HBSS (HyClone) and cut into 0.5-cm pieces. The epithelium was stripped by incubation in complete medium (CM; RPMI 1640 medium (BioWhittaker) supplemented with 2 mM t-glutamine, 10 nM HEPES buffer, 10 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies (BioWhittaker)) containing 1 mM DTT and 1 mM EDTA (both from Sigma-Aldrich) for 1 h under vigorous shaking at 4°C. Remaining colonic tissue was digested in CM containing 400 U/ml collagenase D (Boehringer Mannheim) in a shaking incubator at 37°C. Resulting single-cell suspensions were pelleted and centrifuged through a discontinuous gradient of 100% to 50% Ficoll-Paque PLUS (GE Healthcare Bio-Sciences; “100% Percoll” = 99% Percoll plus 10% PBS; “40% Percoll” is two parts “100% Percoll”; plus three parts PBS). The lymphocyte-enriched population at the 40–100% Percoll interface was collected. Skin cells were isolated by digestion.
of minced skin fragments in CM with collagenase D (400 U/ml) (Boehringer Mannheim) and DNase (30 μg/ml) (Roche) for 60 min at 37°C with agitation and subsequent pipetting and filtering through nylon mesh to obtain a single-cell suspension.

Cell culture and stimulation

LPMCs (1 × 10⁶/ml) and skin cells (2 × 10⁶/ml) were cultured in CM at 37°C under the following stimulation conditions: 1) plate-bound anti-CD3 (10 μg/ml) in carbonate buffer (pH 9.6) overnight at 4°C and soluble anti-CD28 (10 μg/ml) for 48 h; 2) recombinant murine CD40-ligand trimer (10 μg/ml) (Genzyme); 3) recombinant murine IFN-γ (100 ng/ml) (Genzyme) for 18 h, followed by heat-killed, formalin-fixed "Staphylococcus aureus", Cowan strain 1 (SAC, 0.02%; EMD Biosciences) for 24 h. Where indicated, cells were preincubated with anti-CD3 or control IgG (both at 25 μg/ml) for 1.5 h.

Assessment of cytokine production

Cytokine levels were determined in serum and culture supernatant by specific sandwich ELISA for IL-12p40, IL-12p70, IFN-γ (BD RiboQuant System; BD Biosciences), according to manufacturer’s instructions. Quantification of bands was done by densitometry (Molecular Dynamics; Amersham Biosciences) and normalized to bands for GAPDH.

Intracellular calcium release assay

The DC line D1 (DC11b⁺ spleen DC on a C57BL/6 background) (18) was a gift from M. Rescigno (European Institute of Oncology, Milan, Italy) and was cultured in CM supplemented with 30% conditioned supernatant from DCCM cells (GM-CSF-transfected NIH/3T3 fibroblasts) at a final concentration of 10 ng/ml GM-CSF as determined by ELISA (Quantikine; R&D Systems). D1 cells were suspended in phenol red-free HEPES-buffered (50 mM) complete DMEM to a concentration of 1 × 10⁵ cells/ml. Fura 2-AM (2 μM; Molecular Probes) was added, and cells were incubated at 37°C for 40 min in the dark. The suspension was divided into aliquots of 2 ml and placed in the dark at room temperature. Immediately before measurement, each aliquot of cell suspension was washed once with HEPES buffer, [Ca²⁺]i measurement and corresponding excitation scans were conducted using an aliquot of 2 ml of cell suspension each, continually and gently stirred. With a 10-mm quartz cuvette, the fluorescence intensities at emission wavelength 510 nm (5-nm slit) were collected at alternating excitation wavelengths of 340 nm (F₃₄₀/₃₈₀) and 380 nm (F₃₄₀/₃₈₀) for each [Ca²⁺]i measurement and corresponding excitation scans were conducted using an aliquot of 2 ml of cell suspension each, continually and gently stirred. With a 10-mm quartz cuvette, the fluorescence intensities at emission wavelength 510 nm (5-nm slit) were collected at alternating excitation wavelengths of 340 nm (F₃₄₀/₃₈₀) and 380 nm (F₃₄₀/₃₈₀) for each [Ca²⁺]i measurement and corresponding excitation scans were conducted using a fluorescence spectrophotometer (model F-4500; Photon Technology International). The following Abs were added to the cells in agitation: control rat IgG, anti-CD11c/CR4 (αX/β2) (BD Pharmingen), anti-CD11c/CR4 (αX/β2) (BD Pharmingen), anti-CD11c/CR4 (αX/β2) (BD Pharmingen).
or anti-CR3 (M1/70). A dose response was done for each Ab, ranging between 0.25 and 25 µg/ml.

Statistical analysis

Statistical significance was determined by the Student’s t test where indicated. Kaplan-Meier survival curves were evaluated for significant differences between the treatment groups by the log-rank test. Statistical analysis was performed using Prism (Prism).

Results

Therapeutic efficacy of anti-CR3 Ab in TNBS-colitis

In initial studies, we evaluated the capacity of anti-CR3 to treat established colitis induced by the intrarectal administration of the haptenating agent, TNBS. Disease in this model is mediated to a large degree by Th1 cells and can be induced preferentially in SJL/J mice because of susceptibility loci on chromosomes 9 and 11 (15). The locus on chromosome 11 contains a gene that encodes enhanced IL-12p70 responses that is likely to be one of the genes involved in colitis susceptibility (19). For our evaluation of anti-CR3, we chose a nondepleting anti-murine CR3 (anti-CD11b, clone M1/70) since this Ab does not react with the I-domain of CR3 and does not block transendothelial leukocyte migration (20). This allowed us to study a direct effect of anti-CR3 on cell function rather than on cell migration.

Anti-CR3 was given to SJL/J mice between day 4 and 7 after initiation of colitis by intrarectal TNBS, well after colitis had been established (Fig. 1). For comparison, we administered anti-IL-12/IL-23 (p40 subunit), an Ab shown previously to be an effective treatment of TNBS-colitis, and control IgG to identical groups of SJL/J mice with established colitis. Mice with TNBS-colitis given control IgG displayed thickened and shortened colons typical of inflammatory colitis (data not shown) and on microscopic evaluation had severe colonic inflammation (Fig. 1a). In contrast, anti-CR3- and anti-IL-12-treated mice had less severe colonic inflammation characterized by mild lymphocyte infiltration and an intact epithelial cell layer. These findings were reflected in histological colitis scores that were significantly lower in anti-CR3-treated mice than in control IgG-treated mice 48 h after treatment (Fig. 1b) and throughout the course of the study (data not shown).

In addition, colitic mice given control IgG suffered significantly higher mortality (mean survival 35%) than anti-CR3 (62% survival)- or anti-IL-12-treated mice (73% survival; p < 0.05 for both treatments; Fig. 1c and data not shown). Furthermore, surviving anti-CR3-treated, as well as anti-IL-12-treated, mice recovered their initial weight, whereas surviving control IgG-treated mice exhibited no weight gain (p < 0.005; Fig. 1d). Finally, analysis of the inflammatory infiltrate in the various treatment groups by flow cytometry revealed that 48 h after treatment, colitic mice treated with IgG had a significantly elevated proportion of LPMCs expressing CD4 (mainly T cells), CD11c (mainly DCs), and Gr-1 (mainly neutrophils and macrophages), whereas anti-CR3-treated mice showed significantly less colonic infiltration by these subsets of cells (Fig. 1e). A reduction in mucosal neutrophils by anti-CR3 treatment was also supported by studies demonstrating a decrease in the number of cells staining for MPO in situ (Fig. 1f). Taken together, these studies demonstrated that anti-CR3 was efficacious in the treatment of established TNBS-colitis.

Therapeutic efficacy of anti-CR3 Ab in cell-transfer colitis

In further studies, we used a newly developed cell-transfer model to study the therapeutic effects of anti-CR3 Ab on both chronic intestinal inflammation and psoriasisform skin disease. To establish this model we adoptively transferred C45RB<sup>high</sup>CD4<sup>+</sup> T cells from WT C57BL/10 mice to recipient C57BL/10-RAG-2<sup>−/−</sup> mice by i.v. injection. The C57BL/10 strain was chosen because previous studies had shown that C57BL/10 mice, while sharing significant genetic identity with C57BL/6 mice, have a locus on chromosome 11 not present in C57BL/6 mice that contains a gene termed <i>tnbs1</i> (alluded to above in relation to TNBS-colitis) encoding increased susceptibility to TNBS-colitis and increased IL-12p70 response to LPS (19); thus, cell-transfer colitis in C57BL/10 mice was expected to result in more widespread and/or more severe inflammation than in C57BL/6 mice.

We observed that 60% of adoptively transferred mice developed colitis 4–6 wk after transfer of cells and 80% of mice developed colitis 7–9 wk after transfer. As in previous studies of CD4<sup>+</sup>CD45RB<sup>high</sup> transfer models, colitis correlated with the development of weight loss and the appearance of mononuclear cell infiltration of the lamina propria (data not shown). Parallel studies in which colitis induction was achieved by C57BL/6-cell transfer to C57BL/6-RAG-1<sup>−/−</sup> mice yielded similar degrees of intestinal inflammation (data not shown); however, the frequency of mice developing colitis (~20%) was much less than in the adoptively transferred C57BL/10 mice.

To evaluate the capacity of anti-CR3 to treat intestinal inflammation in this model, we administered either anti-CR3 Ab or control rat IgG or PBS to randomized groups of mice with established colitis 6–8 wk after adoptive transfer. Three weeks later, the mice were evaluated for evidence of colitis (Fig. 2). Mice that received control rat IgG (Fig. 2) or PBS (data not shown) exhibited severe transmural colitis as described above with an inflammation score...
of >3.5. In contrast, mice that received anti-CR3 Ab exhibited significant amelioration of colitis with an inflammation score of <2. These results indicate that anti-CR3 has efficacy in the treatment of both short-lived (TNBS-colitis) and a long-lived (cell-transfer colitis) colonic inflammation.

**Therapeutic efficacy of anti-CR3 Ab in a novel model of PD**

In contrast to C57BL/6 mice, the development of colitis in C57BL/10 mice was associated with the appearance of dermatitis in ~25% of mice. Dermatitis only occurred after the onset of weight loss, with skin lesion appearing 5–9 wk after transfer. The dermatitis in this model was found to be similar to human psoriasis (21). Macroscopically it consisted of areas of alopecia and scaly dermatitis affecting some or all of the following areas: abdomen, back, legs, neck, forehead, tail, ear, and eyelid (Fig. 3a). Microscopically, it was characterized by hyperkeratosis, increased epidermal thickness, dermal inflammation, acanthosis (Fig. 3, b and c), and the occasional presence of neutrophilic abscesses (Fig. 3d). In addition, areas of parakeratosis (Fig. 3e) and the presence of CD4+ cells in the epidermis (Fig. 3f) could be identified. The increased epidermal thickness was caused by an excessive proliferation of basal keratinocytes, as revealed by in vivo labeling of proliferating cells with BrdU (Fig. 3g), which occurred only in areas of adjacent dermal inflammation (Fig. 3h).

H&E and MPO staining of tissue sections (Figs. 3 and 4) and flow cytometric analysis of isolated cells (data not shown) revealed that the infiltrating cells were mainly neutrophils and CD4+ lymphocytes. In addition, RPAs performed with skin biopsies showed an increase of mRNA for IFN-γ and IL-1β in affected skin of four of six mice tested (Fig. 3i), similar to human psoriasis (22), whereas mRNA for IL-12/IL-23p40, IL-12p35, IL-18, IL-2, IL-1α, IL-1Ra, and IL-6 were found increased in less than half of the lesions examined (data not shown). Th2 cytokines (IL-4, IL-5, and IL-13) were undetectable in lesions from both treated and untreated mice, and IL-10, IL-15, IL-9, and MIF were normal or undetectable in affected skin (data not shown). Infection was excluded by microbiological culture, revealing only normal flora, and by negative fungal and bacterial staining of affected skin. There

**FIGURE 3.** PD is present in C57BL/10 RAG-2−/− mice transferred CD45RBhigh T cells. a. Representative photographs of affected skin in a RAG-2−/− C57BL/10 mouse 3 wk after the onset of the lesion (8 wk after reconstitution with CD4+CD45RBhigh T cells) displaying alopecia, erythema, and scales. b. Representative H&E stains of fixed tissue sections of the tail and ear, and (c) dorsal skin, of a normal mouse (no PD), and an affected mouse 5 wk after the onset of the skin lesions (PD), illustrating hyperkeratosis, increased skin thickness (acanthosis), dermal inflammation, and invagination into the dermis (rete pegs) of the PD. d. H&E-stained tissue section showing the presence of an intradermal neutrophilic abscesses (arrow; 3-wk-old lesion). e. Double staining of CD4 (Cy3, red) and nuclei (Hoechst 33258, blue) superimposed on a white-light image of this fragment of affected skin, revealing the presence of nucleated cells in the corneal layer of the epidermis (parakeratosis). f. Small, round CD4+ cells, likely T cells, in the epidermis, while larger and irregularly shaped CD4+ cells (likely macrophages and/or DCs) are present in the dermis. g. Anti-BrdU staining of proliferating cells in normal skin (no PD) and affected skin from mice transferred CD4+CD45RBhigh T cells (PD). The staining demonstrates the hyperproliferation of basal keratinocytes in PD; nontransferred RAG-2−/− and mice cotransferred CD4+CD45RBhigh and CD4+CD45RBlow cells were similar to the WT control (data not shown). h. H&E-stained tissue section showing epidermal hyperproliferation only in areas with adjacent dermal inflammation (arrow indicates limit between normal and inflamed tissue; 3-wk-old lesion). i. GAPDH-normalized relative values of mRNA for IFN-γ and IL-1β present in full thickness skin tissue from different groups of mice (NT, nontransferred RAG-2−/−; no PD, CD4+CD45RBhigh-transferred RAG-2−/− mice that did not develop PD; PD, CD4+CD45RBhigh-transferred RAG-2−/− mice with PD), as assessed by RNase protection assay. **, p < 0.05; ***, p < 0.005. j. Incidence of PD in normal RAG-2−/− mice (nontransferred), RAG-2−/− mice transferred both CD4+CD45RBhigh and CD4+CD45RBlow cells, and RAG-2−/− mice transferred only CD4+CD45RBhigh cells over the course of 12 wk after transfer. **, p < 0.05.
was no evidence of epidermal necrosis, in agreement with the absence of GVHD in this syngeneic transfer model. Finally, both the colitis and dermatitis were completely prevented by the cotransfer of regulatory lymphocytes contained in the CD45RB<sup>low</sup> fraction (Fig. 3j).

None of the nontransferred RAG<sup>2−/−</sup> (n = 15) or WT CD57BL/10 (n = 20) mice developed colitis or dermatitis disease over the 11–12 wk observation period of these studies. Interestingly, there appeared to be an inverse correlation between the severity of colitis and dermatitis in that those with colitis only lost an average of 8.5% of body weight by week 6–7 after transfer, whereas those with colitis and dermatitis lost 2.2% of body weight at this time point. This was in contrast to nontransferred mice, which gain weight over this time frame.

We next evaluated the therapeutic effect of anti-CR3 on both the prevention and treatment of the PD induced in this cell-transfer model. As in the studies of colitis described above, we first induced colitis in BL10-RAG<sup>2−/−</sup> mice, and then, 6–8 wk after transfer, before the development of dermatitis, we administered three doses of anti-CR3 Ab or control rat IgG or PBS over a 2-wk period. Mice were monitored for the development of PD for 4 wk. Whereas no mice treated with anti-CR3 (0 of 7) subsequently developed skin lesions, 33% of mice given rat IgG or PBS (9 of 27) developed skin lesions (data not shown).

To determine the effect of anti-CR3 treatment on established skin disease (Fig. 4), mice with PD were given three doses of anti-CR3, control IgG, or PBS (data not shown), and lesions were followed for an additional 4 wk. While all control IgG or PBS-treated mice had stable or progressive skin inflammation over this time frame, anti-CR3 treatment resulted in a partial response (macroscopic improvement in lesion severity and a reduction in size of the lesions) in 40% of treated mice, and a total response (macroscopic normalization with hair regrowth, and histological resolution with reduced acanthosis, epidermal thickness, and inflammation in affected areas) in 30% of treated mice (Fig. 4a). This resolution was also accompanied by near normalization of MPO<sup>+</sup> cells and CD4<sup>+</sup> cells in the formerly affected skin areas (Fig. 4b). Only 28% of mice with partial or complete resolution relapsed with worsening skin lesions 4 wk after anti-CR3 treatment, indicating that in many cases the effect of therapy was durable.

**Mechanism of action of anti-CR3 Ab treatment**

Finally, we addressed the possible mechanisms by which anti-CR3 administration resulted in the treatment of established colitis and dermatitis. First, as previously mentioned, treatment with anti-CR3 reversed the inflammatory process in mice with TNBS colitis with a loss of CD4<sup>+</sup> T cells, CD11c<sup>+</sup> DCs, and granulocytic infiltration into the colonic lamina propria (Fig. 1, f and g). This was accompanied by a suppression of the production of IFN-γ (Fig. 5a) and IL-12p70 (data not shown) production by LPMCs, similar to what was seen with anti-IL-12 treatment of established TNBS-colitis (14). In addition, treatment of mice with colitis in either model with anti-CR3 resulted in a suppression of serum levels of IL-12p40 and p70 (Fig. 5b). The suppression of serum IL-12 levels occurred very shortly (48 h) after anti-CR3 treatment in TNBS colitis, a time at which significant numbers of inflammatory cells were still present in the colonic lamina propria in these mice, indicating that anti-CR3 treatment likely had direct effects on IL-12 production. Furthermore, anti-CR3 induced a calcium flux upon cell binding (Fig. 5c), indicating that it provides an active signal to cells. Finally, anti-CR3 inhibited the in vitro promotion of IL-12/IL-23p40 by a murine DC line (Fig. 5d) and by murine peritoneal macrophages (Fig. 5e), which is consistent with prior studies (10, 11).

**Discussion**

In this study, we demonstrated the ability of Abs to CR3 (CD11b/CD18 and Mac-1) to treat established colitis in two animal models and to prevent and treat skin inflammation in a novel model of psoriasis. Both colitis models have some features of Crohn’s disease in humans and represent two paradigms of abnormal intestinal Th1 responses, one due to excessive Th1 effector-cell activity (TNBS-colitis), and the other due to a lack of regulatory T cell counterregulation (CD45RB<sup>high</sup> transfer) (15). In addition, both models have been used successfully to test novel therapeutic strategies for chronic intestinal inflammation, such as the use of anti-IL-12 Abs (14, 23), recently shown to be safe and possibly efficacious in humans (24).

The idea to pursue the therapeutic use of anti-CR3 stemmed from prior studies demonstrating that certain monoclonal anti-CR3 Abs can prevent IL-12 production by human monocytes in vitro and can block the induction of both IL-12 and IFN-γ following LPS challenge of mice in vivo. It is further based on studies demonstrating that, as in the case of Crohn’s disease in humans, both TNBS and adoptive transfer colitis are mediated in large measure by Th1 cells that are dependent on IL-12 for their induction (15). Importantly, the cell-transfer colitis...
on the C57BL/10 background is associated with high levels of IFN-γ but low to undetectable levels of IL-17 or IL-12p19 in the colitic tissues as determined quantitative RT-PCR (data not shown). Therefore, while the effects of anti-CR3 may affect IL-23 expression, as well as IL-12p70, the primary effects in the adoptive transfer model of colitis on the C57BL/10 background are likely suppression of IL-12p70 and subsequent effects mediated by Th1 cells. Finally, there are theoretical reasons why anti-CR3 could be better for treating Th1-mediated inflammatory disease in humans than Abs directed to IL-12p40. First, in addition to inhibiting IL-12 production, anti-CR3 can block the induction of IFN-γ by human monocytes in vitro (10). Since IFN-γ can directly activate Stat-4 in human (but not mouse) cells (25, 26), similar to IL-12, IFN-γ may provide an additional stimulus for Th1 differentiation that would be blocked by anti-CR3 treatment. Second, certain anti-CR3 Abs (although not those used in the present study; see below) can block the activation of inflammatory cells, such as neutrophils and macrophages, or their trafficking into tissues so that anti-CR3 could potentially affect additional inflammatory cell functions unrelated to Th1 differentiation. Indeed, anti-CR3 Abs with a proven capacity to block leukocyte migration have been successfully used to ameliorate inflammation in murine models of experimental autoimmune encephalitis (27, 28) and to prevent, but not treat, inflammation in the colon of rats given TNBS (29).

We extended therapeutic studies of anti-CR3 to skin inflammation following the discovery that the transfer of CD45RB high T cells into mice on a C57BL/10 background resulted in the development of psoriasis-like skin lesions when compared with cell transfers in C57BL/6 mice. Although both strains of mice were susceptible to the development of colitis on the C57BL/10 background was also significantly higher that with C57BL/6 mice. Interestingly, this difference may be due to the fact that C57BL/10 mice produce high levels of IL-12, at least in response to LPS, when compared with C57BL/6 mice, a trait that has been genetically mapped to chromosome 11 (19). Thus, a combination of IL-12 overproduction with the lack of CD25+ regulatory T cells may account for the increased susceptibility to colitis and skin inflammation in this model.
Several other aspects of this model are of interest. First, the inflammatory skin disease shares many features of human psoriasis, such as the presence of acanthosis, hyperkeratosis, and the accumulation of a mixed inflammatory cell infiltrate (22). Second, CD4⁺ T cells are sufficient to induce skin disease, as has been observed previously in studies of the adoptive transfer of T cells into SCID mice engrafted with human psoriatic skin (30) and with adoptive transfer of naive T cells into immunodeficient hosts with minor histocompatibility Ag mismatches (31, 32). Third, the cotransfer of CD4⁺CD45RB⁺ T cells, which contain CD4⁺CD25⁺ regulatory T cells, with CD4⁺CD45RB⁺ T cells prevented the development of skin lesions, demonstrating that this cutaneous inflammatory process is subject to control by regulatory T cells, similar to what has been suggested for human psoriasis (33). Fourth, the psoriasis-like skin lesions occurred simultaneous with colitis, which reflects the reported (albeit weak) association of psoriasis with Crohn’s disease in humans (34, 35). Fifth, and finally, while this model shares many characteristics of the skin and intestinal inflammation that occurs following adoptive transfer of naive CD4⁺ T cells into immunodeficient mice with minor histocompatibility Ag mismatches (31, 32, 36, 37), including the control of lesions by CD4⁺CD45RB⁺ T cells, it occurs in a fully syngeneic system. Therefore, it is likely that stimuli normally present on the skin can induce inflammation in the absence of appropriate T cell regulation and the presence of certain genetic factors, such as the high sensitivity to TLR-driven IL-12.

There are two possible mechanisms by which anti-CR3 may suppress IL-12/IL-23p40 and IL-12p70 production. One possibility is related to the fact that CR3 has a LPS-binding site on the β2 chain (CD18) (38, 39) and contributes to the LPS binding complex in membrane lipid rafts (38). Accordingly, CR3 has been shown to provide a positive signal for the production of IL-12 induced by low-dose LPS (40), and this could be blocked by anti-CR3. Our data demonstrating that anti-CR3 inhibits IL-12 and IFN-γ production to systemically administered LPS are consistent with these findings. Given the fact that LPS may be an important stimulus to the initiation of Th1 responses (e.g., by providing an impetus for early IL-12 induction), anti-CR3 can be inhibiting Th1 inflammation by inhibiting LPS effects.

A second possible mechanism of the effect of anti-CR3 on IL-12 production is that anti-CR3 provides a direct inhibitory signal for the production of IL-12. Several lines of evidence support this possibility. First, CR3 is not required for IL-12 production, in that cells from CR3-deficient mice produce normal levels of IL-12p40 or p70 in response to high doses of LPS, other TLR stimuli (Ref. 40; data not shown), or to CD40L and IFN-γ (Fig. 5e). Furthermore, CR3-deficient mice mount normal Th1 responses to Mycobacterium tuberculosis (41). Second, the anti-CR3 Ab used in the present study induces a calcium flux in exposed DCs (Fig. 5c), indicating its ability to provide a direct signal, rather than simply acting to block signaling to a competing CR3 ligand. Third, anti-CR3 can inhibit IL-12 production to ligands other than LPS, including additional TLR-ligands and CD40-ligand, which are not likely directly binding to CR3 (10, 11). Fourth, anti-CR3 acts to selectively suppress IL-12 production, as TNF-α and IL-10 production are not affected (10, 11). Therefore, anti-CR3 is not simply blocking all positive signals through this receptor. Fifth, and finally, other “activating” CR3 ligands, such as Histoplasma capsulatum, and iC3b-opsonized erythrocytes can also inhibit the production of IL-12 in response to diverse IL-12 inducers (10, 11). Therefore, we propose that anti-CR3 can act to regulate IL-12 production in two ways, one by blocking signaling in response to low doses of LPS, and another by providing a direct inhibitory signal to IL-12-producing cells.

As alluded to above, CR3 has a complex role in the function of APCs and neutrophils, and thus, the ability of anti-CR3 to ameliorate inflammation is not necessarily attributable only to its ability to down-regulate production of key Th1 cytokines. Another possible mechanism of action relates to the role of CR3 in transendothelial migration and the potential ability of blocking anti-CR3 to inhibit such migration. This is particularly relevant to the function of neutrophils, cells that use the 1 domain of β2 integrins (such as CR3) to bind to endothelial ICAMs (42), which is thought to contribute to the ability of these cells to penetrate the endothelial barrier and enter sites of inflammation (43, 44). In addition, CR3 is a costimulatory molecule in C5a-dependent superoxide production by neutrophils and thus has a key role in one mechanism through which neutrophils cause tissue damage. Here again, blocking anti-CR3 has a down-regulatory role in that it inhibits such superoxide production. It is important to remember, however, that the Ab used in these studies, M1/70, does not react with and block the domain of CR3 involved in neutrophil migration and may not react with and block the domain relating to superoxide formation. However, it is possible that other mAbs will be found that have the function of M1/70 as well as the capacity to block other inflammatory functions of CR3.

In summary, in this study we have established that anti-CR3 can function as a potent inhibitor of Th1 responses underlying two major types of experimental inflammation, acute and chronic colitis and PD. While we present evidence supporting the possibility that in the mouse models the mechanism of this effect is the down-regulation of IL-12/IL-23p40 and IL-12p70 production, in humans, the potential of anti-CR3 to decrease IFN-α production may also play a role. In the course of these studies, we described a novel model of intestinal inflammation and PD in C57BL/10 mice mediated by CD4⁺ T cells and controlled by CD4⁺CD25⁺ regulatory T cells. This model should prove useful in the further evaluation of these forms of inflammation.

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Disclosures
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6982 ANTI-CR3 TREATMENT OF GUT AND SKIN INFLAMMATION
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