

Blocking the CD40L-CD40 Interaction In Vivo Specifically Prevents the Priming of T Helper 1 Cells through the Inhibition of Interleukin 12 Secretion

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Summary

The recent finding that CD40L on activated T cells induces interleukin (IL) 12 secretion in human peripheral blood monocytes in vitro suggests that the CD40L-CD40 interaction may be of importance in the priming of T helper (Th) 1-type T cells. We therefore investigated the in vivo relevance of this interaction in an experimental model for a Th1-mediated disease, the hapten reagent (2,4,6-trinitrobenzene sulfonic acid [TNBS])-induced colitis. The administration of anti-gp39 (CD40L) antibodies during the induction phase of the Th1 response prevented interferon γ production by lamina propria CD4⁺ T cells and also clinical and histological evidence of disease. In contrast, the secretion of IL-4, a Th2-type cytokine, was increased after anti-gp39 treatment. In further studies we showed that the prevention of disease activity was caused by an inhibition of IL-12 secretion, as demonstrated by immunohistochemistry. In addition, the injection of recombinant IL-12 p70 heterodimer into TNBS + anti-gp39-treated mice reversed the effect of anti-gp39 and resulted in severe disease activity. When anti-gp39 was given after the disease was established, no effect on the disease activity was observed. In conclusion, we demonstrated that the CD40L-CD40 interaction is crucial for the in vivo priming of Th1 T cells via the stimulation of IL-12 secretion by antigen-presenting cells (APC).

The presence of specific cytokines is the major factor that influences naive T cells to become a Th1 (IL-2, IFN- γ) or a Th2 T cell (IL-4, IL-5, IL-6, IL-10, and IL-13) (for review see reference 1). Thus, IL-4 plays a critical role in the priming of Th2 cells (2), and IL-12 alone (3) or together with IFN- γ (4) drives naive T cells into the Th1 lymphokine-producing phenotype. Various other aspects of APC-T cell interactions, however, may also influence Th1/Th2 T cell development, either directly or via an effect on cytokine secretion. The first aspect suggests differential signaling through the costimulatory molecules B7-1 and B7-2, with B7-1 costimulation leading to a Th1 phenotype and signaling through B7-2 resulting in a Th2 phenotype (5, 6). The second aspect involves the density of specific peptide-MHC class II complexes on the membrane of an APC, with a high density of these complexes favoring Th1 priming and low densities causing Th2 priming (7). Third, the interaction of CD40L on activated T cells with CD40 on APC has been implicated to be important in T cell priming: a very recent in vitro study by Shu and co-workers demonstrated that this interaction leads to the secretion of IL-12 by human monocytes, which could favor the priming of Th1 cells (8).

We investigated the in vivo relevance of the CD40L-CD40 interaction for the priming of Th1 T cells in a recently developed animal model for chronic intestinal inflammation

in the mouse, the hapten reagent (2,4,6-trinitrobenzene sulfonic acid [TNBS])-induced colitis. This experimental animal model has been shown to be a Th1-mediated disease by the fact that the infiltrating CD4⁺ T cells secrete predominantly IFN- γ . In addition, the treatment of TNBS-induced colitis with antibodies against IL-12, which regulates IFN- γ secretion as mentioned above, abrogated the disease. Finally, the colitis was not inducible in IFN- γ ^{-/-} gene-targeted mice (9). In this study we report that treatment with anti-gp39 (CD40L) antibodies during the induction of Th1-mediated colitis prevented the disease as well as the occurrence of IFN- γ -secreting T cells in the lamina propria (LP). We further observed a decrease in IL-12 production by infiltrating cells after this antibody treatment. If we injected rIL-12 into TNBS + anti-gp39-treated mice, the effect of anti-gp39 was reversed, suggesting that in our experimental model for a Th1-mediated disease, the interaction between CD40L on activated T cells and CD40 on APC is of crucial importance for the priming process during the differentiation of Th1 T cells but not for later differentiation stages of Th1 effector cells.

Material and Methods

Induction of Colitis. Specific pathogen-free 2-4-mo-old female SJL/J mice (National Cancer Institute, Bethesda, MD) were in-

trarectally injected with 0.5 mg of the hapten reagent TNBS (Sigma Chemical Co., St. Louis, MO) in 50% ethanol as previously described (9). In control experiments, mice received 50% ethanol alone. The total injection volume was 100 μ l in both groups.

Treatment with Anti-gp39 (CD40L) and IL-12. After induction of colitis with TNBS, the mice were either treated with 0.5 mg of monoclonal anti-gp39 (CD40L) antibody (MR-1) (10) or hamster IgG (haIgG) as control (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) on days 0 and 2. Mouse rIL-12 p70 heterodimer (100 ng) (kindly provided by Dr. M. Gately, Hoffman-LaRoche, Nutley, NJ) was injected into some of the anti-CD40L-treated mice on days 0, 2, and 4. In addition, to investigate late effect of anti-gp39 treatment, we treated a second group of mice with anti-gp39 antibodies at days 7 and 9.

Immunohistochemistry and Hematoxylin and Eosin (H&E) Staining. Tissue samples were frozen on dry ice, and 7- μ m cryosections were cut according to standard procedures. Sections were fixed in cold acetone. Some sections subsequently underwent the standard procedures for H&E staining. Others were rehydrated in PBS, blocked with 2% milk powder in PBS, and incubated with anti-IL-12 p40 subunit (C17.8; kindly provided by G. Trinchieri, Wistar Institute, Philadelphia, PA) for 60 min. After the incubation with biotinylated rabbit anti-rat antibody and with the ABC-HRPO solution (ABC-Kit, Elite; Vector Laboratories, Inc., Burlingame, CA), the color reaction was performed with the diaminobenzidine substrate for alkaline phosphatase (Vector Laboratories, Inc.), and the sections were counterstained in 5% methyl green, air dried, and mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ).

Isolation and Culture of LP CD4⁺ T Cells. LP lymphocytes were isolated from freshly obtained colonic specimens using a modification of the technique described by van der Heijden and Stok (11). Enriched CD4⁺ T cell populations were obtained by negative selection using mouse CD4⁺ T cell isolation columns (Isocell; Pierce Chemical Co., Rockford, IL). The resultant cells when analyzed by flow cytometry (FACSscan[®]; Becton Dickinson & Co., Mountain View, CA) contained >85% CD4⁺ cells. Cell cultures of LP CD4⁺ T cells were performed as previously described (9).

Reagents and mAbs. Unconjugated and biotinylated rat anti-mouse IL-4 (BVD4-1D11/BVD6-24G2), and IFN- γ (R4-6A2/XMG1.2) mAbs were obtained from PharMingen (San Diego, CA). IL-4 (10⁷ U/mg by CTL2-2.4 assay) and IFN- γ (10⁷ U/mg) were purchased from PharMingen and Genzyme Corp. (Cambridge, MA), respectively. Purified hamster anti-mouse CD3 ϵ (clone 145-2C11) and hamster anti-mouse CD28 (clone 37.51) antibodies were bought from PharMingen.

Cytokine Assays. To measure cytokine production, 10⁵ LP CD4⁺ T cells were placed in anti-CD3-precoated wells or in uncoated control wells, and 1 μ g/ml soluble anti-CD28 antibody was added to the cells in the anti-CD3 ϵ -coated wells. Culture supernatants were removed after 48 h and assayed for cytokine concentration. Cytokine concentrations were determined by specific ELISA according to the manufacturer's recommendations (PharMingen). ODs were measured on an ELISA reader (model MR 5000; Dynatech Laboratories, Inc., Chantilly, VA) at a wavelength of 490 nm.

Results and Discussion

In Vivo Administration of Anti-gp39 Antibodies in Mice Prevents TNBS-induced Colitis but Does Not Abrogate Established Disease. Previous studies in our laboratory have estab-

lished that similar to the situation in the rat (12, 13), intrarectal instillation of TNBS in mice causes a severe chronic intestinal inflammation marked by massive diarrhea, weight loss, and anal prolapse that mimics some aspects of Crohn's disease in humans (9). This colitis is mediated by Th1-type T cells since the infiltrating CD4⁺ T cells produce increased amounts of IFN- γ and decreased amounts of IL-4 compared with control animals. In addition, IFN- γ ^{-/-} gene-targeted mice did not develop colitis after the application of TNBS, and in vivo administration of anti-IL-12, which negatively regulates IFN- γ expression (14), abolished the disease (9). One as yet unanswered question concerning this animal model for a Th1-mediated disease is the nature of the stimuli responsible for the induction of IL-12 secretion underlying the disease. Based on recent evidence that CD40L on activated T cells induces IL-12 secretion by peripheral blood monocytes in vitro (8), we investigated if the CD40L-CD40 interaction in this murine model for Th1-mediated colitis results in the production of IL-12 by APC and thus is involved in the in vivo priming of Th1 T cells. We therefore treated mice with either 0.5 mg of the anti-gp39 mAb (MR-1), 0.5 mg of control haIgG, or PBS 0 and 2 d after the administration of TNBS and then assessed disease activity by monitoring weight changes, histological evidence of inflammation, and cytokine production. As depicted in Fig. 1 A, the administration of anti-gp39 on days 0 and 2 clearly prevented the weight loss caused by the rectal instillation of TNBS; thus treated mice also appeared healthy as assessed by their activity and absence of diarrhea. In addition, the typical lymphocytic infiltrates of TNBS-induced colitis (Fig. 1 B), which were previously shown to contain predominantly CD4⁺ T cells (9), were absent in anti-gp39-treated mice (Fig. 1 C). Finally, as depicted in Fig. 1 D, IFN- γ secretion by anti-CD3/CD28-stimulated CD4⁺ T cells of the LP, which is typically upregulated in TNBS-induced colitis, was reduced to normal levels after the administration of anti-gp39, whereas, as illustrated in Fig. 1 E, the secretion of IL-4 was upregulated about five-fold compared with cells from colitic mice treated with control haIgG. This effect of anti-gp39 cannot result from its possible inhibitory effect on specific anti-TNP antibody production, because in our previous studies, treatment with anti-IL-12 increased the serum anti-TNP levels but nonetheless abolished the disease (Neurath, M., and E. Stüber, unpublished observation). Thus, in the TNBS-induced colitis model, antibody production does not seem to have clinical relevance. In contrast to the above-mentioned findings, mice administered anti-gp39 antibody on day 7 continued to manifest colitis marked by the presence of weight loss, diarrhea, and colonic inflammation (data not shown).

The Inhibition of the CD40-CD40L Interaction by Anti-gp39 Antibodies Prevents Increased IL-12 Secretion in TNBS-induced Colitis. The effect of the blockage of the CD40L-CD40 interaction by anti-gp39 could result from a lack of IL-12 production. To examine this question directly, we first performed in situ immunohistochemistry for IL-12 on frozen gut sections from untreated or anti-gp39-treated

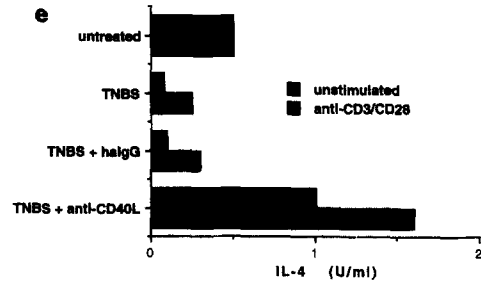
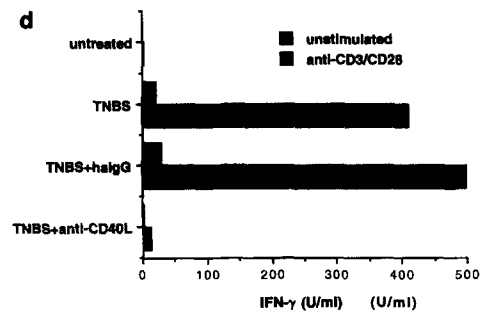
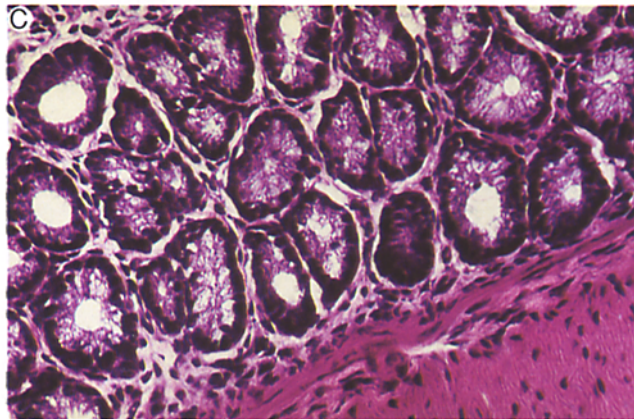
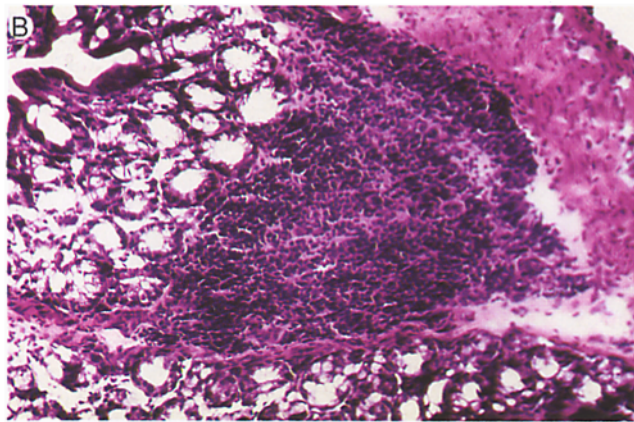
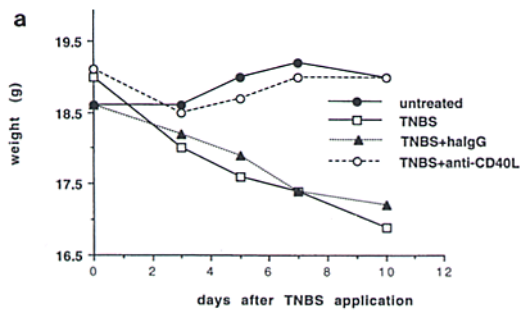


Figure 1. The administration of anti-gp39 prevents disease activity in the TNBS-colitis model in mice. (A) TNBS colitis was induced in three SJL/J mice and treated with haIgG, anti-CD40L (gp39), or not treated at all. Weight changes were monitored for the subsequent 10 d. Treatment with 50% ethanol alone does not cause colitis or wasting disease (data not shown). (B and C) Colon from mice treated with TNBS plus haIgG (B) or anti-CD40L (C) were removed on day 7 after the rectal instillation of TNBS, frozen on dry ice, and H&E stains from frozen sections were performed according to standard techniques. (D and E) Animals were treated as described in A. On day 7 after TNBS administration, colons were removed and CD4⁺ T cells from the LP were prepared as described in Materials and Methods. The T cells were subsequently stimulated with anti-CD3/CD28 or were not stimulated at all, and the concentrations of IFN-γ (D) or IL-4 (E) were determined after 48 h of culture.

mice with TNBS-induced colitis. Second, we injected rIL-12 p70 heterodimer into mice that were also treated with TNBS and anti-gp39. As shown in Fig. 2, A and B, whereas in control haIgG-treated mice, IL-12 p40 expression could clearly be detected within the lymphocytic infiltrates, no such expression could be found in anti-gp39 antibody-treated mice. Second, when we administered IL-12 to TNBS- and anti-gp39-treated mice, we observed the same disease pattern as in mice treated with TNBS and haIgG alone, as assessed by weight loss (Fig. 2 C) and by the appearance of lymphocytic infiltrates (data not shown). IL-12 alone given without TNBS did not induce colitis. Finally, as illustrated in Fig. 2, D and E, we found that IL-12 reverses the effect of the anti-gp39 antibody treatment not only in terms of disease activity and histological changes, but also in terms of IL-4 and IFN-γ secretion by LP-CD4,

with the latter even higher in the IL-12/anti-gp39-treated animals compared with the control haIgG-treated TNBS mice.

Recent studies on the relevance of the CD40L-CD40 interaction revealed that blocking this interaction abolished the B cell response to T cell-dependent antigens, the formation of germinal centers, and the development of B cell memory (15). It also was demonstrated that this treatment impairs T cell functions in the murine collagen type II-induced arthritis model (16), in acute semiallogeneic GVHD (17), and in the allospecific CTL reaction (18), that is, reactions that are believed to be mediated by Th1-type T cells. On the other hand, the administration of anti-gp39 antibody did not influence Th2 T cell functions (19).

The explanation for the impairment of T cell function that occurs in the absence of a normal CD40-CD40L in-

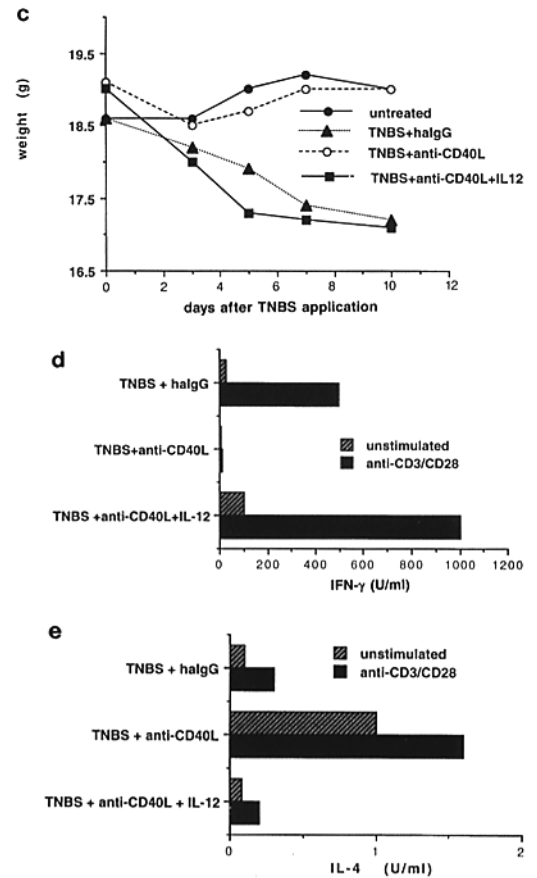
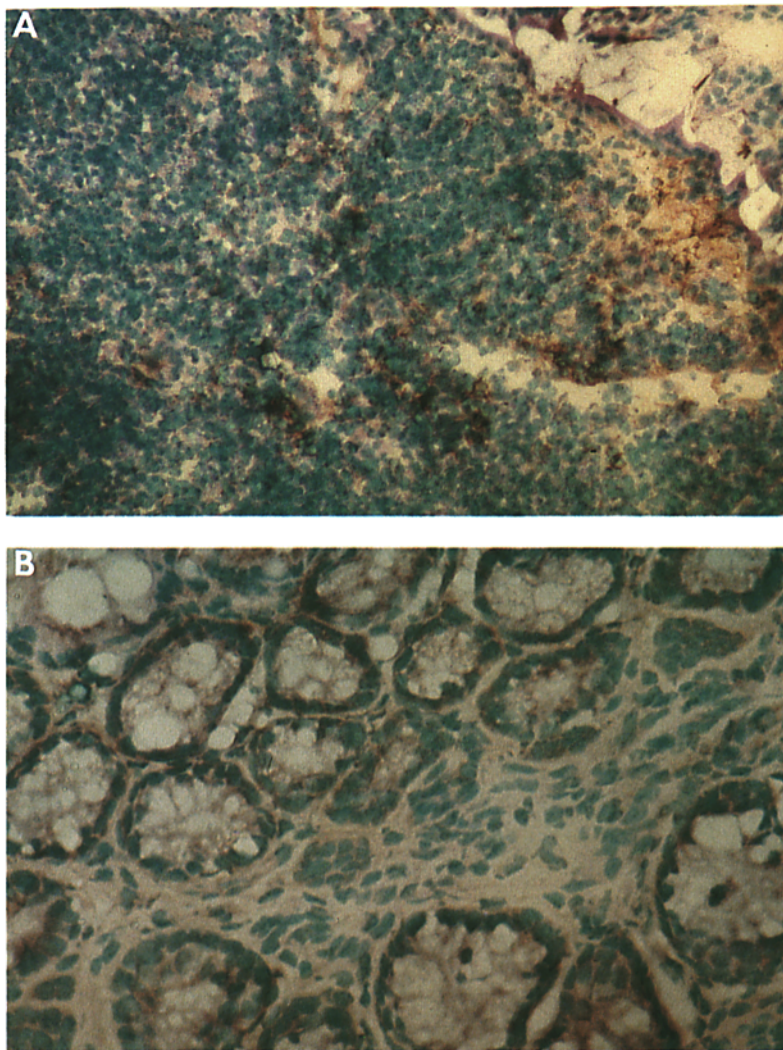


Figure 2. The effect of anti-gp39 on TNBS-induced colitis in mice is mediated through an inhibition of IL-12 secretion and is reversed by the injection of rIL-12. (A and B) SJL/J mice were treated with TNBS plus haIgG (A; 200 \times) or anti-CD40L (gp39) (B; 400 \times), and the large bowels were removed on day 7. Cryosections were stained for the IL-12 p40 subunit as described in Materials and Methods. (C) TNBS colitis was induced in three SJL/J mice and additionally treated with haIgG, anti-CD40L (gp39), or anti-CD40L plus rIL-12 (100 ng every 2nd d). Weight changes were monitored for the subsequent 10 d. (D and E) Animals were treated as described in C. On day 7 after TNBS administration, colons were removed, and CD4⁺ T cells from the LP were prepared as described in Materials and Methods. The T cells were subsequently stimulated with anti-CD3/CD28 or not stimulated at all, and the concentrations of IFN- γ (D) or IL-4 (E) were determined after 48 h of culture.

teraction could be caused by a diminished B7 expression on APC and thus an inefficient costimulation of T cells (20, 21). Although this mechanism might be operative in some situations, however, it does not explain the fact that blocking of the CD40L-CD40 interaction affects predominantly Th1 T cell functions but leaves Th2 T cell responses intact. An alternative explanation for the T cell defect observed in the absence of a functional CD40L-CD40 interaction is provided by the results reported here. In our model of TNBS-induced colitis, the administration of anti-gp39 antibodies resulted in an inhibition of IL-12 production and consequently the lack of Th1 T cell priming. Nevertheless, priming of Th2 T cells, as assessed by IL-4 secretion, was increased, which argues against an impaired costimulation

of T cells. The link between the decrease in IL-12 production and the lack of Th1 T cell priming was further demonstrated by the fact that the effect of anti-gp39 antibody was totally reversible by the coadministration of rIL-12 p70 heterodimer. Taken together, these data strongly suggest that the selective T cell functional impairment occurring after the disruption of the CD40L-CD40 interaction is mainly caused by its effect on IL-12 secretion. Importantly, we did not observe an effect of anti-gp39 antibody on TNBS-induced colitis when it was given after the onset of the disease, suggesting that the CD40L-CD40 interaction is only crucial for the priming of Th1 cells and not for their expansion and secretion of IFN- γ . The most likely explanation for this is that IL-12 production, which has also been shown

to be necessary for these latter processes, can be directly stimulated by IFN- γ and therefore might not be dependent on cell-cell interactions (14).

These data and conclusions have several clinical implications. For instance, if the findings of this study can be repeated in other animal models of Th1-mediated (autoimmune) diseases, one might speculate that the chances for a successful treatment with anti-human CD40L in established human autoimmune diseases are not as good as initially hoped. However, such treatment might still be of importance in the prevention of transplant rejection, because in this specific situation the priming of T helper subsets can be influenced. In addition, this new insight into the mech-

anism for the priming of Th1-type cells also opens a novel potential therapeutic strategy for the treatment of opportunistic infections (*Pneumocystis carinii* and intestinal *Cryptosporidium*) in hyper-IgM patients (22). Thus, from the data presented, one would predict that short-term IL-12 treatment of such patients during a period of infection should be sufficient for the priming of a Th1 T cell response crucial for host defense. Such priming would not be necessary later because the CD40L-CD40 interaction does not seem to be critical in maintaining a Th1 T cell response. In conclusion, these data underscore the crucial role of the CD40L-CD40 interaction during the cell-cell contact between activated T cells and APC in the priming of Th1 cells.

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