

Nonlymphocyte-derived Tumor Necrosis Factor Is Required for Induction of Colitis in Recombination Activating Gene (RAG)2^{-/-} Mice upon Transfer of CD4⁺CD45RB^{hi} T Cells

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Summary

In this study, we addressed the role of tumor necrosis factor (TNF)- α and lymphotoxin (LT)- α in the development of colitis and defined the cellular sources (T cells versus non-T cells) of TNF (TNF- α and LT- α) relevant to disease development. After adoptive transfer of TNF^{+/+}CD4⁺CD45RB^{hi} splenocytes into TNF^{+/+} recombination activating gene (RAG)2^{-/-} mice, the recipients develop massive inflammation of the large intestinal mucosa concurrent with massive weight loss. In contrast, clinical signs of disease are completely absent in TNF^{-/-}RAG2^{-/-} recipients of TNF^{-/-}CD4⁺CD45RB^{hi} T cells, although elevated numbers of interferon- γ -producing cells are present in the colonic mucosa. Surprisingly, upon transfer of TNF^{-/-}CD4⁺CD45RB^{hi} T cells into TNF^{+/+}RAG2^{-/-} recipients, colitis develops with kinetics similar to those upon transfer of TNF^{+/+}CD4⁺CD45RB^{hi} donor cells. In contrast, no clinical signs of colitis are observed in TNF^{-/-}RAG2^{-/-} recipients of TNF^{+/+}CD4⁺CD45RB^{hi} T cells. This protection from colitis is not a consequence of the absence of LT- α , as TNF- α ^{-/-}RAG2^{-/-} recipients of TNF- α ^{-/-}CD4⁺CD45RB^{hi} T cells are also protected from colitis induction. These results demonstrate the importance of TNF production by non-T cells of the colonic mucosa in the pathogenesis of colitis and provide direct evidence for a nonredundant role of TNF- α in this mouse model of colitis.

Key words: inflammatory bowel disease • mucosal immunity • intestinal inflammation • proinflammatory cytokines

Over the last few years, several experimental mouse models for inflammatory bowel disease (IBD),¹ which includes ulcerative colitis and Crohn's disease in human patients, have been developed (1). Most of these mouse strains that spontaneously develop an ulcerative colitis- or Crohn's disease-like disorder were generated by targeted disruption of genes crucially involved in the maintenance of well balanced immune responses, such as the genes encoding TCR chains (2), MHC class II (2), IL-2 (3), IL-10 (4), or TGF- β (5). In these gene-deficient mice, clinical onset of disease is accelerated when mice are kept under conventional rather than specific pathogen-free conditions of maintenance (6, 7). This clearly illustrates the requirement of a balanced immune response against antigens derived primarily from the gut lumen to maintain local tissue homeostasis in the intestinal mucosa. This assumption is further supported by two other mouse models of colitis, where transfer of bone marrow from wild-type mice into immunocompromised (tg ϵ 26)

CD3 ζ -transgenic mice (8) and of CD4⁺CD45RB^{hi} T cells into SCID mice (9, 10) leads to histopathological and clinical signs of colitis. The preferential differentiation of transferred cells into functional Th1 CD4 T cells in the absence of functionally heterogeneous lymphoid cell subsets of the recipient are believed to represent a main pathogenetic factor in the initiation and progression of the disease in these mouse models of colitis (11–13).

In the absence of a tightly regulated local immune system, the constant exposure of immune effector cells to antigens derived from the gut lumen may lead to an exacerbating immune response in the colonic mucosa characterized by the extensive production of proinflammatory cytokines, such as TNF- α , IFN- γ , and chemokines. An overproduction of TNF- α in the affected intestinal mucosa has been well documented in different animal models of colitis (14–16) and in human patients with active Crohn's disease (17–19). In addition, administration of neutralizing anti-TNF- α and lymphotoxin (LT)- α antibodies in human patients with active Crohn's disease leads to a transient improvement of the disease (19, 20) and attenuates the development of colitis in some experimental mouse models (15, 16). Markedly in-

¹Abbreviations used in this paper: IBD, inflammatory bowel disease; LPL, lamina propria lymphocytes; LT, lymphotoxin; RAG, recombination activating gene.

creased production of TNF- α by macrophages isolated from lamina propria of affected colons has been reported in patients with active IBD (18, 21) and also in several animal models of colitis (22, 23). In addition, infection of monolayers of colonic epithelial cell lines with invasive bacteria leads to the upregulation of proinflammatory cytokines, including TNF- α (24).

TNF- α is synthesized as a type II membrane-anchored protein and released in secreted form by a TNF- α converting enzyme (25). Secreted TNF- α is biologically active as a trimer and binds to two plasma membrane receptors, TNFR p55 and TNFR p75. The two receptors also bind secreted trimers of the structurally related LT- α (formerly also termed TNF- β). Both receptors have the ability to engage a variety of overlapping signaling pathways, which may lead to cell activation or proliferation or apoptosis depending on the nature and the differentiation state of the target cell. LT- α can also bind to a recently defined receptor, Herpes simplex virus entry mediator (26), and forms heterotrimers with the membrane-anchored LT- β . LT heterotrimers can also bind to an additional receptor, LT- β R. TNF- α and LT- α exert overlapping but distinct functions, as exemplified by the distinct effects of TNF- α , LT- α , and LT- β deficiencies on the presence of secondary lymphoid organs and their architecture (27, 28).

The aims of this study were to determine (a) if TNF- α /LT- α (hereafter referred to as TNF) plays an essential, non-redundant role in the pathogenesis of colitis in the CD4⁺CD45RB^{hi} T cell transfer model of colitis induction, (b) if TNF production by CD4 T cells is required for disease induction, and (c) if, in the absence of donor T cell-derived TNF, secretion of TNF by recipient-derived non-T cells is sufficient for induction of severe colitis. To this end, syngeneic CD4⁺CD45RB^{hi} T cells from wild-type and TNF-deficient mice, respectively, were adoptively transferred into immune-deficient recombination activating gene (RAG2)^{-/-} or TNF-deficient RAG2^{-/-} mice. The results obtained clearly demonstrate that TNF is required for induction of severe colitis in this adoptive cell transfer model. Furthermore, TNF production by disease-inducing CD4 T cell subsets is not required for onset of clinically overt colitis, whereas TNF produced by non-T cells of the recipients is essential for the induction of colitis.

Materials and Methods

Animals. C57BL/6J \times 129/SvEv-Gpi1c RAG2^{-/-} mice (derived from the ES cell line EK.CCE 129/SvEv-Gpi1c; reference 29) were provided by Drs. H. Bluethmann and E. Wagner (Basel Institute for Immunology). TNF^{-/-} C57BL/6J \times 129/SvEv mice were generated using the ES cell line GS1 as previously described (28). C57BL/6J \times 129SvJ mice were obtained from Dr. H. Bluethmann and the Biotechnology and Animal Breeding Division of RCC (Füllinsdorf, Switzerland). TNF^{-/-} mice were backcrossed with (C57BL/6J \times 129/SvEv-Gpi1c) RAG2^{-/-} mice in our animal facility. PCR procedures were used to determine RAG2 and TNF genotypes. Three different primers were used to characterize the RAG2 genotype: Rag1, 5'-GGGAGGACTCACTTGCCAGTA-3'; Rag2, 5'-AGTCAGGAGTCTCCATCT-

CACTGA-3'; and Ragneo, 5'-CGGCCGGAGAACCCTGCGTGCAA-3', to yield a 350-bp fragment for the mutated and a 263-bp fragment for the wild-type allele. The TNF genotype was defined using the TNF10 (5'-CCTCAGCAAACCACCAAGTGA-3') and TNF12 (5'-TTGGGCAGATTGACCTCAGCG-3') primers, where a 350-bp fragment corresponds to wild-type allele, and no band is detected for the mutant allele. As the RAG2^{-/-}, TNF^{-/-}, and 129 \times B6 mice were obtained from different sources and the number of backcrosses to C57BL/6J mice was not documented for the two gene-deficient mouse lines, several control experiments were performed before using these mouse lines in the CD4 T cell transfer model of colitis to exclude potential graft-versus-host reactions. These controls included mixed lymphocyte reactions using splenocytes from donor and recipients as responders and, upon irradiation (2,000 rads), as stimulators, as well as the careful histopathological examination of small intestine, skin, and liver from all experimental animals. Both the mixed lymphocyte reactions and the histopathological analyses revealed no indications of minor histoincompatibilities or graft-versus-host reactions in all of the different donor-recipient combinations used (data not shown).

TNF- α ^{-/-} mice (C57BL/6) were obtained from Dr. M. Marino (Ludwig Institute for Cancer Research, New York, NY) for subsequent backcrossing to C57BL/6J RAG2^{-/-} mice (>10 backcross generations; obtained from E. Wagner, Basel Institute for Immunology). The TNF- α genotype was defined using three different primers (TNF- α 1, 5'-AGATAGCAAATCGGCTGACGG-3'; TNF- α 2, 5'-ATCAGTTCTATGGCCAGACC-3'; and TNF- α neo, 5'-CCTTCTATCGCCTTCTTGACG-3'), where TNF- α 1/TNF- α neo yielded a band of 1,200 bp for the mutant allele, and the TNF- α 1/TNF- α 2 primers yielded a band of 750 bp for the wild-type allele. Mice were kept at specific pathogen-free conditions in the central animal facility of the Medical School, University of Bern, Bern, Switzerland.

mAbs Used for Cell Sorting and Enzyme-linked Immunospot Assay. PE-conjugated anti-CD4 (clone GK1.5), FITC-conjugated anti-CD45RB (clone 16A), biotin-conjugated anti-TNF- α pAb, biotin-conjugated anti-IL-4 mAb (clone BVD6-24G2), anti-TNF- α mAb (clone MP6-XT22), and anti-IL-4 mAb (BVD4-1D11) were purchased from PharMingen. Protein G-purified antibodies from the hybridoma supernatants anti-B220 (clone RA3-6B2), anti-macrophage-specific mAb (clone F4/80), anti-CD8 α (clone 53-6.7), anti-IFN- γ mAb (clone AN181724), anti-Mac-1 mAb (clone M1/70), and anti-IFN- γ mAb (clone OIE703B2) were biotinylated or FITC conjugated according to standard protocols.

Isolation of Intestinal Lamina Propria Lymphocytes. Lamina propria lymphocytes (LPLs) from small and large bowel were isolated as previously described (30). In brief, small pieces of appropriate intestinal tissues were incubated for 20 min at 37°C in Ca²⁺- and Mg²⁺-free HBSS containing 2% horse serum (GIBCO-BRL Life Technologies), 1 mM dithiothreitol, and 0.5 mM EDTA. After washing pieces of intestine with HBSS containing 5% horse serum, the tissues were incubated with 100 U/ml collagenase (Sigma Chemical Co.) and 10 mg/ml DNase (Boehringer Mannheim) twice for 60 min each at 37°C. Cells were passed through 70- and then 40- μ m nylon cell strainers and further fractionated on a 40-70% Percoll gradient (Pharmacia Biotech; 15 min, 800 g, room temperature). For the sorting of macrophages and CD4 cells, isolated cells from small and large bowel were stained with PE-conjugated anti-CD4 mAb, FITC-conjugated anti-F4/80, and FITC-conjugated anti-Mac-1 mAb for subsequent sorting on a FACS VantageTM (Becton Dickinson).

Isolation and Purification of CD4⁺CD45RB^{hi} versus CD4⁺CD45RB^{lo} Splenocytes. After osmotic lysis of erythrocytes, splenocytes were

incubated with 0.5–1 μg biotinylated anti-CD8 α and anti-B220 mAb per 10^6 cells for 15 min on ice. Avidin magnetic beads (Miltenyi Biotec) were used as second step reagents, and the CD8 α^+ and B220 $^+$ splenocytes attached to the beads were removed by magnetic separation. The negative fraction, enriched for CD4 T cells, was stained using FITC-conjugated anti-CD45RB mAb and PE-conjugated anti-CD4 mAb. Subsequently, CD4 T cells were sorted according to the expression of CD45RB on a FACS VantageTM. CD4 T cells were divided into three different subpopulations (low, intermediate, and high) according to expression level of CD45RB. The CD4 cell subset with lowest expression of CD45RB was collected as CD45RB^{lo}, and the subset with highest expression of CD45RB was termed CD45RB^{hi}. CD4 $^+$ T cells with intermediate cell surface expression of CD45RB were discarded.

Reconstitution of RAG2^{-/-} Mice with CD4 T Cell Subpopulations. Sorted CD4 $^+$ CD45RB^{hi} and CD4 $^+$ CD45RB^{lo} T cells were washed and resuspended at 10^6 cells/ml in sterile PBS. 2×10^5 cells were injected intraperitoneally into each of 8–12-wk-old recipient mice. Upon transfer, body weight of recipient mice was measured every other day. Mice were killed and analyzed either on day 10 or between 3 and 4 wk after adoptive transfer when the mice showed a weight loss between 20 and 30% of their initial weight or 6 wk after transfer in experimental groups where no sign of colitis was observed.

Histology. Intestinal tissue sections from large and small bowel were fixed in 4% paraformaldehyde (in $1 \times$ PBS) for subsequent paraffin embedding or frozen in O.C.T. compound (Bayer AG) for preparation of cryostat sections. Paraffin-embedded sections were cut and stained with hematoxylin and eosin. To assess the histopathological alterations in the colon present after adoptive transfer of CD4 T cell subpopulations from different donors, a scoring system was established using the following parameters: (a) cellular infiltration in the lamina propria of the large bowel (score from 0 to 3); (b) mucin depletion (score from 0 to 2); (c) crypt abscesses (score from 0 to 2); (d) epithelial erosion (score from 0 to 2); (e) hyperemia (score from 0 to 3); and (f) thickness of colonic mucosa (score from 1 to 3). Hence, the range of histopathological scores was from 1 (no alteration) to 15 (most severe signs of colitis).

Preparation of ³⁵S-labeled RNA Probes. A 1,108-bp cDNA fragment of the murine TNF- α gene (position 1–1,108; provided by Genentech Inc.) and a 900-bp EcoRI–HindIII cDNA fragment of the IFN- γ gene (provided by Dr. K. Arai, DNAX, Inc., Palo Alto, CA) were subcloned into pGEM-2 (Promega Corp.). After linearization of both plasmids, sense and antisense RNA probes were prepared using the appropriate RNA polymerase as previously described (31).

In Situ Hybridization. Serial frozen sections of intestinal tissue were hybridized in situ with an antisense RNA probe specific for the TNF- α and IFN- γ genes. In situ hybridizations were performed as previously described (31). In brief, cryostat sections were fixed in 4% paraformaldehyde in PBS for 20 min, washed in PBS, and incubated in the presence of 1 $\mu\text{g}/\text{ml}$ proteinase K (Boehringer Mannheim) at 37°C for 30 min. After postfixation and acetylation, the hybridization was performed with 2×10^5 cpm of ³⁵S-labeled RNA probe per microliter of hybridization solution for 18 h at 48°C. After digestion of nonhybridized single-stranded RNA and washing, the slides were dipped in NTB2 nuclear track emulsion (Eastman Kodak Co.). The slides were exposed for 3 wk in the dark at 4°C, developed, and counterstained with nuclear fast red (Sigma Chemical Co.) by standard techniques.

Enzyme-linked Immunospot Assay. To determine the number of TNF- α -, IL-4-, and IFN- γ -producing cells in the LPLs of large versus small intestine, an enzyme-linked immunospot (ELISPOT)

assay was performed as previously described (32). In brief, nitrocellulose-backed microtiter plates (96-well; Millipore Corp.) were coated at 4°C overnight with anti-TNF- α , anti-IFN- γ , and anti-IL-4 mAbs and subsequently blocked with TBS containing 5% BSA. After washing the plate with TBS containing 0.025% Tween-20, freshly isolated LPLs or sorted CD4 T cells and macrophages were incubated in IMDM containing 10% FCS in a CO₂ incubator at 37°C overnight. The plates were extensively rinsed with TBS plus 0.025% Tween-20 and incubated for 2 h at 37°C with biotinylated anti-TNF- α pAb, anti-IFN- γ mAb, and anti-IL-4 mAb, respectively. The plate was subsequently rinsed and incubated for 2 h at 37°C with avidin-alkaline phosphatase (Sigma Chemical Co.), diluted 1:1000. 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NTB) solution (Kirkegaard and Perry Labs., Inc.) was used as a substrate for the alkaline phosphatase. Positive spots were counted under a dissecting microscope.

Results

When 2×10^5 CD4 $^+$ CD45RB^{hi} T cells from B6 \times 129 donor animals (TNF^{+/+}) were transferred into sex-matched, 8–10-wk-old syngeneic RAG2^{-/-} mice, the recipients rapidly started to lose weight 12–14 d after adoptive T cell transfer. This dramatic wasting disease led to an average weight loss of 25–30% 24 d after transfer and is associated with clinical signs of severe colitis, in particular, persistent diarrhea and occasionally also bloody stool and anal prolapses. RAG2^{-/-} recipients of 2×10^5 CD4 $^+$ CD45RB^{lo} T cells did not, however, show weight loss, and clinical signs of colitis were completely absent throughout the entire observation period (Fig. 1 A).

To assess the contribution of TNF- α and the structurally related LT- α (TNF- β) to the course of disease in this mouse model of colitis, B6 \times 129 RAG2^{-/-} mice were first backcrossed to TNF-deficient B6 \times 129 mice (TNF^{-/-}) to obtain TNF^{-/-}RAG2^{-/-} mice. The consequences of an adoptive transfer of CD4 $^+$ CD45RB^{hi} T cells from TNF^{-/-} donors into TNF^{-/-}RAG2^{-/-} mice on the body weights of the recipients is depicted in Fig. 1 B. Throughout the entire observation period of 42 d, no sign of weight loss was observed. Clinical signs of colitis, such as persistent diarrhea, or more severe signs, such as anal prolapse or bloody stool, were absent in all animals throughout the entire observation period. No difference in the kinetics of body weight or clinical signs of colitis were apparent when TNF^{-/-}RAG2^{-/-} recipients of TNF^{-/-}CD4 $^+$ CD45RB^{hi} and CD4 $^+$ CD45RB^{lo} T cells were compared (Fig. 1 B).

When recipients of CD4 $^+$ CD45RB^{hi} T cells had lost between 20 and 30% of their initial body weight or at the end of the observation period on day 42 after transfer, all mice were killed and part of each colon was paraffin embedded for subsequent detailed histopathological analyses. To quantitate the histological alterations present in the large intestines of recipient mice, a semiquantitative colitis score system ranging from 1 (no histopathological alterations) to 15 (most severe histopathological alterations) was established (see Materials and Methods). The results of these microscopic analyses of colonic tissue sections are summarized in Fig. 2 A. TNF^{+/+}RAG2^{-/-} recipients of TNF^{+/+}

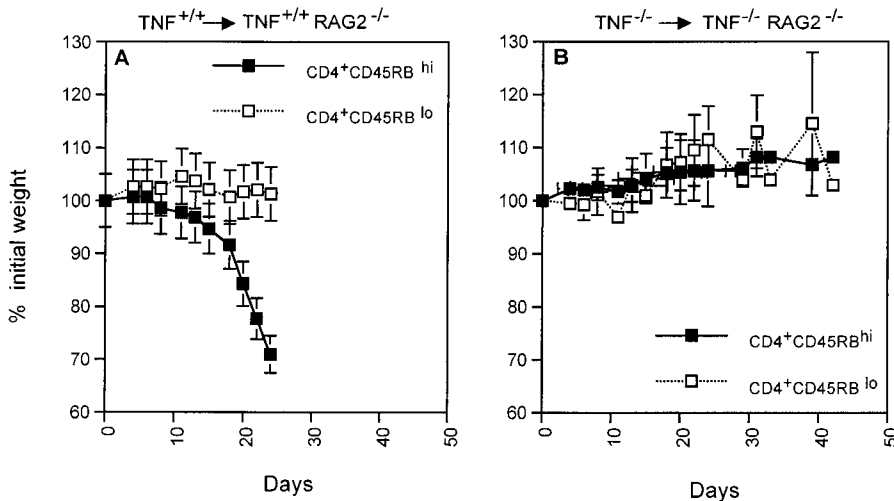


Figure 1. TNF- α /LT- α is essential to induce wasting disease. Reconstitution of RAG2^{-/-} mice with 2×10^5 TNF^{+/+} CD4⁺CD45RB^{hi} T cells induces colitis characterized by weight loss (A), whereas no weight loss is detected in TNF^{-/-} recipients reconstituted with 2×10^5 TNF^{-/-} CD4⁺CD45RB^{hi} T cells (B). As negative controls, mice were injected with 2×10^5 CD4⁺CD45RB^{lo} T cells. The change in weight is expressed as percentage of the weight at the time of cell transfer. Data represent the mean values \pm SD of four to five mice per group. Experiments were repeated at least three times.

CD4⁺CD45RB^{hi} T cells revealed massive alterations of the colon, with colitis scores between 12 and 15 (mean 13.2) for individual animals. Control TNF^{+/+} RAG2^{-/-} mice reconstituted with TNF^{+/+}CD4⁺CD45RB^{lo} splenocytes, however, showed only minimal signs of histopathological alterations (mean 4.5). Histological changes of the colon in TNF^{-/-}RAG2^{-/-} mice after adoptive transfer of TNF^{-/-}CD4⁺CD45RB^{hi} T cells were minimal to moderate (mean 5.8) and comparable to the colitis scores observed in TNF^{-/-}RAG2^{-/-} recipients of the corresponding TNF^{-/-}CD4⁺CD45RB^{lo} T cells (mean 4.5; Fig. 2 A). Representative examples of cross-sections of the colon from a TNF^{+/+} RAG2^{-/-} recipient of colitis-inducing TNF^{+/+}CD4⁺CD45RB^{hi} T cells (colitis score 14) and from a TNF^{-/-} RAG2^{-/-} recipient of TNF^{-/-}CD4⁺CD45RB^{hi} T cells (colitis score 5) are shown in Fig. 2, B and C, respectively. Extensive inflammatory cell infiltrates, glandular elongation, loss of goblet cells, epithelial erosion, and crypt abscesses were observed in colonic tissue sections of mice affected with wasting disease (Fig. 2 D). In contrast, minimal colonic pathology was observed in tissue sections from TNF^{-/-}CD4⁺CD45RB^{hi} T cells \rightarrow TNF^{-/-}RAG2^{-/-} mice. Limited cellular infiltration, together with minimal glandular elongation and minimal loss of goblet cells, was present in the lamina propria and submucosa, whereas epithelial erosion and crypt abscesses were completely absent in these mice (Fig. 2 E).

These experiments clearly demonstrate that TNF is required for the induction of clinical and histopathological signs of severe colitis and epithelial erosion of the intestinal wall. Thus, we attempted to determine if TNF production by the transferred colitogenic CD4⁺CD45RB^{hi} T cells is required for the development of colitis or whether local production of TNF by resident non-T cells of the recipients is sufficient for disease induction and perpetuation. To this end, two reciprocal donor-recipient combinations were analyzed: transfer of TNF^{+/+} CD4 T cell subsets into TNF^{-/-}RAG2^{-/-} recipients and transfer of TNF^{-/-} CD4 T cell subsets into TNF^{+/+}RAG2^{-/-} recipients. The consequences of adoptive transfer of TNF^{-/-}CD4⁺CD45RB^{hi}

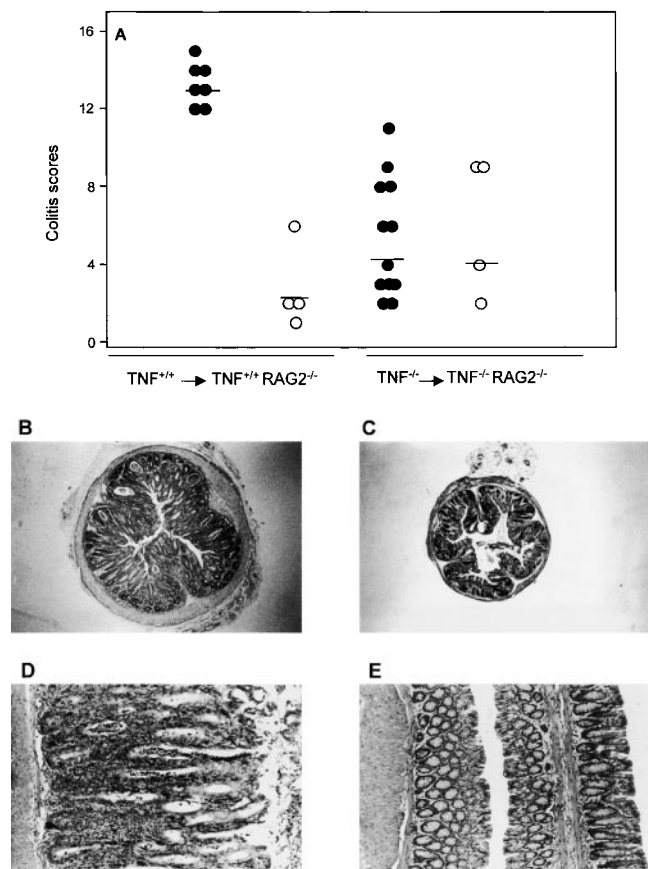


Figure 2. Colitis scores and histopathology of the colons from TNF^{+/+} RAG2^{-/-} recipients of TNF^{+/+}CD4⁺CD45RB^{hi} T cells (●) and CD4⁺CD45RB^{lo} T cells (○) and from TNF^{-/-} RAG2^{-/-} recipients of TNF^{-/-}CD4⁺CD45RB^{hi/lo} T cells. (A) The colitis scores shown for individual mice represent the total of individual scores for cellularity, mucin depletion, crypt abscess, epithelial erosion, hyperemia, and thickness of the colon as described in Materials and Methods. (B and D) Tissue sections of the colon from TNF^{+/+}RAG2^{-/-} recipients of TNF^{+/+}CD4⁺CD45RB^{hi} T cells (colitis score 12) and (C and E) from TNF^{-/-}RAG2^{-/-} recipients of TNF^{-/-}CD4⁺CD45RB^{hi} T cells (colitis score 5). Hematoxylin and eosin staining; magnifications: B and C, 9 \times and D and E, 60 \times .

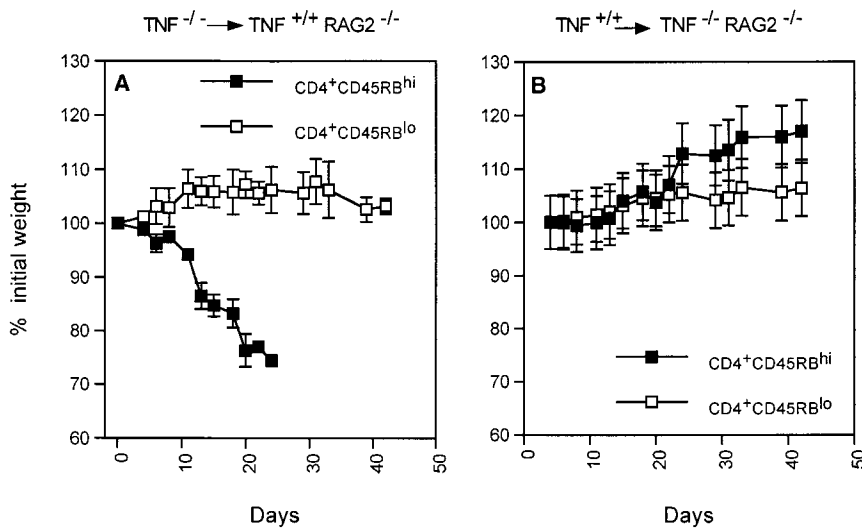


Figure 3. TNF- α /LT- α produced by recipient non-T cells is required and sufficient to induce clinical signs of colitis. Reconstitution of TNF^{+/+} RAG2^{-/-} mice with 2×10^5 TNF^{-/-} CD4⁺CD45RB^{hi} T cells induces colitis characterized by weight loss (A), whereas no weight loss is observed in TNF^{-/-} recipients reconstituted with 2×10^5 TNF^{+/+}CD4⁺ CD45RB^{hi} T cells (B). As negative controls, mice were injected with 2×10^5 CD4⁺CD45RB^{lo} T cells. Data represent the mean values \pm SD of four to five mice per group. Experiments were repeated at least three times.

T cells into TNF^{+/+}RAG2^{-/-} mice on body weights are illustrated in Fig. 3 A. Similar to when TNF^{+/+}CD4⁺CD45RB^{hi} T cells were transferred into TNF^{+/+}RAG2^{-/-} mice, recipients started to lose weight 12–14 d after adoptive T cell transfer. This weight loss paralleled with clinical signs of colitis such as persistent diarrhea, anal prolapse, and bloody stool. TNF^{-/-}RAG2^{-/-} recipients of TNF^{+/+} CD4⁺CD45RB^{hi} T cells, however, did not show any clinical signs of colitis during the entire observation period (Fig. 3 B).

The results of the semiquantitative histopathological analysis of the colon in these two different donor–recipient combinations are shown in Fig. 4 A. Massive alterations of the colon with colitis scores ranging from 10 to 14 for individual mice (mean colitis score 11.7) were observed in tissue sections from TNF^{+/+}RAG2^{-/-} recipients of TNF^{-/-} CD4⁺CD45RB^{hi} T cells. A representative cross-section of an affected colon from this donor–recipient combination is shown in Fig. 4 B (colitis score 12). Hence, CD4⁺CD45RB^{hi} T cells from TNF^{-/-} donors induce histological alterations of the colonic mucosa comparable to those of CD4⁺CD45RB^{hi} T cells from TNF^{+/+} donors upon transfer into TNF^{+/+}RAG2^{-/-} mice (Fig. 4 D). In contrast, only minimal colonic pathologies were observed in TNF^{-/-}RAG2^{-/-} mice reconstituted with TNF^{+/+}CD4⁺CD45RB^{hi} splenocytes (Fig. 4, A, C, and E), demonstrating that TNF produced exclusively by transferred CD4 T cells is not sufficient to induce wasting disease. The absence of a wasting disease is associated with only minimal to moderate histopathological signs of colitis, with scores ranging from 2 to 8 for individual animals of this group (mean colitis score 4.7). Control animals of both groups reconstituted with the corresponding CD4⁺CD45RB^{lo} T cells showed only mild to moderate histopathological alterations of the colonic mucosa (Fig. 4 A).

Whereas protection from onset of severe colitis in TNF^{-/-}RAG2^{-/-} mice reconstituted with TNF^{-/-}CD4⁺CD45RB^{hi} T cells can be attributed to the complete absence of TNF production in both transferred T cells and recipient cells, the absence of clinical signs of colitis and severe histopatho-

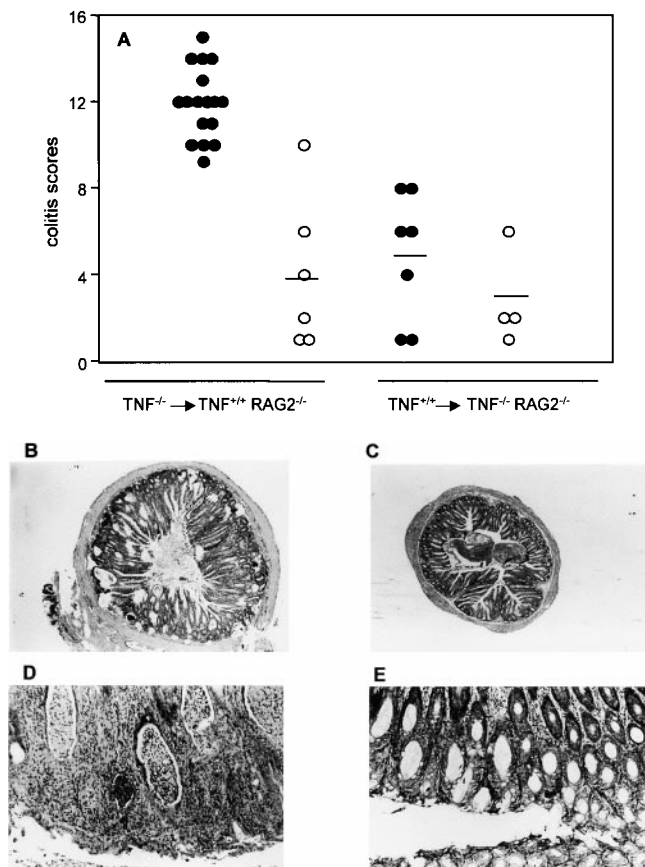


Figure 4. Colitis scores and histopathology of colons from TNF^{+/+}RAG2^{-/-} recipients of TNF^{-/-}CD4⁺CD45RB^{hi} T cells (●) and CD4⁺CD45RB^{lo} T cells (○) and from TNF^{-/-}RAG2^{-/-} recipients of TNF^{+/+}CD4⁺CD45RB^{hi/lo} T cells (A). (B and D) Tissue sections of the colon from a TNF^{-/-}CD4⁺CD45RB^{hi}→TNF^{+/+}RAG2^{-/-} mouse (colitis score 12) and (C and E) from a TNF^{+/+}CD4⁺CD45RB^{hi}→TNF^{-/-}RAG2^{-/-} mouse (colitis score 4). Hematoxylin and eosin staining; magnifications: B and C, 9 \times and D and E, 120 \times .

logical alterations in the colons of $TNF^{-/-}$ $RAG2^{-/-}$ mice reconstituted with potentially TNF -producing $TNF^{+/+}$ $CD4^{+}CD45RB^{hi}$ T cells is less obvious. Thus, we defined the frequency of TNF - α -producing cells in the colon in the four different donor-recipient combinations to assess whether protection from colitis in the $TNF^{+/+}CD4^{+}CD45RB^{hi}$ T cells into the $TNF^{-/-}$ $RAG2^{-/-}$ group is associated with low production of TNF - α by donor-derived T cells. To this end, inflammatory cells were isolated from the colonic mucosa for subsequent analysis in an ELISPOT assay. The results obtained clearly show that TNF - α is produced by a massive number of inflammatory cells isolated from the colons of $TNF^{+/+}$ $RAG2^{-/-}$ mice, transferred with either $TNF^{+/+}$ or $TNF^{-/-}$ $CD4^{+}CD45RB^{hi}$ T cells 24 d after adoptive transfer of the respective $CD4^{+}CD45RB^{hi}$ T cell subset. In contrast, in the experimental group that did not develop colitis, i.e., in $TNF^{-/-}$ $RAG2^{-/-}$ mice reconstituted with $TNF^{+/+}CD4^{+}CD45RB^{hi}$ T cells, TNF - α was produced only by very few infiltrating cells isolated from the large intestinal mucosa. This demonstrates that only a small fraction of transferred $CD4$ T cells produces TNF - α in the colonic mucosa of $TNF^{-/-}$ $RAG2^{-/-}$ recipients (Fig. 5). To further confirm that absence of colitis induction and perpetuation of disease correlate with the absence of significant numbers of TNF - α -producing cells in the colonic mucosa, we also determined the frequency of TNF - α -producing cells in the colonic mucosa of recipients of $CD4^{+}CD45RB^{lo}$ T cells, i.e., in the control groups that did not show signs of clinical or histopathological colitis. As seen in Fig. 5, right panels, absence of colitis induction always correlates with the absence of TNF - α production by isolated colonic cells from recipients of $CD4^{+}CD45RB^{lo}$ splenocytes.

To determine if the absence of TNF production by host-derived cells affects the induction of TNF production in the transferred $CD4$ T cells, thus resulting in the low frequency of TNF - α -producing T cells detected in $TNF^{-/-}$ $RAG2^{-/-}$ recipients of $TNF^{+/+}$ colitogenic T cells (Fig. 5), we assessed the frequency of TNF - α -producing cells in fractionated $CD4$ T cells and, as a main TNF - α -producing, recipient-derived cell type, in macrophages isolated from the affected colonic mucosa of $TNF^{+/+}$ $RAG2^{-/-}$ recipients of $TNF^{+/+}CD4^{+}CD45RB^{hi}$ T cells. 8.7% of the macrophages isolated from the affected colons produced TNF - α , whereas only 0.17% of isolated colonic $CD4$ T cells produced TNF - α in an ELISPOT assay. The frequency of TNF - α -secreting macrophages in the colonic mucosa in diseased animals was ~ 10 -fold higher than in macrophages isolated from the small intestinal mucosa of the same animals, where no signs of histopathological alterations were observed (8.7 and 0.8% TNF - α -producing macrophages, respectively).

To assess how the presence or absence of TNF production by recipient cells and donor-derived $CD4$ T cells might affect the cytokine pattern produced in the colonic mucosa, ELISPOT assays were performed to determine the frequency of IL -4- and IFN - γ -secreting cells in the isolated inflammatory cells from the colonic mucosa. In all four

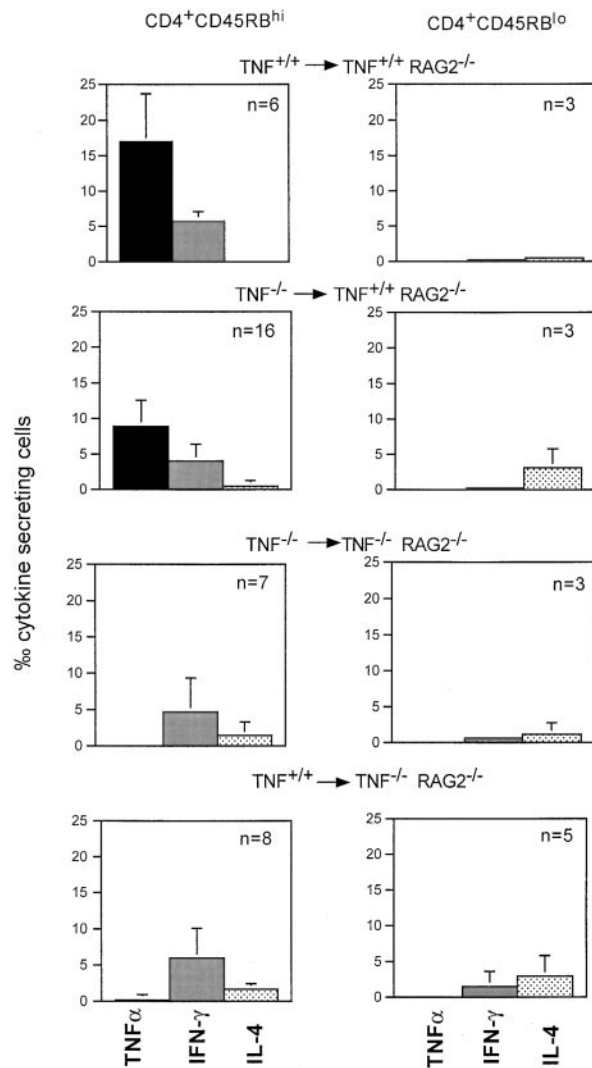


Figure 5. Cytokine production by inflammatory cell infiltrates in the colonic mucosa of the four different donor-recipient combinations after transfer of $CD4^{+}CD45RB^{hi}$ and $CD4^{+}CD45RB^{lo}$ T cells. The frequency of TNF - α -, IFN - γ -, and IL -4-producing cells (in %) as determined by ELISPOT, with isolated cells of the colonic mucosa indicated as a mean values \pm SD; n = number of mice analyzed per group.

combinations of transfer, $CD4^{+}CD45RB^{hi}$ T cells preferentially induced production of the Th1 cytokine IFN - γ in the colonic mucosa. Similar frequencies of IFN - γ -producing infiltrating $CD4$ T cells were detected in experimental groups of mice that developed severe colitis and in recipient mice that were protected from onset of severe disease (Fig. 5). Hence, production of IFN - γ does not correlate with the presence of clinical signs of colitis but correlates with the transfer of $CD4^{+}CD45RB^{hi}$ T cells, as the frequency of IFN - γ -producing cells is markedly reduced in recipients of $CD4^{+}CD45RB^{lo}$ T cells from both $TNF^{+/+}$ and $TNF^{-/-}$ donor mice. Colonic IL -4-producing cells, however, were less frequent in recipients of $CD4^{+}CD45RB^{hi}$ T cells when compared with recipients of $CD4^{+}CD45RB^{lo}$ T cells. The differences in IL -4 production by infiltrating $CD4$ T cells

were only minimal among the four recipient groups of CD4⁺CD45RB^{hi} T cells (Fig. 5).

To assess not only the frequency but also the histological distribution of TNF- α -producing cells, in situ hybridization of cryostat sections of the affected colonic mucosa (day 24 after cell transfer) with TNF- α gene probes were performed. As shown in Fig. 6, A and E, high frequencies of TNF- α mRNA-expressing cells were detected on colonic tissue sections of TNF^{+/+}RAG2^{-/-} recipients of TNF^{+/+}CD4⁺CD45RB^{hi} T cells (Fig. 6 A; colitis score 12) and

TNF^{+/+}RAG2^{-/-} recipients of TNF^{-/-}CD4⁺CD45RB^{hi} T cells (Fig. 6 E; colitis score 14). In both groups, TNF- α mRNA-expressing cells were not randomly distributed over the entire colonic mucosa but were preferentially clustered in the apical areas of colonic crypts proximal to the gut lumen and also at sites close to epithelial erosions of the colonic mucosa. In contrast, only a limited number of TNF- α mRNA CD4 T cells were detected in TNF^{-/-}RAG2^{-/-} recipients of TNF^{+/+}CD4⁺CD45RB^{hi} T cells (Fig. 6 G). In these mice, rare TNF- α mRNA-expressing

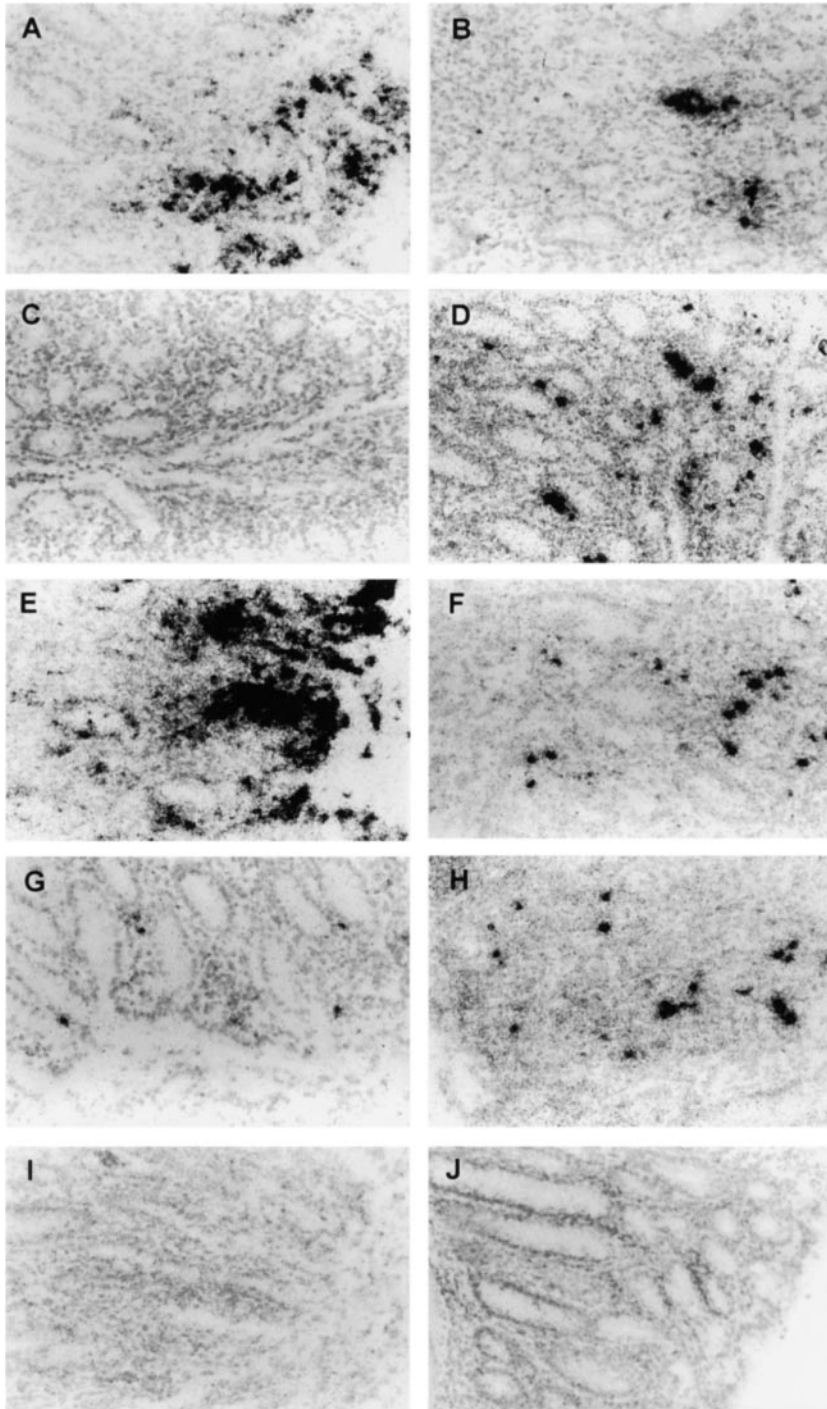


Figure 6. In situ hybridization of cryostat sections from colonic specimens of the four different donor-recipient combinations 24 d after adoptive cell transfer. (A and B) TNF^{+/+}CD4⁺CD45RB^{hi}→TNF^{+/+}RAG2^{-/-} mice; (C and D) TNF^{-/-}CD4⁺CD45RB^{hi}→TNF^{-/-}RAG2^{-/-} mice; (E and F) TNF^{-/-}CD4⁺CD45RB^{hi}→TNF^{+/+}RAG2^{-/-} mice; and (G and H) TNF^{+/+}CD4⁺CD45RB^{hi}→TNF^{-/-}RAG2^{-/-} mice, using ³⁵S-labeled antisense mRNA probes of TNF- α (A, C, E, and G) and IFN- γ (B, D, F, and H). TNF- α mRNA-expressing cells are preferentially located proximal to the lumen of the colon in affected mice (A and C). In situ hybridization with sense probes of the TNF- α (I) and IFN- γ genes (J) were used as negative controls. Magnification 120.

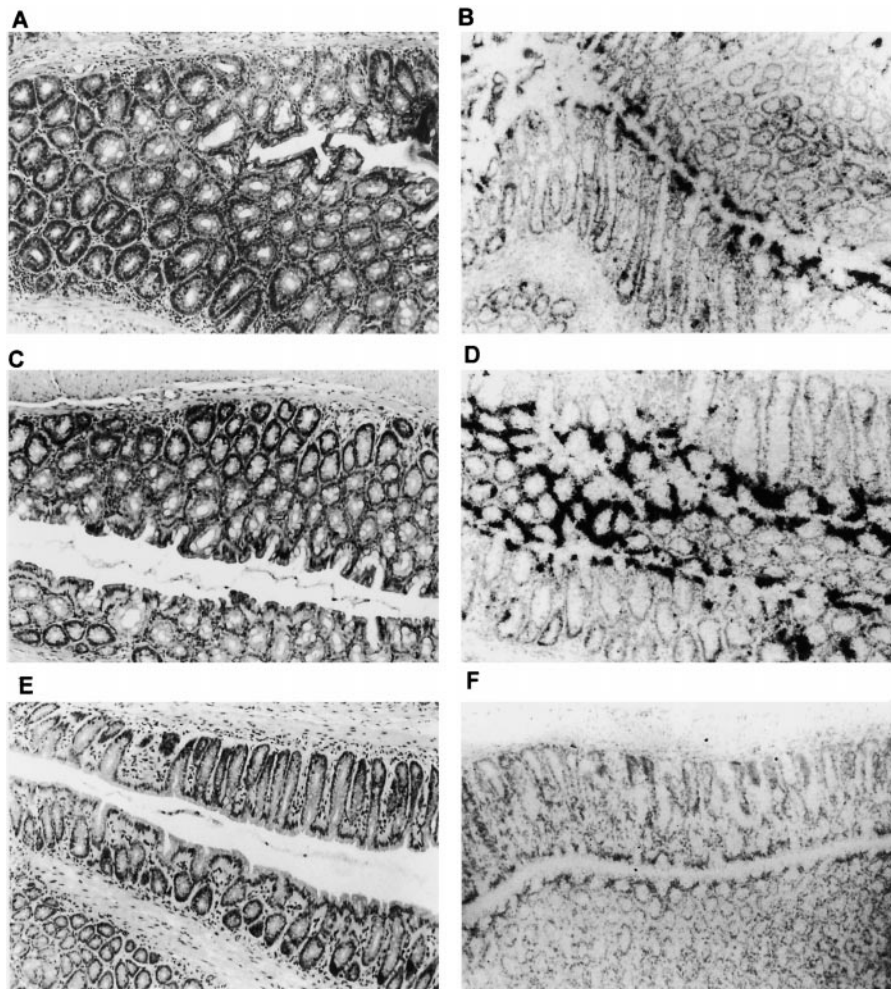


Figure 7. Histopathological alterations and TNF- α mRNA expression before clinical onset of colitis. Day 10 after transfer of TNF^{+/+}CD4⁺CD45RB^{hi} T cells into TNF^{+/+}RAG2^{-/-} recipients (A and B); TNF^{-/-}CD4⁺CD45RB^{hi} T cells into TNF^{+/+}RAG2^{-/-} recipients (C and D); and TNF^{+/+}CD4⁺CD45RB^{hi} T cells into TNF^{-/-}RAG2^{-/-} recipients (E and F). A, C, and E, hematoxylin and eosin staining; B, D, and F, in situ hybridization; magnification 60.

CD4 T cells were almost randomly distributed in the colonic lamina propria. In situ hybridization with an IFN- γ -specific probe revealed strong IFN- γ mRNA expression in the colonic mucosa of all groups of recipients of CD4⁺CD45RB^{hi} T cells (Fig. 6, B, D, F, and H). In the two donor-recipient combinations in which no severe colitis is induced, TNF^{-/-}RAG2^{-/-} recipients of TNF^{+/+} (Fig. 6 H) and TNF^{-/-}CD4⁺CD45RB^{hi} T cells (Fig. 6 D), the strong IFN- γ mRNA expression observed in some mice was always associated with elevated cellular infiltration of the colonic mucosa (colitis score ≥ 7). IFN- γ mRNA⁺ cells were not, however, restricted to the sites proximal to the lumen of the colon but were focally distributed throughout the entire colonic mucosa (Fig. 6). As a negative control, affected intestinal colonic tissues were hybridized with sense probes of the TNF- α (Fig. 6 I) and IFN- γ genes (Fig. 6 J). To further determine if the correlation between absence of clinical signs of colitis and absence of TNF- α -expressing cells is also true at early time points of disease induction where histopathological signs of a deleterious inflammation of the colon are still absent, in situ hybridization for the localization of TNF- α mRNA-expressing cells were performed on colonic tissue obtained on day 10 (Fig. 7). Despite the absence of significant histopathological alterations

of the colonic mucosa 10 d after transfer in the two groups that later developed colitis (TNF^{+/+}CD4⁺CD45RB^{hi} \rightarrow TNF^{+/+}RAG2^{-/-} (Fig. 7, A and B) and TNF^{-/-}CD4⁺CD45RB^{hi} \rightarrow TNF^{+/+}RAG2^{-/-} mice (Fig. 7, C and D), the frequency of TNF- α mRNA-expressing cells was comparable to that observed on day 24 after cell transfer with a preferential localization of TNF- α mRNA in cells close to, or even facing, the intestinal lumen. In TNF^{-/-}RAG2^{-/-} recipients of TNF^{+/+}CD4⁺CD45RB^{hi} T cells that did not develop clinical colitis, TNF- α mRNA-expressing cells were almost completely absent on day 10 (Fig. 7 F).

To exclude the possibility that the failure of TNF^{+/+}CD4⁺CD45RB^{hi} T cells to induce colitis in TNF^{-/-}RAG2^{-/-} mice was due to the absence of peripheral lymph nodes and Peyer's patches or the aberrant splenic architecture found in mice deficient for LT- α (28), we decided to back-cross C57BL/6J TNF- α ^{-/-} mice to the syngeneic RAG2^{-/-} background for subsequent transfer experiments using TNF- α ^{-/-} mice as donors. As depicted in Fig. 8 A, transfer of the CD4⁺CD45RB^{hi} T cells initially leads to a slight decrease in body weight around day 10 after cell transfer, but thereafter, recipients begin to gain weight to reach the body weight of the control animals transferred with the same number of CD4⁺CD45RB⁰ T cells from

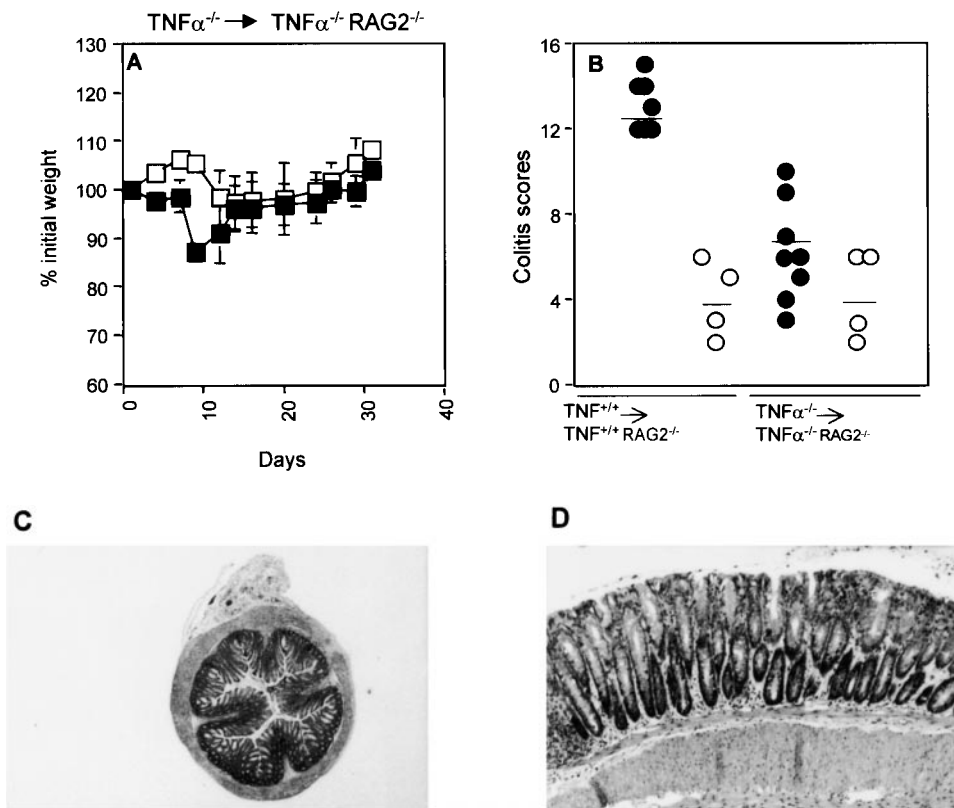


Figure 8. TNF- α plays a nonredundant role in the development of colitis. Kinetics of the body weight of TNF- $\alpha^{-/-}$ (LT- $\alpha^{+/+}$) RAG2 $^{-/-}$ mice upon transfer of 2×10^5 TNF- $\alpha^{-/-}$ (LT- $\alpha^{+/+}$) CD4 $^{+}$ CD45RB hi T cells (■) and 2×10^5 TNF- $\alpha^{-/-}$ (LT- $\alpha^{+/+}$) CD4 $^{+}$ CD45RB lo T cells (□). Data are the mean \pm SD of eight CD4 $^{+}$ CD45RB hi and four CD4 $^{+}$ CD45RB lo mice (A). Colitis scores of colons from TNF- $\alpha^{-/-}$ RAG2 $^{-/-}$ recipients of TNF- $\alpha^{-/-}$ CD4 $^{+}$ CD45RB hi T cells (●) and from CD4 $^{+}$ CD45RB lo T cells (○) are compared with colitis scores of TNF $^{+/+}$ RAG2 $^{-/-}$ recipients of TNF $^{+/+}$ CD4 $^{+}$ CD45RB hi and CD4 $^{+}$ CD45RB lo T cells (B). Tissue sections of the colon from a TNF- $\alpha^{-/-}$ CD4 $^{+}$ CD45RB hi →TNF- $\alpha^{-/-}$ RAG2 $^{-/-}$ mouse (colitis score 5) (C and D). Hematoxylin and eosin staining; magnifications: C, 9 \times and D, 60 \times .

the same donor animals. During the entire observation period of 35 d, the recipients of CD4 $^{+}$ CD45RB hi T cells did not show any signs of clinical colitis such as loose stool or even diarrhea or anal prolapse. The histopathological analyses performed 35 d after adoptive cell transfer demonstrated only mild signs of colitis, in particular focal accumulation of inflammatory cells. The histopathological scores determined for recipients of CD4 $^{+}$ CD45RB hi and CD4 $^{+}$ CD45RB lo T cells were not markedly different and even overlapped (Fig. 8 B). Microphotographs of the colon from a TNF- $\alpha^{-/-}$ RAG2 $^{-/-}$ recipient of TNF- $\alpha^{-/-}$ CD4 $^{+}$ CD45RB hi T cells 35 d after adoptive transfer are shown in Fig. 8, C and D.

Discussion

In recent years, several studies attempted to elucidate the role of TNF- α in IBD. Overproduction of TNF- α has been well documented in the affected intestinal mucosa in different animal models (14, 16, 33) and human patients with active Crohn's disease (17, 19). After administration of anti-TNF- α /LT- α (TNF) antibodies, an attenuation of disease progression has been observed in different mouse models of colitis and patients with IBD (15, 16, 19). However, the beneficial effects of an anti-TNF treatment are often transient (19). The reasons for the limited effects are not entirely understood and can possibly be attributed to a variety of factors, including the limited bioavailability of TNF-neutralizing agents at the site of mucosal lesions and redundant effects mediated by TNF at various stages of disease. Furthermore, recent results suggest that neutralizing

antibodies to TNF can reverse suppressive effects mediated by TNF- α on T cells in vivo (34), thus leading to higher functional and proliferative activity of disease-promoting T cells. Such an antiinflammatory effect of TNF- α has been demonstrated by Liu et al. in experimental allergic encephalomyelitis in mice where TNF- α can limit the extent and duration of severe central nervous system pathology (35). Hence, the accelerated onset of weight loss and clinical signs of colitis in TNF $^{+/+}$ RAG2 $^{-/-}$ mice after transfer of CD4 $^{+}$ CD45RB hi T cells from TNF $^{-/-}$ rather than TNF $^{+/+}$ donors (Fig. 3) is thus in agreement with the notion that systemic TNF- α may attenuate T cell functions in vivo. On the other hand, our study also unambiguously demonstrates the nonredundant role of TNF in the induction of colitis in this mouse model.

The nature of the TNF-producing cells (T cells versus non-T cells) relevant for the development of colitis in experimental animal models and human patients with IBD has not been directly determined so far. Several studies (22, 36) found large numbers of TNF- α -secreting macrophages in the inflamed colonic mucosa. Other studies reported high frequencies of TNF-producing T cells, in particular CD4 $^{+}$ T cells, in the affected colonic mucosa (14, 37), suggesting that TNF produced by T cells may represent a critical factor directly affecting the extent of disease. Our results, however, clearly indicate that CD4 $^{+}$ T cell-produced TNF is neither sufficient nor required for induction of disease in this mouse model of colitis and that TNF production by recipient non-T cells is essential and sufficient for induction of severe histopathological alterations and onset

of clinical signs of colitis. The observed preferential TNF- α production by colonic macrophages and their nonrandom distribution close to sites with epithelial erosion (data not shown) implies an important role of this cell subset in the pathogenesis of colitis. However, it remains to be determined if macrophages are the only recipient-derived cellular source of TNF- α crucial for the development of clinical signs of disease. In particular, as intestinal epithelial cells have also been demonstrated to produce TNF- α (24), it is possible to speculate that epithelial cells are induced directly or indirectly by activated CD4 T cells to produce TNF- α . This assumption is supported by the results of in situ hybridizations of tissue specimens from the affected colon, where TNF- α mRNA-expressing cells are consistently observed in the intestinal epithelium even at early stages of disease when only minimal infiltration of the colon with inflammatory cells is observed (Fig. 7). Although the number of cells recovered from the colonic mucosa from animals at the time of initial signs of weight loss, i.e., between days 10 and 14 after cell transfer, was too low for a quantitative assessment of the frequency of TNF- α -secreting cells, the results of the in situ hybridizations clearly indicate that TNF- α mRNA expression is detectable at levels similar to those observed on day 24, during the most active stage of disease. This indicates that elevated TNF- α expression is not the consequence of the strong inflammatory cell infiltrates but in fact precedes the severe histopathological alterations. As comparable numbers of TNF- α mRNA-expressing cells are detected in TNF^{+/+}RAG2^{-/-} mice reconstituted with either TNF^{+/+} or TNF^{-/-} CD4⁺CD45RB^{hi} T cells, we conclude that recipient non-T cells are already the main TNF-producing cell type at the very early stages of disease. Similar findings have been reported in IL-2-deficient mice where, in the intestines of 10-d-old mice, 20-fold higher TNF- α mRNA expression levels were observed compared with older mice (24). This increase, however, was found both in the small and the large intestines, whereas in our model, elevated expression of TNF- α mRNA as detected by in situ hybridization was restricted to the colon (data not shown).

The precise effects mediated by locally produced TNF- α during development of colitis must remain speculative. The location of TNF- α -producing cells close to or even in the colonic epithelium concomitant with the appearance of histopathological alterations and subsequent clinical onset of severe colitis suggests that TNF- α may have a direct cytotoxic effect on epithelial cells by inducing an accelerated apoptosis of epithelial cells. Induction of TNF- α -mediated apoptosis via TNFR p55 in intestinal epithelial cells, first at the top of the villi and subsequently extending along the villus axis towards the intestinal crypts, has been recently reported by Guy-Grand et al. (38) and Pigué et al. (39). Such an enhanced apoptosis of colonic epithelial cells may lead to the extensive erosion of the epithelium with subsequent severe inflammatory reactions, as observed during later stages of disease. Furthermore, TNF- α can induce the production of other inflammatory mediators such as free radicals, tissue degrading enzymes such as matrix metallo-

proteases (40, 41), and further proinflammatory cytokines, including IL-1 β (42). TNF- α can further amplify a local immune reaction through its effects on professional APCs such as dendritic cells, which are activated by TNF- α to become potent inducers of immune reactions (43).

Because LT- α , in addition to TNF- α , either as a homotrimer or heterotrimer, also binds to TNFR p55 and TNFR p75, we decided to use donor and recipient mice that were deficient for both TNF- α and LT- α (TNF^{-/-}). The protection of TNF^{-/-}RAG2^{-/-} recipients of TNF^{+/+}CD4⁺CD45RB^{hi} T cells from developing severe colitis clearly demonstrates that not only CD4 T cell-derived TNF- α but also CD4 T cell-produced LT- α is not sufficient for disease induction, nor is it required for initiation and progression of disease. The observed protection of TNF- α ^{-/-}LT- α ^{+/+}RAG2^{-/-} mice from onset of colitis upon transfer of TNF- α ^{-/-}CD4⁺CD45RB^{hi} T cells directly demonstrates that TNF- α plays a nonredundant role in the development of colitis, and its functions cannot be compensated for by the action of LT- α . These findings confirm the results of a previous report on the respective roles of TNF- α and LT- α in a mouse model of experimental allergic encephalomyelitis in which no redundancy in the functions of TNF- α and LT- α was observed in vivo (44). The observed protection of TNF- α ^{-/-} recipients from colitis induction seems to be in contrast to the reported attenuation of disease when experimental mice are treated with a soluble LT- β R-human Fc γ 1 fusion protein in two models of colitis, CD4⁺CD45RB^{hi} T cells \rightarrow SCID mice and bone marrow-transplanted tg ϵ 26 mice (45). However, it cannot be excluded that binding of the soluble LT- β R-human Fc γ 1 fusion protein to activated, LT-bearing transferred T cells or recipient cells may affect the subsequent functional differentiation and/or survival of these LT- β ⁺ cells. Evidence for opsonizing effects of receptor-Fc γ fusion proteins bound to cell surface ligands has recently been provided by the recombinant cell surface expression of human Fc γ in a reversed opsonization (46).

TNF- α has also been previously found to affect the trafficking of T cells into distinct organs such as the central nervous system (47). However, when we examined the presence of CD4 T cells in the colonic mucosa at early time points (10 d) after adoptive cell transfer, in all donor-recipient combinations, CD4 T cells were found in the lamina propria of the small bowel and also, in slightly lower numbers, in the colon. 24 d after adoptive cell transfer, the frequency of colonic CD4 T cells recovered from the recipients of CD4⁺CD45RB^{hi} T cells varied considerably among individual animals. However, no clear correlation between the frequency of CD4 T cells recovered from the inflammatory infiltrates of the colon and development of or protection from colitis was found. A possible explanation for the presence of CD4 T cells in the intestinal lamina propria might be the TNF-independent expression of MAdCAM (mucosal addressin cellular adhesion molecule) 1 on endothelial cells in the small intestine, which we also observed in TNF^{-/-}RAG2^{-/-} mice before adoptive cell transfer (data not shown).

Although clinical signs of colitis are completely abrogated in the absence of TNF, histopathological analyses revealed in some recipients moderate alterations of the large intestinal mucosa. The enhanced cellularity of the colonic mucosa (colitis score ≥ 7) was always associated with enhanced expression of IFN- γ at a protein and mRNA level. This is in agreement with previous reports that indicated an involvement of IFN- γ in the induction of inflammatory reactions in the colonic mucosa (6, 15, 48). The function of IFN- γ in the pathogenesis of colitis, however, seems to be nonessential, as described by the recent report by Simpson et al., who clearly demonstrated a redundant role of IFN- γ in the CD4⁺CD45RB^{hi} transfer model and the bone marrow transplantation into t ϵ 26 transgenic mouse model of colitis (23). In agreement with this notion is our calculation of the absolute numbers of IFN- γ -producing cells isolated

from the colons of recipients of CD4⁺CD45RB^{hi} T cells 24 d after transfer that revealed no clear correlation between the number of IFN- γ -secreting cells and presence or absence of severe colitis (data not shown).

In conclusion, we provide evidence that (a) resident non-T cells are induced in situ directly or indirectly by CD4 T cells to produce TNF, which is essential for the development of clinical signs of colitis; (b) TNF produced by CD4 T cells alone is not sufficient to induce colitis; (c) activation of resident non-T cells by CD4 T cells is not TNF dependent, and hence, other mechanisms, either cell-cell contact such as CD40-CD40 ligand interactions (49) or soluble mediators are involved in the induction of TNF- α production in non-T cells of the affected colonic mucosa; and (d) the functions exerted by TNF- α in the development of colitis can not be compensated for by LT- α .

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