Abrogation of Experimental Colitis Correlates with Increased Apoptosis in Mice Deficient for CD44 Variant Exon 7 (CD44v7)

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Abstract

Experimental colitis in mice is characterized by infiltration of activated T helper (Th) cells and macrophages into the lamina propria. Particularly, these cells expressed CD44 variant exon 7 (CD44v7)-containing isoforms. Upregulation of CD44v7 isoforms was induced by CD40 ligation, an inflammation-driving interaction between activated Th cells and macrophages. To define the role of CD44v7 in colitis, mice bearing a targeted deletion for exon v7 were generated. In trinitrobenzene sulfonic acid–induced colitis, wild-type mice developed severe signs of persistent inflammation. Mice lacking CD44v7 initially showed unspecific inflammation, then recovered completely. The pathogenic origin was shown to reside in bone marrow–derived CD44v7$^+$ cells, because adoptive transfer experiments demonstrated an absolute requirement for CD44v7 on hematopoietic cells for maintenance of colitis. Interleukin (IL)-10–deficient mice, which develop a chronic Th1-driven enterocolitis, were crossbred with CD44v6/v7 null mice. In IL-10 × CD44v6/v7 double deficient mice, intestinal inflammation developed only weakly and at an older age. Analysis of cell death in the inflamed lesions revealed that mononuclear cells in the CD44v7 null infiltrates had higher rates of apoptosis than those from wild-type mice. Thus, the region encoded by CD44v7 appears to be essential for survival of effector lymphocytes, resulting in persistence of inflammation.

Key words: experimental colitis • CD44 knockout • IL-10 knockout • TNBS • apoptosis

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Introduction

In inflammatory bowel disease (IBD),[1] such as Crohn's disease and ulcerative colitis (1–3), an aberrant mucosal homeostasis is observed accompanied by an upregulation of proinflammatory cytokines (4, 5). Similar findings have been described in mouse models of colitis, e.g., in a chemically induced colitis in which the contact sensitizer trinitrobenzene sulfonic acid (TNBS) is locally administered, in transgenic mice bearing targeted deletions for cytokines and receptors important in T cell development or activation and in immunodeficient mice receiving defined T cell subpopulations (6–8).

Pathogenic cells in inflamed lesions of autoimmune diseases have an activated phenotype and are known to express high levels of CD44 that appear to be pivotal for extravasation into inflamed tissues (9). Furthermore, the transmembrane glycoprotein CD44 has been proposed to mediate costimulatory functions (10). A large number of isoforms generated by strictly controlled alternative splicing (11, 12) is found in association with activated lymphocytes, hematopoiesis, and tumor progression (13). These findings prompted us to evaluate whether blockade of defined CD44 variant (CD44v) isoforms might provide a specific and effective therapeutic strategy for inflammatory diseases.

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[1]*Abbreviations used in this paper:* AICD, activation-induced cell death; BL/6, C57BL/6; ES, embryonic stem; HPRT, hypoxanthine ribosyltransferase; IBD, inflammatory bowel disease; LPMC, lamina propria mononuclear cell; RT, reverse transcription; TNBS, trinitrobenzene sulfonic acid; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling; v, variant.

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In fact, administration of an mAb specific to CD44v7 efficiently cured TNBS-induced colitis in mice (14, 15). However, it was not clear from these studies whether the antibody blocked CD44 receptor functions or delivered a transmembrane stimulus. CD44v7 has recently been demonstrated to be upregulated in peripheral blood leukocytes of patients with chronic IBD (16).

We generated mice deficient for CD44 variant exons 6 and 7, known to be upregulated in leukocyte activation and hematological malignancies in humans, rats, and mice (13). Stimulation of CD40 ligation caused upregulation of CD44v7 isoforms. Deficiency for CD44v6/v7 prevented susceptible mice from developing experimental colitis. The inflammation promoting potential resided on $CD44v7^+$ bone marrow cells. These cells appeared to sustain the inflammatory process by inhibiting apoptosis.

Materials and Methods

Vector Construction and Generation of CD44v7- and CD44v6/v7-deficient Mice. The mouse CD44 variant region was isolated from a 129SV genomic library. (The mouse CD44 full-length cDNA sequence is available from EMBL/GenBank/DDBJ under accession no. AJ251594.) Two 34-bp loxP sites were inserted in direct repeats into a single SacI site 3′ of exon v6 and at the 5′ end of the neo^r cassette, which was then inserted into the single Bst1107I site 5′ of exon v6. The HSV-tk cassette was blunt ligated into the BamHI site. For electroporation, the targeting vector was linearized with PvuI and 20 μg was transfected into 10^7 R1 embryonic stem (ES) cells. ES cells were maintained on a feeder layer of embryonic fibroblasts in the presence of leukemia inhibitory factor. After selection with G418 (300 μg/ml) and gancyclovir (10^{-6} M), 415 clones were analyzed by Southern blot using a 5′ external probe (StuI–EcoRI; see probe A in Fig. 1 C). SacI digests revealed that two clones showed homologous recombination (frequency 1/200). Positive clones were injected into C57BL/6 (BL/6) blastocysts and chimeric male offspring were mated with 129SV and BL/6 females. Heterozygous 129SV mice were intercrossed to produce homozygous 129SV mice, targeted with loxP sites flanking exon v6. Heterozygous offspring of the BL/6 females was backcrossed for 10 generations onto the BL/6 background. As reverse transcription (RT)-PCR analyses revealed that homozygous v6 loxP-targeted mice do not express exon v7 (data not shown), further Southern blot analyses were performed using probes 3′ of exon v6. Probe B (EcoRI–EcoRV) indicated loss of 1.6 kb in the region of exon v7, which only occurred in one of the two positive ES clones.

To generate v6-deficient mice, loxP-positive ES clones were transiently transfected with pBS185, a plasmid expressing cre recombinase (17). Cells were grown in the absence of G418, and clones obtained were tested for the loss of G418 resistance. Southern blotting of SacI-digested genomic DNA indicated that one clone (no. 126/28) showed the correct genotype. ES cells were again injected into BL/6 blastocysts, and male chimeric offspring were mated with 129SV, BL/6, or BALB/c females. Heterozygous 129SV mice were intercrossed to produce homozygous 129SV mice, deficient for exon v6. Heterozygous offspring of the BL/6 females was backcrossed for 10 generations onto the BL/6 background. Heterozygous BALB/c females were backcrossed for six generations onto the BALB/c background. Mice were kept under specific pathogen-free conditions; 10–20-wk-old mice were used for inducing colitis with TNBS, all of them on either 129SV or BALB/c background.

$IL\text{-}10^{-/-}$ mice backcrossed to BL/6 for eight generations were provided by M. Kopf (Basel Institute for Immunology) with permission of W. Müller (Institute for Genetics, Cologne, Germany; 18) and cross-bred with $CD44v6/v7^{-/-}$ BL/6 mice. Groups of 6–8 mice at an age of 10–13 wk (a total of 94 mice) were placed into normal housing conditions and investigated weekly for overall health and weight. All mouse experiments were performed in accordance with Swiss and institutional animal care guidelines.

Generation of Antibodies Specific for Mouse CD44v. The CD44v6/v7-deficient mice were also used to obtain mAbs with specificity for v6 (LN6.1) and v7 (LN7.1 and LN7.2). Mice ($CD44v6/v7^{-/-}$) or rats were immunized with 100–200 μg of CD44v4–10/glutathione *S*-transferase (GST) fusion protein, three times over a period of 12 d. 2 d after the last immunization, the regional LNs were taken and cell fusion was performed using the cell line X63-Ag8. The following mAbs were obtained: anti-CD44v10 (termed LN10.1, rat IgG_1), anti-CD44v7 (LN7.1, mouse IgG_1; LN7.2, mouse IgG_{2a}; and LN7.3, mouse IgM), and anti-CD44v6 (LN6.1, mouse IgG_{2a}). The epitopes recognized by the mAbs were mapped using 16-mer peptides (overlapping by eight amino acids) covering the region v4–v10 in ELISAs coated with CD44v4–10/GST, yielded the following specificities: FQNGWQGKNPPTPSED (LN6.1), QEDVSWTDFFDPISHP (LN7.1), and FFDPISHPMGQGHQTE (LN7.2). A CD44v10-specific mAb had already been generated by immunizing rats. It recognizes the sequence PTDTTTSVEGYTFQYPDTMENGTL (LN10.1).

mAbs were purified from cell culture supernatant using protein G–Sepharose columns (Amersham Pharmacia Biotech), and isotypes of the mAbs were determined using commercially available kits (Serotec; Roche Molecular Biochemicals). The purified antibodies were dialyzed and concentrated using Centricon concentrators (Amicon) and labeled with biotin-*N*-hydroxy-succinimide ester (Sigma-Aldrich). Secondary antibodies with enzymatic or fluorescent labels were purchased from Southern biotechnology Associates, Inc., and titrated for use at optimal concentrations. Biotinylated isotype control mAbs (rat IgG_1, mouse IgG_1, mouse IgG_{2a}) were purchased from BD PharMingen and used at identical concentrations as the respective mAbs. Data were acquired in a FACSCalibur™ (Becton Dickinson) and analyzed with the CELLQuest™ program (Becton Dickinson).

Induction of TNBS Colitis. 1 wk before induction of colitis, mice were transferred from specific pathogen-free to conventional conditions. Mice at an age of 10–16 wk were separated into groups of six to eight mice. In the colitis group, mice were given 200 μl TNBS (2.5% wt/vol; Sigma-Aldrich) in 50% ethanol. Control mice received identical injections of 50% ethanol in PBS. A 2.5 F catheter was inserted into the colon up to the appendix, the hapten was applied, and the animals were kept in a vertical position for 30 s.

Histological and Immunohistological Analysis. Colons were macroscopically investigated, and small pieces were embedded in Tissue-Tek (Sakura), frozen on dry ice, and stored at −70°C. Cryostat sections (6 μm) were air dried and stained with hematoxylin and eosin according to standard protocols. The histological changes were graded semiquantitatively into no signs of inflammation (grade 0); low level of leukocyte infiltration (grade 1); high level of leukocyte infiltration, thickening of the colon wall (grade 2); and transmural infiltration of leukocytes with loss of goblet cells, thickening of the colon wall (grade 3).

For immunohistological evaluations, sections were fixed in

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chloroform/acetone for 2 min. Sections were incubated in PBS containing 0.2% FCS and 2% H_2O_2 to quench endogenous peroxidase activity, followed by incubation with human serum (10% in PBS) to block nonspecific binding of antibodies. Tissue was washed twice with an isotonic sodium chloride buffer and then incubated for 30 min with the biotinylated antibodies LN6.1 (anti-v6) and LN7.2 (anti-v7). Detection of immunolabeled cells was performed with streptavidin-biotinylated horseradish peroxidase (Amersham Pharmacia Biotech), and visualized by 3-amino-9-ethylcarbazol (ICN Biomedicals). Negative controls were processed identically, apart from omitting the primary antibody.

Lamina Propria Mononuclear Cell Preparation and Cultivation. Mice were killed by CO_2 at days 2, 5, and 9 after TNBS application. The colon was trimmed of fat, mesenteric tissue, and Peyer's patches. The colon was opened longitudinally and cut into 3-mm pieces. Intraepithelial lymphocytes were removed by incubation in HBSS containing 5 mM EDTA for 30 min at room temperature under vigorous agitation. After three washing steps with PBS, the tissue was incubated for 45 min at 37°C in RPMI 1640 medium, supplemented with 5% FCS and 0.2 mg/ml collagenase D (Roche Molecular Biochemicals) and 0.1 mg/ml DNAse I (Sigma-Aldrich). Cells were separated from tissue debris by filtration through a nylon gauze followed by purification through a discontinuous 40/70% Percoll gradient (Amersham Pharmacia Biotech) for 60 min at 1,500 *g*. This procedure yielded 0.5–1 $\times$ 10^6 cells/mouse.

Cell Culture. LNs from wild-type and $v6/v7^{-/-}$ mice were isolated and single cell suspensions were prepared. Cells were incubated overnight in RPMI medium, and supplemented with 10% FCS and 20 U/ml IL-2. Activation was induced by 1 μg/ml PHA (Wellcome) or coculture with CD40L secreting J558 myeloma cells in transwells (Costar) overnight.

Reconstitution of Lethally Irradiated 129SV Mice with CD44v7-deleted Lymphocytes. 129SV mice were lethally irradiated with 8 Gy followed by reconstitution with 5×10^6 bone marrow cells from either $CD44v7^{-/-}$ ($n = 10$) or $CD44v7^{+/+}$ ($n = 5$) mice (129SV or BALB/c background) intravenously. 6 wk after reconstitution, mice were treated with a single application of TNBS as described above.

Semiquantitative Analysis of CD44 Isoforms by RT-PCR. Mononuclear cells prepared from inflamed and noninflamed colon mucosa were resuspended in guanidium isothiocyanate (GITC), and cDNA was prepared as described previously (19). For semiquantitative analysis, cDNA amounts were equilibrated by standardizing to the mouse hypoxanthine ribosyltransferase (HPRT) gene product. The HPRT primers GCCCCAAAATGGTTAAGGTTGC and GACAACGATTTACTGAAAGTGGG yield a product of 701 bp. Identical amounts of cDNAs were then used to perform CD44v-specific PCR. The primers used for CD44 amplification were CCGGAGCACCTTGGCCACC and CCTGGTTCGCACTTGAGTGTC, which are flanking the alternatively spliced variant region yielding a product of 107 bp for CD44s when no variant exons are included (see Fig. 1 B). The PCR regime was 10 s at 96°C, 15 s at 55°C (for HPRT) or 60°C (for CD44), 1 min at 72°C for 30 cycles, followed by a final 10 min at 72°C in a Hybaid OmniGene machine.

Products were separated on 1.2% agarose gels and alkaline-transferred onto Hybond N^+ membranes (Amersham Pharmacia Biotech). For specific detection, PCR products from CD44v exons were prepared, gel purified, and ^{33}P-labeled using the Megaprime DNA labeling system from Amersham Pharmacia Biotech. After stringent hybridization and washing, blots were exposed to BioMax films (Eastman Kodak Co.) for 6 h.

Results

CD44 Isoform Expression in Colitis and Generation of CD44v7- and CD44v6/v7-deficient Mice. Activation of human and rat lymphocytes causes a preferential upregulation of CD44v6-containing isoforms (19, 20). Immunohistochemical analyses revealed that CD44v7-containing isoforms were also present in the weakly inflamed infiltrates in the colonic mucosa of TNBS-treated BALB/c mice (Fig. 1 A, top left). About 50% of the cells in the infiltrates with severe colitis were positive for CD44v6 (not shown) and v7 (Fig. 1 A, bottom right). Lamina propria mononuclear cells (LPMCs) from inflamed colons were mostly $CD4^+$ T cells (40–70%) and $CD11b^+$ macrophages (10–30%) (data not shown).

To analyze the composition of CD44 splice variants, we performed RT-PCR analyses of LPMCs from inflamed and noninflamed mucosa (Fig. 1 B). Whereas in LPMCs from noninflamed mucosa only exons v3 and v10 were expressed (included into the standard framework either as a single exon or, for v10, combined with exons v8 and v9) and no exon v7, in LPMC inflamed mucosa, especially variant isoforms containing exon v7 were detected (Fig. 1 B). RT-PCR analysis of Th1-polarized $CD4^+$ splenic T cells revealed a very similar pattern as LPMCs from inflamed mucosa. In contrast, the pattern of LPMCs in noninflamed mucosa resembled one of the naive T cells and Th2-polarized $CD4^+$ splenic T cells (19; and Johansson, B., and U. Günthert, manuscript in preparation).

The v6 exon of the CD44 gene (Fig. 1 C) was targeted in murine R1 ES cells with flanking loxP sites and 5′ insertion of a neo expression cassette. Detailed Southern blot analysis indicated that a deletion occurred in the region of exon v7, most likely during homologous recombination in one of the two ES clones. Thus, v6 loxP-targeted mice were devoid of exon v7, but retained expression of the other isoforms. The loxP-targeted ES cell clones were transiently transfected with a cre recombinase–expressing plasmid, pBS165 (17), and a neo-sensitive clone revealed proper excision of the loxP-targeted region, R1-126/28. Heterozygous 129SV mice were backcrossed with BL/6 mice for 10 generations and with BALB/c mice for 6 generations. Homozygous mice were born at the expected frequency on all backgrounds. Female and male mutant mice ($v7^{-/-}$ and $v6/v7^{-/-}$) were fertile and did not show obvious abnormalities. The distribution of subpopulations of hematopoietic cells in central and peripheral lymphoid organs appeared unaltered (data not shown).

Activation of LN cells from BALB/c mice with PHA in vitro led to a 12-fold increase of CD44v6 and v10 (not shown) surface expression, but v7 expression was almost unchanged (Fig. 1 D). Cocultivation of LN cells with CD40L-secreting J558 cells (21) increased the CD44v7 expression sixfold, but did not affect v6 (Fig. 1 D). Similar upregulation of v7 was achieved by treatment of LN cells with anti-CD40 antibodies (data not shown). Neither PHA nor CD40L induced expression of v6 and v7 in CD44v6/$v7^{-/-}$ mice. Notably, CD44's expression was unaffected in

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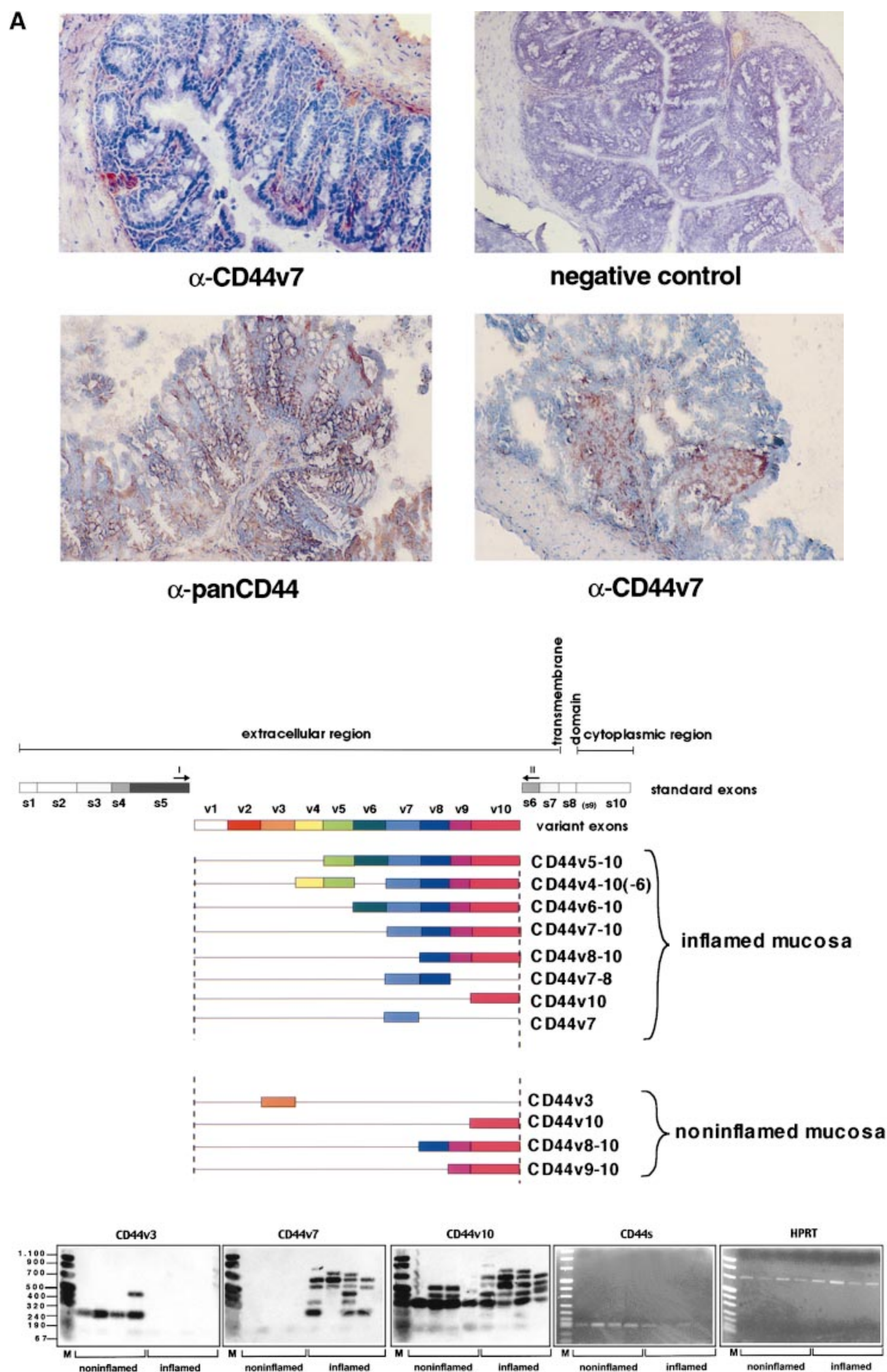

Figure 1. *(continued on facing page).* Expression of CD44v7 and generation of CD44v6/v7$^{-/-}$ mice. (A) Immunohistochemical analysis of CD44v7 isoforms in inflamed mucosa showing expression of slightly inflamed mucosa (top left) as well as in large transmural infiltrates (bottom right). IM7.8.1 (anti–pan-CD44) reveals broad staining (bottom left). Original magnifications: ×200; negative control, ×100. (B) cDNA was prepared from LPMCs of noninflamed and inflamed large intestines of TNBS colitis, and semiquantitative RT-PCR of CD44 variant isoforms was performed using primers I and II located within the standard region, flanking the variant part. Amounts of cDNA were equilibrated to HPRT- and CD44-specific reactions blotted and hybridized with exon-specific probes. Reactions for v3, v7, v10, and the standard region (CD44s) are shown in the bottom panel. Presence and size of a signal allowed for the composition of the variant isoforms expressed, which are indicated in the scheme above. LPMCs from inflamed colons express a variety of CD44v isoforms, mostly including v7, whereas LPMCs from noninflamed tissue express exons v3 and v10 as a single exon, as well as v10 in combination with v8 and v9. The gray bars in the upper region of the scheme represent the CD44 standard region, which is expressed with similar intensity in all preparations. (C) Genomic targeting of CD44 exons v6 and v7. Analysis of ES clones by Southern blotting using a 5′ external probe (StuI–EcoRI; probe A). Southern blot analyses using probe B (EcoRI–EcoRV) indicated loss of 1.6 kb in the region of exon v7, which only occurred in one of the two

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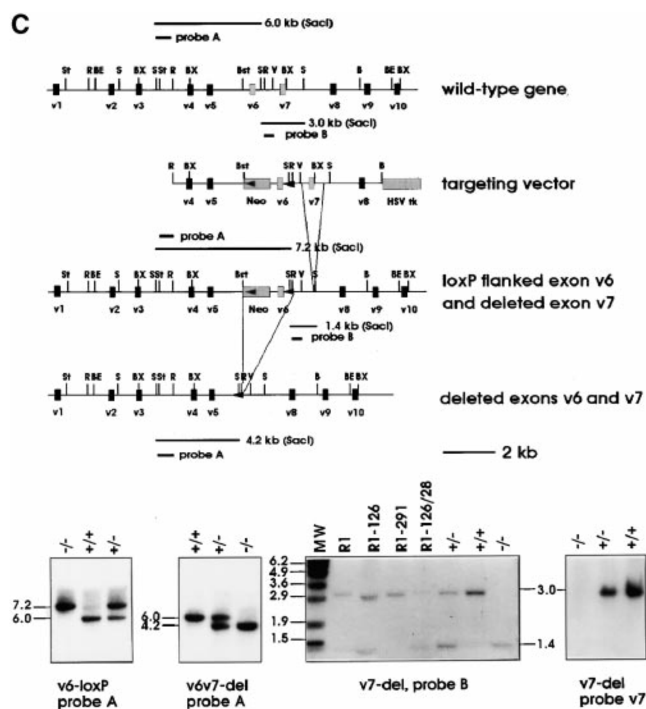
the v6/v7$^{-/-}$ mice and similarly strong as in the wild-type mice (Fig. 1 D).

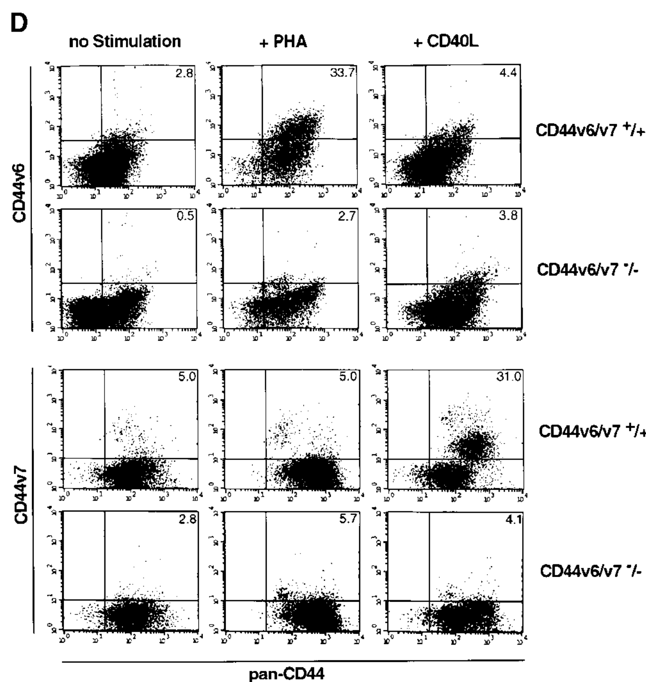
CD44v7-deleted Mice Do Not Develop Colitis. A small number of either wild-type 129SV (19%, 10/52) or CD44v7$^{-/-}$ mice (18%, 11/61) died within 2 d after rectal administration of TNBS in ethanol (Fig. 2 A). However, all CD44v7$^{-/-}$ mice that survived the acute injury were protected from colitis and were clinically normal at day 9. Similar findings (survival rate 78%, 32/41) accounted for CD44v6/v7$^{-/-}$ mice. In contrast, only 44% of wild-type mice survived, and 92% of these developed severe colitis with wasting disease. The clinical data were consistent with the histological findings. CD44v7$^{-/-}$ and CD44v6/v7$^{-/-}$ mice responded to the vehicle (50% ethanol) injection with an acute mucosal injury, ulceration, and hemorrhagic infiltrates within the first 2 d (Fig. 2 B). They completely recovered thereafter with macroscopically and histologically normal colons on day 9, when those of wild-type mice still showed severe strictures and inflammation (Fig. 2 B). Mice heterozygous for CD44v7 (or CD44v6/v7, not shown) did not differ from normal wild-type mice (Fig. 2 B). Thus, CD44v7 is essential for exacerbation of experimental colitis.

Analysis of cytokine production of LPMCs (protein and mRNA levels) during the 9-d course of TNBS treatment revealed a downregulation of Th1-type cytokines and chemokines (IL-12, IFN-γ, and macrophage inflammatory protein 1α and 1β) and a corresponding upregulation of Th2-type cytokines (IL-4, IL-10, and TGF-β) in CD44v7$^{-/-}$ and CD44v6/v7$^{-/-}$ mice in contrast to wild-type mice (or CD44v7$^{+/-}$ and CD44v6/v7$^{+/-}$ mice) (data not shown).

Resistance to TNBS Colitis Can Be Transferred from CD44v7-deficient Mice into Lethally Irradiated Susceptible Mice. As expression of CD44v7 and v6/v7 isoforms on both epithelial cells and leukocytes was deleted in our targeted mice, either cell type might be participating in disease progression. Therefore, lethally irradiated 129SV mice were reconstituted with bone marrow from either CD44v7$^{-/-}$ or CD44v7$^{+/+}$ mice (129SV). All 129SV mice that received bone marrow from 129SV CD44v7$^{-/-}$ mice survived a single application of TNBS given 6 wk after transplantation, whereas mice given bone marrow from 129SV CD44v7$^{+/+}$ mice developed colitis (42% survival; Fig. 3). Similar data were obtained by transfer of CD44v7$^{-/-}$ and v7$^{+/+}$ (BALB/c) bone marrow into BALB/c mice (88 vs. 22% survival). Hence, development of the inflammatory reaction requires CD44v7-proficient hematopoietic cells.

Targeted Deletion of CD44v6/v7 Exons Delays Onset and Ameliorates the Intensity of Chronic Enterocolitis in IL-10$^{-/-}$ Mice. The importance of IL-10 in the regulation of mucosal inflammation has been demonstrated in several animal models (22). IL-10$^{-/-}$ mice spontaneously develop a generalized enterocolitis under conventional housing conditions, and the inflammation can be prevented by the





positive ES clones. The restriction sites are: St, StuI; R, EcoRI; BE, BstEII; S, SacI; BX, BstXI; Bst, Bst1107I; V, EcoRV; and B, BamHI. (D) LN cells were prepared and stimulated overnight with PHA or cocultured with CD40L-transfected J558 cells in transwell plates (Costar). Surface staining was performed using pan-CD44–specific mAb (clone IM7.8.1)–FITC, and biotinylated CD44v6 (LN6.1 or BMS145; Bender MedSystems) specific and v7 (LN7.1) specific antibodies. Avidin-PE was used for detection of the CD44v expression. Percentage of double labeled cells is indicated in the upper right quadrant.

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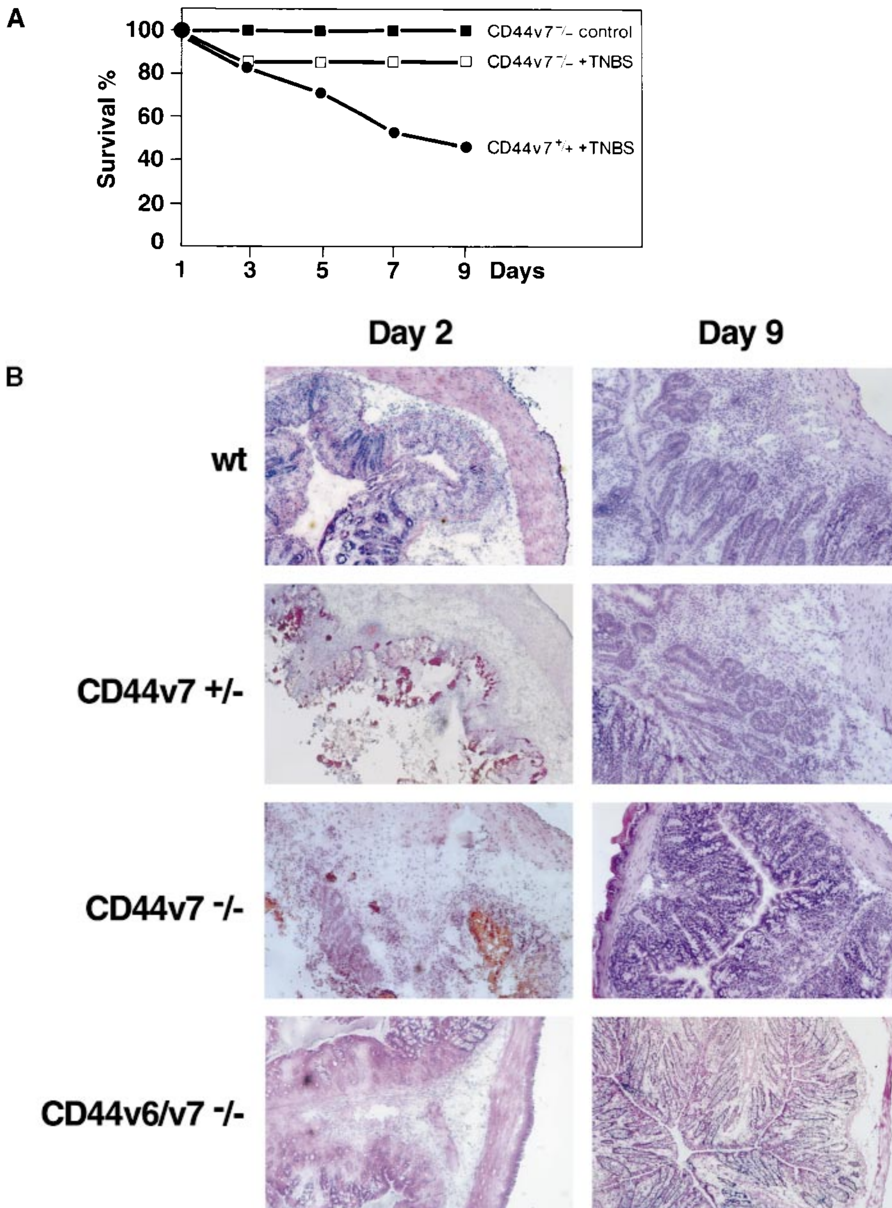

Figure 2. CD44v7- and CD44v6/v7-deficient mice overcome TNBS-induced colitis. (A) Survival of mice treated with TNBS. Mice were kept under normal housing conditions and at an age of 10–16 wk, were separated into groups of 6–8 mice (a total of 61 CD44v7$^{-/-}$ mice and 52 129SV wild-type [wt] mice), and either given 200 μl TNBS (2.5% wt/vol; Sigma-Aldrich) in 50% ethanol, or 200 μl 50% ethanol in PBS (control). (B) Histology of CD44v7 and CD44v6/v7$^{-/-}$ mice treated with TNBS. Frozen sections of colons 2 and 9 d after TNBS treatment were stained with hematoxylin and eosin. Original magnifications: ×100.

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administration of recombinant IL-10 (18). Intestinal inflammation in IL-10$^{-/-}$ mice appears to be due to the lack of the general suppressive effects of IL-10 on cytokine production by macrophages and Th1 cells (23). IL-10 treatment also ameliorates inflammation in TNBS-induced colitis and in a T cell subset transfer model (24, 25). In addition, local administration of IL-10 has been beneficial for patients with Crohn's disease (26).

IL-10 is upregulated in TNBS-treated CD44v6/v7$^{-/-}$ mice and anti-v7–treated wild-type mice (14, 15; data not shown). To investigate the role of CD44v6/v7 in the chronic enterocolitis that spontaneously develops in IL-10$^{-/-}$ mice (18), we intercrossed both strains. Groups of six mice, each with different genotypes, were kept under normal housing conditions. All IL-10$^{-/-}$ mice (either CD44v6/v7$^{+/+}$ or CD44v6/v7$^{+/-}$) developed chronic colitis with macroscopically obvious anal prolapses at 12–18 wk of age, and weight loss starting around 25 wk of age (Fig. 4 A). A substantial mucosal infiltrate of mononuclear cells was present in 12-wk-old animals, whereas at 31 wk infiltrates were detectable in both mucosa and submucosa with transmural ulcerations (Fig. 4 B). At this age, disease was observed in 100% of the IL-10$^{-/-}$ (CD44v6/v7$^{+/+}$ or $^{+/-}$) mice. Control mice (IL-10$^{+/+}$ and any CD44v6/v7 genotype) did not spontaneously develop colitis and continually gained weight within the observation period (Fig. 4 A). The colons of these mice showed normal morphology (Fig. 4 B). Double deficient mice (IL-10$^{-/-}$ CD44v6/v7$^{-/-}$) developed well until an age of 24 wk, then they slowly started to lose weight (Fig. 4 A). Histological evalu-

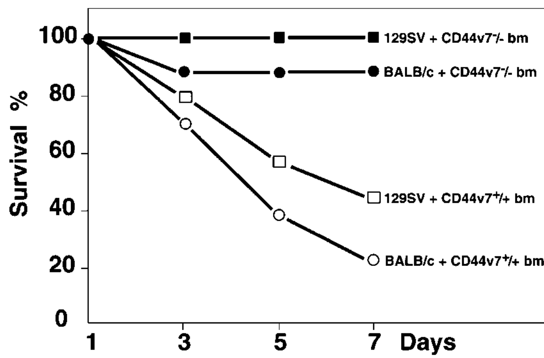
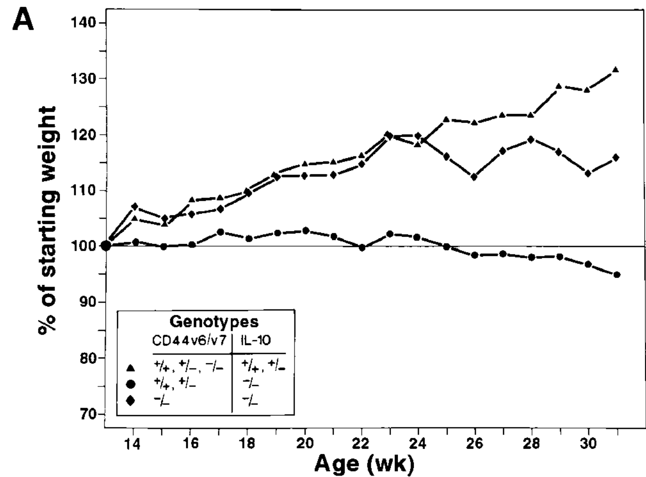


Figure 3. Resistance against TNBS colitis can be transferred by bone marrow from CD44v7$^{-/-}$ mice. 129SV and BALB/c mice were lethally irradiated with 8 Gy and reconstituted with 5×10^6 bone marrow cells (bm) from either CD44v7$^{-/-}$ ($n = 10$) or CD44v7$^{+/+}$ ($n = 5$) mice (129SV or BALB/c background). TNBS was applied 6 wk after reconstitution.

ation revealed only small areas of inflammation in the mucosae (Fig. 4 B). These double deficient mice did not have the shortened life span typical of IL-10$^{-/-}$ CD44v6/v7$^{+/+}$ mice. Analysis of mucosal leukocytes showed a strongly diminished IL-12 production in the double deficient mice in contrast to IL-10$^{-/-}$ CD44v6/v7$^{+/+}$ mice (protein and mRNA levels; data not shown). A total of 52 double deficient mice were analyzed, and all showed strongly reduced inflammation. Thus, CD44v6/v7 is required for the initiation and maintenance of the disease that spontaneously develops in IL-10$^{-/-}$ mice.

Increased Apoptosis of Leukocytes in Inflamed Regions of CD44v6/v7-deficient Mice. As described previously, interactions involving CD40 and CD40L result in an immediate and strong increase of pan-CD44 expression on Th1 cells as well as on the APCs (10). Using specific reagents, we now observed that CD40 ligation induces an upregulation of CD44v7 expression on mouse LPMCs, which may be important for the interaction between Th1 cells and macrophages to sustain the inflammatory reaction. Blocking of the CD40–CD40L interaction, which is pivotal for Th1 cell priming and induction of IL-12 secretion in monocytes/macrophages (27), prevents TNBS-induced colitis (28). Deficiency for the CD44v6/v7 isoforms could thus abrogate the Th1–macrophage interaction and prevent the development of a transmural colonic inflammation with massive accumulation of LPMCs.

A further important feature of CD40–CD40L interactions is the enhancement of clonal T cell expansion and the delay in their activation-induced cell death (AICD) (29–31). Furthermore, there is strong evidence that a balance between the production of autoreactive T cells and their rapid elimination by AICD is perturbed in autoimmune diseases (32). Therefore, we analyzed apoptotic activity in early inflammatory lesions of wild-type and CD44v7-deficient mice. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays revealed three- to fourfold higher numbers of apoptotic cells in CD44v7$^{-/-}$ or CD44v6/v7$^{-/-}$ colons compared with those



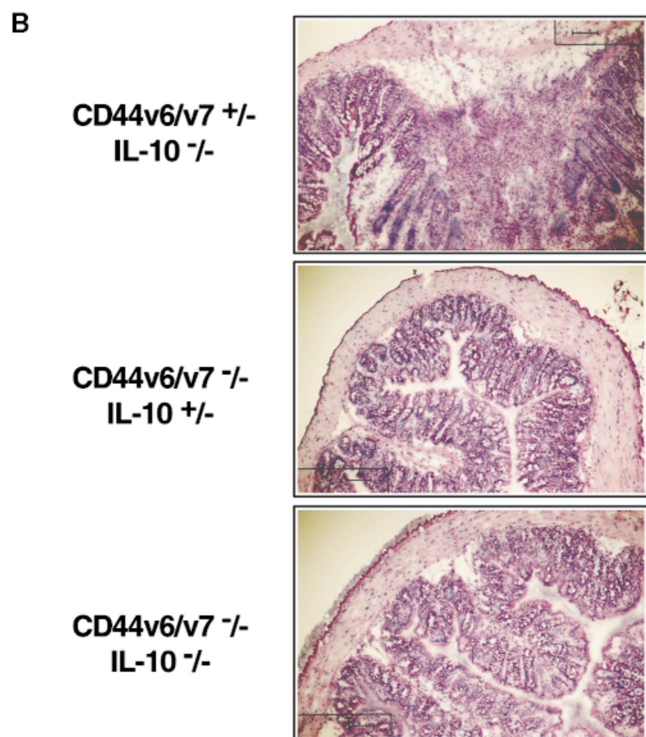


Figure 4. CD44v6/v7 deficiency can largely rescue colitis in IL-10$^{-/-}$ mice. (A) Mice (six per group) proficient for IL-10 (+/+ or +/−) and any CD44v6/v7 genotype (▲), IL-10 (−/−) and CD44v6/v7 (+/+ or +/−) mice (●), and mice deficient for both markers (◆) were weighed weekly (reference 23). (B) Histology of CD44v6/v7 × IL-10 mice. Frozen sections of colons from three different genotypes of mice taken from the experiment in A at 31 wk of age were stained with hematoxylin and eosin. Sections show transmural colitis in a v6/v7$^{+/-}$ IL-10$^{-/-}$ mouse; no signs of colitis in a v6/v7$^{-/-}$ IL-10$^{+/-}$ mouse; and low levels of leukocyte infiltration in a v6/v7$^{-/-}$ IL-10$^{-/-}$ mouse. Bar, 40 μm; original magnifications: ×100.

from similarly TNBS-treated wild-type mice (Fig. 5 A). However, in noninflammatory untreated CD44v6/v7$^{-/-}$ mice, such an increase in apoptotic nuclei was not observed (Fig. 5 A). Counts of apoptotic nuclei per field were 52 (± 20) for CD44 wild-type; 23 (± 11) for CD44v6/v7$^{-/-}$ control; 192 (± 43) for CD44v6/v7$^{-/-}$; and 183 (± 49) for

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CD44v7$^{-/-}$. Concomitantly, LPMCs from CD44v7$^{-/-}$ and CD44v6/v7$^{-/-}$ inflamed lesions revealed more apoptotic cells compared with LPMCs from TNBS-treated wild-type mice by assaying for annexin V binding and propidium iodide uptake (Fig. 5 B).

Discussion

Targeted deletion of exons 6 and 7 of the variant region of CD44 in mice allowed us to demonstrate in hapten-induced experimental colitis, by adoptive transfer as well as by crossing the mutants with IL-10$^{-/-}$ mice, that expression of these CD44v regions on activated hematopoietic cells, particularly of inflamed mucosa, is essential for promoting and maintaining unbridled Th1 reactions in mouse models for IBD. The requirement for CD44v6/v7 was clearly pointed out due to the fact that the isoform-specific mutant mice were not affected in the expression of the predominant hematopoietic CD44 isoform. The region encoded by exon v7 appears pivotal in these diseases, as treatment with anti-CD44v7 antibodies led to complete remission in TNBS-induced colitis, in contrast to v6-specific therapy (14). Furthermore, (a) CD44v7- and CD44v6/v7-deficient mice were equally resistant to TNBS colitis; (b) CD44v7-deficient bone marrow cells were able to fully protect against TNBS colitis in adoptive transfer experiments; and (c) both CD44v7- and CD44v6/v7-deficient mice exhibited a similar degree of apoptotic cells in the early inflamed lesions.

Immunomodulatory therapies, such as treatment with anti–IL-12, anti-CD40, and recombinant IL-10, have been applied successfully in experimental colitis (6, 28, 33). We have observed a strong downregulation of IL-12 in CD44v7- and CD44v6/v7-deficient mice when challenged with TNBS or when cross-bred with IL-10–deficient mice. The Th1-polarizing cytokine IL-12 is known to play a major role in both TNBS-induced colitis and Crohn's disease as well as sustaining the chronic phase in

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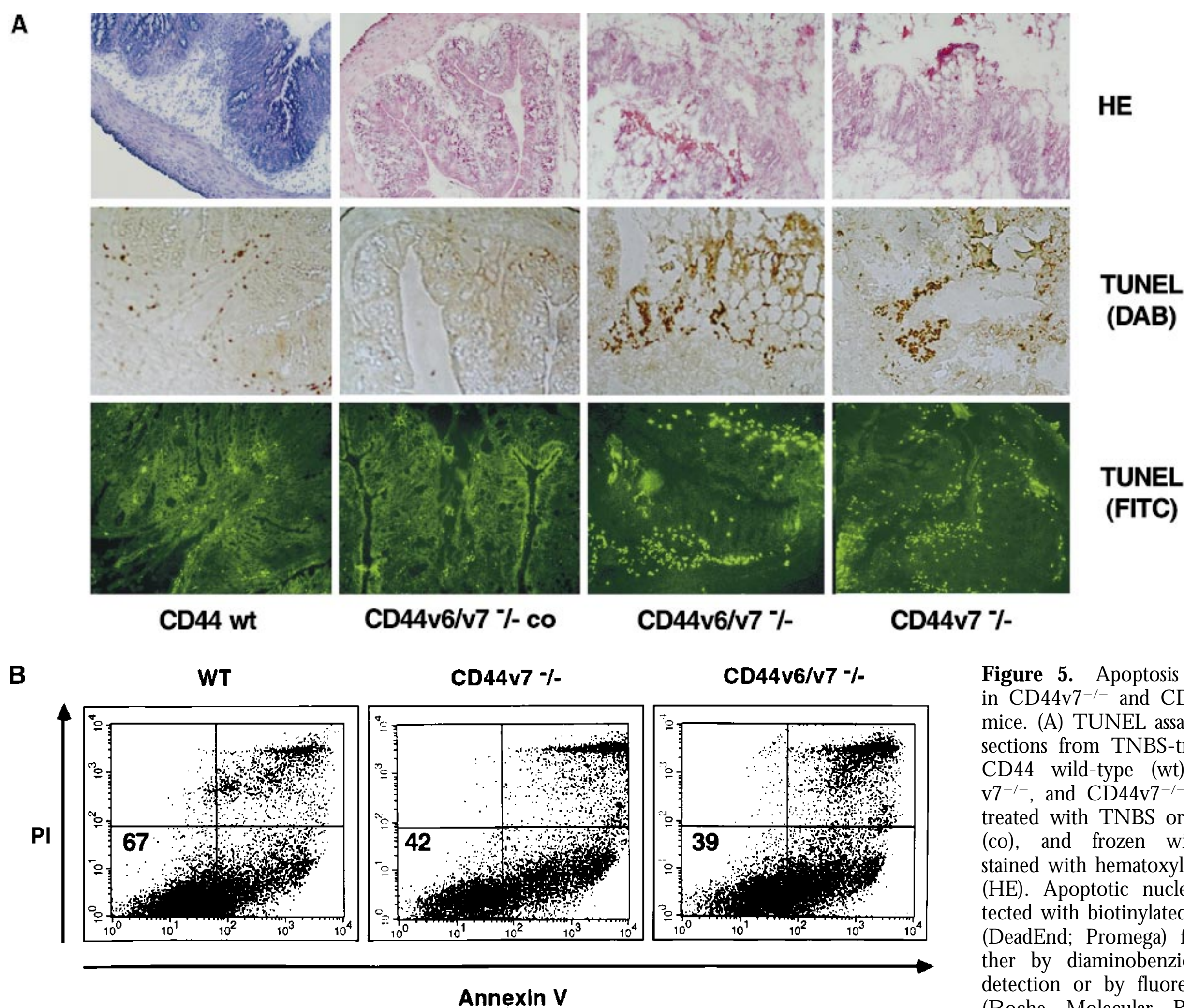

Figure 5. Apoptosis is increased in CD44v7$^{-/-}$ and CD44v6/v7$^{-/-}$ mice. (A) TUNEL assay of colonic sections from TNBS-treated mice. CD44 wild-type (wt), CD44v6/v7$^{-/-}$, and CD44v7$^{-/-}$ mice were treated with TNBS or not treated (co), and frozen with sections stained with hematoxylin and eosin (HE). Apoptotic nuclei were detected with biotinylated nucleotides (DeadEnd; Promega) followed either by diaminobenzidine (DAB) detection or by fluorescein-dUTP (Roche Molecular Biochemicals) (FITC). (B) LPMCs from TNBS-treated wild-type (WT), CD44v7$^{-/-}$, and CD44v6/v7$^{-/-}$ mice were incubated with annexin V–FITC (CLONTECH Laboratories, Inc.) and propidium iodide (PI). The percentage of live cells is indicated for one of four experiments.

IL-10–deficient mice (33–36). Administration of anti-CD40L antibodies prevents the priming of Th 1 cells due to an inhibition of IL-12 secretion (28). As shown here, CD40–CD40L ligation specifically induced CD44v7 expression on mouse LN cells. Similarly, CD44 variant isoforms were upregulated on human leukocytes upon CD40 stimulation (our unpublished data).

In this study, the strong reduction of the initial inflammatory response in the CD44v7$^{-/-}$ mice is correlated with increased apoptotic activity in the lesions. CD44v7, which is upregulated upon CD40 ligation, thus appears to protect activated leukocytes from AICD. There is compelling evidence that pan-CD44 promotes survival of activated lymphocytes, fibroblasts, and carcinoma cells (37–41). CD44 may thus have a major impact in promoting the persistence of inflammation in autoimmune diseases and possibly in preventing tumor cells from undergoing apoptosis. The increased apoptotic activity of activated leukocytes results in a rapid clearing of the inflammation by phagocytosis of the dead cells. This process may lead to systemic tolerance due to presentation of antigens from the dead cells by the phagocytosing APCs and reduction of the inflammation by production of IL-10 from the dying lymphoid cells (42).

Analyses of surface molecules from inflamed regions of patients or mice with Th1-type autoimmune disease or chronic inflammations, such as systemic lupus erythematosus, rheumatoid arthritis, experimental autoimmune encephalomyelitis, insulin-dependent diabetes mellitus, and delayed-type hypersensitivity reactions all correlate with high expression levels of CD44 (43–47). Using CD44 reagents specific for the variant isoforms, disease scores in experimental autoimmune encephalomyelitis could be strongly reduced (48). By specifically deleting exons v6 and v7 in mice, without affecting the expression of the other exons, we could now unequivocally demonstrate that the region encoded by exon v7 rather than the standard region of CD44, whose expression is unaffected by the mutation, is causally involved in chronic bowel inflammation.

In contrast to the CD44 standard isoform, devoid of any variant regions, which is expressed ubiquitously, we now demonstrate that CD44v7 expression is restricted to lymphocytes of inflammatory lesions in Th1-polarized experimental colitis. The same finding was observed in Th1- but not Th2-polarized CD4^{+} splenic T cells (Johansson, B., and U. Günthert, manuscript in preparation). In addition, in an earlier study we have shown that stimulation with IFN-γ in vitro results in an upregulation of CD44 variant isoforms in human leukocytes (49). Thus, CD44v7 appears to be a new marker for Th1-polarized activated T cells.

Our ongoing research in mouse models for rheumatoid arthritis, experimental autoimmune encephalomyelitis, and delayed-type hypersensitivity reactions indicates that the CD44 variant region is also of major importance in maintenance of the inflammatory reactions in these diseases. This finding may well open a new strategy of therapeutic intervention in autoimmune diseases by interfering with CD44v7 as a potent inhibitor of apoptosis. Therapeutic intervention strategies promoting cell death by interfering with inhibitors of apoptosis have been suggested in the treatment of patients with autoimmune disease and tumors (50).

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