On the pathophysiological significance of CD154/CD40-mediated leukocyte-endothelial cell interaction

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Abbreviations

AAP	abridged anchor primer
APC	antigen-presenting cell
AUAP	abridged universal amplification primer
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxynucleotide acid
dNTP	deoxynucleotide triphosphate
ECGS	endothelial cell growth supplement
EDTA	ethylendinitrilo-N, N, N', N'-tetra-acetate
EF	elongation factor
FCS	fetal calf serum
HBSS	Hank's buffered salt solution
HEPES	2-(4-2-hydroxylethyl)-poperazinyl-1-ethansulfonat
HIGM	hyper-IgM syndrome
HUVEC	human umbilical vein endothelial cell
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon gamma
Ig	immunoglobin
IL	interleukin
IRF-1	interferon regulatory factor-1
mAb	monocolonal antibody
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein-1 alpha
mRNA	messenger RNA

ODN	oligodeoxynecleotide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
rGSP	rat gene specific primer
RNase	ribonuclease
RPL	ribosomal protein large unit
rSMC	rat smooth muscle cell
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
s.e.	standard error
SOCS	suppressor of cytokine signaling
STAT-1	signal transducer and activator of transcription-1
TGF-β	transforming growth factor-beta
TNBS	trinitrobenzene sulphonic acid
TNF-α	tumor necrosis factor-alpha
Tris	Tris-(hydroxymethyl)-aminomethan
U	unit
VCAM-1	vascular cell adhesion molecule-1

1. Introduction

1.1 CD40 and CD154

CD40 is a cell surface receptor which was first identified and functionally characterized in B-lymphocytes. Its ligand, CD154 (CD40L, gp39, T-BAM or TRAP), is mainly expressed on activated $CD4^+$ T cells (van Kooten et al. 2000, Schoenbeck et al. 2001). Both CD40 and CD154 belong to an emerging receptor-ligand family that includes the TNF receptor and TNF family, respectively. This family is characterized by structural homologies, clustered chromosome location, shared signal transduction pathways and overlapping biological activities in processes such as cell growth, differentiation and programmed cell death.

1.1.1 Expression of CD40 and CD154

CD40 is principally expressed on B-cells and professional antigen-presenting cells (APCs), like dendritic cells and monocytes. Recently, it has become clear that CD40 is expressed much more broadly, including endothelial cells, vascular smooth muscle cells and fibroblasts (van Kooten et al. 2000, Schoenbeck et al. 2001). Although CD40 expression is generally low in these cells, the protein is clearly up-regulated under various pathological conditions. The combination of TNF-α and INF-γ has been shown to be the most potent stimulus for CD40 expression. Research in our group has shown that activation of signal transducer and activator of transcription-1 (STAT-1) is crucial for the cytokine-induced expression of CD40 in rat vascular smooth muscle cells as well as in human endothelial cells (Krzesz et al. 1999, Wagner et al. 2002). With regard to the latter, however, STAT-1 mediates CD40 expression either directly or indirectly through *de novo* synthesis of interferon regulatory factor-1 (IRF-1).

The expression of CD154 on activated T-cells is transient and tightly regulated. CD154 expression can be seen on a subset of $CD4^+$ memory T-cells as early as 5-15 minutes after APC-induced activation through the T-cell receptor complex (Casamayor-Pellejia et al. 1995). Such surface expression does not require *de novo* protein synthesis and is probably due to preformed CD154. A second wave of CD154 expression, preceded by an increase in the RNA level, occurs 1-2 hours after activation, reaches the peak after 6-8 h and gradually declines thereafter.

This transient expression help to ensure that CD154 expression remains restricted in time. Moreover, CD154 expression has also been detected on $CD8^+$ T-cells, B-cells, NK cells, monocytes and dendritic cells under certain conditions (van Kooten et al. 2000, Schoenbeck et al. 2001). Especially, it has recently been reported that platelets express CD154 within seconds of thrombin activation *in vitro* and in the process of thrombus formation *in vivo* (Henn et al. 1998). In addition, despite the membrane-bound form (protein of 32 - 33 kDa), there are two shorter versions of CD154 (31 kDa and/or 18 kDa). These shorter soluble forms retain their ability to form trimers to bind CD40, and to transduce biological signals (Graf et al. 1995, Mazzei et al. 1995). This indicates that CD154 might also act as a bona fide cytokine. Qualitative differences between the soluble and membrane-bound forms may exist, too.

1.1.2 CD154/CD40 interactions in immune responses

CD154 interacts with CD40 expressed on B-cells, macrophages, dendritic cells, fibroblasts and endothelial cells to induce and regulate immune responses. The important biological role of CD154/CD40 interactions has been firmly established. *In vivo*, deletion or mutations of either the CD40 or CD154 gene leads to severe immunodeficiency, in both man and mouse, characterized by hyper-IgM syndrome (HIGM) as well as T-cell defects in cell-mediated immunity (Castigli et al. 1994, Renshaw et al. 1994, Razanajaona et al. 1996).

For humoral immunity: CD40 emerges early on $CD34^+$ B-cell precursors in the bone marrow before immunoglobin gene rearrangement. It is expressed on B-cells until their terminal differentiation into plasma cells. Direct effects of CD40 activation on B-cells include cytokine production (IL-6, IL-10, TNF-α, LT-α), expression of adhesion molecules and costimulatory receptors (ICAM-1, CD23, CD80, CD86) and increased expression of MHC I and MHC II (Khanna et al. 1997, Dadgostar et al. 2002). These molecules all contribute to the biological function of B-cells. Extensive *in vitro* studies have demonstrated that CD40 activation has major effects on many steps of the B-cell nature history, including rescue from apoptosis, differentiation into germinal center cells, Ig isotype switching and maturation into memory cells (Kehry 1996, Klaus et al. 1997). Considering the key role of the CD154/CD450 pathway in antibody formation, it is evident that blockade of this pathway may provide a novel means of inhibiting pathogenic auto-

antibodies or unwanted antibodies that may arise during the exogenous administration of foreign antigens.

For cellular immunity: CD154-induced triggering of CD40 on the surface of other APCs including dendritic cells and macrophages may ultimately control both the afferent phases of immune recognition by T-cells as well as important effector mechanisms of cell mediated immunity (Grewal 1998, van Kooten et al. 2000). CD40 activation augments the antigen presenting functions of dendritic cells by inducing maintenance of high levels of MHC class II antigens and marked upregulation of accessory molecules including CD58. In addition, CD40 activation also induces the expression of key co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2). Through interaction with CD28, they provide the essential second signal required for full T-cell activation. In the absence of CD80/CD86 triggering of CD28, anergy or tolerance rather than T-cell activation ensues as a consequence of antigen triggering. These data suggest that the CD154/CD40 pathway governs the T-cell decision whether immunity or tolerance is developed in response to exogenous antigens. In this regard, recent *in vivo* studies in mice strongly suggest that antibody-based blockade of CD154/CD40 interactions induces tolerance. For example, the anti-CD154 mAb (MR1) alone has allowed indefinite islet allograft survival in recipient mice and significantly inhibits heart, skin and kidney allograft rejection (Reul et al. 1997, Larsen et al. 1997, Elwood et al. 1998).

In addition to up-regulating co-stimulatory molecules, CD154/CD40 interactions also induce APCs to secrete an important set of cytokines and chemokines including IL-12, IL-8, TNF-α and MIP-1α (Kiener et al. 1995, Cella et al. 1996). The effect of CD40 ligation on IL-12 secretion is of interest because IL-12 itself up-regulates CD154 expression on T-cells and synergizes with IL-2 as well as with other co-stimulatory molecules, including CD80 and CD86, to maximize CD154 expression (Peng et al. 1998). It is well known that IL-12 functions as a major cytokine governing the differentiation of $CD4^+$ T-cell subsets by promoting the differentiation of the Th1 subset. Moreover, it has been shown that CD154/CD40 interactions are crucial for the IL-12-dependent priming of Th1-cells *in vivo* (Kelsall et al. 1996). Blockade of the CD154/CD40 pathway prevents a variety of immune disorders in mice mediated by $CD4^+$ Th1-cells including type I collagen-induced arthritis, experimental autoimmune encephalitis and the NOD model of diabetes mellitus (Desai-Mehta et al. 1996, MacDonald et al. 1997, Markees et al. 1999).

1.1.3 CD154/CD40 interaction in endothelial cells

As noted above, CD40 expression is generally low in endothelial cells, but clearly up-regulated under pro-inflammatory conditions, especially by the combination of TNF-α plus INF-γ. As the site for leukocyte adhesion and extravasation, the endothelium plays an important role in chronic inflammatory diseases. Likewise, CD154/CD40 interactions between activated T-cells and endothelial cells are in concert with other ligand-receptor interactions and cytokine release (Fig. 1). A series of studies have demonstrated that CD40 activation induce endothelial cells to express ICAM-1, VCAM-1 and E-selectin (Hollenbaugh et al. 1995, Karmann et al. 1995). These adhesion molecules are involved in the binding of inflammatory cells to the endothelium and the subsequent emigration of these cells to the site of inflammation. Functional studies have provided further evidence that blockade of CD154/CD40 interaction will retard migration of leukocytes through endothelial cell barriers. In this regard, anti-CD154 antibodies have been shown to effectively block the appearance of pro-inflammatory cells at the site of inflammation in a variety of animal models of autoimmunity.

Moreover, CD154/CD40 interaction on the surface of endothelial cells induces secretion of tissue factor, as well as the release of cytokines and chemokines (IL-6, IL-8, IL-12 and MCP-1) (Millar et al. 1998). All of these will facilitate the recruitment and activation of more leucocytes. Interestingly, CD154 is the most potent stimulus for endothelial cells to release IL-12, since TNF-α, which belongs to the same superfamily as CD154, has no such effect. The CD154 stimulated IL-12 release can be further enhanced by IFN-γ, a typical Th1 cytokine (Lienenlueke et al. 2001). Therefore, a positive feed-forward loop seems to exist between leukocytes and endothelial cells, and CD154/CD40 interactions may play a pivotal role in this process. In addition to expressing CD40, as noted above, endothelial cells also have been reported to express CD154. Although the precise biological function of CD154 expressed on the endothelial cell surface is not known, it is of major interest that blockade of the CD154/CD40 pathway exerts a profound inhibitory effect in animal models of atherosclerosis (Mach et al. 1998).

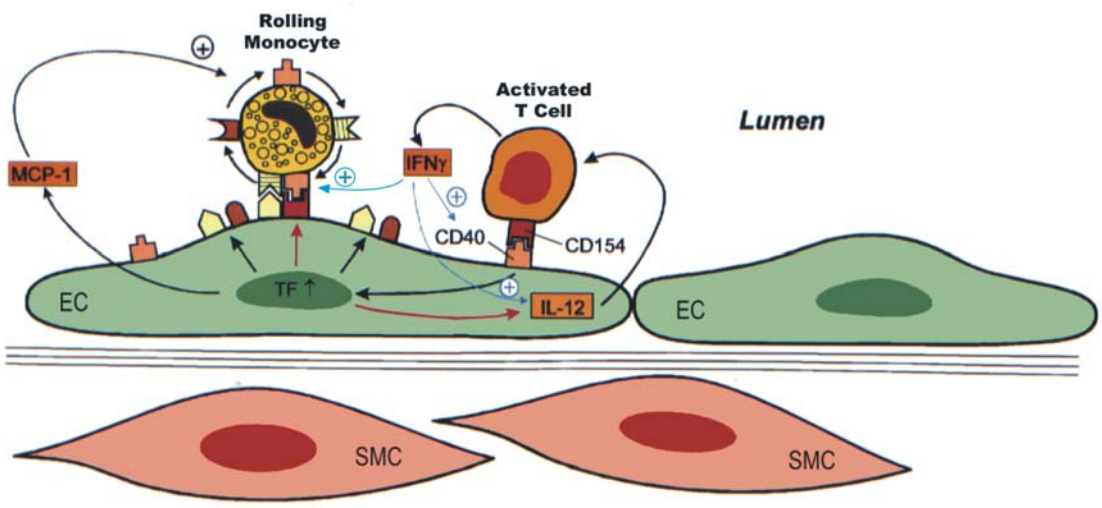


Figure 1. CD154/CD40 mediated interactions between activated T-cell, monocyte and endothelial cell. The activation of endothelial cell by CD154 is associated with an increase in activity of a variety of other ligand-receptor interactions and the release of cytokines, especially IL-12. This indicates that CD154/CD40 interactions drive a positive pro-inflammatory feed-forward loop between these cells. (EC: endothelial cell, SMC: smooth muscle cell, TF: transcription factor)

1.2 Inflammatory bowel disease

Inflammatory bowel disease (IBD), mainly including Crohn's disease and ulcerative colitis, is characterized by chronic inflammation of the gastrointestinal tract with variable location, extent and pathological features (Sartor 1997, Fiocchi 1998, Podolsky 2002). Patients with IBD often lose weight and experience a change on bowel habits, mostly diarrhea. They also experience cramping abdominal pain, fever, joint pain and skin lesions. In industrialized nations of the western hemisphere, approximately 0.2% of the population develops IBD. However, the etiology of IBD remains unknown up to now and further study is necessary to provide efficient therapy.

1.2.1 Clinical pathology of IBD

Crohn's disease is an inflammation that extends into the deeper layer of the intestinal wall. It is found most often in the ileum and the cecum, which is

sometimes referred to as the ileocecal region. However, it can develop in any part of the gastrointestinal tract, including the anus, stomach, esophagus, and even the mouth. Crohn's disease may affect the entire colon or form a string of continuous ulcers in one part of the colon or develops as multiple scattered clusters of ulcers skipping healthy tissue in between. Unlike Crohn's disease, ulcerative colitis is an inflammatory disease of the large intestine. It is characterized by a more superficial, continuous inflammation that affects the inner lining or mucosa of the colon. The inflammation is usually most severe in the sigmoid and rectum and diminishes higher in the colon. The disease develops uniformly and consistently until, in some cases, the colon becomes rigid and foreshortened. Both diseases increase the risk of developing bowel cancer and cause a greatly diminished quality of life in affected individuals, which mostly lasts throughout their disease.

1.2.2 The important role of the immune system in chronic IBD

In Crohn's disease, it was found that the mucosa of the affected patients is dominated by $CD4^+$ lymphocytes with a Th1-type, characterized by the production of IFN-γ and IL-2. In contrast, the mucosa in patients with ulcerative colitis is dominated by Th2-cells, characterized by the production of transforming growth factor-β (TGF-β) and IL-5 but not IL-4 (Neurath et al. 2002). This is not to say that Th2 cytokines are not present in Crohn's disease and that Th1 cytokines are not present in ulcerative colitis, but there appears to be a polarization of one group of cytokines over the other. In fact, activation of these central immune cells is eventually accomplished by the production of a wide variety of nonspecific mediators of inflammation. These mediators enhance the inflammatory process itself and tissue destruction, which ultimately results in the clinical manifestation of disease.

In IBD, chronic recurrent intestinal inflammation appears to derive from stimulation of the mucosal immune system by products of commensal bacteria in the lumen (Fig. 2). Stimulation may occur as a result of the penetration of bacterial products through the mucosal barrier, leading to their direct interaction with immune cells, especially dendritic cells and lymphocyte populations, to promote a classic adaptive immune response. Alternatively, bacterial products may stimulate the surface epithelium, possibly through receptors that are components of the innate immune-response system. The epithelium can, in turn, produce cytokines and

chemokines that recruit and activate mucosal immune cells. Activation of classic antigen-presenting cells, such as dendritic cells, or direct stimulation through pattern-recognition receptors promotes the differentiation of Th1-cells in patients with Crohn's disease (shown here) or possibly, atypical Th2-cells in patients with ulcerative colitis. The stereotypical cytokines activate macrophages which in turn produce IL-12 and IL-18 and thus further stimulate Th1-cells in a self-sustaining cycle. In addition, the activated macrophages also produce a potent mix of pro-inflammatory cytokines including IL-1, IL-6 and most notably TNF-α, which activate a broad variety of other immune cells. All of these will destroy the balance between anti-inflammatory and pro-inflammatory cytokines in the mucosa and finally lead to an inappropriate and sustained activation of the mucosal immune system.

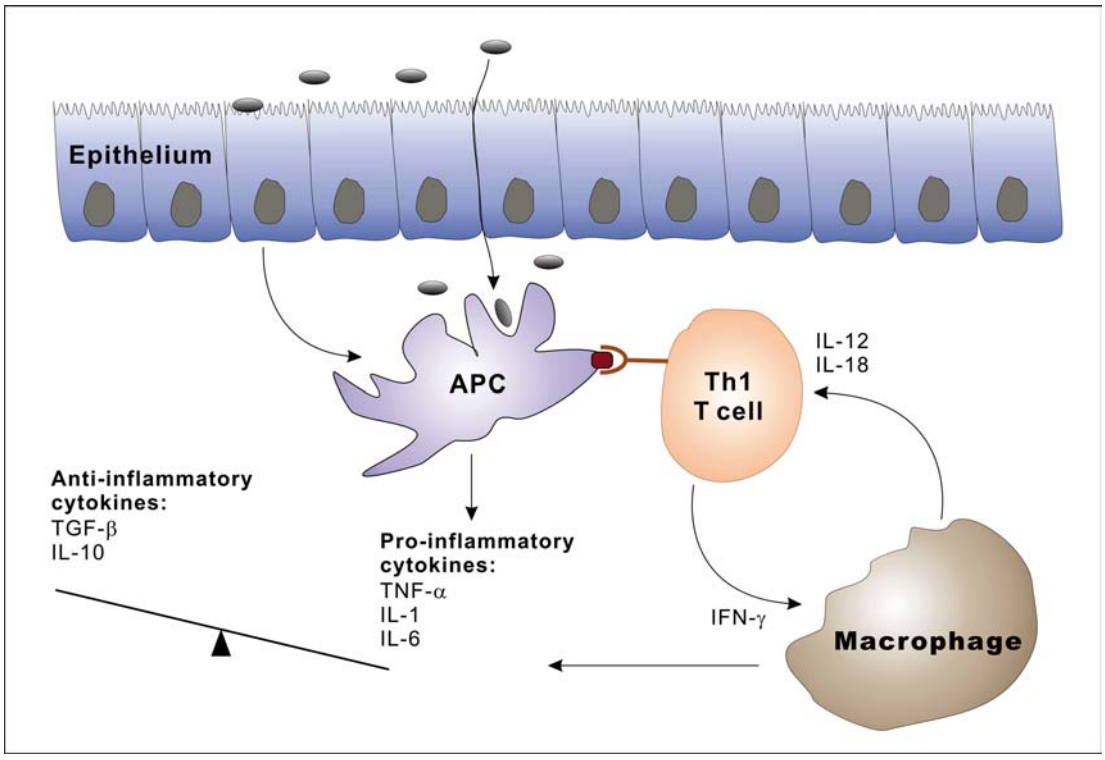


Figure 2. Pathogenesis of IBD.

Therefore, IBD might result from defects in both the barrier function of the intestinal epithelium and the mucosal immune system. Genetic and environmental factors may be involved with the abnormal immune response as the ultimate result. Advances in the understanding of the mediators involved in this process, even if still incomplete, have lead to great interest in the evaluation of a variety of new therapeutic agents with novel actions such as anti-TNF-α, IL-10, anti-α_4

integrin, anti-IL12, anti-CD154 and anti-ICAM-1 (Rutgeerts 2002, Rijcken et al. 2002, Jong et al. 2000). As noted above, the chronic inflammatory response in Crohn's disease is thought to be mediated by Th1-cells and CD154/CD40 interaction is crucial for the *in vivo* priming of Th1-cells through stimulation of IL-12 secretion by APCs. In addition, CD154/CD40 interaction also contributes to the enhanced expression of adhesion molecules on endothelial cells, which is especially important in maintaining inflammation. Considering all of these, blockade of CD154/CD40 interaction may have plausible therapeutic effects for Crohn's disease.

1.3 Aims of the work

Based on the immune-regulatory features of CD154/CD40 interaction and its role in the pathogenesis of autoimmune diseases, interfering with this costimulation has been considered to exert pronounced therapeutic effects. Although administration of anti-CD154 antibodies has been demonstrated to effectively inhibit inflammatory responses in a variety of animal models, adverse side effects observed both in experimental animals and in patients may limit their use in the treatment of chronic inflammatory diseases. Suppression of CD40 expression in CD154 target cells may thus provide a feasible therapeutic alternative. In this study, the possibility of interfering with CD154/CD40 interactions by using CD40 antisense ODNs was investigated both *in vitro* and *in vivo*.

2. Materials and methods

2.1 Elucidation of the rat CD40 mRNA sequence

Depending on the partial sequence of the rat CD40 mRNA, the Rapid Amplification of cDNA Ends (RACE) method was employed to elucidate the coding sequence of its 5' -end including the transcription start site. It is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and unknown sequences. An overview of the procedure is shown in Fig. 3. The 5'-RACE kit, (Life Technologies, Karlsruhe) was used for these experiments.

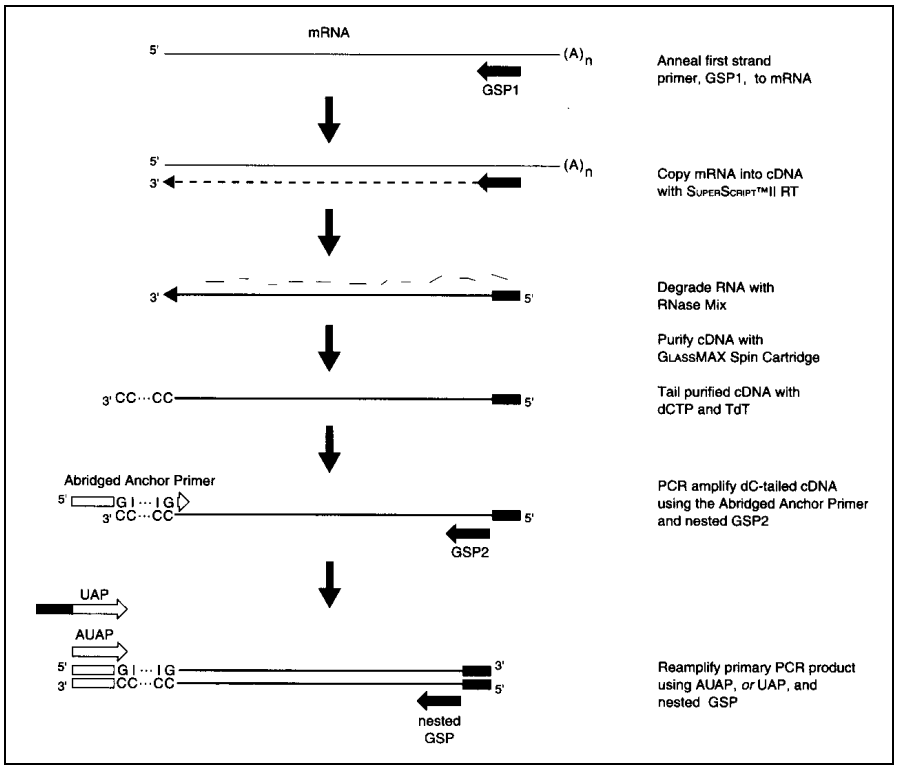


Figure 3. Overview of the 5'-RACE procedure.

The sequences of abridged anchor primer (AAP), abridged universal amplification primer (AUAP) and rat CD40 gene specified primers (rGSP) were as follows:

AAP: 5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG –3'

AUAP: 5'-GGCCACGCGTCGACTAGTAC -3'

rGSP1: 5'-ATCCTCACAGCTTGTCCA-3'

rGSP2: 5'-ACATGCCTCGCAATCCTT GC-3'

rGSP3: 5'-GAGGCAAAGACACCAT-3'

2.1.1 Total RNA extraction from animal tissue

Total RNA was isolated by Rneasy Mini Kit (Qiagen, Germany). Rat spleen was freshly removed from sacrificed animals, and immediately frozen in liquid nitrogen. With a mortar and pestle, tissues were grinded thoroughly in liquid nitrogen. The tissue powder (about 20 mg) was lysed in 350 μl buffer RLT and homogenized by 5 times passing through a 20-gauge needle. Then, 350 μl 70% ethanol were added and mixed immediately by pipetting. The mixture (about 700 μl) was applied to an Rneasy mini column to bind the RNA by centrifugation at 13,000 rpm for 15 s. The column was washed once with 700 μl buffer RW1, and twice with 500 μl buffer RPE. After the last washing step, an additional centrifugation at 13,000 rpm for 1 min was performed to dry the membrane. RNA was eluted into 40 μl RNase-free water and kept at -70°C.

2.1.2 First strand cDNA synthesis

Total RNA isolated from rat spleen (~5 μg) was mixed with 2.5 pmol rGSP1 (1 pmol/μl) in a total volume of 15.5 μl. The mixture was incubated 10 min at 70°C to denature RNA, then chilled 1 min on ice. After a brief centrifugation, it was mixed with 9.5 μl first strand cDNA synthesis mix [20 mM Tris/HCl (pH 8.4); 50 mM KCl; 2.5 mM $MgCl_2$; 10 mM DTT; 0.4 mM dNTP and 200 U SUPERSCRIPT™ II RT, final concentration] and incubated for 50 min at 42°C. The reaction was terminated by incubation at 70°C for 15 min. Subsequently, the sample was treated with 1 μl of RNase mix, a mixture of RNase H and RNase T1, at 37°C for 30 min to degrade the RNA. The sample was kept on ice until the purification step.

2.1.3 Purification of cDNA

Excess nucleotides and rGSP1 must be removed from the first strand product. Otherwise, the large amount of rGSP1 relative to cDNA product will influence the tailing step. The sample was mixed with 120 μl of binding solution (6M NaI) and transferred to a GlassMAX spin cartridge. cDNA was bound to the column by centrifugation at 13,000×g for 20 s. The column was washed four times with 400 μl of pre-chilled 1×washing buffer (4°C) and two times with 400 μl of pre-chilled 70% ethanol (4°C). After removing the final 70% ethanol wash from the tube, an additional centrifugation at 13,000×g for 1 min was performed to dry the column.

The purified cDNA was recovered in 50 μl sterilized water (pre-heated to 65°C).

2.1.4 TdT tailing of cDNA

Purified cDNA sample (10 μl) was mixed with 14 μl tailing mix [10 mM Tris/HCl (pH 8.4); 25 mM KCl; 1.5 mM $MgCl_2$; 200 μM dCTP, final concentration]. The mixture was incubated for 3 min at 94°C and then cooled down for 1 min on ice. After a brief centrifugation, 1 μl TdT was added to the mixture and incubated for 10 min at 37°C. The reaction was terminated by heating for 10 min at 65°C. The dC-tailed cDNA was stored at -20°C.

2.1.5 PCR of dC-tailed cDNA

The first PCR amplification of the rat CD40 cDNA was performed with primers of AAP and rGSP2. To get rid of the possible mismatching, *pfu* DNA polymerase was used in the reaction. dC-tailed cDNA (5 μl) was mixed with 45 μl PCR Mix [20 mM Tris/HCl (pH 8.4); 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM dNTP, 400 nM rGSP2, 400 nM AAP, 2.5 U pfu, final concentration]. PCR parameters were as follows: pre-denaturation at 94°C for 2 min; denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 76°C for 2 min (35 cycles); final extension at 76°C for 10 min.

Subsequently, a nested PCR was performed with primers of rGSP3 and AUAP, using the dilution of the first PCR product (1:1000) as a template. PCR was programmed as described above. PCR products were checked in 1% agarose gel with ethidium bromide staining.

2.1.6 Purification of PCR product from agarose gel

The DNA fragment was excised from the gel and purified with QIAEX II kit (Qiagen, Germany). Three volumes of binding and solubilization buffer (QX1) and 10 μl QIAEX II solution were added to 1 volume of gel. To bind DNA, the solution was incubated at 50°C for 10 min with occasionally vortexing. After centrifugation at 13,000×g for 30 sec, the pellet was washed once with QX1 buffer and once with PE buffer. After removing the washing buffer of the last step, the pellet was completely dried at room temperature (for about 15 min). Then, the pellet was

resuspended in 20 µl H_2O and incubated for 5 min at room temperature. After centrifugation at 13,000×g for 1 min, the supernatant, which contains the DNA fragments, was collected into a new tube.

2.1.7 Sequencing of the PCR product

A 10 µl sequencing reaction was set up by mixing 5 µl purified PCR product (100 ng), 3 µl BigDye Terminator DNA-sequencing-mix (Applied Biosystems, Damstadt), 1 µl rGSP3 primer (1 pmol/µl) and 2 µl sterilized water. Sequencing-PCR was performed for 25 cycles as follows: denaturation at 94°C for 30 sec, annealing at 55°C for 15 sec, extension at 60°C for 4 min.

After the sequencing-PCR, the products were precipitated by adding 80 µl H_2O, 15 µl 2M NaAC (pH 4.0), 250 µl 100% ethanol and centrifugation at 14,000×g for 20 min. The pellet was washed once with 250 µl 70% ethanol and then dissolved in 5 µl H_2O. Samples were sent for sequencing reading with 3100 Genetic Analyser (Applied Biosystems, Damstadt).

2.2 Cell biology methods

2.2.1 Medium and substances for cell culture

M199	Life Technologies	Karlsruhe
RPMI 1640	Life Technologies	Karlsruhe
Waymouth	Life Technologies	Karlsruhe
HBSS	Life Technologies	Karlsruhe
0.05% Trypsin/0.2% EDTA	Life Technologies	Karlsruhe
TES	Fluka	Buchs/Swiss
HEPES	Roth	Karlsruhe
Dispase	Roche Diagnostics	Mannheim
Nystatin	Life Technologies	Karlsruhe
Penisillin	Life Technologies	Karlsruhe
Streptomycin	Life Technologies	Karlsruhe
FBS (Fetal Bovine Serum)	Life Technologies	Karlsruhe
ECGS	c.c.pro	Neustadt/W.

(Endothelial Cell Growth Supplement)

Heparin	Sigma-Aldrich	Deisenhofen
Gelatine	Sigma-Aldrich	Deisenhofen
Ficoll-Paque®	Amersham	Freiburg

2.2.2 Culture of rat vascular smooth muscle cells (rSMCs)

Vascular smooth muscle cells were isolated from male Wistar rats as follows (200g body weight; Winkelmann, Borchen/Westfalen, Germany). After sacrifice of the animal by an overdose of pentooooo, the aorta was gently isolated and rinsed with Waymouth medium. After washing off the contaminating blood and completely removing the surrounding fat and connective tissue, the aorta was opened longitudinally to scrape off the endothelial cells. Then, the aorta was seized into small pieces (about 4 mm^2), which were placed in a 60mm gelatin-coated dish containing 1.5 ml growth medium (Waymouth medium supplemented with 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin and 1 µg/mL heparin). The aorta pieces were cultured under 5% CO_2, at 37°C. After about 3 days, smooth muscle cells started to grow out of the explants which were subsequently removed. The cells usually formed a confluent monolayer within one week after explantation. The identity of the cells was confirmed by immunofluorescence staining with anti-α-actin antibody.

For passaging, cells were washed twice with HBSS, and then digested with 0.5% trypsin/0.2% EDTA for 5 min at 37°C. Trypsin was inactivated by medium containing 10% FBS. Cells were pelleted by centrifugation at 126×g for 5 min and resuspended in Waymouth medium. Cells of passage 3 were used for the experiments.

2.2.3 Culture of human umbilical vein endothelial cells (HUVECs)

HUVECs were isolated from freshly collected umbilical cords (less than 24 hours) by enzymatic digestion under sterile conditions. After cannalating the umbilical vein from both ends, 50 ml HBSS were passed through to get rid of remaining blood. Then, the vein was filled with Dispase solution (0.3%) and incubated for 30 min at 37°C. The endothelial cells were detached from the vessel wall by gentle massage and collected into 50 ml Facon tubes. After centrifugation at 126×g for 5 min, the pelleted cells were resuspended in the growth medium (M199 medium

containing 20% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 10 U/mL nystatin, 5 mM HEPES and 5 mM TES, 1 µg/mL heparin and 40 µg/mL ECGS). Cells were seeded in gelatin-coated 6-well plates and cultured under 5% CO_2 at 37°C. Medium was changed on the second day to get rid of contaminating blood cells. Cells were grown to 90-100% confluence within 4 to 5 days.

2.2.4 Culture of mouse myeloma cells

The mouse myloma cell line P3xTB.A7 stably expressing human CD154, together with the non-transfected P3x63Ag8.653 control cells were gifts from Prof. R. Kroczek (Robert-Koch-Institute, Berlin). They were cultured in RPMI 1640 medium with 10% FBS under 5% CO_2 at 37°C. Every three months, cells were selected with gentamycin (G-418 sulphate, Gibco-BRL, Life Technologies, Karlsruhe) to maintain expression of the transgene.

2.2.5 Western blot analysis

For Western blot analysis of CD40 expression, cells were scraped off the plate into 1 ml HBSS and then pelleted by centrifugation at 500×g for 5 min. The cell pellet was resuspended in lysis buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1 mM DTT, with Proteinase Inhibitor Mix, 30 µl/well] and lysed by 5 cycles of freeze/thawing in liquid nitrogen and at 37°C, respectively. After centrifugation at 13000×g for 5 min, the supernatant was collected into a new tube. Protein concentration of cell extracts was measured with Protein Assay Buffer (Bio-Rad, Muenchen).

Sample protein (30 µg) was denatured by heating to 94°C for 5 min in 1× loading buffer (Roth, Karlsruhe), and then separated on 12% denaturing polyacrylamide gel. Electrophoresis was performed at constant current (20 mA per gel) for approximately 2 h. Subsequently, the separated proteins were electronically transferred to a BioTrace polyvinylidene fluoride transfer membrane (Pall, Dreieich) with Mini-Trans Blot system (Bio-Rad, Muenchen) at 350 mA for 45 min.

The membrane was blocked with 5% non-fat milk in PBS at room temperature for 1 h followed by incubation with primary antibody at 4°C overnight. Human CD40 was detected by using polyclonal rabbit anti-human CD40 antibody (Research Diagnostics, Flanders, NJ) at 1:2000 dilution. Rat CD40 was detected by using a

polyclonal rabbit anti-rat CD40 antibody (Biovision, Sandiego, USA) at 1:500 dilution. HRP-conjugated goat anti-rabbit IgG antibody (Sigma, Germany) was used as the secondary antibody at 1:10,000 dilution. The detected bands were visualized by SuperSignal® Chemiluminescent Substrate (Pierce, Rockford, USA) followed by exposure of the membrane to X-ray film (Koda, Germany) for 1 min.

2.3 Molecular biology methods

2.3.1 Transfection with antisense ODNs

Cells were grown in 6-well plates to approximately 90% confluence at the time of transfection. For each well, 400 pmol antisense ODNs was diluted in 100 µl M199 medium. In parallel, 2 µg Lipofectin (Life Technologies, Karlsruhe) was diluted in 100 µl M199 medium. Both solutions were left at room temperature for 30 min. Thereafter, the solutions were combined and incubated at room temperature for another 15 min. During this time, cells were washed once with M199 medium and 800 µl M199 medium were added in each well. The transfection mixture was poured onto the cells followed by gentle mixing. Cells were then incubated with the transfection medium for 4 h (rSMCs) or 2 h (HUVECs) in the incubator before changing back to the normal growth medium.

2.3.2 Semi-quantitative RT-PCR

Cells cultured in 6-well plates were washed twice with PBS before being disrupted by the addition of RLT buffer (350 µl/well). Total RNA was isolated as described in 2.1.1. For each sample, 1 µg RNA was mixed with 1 µl oligo-$dT_{(15)}$ (500 µg/µl) in a total volume of 14 µl and denatured by incubation at 70°C for 10 min. After brief centrifugation, 6 µl cDNA-Synthesis-Mix [50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$. 10 mM DTT, 0.5 mM dNTP and 200 U MMLV RT, final concentration] was added and cDNA synthesis continued for 50 min in a water bath at 42°C. The reaction was stopped by heating to 70°C for 10 min. Each cDNA sample was diluted with 180 µl H_2O and stored at -20°C.

Semi-quantitative PCR analysis was performed by normalizing to the relative amount of cDNA of a house-keeping gene. For this purpose, elongation factor 2

(EF-2) and the ribosomal protein L32 (Rpl32) were chosen as intern controls for the rSMCs and HUVECs, respectively. The reagents for PCR amplification were premixed to ensure identical conditions in each reaction [20 mM Tris/HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 20 pmol primers (for both forward and reverse primers) and 1 U Taq DNA polymerase, in a total volume of 50 µl]. The PCR reaction was performed in a model thermocycler (Biometra, Goettingen) programmed as follows:

Pre-denaturation:	2 min at 94°C
Denaturation:	30 sec at 94°C
Annealing:	45 sec at the temperatures shown in Table 2
Extension:	1 min at 76°C
Cycles:	primer-specific number as shown in Table 2
Final extension:	5 min at 76°C

After the amplification, PCR products were separated on 1.5% agarose gels with ethidium bromide staining. The density of the detected bands was determined by using PhotoFinish® imaging system (WordStar Atlanta Technology Center, USA) and One-Dscan® (software, Scananlytics, Billerica, USA).

Table 1. Primers and PCR conditions used.

PCR-product (species)	Product length	Num. of cycles	Annealing Temp.	Sequence forward/reverse (5' → 3')	GenBank-No (position)
CD154 (Rat)	319	32-36	58°C	TCCTTGCTCAACTGTGAGGAG CGTTGACTCAAAGGTTCCCGA	AF013985 (238-557)
EF-2 (Human)	218	19-24	58°C	GACATCACCAAGGGTGTGCAG GCGGTCAGCACAATGGCATA	Z11692 (1990-2207)
IL-12 p40 (Human)	281	30-35	62°C	GTACTCCACATTCCTACTTCTC TTTGGGTCTATTCCGTTGTGTC	NM_002187 (801-1082)
Rpl32 (Human)	368	18-22	60°C	GTTCATCCGGCACCAGTCAG ACGTGCACATGTGCTGCCTAC	X03342 (91-459)
VCAM-1 (Human)	1104	25-30	53°C	TGGAGCAAGAAATTAGATAATGG CACATGTACAGGAGATGATGAC	X53051 (729-1252)

The identity of the PCR products was confirmed by sequencing as described in 2.1.7.

2.4 Rat colitis model

Rat CD40 antisense ODNs were administrated to TNBS/ethanol treated rats according to the study protocol (Fig. 4). Its therapeutic effects were evaluated by macroscopic scoring, RT-PCR analysis, histology and immunohistochemistry.

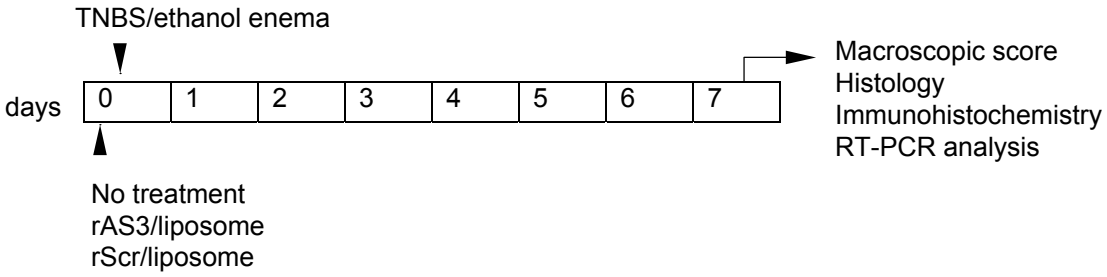


Figure 4. Scheme of the study protocol.

2.4.1 Animals

Non-fasted male Wistar rats (Winkelmann, Borchen/Westfalen, Germany) weighting 200-250 g were used for the experiments. All animals were fed standard rat chow and had free access to water and food. The animals were kept according to the German legislation on the protection of animals with a 12 h day and night rhythm.

2.4.2 The TNBS-induced colitis model of the rat

The inducing mixture was prepared by dissolving 20 mg TNBS (Sigma, Heidelberg) in 35% ethanol with 0.9% NaCl in a total volume of 136 μl. Rats were placed under light ether anaesthesia. The mixture was applied by an intracolonic injection through an 8 cm long catheter inserted through the anal canal into the descending colon. Expelling of the total volume was ensured by additional air injection. After removing of the catheter, rats were holding in a headfirst position for 30 sec to avoid flowing out of the enema. Rats were kept in normal condition after the intervention.

For application of the antisense and scrambled ODNs, the mixture of ODNs and liposome (novosom® AG, Halle) was prepared freshly and applied 4 hours prior to the TNBS/ethanol enema via the same route.

2.4.3 Macroscopic analysis

Seven days after induction of colitis, the animals were sacrificed in deep ether anaesthesia. The colon was removed and opened longitudinally. Colonic inflammation and damage were assessed by macroscopic evaluation. This was done using a score originally described by McCafferty et al. (1994) with some modifications (Table 3).

Table 2. Criteria for macroscopic scoring of colonic damage

Parameter	***Score***
Diarrhea	
No	0
Yes	1
Continuous	2
General condition	
Normal	0
Reduced	1
Adhesion	
None	0
Minor (colon can be easily separated from other tissue)	1
Moderate	2
Major	3
Perforation	
No	0
Yes	1
Necrosis	
None	0
Minor	1
Moderate	2
Major	3
Severe (necrosis at two or more sites or extending >1 cm)	4
Ulceration	
Normal appearance	0
Focal hyeremia, no ulcers	1
Ulceration without hyperemia or bowel wall thickening	2
Ulceration with inflammation at one site	3
Ulceration at two or more sites or extending > 1 cm	4
Edema	
No	0
Yes	1
Megacolon	
No	0
Yes	1
Total score	0 – 17

2.4.4 Semi-quantitative RT-PCR analysis

Total RNA was extracted from the affected part of the descending colon as well as from unaffected sites of the ascending colon of each animal. CD154 and VCAM-1 mRNA expression were checked by semi-quantitative RT-PCR analysis using the house-keeping gene, EF-2, as an internal control. The procedure was as described in 2.3.2.

2.4.5 Histological analysis

Histological analyses of both the ascending and descending colon of each animal were done as follows. Tissue samples were freshly fixed with 4% formaldehyde in PBS (pH 7.4) for 12 hours. Then the samples were trimmed into proper size and dehydrated by subsequently passing through 75% (twice), 95% (twice) and 100% (three times) alcohol for 2 h each. Clearing of alcohol from the tissue was performed using xylol. Then, specimens were embedded in liquid paraffin (55°C). After solidification 5 µm thick sections were cut and mounted onto 'Superfrost plus' glass slides (Menzel-Glaeser, Braunschweig). For paraffin removal, sections were allowed to dry at 60°C for 1 h. Then, slides were subsequently placed in xylol for 2× 7 min, propanol for 5 min, 98% alcohol for 5 min, 75% alcohol for 5 min, 60% alcohol for 5 min and finally distilled water for 5 min.

Hematoxylin/eosin staining: After the dewaxing, sections were stained for 5 min in hematoxylin and eosin solution (Merck, Darmstadt) each. After passing through 60%, 75%, 98% alcohol, propanol and xylol (twice), the slides were fixed with cover-slips. The colonic damages of each section were blindly assessed under microscope according to the criteria described in Table 3.

Immunohistological staining: For immunohistological staining, the sections were incubated 6 times for 5 min in 0.01 M citrate buffer (pH 6.0) in a microwave oven set to high power (800 W). Thereafter, they were incubated in 3% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity. Then, the sections were incubated for 1 h with blocking buffer (10% BSA in PBS), followed by an overnight incubation at 4°C with a mouse anti-rat ED-1 antibody (1:750 dilution, Dako, Hamburg) or mouse anti-rat CD3 antibody (1:75 dilution), respectively. To visualise the bound primary antibody, the following detector components were applied at the recommended working dilutions of the manufacturer (Dako, Hamburg): biotinylated secondary mouse-anti-goat/sheep or

swine-anti-rabbit antibody as a linker, peroxidase-conjugated avidin as a label and 3-amino-9-ethylcarbazole as a chromogen.

Granulocyte staining: Granulocytes were specifically stained by using the Esterase Kit (Sigma Diagnostics, Louis, USA). Cellular esterases are ubiquitous, apparently representing a series of different enzymes acting upon select substrates. Under defined reaction conditions, it is possible to distinguish granulocytes from other cell types, using specific esterase substrates. The staining solution was prepared freshly as follows: 1 ml sodium nitrite solution (0.1 mol/L) was gently mixed with 1 ml Fast Red Violet LB Base solution (15 mg/mL, in 0.4 mol/L hydrochloric acid with stabilizer) and incubated for 2 min at 37°C. Thereafter, the mixture was dissolved in 40 ml pre-warmed deionised water and subsequently adding 5 ml TRIZAMAL buffer (1 mol/L with surfactant, pH 6.3) and 1 ml naphthol AS-D chloroacetate solution (8 mg/mL with stabilizer). The dewaxed sections were incubated in the staining solution for 15 min at 37°C protected from light and then rinsed thoroughly in deionised water for 5 min. After counterstained with hematoxylin for 2 min followed by rinsing in tap water, the slides were fixed with cover-slips.

Table 3. Criteria for microscopic scoring of colonic damage.

Parameters	Score
Ulceration	
No	0
Minor	1
Major	2
Inflammation	
None	0
Minor	1
Major	2
Severe	3
Depth of lesion	
None	0
Superficial	1
One third	2
Two third	3
Transmural	4
Fibrosis	
None	0
Minor	1
Major	2
Lymphocyte infiltration	
No	0
Yes	1
Total score	0 – 12

2.5 Statistical analysis

Unless indicated otherwise, all data in the figures and text are expressed as means±s.e. of *n* independent observations (i.e., samples from different animals). Statistical evaluation was performed either by one-way analysis of variance followed by Bonferroni multiple comparisons test (comparison of three or more groups) or unpaired two-tailed Students *t*-test (comparison of two groups) where appropriate with the Instat for Windows™ statistics software package (GraphPad Software). A *P* value < 0.05 was considered statistically significant.

3. Results

3.1 Sequencing of the rat CD40 gene

To be able to design an antisense ODN against rat CD40 mRNA, first the 5'-end of this RNA had to be characterized as the gene sequence was not available from public data base in GenBank. A partial sequence located in the middle part of rat CD40 mRNA, was previously sequenced in our group (Krzesz R. et al. 1999). Based on the known sequence, three specific primers were designed (as shown in 2.1). Using the total RNA extracted from rat spleen as a template, the 5'-end of rat CD40 mRNA was subsequently amplified by using the 5'-RACE method.

As shown in Fig. 5, although no specific band was detected after the first round of PCR (lane 1), using the dilution (1:500) of this PCR product as a template, a band of about 400 bp appeared after the nested PCR (lane 2). The identity of this fragment was confirmed by running a control PCR (lane 3). Thus, the expected 220 bp fragment could be amplified with a pair of rat CD40 primers. Then, the 400 bp fragment was purified from the nested PCR product after running an agarose gel and sequenced with a rat CD40 primer (rGSP3). The result is shown in Fig. 6. It revealed that this fragment contains 220 bp same sequence of the previously known region of rat CD40 mRNA. Moreover, it shares 86% homology with mouse CD40 gene, thus further confirmed it as the sequence of rat CD40 gene.

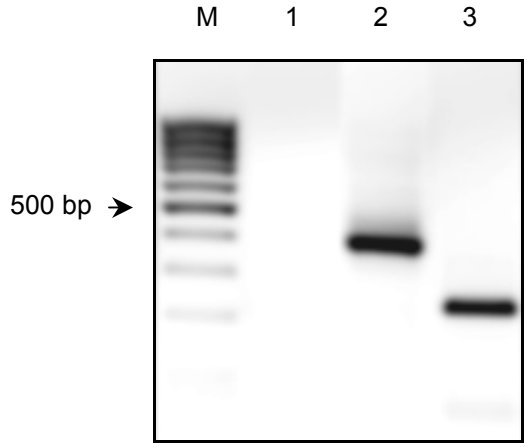
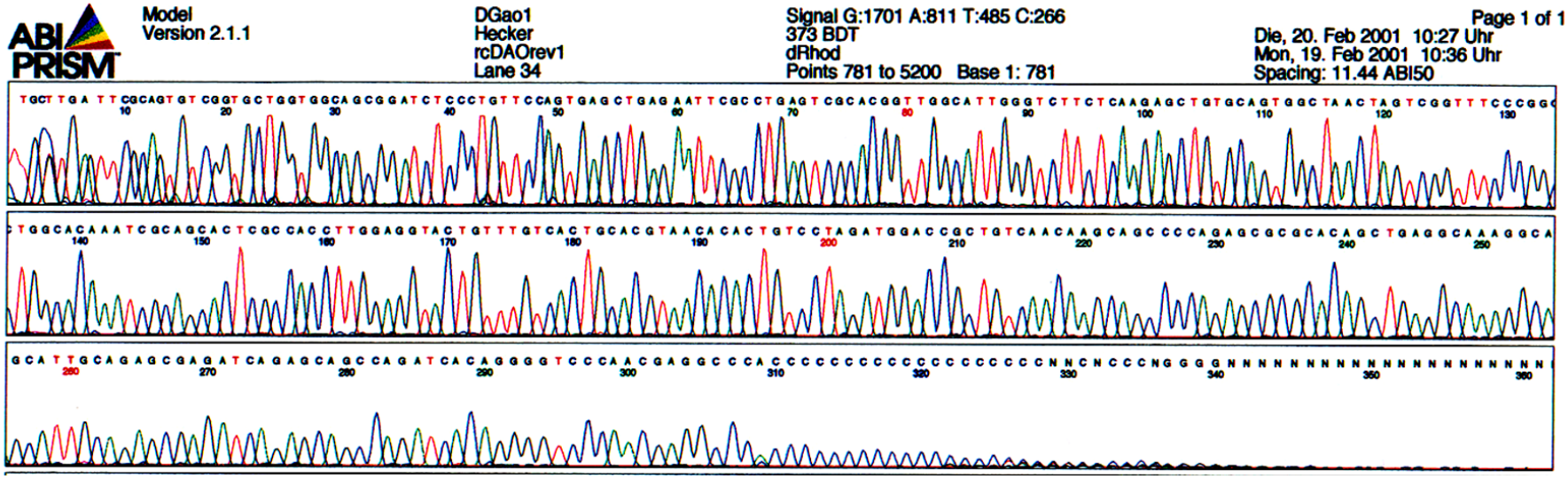


Figure 5. Specific amplification of rat CD40 by 5'-RACE. Using the total RNA from rat spleen as a template, 5'-end unknown region of rat CD40 was amplified by the 5'-RACE method. The PCR products from different steps were separated on a 1.5 % agarose gel. M: 100 bp length DNA Marker; 1: Products from the first PCR using rGSP2 and abridged anchor primer (AAP); 2: Products from the nested PCR using rGSP3 and abridged universal amplification primer (AUAP); 3: Products from the control PCR.

A.



B.

tgggacccct gtgatctggc tgctctgatc tcgctctgca ATGCTGCCTT TGCCTCAGCT GTGCGCGCTC TGGGGCTGCT TGTTGACAGC GGTCCATCTA

rAS1 rAS2 rAS3

GGACAGTGTG TTACGTGCAG TGACAAACAG TACCTCCAAG GTGGCGAGTG CTGCGATTTG TGCCAGCCGG GAAACCGACT AGTTAGCCAC

rAS4

TGCACAGCTC TTGAGAAGAC CCAATGCCAA CCGTGCGACT CAGGCGAATT CTCAGCTCAC TGGAACAGGG AGATCCGCTG CCACCAGCAC

rAS5

CGACACTGCG AACTCAATCA AGGGCTTCAG GTTAAGAAGG AGGGCACCGC GGTNTCAGAC ACTGTTTGTA CCTGCAAGGA AGGGCAGCAC

TGCGCCAGCA AGGAGTGCGA GACGTGCGCT CAGCACAGGC CCTGTGGCCC TGGCTTTGGA GTCGTGCAGA TGGCCACTGA GACTACTGAT

ACCGTCTGCC AACCCTGCCC GGTCGGATTC TTCTCCAATG GGTCATCACT TTTTGAAAAG TGTCATCCAT GGACAAGCTG TGAAGAT

Figure 6. The partial sequence of rat CD40 mRNA. (A) Sequence of the purified nested PCR product with rGSP3. (B) Rat CD40 partial sequence with the positions of the antisense ODNs (underlined). Capital characters denote the translated region.

3.2. Design of rat CD40 antisense ODNs

After obtaining the 5'-end of rat CD40 mRNA, antisense ODNs were generated from different locations along this region (Table 4). Selection criteria were as follows: The antisense ODN should posses 16 - 19 bases with a guanine/cytosine (GC) content between 40% and 65%. To avoid destruction in lysosomes and degradation by the various endo- and exonucleases present in the course of experiments, the ODNs were protected by nuclease-resistant phosphorothioate bonds in the last three nucleotides at the 5'- and 3'-end, respectively.

Table 4. Antisense ODN against rat CD40 mRNA (including the scrambled control).

ODN name	Sequence	Location
rAS1	A*A*G*GCAGCATTGCA*G*A*	-6 — +10
rAS2	A*C*C*GCTGTCAACAAGCA*G*C*	+35 — +53
rAS3	T*C*C*TAGATGGACCGCT*G*T*	+46 — +63
rAS4	T*A*A*CACACTGTCCT*A*G*	+58 — +73
rAS5	T*C*T*CCCTGTTCCAG*T*G*	+208 — +223
rScr	C*T*C*TCACGTCGAGGTG*T*A*	Scrambled ODN

* phosphorothioate modified nucleotide

3.3 Effects of CD40 antisense ODN in rat vascular smooth muscle cells (rSMCs)

3.3.1 Functional expression of CD40 in rSMCs

In cultured rSMCs, a certain level of CD40 expression was detected under basal conditions. This CD40 expression was markedly increased by stimulation with TNF-α (1000 U/ml) plus IFN-γ (100 U/ml). Interestingly, CD40 stimulation itself (through CD154) also up-regulated CD40 expression on both the mRNA and protein level (Fig. 7). It has been reported that CD40 activation enhances the expression of adhesion molecules and pro-inflammatory cytokines, including VCAM-1, E-selectin and MCP-1 in vascular smooth muscle cells (van Kooten and Banchereau 2000). Therefore, rSMCs were chosen as target cells and CD40-mediated VCAM-1 expression as read-out to judge the effects and specificity of the antisense ODNs *in vitro*.

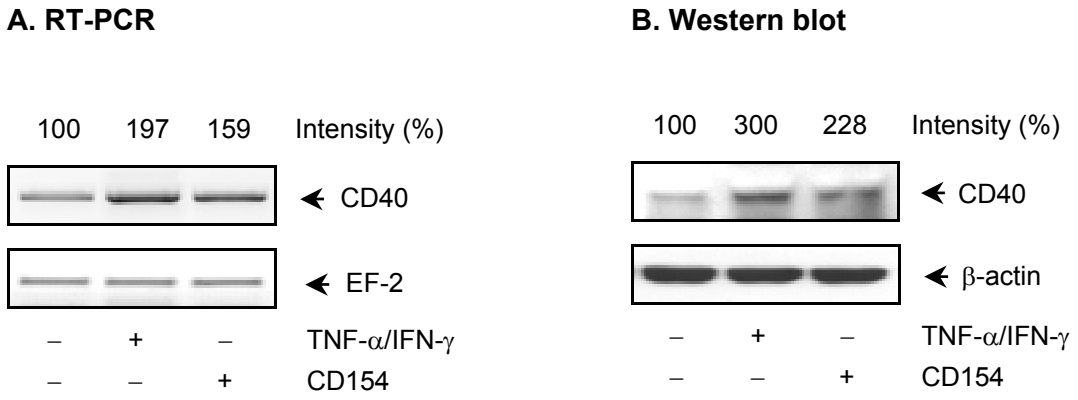


Figure 7. CD40 expression in rSMCs. (A) The cells were stimulated with TNF-α (1000 U/ml) plus IFN-γ (100 U/ml) or co-cultured with $CD154^+$ P3xTB.A7 cells (2×10^6 cells/ml) for 12 h. CD40 expression was measured by semi-quantitative RT-PCR, using a EF-2 mRNA as an internal control. (B) CD40 protein expression in rSMCs was measured by Western blot analysis 24 h after the stimulation. The equal loading of each sample was verified by reprobing the membrane with an anti-β-actin antibody. The relative intensity (% of control) was measured by densitometry, indicated at the top. The figure depicts the result of one representative experiment. Similar results were obtained in 2-3 additional experiments with different batches of rSMCs.

3.3.2 Effects of the different antisense ODNs on CD40 expression

With the help of Lipofectin, the antisense ODNs were transfected into rSMCs. Their effect on CD40 expression was measured at the protein level. As expected, antisense ODNs against the different regions of rat CD40 mRNA showed different efficiency (Fig. 8). The rAS1, encompassing the transcription initiation codon, was inactive and rAS5 had no effect either. The other three antisense ODNs tested in this study showed significant inhibition on CD40 expression as compared to the untreated control. Especially, rAS3, which encompasses position +46 to +63 of the rat CD40 mRNA, effectively inhibited CD40 expression. Its blocking effect on CD40 expression was characterized further in the following experiments.

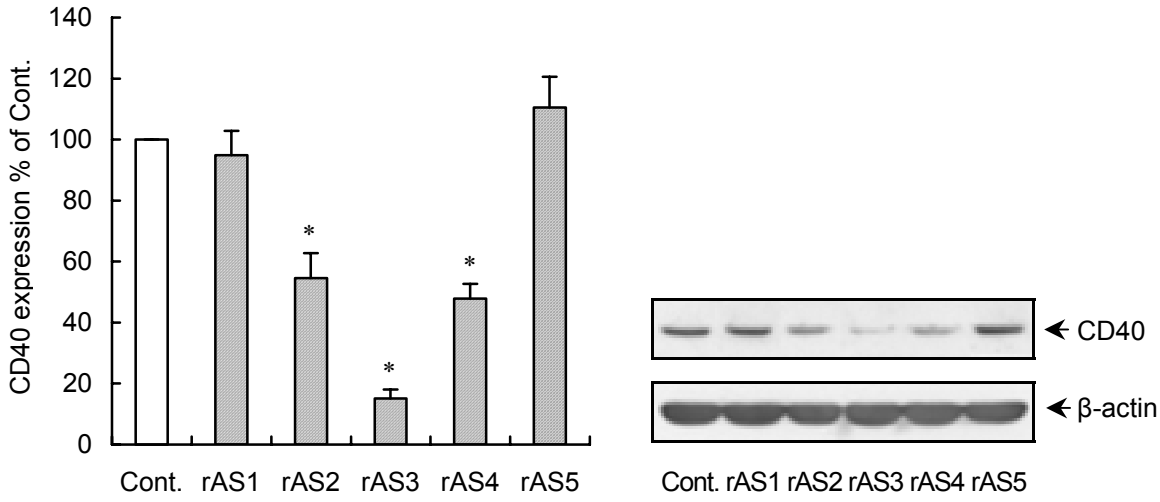


Figure 8. Effects of the different antisense ODNs on CD40 expression. The cultured rSMCs were transfected with different antisense ODNs at the same concentration (1 μM). Cells were harvested 24 h after transfection for Western blot analysis. CD40 expression was estimated by densitometry and expressed as relative intensity (%) compared with untreated cells. The statistical summary of the effects of the different antisense ODNs on CD40 expression is shown on the left (n=3, $^{*}P<0.01$ versus untreated control). A typical Western blot result is shown on the right. The equal loading of each sample was verified by reprobing the membrane with an anti-β-actin antibody.

3.3.3 Characterization of the blocking effects of CD40 antisense ODNs

Subsequently, rSMCs were transfected with different concentrations of rAS3 (200, 400, 600, 800 and 1000 nM). Changes in CD40 expression were monitored as described above. It was found that rAS3 inhibited CD40 expression in a concentration-dependent manner. A significant inhibition was observed even at the lowest concentration (200 nM) tested in this experiment (50.5% ± 6.2% of untreated control). At a concentration of 1000 nM, rAS3 achieved 85% reduction of CD40 expression compared with the untreated control (Fig. 9A). Moreover, to study the stability of the blocking effect, the rSMCs were transfected with rAS3 (1000 nM) and the change in CD40 expression was measured for up to 6 days after transfection. It was found that the inhibition of CD40 expression maintained the same level for three days (Fig. 9B). The effect appeared to diminish on the fourth day probably due to nuclease degradation of the antisense ODN and/or proliferation of the cells.

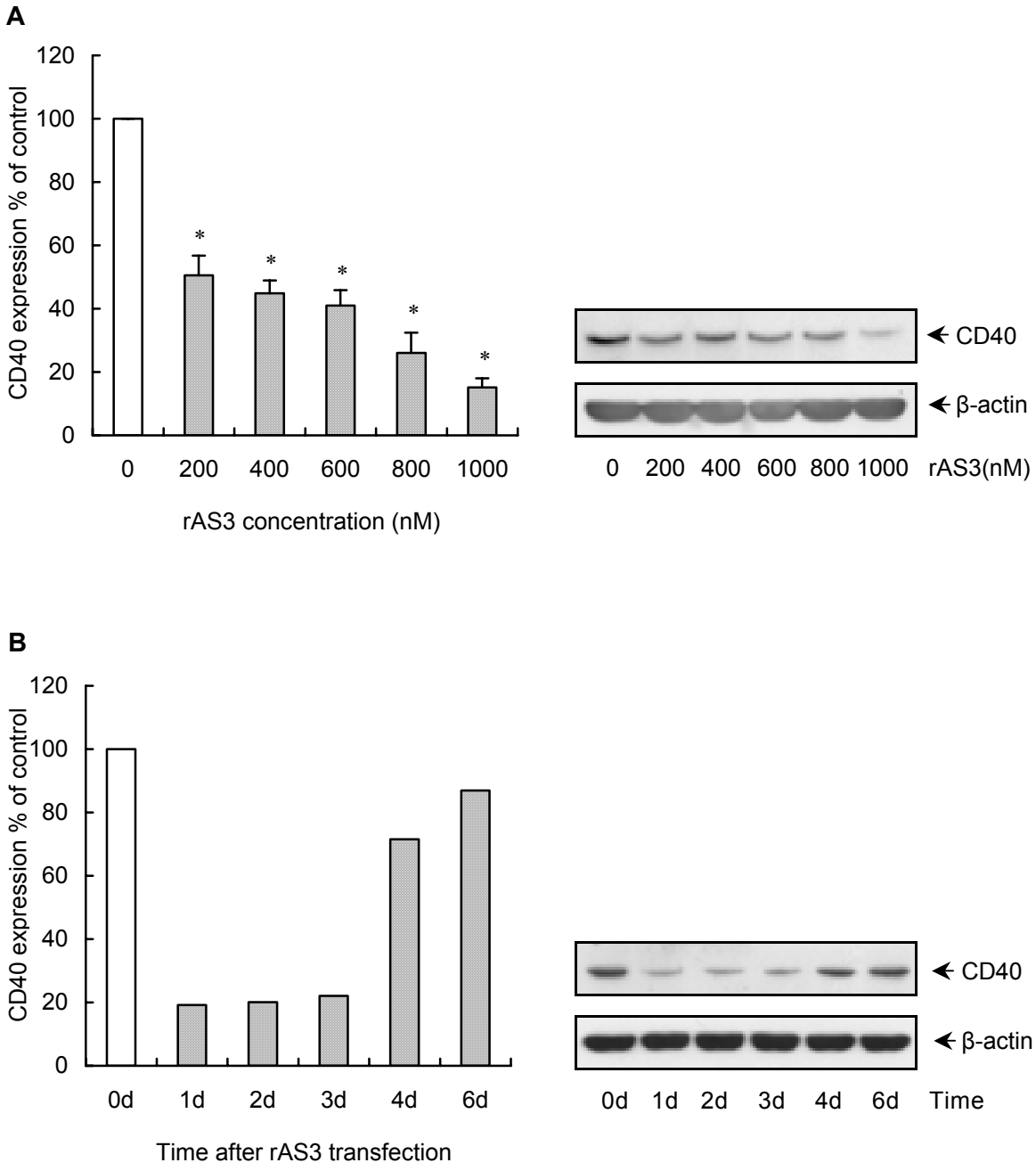


Figure 9. Blocking effects of rAS3 on CD40 expression. (A) The rSMCs were transfected with different concentration of rAS3 (0, 200, 400, 600, 800 or 1000 nM, separately). CD40 expression (expressed as % of control, i.e. no antisense ODN treatment) was measured after 24 h. Statistical summary of CD40 expression is shown on the left with a typical Western blot result on the right (n=3, $^{*}P$<0.01 versus 0 nM). (B) The rSMCs were transfected with rAS3 (1000 nM) and then harvested after 1, 2, 3, 4, or 6 days, separately. The mean of two experiments is shown on the left with one Western blot analysis on the right. Equal loading of samples was verified by reprobing the membrane with an anti-β-actin antibody.

3.3.4 Inhibition of CD154-induced VCAM-1 expression in rAS3-treated rSMCs

Having established that rAS3 has the most potent inhibitory effect on CD40 expression, investigations were extended to determine whether it could functionally block CD40 activation in rSMCs. In these experiments, VCAM-1 expression was used as a read-out for CD40 stimulation. Moreover, CD40 is a member of the TNF-receptor family and, as such, shares some common signaling pathways with the TNF-α receptor. To demonstrate the specificity of rAS3 for the CD40 pathway, its effects on TNF-α-mediated activation were also measured. A scrambled ODN was used as a control to indicate any nonspecific effects. As shown in Fig. 10, VCAM-1 expression in the rSMCs was markedly increased after stimulation with TNF-α (1000 U/ml) or CD154. After treatment with rAS3, CD154 stimulated VCAM-1 expression was significantly inhibited, while the scrambled ODN had no effect. Furthermore, neither rAS3 nor the scrambled ODN had any effect on TNF-α induced VCAM-1 expression, indicating a highly specific effect of rAS3 on the CD40 signaling pathway. These results verify that rAS3 is a specific blocker for CD154/CD40 interactions, and encouraged us to investigate its potential therapeutic effects *in vivo*.

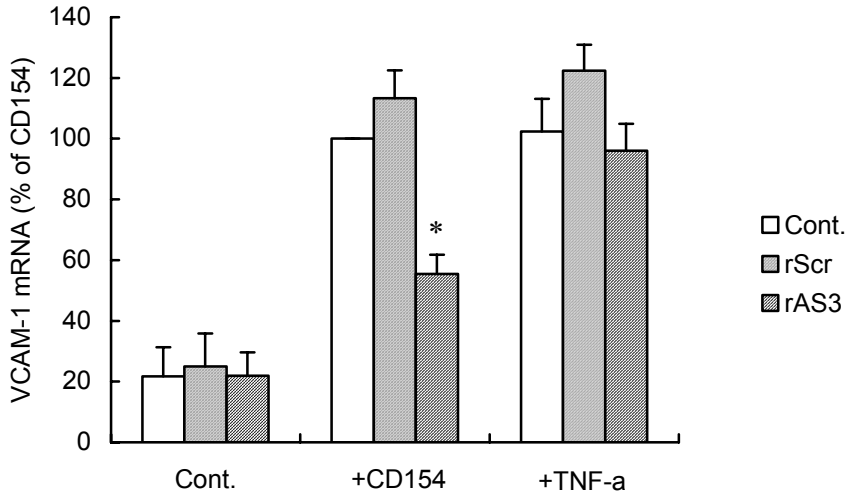


Figure 10. Blocking effect of rAS3 on CD40-dependent VCAM-1 expression in rSMCs. The cells were transfected with rAS3 or rScr (1 μM). After 24 h, they were stimulated with TNF-α (1000 U/ml) or by co-culturing with $CD154^+$ P3xTB.A7 cells (2×10^6 cells/ml) for 12 h. VCAM-1 mRNA abundance was measured by semi-quantitative RT-PCR, using EF-2 as an internal control. Statistical summary of VCAM-1 mRNA abundance is expressed as percentage of the CD154-stimulated control (n=3-5, $^*P<0.01$ versus control in the same group).

3.4 Therapeutic effects of the CD40 antisense ODN in TNBS-induced colitis

To further study the therapeutic effects of the CD40 antisense ODNs in vivo, rAS3 was administrated in the TNBS-induced colitis model of the rat. Previous research in our group has shown that a single enema consisting of 20 mg TNBS in 35% ethanol reproducibly induces a transient Crohn's disease-like colitis in Wistar rat. In this model, the maximum inflammatory response was observed at day 3 to 5 and spontaneous healing occured after approximately 4 weeks. Enhanced expression CD154 could be detected in the affected mucosa, indicating the involvement of CD154/CD40 interactions in the inflammatory reaction (Lienenlueke et al. 2001). For this study, the CD40 antisense ODNs (rAS3) and the scrambled control ODNs (rScr) were packed into liposomes (novosom®AG) and administrated by intra colonic injection 4 h before the TNBS/ethanol enema. The degree of inflammation and damage to the colon was evaluated 7 days post colitis induction by macroscopic scoring (as described in the methods section, see also Lienenlueke et al. 2001), histological staining, immunohistochemistry and RT-PCR analysis.

3.4.1 CD40 antisense treatment prevents the development of severe colitis

Seven days after the administration of TNBS/ethanol, significant edema formation together with focal ulcerations, necrosis and adhesion was observed in the descending colon of almost all control animals. In the rAS3 treatment group, however, the overall damage to the colon (macroscorpic score) was significantly reduced as compared to the untreated group (Table 5).

Table 5. Effects of rat CD40 antisense ODNs on macroscopic appearance. Stated are the mean ± s.e. score values rounded to one decimal. (*P<0.01 versus no treatment)

Parameter (range)	*No treatment*	*rAS3 treatment*	*rScr treatment*
Diarrhea (0-2)	0.1 ± 0.4	0.0 ± 0.0	0.4 ± 0.5
General condition (0-1)	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
Adhesions (0-3)	2.1 ± 0.6	1.0 ± 0.5*	2.1 ± 0.7
Perforation (0-1)	0.3 ± 0.5	0.0 ± 0.0*	0.4 ± 0.5
Necrosis (0-4)	2.9 ± 0.6	1.3 ± 1.3*	3.4 ± 0.5
Ulceration (0-4)	3.6 ± 0.5	2.5 ± 0.5*	3.9 ± 0.4
Edema (0-1)	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Megacolon (0-1)	0.3 ± 0.5	0.1 ± 0.4*	0.3 ± 0.5
Total score	10.6 ± 2.3	5.9 ± 2.2*	11.6 ± 2.4
Number of animals	8	8	7

Of note was that a significant reduction in the most severe types of damages i.e. necrosis, ulceration and adhesion, as well as a decrease in perforation and megacolon formation, was observed in the rAS3 treatment group. On the other hand, treatment with the scrambled control ODN (rScr) did not result in any improvement regarding the colonic inflammation.

Histologically, damage to the bowel wall appeared to be discontinuous with areas of normal mucosa next to severely necrotic ones. Sections from each sample were evaluated according to the criteria described in 2.4.5 after haematoxylin/eosin staining (Fig.11). In affected areas, the mucosa propria was usually lost and the bowel wall was thickened because of the prominent infiltration of leukocytes and moderate fibrosis (Fig. 12B). Treating rats with rAS3, but not with rScr, resulted in a significant improvement in colonic inflammation. The pathological changes in bowel wall structure of rAS3-treated animals were much less and usually showed a normal appearance in most areas (Fig. 12D).

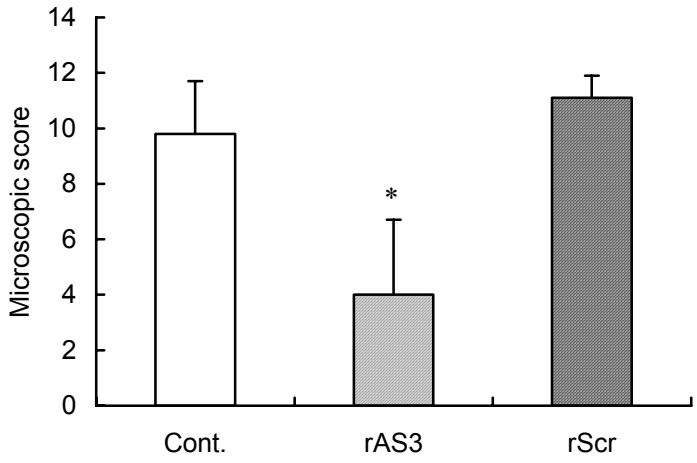


Figure 11. Effects of rat CD40 antisense ODN on microscopic appearance. Sections from the affected descending colon of each animal were stained with hematoxylin/eosin. Inflammation and damage of the bowel wall were evaluated according to the criteria described in 2.4.5. Treatment of CD40 antisense ODN (rAS3) but not the scrambled control ODN (rScr) significantly attenuated the development of inflammation in the TNBS-induced colitis model (n=7-8. **P*<0.01 versus non-treated control).

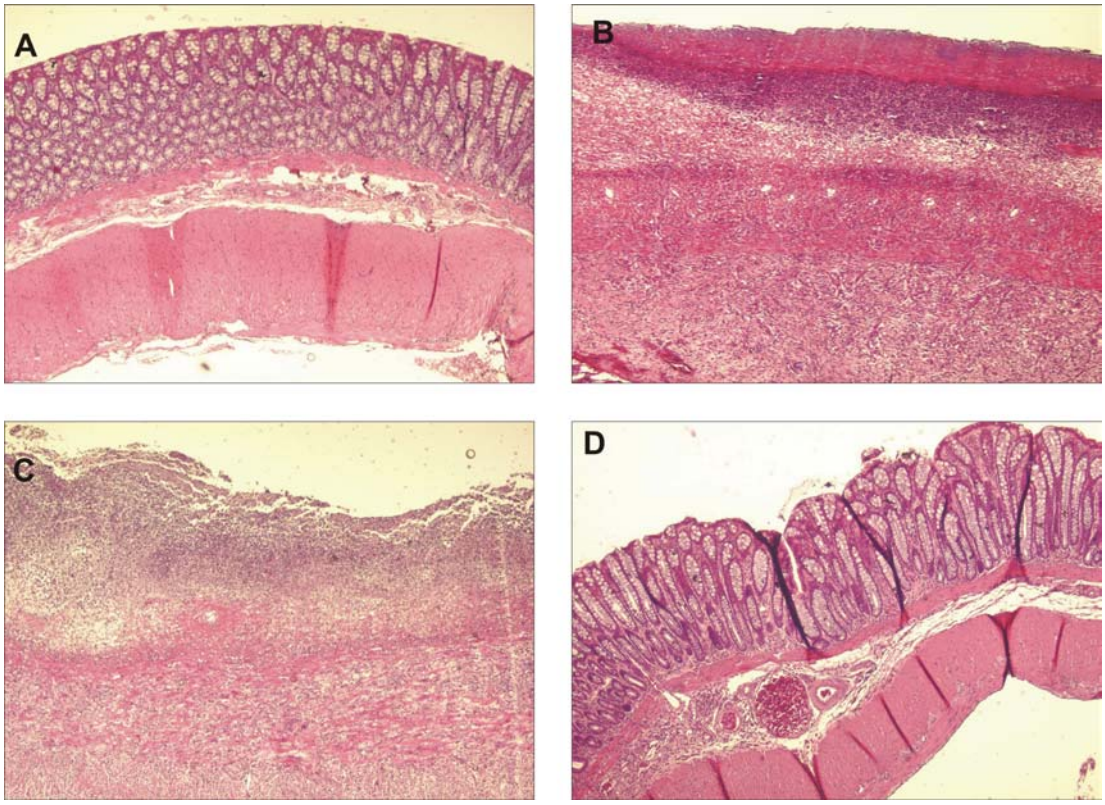


Figure 12. Histological analysis of CD40 antisense ODN effects in TNBS-induced colitis 7 days after the enema. Normal structure of the bowel wall in the unaffected ascending colon (A), but major changes in the affected descending colon of control animals (B), revealed by necrosis and thickening of bowel wall as well as a prominent leukocyte infiltrate. Treatment with rAS3 (D), but not with rScr (C), resulted in a significant protection against colitis development. Representative histology data from each group; n=7-8; hematoxylin/eosin staining; original×50.

Furthermore, immunohistochemistry results revealed the prominent infiltration of granulocytes in the affected colon of both control and rScr-treated rats, especially in the most severe damaged region (Fig.13B, C, respectively). In contrast, in colonic sections from rAS3-treated animals, only few granulocytes were observed in the mucosa and submucosa propria (Fig.13D). However, CD3 positive-stained cells (T-cells) were mostly located in the less inflamed regions. No significant decrease of these cells was observed with the rAS3-treatment, as judged by immuno- histochemistry (Fig. 14).

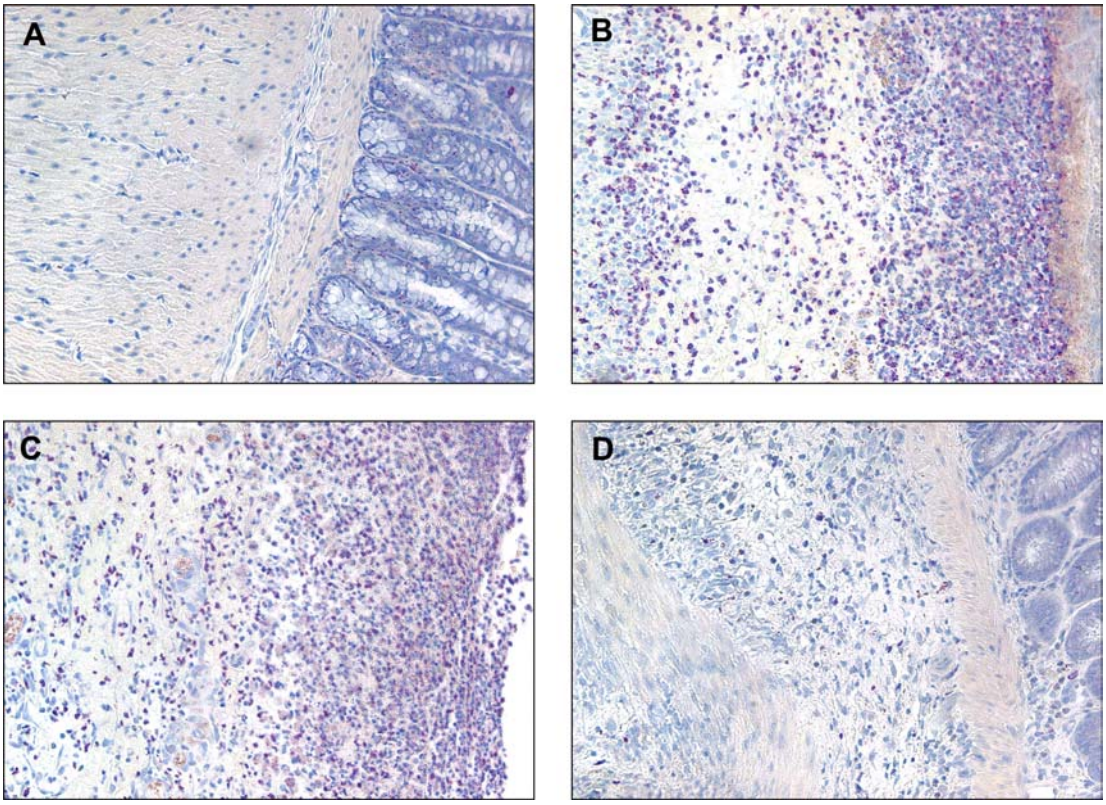


Figure 13. CD40 antisense ODN effects on granulocyte infiltration in the colon of animals. The sections were stained with Fast Red Violet LB (Sigma Diagnostics) for granulocyte in bright red colour. Only a few cells were positively stained in the unaffected ascending colon (A), while a great number of granulocytes were observed in the descending colon of control animals (B). Treatment with rAS3 (D), but not with rScr (C) resulted in a significant decrease in the granulocytes in the affected descending colon of animals. Representative data from each group; n=7-8; original×200.

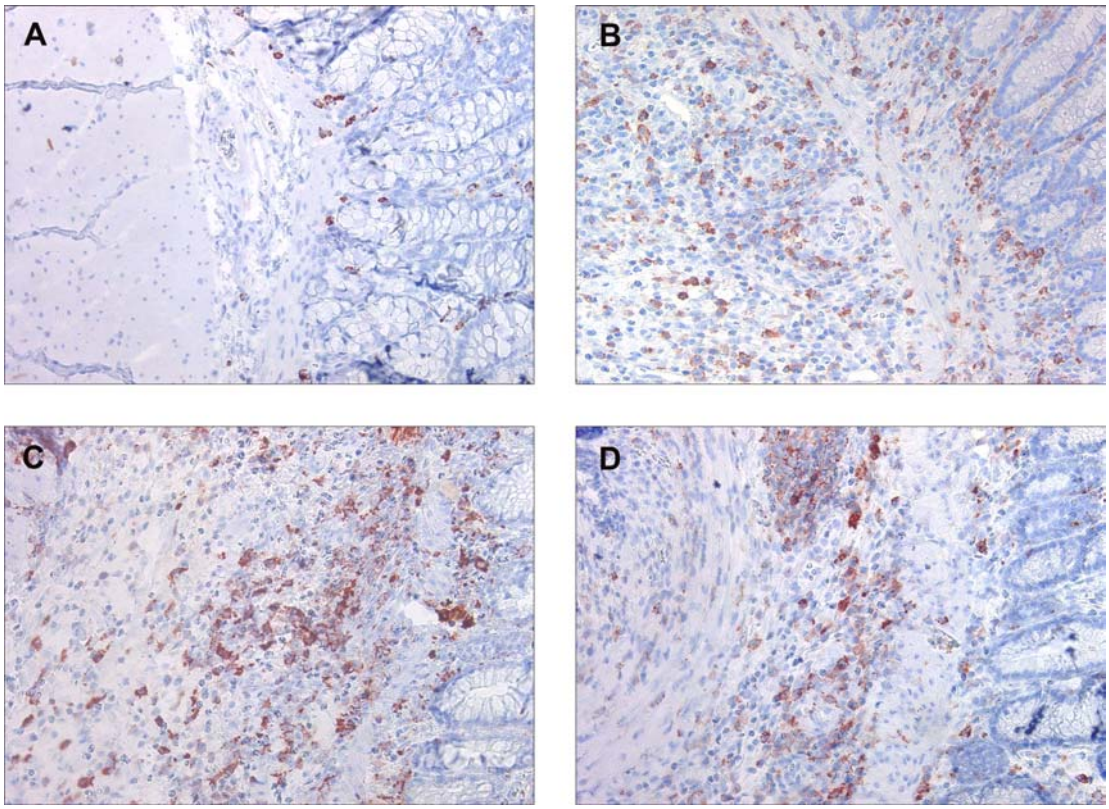


Figure 14. CD40 antisense ODN effects on T-cell infiltration in the colon of animals. The sections were stained with anti-CD3 antibody. Only a few cells were positively stained in the unaffected ascending colon (A), while a large number of CD3+ cells was observed in the descending colon of control (B), rScr-treated (C) and rAS3-treated animals (D). Representative histology data from each group; n=7-8; haematoxylin counterstaining, original×200.

3.4.2 CD40 antisense ODN inhibits enhanced VCAM-1 expression *in vivo*

According to RT-PCR analysis, expression of CD154 and VCAM-1 was significantly increased in the descending colon of TNBS-treated animals as compared to the unaffected ascending colon (Fig. 15A). This indicated the ongoing inflammatory response in TNBS-treated animals. The rAS3 treatment significantly reduced the increase in VCAM-1 mRNA abundance, while CD154 expression was not affected (Fig. 15B). In rScr-treatmed animals, both VCAM-1 and CD154 mRNA expression were even more pronounced than in the untreated control animals.

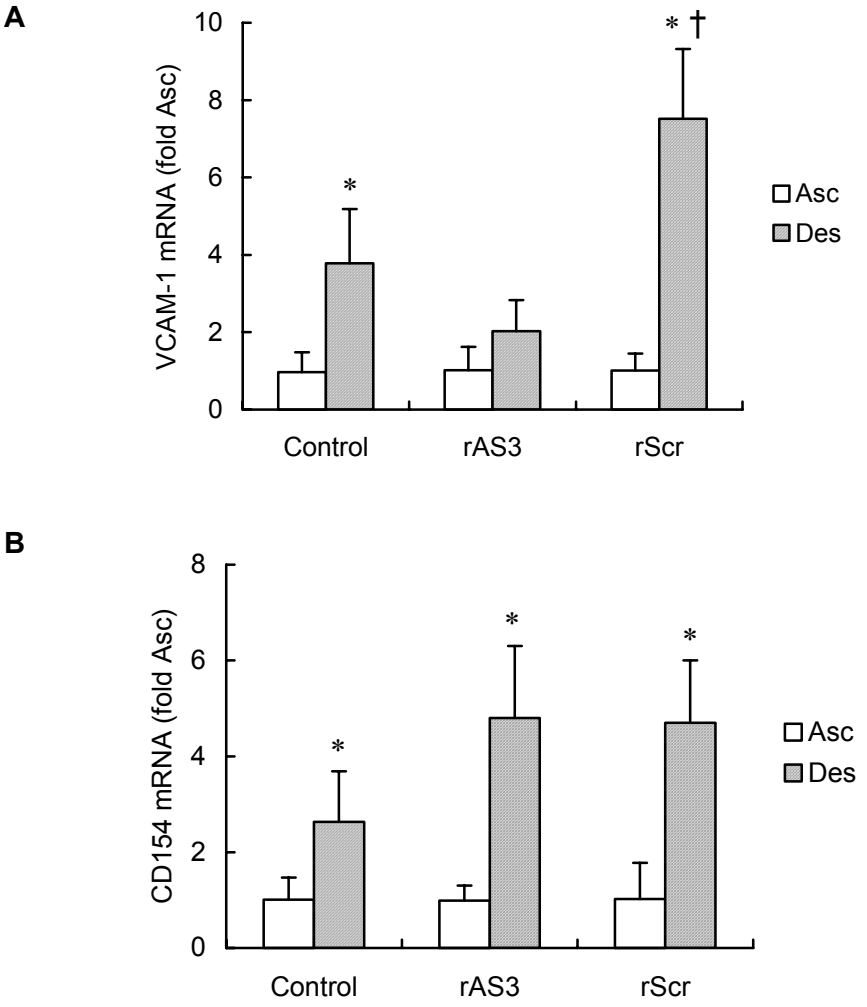


Figure 15. Effects of CD40 antisense ODNs on VCAM-1 (A) and CD154 (B) mRNA abundance in the colon of TNBS-treated rat. Samples of both the ascending (Asc; i.e. unaffected region) and descending colon (Des; i.e. affected region) were collected 7 days post colitis induction. The mRNA abundance (relative to the mRNA level of the house-keeping gene, EF-2) is statistically summarized (n=8, ∗P<0.01 versus Asc, †P<0,01 versus rAS3 Des).

3.5 Experiments with human CD40 antisense ODNs

3.5.1 Design of human CD40 antisense ODNs

Having demonstrated that blockade of CD40 expression by anti-rat CD40 ODNs led to functional effects both *in vitro* and *in vivo*, it was of special interest if antisense ODN-mediated CD40 suppression was also effective in human cells. The sequence of human CD40 cDNA was obtained from GenBank (Genbank No. X60592). Similar to the design of rat CD40 antisense ODNs, several antisense ODNs were selected from the 5'-end of the mRNA (Tab. 6). The antisense ODNs were 16 – 18 bases in length and flanked by three phosphorothioate modified nucleotides at both ends to provide more resistance against nucleases.

Table 6. Antisense ODNs (including the scrambled control) against human CD40.

Human CD40	Sequence	Location
hAS1	G*A*C*CAGGCGGCAGGAC*C*A*	-28 — -11
hAS2	A*G*G*ACG ACG AAC CA*T*G*	-1 — +15
hAS3	C*A*G*CGGTCAGCAAGCA*G*C*	+35 — +52
hAS4	C*A*T*GCAGTGGGTGG*T*T*	+60 — +75
hAS5	C*A*C*TGACTGTTTATTA*G*G*	+93 — +110
hScr	C*A*C*GACAGGAATCG*A*G*	Scrambled ODN

* phosphorothioate modified nucleotide

3.5.2 Blocking effects of human CD40 antisense ODNs in human endothelial cells

For checking effects of these human CD40 antisense ODNs, primary cultured human umbilical vein endothelial cells (HUVECs) were selected as the target cell. As noted in the introduction, though basal expression of CD40 in endothelial cells is usually low, expression of the gene is markedly induced under pro-inflammatory conditions (especially with TNF-α 100 U/ml plus IFN-γ 1000 U/ml). This stimulation of CD40 expression occurs at the transcriptional level. The effects of human CD40 antisense ODNs were investigated both under basal and pro-inflammatory conditions in this study. As in rSMCs, not all antisense ODNs showed the same

effects on CD40 expression (Fig. 16). Among them, the hAS4 encompassing +60 to +75 of the human CD40 mRNA, showed the most potent inhibition under both basal as well as stimulated conditions. The other antisense ODNs either had no effect (hAS1 and hAS2), or were not as effective as hAS4 (hAS3 and hAS5).

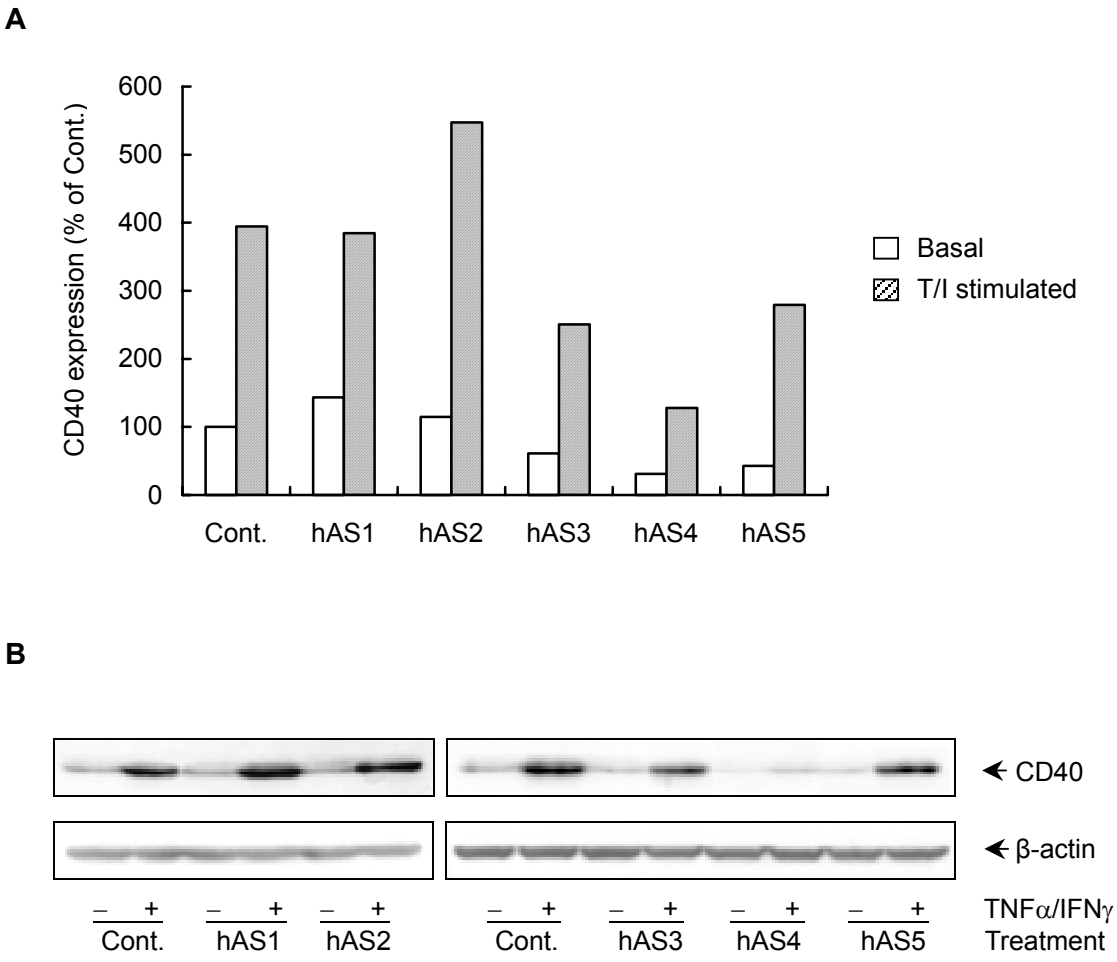


Figure. 16 Effects of CD40 antisense ODNs on CD40 expression in HUVECs. The cultured HUVECs were transfected with the antisense ODNs (400 nM) and then stimulated with TNF-α (100 U/ml) plus IFN-γ (1000 U/ml) or left untreated. Changes in CD40 expression were measured by Western blot analysis after 24 h. (A) CD40 expression is shown in relative intensities (% of treated control, mean of two experiments). (B) Representative result of Western blot analysis. Equal protein of each sample was verified by reprobing the membrane with an anti-β-actin antibody.

Further application of hAS4 at different concentrations revealed a concentration-dependent decrease in CD40 expression both under basal and stimulated conditions (Fig. 17). Significant inhibition was attained already at a concentration of 100 nM.

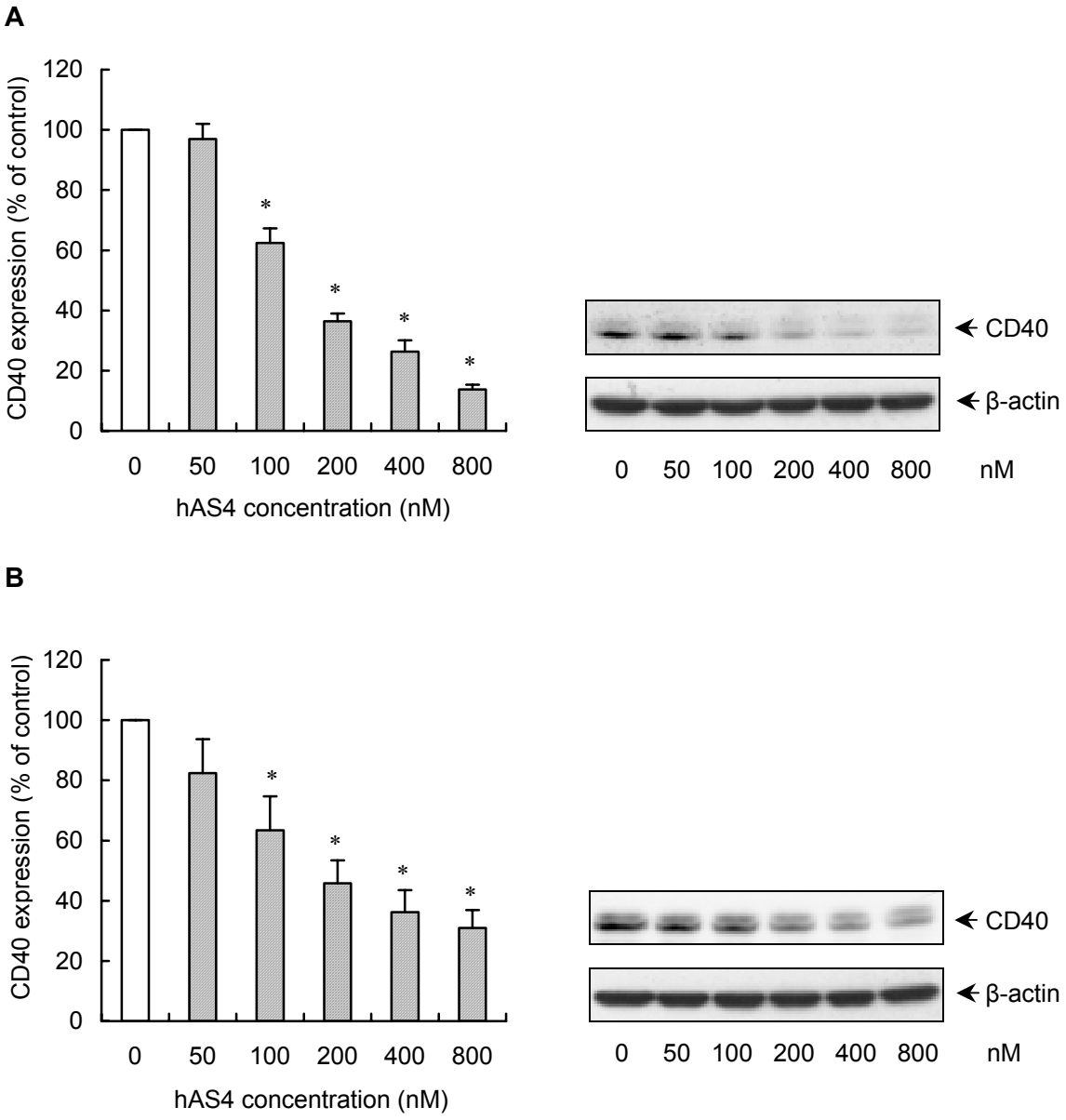


Figure. 17 CD40 expression in human endothelial cells after hAS4 transfection. The cultured HUVECs were transfected with different concentrations of hAS4. Basal CD40 expression (A) as well as TNF-α (100 U/ml) plus IFN-γ (1000 U/ml) stimulated CD40 expression were measured by Western blot analysis after 24 h. CD40 expression is expressed as percentage of that in untransfected control cells. (n=3, $^{*}P<0.01$ *vs*. untransfected control). Equal protein loading of each sample was verified by reprobing the membrane with an anti-β-actin antibody.

3.5.3 Inhibition of CD154-mediated IL-12 p40 expression in hAS4-treated HUVECs

With the establishment of the most potent antisense ODN against human CD40, the study was continued by evaluating its blocking effects on CD40 function. Activation of CD40 in HUVEC enhances the expression of adhesion molecules (ICAM-1, VCAM-1 and E-selectin), tissue factor and the release of cytokines and chemokines (IL-6, IL-8 and MCP-1). Notably, CD154 also stimulates expression of IL-12 p40, which forms a heterodimer with IL-12 p35 and plays an essential role in the differentiation of Th1-cells. This stimulated IL-12 p40 expression can be further enhanced by IFN-γ. Interestingly, *de nove* IL-12 p40 expression appears to be a special feature of CD154 stimulation in HUVEC, since TNF-α, which belongs to the same superfamily as CD154, has no such effect (Fig. 16). Therefore, IL-12 p40 was selected as a read-out for CD40 function in HUVEC.

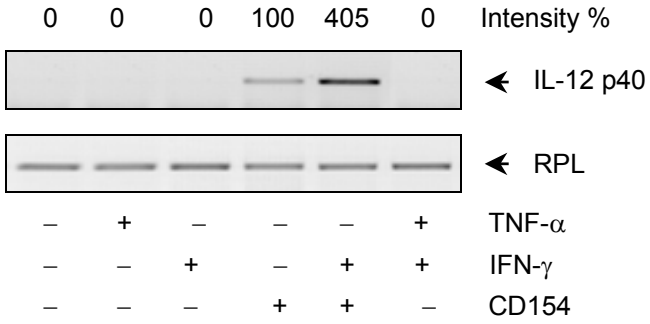


Figure 18. IL-12 p40 expression upon CD154 stimulation in HUVECs. Cells were stimulated by TNF-α (1000 U/ml), or co-cultured with $CD154^+$ P3xTB.A7 cells (2×10^6 cells/ml) in the presence and absence of IFN-γ (1000 U/ml) for 12 h. IL-12 p40 mRNA expression was measured by semi-quantitative RT-PCR, using Rpl32 mRNA levels as the internal control. Relative intensities are expressed as percentage of CD154-stimulated IL-12 p40 expression in control cells. Representative result of 3 individual experiments.

After treatment of the cultured HUVECs with hAS4, CD154-induced IL-12 p40 expression was not detectable by RT-PCR analysis (Fig. 19). In the presence of IFN-γ which markedly augments CD154-induced IL-12 p40 expression, IL-12 p40 expression was also significantly inhibited in hAS4-treated cells. As in the rat cultured smooth muscle cells, the scrambled control ODN was inactive, demonstrating that antisense ODN inhibition of both CD40 expression and function is sequence-specific.

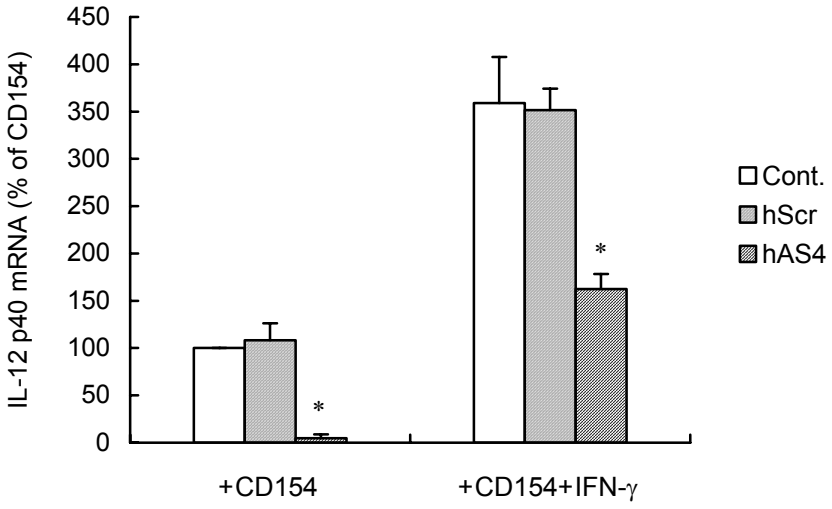


Figure 19. Effect of CD40 antisense ODN on IL-12 p40 expression in cultured HUVECs. Cells were transfected with hScr (400 nM) or hAS4 (400 nM). After 24 h they were co-cultured with $CD154^+$ P3xTB.A7 cells (2×10^6 cells/ml) in the absence or presence of IFN-γ (1000 U/ml) for 12 h. IL-12 p40 expression was measured by semi-quantitative RT-PCR, using Rpl32 as the internal control (n=3, **P*<0.01 versus CD154 or CD154 plus IFN-γ stimulated control).

4. Discussion

In recent years, there is increasing evidence that CD154/CD40 interactions are involved in the pathogenesis of many autoimmune diseases, where T or B cells have a prominent role. As noted above, administration of anti-CD154 antibodies has been demonstrated to effectively inhibit inflammatory responses in a variety of animal models. Although it has been reported that these antibodies only need to be administrated over a short period of time, major thromboembolic complications have been reported for this treatment both in experimental animals (Kawai et al. 2000) and in patients (Vincent 1999). In addition, the humanized antibodies are expensive to produce and prolonged use can lead to sensitization and concomitant loss of therapeutic effect. It would be helpful, therefore, to provide other means of interfering with CD154/CD40 interactions.

The approach of targeting CD40 expression in CD154-responsive cells may provide such an alternative. However, low-molecular weight antagonists for CD40 are not yet available and antibodies against CD40 activate rather than inhibit CD154 signaling (Sakata et al. 2001). Here, the possibility of blocking CD154/CD40 pathway with CD40 antisense ODNs was examined.

4.1 Antisense technology

Antisense molecules are usually short, 15-25 bases long, single stranded DNA molecules, which comprise the complementary sequence against their target mRNA. The specificity of antisense molecules has been reported to be extremely high so that translation of two mRNAs that differ by mutation of a single base can be differentially inhibited (Monia et al. 1992). This qualifies antisense ODNs as an ideal tool to mechanistically dissect one pathway from the multitude pathways involved in chronic inflammatory diseases. In this study, the antisense technology was employed for blocking CD40 expression and thus interfere with CD154/CD40 interactions.

To know the sequence of a target mRNA is the first requirement for antisense ODN design. Unfortunately, the complete rat CD40 mRNA sequence was not available in GenBank. Moreover, it has been reported that multiple isoforms of CD40 mRNA, generated by alternative splicing, exist in both human and mouse (Tone et al. 2001). This may also happen to rat CD40 mRNA. The mouse CD40 splicing

variances occur in the region between exon 5 and exon 9 of the gene. The major alternative isoform lacks the membrane-associated endodomain and seems to reduce the amount of the signal transducing form on the cell surface. The exact role of these splice variants in CD40 signaling and their distribution in other primary cells still need to be investigated. Therefore, we focussed on the 5'-end of rat CD40 mRNA and the sequence of this part was obtained by employing the 5'-RACE method.

Antisense molecules must arrive at their target mRNA intact to avoid destruction in lysosomes and degradation by the various endo- and exonucleases present intracellularly and within serum and tissues (Thierry et al. 1992). Several chemical modifications can be incorporated into antisense molecules to boost their nuclease resistance. Among them, the phosphorothioate-modified and 2'-O-methyl oligonucleotides are most commonly used. The phosphorothioate modification, in which one of the non-bridging oxygen atoms in the nucleotide backbone is replaced with sulfur, fully supports antisense activities (Mayers and Dean 2000). However, the mRNA binding affinity of this modified ODNs is also reduced. Here, the antisense ODNs were flanked on both sides by three phosphorothioated nucleotides to give them resistance to nuclease without interfering with their mRNA binding affinity too much.

4.2 Application of anti-rat CD40 antisense ODNs *in vitro*

Antisense ODNs were initially selected with a GC content between 40% and 65% from the obtained partial sequence of rat CD40. They must hybridize to target mRNA with sufficient affinity and specificity, and evoke a mechanism of action leading to mRNA inactivation or destruction. Inhibition of protein expression by antisense ODN is achieved by a variety of mechanisms including RNase H activation, transcription arrest, splicing inhibition and blocking translation (Fig.20) (Myers et al. 2000). The most widely recognized mechanism involves RNase H mediated cleavage of the target mRNA. RNase H is an endonuclease that recognizes RNA-DNA duplex molecules and selectively cleaves the RNA strand. The process is catalytic, i.e. once an RNA molecule is cleaved, the antisense ODN dissociates from the duplex and is free to bind to a second target mRNA (Kanaya and Ikehara 1995).

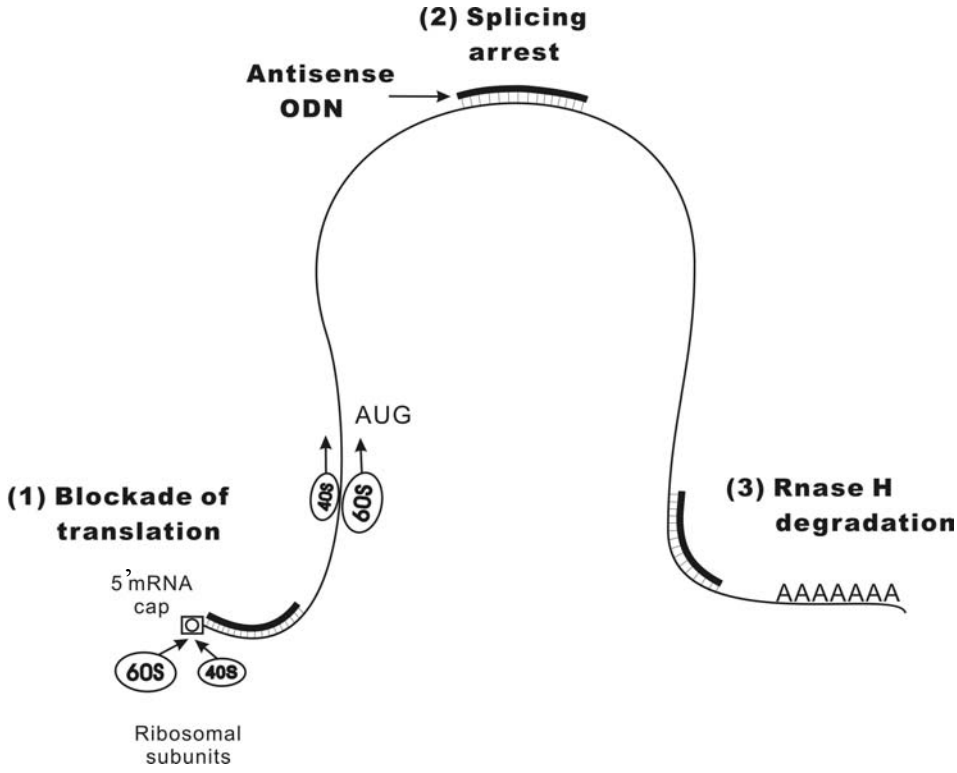


Figure 20. The mechanisms by which antisense ODNs disrupt protein synthesis (adopted from Myers et al. 2000). (1). The blockade of ribosomal subunit attachment to mRNA at the 5' cap site. (2). Interference with proper mRNA splicing through antisense binding to splice donor or splice acceptor sites. This should happen in the nucleus with hnRNA. (3) RNase H mediated degradation of DNA-mRNA hybrid. Hybridization can occur anywhere in the mRNA where an antisense ODN binds with sufficient affinity.

In fact, antisense ODNs encompassing different region of an mRNA molecule usually behave differently in blocking their target gene expression. An antisense ODN can access and bind only to few regions of the mRNA molecule, i.e. those are positioned outside its three-dimensional structure. For finding out the most effective antisense for the rat CD40, five antisense ODNs, which hybridize to the different parts of the mRNA, were designed. Their blocking effects on CD40 expression were investigated in rat vascular smooth muscle cells. One of them (rAS3), located at position +46 - +63 proved to be an effective inhibitor of CD40 protein expression. A recent report on the use of antisense ODNs against porcine CD40 resulted in the characterization of an effective antisense ODN targeting the 3'-untranslated region of the mRNA molecule (Rushworth et al. 2002), indicating that multiple antisense ODN-sensitive sites might exist in one mRNA molecule.

Further characterization of rAS3 showed that the antisense ODN inhibited CD40 expression in a dose-dependent manner, attaining about 90% inhibition at a concentration of 1 μM. Moreover, its blocking effect on CD40 expression lasted up to 3 days after transfection *in vitro*. The transient nature of this inhibitory effect is presumably due to degradation of the antisense ODNs and/or the decrease in concentration during cell proliferation. Elucidating the time-course and required concentration to obtain an optimal inhibitory effect of rAS3 was important, as the final goal of the study was to use rAS3 as an experimental drug *in vivo*. However, it is difficult to predict how long CD40 expression must be down-regulated *in vivo* to observe therapeutic effects. Interestingly, most therapeutic stratgies using anti-CD154 antibodies revealed that only a brief treatment regimen was necessary to achieve beneficial effects (Liu et al. 2000, Cong et al 2000).

Moreover, by virtue of the decrease in CD40 expression rAS3 also blocked CD154/CD40 interactions functionally as demonstrated by the decrease in VCAM-1 expression following CD154 stimulation in rAS3-transfected rSMCs. The specificity of rAS3 to interfere with CD154/CD40 signaling was demonstrated by its inability to TNF-α-induced changes in gene expression. Moreover, the inactivity of the scrambled control ODN demonstrated that rAS3 reduces CD40 expression through a sequence-dependent mechanism. Collectively these data confirmed that rAS3 is not only a valuable tool to specifically interfere with CD154/CD50 interactions, but also to investigate the pathological role of CD40 in chronic inflammatory diseases *in vivo*.

4.3 Application of anti-rat CD40 antisense ODNs *in vivo*

In this study, the TNBS-induced rat colitis model was employed to evaluate the therapeutic effects of rAS3. In this model, colonic injury and inflammation are induced by intrarectal administration of a TNBS/ethanol enema. It is thought that ethanol temporarily disrupts the mucosal epithelial barrier and enables TNBS to bind covalently to proteins of colonic epithelial cells and modify them. Subsequently, these TNBS-modified self-antigens are presented to the immune system, leading to a delayed-type hypersensitivity reaction. The TNBS-induced colitis model has marked similarities to human Crohn's disease because it is also characterized by Th1-cell mediated inflammation (Singh et al. 2001). The involvement of CD154/CD40 interactions in this colitis model has been revealed by

elevated expression of both CD154 and CD40 in the affected mucosa and the therapeutic effects achieved by administration of an anti-CD154 antibody (Lienenlueke et al. 2001, Stueber et al. 1996).

For successful administration of the antisense ODNs, they must reach a sufficient concentration in the affected tissue. The mechanism by which oligonucleotides enter cells remains controversial but probably involves fluid phase pinocytosis, receptor-mediated endocytosis, or both (Gewirtz et al. 1996, Bennett et al. 1992). For most cells, delivery systems need to be used such as inactivated viruses, liposomes or electroporation. Antisense ODN uptake *in vivo* may be even more complex and involve different mechanisms than those in cultured cells through possible interactions with a variety of intra- and extracellular proteins. The usual route of antisense ODN administration is intravenous injection, which was used, e.g., in most studies employing antisense ODN against TNF-α, ICAM-1 and VCAM-1 in IBD models (Myers et al. 2003, Rijcken et al 2002). After intravenous injection, phosphorothioate-modified oligonucleotides distribute rapidly to peripheral tissues with high amounts going to the kidney and liver. However, a significant accumulation also occurs in bone marrow, skeletal muscle, skin and the intestine. Besides causing unexpected side effects, this wide distribution also lead to high costs because extremely high dosage of the antisense ODN is necessary for efficient therapy. In addition, DNA binding to serum proteins could lead to increases in blood clotting time and complement activation (Galbraith et al 1994, Henry et al. 1997). Therefore, local administration may offer several advantages including less side effects and higher local concentration of the antisense ODN. In this study, rAS3 was first packed into liposomes followed by administration through intracolonic injection.

Single application of the liposomal antisense ODN greatly attenuated the severe inflammation following the TNBS/ethanol enema, as judged by both macroscopic and histology analysis. As noted above, CD40 is mainly expressed by APCs and endothelial cells, which most likely are the target cells of the antisense ODNs in this experiment. Change in CD40 expression in these cells, however, could not be revealed due to the lack of an anti-rat CD40 antibody suitable for immunohistochemistry. On the other hand, it is well known that CD154/CD40 interactions between activated Th1-cells and APCs/endothelial cells in the affected mucosa, greatly contribute to the secretion of pro-inflammatory cytokines including IL-12, IFN-γ and TNF-α (Cong et al. 2000, Stueber et al, 1996). At the same time,

the expression of adhesion molecules on endothelial cells such as VCAM-1 is also enhanced. This may result from exposure to the pro-inflammatory cytokines and/or CD154/CD40 interactions directly. RT-PCR analysis demonstrated that the rAS3 treatment significantly reduces the increased VCAM-1 expression in the affected mucosa, which in turn may have contributed to the decrease in leukocytes infiltration revealed by histological staining. In any case, these findings strongly suggest that the CD40 antisense ODN attenuates CD154/CD40 interactions *in vivo*, resulting in a decreased mucosal inflammation in this model of chronic IBD.

4.4 Pathophysiological role of CD154/CD40 interactions in chronic IBD

There is increasing evidence that CD154/CD40 interactions play a role in the pathogenesis of chronic IBD. In CD154 transgenic mice, lethal IBD was developed spontaneously and characterized by the infiltration of $CD154^+$ T-cells and $CD40^+$ cells into diseased tissues (Xu et al. 1994). Actually, in patients with Crohn's disease increased numbers of T-cells expressing CD154 have been detected in the lamina propria and several $CD40^+$ cells have also been found in the same areas (Battaglia et al. 1999). Most of these $CD40^+$ cells co-stained with CD20, thus revealing them as B-cells, and only a few were $CD14^+$ macrophages. Interestingly, several $CD40^+$ cells were also positive for von Willebrand factor, an endothelial cell-specific marker. This finding is in line with the view that the vascular endothelium plays an important role in the maintenance of intestinal inflammation and indicates that CD154/CD40 interaction on endothelial cells may contribute to the pathogenesis of Crohn's disease as well.

The CD40-mediated interactions between APCs, T cells and endothelial cells in the pathogenesis of chronic IBD were summarized in Fig. 21. CD154/CD40 interactions induce APCs to secrete an important set of cytokines and chemokines including IL-12, TNF-α and IL-8. Especially, IL-12 is known as the major cytokine governing the differentiation of $CD4^+$T cells by promoting the differentiation towards Th1 subsets, of which the secretion of IFN-γ is characteristic. Interestingly, IL-12 itself up-regulates CD154 expression in T-cells and IFN-γ enhances CD40 expression on APCs. Such a feed-forward loop may exist between T helper cells and endothelial cells as well. In addition, CD154/CD40 interactions on endothelial cells also induce the expression of adhesion molecules, thereby generating signals for the recruitment and extravasation of leukocytes at the site of

inflammation. Although a great portion of CD40 positive cells in the affected mucosa is found to be B-cells, their pathological role in IBD is still not clear.

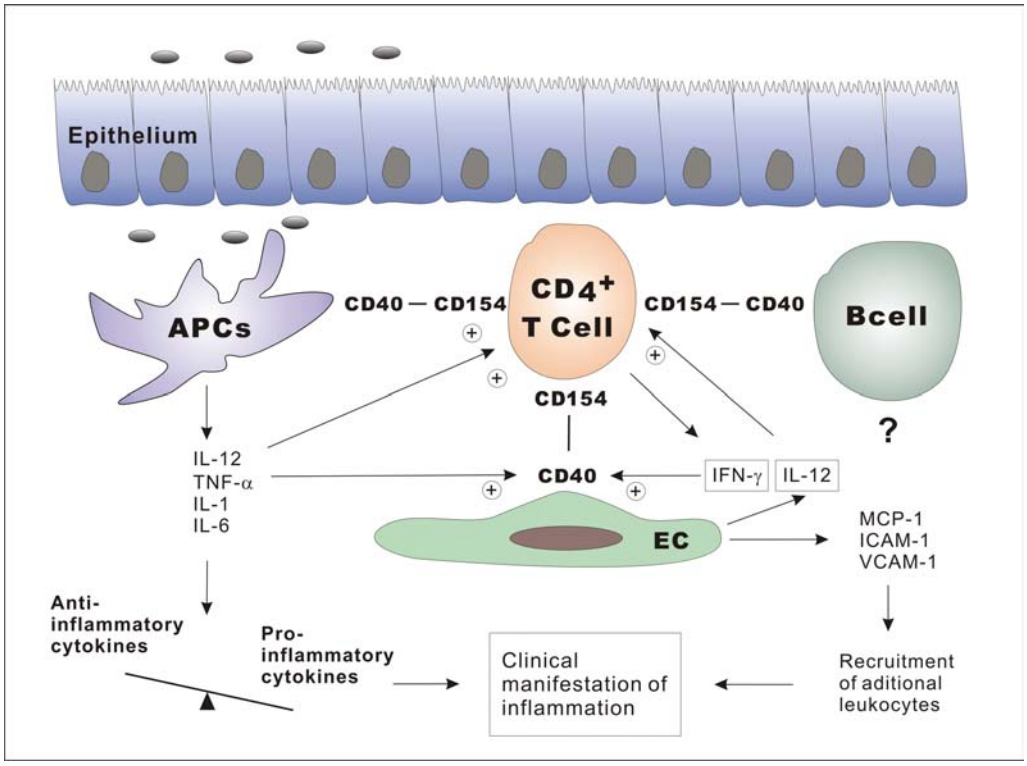


Figure 21. Pathophysiological role of CD154/CD40 interactions in IBD.

An *in vivo* study has shown that anti-CD154 antibody (MR1) treatment prevented the onset and progression of colitis as revealed by abrogated leukocyte infiltration, decreased CD54 expression and strongly diminished mRNA levels of the pro-inflammatory cytokines IFN-γ, TNF-α and IL-12 (Liu et al. 2000). Even with a delayed treatment, MR1 still led to significant clinical and histological improvement and down-regulated secretion of pro-inflammatory cytokines. This suggests that the maintenance of intestinal inflammation requires continuous or at least repeated CD154/CD40 interactions. However, no response to administration of anti-CD154 antibodies was seen in an established TNBS/ethanol-induced murine colitis model (Stueber et al. 1996). Therapeutic effects of the CD40 antisense ODNs were only demonstrated by treatment prior to colitis induction in this study. It still necessary, therefore, to evaluate whether treatment with the antisense ODN also has a beneficial effect on the ongoing inflammation when this has already been initiated, i.e. causes regression of the symptoms.

4.5 Decoy technology

Regarding the increase in CD40 expression under pro-inflammatory conditions, especially in endothelial cells, a decoy oligonucleotide strategy was also tested in the colitis model. Compared with the antisense ODN, decoy ODNs represent a more recently developed tool, targeting gene transcription rather than translation (Morishita et al. 1998). Decoy ODNs are composed of two short complementary DNA strands mimicking the consensus binding sequence of certain transcription factors (TF). Once inside the cell, decoy ODNs effectively neutralize their target transcription factor, thereby preventing it from inducing the expression of its target genes. The mechanism of action of decoy ODNs is shown in Fig. 22.

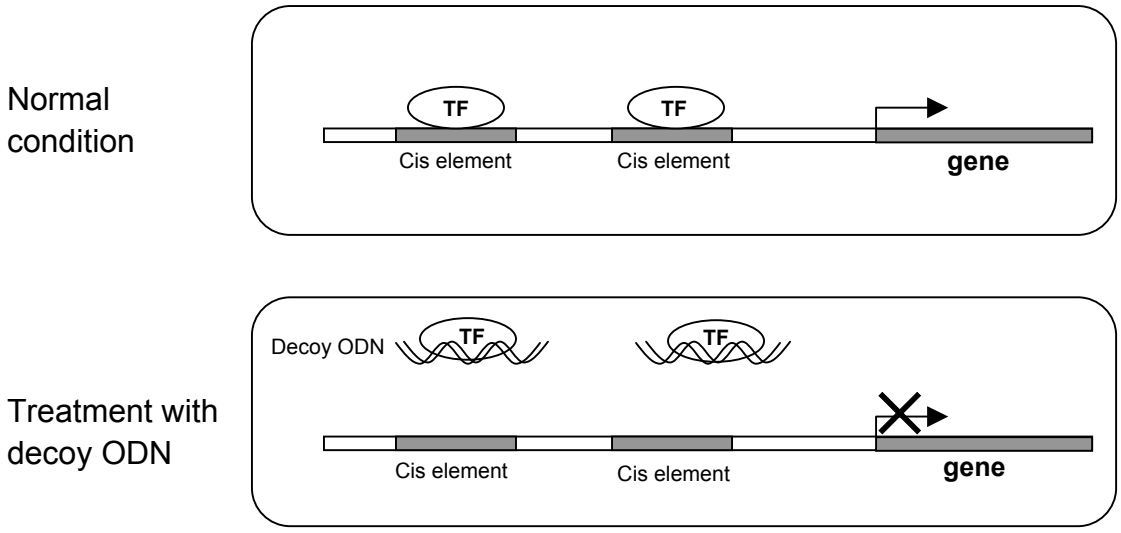


Figure 22. The mechanism by which decoy ODNs disrupts gene expression. Under normal condition, transcription factors (TF) bind to *cis* elements, resulting in continuous activation of target gene expression. Decoy ODNs prevent the binding of transcription factors to their target *cis* elements, resulting in blockade of gene expression.

Previous work in our group has shown that the transcription factor STAT-1 is a key molecule in mediating cytokine-induced CD40 expression in rat vascular smooth muscle cells (Krzesz et al. 1999). Treatment with STAT-1 decoy ODNs revealed profound therapeutic effects in mouse and rat models of allergen-induced asthma, antigen-induced arthritis and transplant rejection. However, application of STAT-1 decoy ODNs, either in liposomal form or as naked DNA, in the TNBS-induced colitis model in this study failed to show any therapeutic effect. Probably, this lack of effect was due to the inefficient delivery of the double stranded DNA molecules. Decoy ODNs are thought to require nuclear localization if they are to prevent the

transactivation of their target genes. There is increasing evidence showing that the uptake of double-stranded ODNs is different from that of single-stranded ODNs (Mann and Dzau 2000). The development of a more efficient delivery system is still a great challenge in the field of synthetic ODN application *in vivo*. Moreover, it was found that increase in STAT-1 expression and activation in mucosal samples from Crohn's disease is not as high as from patients with ulcerative colitis (Schreiber et al. 2002). At the same time, high level of suppressor of cytokine signaling (SOCS)-3 expression, an inhibitor of STAT activation, was observed in Crohn's disease. Therefore, STAT-1 might neither be the right target molecule in the TNBS-induced rat colitis model nor in human Crohn's disease. However, expression and activation of STAT-1 still needs to be investigated in this model.

4.6 The prospect of human CD40 antisense ODN

The possibility of blocking human CD40 expression by antisense ODNs was also investigated in this study. The most effective antisense ODN designed (hAS4) locates to position +60 to +75 of the human CD40 mRNA. It significantly inhibited CD40 expression under both basal and pro-inflammatory conditions and could further down-regulate of CD154-stimulated IL-12 expression in primary cultured human endothelial cells. Up to now, there is only one therapeutic antisense ODN that has been approved for therapeutic use by the Food & Drug Administration in the U.S.A. This antisense ODN is complementary to the major immediate early region 2 of cytomegalovirus (CMV) and has been shown to be an effective against CMV-induced retinitis in AIDS patients (de Smet et al. 1999). However, there are approximately 15 other antisense ODNs in various stages of clinical development that are directed against cancer, inflammation and viral targets. In patients with Crohn's disease, an antisense ODN against ICAM-1 (ISIS 2302) has been evaluated considering the importance of adhesion molecules in maintaining the inflammatory reaction. Although the first-in-man study failed to demonstrate efficacy, pharmacodynamic modeling suggests that this antisense ODN may be an effective therapy for steroid-dependent Crohn's disease (Yacyshyn et al 2002). The evaluation of higher doses of ISIS 2302 in Crohn's disease is currently underway. Moreover, an antisense ODN against protein kinase A, which is currently in phase II clinical trial for the treatment of cancer, has recently been reported to have anti-tumor activity in mice with oral delivery (Tortora et al. 2000).

Because drug delivery is touted as the biggest hurdle to the general use of antisense ODNs as effective drugs, these developments are particularly exciting. It would perhaps be worthwile to evaluate this delivery system with CD40 antisense ODNs in IBD patients in the future.

5. Summary

CD154/CD40 interactions play a pivotal role both in humoral and cellular immune responses and, as such, represent an attractive target for immune modulation in a number of diseases. Over recent years, administration of anti-CD154 antibodies has been shown to effectively inhibit inflammatory responses in a variety of animal models such as collagen-induced arthritis, experimental allergic encephalomyelitis, murine lupus erythematosm, or experimental colitis in SCID mice. However, it has recently been reported that platelets present CD154 on the cell surface within seconds of thrombin activation *in vitro* and in the process of thrombus formation *in vivo.* Especially, soluable CD154 (shed from CD154-expressing cells including thrombocytes) is required for stabilization of existing thrombi. These may be relevant to the unusually high incidence of thromboembolic complications observed in anti-CD154 antibody treated animals and patients alike. In this study, an alternative approach to blocking CD154/CD40 interactions by employing a CD40 antisense oligonucleotide (ODN) was explored.

After obtaining the sequence of rat CD40 mRNA by 5'-RACE (rapid amplification of cDNA ends), five antisense oligoneclotides were designed against different regions of the target mRNA. One of them (rAS3, targeting position + 46 to + 63 of rat CD40 mRNA) proved highly efficient at a relatively low dose in attenuating CD40 expression in rat cultured vascular smooth muscle cells. Its effect lasted for about three days *in vitro*. Moreover, inhibition of CD40 expression by rAS3 also led to a significant decrease in CD154-stimulated pro-inflammatory gene expression (e.g. vascular cell adhesion molecule-1, VCAM-1) in these cells. The inability of rAS3 to block tumor necrosis factor-α induced changes in gene expression validated it as a specific blocker for CD154/CD40 interactions.

The potential therapeutic effect of the CD40 antisense ODN was investigated further in a Crohn's disease-like colitis model in rats. Single treatment with the antisense ODN strongly attenuated the trinitrobenzene sulphonic acid/ethanol-induced colonic inflammation in this model. RT-PCR analysis demonstrated that the antisense ODN treatment significantly reduced the increase in VCAM-1 expression in the affected mucosa, which in turn may have contributed to the decrease in leukocyte infiltration.

Furthermore, an efficient antisense ODN approach against human CD40 (hAS4) was also designed in this study. Due to its inhibition of CD40 expression, hAS4

attenuated CD154-stimulated interleukin-12 p40 expression in human umbilical vein cultured endothelial cells. When compared to anti-CD154 antibodies, CD40 antisense ODNs may provide the following advantages: (1) no thromboembolic complications; (2) ease of handling; (3) improved stability; and (4) low costs.

In summary, the results of this study suggest that CD40 antisense ODNs interfere with CD154/CD40 interactions with high potency and specificity both *in vitro* and *in vivo*. Therefore, they may provide an attractive therapeutic approach for Type I T helper cell-mediated chronic inflammatory diseases including Crohn's disease.

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