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Regulation of T Cell Activation In Vitro and In Vivo by Targeting the OX40-OX40 Ligand Interaction: Amelioration of Ongoing Inflammatory Bowel Disease with an OX40-IgG Fusion Protein, But Not with an OX40 Ligand-IgG Fusion Protein¹

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OX40 is a member of the TNFR superfamily, and is found predominantly on activated CD4-positive T cells. In vitro an OX40-IgG fusion protein inhibits mitogen- and Ag-driven proliferation and cytokine release by splenocytes and lymph node T cells. In contrast, an OX40 ligand-IgG fusion protein enhanced proliferative responses. In normal mice, OX40-positive cells are observed only in lymphoid tissues, including Peyer's patches of the gut. In mice with hapten-induced colitis or IL-2 knockout mice with spontaneous colitis, OX40-positive cells are found infiltrating the lamina propria. Administration of the OX40-IgG fusion protein to mice with ongoing colitis (but not the OX40 ligand-IgG) ameliorated disease in both mouse models of inflammatory bowel disease. This was evidenced by a reduction in tissue myeloperoxidase; reduced transcripts for TNF- α , IL-1, IL-12, and IFN- γ ; and a reduction in the T cell infiltrate. Targeting OX40 therefore shows considerable promise as a new strategy to inhibit ongoing T cell reactions in the gut. *The Journal of Immunology*, 1999, 162: 486–493.

Interaction between members of the TNF ligand and TNFR superfamilies is intrinsic to a host of functions ranging from cellular proliferation, activation, and death (1, 2). Members of the TNFR superfamily include TNFR, CD95, CD40, CD30, and OX40, and are structurally related type I transmembrane proteins with homology restricted to the cysteine-rich extracellular domain. The corresponding ligands are type II transmembrane proteins and include TNF, FasL,³ CD40L, CD30L, and OX40L (1–5). OX40 and OX40L demonstrate a one receptor:one ligand binding principle. OX40 was first identified on activated CD4⁺ T cells in the rat (6) and has since been detected on human and murine CD4⁺ T cells and, to a lesser extent, CD8⁺ T cells (7), with expression mainly in the organized lymphoid tissues. The ligand for OX40 has a broader cellular and tissue distribution and has been identified on activated T cells and B cells, endothelial cell lines, and dendritic cells (7–10). Signaling through OX40 generates costimulatory signals, resulting in enhanced Con A-induced T cell proliferation (6) and enhanced cytokine production after ligation of TCR $\alpha\beta$ (8). The cytoplasmic tail of OX40 interacts with the TNFR-associated

factor 2 and 3, which regulate activation of nuclear factor- κ B (11). Blocking OX40L with an OX40-IgG fusion protein has been shown to inhibit these responses (8). Signaling also occurs through OX40L and is important in T cell-dependent terminal differentiation of activated B cells (12). In addition, the expression of OX40L on vascular endothelium suggests the involvement of OX40 in T cell migration into tissues (13). The therapeutic potential of targeting OX40 lies in its limited cellular expression, predominantly on activated CD4⁺ T cells, which are thought to be central to the pathogenesis of many human diseases, including inflammatory bowel disease (IBD), multiple sclerosis, rheumatoid arthritis, and graft-versus-host disease (14–17). The efficacy of anti-OX40 Abs in the treatment of the rat experimental allergic encephalomyelitis model of MS has been demonstrated, in which the use of a ricin-A anti-OX40 immunotoxin ameliorated disease (18).

In this study, we have constructed OX40-IgG and mOX40L-IgG fusion proteins and have examined their effect on the in vitro proliferation and cytokine production of T cells to mitogenic and antigenic stimulation. In addition, we have tested their efficacy at inhibiting the ongoing Th1-type responses responsible for colonic tissue injury in hapten-induced colitis and IBD in IL-2 knockout mice (19, 20). In both models, disease is mediated by Th1 type T cells through massive TNF- α , IL-1, and IFN- γ release (19, 21).

Materials and Methods

Animals

Female BALB/c mice (8–10 wk old) were obtained from A. Tuck & Sons (Southend-on-Sea, U.K.). IL-2^{+/-} C3H mice (20) were bred under standard conditions, and mice homozygous for the null mutation were identified by genotyping, as described previously (21). All mice were housed under standard conditions with free access to food and water.

Induction of colitis

BALB/c mice were weighed before procedure. Trinitrobenzene sulfonic acid (TNBS; Fluka, Gillingham, U.K.) was prepared in a 50% ethanol

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³ Abbreviations used in this paper: L, ligand; h, human; IBD, inflammatory bowel disease; KLH, keyhole limpet hemocyanin; m, murine; MLN, mesenteric lymph node; MPO, myeloperoxidase; TNBS, trinitrobenzene sulfonic acid.

solution diluted to give a final concentration of 2 mg TNBS in 75 μ l total volume. Mice were lightly anesthetized using 200 μ l of a 1/10 aqueous dilution of Hypnorm (Janssen-Cilag, High Wycombe, U.K.). Colitis was induced by intrarectal administration of 75 μ l of the TNBS solution using a plastic catheter. Control mice received 50% aqueous ethanol only. Mice were checked daily with respect to general condition and body weight.

Myeloperoxidase assay

Myeloperoxidase (MPO) was measured in snap-frozen samples of colonic tissue (22). Tissue (75–150 mg) was homogenized in 400 μ l of cold 1% (w/v) hexadecyl trimethyl ammonium bromide (Sigma, Poole, U.K.) in phosphate buffer, pH 6. The homogenate was then sonicated for 15 s. After snap freezing (in liquid nitrogen) and thawing three times, the homogenate was centrifuged for 15 min at 12,000 \times g at 4°C. The supernatant was then removed for MPO assay. To 10 μ l of supernatant in a flat-bottom 96-well microtiter plate (Philip Harris, London, U.K.), 200 μ l of 50 mM phosphate buffer, pH 6, containing 0.4 mg/ml of substrate *o*-phenylenediamine (Sigma) and 0.05% H₂O₂ (Sigma) was added. After 20 min, the reaction was stopped by the addition of 50 μ l 0.4 M H₂SO₄, and absorbance at 490 nm was determined using a plate reader (Titertek Multiscan, Eflab, Finland). Sample enzyme activity was measured from a standard curve of horseradish peroxidase activity (Boehringer Mannheim, Lewes, U.K.). Assay sensitivity was 10⁻⁷ U/ μ l.

Generation of fusion proteins

For the hOX40-hIgG1 fusion protein, the construct was as described previously (8). This construct was used to transfect Chinese hamster ovary cells, and positive clones were selected using G418. Fusion protein secretion was detected by incubation of supernatants with OX40L-transfected Sp2/0 cells and detection of binding by flow-cytometric analysis. Cells secreting high levels of fusion protein were expanded, and fusion protein from the supernatant was purified on a protein G-Sepharose column. Eluted material was electrophoresed on SDS-PAGE (12%), and the gel was stained with Coomassie blue to confirm purity. The mOX40L-hIgG1 fusion protein was prepared by a similar method (all enzymes were purchased from New England Biolabs, Hitchin, U.K.). A fragment encoding the extracellular domain of mOX40L was PCR cloned with the introduction of *Pst*I and *Hind*III sites at the 5' and 3' ends, respectively. To form the hIgG1-mOX40L fusion construct, this fragment was ligated into *Pst*I-digested plasmid that encoded for the hinge CH2 and CH3 domains of hIgG1. This gene was then isolated as a *Hind*III fragment and transferred to the pCR3 expression vector (Invitrogen, Abingdon, U.K.) containing the hCMV promoter and neoR selectable marker. Clones were screened for inserts in the correct orientation, and then grown up for transfection and expression experiments, as above.

Treatment with fusion proteins

TNBS colitic mice and ethanol-treated controls were injected i.p. with hOX40-IgG (100 or 10 μ g) or mOX40L-IgG (100 μ g) on days 4, 5, and 6 after induction of colitis, or one single dose of hOX40-IgG (100 μ g) on day 4. All mice were killed on day 7. IL-2-deficient mice, aged over 35 days, showing physical signs of deterioration and weight loss, indicative of colitis, were treated with three consecutive daily doses of hOX40-IgG (100 μ g) and were killed one day later. Since IL-2 knockout mice developed disease unpredictably, the choice of treating a mouse with weight loss with either IgG or OX40-IgG was decided by tossing a coin. In all cases, hIgG (100 μ g) (Sigma) was used as a control.

RNA extraction and quantitative RT-PCR

Constructs encoding standard RNAs (pMCQ1, pMCQ2, pMCQ3, and pMCQ4) kindly provided by Dr. M. F. Kagnoff (Department of Medicine, University of California, San Diego) (23) were used for quantitative competitive RT-PCR. To generate standard RNA, plasmids were linearized with *Sa*II (pMCQ1) or *Not*I (pMCQ2, 3, 4) and transcribed in vitro using T7 RNA polymerase, under conditions recommended by the supplier (Promega, Southampton, U.K.).

Gut tissue and cell pellets were snap frozen in liquid nitrogen and stored at -70°C. Total cellular RNA was isolated by homogenizing tissue or cells in TRIzol (Life Technologies, Paisley, U.K.) and incubating at room temperature for 5 min. RNA was extracted using chloroform (Sigma), followed by centrifugation for 15 min at 12,000 \times g at 4°C. The aqueous phase was precipitated with an equal volume of isopropanol (Sigma), followed by centrifugation for 15 min at 12,000 \times g at 4°C. The pellet was washed with 70% ethanol and resuspended in 50 μ l water. Total RNA was determined by spectrometric analysis.

RT-PCR amplification

Serial 10-fold dilutions of standard RNA (1 pg to 1 fg) were co-reverse transcribed with total cellular RNA (2 μ g) at 42°C for 50 min in a 20 μ l reaction volume containing 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 3 mM DTT, 10 mM dNTP mix, and 0.5 μ g oligo(dT) (Pharmacia Biotech, Herts, U.K.), using 100 U of reverse transcriptase (Superscript II RNase H⁻; Life Technologies). The reaction was terminated by heat inactivation at 70°C for 10 min. PCR amplification was conducted routinely in 50 μ l reaction volumes (10 mM Tris, pH 9, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M dNTPs, 10 pmol 5' and 3' primers, as described elsewhere (23), and 1 U *Taq* polymerase (Pharmacia Biotech, U.K.)). Forty amplification cycles of 45-s denaturation at 94°C, 45-s annealing at 58°C, and 75-s extension at 72°C were used.

After amplification, PCR products were analyzed on 1% agarose gels and bands were visualized by ethidium bromide staining. Band intensities were quantified by densitometry (Seescan, Cambridge, U.K.). The sensitivity of this technique enables the detection of >10³ mRNA transcripts per μ g of total RNA.

Immunohistochemistry

Three-step avidin peroxidase staining was performed on 5- μ m frozen sections, as described previously (24), using mAbs 145-2C11 (anti-CD3), YTS 191 (anti-CD4), YTS 169 (anti-CD8), and OX86 (anti-mOX40). Biotin-conjugated rabbit anti-rat IgG (Dako, High Wycombe, U.K.) and goat anti-hamster IgG (Vector Laboratories, Peterborough, U.K.) were used at a 1/50 dilution in TBS, pH 7.6, containing 4% (v/v) normal mouse serum (Harlan Seralab, Oxon, U.K.). Avidin peroxidase (Sigma) was used at a dilution of 1/200 in TBS. Peroxidase activity was detected with 3,3'-diaminobenzidine-tetra-hydrochloride (Sigma) in 0.5 mg/ml Tris-HCl, pH 7.6, containing 0.01% H₂O₂. The density of positive cells in the lamina propria was determined by image analysis, as described previously (21).

Ag-specific T cell responses

Female BALB/c (2–4 mo old) mice were immunized (s.c.) with keyhole limpet hemocyanin (KLH; 100 μ g; Sigma) in 200 μ l CFA (Sigma). Draining lymph nodes and spleens were removed 14 days postimmunization.

Preparation of cells and proliferation assays

Single cell suspensions of mesenteric lymph node (MLN) and spleen cells were prepared by gently teasing apart in RPMI cell culture medium, supplemented with 10% FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin, using sterile forceps. Cell aggregates were removed by passing suspensions through sterile cell strainers (Falcon, London, U.K.). Single cell suspensions were washed three times with RPMI/FCS. A total of 200 μ l cell suspensions (5 \times 10⁵ cells/ml) was incubated in a 96-well microtiter plate with or without Con A (5 μ g/ml) or KLH (100 μ g/ml). hOX40-IgG, mOX40L-IgG, or hIgG was added at 5, 25, or 50 μ g/ml to cell cultures. Con A-stimulated cells were incubated for 3 days, and KLH-stimulated cells for 5 days at 37°C, and were pulsed with 1 μ Ci per well of [³H]thymidine during the last 15 h before being harvested onto filters.

Isolation of lamina propria cells and flow cytometry

Lamina propria lymphocytes were prepared by enzymic digestion. Briefly, colon was washed out with HBSS (Sigma) and was cut into 0.5-cm pieces. Epithelial cells were removed by incubating gut segments in 25 ml HBSS, without Ca/Mg, and supplemented with 1 mM EDTA (Sigma), for 20 min at 37°C. After removing supernatant, gut segments were washed with HBSS resuspended in RPMI/FCS containing collagenase (90 U/ml; Sigma) and dispase (2.5 U/ml; Sigma), and tissue was left to digest for 1 h at 37°C, with stirring. The resultant cell suspension was passed through a sterile cell strainer and was washed twice with RPMI/FCS.

OX40 surface expression was determined using mAb OX86 (5) with secondary FITC-conjugated goat anti-rat IgG (Sigma). Rat IgG (Sigma) was used as a control. Briefly, 400 μ l OX86 supernatant or rat IgG (1/50 dilution in PBS containing 0.1% NaN₃) was incubated with 500,000 cells for 60 min on ice. Cells were washed once with PBS, pH 7.4, and resuspended in FITC-conjugated secondary Ab (1/50 dilution in PBS/NaN₃ containing 4% normal mouse serum) for 30 min on ice. Cells were washed again and resuspended in 1% paraformaldehyde/PBS for counting. Single color flow cytometry was conducted using a FACScan (Becton Dickinson Immunocytometry Systems, Oxford, U.K.).

Statistics

The significance of differences between means was determined using the Mann-Whitney *U* test.

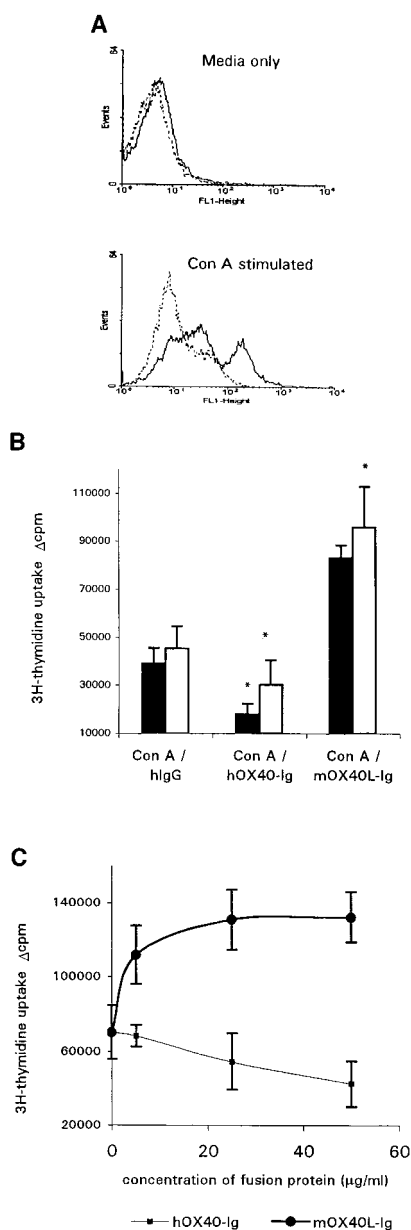


FIGURE 1. A, Up-regulation of OX40 on Con A-stimulated cells. FACS analysis of OX40⁺ cells after 60-h incubation in media alone or with Con A; dashed line represents staining with control Ab, and filled line represents OX86 staining. B, Con A stimulation in the presence of OX40-Ig or OX40L-Ig. Proliferation, after 60 h, of splenocytes (filled bars) and MLN cells (open bars). *, $p < 0.05$. C, Dose response of fusion proteins on Con A-stimulated cells after 60 h. Results are expressed as mean \pm SE and are representative of three experiments. Background cpm was <1000 .

Results

The effect of hOX40-IgG and mOX40L-IgG on the proliferation of Con A-stimulated cells

To examine the expression of OX40 on activated T cells, flow cytometry using the mAb OX86 was performed on splenocytes stimulated for 60 h with Con A. A peak for OX40-positive cells was observed, while no expression was detectable on resting cells. There was a double peak for stimulated cells, suggesting a population of cells with high and low expression of OX40 (Fig. 1A). OX40L and OX40 fusion proteins were then examined for their effect on mitogen activation of splenocytes and MLN cells (Fig.

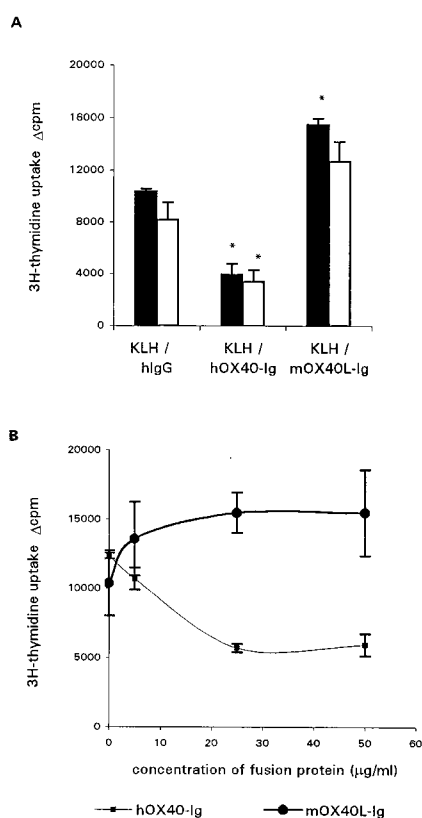


FIGURE 2. A, KLH stimulation in the presence of OX40-Ig or OX40L-Ig. Proliferation, after 96 h, of splenocytes (filled bars) and DLN cells (open bars). *, $p < 0.05$. B, Dose response of fusion proteins on KLH-stimulated cells after 96 h. Results are expressed as mean \pm SE and are representative of three experiments. Background cpm was <1000 .

1B). Cross-linking of OX40 using the OX40L fusion protein was shown to be costimulatory, enhancing proliferation by up to 100% in both splenocytes and MLN cells at all concentrations examined (Fig. 1C). The OX40 fusion protein inhibited proliferation by up to 50% at a concentration of 50 $\mu\text{g/ml}$ for MLN and spleen cells. The dose response indicated that OX40-IgG had a reduced effect on stimulation at lower concentrations (Fig. 1C). Addition of OX40-IgG 15 h before harvesting also resulted in an inhibition of proliferation (data not shown). No effect with either fusion protein or control hIgG was observed on resting cells.

The effects of hOX40-IgG and mOX40L-IgG on an Ag-specific recall response

To examine the effects of OX40-IgG and OX40L-IgG on an Ag-specific response, mice were immunized with 100 μg KLH in 200 μl CFA s.c., and the in vitro proliferation of draining LN cells and splenocytes challenged with KLH was examined. Ag-induced proliferation was reduced by using the OX40 fusion protein at 50 $\mu\text{g/ml}$, whereas OX40L-IgG was costimulatory (Fig. 2A). Lower concentrations of OX40L-IgG were, as before, sufficient to promote proliferation (Fig. 2B). We wanted to eliminate the possibility that the OX40-IgG fusion could be causing complement-mediated lysis of cells expressing OX40L. We therefore examined the effect of OX40-IgG on B cells as these are the principal cell type expressing OX40L. Following in vitro Con A-induced activation of splenocytes in the presence of OX40-IgG there was no diminution of B cells compared to controls as shown by trypan blue

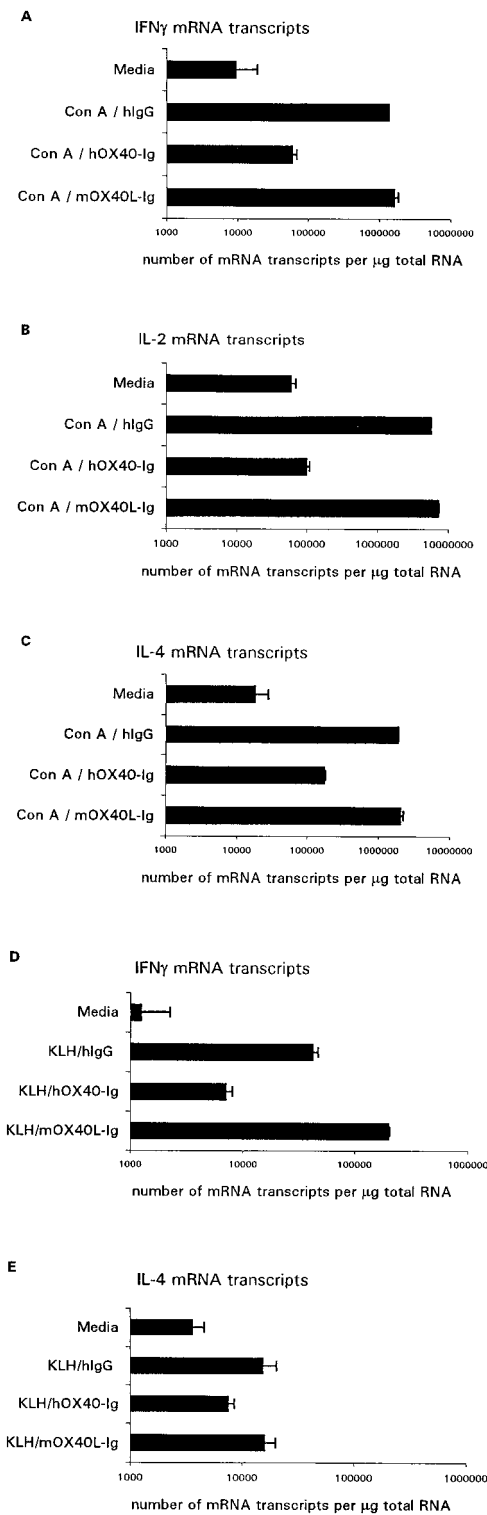


FIGURE 3. Cytokine mRNA transcripts in spleen cells stimulated in vitro with Con A or KLH. All results represent a dose of 50 μ g/ml of fusion protein when maximal effect was observed. Results are expressed as mean \pm SE and are representative of two experiments. A–C, Con A-stimulated cells incubated with fusion proteins and control IgG. D–E, KLH-stimulated cells incubated with fusion proteins and control IgG.

exclusion and FACS analysis for B cell numbers. In addition, neither freshly isolated mouse serum nor commercial rabbit complement were capable of lysing B cells in the presence of OX40-IgG (data not shown).

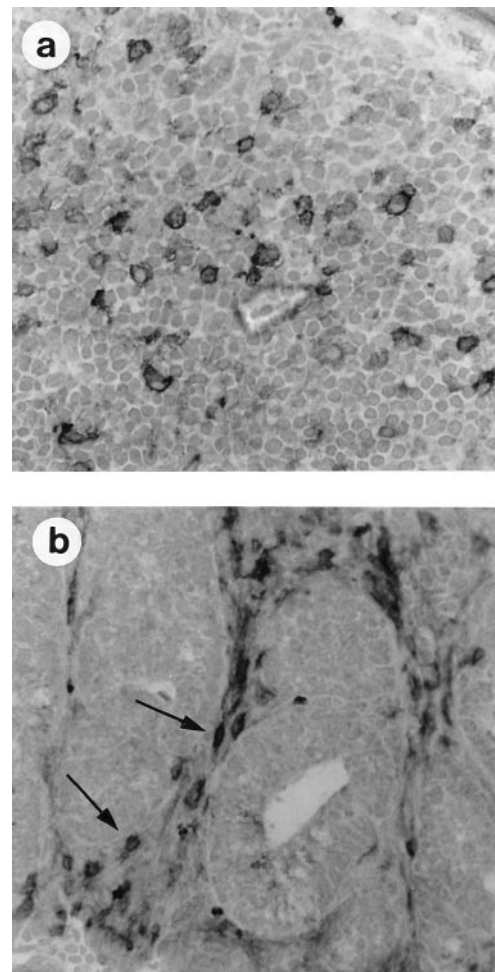


FIGURE 4. OX40-positive cells in TNBS-induced colitic mice on day 7 shown by immunoperoxidase immunohistochemistry using mAb OX86 in Peyer's patch (a) and infiltrating the lamina propria (b), indicated by arrows. Control mice had no positive cells in the lamina propria (original magnification \times 400).

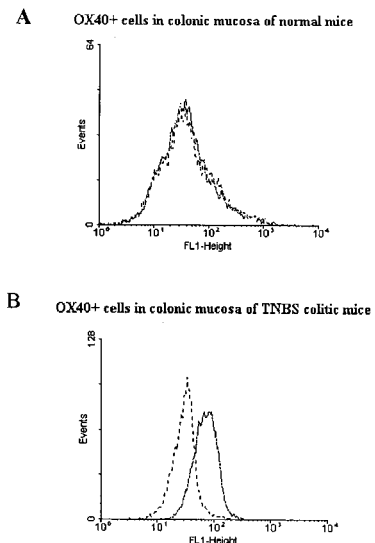


FIGURE 5. FACS analysis for OX40 expression in lamina propria MNCs of normal BALB/c mice (A) and TNBS colitic mice (B) on day 7 postinduction of colitis. Cells were pooled from four mice in each group; dashed line represents control Ab, and filled line represents OX86 staining.

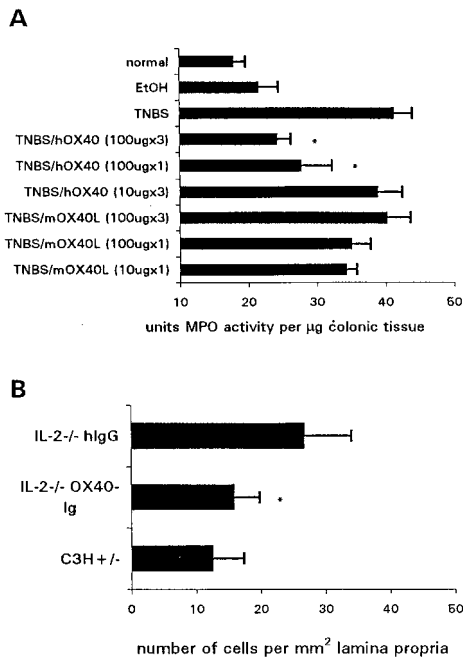


FIGURE 6. A, MPO activity in frozen colonic tissue of TNBS colitic mice and control mice. Activity in mice that received hIgG, hOX40-Ig, or mOX40L-Ig is also given; three daily doses of 100 μg (100 $\mu\text{g}\times 3$); one single dose of 100 μg or 10 μg (100 $\mu\text{g}/10 \mu\text{g}\times 1$); each group represents six mice (mean \pm SE; *, $p < 0.05$). B, Endogenous peroxidase-containing cells in the lamina propria of IL-2^{-/-} mice that received hIgG or hOX40-Ig (100 $\mu\text{g}\times 3$) and control wild-type mice (C3H^{+/+}); each group represents five mice (mean \pm SE; *, $p < 0.05$).

Alteration in cytokine profiles

Increased transcripts for IL-2, IFN- γ , and IL-4 were found in Con A-activated cells. OX40-IgG dramatically reduced cytokine transcripts. However, despite the much greater proliferative response, OX40L-IgG did not enhance cytokine production (Fig. 3, A–C). Similar results for KLH-stimulated cells were also seen (Fig. 3, D–E). IL-2, IFN- γ , and IL-4 ELISAs were performed on supernatants from Con A-stimulated splenocytes in the presence of OX40-IgG, and a similar reduction in protein compared with controls was observed for each cytokine. IL-2 was reduced from 9.1 to 2.1 U/ml, IFN- γ from 10.8 to <1 ng/ml, and IL-4 from 4.3 to <1 pg/ml (data not shown).

In vivo expression of OX40 in TNBS colitis and IL-2-deficient colitis

Since activated CD4⁺ T cells play an important role in both TNBS colitis and IL-2 knockout mice with colitis (19, 21), we examined the expression of OX40 in vivo in the gut by immunohistochemistry. In normal BALB/c and C3H mice, OX40-positive cells were only observed in lymphoid tissue, including Peyer's patches and MLN. Positive cells were, however, seen by immunohistochemistry in the lamina propria of both TNBS (Fig. 4) and IL-2 knockout mice with colitis, but not in controls. Positive cells were also observed by FACS analysis of cells isolated from the lamina propria of mice with TNBS colitis (Fig. 5).

In vivo administration of OX40 and OX40L fusion proteins in TNBS colitis

TNBS colitis was induced in female BALB/c mice. The disease profile (not shown) was such that an initial severe acute colitis took place 1–3 days postinduction, followed on day 4 by T cell infil-

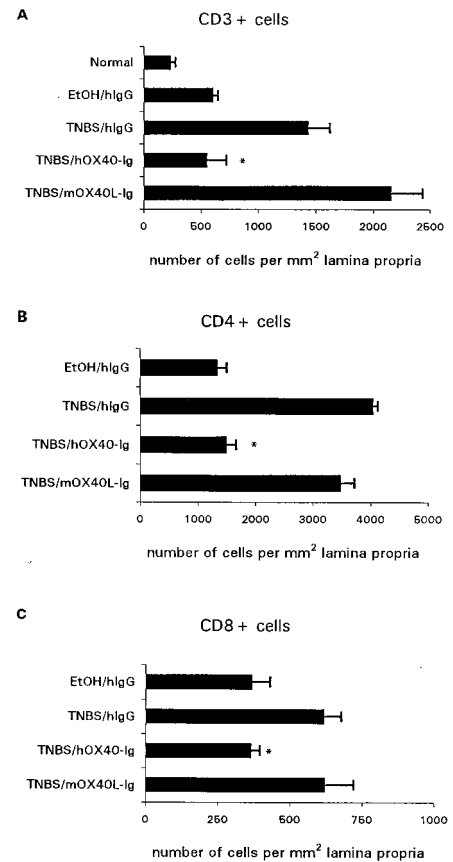


FIGURE 7. Cell counts for CD3-, CD4-, and CD8-positive cells infiltrating the lamina propria in TNBS mice (day 7) treated with either hOX40-Ig, mOX40L-Ig, or hIgG (100 μg on days 4, 5, and 6), and EtOH control group treated with hIgG. Each group represents six mice (mean \pm SE; *, $p < 0.05$).

tration, which was maximal at day 7, after which the colitis subsided. Day 4 was chosen to begin treatment with the fusion proteins. The results shown represent one experiment ($n = 6/\text{group}$). Similar results have been shown in two additional experiments in which $n = 5/\text{group}$ (data not shown).

MPO activity in TNBS mice was significantly higher than in ethanol control mice. MPO in mice that had received three daily doses, or one single dose, of 100 μg of hOX40-IgG was comparable with the ethanol control. Treatment with the lower dose of 10 μg of hOX40-IgG, or with OX40L fusion protein at either 10 or 100 μg had no effect (Fig. 6).

T cell and macrophage infiltration in the mucosa of TNBS mice was assessed by immunohistochemistry for CD3-, CD4-, and CD8-positive cells in the colonic lamina propria of all mice. A striking reduction in all three cell types was observed in mice treated with three doses of 100 μg of OX40-IgG, while ligand-treated mice had similar cellular infiltrate to TNBS colitic mice (Fig. 7).

The cytokine profile in the distal colons of TNBS mice was determined by competitive quantitative PCR. Mice with TNBS colitis have increased transcripts of IL-1, TNF- α , IL-12, and IFN- γ in their gut. OX40-IgG-treated mice showed a dramatic reduction in all of these cytokines (Fig. 8, A–D). Transcripts for Th2-type cytokines in the colon of OX40-IgG-treated mice were not significantly different from normal mice (Fig. 8, E–F). A single dose of 100 μg of OX40-IgG on day 4 had similar effects to the three daily doses, but mice given 10 μg still showed marked elevation in

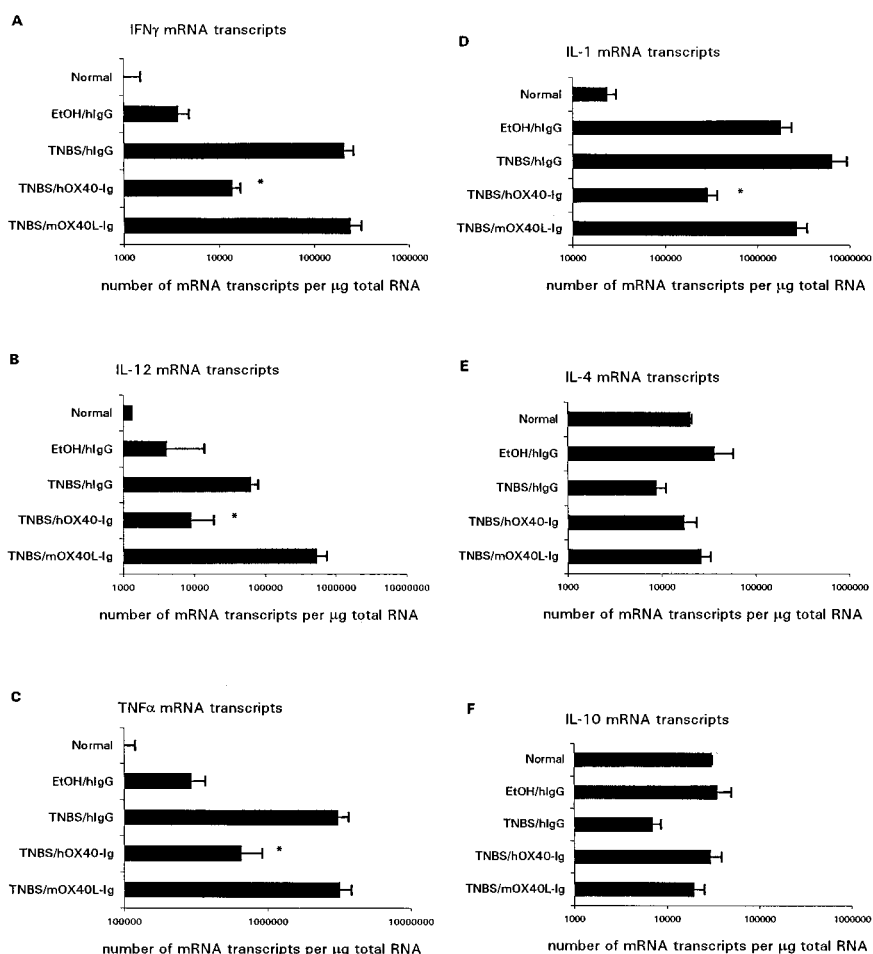


FIGURE 8. Cytokine mRNA transcripts in gut tissue of TNBS mice with colitis treated with fusion proteins (all 100 μ g), together with EtOH control mice and normal BALB/c mice. Each group represents six mice (mean \pm SE, *, $p < 0.05$). A, IFN γ ; B, TNF α ; C, IL-12; D, IL-1; E, IL-4; F, IL-10.

proinflammatory and Th1 cytokines (data not shown). Treatment with mOX40L-IgG did not reduce cytokine transcripts, and in one to two mice, per experiment, treated with three daily doses of 100 μ g, they were markedly elevated.

In vivo administration of OX40 fusion proteins in IL-2 knockout mice with colitis

IL-2 knockout mice with colitis were given three 100 μ g doses of OX40-IgG or hIgG once the first signs of deterioration were apparent. On day 4 posttreatment, the mice were killed. A massive infiltration of T cells and macrophages takes place in IL-2 knockout mice with colitis when compared with normal wild-type mice. Counts for CD3-, CD4-, and CD8-positive cells were all reduced dramatically in mice treated with hOX40-IgG (Fig. 9). A significant reduction in peroxidase-containing cells was also observed (Fig. 6B).

Likewise, transcripts for IFN- γ , IL-12 and proinflammatory cytokines, IL-1, and TNF- α were markedly increased in IL-2 knockout mice with colitis treated with control IgG, but were reduced significantly in mice treated with OX40 fusion protein (Fig. 10).

Discussion

In the present study, we have shown the importance of the OX40-OX40L interaction in ongoing T cell-mediated reactions in the gut. First, we demonstrate *in vitro* that an OX40-IgG fusion protein can reduce activation of mitogen- and Ag-stimulated T cells, and that an OX40L-IgG fusion protein can enhance costimulation. Second, we demonstrate that OX40-positive cells, normally only observed

in lymphoid tissue, are present in the lamina propria of mice with TNBS colitis and IL-2 knockout mice with spontaneous colitis. Third and finally, we show that the T cell-mediated colitis in the two murine models of IBD can be ameliorated by the administration of OX40-IgG, but not of mOX40L-IgG.

The *in vitro* enhancement of mitogen-induced proliferation by costimulation through OX40 is in agreement with previous studies using anti-OX40 Abs (6) or OX40L transfectants (7, 8). The use of an OX40 fusion protein to block the OX40-OX40L interaction between purified CD4⁺ T cells and OX40L transfectants has also been reported (8). In addition, we have shown that the OX40-OX40L interaction is important in an Ag-specific response that is in agreement with studies showing enhanced proliferation in an MLR in the presence of an anti-OX40 Ab (6). Dose dependency was observed for blocking OX40L, and may be indicative of high OX40L expression in a mixed population of cells in contrast to limited expression of OX40 on activated T cells.

In vivo the success of OX40-IgG was also dose dependent and was highly efficient at blocking T cell responses in the gut. Although in certain individual mice treated with OX40L-IgG, colitis did appear to be heightened with respect to MPO and cytokine production, the overall result was not significantly greater than hIgG-treated controls. Transcripts for IL-4 and IL-10 in OX40-IgG-treated groups were not increased when compared with normal mice, demonstrating that OX40-IgG therapy did not result in immune deviation toward a Th2 response. *In vitro*, an increase in cytokine production did not take place with mOX40L-IgG, which, considering the proliferation data, was surprising. It suggests that

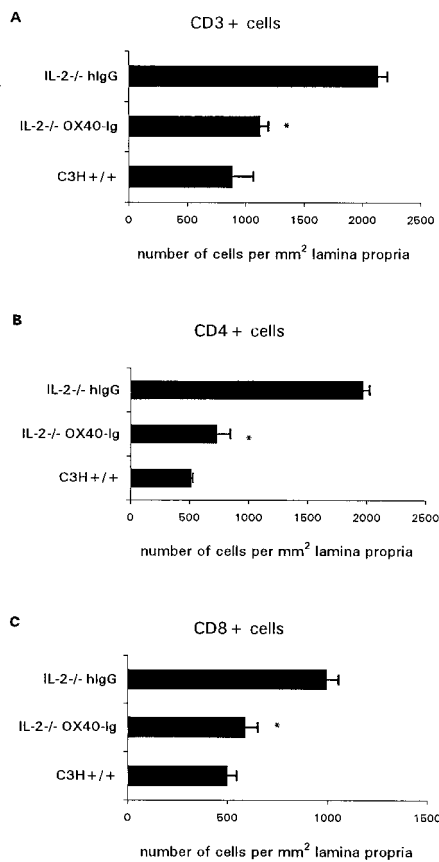


FIGURE 9. Cell counts for CD3⁺, CD4⁺, and CD8⁺ cells infiltrating the lamina propria in IL-2^{-/-} colitic mice treated with either hOX40-Ig or hIgG together with wild-type C3H mice. Each group represents five mice (mean \pm SE; *, $p < 0.05$).

the OX40L fusion protein was able to enhance proliferation through cross-linkage of OX40, but was not enough to induce cytokine production, or it may be that an upper limit in detection of such high concentrations of cytokine transcripts was reached. In

addition, *in vivo* treatment with OX40L-IgG did not result in increased cell infiltration. It is clear, however, that endogenous OX40-OX40L interactions play a crucial role in regulating T cell-mediated damage. Many recent studies have dealt with the expression and function of OX40L on B cells, dendritic cells, and endothelial cells (7–10), and from these studies, it is evident that whatever the cell type expressing the ligand, or indeed one cannot not exclude the existence of a soluble form of ligand, all such interactions are crucial for cross-talk with activated T cells.

The OX40-OX40L interaction is a good example of bidirectional signaling. Cross-linking of OX40L on B cells results in B cell proliferation and secretion of all Ig isotypes and is critical for T cell-dependent terminal differentiation of B cells (12). In addition, signaling through OX40L is also important for the proliferation of activated naive dendritic cells (10). Another possible mechanism for OX40-IgG therapy, which is not related to activation, but rather to homing, is the prevention of recruitment of OX40⁺ cells to sites of inflammation through OX40L expression on endothelial cells. It has been shown that cells from patients with adult T cell leukemia adhere to HUVECs through OX40-OX40L interaction (9). In human IBD, OX40L endothelial cells have been seen, but OX40L is also expressed on as yet unidentified lamina propria cells (H. Souza and J. Spencer, personal communication). The expression of OX40L on murine endothelial cells has not been reported.

IBD in mice is a result of immune dysregulation. Mice with a variety of T cell defects including mice with a deleted gene for IL-2 (20), IL-10 (25), TCR α or TCR β (26), and G α_{12} (27) as well as T cell-reconstituted tg ϵ 26 mice transgenic for the human CD3 ϵ gene (28) and mice transgenic for IL-7 (29) develop chronic IBD. Disease is mediated principally by activated CD4⁺ T cells. Therapeutic strategies have aimed either at blocking cytokines produced by these and accessory cells, as demonstrated by anti-IL-12 (19), anti-TNF- α (30), or IL-10 (31) treatment, or at inhibiting signal transduction (32), or at preventing costimulatory signals between T cells and APCs. Many costimulatory interactions are regulated by molecules that are normally absent or have low expression, but are up-regulated upon activation. These include CD25, CD28, CD40L, OX40, and 4-1BB, all considered as markers of T

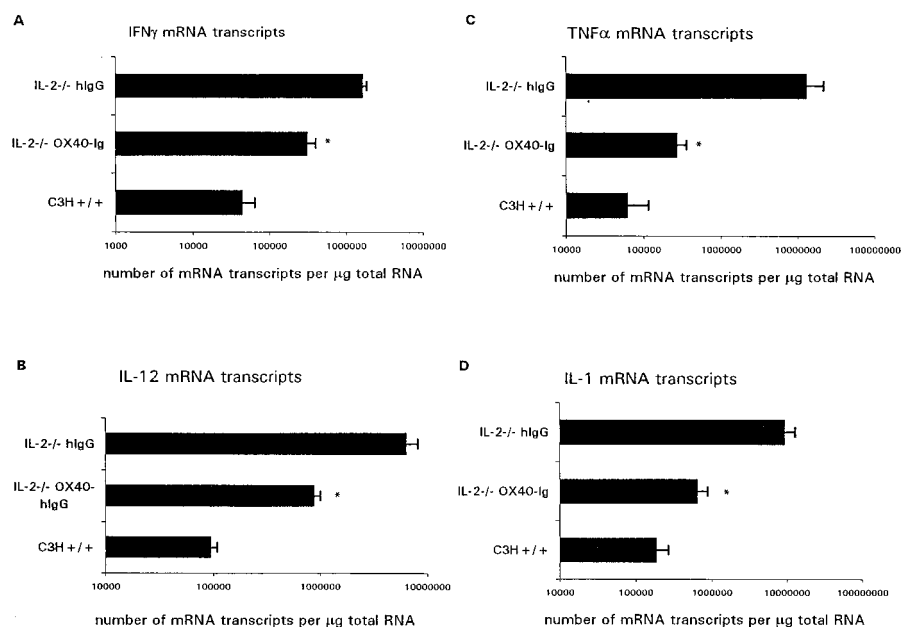


FIGURE 10. Cytokine mRNA transcripts measured by RT-PCR in colonic tissue of treated IL-2-deficient colitic mice together with wild-type nontreated C3H mice. Each group represents five mice (mean \pm SE, *, $p < 0.05$). A, IFN- γ ; B, IL-12; C, TNF- α ; D, IL-1.

cell antigenic stimulation, and therefore viable targets in immunotherapy. The CD40-CD40L interaction, for example, has been blocked in the treatment of experimental IBD (33). Negative regulation of T cell activation through CTLA4 has also been used in the down-regulation of aberrant T cell responses, as shown by the inhibition of the CD28-B7 interaction in transplantation (34) and experimental multiple sclerosis (35).

There is a plethora of molecules, including OX40, that can play a role in enhancing the efficiency of activation and proliferation of T cells, and it has been demonstrated with genetically mutant mice that not all of these are essential, indicating that a certain degree of compensation can occur. It is clear that different molecules are important at different stages in the pathway to a fully activated T cell, and that cooperation is needed for a fully efficient response. The success of blocking T cell responses by inhibiting these interactions continues to show promise in the treatment of autoimmune disease, graft rejection, and lymphoma. The advantage of using OX40-IgG in therapy lies in the selective expression of OX40 predominantly on activated CD4⁺ T cells for a transient period in vivo.

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