Infliximab but not Etanercept Induces Apoptosis in Lamina Propria T-Lymphocytes From Patients With Crohn's Disease

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Background & Aims: **Steroid-refractory Crohn's disease responds to therapy with the chimeric anti–tumor necrosis factor (TNF)-α antibody infliximab. Etanercept, a recombinant TNF receptor/immunoglobulin G fusion protein, is highly effective in rheumatoid arthritis but not in Crohn's disease. Because both infliximab and etanercept are TNF-α–neutralizing drugs, we investigated the differences in TNF-α–neutralizing capacity and human lymphocyte binding and apoptosis-inducing capacity of both molecules.** ***Methods:*** **We used a nuclear factor κB reporter assay and a cytotoxicity bioassay to study TNF-α neutralization by infliximab and etanercept. Lymphocyte binding and apoptosis-inducing capacity was investigated using fluorescence-activated cell sorter analysis, annexin V staining, and cleaved caspase-3 immunoblotting using mixed lymphocyte reaction–stimulated peripheral blood lymphocytes (PBL) from healthy volunteers and lamina propria T cells from patients with Crohn's disease.** ***Results:*** **Both infliximab and etanercept neutralized TNF-α effectively. Infliximab bound to activated PBL and lamina propria T cells, whereas binding of etanercept was equal to a nonspecific control antibody. Infliximab but not etanercept induced peripheral and lamina propria lymphocyte apoptosis when compared with a control antibody. Infliximab activated caspase 3 in a time-dependent manner, whereas etanercept did not.** ***Conclusions:*** **Although both infliximab and etanercept showed powerful TNF-α neutralization, only infliximab was able to bind to PBL and lamina propria T cells and subsequently to induce apoptosis of activated lymphocytes. These data may provide a biological basis for the difference in efficacy of the 2 TNF-α–neutralizing drugs.**

Tumor necrosis factor (TNF)-α is believed to play a central role in the initiation and amplification of Crohn's disease (reviewed by Papadakis and Targan[1] and Van Deventer[2]). TNF-α is first produced as a 26-kilodalton transmembrane form with an intracellular tail, which is cleaved to the secreted 17-kilodalton soluble form by the metalloproteinase-desintegrin TNF-α converting enzyme.[3,4] The 17-kilodalton form of TNF-α then aggregates to trimolecular complexes (trimers), which bind and activate their receptors.[1] Soluble TNF-α is predominantly produced by activated macrophages and lymphocytes (reviewed by Vassalli[5]). The number of lamina propria soluble TNF-α–producing T cells in patients with Crohn's disease is increased,[6,7] and high concentrations of soluble TNF-α can be detected in the stool of patients with active immune responses.[8,9] Several TNF-α–neutralizing antibodies and fusion proteins have been reported to be clinically beneficial in chronic inflammatory diseases (reviewed by Sandborn and Hanauer[10] and Feldmann and Maini[11]). In particular, infliximab, a chimeric monoclonal antibody with murine variable regions and human immunoglobulin (Ig) G1 constant regions,[12] is highly effective in the treatment of steroid-refractory Crohn's disease, inducing both clinical remission and endoscopic healing.[13,14] Thus, it has been suggested that neutralization of soluble TNF-α is clinically useful in treating Crohn's disease.

However, recent data question the role of soluble TNF-α neutralization in infliximab action in Crohn's disease. Infliximab can induce long-term remission, which cannot be explained by soluble TNF-α neutralization only, considering the pharmacodynamics of infliximab.[10] Furthermore, observations obtained with a TNF-α–neutralizing fusion protein, etanercept, raise further questions. Etanercept is a dimeric fusion protein consisting of the extracellular portion of the p75 TNF

Abbreviations used in this paper: DC, dendritic cells; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; IMDM, Iscoves Modified Dulbecco's medium; LPMNC, Lamina propria mononuclear cells; Mem TNFα, transmembrane TNFα; MLR, mixed lymphocyte reaction; NF-κB, nuclear factor κB; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; SolTNFα, soluble TNFα; TNFα, tumor necrosis factor α.

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receptor linked to the F_c domains of a type 1 human immunoglobulin (IgG1).[15] In rheumatoid arthritis, treatment with etanercept led to significant reductions in disease activity,[16] with effects comparable to those observed with infliximab.[17] Surprisingly, in Crohn's disease, etanercept failed to induce clinical responses.[18] It has been suggested that infliximab-induced apoptosis of immune effector cells may explain the therapeutic effect of the drug. In the uninflamed, normal intestinal mucosa, lamina propria T cells are susceptible to apoptosis mainly through activation-induced cell death (the CD95 system), which serves to suppress uncontrolled lymphocyte proliferation.[19] Lamina propria T cells from patients with Crohn's disease are resistant to the induction of apoptosis by a variety of stimuli.[20,21] We have recently reported that infliximab induces apoptosis of lamina propria T cells in vivo and of a CD3/CD28-activated T-cell line (Jurkat cells).[22] In addition, infliximab was shown to bind to monocytes and induce apoptosis in a Fas-independent manner.[23]

To understand the differences in clinical efficacy between both drugs, we sought to investigate differences of both antibodies in soluble TNF-α–neutralizing capacity and the ability to bind to activated lymphocytes and induce apoptosis. In the current report, we show that although both infliximab and etanercept effectively neutralized soluble TNF-α, only infliximab was capable of binding activated lymphocytes, inducing apoptosis, and activating caspase 3. Thus, our results suggest that these unique abilities of infliximab may mediate the therapeutic effect in Crohn's disease.

Materials and Methods

Cell Culture

All cells were cultured at 37°C, 5% CO_2, and 99% humidity. Three culture media were used. Complete Iscove's modified Dulbecco's medium (Life Technologies, Paisley, Scotland) contained gentamicin (86 μg/mL; Duchefa, Haarlem, The Netherlands), 1% heat inactivated fetal calf serum (HyClone, Logan, UT), and L-glutamine (29.2 mg/mL; Gibco, Rhode Island, NY) for monocyte-derived dendritic cells. Complete Dulbecco's modified Eagle medium (BioWhittaker, Verviers, Belgium) was supplemented with 5% fetal calf serum (Gibco), 1% penicillin (10,000 U/mL; Sigma, Munich, Germany), 1% streptomycin (10,000 μg/mL; Sigma), and L-glutamine (29.2 mg/mL; Gibco) for WEHI cell culture. Complete RPMI 1640 (Gibco) for HeLa cell culture contained 10% fetal calf serum and the other supplements as in Dulbecco's modified Eagle medium.

Antibodies and Reagents

Infliximab (Remicade; Centocor, Malvern, PA) and the control chimeric antibody directed against hEp-CAM, C17-1a, were a gift from David Shealy (Centocor). Etanercept (Enbrel) was from Immunex Corp. (Seattle, WA). Other antibodies used were against cleaved caspase 3 (Asp175; Cell Signaling Technology, Beverly, MA), anti-CD3 and anti-CD28 (CLB, Amsterdam, The Netherlands), and polyclonal fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-human IgG1-2 (DAKO, Glostrup, Denmark). FITC-labeled annexin V was obtained from Nexins Research (Kattendijke, The Netherlands), and VIA Probe/7 AAD was from Pharmingen (San Diego, CA). Recombinant human TNF-α was obtained from RDI Inc. (Flanders, NJ). Streptavidin-FITC was from Dako (Glostrup, Denmark), and anti-human CD3-Cy3 was from Pharmingen.

Nuclear Factor κB Reporter Assay

HeLa cells were stably transfected with a nuclear factor κB (NF-κB)-d_2E GFP construct (Clontech, Palo Alto, CA). HeLa cells were preincubated for 30 minutes with increasing concentrations of infliximab or etanercept and stimulated overnight after addition of 50 ng/mL TNF-α. NF-κB–mediated GFP expression in the harvested cells was analyzed using fluorescence-activated cell sorter (FACS) analysis.

WEHI Bioassay

WEHI 164 cells clone 13, a mouse fibrosarcoma line kindly provided by Prof. Marc Feldmann (The Kennedy Institute of Rheumatology, London, England), were seeded in 96-well plates and incubated with various concentrations of TNF-α in Dulbecco's modified Eagle medium in the presence or absence of infliximab or etanercept as appropriate; cytotoxicity was determined as described by Espevik and Nissen-Meyer.[24]

In Vitro Generation and Maturation of Monocyte-Derived Dendritic Cells

Dendritic cells were generated as described by Sallusto and Lanzavecchia[25] and Vieira et al.[26] In brief, monocytes were isolated from peripheral blood mononuclear cells using density centrifugation. Immature dendritic cells were generated by culturing monocytes for 6 days in Iscove's modified Dulbecco's medium supplemented with granulocyte-macrophage colony–stimulating factor (500 U/mL; a gift from Schering-Plough, Uden, The Netherlands) and interleukin 4 (250 U/mL; Pharma Biotechnologie, Hannover, Germany). At day 6, maturation was induced by culturing the cells for 2 days with recombinant human interleukin 1β (5 ng/mL; Boehringer, Mannheim, Germany), recombinant human TNF-α (25 ng/mL; PBH, Hannover, Germany), and lipopolysaccharide (100 ng/mL; Sigma Chemical Co., St. Louis, MO). The maturation state of the dendritic cells was confirmed using CD1a, CD83, and HLA-DR antibodies and an FITC-labeled goat anti-mouse antibody as a secondary antibody for FACS analysis. On day 8, dendritic cells were harvested and labeled for FACS analysis or used for experimentation.

Isolation of Peripheral Blood Lymphocytes

Venous blood from healthy donors was collected by venipuncture in sodium/heparin-containing tubes. Peripheral blood mononuclear cells were isolated by density centrifugation with Lymphoprep (Nycomed, Torshov, Norway). Subsequently, peripheral blood mononuclear cells were centrifuged on a Percoll gradient (Pharmacia, Uppsala, Sweden) to isolate the peripheral blood lymphocytes (PBL).

Isolation of Lamina Propria Mononuclear Cells

The intestinal specimens were obtained from consenting patients with Crohn's disease who underwent bowel resection. Immediately after resection and macroscopic investigation by the pathologist, the tissue was transported to the laboratory in ice-cold Hank's balanced salt solution (HBSS). Lamina propria mononuclear cells were isolated from freshly resected mucosal disease using a dithiothreitol/ethylenediaminetetraacetic acid/collagenase method as described by Bull and Bookman[27] with slight modifications described by van Tol et al.[28] In brief, after washing in HBSS free of calcium and magnesium (Gibco), strips of mucosa were carefully dissected from the submucosal layer. Small pieces of mucosa of 0.5–1 cm^2 were incubated in 1 mmol/L dithiothreitol (Sigma Chemical Co.) in HBSS supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B to remove the mucus layer. The tissue was collected, washed extensively in complete HBSS free of calcium and magnesium, and cut into smaller fragments. Four or 5 sequential incubations with 0.75 mmol/L ethylenediaminetetraacetic acid in complete HBSS free of calcium and magnesium (Merck, Darmstadt, Germany) were performed at 37°C for 1-hour periods in a Wheaton chamber (Wheaton Science, Melville, NJ) to remove epithelial cells. The remaining lamina propria tissue fragments were washed twice for 15 minutes in culture medium (RPMI supplemented with 10% fetal calf serum and antibiotics followed by overnight incubation at 37°C in culture medium supplemented with 20 U/mL collagenase (Wothington, Freehold, MA) and 278 U/mL deoxyribonuclease (Wothington). Hereafter the cell suspension was filtered through a nylon mesh (50 μm), washed, and counted for cell viability using trypan blue dye. The heterogeneous population was further separated by density gradient centrifugation on Ficoll-Isopaque for 20 minutes at room temperature. The interface was harvested, washed thoroughly, and counted for viability.

Mixed Lymphocyte Reaction

PBL and lamina propria mononuclear cells were activated using a mixed lymphocyte reaction as described by Genestier et al.[29] and Kalinski et al.[30] Dendritic cells were harvested in complete Iscove's modified Dulbecco's medium at a concentration of 2.5×10^4 dendritic cells/mL and cocultured with either 10×10^4 PBL/mL allogenic nonadherent PBL or 10×10^4 lamina propria mononuclear cells/mL in 96-well round-bottom microtiter plates (25,000 cells per condition).

Characterization of Infliximab and Etanercept Lymphocyte Binding

PBL were isolated as previously described. PBL were cultured for 3 days and activated with anti-CD3 and anti-CD28 (concentration, 1:1000) for 24 and 48 hours. Activated lymphocytes were washed with ice-cold phosphate-buffered saline and incubated (1×10^6cells/mL) for 30 minutes on ice with no antibody, infliximab, etanercept, or a control chimeric antibody c17-1a (all at 10 μg/mL) in FACS buffer (phosphate-buffered saline supplemented with 0.01% NaN_3, 0.5% bovine serum albumin, and 0.3 mmol/L ethylenediaminetetraacetic acid) and an FITC-conjugated goat $F(ab')_2$ anti-human antibody (concentration, 1:100) as a secondary antibody for FACS analysis.

Lamina propria mononuclear cells were isolated as previously described, stimulated with 100 ng/mL phorbol myristate acetate and 100 ng/mL ionomycin (both from Sigma-Aldrich, Zwijndrecht, The Netherlands) for 24 hours, and washed extensively in ice-cold FACS buffer. First, the activated lamina propria mononuclear cells were incubated for 30 minutes on ice in FACS buffer with serial dilutions of 1 μg/mL, 10 μg/mL, and 100 μg/mL of biotinylated infliximab, etanercept, and c17-1a and no first antibody as a negative control. Second, streptavidin-FITC in a concentration of 1:100 was incubated for 20 minutes on ice. Third, the lamina propria mononuclear cells were labeled with anti-human CD3-cychrome. After each step, all of the conditions were washed twice with ice-cold FACS buffer.

Biotinylation of Infliximab, Etanercept, and c17-1a

Infliximab, etanercept, and the control antibody c17-1a were biotinylated according to Brown et al.[31] The antibodies were dissolved at stock concentrations of 2 mg/mL in phosphate-buffered saline. A total of 20 μL of 3 mg/mL stock long-chain biotin NHS ester (LCB-NHS biotin; Pierce, Rockford, IL) in dimethyl sulfoxide (Merck, Hohenbrunn, Germany) was incubated with 1 mL antibody stock concentration for 50 minutes at room temperature. The reaction was terminated with 1 mol/L Tris buffer. The solution was dialyzed for 48 hours at 4°C (dialysis membrane MWCO 12,000–14,000; Spectrum Laboratories, Rancho Dominguez, CA) to remove the excess of biotin and stored at 4°C with 0.1% sodium azide.

Contribution of Receptor-Bound TNF-α to Antibody Binding

PBL were either preincubated with or without an excess of recombinant human TNF-α on ice. Hereafter the unstimulated cells were stained according to the previously described staining of PBL for binding patterns of the 3 different antibodies for FACS analysis.

Antibody-Dependent Cell-Mediated Cytotoxicity

Supernatants of the PBL/dendritic cell mixed lymphocyte reaction were analyzed for levels of granzyme B using the granzyme B enzyme-linked immunosorbent assay kit (CLB, Amsterdam, The Netherlands) according to the procedure recommended by the manufacturer.

Caspase-3 Activation and Annexin V/7AAD Apoptosis Assay

Caspase-3 activation was measured in lymphocytes in the mixed lymphocyte reaction. For this purpose, the mixed lymphocyte culture was incubated with infliximab, etanercept, or c17-1a (all at a concentration of 10 μg/mL) for 0, 24, and 48 hours. After incubation, nonadherent cells (in total 1×10^6 lymphocytes) were collected and lysed in protein sample buffer (125 mmol/L Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 3% mercaptoethanol, 20% glycerol, and 1 mg/L bromophenol blue). Caspase-3 activation was assayed using immunoblotting and a cleaved (active) caspase-3 antibody according to routine Western blot procedures. A β-actin antibody was used as a loading control. After enhanced chemiluminescence using Lumilight+ substrate (Roche, Mannheim, Germany), antibody binding was visualized and relative expression levels were quantified using a Lumi-Imager (Boehringer Mannheim). Alternatively, the nonadherent cells (in total 25,000 lymphocytes) were incubated for 15 minutes with annexin V/FITC and subsequently for 5 minutes with 7AAD, both on ice in the dark, and analyzed by FACS.

Results

Infliximab and Etanercept Neutralize Recombinant Human TNF-α Effectively

A reason for the difference between infliximab and etanercept in Crohn's disease could be related to a difference in TNF-α–neutralizing potential between the 2 drugs. Hence, we assayed the TNF-α–neutralizing potential of infliximab and etanercept both with respect to TNF-α–dependent transactivation of a NF-κB–driven reporter construct as well as in the WEHI assay for TNF-α–induced cell death. Infliximab abolished TNF-α–induced GFP expression in NF-κB reporter construct transfected HeLa cells at a concentration of 1 μg/mL, and etanercept neutralized TNF-α at a concentration of 0.25 μg/mL (Figure 1). In our bioassay, the concentration of TNF-α that induced 50% cytotoxicity in targeting WEHI 164 cells was 100 pg/mL. TNF-α–mediated toxicity was neutralized by both infliximab and etanercept at concentrations of 40–200 ng/mL and 80 ng/mL, respectively (Figure 2). Thus, both infliximab and etanercept neutralized TNF-α and, in both assays, etanercept was even more potent than infliximab.

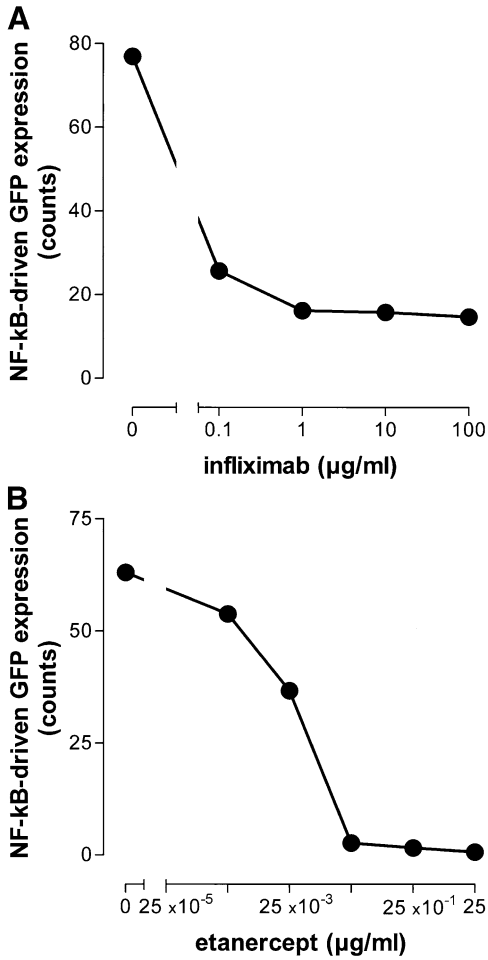

Figure 1. Both (*A*) infliximab and (*B*) etanercept neutralized recombinant human TNF-α effectively at concentrations of 1 μg/mL and 0.25 μg/mL, respectively, as assayed by transcription of an NF-κB–driven reporter construct. Both graphs are representative of 3 experiments.

Infliximab but not Etanercept Binds to Activated Peripheral Lymphocytes From Healthy Volunteers and Lamina Propria T Cells From Patients With Crohn's Disease

Differences in binding between infliximab and etanercept to a murine myeloma cell line transfected with uncleavable transmembrane TNF-α has been described.[32] To investigate the possibility that infliximab and etanercept differ with respect to their affinity to transmembrane TNF-α expressed by activated lymphocytes, we tested their binding to isolated PBL from healthy volunteers. For activation, these PBL were stimulated with CD3/CD28. Binding of infliximab and etanercept to the activated lymphocytes was measured using FITC-labeled anti-human IgG $F(ab)_2$ fragments. Infliximab clearly bound to activated PBL, but we were not able to detect specific binding of etanercept to acti-

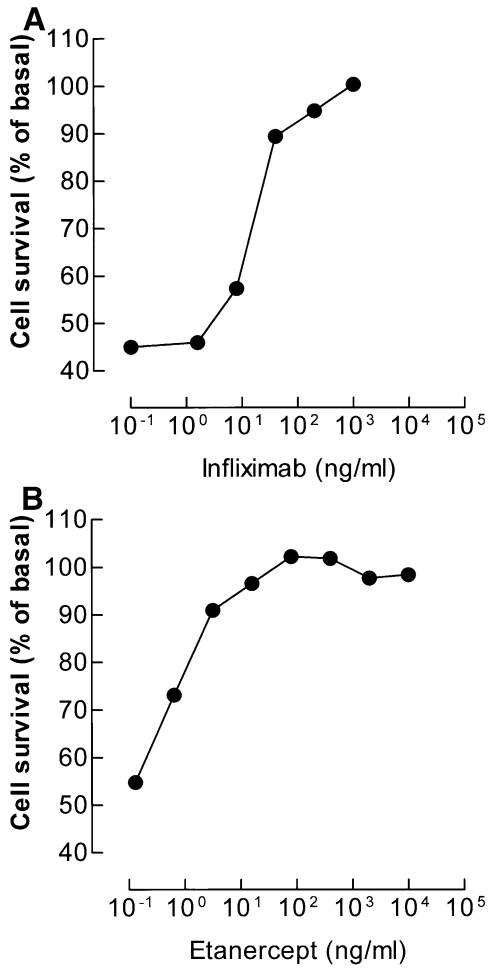

Figure 2. WEHI cells were incubated with serial dilutions of (*A*) infliximab and (*B*) etanercept. Both graphs are representative of 3 experiments. First, serial dilutions of TNF-α were tested from 18,000 to 0.36 pg/mL to induce cell cytotoxicity. TNF-α induced 60% cell death at the highest concentration of 18,000 pg/mL. Cytotoxicity is expressed as value related to TNF-α–induced cell death.

vated PBL (Figure 3). Some nonspecific binding of the irrelevant chimeric antibody c17-1a and, to a weaker extent, etanercept was observed at 24 hours but not at 48 hours. Finally, we addressed the possible contribution of receptor-bound TNF to infliximab binding to activated T cells, but no major contribution was detected; hence, infliximab binds mainly to transmembrane TNF (Figure 3*D*).

Also, using lamina propria T cells of patients with Crohn's disease, phorbol myristate acetate/ionomycin activated for 24 hours, and investigated with biotinylated infliximab, etanercept, and c17-1a, only infliximab was capable of binding to the cells. Double stainings with an anti-CD3 antibody confirmed that the infliximab-binding cells represented T lymphocytes (Figure 4, upper right quadrant).

Infliximab but not Etanercept Induces Apoptosis of Activated Peripheral Blood and Lamina Propria Lymphocytes

To investigate the functional consequences of the binding of infliximab to lymphocytes, PBL were activated using a mixed lymphocyte reaction and treated with infliximab, etanercept, or an isotype control antibody to exclude nonspecific effects. Lymphocyte apoptosis was determined using annexin V (binds to externalized phosphatidyl serine, an early marker of apoptosis) and 7AAD (stains nucleus of necrotic cells) double staining. In this assay, the annexin V–positive, 7AAD-negative cells represent the apoptotic cells. Addition of infliximab induced significant apoptosis in activated lymphocytes, but etanercept and the isotype control did not (Figure 5). Similarly, mixed lymphocyte reaction–stimulated lamina propria cells obtained from patients with Crohn's disease showed significant apoptosis when incubated with infliximab, whereas etanercept was inactive in this respect (Figure 6). Apparently, the absence of binding of etanercept to activated T cells correlated with an inability to induce apoptosis in these cells.

Infliximab Activated Caspase 3

For further characterization of the effects of infliximab binding to activated lymphocytes in the mixed lymphocyte reaction, we investigated the activation state of caspase 3 after incubation with infliximab, etanercept, or the c17-1a control antibody. As is evident from Figure 7, infliximab caused activation of caspase 3 at 24 and 48 hours but etanercept and the control antibody had no effect. These results show that the induction of apoptosis by infliximab correlates with activation of caspase 3. Thus, binding of infliximab to activated lymphocytes correlates with induction of apoptosis, whereas soluble TNF-α–neutralizing capacity does not.

Infliximab-Induced T-Cell Apoptosis Does not Involve Cell-Mediated Immunity or Complement Activation

Infliximab labeling of T cells may well provoke antibody-dependent complement activation or cell-mediated immunity via the activation of natural killer cells. Because our experiments involved human cells in heat-inactivated fetal calf serum–supplemented medium, we considered a major contribution of the complement system to effects observed in this study unlikely. In agreement, replacing fetal calf serum with complement-competent human serum did not increase the effects of

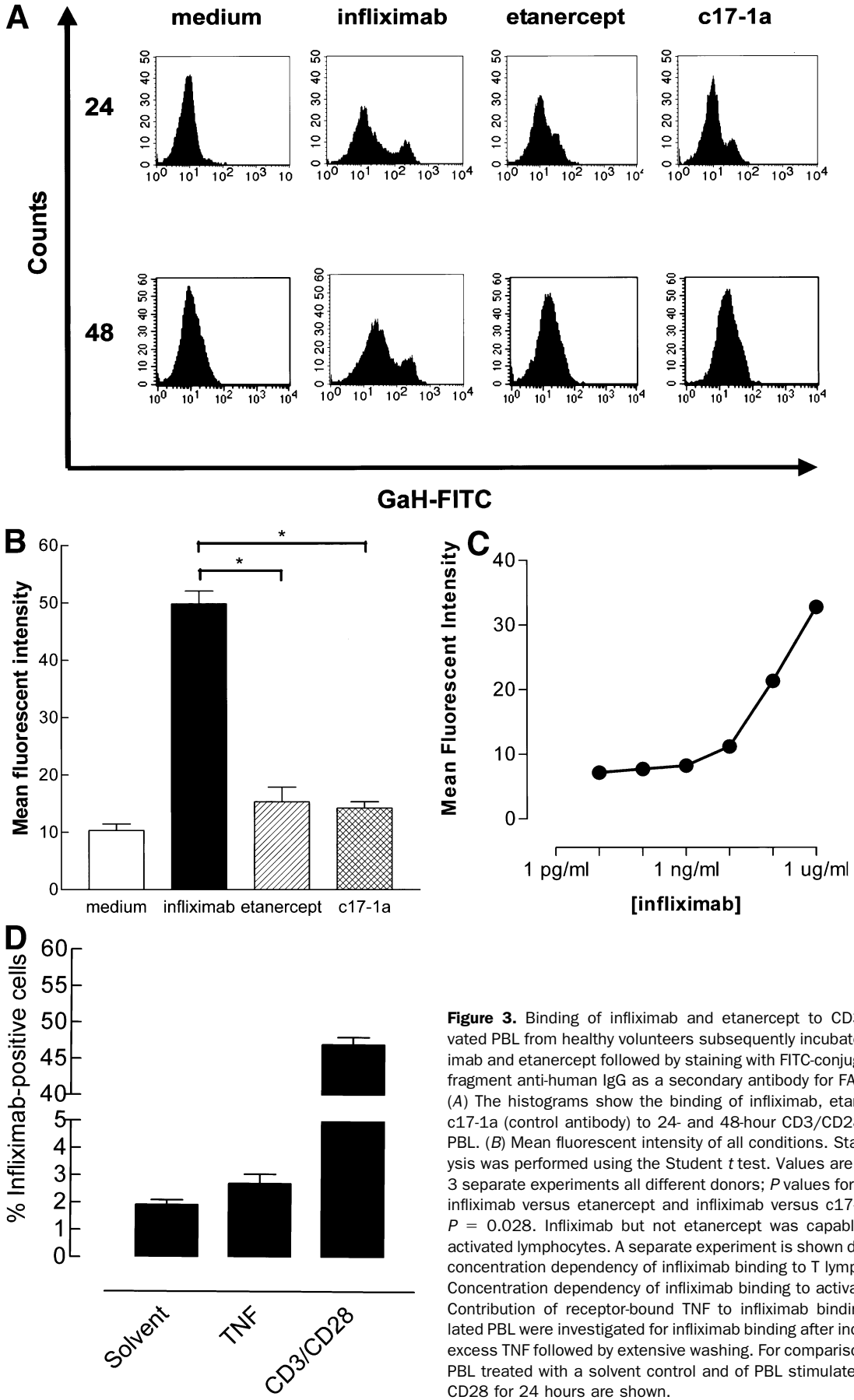

Figure 3. Binding of infliximab and etanercept to CD3/CD28-activated PBL from healthy volunteers subsequently incubated with infliximab and etanercept followed by staining with FITC-conjugated $F(ab')_2$ fragment anti-human IgG as a secondary antibody for FACS analysis. (*A*) The histograms show the binding of infliximab, etanercept, and c17-1a (control antibody) to 24- and 48-hour CD3/CD28 stimulated PBL. (*B*) Mean fluorescent intensity of all conditions. Statistical analysis was performed using the Student *t* test. Values are the mean of 3 separate experiments all different donors; *P* values for the effect of infliximab versus etanercept and infliximab versus c17-1a are both $P = 0.028$. Infliximab but not etanercept was capable of binding activated lymphocytes. A separate experiment is shown displaying the concentration dependency of infliximab binding to T lymphocytes. (*C*) Concentration dependency of infliximab binding to activated PBL. (*D*) Contribution of receptor-bound TNF to infliximab binding. Unstimulated PBL were investigated for infliximab binding after incubation with excess TNF followed by extensive washing. For comparison, results of PBL treated with a solvent control and of PBL stimulated with CD3/CD28 for 24 hours are shown.

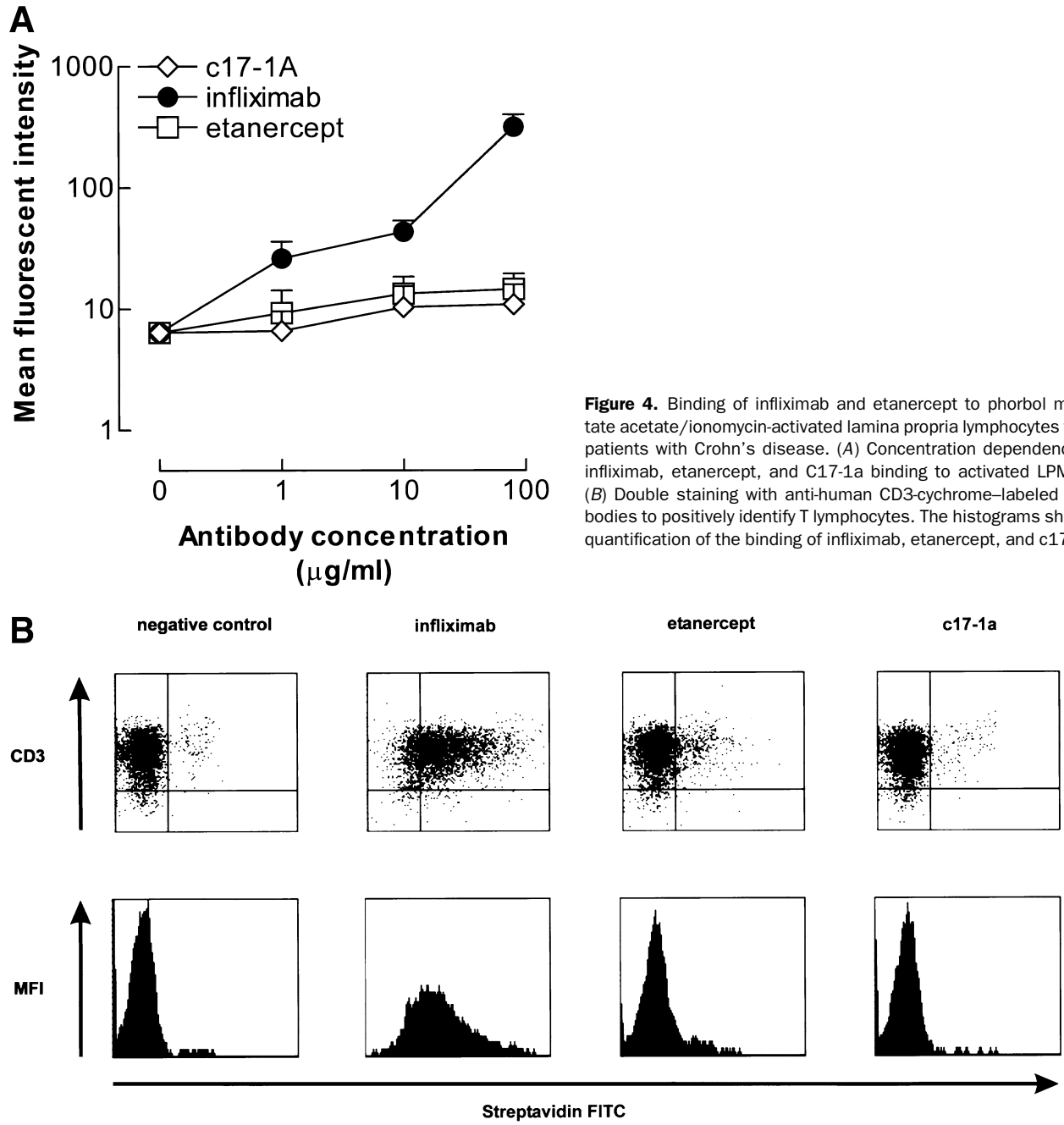

Figure 4. Binding of infliximab and etanercept to phorbol myristate acetate/ionomycin-activated lamina propria lymphocytes from patients with Crohn's disease. (*A*) Concentration dependency of infliximab, etanercept, and C17-1a binding to activated LPMNC. (*B*) Double staining with anti-human CD3-cychrome–labeled antibodies to positively identify T lymphocytes. The histograms show a quantification of the binding of infliximab, etanercept, and c17-1a.

infliximab on apoptosis (Figure 8*A*). Activation of cell-mediated immunity via the activation of natural killer cells involves the release of granzymes and especially granzyme B. Hence, enhanced levels of granzyme B are indicative of natural killer cell activation. Neither infliximab nor etanercept increased granzyme B levels in mixed lymphocyte reaction–activated PBL cultures; however, in the infliximab-treated cultures, apoptosis was concomitantly increased (Figure 8*B*). Hence, the effects of infliximab on apoptosis are not dependent on natural killer cell activation or complement stimulation in our experimental system.

Discussion

The cytokine TNF-α is believed to play a key role in the pathogenesis of acute and chronic inflammatory diseases. TNF-α–neutralizing antibodies have proved to be effective in various animal models; 2 therapeutic agents, infliximab (a chimeric monoclonal anti–TNF-α antibody) and etanercept (a recombinant human TNF receptor fusion protein), have been approved for clinical use. Both TNF-α–neutralizing agents have shown high efficacy in rheumatoid arthritis,[17,33] but only infliximab is efficacious in Crohn's disease.[13,18] These observations

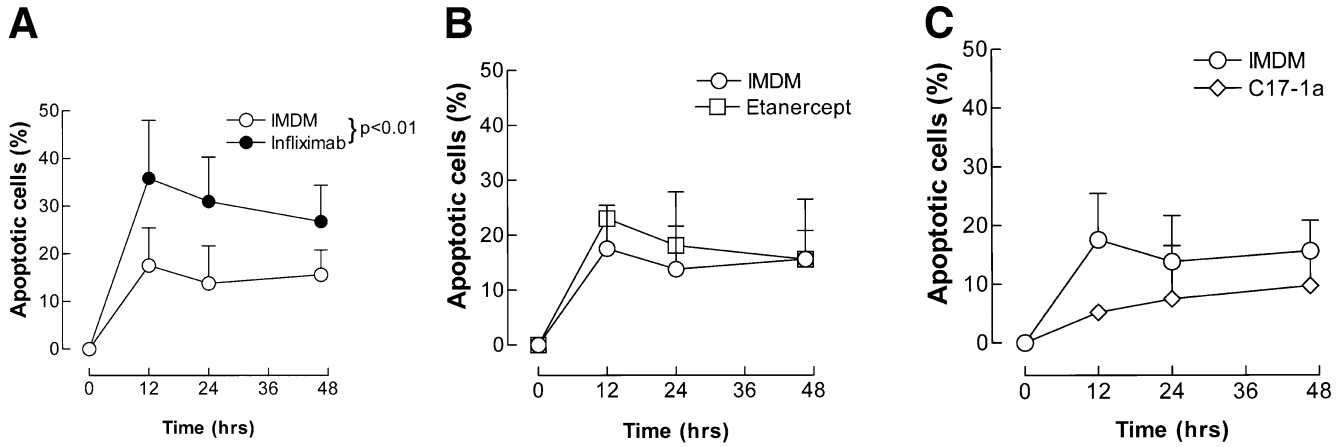

Figure 5. Infliximab, but not etanercept and the control antibody, induces apoptosis in activated PBL as assessed by annexin V/7AAD staining and FACS analysis (maximum at 12 hours, 35% for infliximab vs. 18% in control). Values are the mean of 6 separate experiments, all different donors. Statistical analysis was performed using the Wilcoxon matched pairs test.

suggest that the mechanism of action of these 2 TNF-α–binding proteins differ.

To investigate whether the differences between infliximab and etanercept may be attributed to different potencies with respect to soluble TNF-α–neutralizing capacity, we tested the effect of both proteins on soluble TNF-α–induced NF-κB activation or soluble TNF-α bioactivity in a WEHI assay. These assays showed no major quantitative differences in the potential of both drugs in inhibiting soluble TNF-α. However, we showed that infliximab but not etanercept bound to activated PBL and lamina propria mononuclear cells from patients with Crohn's disease. The nonspecific binding of the antibodies, as shown in Figure 3*A*, could be subtracted by using an irrelevant chimeric antibody c17-1a directed against an epithelial cell adhesion molecule.[34] Transmembrane TNF is expressed on activated normal human T cells in considerable amounts.[35,36] The differential binding of etanercept and infliximab to membrane-expressed TNF-α may be explained by differences in epitope recognition. It is possible that the p75 receptor structure of etanercept has less binding affinity for the extracellular part of transmembrane TNF, whereas the 2 active binding sites of infliximab recognize the extracellular portion of transmembrane TNF-α on activated lymphocytes. Alternatively, infliximab but not etanercept may recognize TNF receptor-bound soluble TNF-α. Soluble TNF-α added to unstimulated PBL increased the binding of infliximab to activated PBL, as shown in Figure 3. This suggests a contributing role for receptor-bound soluble TNF-α in infliximab binding to activated lymphocytes. However, the number of TNF receptors on normal human lymphocytes (approximately 270 per cell[37]) may be too low to account for the differences (8 times higher infliximab binding then etanercept) observed. Further experimental work is needed to establish the exact nature of infliximab binding to lymphocytes.

Infliximab provoked effects that were not observed with etanercept, suggesting that these effects do not depend on neutralization of soluble TNF-α only. We

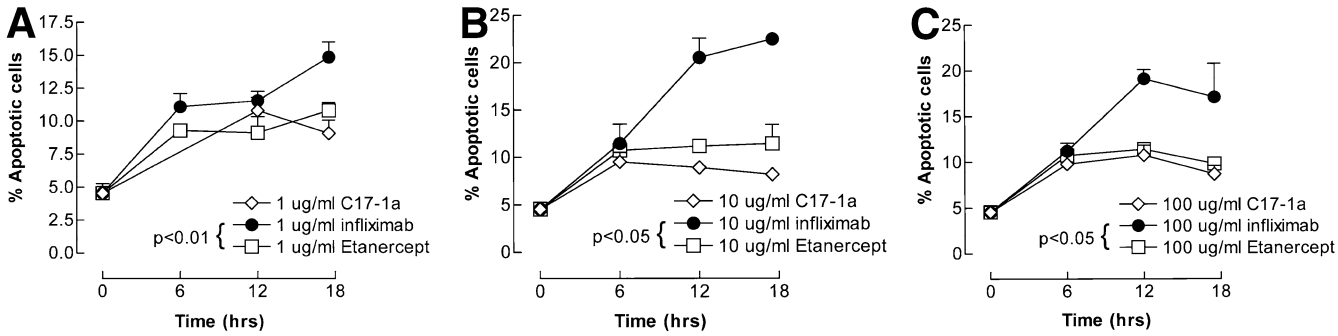

Figure 6. Infliximab, but not etanercept and the control antibody, induces apoptosis in activated lamina propria lymphocytes from patients with Crohn's disease. Apoptosis was assessed by annexin V/7AAD staining and FACS analysis, and the effects of different antibody concentrations were tested in a time curve. The results show that even high concentrations of etanercept do not produce lymphocyte apoptosis, whereas infliximab is highly active in this respect.

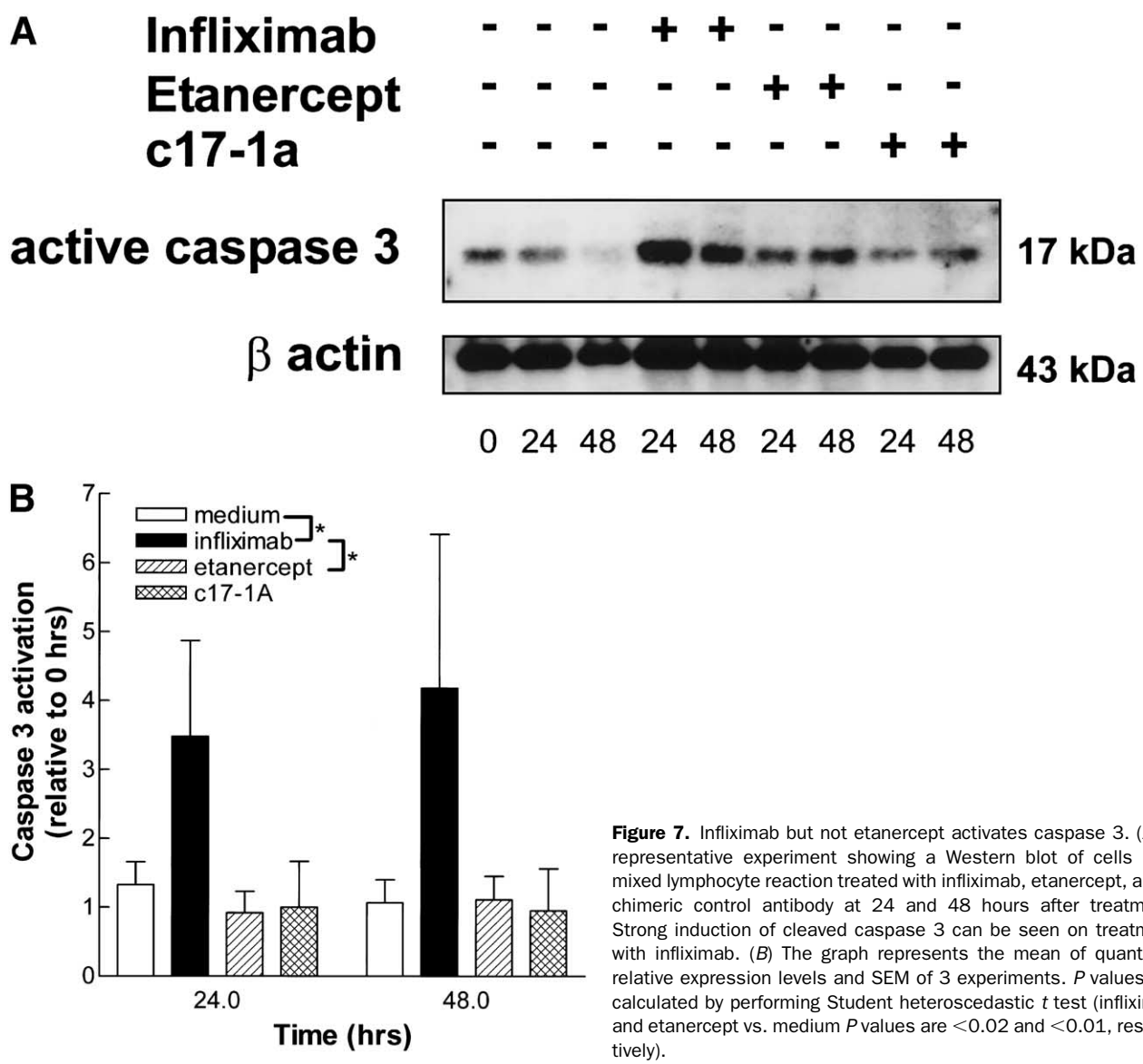

Figure 7. Infliximab but not etanercept activates caspase 3. (*A*) A representative experiment showing a Western blot of cells in a mixed lymphocyte reaction treated with infliximab, etanercept, and a chimeric control antibody at 24 and 48 hours after treatment. Strong induction of cleaved caspase 3 can be seen on treatment with infliximab. (*B*) The graph represents the mean of quantified relative expression levels and SEM of 3 experiments. *P* values are calculated by performing Student heteroscedastic *t* test (infliximab and etanercept vs. medium *P* values are <0.02 and <0.01, respectively).

observed that, whereas infliximab efficiently induced both caspase-3 activation and apoptosis, etanercept had no significant effect on either parameter. We have previously reported that a large proportion of lamina propria lymphocytes becomes apoptotic within 24 hours after administration of infliximab via an unknown mechanism.[22] We now report that infliximab but not etanercept directly induces apoptosis of a significant percentage of activated lamina propria lymphocytes obtained from patients with Crohn's disease. Infliximab-induced apoptosis may depend on its ability to bind to activated lymphocytes, but the molecular mechanism by which infliximab induces apoptosis and activates caspase 3 remains unclear. Although we performed our experiments in medium containing heat-inactivated fetal calf serum, complement activation in vivo by the antibody binding remains a theoretical possibility. However, in vitro experiments in which we compared the effects of infliximab in complement-containing human serum with the effects of infliximab in medium containing heat-inactivated fetal calf serum did not show significant marked differences between both conditions Thus, it is unlikely that infliximab-induced apoptosis is a consequence of complement activation in our mixed lymphocyte reaction model of lymphocyte activation. Nevertheless, in vivo complement activation as a consequence of infliximab binding to transmembrane TNF-α and subsequent immune cell cytolysis as described by Scallon et al.[38] may further contribute to the efficacy of infliximab.

Possible mechanisms underlying infliximab-induced apoptosis include so-called "outside-in" signaling in response to infliximab binding to transmembrane TNF-α.

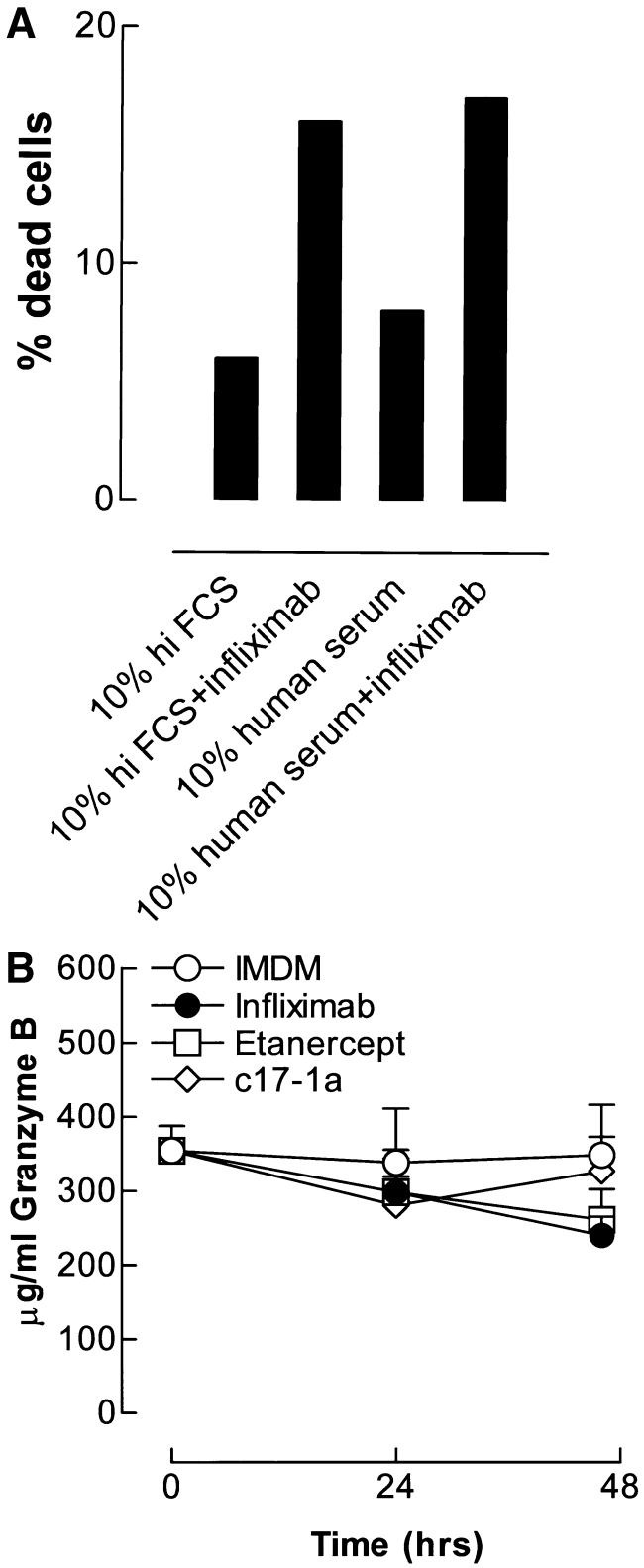

Figure 8. Contribution of complement and cellular cytotoxicity to infliximab-induced apoptosis. (*A*) Effects of infliximab on 24-hour cell survival of PBL in 10% fetal calf serum–containing medium and 10% human serum–containing medium. (*B*) Granzyme B levels in supernatants of the PBL mixed lymphocyte reaction cultures treated with various antibodies for various time periods (average and SEM of 6 cultures).

Infliximab is capable of binding 2 soluble TNF-α molecules (G. Heavner, personal communication), whereas etanercept only binds to an individual soluble TNF-α molecule.[39] Dimerization of transmembrane TNF-α by infliximab may initiate outside-in signal transduction.[40] A recent study convincingly showed that transmembrane TNF-α is capable of reverse signal transduction,[41] and a possibility is that the infliximab-induced apoptosis and caspase-3 activation are the consequence of infliximab-induced transmembrane TNF-α clustering and subsequent reverse signaling.

Another possible mechanism is that infliximab-induced apoptosis is a consequence of diminished biological activity of transmembrane TNF-α. It is now well recognized that interfering with the biological activity of single cytokines may have important effects. Blockade of interleukin-12 and interleukin-6 signaling reduced the severity of experimental colitis by inducing T-cell apoptosis, which is a receptor-mediated process.[42,43] It is conceivable that diminished TNF-receptor stimulation due the blockade of transmembrane TNF may result in diminished antiapoptotic signaling and increased apoptosis. Transmembrane TNF-α is superior to soluble TNF-α in activating the p75 TNF receptor (TNFR2).[44] TNF-R2 engagement leads to NF-κB activation and cell survival[45]; thus, transmembrane TNF-α may exert antiapoptotic effects not shared with soluble TNF-α. Further experimental work is needed to establish whether one of these possibilities is responsible for infliximab-induced apoptosis.

In summary, we observed that both infliximab and etanercept were highly potent in neutralizing soluble TNF-α but that only infliximab was capable of binding to activated lymphocytes from peripheral blood and the lamina propria of patients with Crohn's disease. Interestingly, infliximab potently induced caspase-3 activation as well as apoptosis, whereas etanercept did not. These data suggest that the differences between the effects of etanercept and infliximab may be related to a difference in their ability to bind to activated lymphocytes and induce immune cell apoptosis.

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