Towards therapeutic disease control in inflammatory bowel diseases

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Chapter four

Anti-TNFα antibodies induce regulatory macrophages in an Fc region dependent manner

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Abstract

Anti-TNFα antibodies are effective in Crohn's disease whereas soluble TNFα receptors have failed to show clinical efficacy. The molecular mechanism that underlies the differences between these compounds has not been elucidated. Here we aimed to examine the mechanism of action of the immunosuppressive effect of anti-TNFα antibodies on activated T cells.

We studied the effect of anti-TNFα antibodies infliximab and adalimumab, the soluble TNFα receptor etanercept, pegylated F(ab') fragment certolizumab and certolizumab-IgG on primary activated T cells. T cells were grown in isolation or in a mixed lymphocyte reaction (MLR). Proliferation was measured by ^{3}H thymidine incorporation and apoptosis was examined using Annexin V labeling and a colorimetric assay for activated caspase-3. Macrophage phenotype was assayed by flow cytometry and cytokine secretion.

Infliximab and adalimumab reduced proliferation in an MLR, whereas etanercept and certolizumab did not. This effect was completely abolished after blocking Fc receptors. Infliximab F(ab')2 fragment failed to inhibit proliferation whereas certolizumab-IgG gained the ability to inhibit proliferation. In the MLR anti-TNFs induced a new population of macrophages in an Fc region dependent manner. These macrophages were found to have an immunosuppressive phenotype, in terms of their capacity to inhibit proliferation of activated T cells, production of anti-inflammatory cytokines and the expression of the regulatory macrophage marker CD206.

Regulatory macrophages have immunosuppressive properties and play an important role in wound healing. Our data show that anti-TNFs induce regulatory macrophages in an Fc region dependent manner. This mechanism of action of anti-TNFs may contribute to the resolution of inflammation.

Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease that results from a dysregulated immune response of unknown aetiology. [1-3] Tumour necrosis factor alpha (TNFα), a cytokine produced by activated macrophages, monocytes and T cells, [4] is a key mediator in immune responses and is increased in serum and intestine in CD and synovium in rheumatoid arthritis (RA). [5-7]

Since their introduction in the '90s, anti-TNFα antibodies are commonly used for the treatment of Crohn's disease. Various classes of anti-TNFs have been introduced, and although all classes efficiently neutralize TNFα, they show different efficacy profiles. The antibodies infliximab [8, 9] and adalimumab [10, 11] were shown to be effective in both inducing and maintaining remission in CD patients. On the other hand, two soluble receptors; etanercept and onercept were ineffective in inducing remission in CD patients. [12, 13] Also, the humanized anti-TNFα antibody CDP571 which was designed as an IgG_4 to reduce interaction with Fc receptors in the hope to reduce side effects also failed to show effectiveness in CD. [14] These clinical data strongly suggest that neutralizing TNFα may not be the sole mechanism of action of anti-TNFα treatment in Crohn's disease.

Several effector mechanisms of anti-TNFα treatment have been proposed that are independent of TNFα neutralizing activity. Examples are the induction of apoptosis in T cells and monocytes via binding of membrane bound TNFα (mTNFα), [15-17] antibody-dependent-cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity. [18, 19] Furthermore, a role for Fc receptors has been proposed based on the association between a polymorphism in the Fc gamma receptor IIIa and the biological response to infliximab. [20] Fc receptors bind the Fc region of an antibody or antibody-complex, resulting in myeloid cell activation, phagocytosis and cytokine secretion. The importance of Fc receptors in the mechanism of action of other antibody therapies such as anti-CD20 and anti-Human Epidermal growth factor Receptor 2 (Her2) has been described before. [21, 22] In $Fc\gamma^{-/-}$ mice which are unable to bind Fc regions, both anti-CD20 and anti-Her2 lost their efficacy in reducing tumor size, demonstrating a key role for the Fc receptor in the mechanism of action of these antibodies. Although a number of mechanisms have been suggested, it is still unclear why certain anti-TNFs are effective in Crohn's disease and other anti-TNFs are not. Many studies on the mechanism of action of anti-TNFα focus on binding to mTNFα and reverse signaling in T cells, [18, 23] whereas the effect and importance of binding to Fc receptors has not been established thus far.

In this study, we found that in order for an anti-TNFα to inhibit T cell proliferation *in vitro*, the compound needs to bind to mTNFα on activated T cells and posses an Fc region to interact with the Fc receptor on antigen presenting cells. Upon this binding, a distinct macrophage subset is induced with immunosuppressive capacities, including the production of anti-inflammatory cytokines and inhibition of T cell proliferation.

Material and methods

Antibodies and Reagents

Infliximab, certolizumab, adalimumab and etanercept were prepared according to manufacturers' recommendations. Certolizumab-IgG was obtained from UCB (UCB, Belgium), and IgG_{1k} was obtained from Sigma.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated by Ficoll Paque density-gradient centrifugation. After washing, monocytes were isolated by Percoll density-gradient centrifugation. CD3 positive T cells were isolated from PBMCs using negative magnetic bead separation (Invitrogen). For T cell activation, cells were activated with αCD3/αCD28 antibodies (Sanquin) at the indicated concentration or αCD3/αCD28 beads (Invitrogen) (1 bead/5 cells). PBMCs and T cells were cultured in RPMI 1640 containing 10% heat-inactivated FCS.

Dendritic cells (DCs) were obtained by culturing monocytes with GM-CSF (50 ng/mL, R&D), IL-4 (50 ng/mL, R&D) for 7 days in AIM-V medium. To generate macrophages, monocytes were cultured in 6 wells plates for 5 days.

Infliximab induced macrophages ($M\phi_{ind)}$ were isolated from MLR cultures using CD14 microbeads according to manufacturer's protocol (Miltenyi). Next, cells were cultured in RPMI 1640, containing 10% heat-inactivated FCS. For light microscopy, cells were adhered to poly-L-lysine coated coverslips and stained using DiffQuick.

Binding to membrane TNFα

For mTNFα binding assays, infliximab, certolizumab, adalimumab, etanercept and control IgG were labeled with a fluorescent dye using a commercially available kit according to the manufacturers' instructions (Alexa Fluor 647 protein labeling kit, Pierce). CD3 positive T cells were activated with αCD3/CD28 antibodies for 48 hours. Cells were collected, washed three times in FACS buffer (PBS containing 1% BSA) and incubated with various amounts of labeled anti-TNFα compound or IgG control for 30 minutes on ice. Binding of infliximab F(ab')2 fragment was assessed in a competition assay; activated T cells were incubated with labeled infliximab (10 μg/mL) and increasing concentrations of unlabeled infliximab F(ab')2 fragment at the same time. After washing, binding to mTNFα was analyzed by flow cytometry.

Allogeneic mixed lymphocyte reaction (MLR)

PBMCs from two healthy donors were cultured in a 1:1 ratio in RPMI 1640 culture medium. After 48 hours of activation, cells were treated with the indicated compound (infliximab, adalimumab, certolizumab, etanercept, Certolizumab-IgG or IgG control, all at 10 μg/mL) for up to 7 days where indicated. When appropriate, Fc receptors were saturated by treating MLRs with IgG (10 μg/mL, Sigma) for 6 – 16 hours and next treated with anti-TNF compound or IgG control for 2 days. Finally, proliferation was measured using a ^{3}H-thymidine incorporation assay.

Assays for apoptosis

PBMCs or isolated T cells were activated in an MLR or with CD3/CD28 antibodies for 48 hr, and treated with anti-TNF compound or control (10 μg/mL). Cells were collected, washed three times, and stained with Annexin V and Pi and analyzed by flowcytometry.
For measurement of caspase-3 enzymatic activity, a colorimetric assay was used as described before. [24] Cell lysates were generated from MLR cultures and protein concentration was determined by BCA analysis (Pierce). Lysates were incubated with a saturating concentration of 25μM specific enzyme substrate Ac-Aps-Glu-Val-Asp-AMC (Ac-DEVD-AMC, Bachem, Germany) in 100mM HEPES buffer with 10% sucrose, 10mM dithiothreitol and 0.1% Nonidet-P40. Samples were incubated at 37°C and fluorescent AMC release was monitored (Fluostar Optima plate reader).

FACS analysis

Human monocytes and DCs were plated in 6 well plates (2 x 10^6 cells/well) and cultured with or without LPS (Sigma, 100 ng/mL) for 16 hours, and treated with anti-TNFα compound or IgG control (Sigma, 10 μg/mL) for 25 – 48 hours. Cells were harvested, washed, and stained for αCD14-FITC, αCD40-FITC, αCD80-Pe, αCD83-APC, αCD86-APC, αHLA-DR-FITC and appropriate controls (all BD) for 30 minutes on ice. For analysis of marker expression on monocytes, macrophages and $M\phi_{ind}$, cells were cultured in 6 wells plates and stained for αCD14-FITC, αCD16-FITC, αCD32-APC, αCD40-FITC, αCD80-Pe, αCD83-APC, αCD86-APC, αCD206-APC, αCD209-Pe or αHLA-DR-FITC (all BD). Finally, expression was analyzed by flow cytometry using a FACS Calibur (BD) and FlowJo software (Treestar Inc, Ashland, OR). Expression was calculated as MFI specific staining – MFI control.

Generation of Infliximab F(ab')2 fragment

Infliximab F(ab')2 fragments were generated using a Fab Preparation Kit according to the manufacturers protocol (Pierce). Purity of the resulting fraction was analyzed by SDS-PAGE followed by Coomassie Blue staining and showed no remaining intact antibodies.

Cytokine detection by cytokine beads array (CBA)

DCs, Mϕ1 or $M\phi_{ind}$ were treated or untreated with LPS for 24 hours, supernatants were collected and stored at -20 until use. A CBA was performed according to manufacturer's protocol. Data were analyzed with FlowJo (Treestar).

Statistical analysis

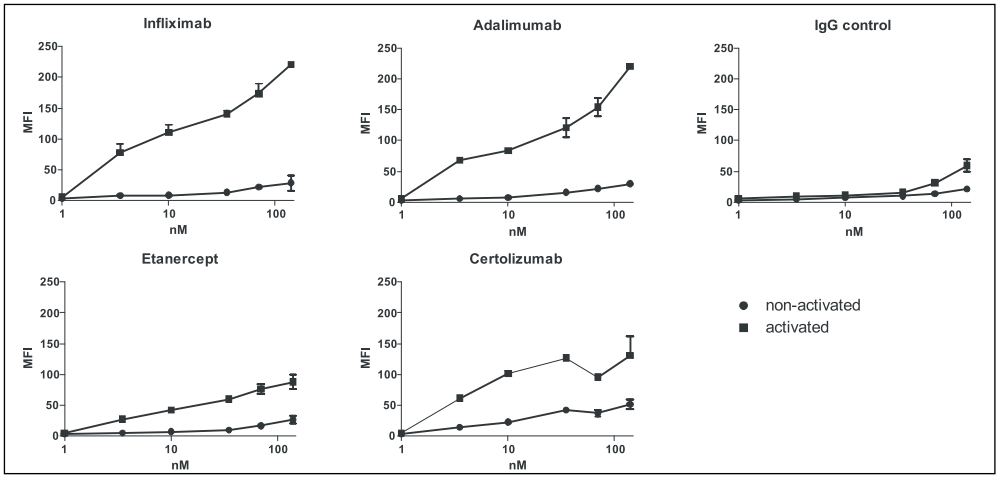
Results are representative for at least three independent experiments and show means ± SEM unless otherwise indicated. For statistical analysis, one way ANOVA was used followed by Bonferroni post test. Results were considered significant when $p < 0.05$.

Results

Anti-TNFα compounds bind TNFα on activated T cells to varying degrees

To assess binding of compounds to mTNFα, anti-TNFα compounds and IgG control were labeled with a fluorescent dye, and binding of fluorescent labeled compounds to activated T-cells was compared to binding to non-activated cells. Binding of infliximab and adalimumab to mTNFα was highly efficient, binding of certolizumab was intermediate and binding of etanercept was low compared to the IgG control (Figure 1).

Figure 1 Anti-TNF compounds bind to mTNF in varying degrees.



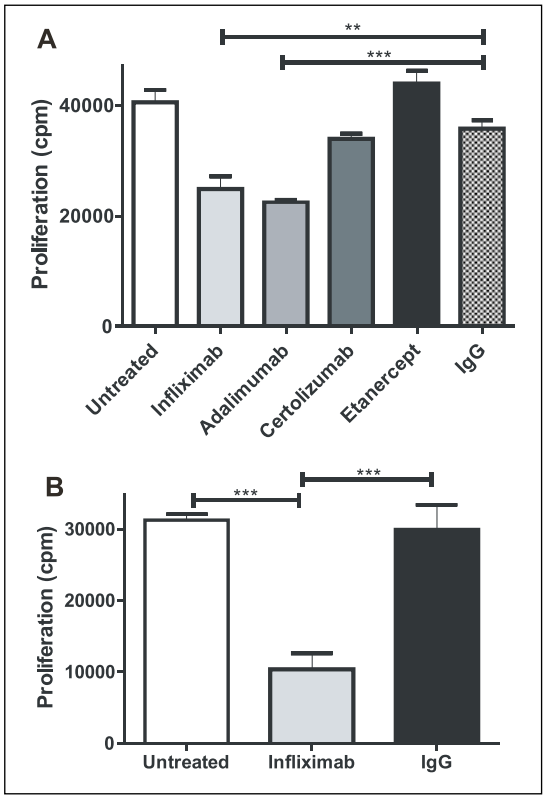
Naïve or CD3/CD28 activated T cells were labeled with a fluorescent dye (Alexa Fluor). Next, cells were incubated with different labeled anti-TNF compounds or IgG control for 30 minutes and binding to activated or non-activated T cells was analyzed by flow cytometry. Data are shown as means ± SD from 3 independent experiments

Anti-TNFα compounds with an Fc region suppress T cell activation but only in an MLR

We examined the effect of various anti-TNFα compounds on the proliferation of activated T cells and found that all anti-TNFα agents slightly inhibited T cell proliferation although this did not reach statistical significance (Supplementary Figure 1*A*). This minor effect was similar for all anti-TNFα compounds and irrespective of their capacity to bind mTNFα, suggesting it may be due to the neutralizing effect on soluble TNFα.

As it has previously been described that anti-TNFs can induce apoptosis in T cells, we examined effects on apoptosis of T cells cultured in isolation or in an MLR. No apoptosis was induced in CD4+ cells treated with anti-TNFs as detected by Annexin V/Pi staining (Supplementary Figure 1*B*). Also, no apoptosis was observed at increasing concentrations, or when we used other cell types or different detection methods (Jurkat cells, lymphocytes, monocytes, Annexin V staining, Caspase 3 activity assay; data not shown). As a result, we hypothesized that anti-TNFs do not have a direct effect on T cells grown in isolation and we established a mixed lymphocyte reaction as a model to further elucidate their anti-inflammatory properties. Again, in this model, no apoptosis was observed (Supplementary Figure

Figure 2 Anti-TNFs differ in their capacity to inhibit proliferation in an MLR.



(A) MLR cultures were treated with anti-TNF compound or IgG control (all 10 μg/mL) for 36 - 48 hours. Proliferation was measured by thymidine incorporation. Only infliximab and adalimumab significantly inhibited proliferation (** $P < 0.01$ and *** $P < 0.001$). (B) CD14+ cells and CD4+ cells from two healthy donors were cocultured for 48 hours. Next, cultures were treated with infliximab or IgG control (10 μg/mL) for 72 hours. Proliferation was measured by thymidine incorporation. Data show means ± SD from 2 independent experiments

1*C*). In addition, no differences were observed in caspase 3 activity whether we used heat inactivated serum or serum without heat inactivation (Supplementary Figure 1*D*). However, a reproducible effect on T cell proliferation was observed with some of the anti-TNFα compounds in the MLR. We found that infliximab and adalimumab inhibit proliferation in this model, whereas etanercept and certolizumab do not (Figure 2*A*). The suppressive effect was much stronger than the effect observed in T cells alone (up to 50% inhibition in an MLR vs 15% inhibition in T cells alone (Supplementary fig 1*A*). These findings suggest that the various anti-TNFα compounds have distinct properties that may result in different pharmacological behavior. To further elucidate the cell types involved in this observed effect, we established an MLR containing purified CD14+ cells and CD4+ T cells. In this assay, infliximab strongly inhibited proliferation compared to the untreated or IgG treated condition (Figure 2*B*). These data show that CD4+ T cells and CD14+ cells are sufficient to induce inhibition of proliferation.

The main difference between the two assays we used is that an MLR contains not only T cells, but also antigen presenting cells (APCs). As these cells may also express low levels of mTNFα, binding of the anti-TNF compounds may affect the activation of the APC and thus decrease its T cell stimulatory capacity. To test this hypothesis, we incubated monocytes and dendritic cells with anti-TNFα compounds in the presence or absence of LPS and analyzed the expression of costimulatory molecules. No effect was found for any of the anti-TNFα compounds tested, either on the expression of costimulatory molecules or on the expression of HLA-DR (Supplementary Figure 2), indicating that infliximab does not affect the activation of APCs.

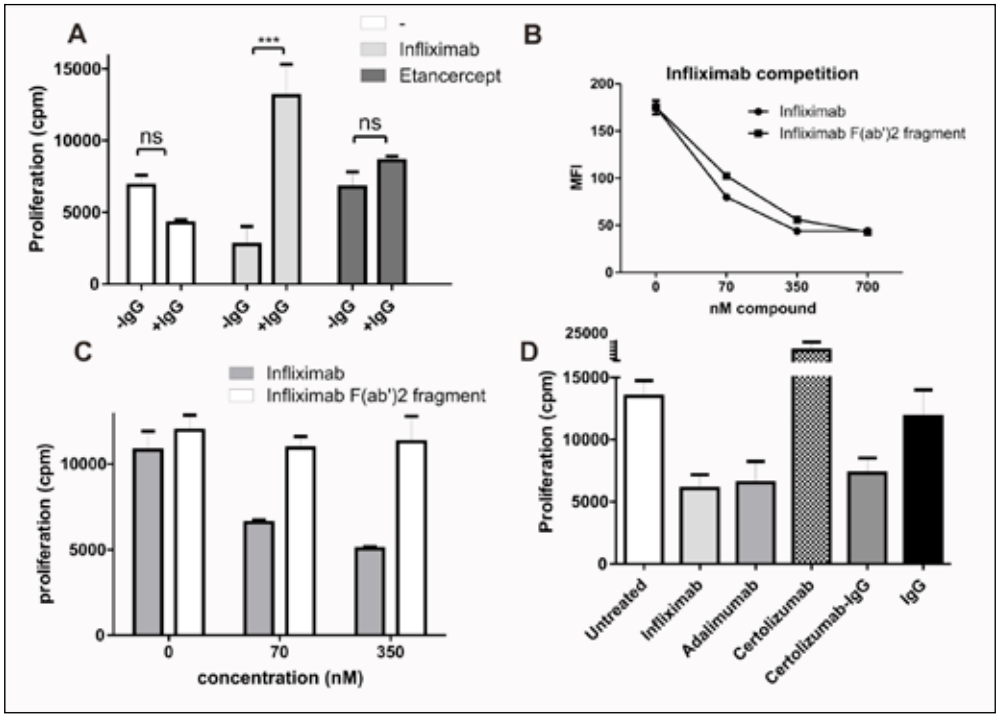
Inhibition of proliferation is abolished when binding to Fc receptors is inhibited

We found that only infliximab and adalimumab (Figure 2) were effective in the MLR, and both of these agents are characterized by efficient binding to mTNFα on the T cells and the presence of an Fc region. In contrast, certolizumab does not contain an Fc region whereas etanercept does, but does not bind efficiently to membrane bound TNFα. We hypothesized that the combination of efficient binding to T cells and the ability to bind and activate an Fc receptor plays an important role in the immunosuppressive function of these compounds in our system. To examine the contribution of the Fc receptor, the ability to inhibit MLR responses was tested after blocking Fc receptors with IgG. Indeed, we found that the inhibition of proliferation by infliximab was completely abolished after saturation of Fc receptors (Figure 3*A*).

To further examine the role of the Fc receptor, infliximab F(ab')2 fragments were generated from infliximab. This compound is identical to the structure of infliximab, except for the fact that it does not contain an Fc region. Successful digestion was confirmed by Coomassie Blue staining (data not shown). To confirm that infliximab F(ab')2 fragment remained capable of binding to mTNF, a competition assay was performed. In this assay, infliximab was labeled with a fluorescent dye, and activated T cells were incubated with a constant concentration of labeled infliximab and increasing concentrations of either infliximab F(ab')2 fragment or infliximab at the same time. Binding of fluorescent infliximab to mTNFα decreases in the presence of a competitor, in this assay infliximab itself or infliximab F(ab')2 fragment. The experiment showed that infliximab F(ab')2 fragment competes with fluorescent infliximab to the same extent as infliximab itself (Figure 3*B* and Supplementary Figure 3), demonstrating that infliximab F(ab')2 fragment binds to mTNF to the same degree as infliximab. In contrast, infliximab F(ab')2 fragment did not inhibit proliferation in an MLR, (Figure 3*C*). This effect was also absent at higher concentrations. These data further support a crucial role for the Fc region in the effects of infliximab and adalimumab on T cells in the MLR.

The previous results indicated that binding to the Fc receptor is necessary for the immunosuppressive effect of anti-TNFα; however, Fc receptor binding alone is not sufficient since IgG and etanercept do not inhibit proliferation although these compounds do contain an Fc region. Therefore, we hypothesized that both binding the Fc receptor and binding mTNFα is required to inhibit T cell proliferation. As shown in figure 1, certolizumab binds to mTNF, albeit at an intermediate level. However, certolizumab does not contain an Fc region and does not inhibit proliferation in an MLR (Figure 2). Strikingly, certolizumab-IgG, a compound containing the mTNFα binding region of certolizumab as well as an Fc region does inhibit proliferation to the same extent as infliximab and adalimumab (Figure 3*D*), fur-

Figure 3 Infliximab induced T cell proliferation is completely abolished when binding to Fc receptors is inhibited.



(A) Fc receptors were saturated by treating MLRs with IgG (10 μg/mL) for 6 – 16 hours following activation. Next, cells were treated with the indicated compound. Proliferation was measured using a 3H-thymidine incorporation assay. *** $p \leq 0.001$ (B) T cells were activated for 48 hours with CD3/CD28 beads. Infliximab was labeled with a fluorescent dye (Alexa Fluor 647). Cells were incubated with infliximab Alexa Fluor (70 nM) and different concentrations of infliximab and infliximab F(ab')2 fragment. Fluorescence was measured with Flow Cytometry. (C, D) Cells in an MLR were treated with the indicated compound for 48 hours. Next, proliferation was measured by thymidine incorporation

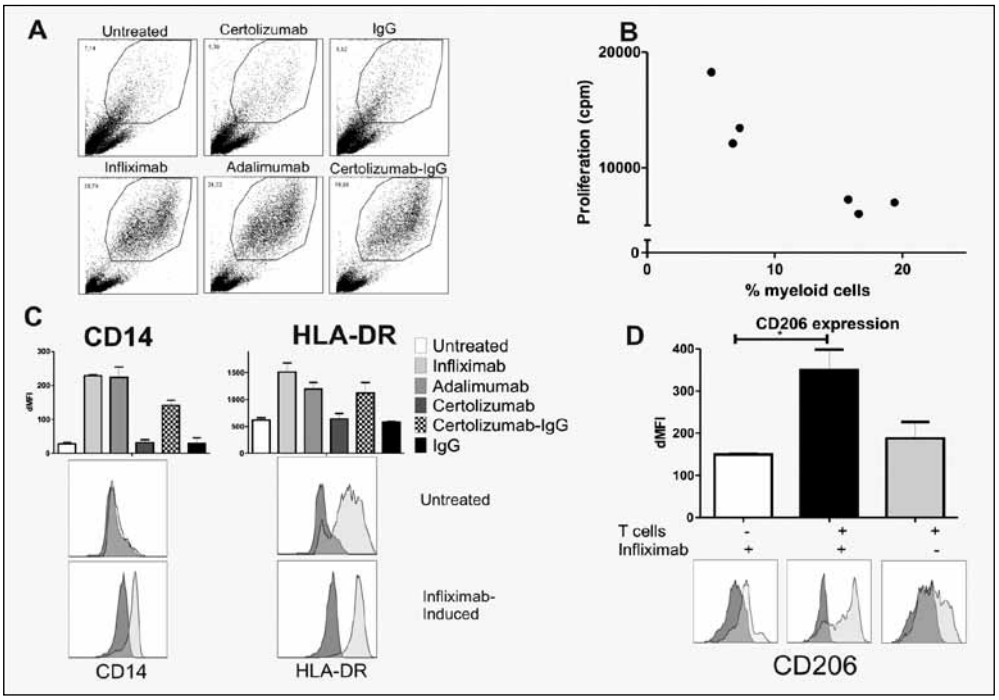
ther confirming that both binding Fc receptor and binding to mTNF is responsible for the immunosuppressive effects of anti-TNF.

Only infliximab, adalimumab and certolizumab-IgG induce a distinct CD14+ and HLA-DR+ cell population in an MLR which correlates with inhibition of T cell activation

To further investigate the mechanisms involved in the inhibition of T cell activation in an MLR, we evaluated the cells by flowcytometry after one week of treatment. We noticed a distinct cell population characterized by high forward scatter and intermediate side scatter upon treatment with infliximab, adalimumab or certolizumab-IgG, which was absent when cells were untreated or treated with certolizumab or IgG (Figure 4*A*). Importantly, the presence of these cells correlates with the degree of inhibition of proliferation induced by the

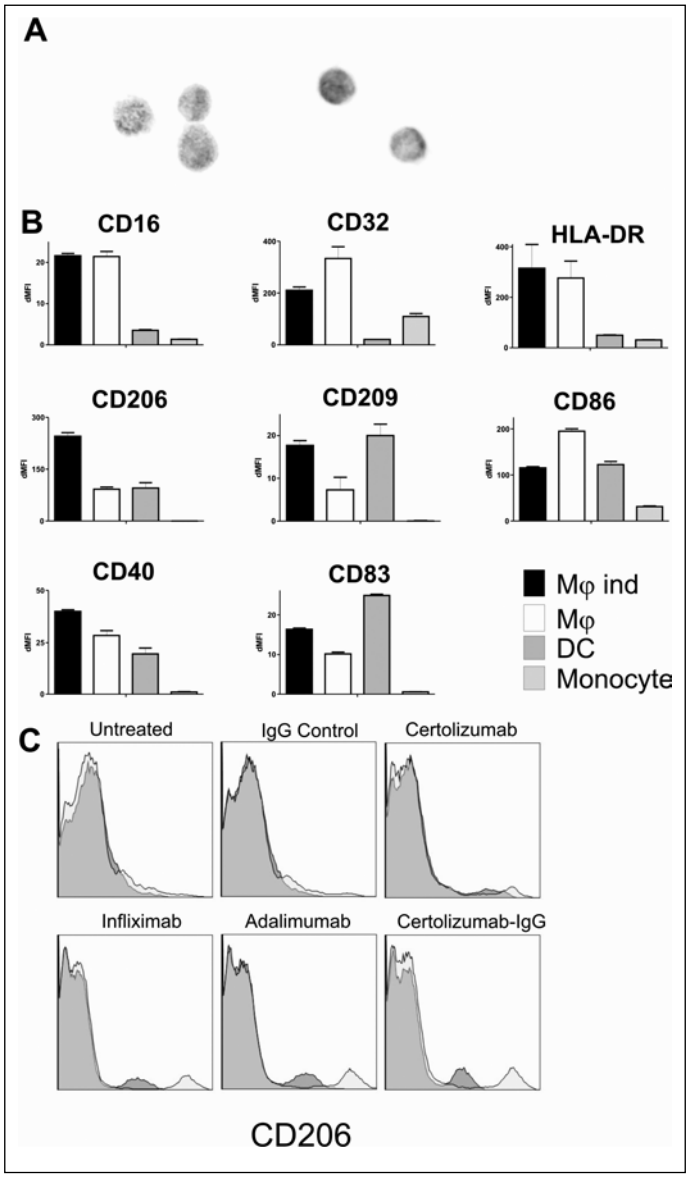
compounds in the MLR, indicating their involvement in the inhibition of T cell activation (Figure 4*B*). Based on the cell size and the position of the cells in the FSC/SSC plot, we hypothesized that these cells might be macrophages. This idea was further supported by the fact that the population expressed the macrophage marker CD14 as well as HLA-DR (Figure 4*C*). This finding also facilitated the isolation of the cell population based on the expression of CD14. Since this induced cell population (further referred to as $M\phi_{ind}$) was absent when cells were treated with certolizumab or IgG, and present upon treatment with infliximab, adalimumab and certolizumab-IgG, this effect seems to be mediated by the Fc region. The characteristics of the different anti-TNFα agents are summarized in Table 1.

Figure 4 Only infliximab, adalimumab and certolizumab-IgG induce a distinct CD14+ and HLA-DR+ population in an MLR.



(A) Cells in an MLR were treated with anti-TNF compound or IgG control (all 10 μg/mL) for 7 days. Cells were analyzed on the FSC/SSC. (B) Correlation between the presence of myeloid cells analyzed on the FSC/SSC and proliferation. (C) Cells in an MLR were treated with anti-TNF compound or IgG control (all 10 μg/mL) for 7 days, stained for HLA-DR and CD14 and analyzed on the FACS. Cells were gated as in 4A. Graphs show MFI ± SD and representative histograms of CD14 and HLA-DR expression of an untreated or infliximab-treated MLR are shown. Grey = isotype, white = expression. (D) T cells are required for the differentiation of $M\phi_{ind}$. CD14+ cells were treated with infliximab in the presence or absence of CD4+ T cells, and CD206 expression was analyzed by flowcytometry. CD206 expression was upregulated only when CD14+ cells were cocultured with CD4+ cells. A representative graph is shown from 2 independent experiments. Upper panel: MFI ± SD. Lower panel: histogram plots. Black = isotype control, grey = CD206 expression

Figure 5 Characterization of Mϕind.



(A) $M\phi_{ind}$ have a macrophage like appearance (40x magnification) Macrophages ($M\phi_{ind}$: left panel, Mϕ1: right panel) were stained using DiffQuick (B) Mϕ1, monocytes, DCs or $M\phi_{ind}$ were stained for different markers and expression was measured on the FACS. (C) Cells in an MLR were treated with anti-TNF compound or IgG control (all 10 μg/mL) for 7 days and stained for CD206 or isotype control

Finally, we examined whether the presence of T cells was required for the differentiation of $M\phi_{ind.}$ To address this question, we isolated CD14+ cells and cultured these cells in the presence or absence of CD4+ T cells, treated the cells with infliximab and analyzed CD206 expression by flowcytometry. We found an upregulation of CD206 when CD14+ cells were cocultured with CD4+ cells. This upregulation was absent when CD14+ cells were cultured without T cells (Figure 4*D*). These data show that T cells are required for the infliximab-induced differentiation of $M\phi_{ind.}$

$M\phi_{ind}$ express costimulatory molecules, Fc receptors and the regulatory macrophage marker CD206

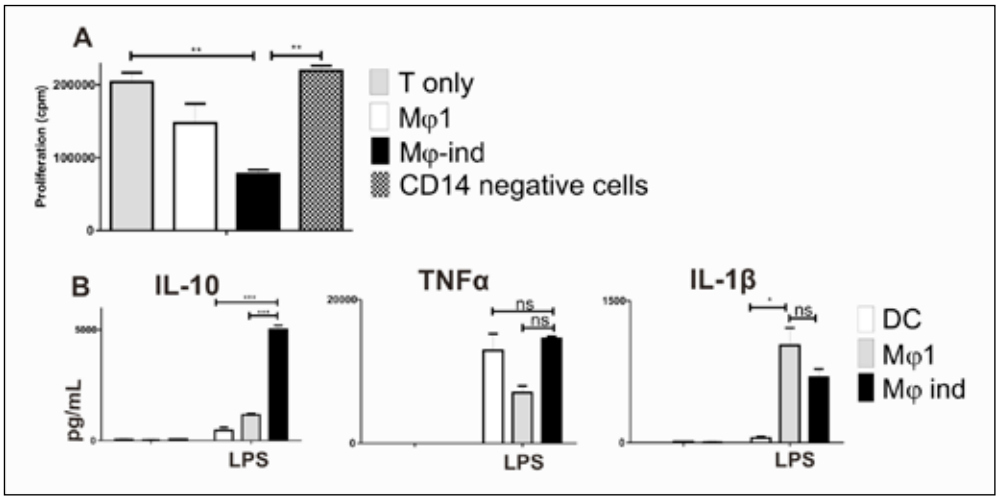
Since the cell population induced by some anti-TNFs expresses CD14 and HLA-DR and looks like macrophages (Figure 5*A*), we sought to further characterize the phenotype of these cells. Therefore we isolated $M\phi_{ind}$ based on CD14 expression and compared the expression level of different markers with the levels on monocytes, DCs and macrophages. Dendritic cells were cultured in the presence of IL-4 and GM-CSF, macrophages in the presence of IFN-γ (further referred to as Mϕ1) and monocytes were freshly isolated from blood. $M\phi_{ind}$ express CD40, CD80, CD83 and CD86 comparable to the expression of these markers on Mϕ1, as well as CD16 (FcγRIII, activating Fc receptor) and CD32 (FcγRIIb, inhibitory Fc receptor) (Figure 5*B*). Furthermore, $M\phi_{ind}$ express CD206 (mannose receptor), a commonly used marker for regulatory macrophages (alternatively activated macrophages, type 2 macrophages or Mϕ2), which was absent on Mϕ1. We confirmed the presence of this marker in the primary MLR and found CD206 expression only in the infliximab, adalimumab or certolizumab-IgG treated conditions (Figure 5*C*). Therefore, we hypothesized that $M\phi_{ind}$ are macrophages with regulatory properties.

$M\phi_{ind}$ have anti-inflammatory properties

Because $M\phi_{ind}$ are only present in the conditions where inhibition of T cell proliferation was observed, we hypothesized that $M\phi_{ind}$ inhibit T cell responses. To test this hypothesis, we isolated the $M\phi_{ind}$ from an MLR and co-cultured these cells with pre-activated isolated T cells from a third donor (secondary MLR). Indeed, we found a strong immunosuppressive effect that was induced by $M\phi_{ind}$ and not by Mϕ1. In addition, this effect was absent when activated T cells were co-cultured with the CD14 negative fraction of the MLR, indicating that $M\phi_{ind}$ mediate the immunosuppressive effect (Figure 6*A*).

To further characterize $M\phi_{ind}$, we evaluated the cytokine profile of these cells in the presence and absence of LPS, and compared this to the cytokine production by DCs and Mϕ1. In accordance with their regulatory properties, $M\phi_{ind}$ produce large amounts of IL-10 in response to LPS (figure 6*B*). Also, $M\phi_{ind}$ secrete less IL-1β in response to LPS compared to Mϕ1. The secretion of TNFα was comparable to that produced by DCs. Taken together, these data indicate that $M\phi_{ind}$ induced by infliximab, adalimumab or certolizumab-IgG have anti-inflammatory properties.

Figure 6 Mϕind have anti-inflammatory properties.



(A) Isolated CD3/CD28 activated T cells from a third donor were co-cultured with $M\phi_{ind}$, Mϕ1, or the CD14 negative fraction of the MLR. Proliferation was measured by thymidine incorporation. (B) $M\phi_{ind}$, Mϕ1 and DCs were cultured in the presence or absence of LPS. Supernatants were collected after 24 hours and cytokine production was measured by CBA. Data are shown as means ± SD from 3 independent experiments. * $p \leq 0.05$, *** $p \leq 0.001$, ns = not significant

Discussion

In the present study, we find that drugs used to target TNFα have different functional properties. The anti-TNFα antibodies bind to mTNFα on activated T cells and inhibit their proliferation but only in the presence of antigen presenting cells. In contrast, a soluble TNFα receptor or F(ab')2 fragments of the anti-TNFα antibodies do not affect T cell proliferation under the same circumstances. Our experiments show that the immunosuppressive properties of the anti-TNFα antibodies are dependent on their Fc region. We find that the antibodies differentiate blood derived monocytes to a regulatory macrophage phenotype ($M\phi_{ind}$). $M\phi_{ind}$ have anti-inflammatory properties since they inhibit proliferation of activated T cells, produce IL-10 and express the regulatory macrophage marker CD206.

It was previously described that infliximab may induce low levels of apoptosis in T cells as determined by TUNEL staining of cytospins of infliximab treated activated Jurkat cells, a lymphoblastoid T cell line. [15] Also, others found that anti-TNFα antibodies induce apoptosis in Jurkat cells that stably overexpress a non-cleavable form of mTNFα using Annexin V-PI staining as a read out. [18, 23] Additionally, apoptosis was described in primary lymphocytes isolated from the lamina propria of CD patients. [16] These data are in contrast to our current results, where none of the anti-TNFα compounds affected either apoptosis or proliferation of Jurkat cells or primary T cells grown in isolation despite extensive attempts. The difference between the previous studies and ours most likely lies in the use of endogenous levels of mTNFα expression in contrast to overexpression and the use of highly viable peripheral

blood lymphocytes rather than lamina propria lymphocytes which had undergone extensive isolation procedures. In line with this is the study by Bedini *et al,* [25] who described that anti-TNFα antibodies suppress activation of T cells in the presence of either immature dendritic cells or monocytes. Indeed, we find similar properties of the anti-TNFα antibodies but not of the other anti- TNFα compounds in the presence of monocytes in an MLR. The results we obtained in this assay are robust and reproducible.

In contrast to some earlier studies, we did not detect binding of etanercept to mTNFα. Importantly, binding of etanercept to mTNFα has only been described in experiments in which a non-cleavable mTNFα was stably overexpressed. [18, 26] In untransfected activated primary lymphocytes it was found that etanercept has weak mTNFα binding activity [16] similar to the results of our current experiments. Thus strong binding of etanercept to mTNFα may only occur in situations in which a non cleavable mTNFα is artificially overexpressed.

An important role for Fc receptor binding has been shown for the mechanism of action of other antibody therapies. Fc receptor binding of rituximab (a chimaeric monoclonal anti-CD20 antibody) was found to be indispensable for the clearance of B cells. [27] In addition, the FcγRIIIA-158V allotype has a higher affinity for binding to IgG_1 than the FcγRIIIA-158F allotype and an association between the *FCGR3A* genotype and clinical response to rituximab was observed in several studies. [27, 28] The *FCGRIIIA* genotype has also been associated with outcome in other antibody therapies, such as cetuximab (chimaeric monoclonal anti-EGFR antibody). [29] In line with this, antibodies with higher Fc receptor affinity have been developed to augment antibody efficacy in animal models. [22, 30] The same polymorphism has been associated with response to infliximab, however with conflicting results. [20, 31] In addition, CDP571, an IgG_4 antibody against TNFα, was found to be ineffective for steroid-sparing in CD. [14] Interestingly, IgG_4 has very low affinity for binding Fc receptors [32] and our data suggest that this could play a causal role in the disappointing performance of CDP571 in the treatment of Crohn's disease.

The effects observed with various anti-TNFs in the MLR may have implications for our understanding of the clinical activity of the compounds in patients with Crohn's disease. It is tempting to speculate that Fc receptor dependent differentiation of alternatively activated macrophages plays a role in mucosal healing in patients with Crohn's disease, since alternatively activated macrophages play a major role in wound healing. [33] Recently, it has been shown that regulatory macrophages cultured *in vitro* reduce colonic inflammation in mice and that patients with active CD have reduced numbers of these cells compared to patients with inactive CD. [34] Also, lamina propria macrophages produce large amounts of IL-10 and have anti-inflammatory properties, indicating that these cells are involved in gut homeostasis. [35] Thus far, our data has been limited to *in vitro* experiments, and it would be highly informative to verify the generation of regulatory macrophages *in vivo* in patients receiving anti-TNF therapy. Furthermore, although we have shown *in vitro* that an Fc region is required for inhibition of proliferation and the induction of regulatory macrophages, the *in vivo* clinical benefit of certolizumab has been shown in several studies. [36, 37] Certolizumab was found to be more effective than placebo to induce and maintain remission in CD patients and anti-TNFs are likely to exert their effects through multiple mechanisms.

The anti-TNFα antibodies and soluble receptors not only differ in their clinical efficacy in Crohn's disease but it has also been reported that the risk of tuberculosis with infliximab or adalimumab was 13 to 17 times higher than with etanercept. [38] Interestingly, mycobacteria such as *M. Tuberculosis* bind stronger to Mϕ2 than to Mϕ1, and have a prolonged survival in Mϕ2. [39] In this light it is interesting to note that Mϕ2 are strongly induced by infliximab and adalimumab in our experiments but significantly less by etanercept (data not shown). In addition, the mannose receptor plays an important role in the phagocytosis of mycobacteria [40] and we found that the mannose receptor CD206 is upregulated on $M\phi_{ind}$. Therefore it is tempting to speculate that the increased risk of tuberculosis in patients treated with infliximab or adalimumab is at least partly mediated by the induction of $M\phi_{ind}$.
In conclusion, our data show that the anti-TNFα antibodies have immunosuppressive properties that are distinct from those of the soluble TNFα receptor etanercept and F(ab')2 fragments of anti-TNFα. These distinct properties may have implications for both the effects and side effects of anti-TNFα compounds in patients *in vivo*. This finding sheds more light on the complex mechanism of action of infliximab and anti-TNFα therapies in general and gives new directions for the future development of new antibody based therapy for CD.

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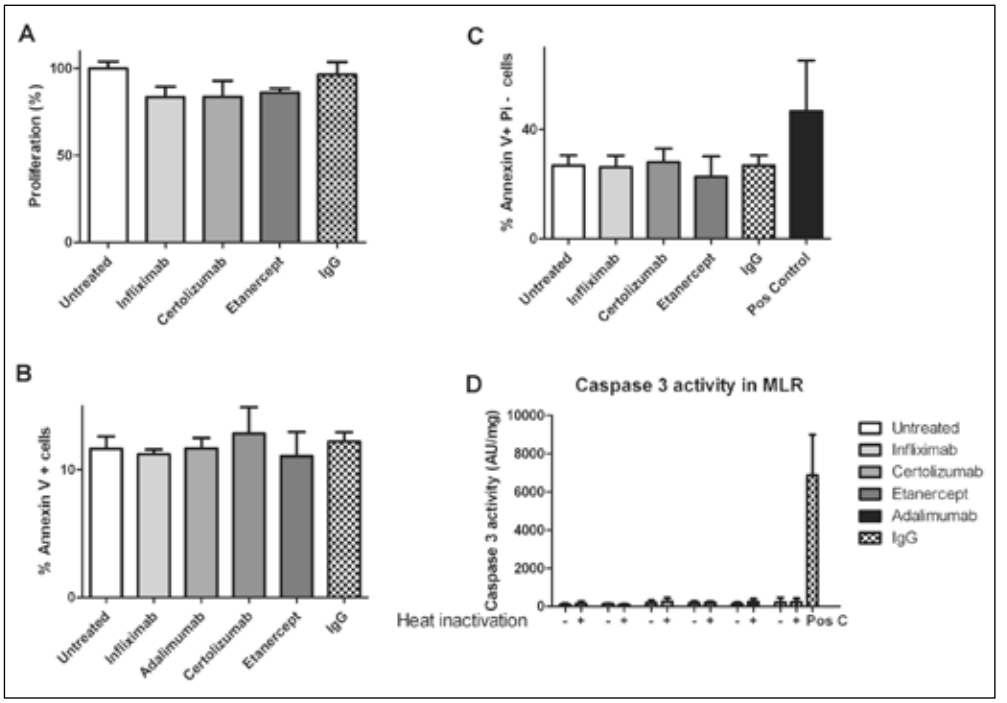
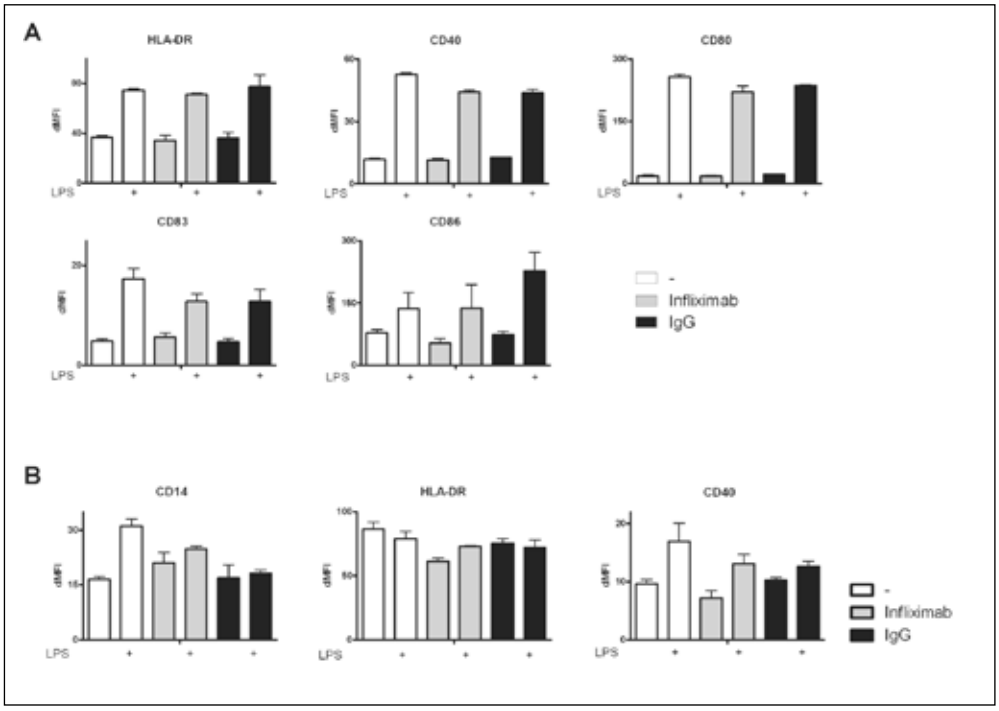
Supplementary Figures

Supplementary Figure 1 Anti-TNFs do not induce apoptosis in activated T cells or an MLR, and do not inhibit proliferation in isolated T cells.



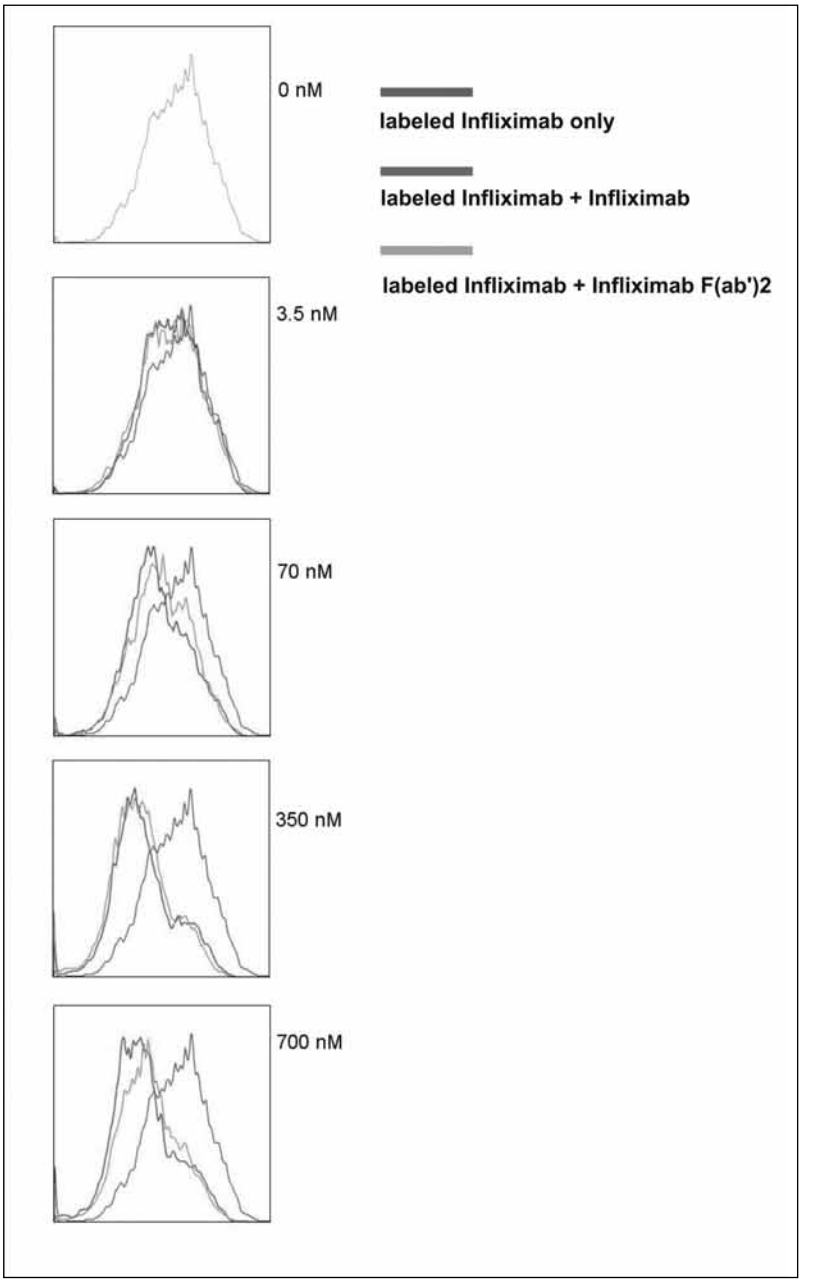
(A) T cells were isolated from PBMCs using negative magnetic bead separation (Invitrogen) and activated with CD3/CD28 antibodies for 48 hr. Next, cells were treated with anti-TNF or IgG control (10ug/mL) for 48 hrs. Finally proliferation was measured by thymidine incorporation. (B) T cells were stained with annexin V and Pi and fluorescence was measured on the FACS. (C) MLR cultures were stained with annexin V and Pi and fluorescence was measured on the FACS. (D) Apoptosis in an MLR with heat inactivated serum and serum without heat inactivation was measured in a caspase 3 activity assay. All data are shown as means ± SD

Supplementary Figure 2 Infliximab has no effect on costimulatory molecules on DCs and monocytes.



MoDCs (A) or monocytes (B) were activated with 100 ng/mL LPS for 16hr, and treated with anti-TNF for 24 hr. Cells were stained with αHLA-DR, αCD40, vCD80, αCD83, αCD86 and isotype control and analyzed by flowcytometry. All data are shown as means ± SD

Supplementary Figure 3 Infliximab F(ab')2 fragment binds to activated T cells.



T cells were activated for 48 hours with CD3/CD28 beads. Infliximab was labeled with a fluorescent dye (Alexa Fluor 647). Cells were incubated with infliximab Alexa Fluor (70 nM) and different concentrations of infliximab and infliximab F(ab')2 fragment. Fluorescence was measured with Flow Cytometry

Reference List

1. Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002;347:417-429.
2. Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 1998;115:182-205.
3. Swidsinski A, Ladhoff A, Pernthaler A, et al. Mucosal flora in inflammatory bowel disease. Gastroenterology 2002;122:44-54.
4. Schreiber S, Nikolaus S, Hampe J et al. Tumour necrosis factor [alpha] and interleukin 1[beta] in relapse of Crohn's disease. The Lancet 1999;353:459-461.
5. Plevy SE, Landers CJ, Prehn J et al. A role for TNF-alpha and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. J Immunol 1997;159:6276-6282.
6. van Deventer SJH. Review article: targeting TNF as a key cytokine in the inflammatory processes of Crohns disease the mechanisms of action of infliximab. Alimentary Pharmacology & Therapeutics 1999;13:3-8.
7. Kontoyiannis D, Pasparakis M, Pizarro TT et al. Impaired On/Off Regulation of TNF Biosynthesis in Mice Lacking TNF AU-Rich Elements: Implications for Joint and Gut-Associated Immunopathologies. Immunity 1999;10:387-398.
8. Targan SR, Hanauer SB, van Deventer SJH et al. The Crohn's Dc. A Short-Term Study of Chimeric Monoclonal Antibody cA2 to Tumor Necrosis Factor {alpha} for Crohn's Disease. N Engl J Med 1997;337:1029-1036.
9. Hanauer SB, Feagan BG, Lichtenstein GR et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. The Lancet 2002;359:1541-1549.
10. Hanauer SB, Sandborn WJ, Rutgeerts P et al. Human Anti-Tumor Necrosis Factor Monoclonal Antibody (Adalimumab) in Crohn's Disease: the CLASSIC-I Trial. Gastroenterology 2006;130:323-333.
11. Sandborn WJ, Hanauer SB, Rutgeerts P et al. Adalimumab for maintenance treatment of Crohn's disease: results of the CLASSIC II trial. Gut 2007;56:1232-1239.
12. Sandborn WJ, Hanauer SB, Katz S et al. Etanercept for Active Crohn's Disease: A Randomized, Double-Blind, Placebo-Controlled Trial. Gastroenterology 2001;121:1088-1094.
13. Rutgeerts P, Sandborn WJ, Fedorak RN et al. Onercept for moderate-to-severe Crohn's disease: a randomized, double-blind, placebo-controlled trial. Clin Gastroenterol Hepatol 2006;4:888-893.
14. Feagan BG, Sandborn WJ, Lichtenstein G et al. CDP571, a humanized monoclonal antibody to tumour necrosis factor-alpha, for steroid-dependent Crohn's disease: a randomized, double-blind, placebo-controlled trial. Aliment Pharmacol Ther 2006;23:617-628.
15. ten Hove T, van Montfrans C, Peppelenbosch MP et al. Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. Gut 2002;50:206-211.
16. Van den Brande JMH, Braat H, van den Brink GR et al. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. Gastroenterology 2003;124:1774-1785.
17. Lügering A, Schmidt M, Lügering N et al. Infliximab Induces Apoptosis in Monocytes From Patients With Chronic Active Crohn's Disease by Using a Caspase-Dependent Pathway. Gastroenterology 2001;121:1145-1157.
18. Mitoma H, Horiuchi T, Tsukamoto H et al. Mechanisms for cytotoxic effects of anti-tumor necrosis factor agents on transmembrane tumor necrosis factor alpha-expressing cells: comparison among infliximab, etanercept, and adalimumab. Arthritis Rheum 2008;58:1248-1257.

19. Arora T, Padaki R, Liu L et al. Differences in binding and effector functions between classes of TNF antagonists. Cytokine 2009;45:124-131.
20. Louis E, El GZ, Vermeire S et al. Association between polymorphism in IgG Fc receptor IIIa coding gene and biological response to infliximab in Crohn's disease. Aliment Pharmacol Ther 2004;19:511-519.
21. Clynes RA, Towers TL, Presta LG et al. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nat Med 2000;6:443-446.
22. Zalevsky J, Leung IW, Karki S et al. The impact of Fc engineering on an anti-CD19 antibody: increased Fcgamma receptor affinity enhances B-cell clearing in nonhuman primates. Blood 2009;113:3735-3743.
23. Mitoma H, Horiuchi T, Hatta N et al. Infliximab induces potent anti-inflammatory responses by outside-to-inside signals through transmembrane TNF-[alpha]. Gastroenterology 2005;128:376-392.
24. de HP, de Bruin EC, Klein-Kranenbarg E et al. Caspase-3 activity predicts local recurrence in rectal cancer. Clin Cancer Res 2007;13:5810-5815.
25. Bedini C, Nasorri F, Girolomoni G et al. Antitumour necrosis factor-alpha chimeric antibody (infliximab) inhibits activation of skin-homing CD4+ and CD8+ T lymphocytes and impairs dendritic cell function. Br J Dermatol 2007;157:249-258.
26. Kaymakcalan Z, Sakorafas P, Bose S et al. Comparisons of affinities, avidities, and complement activation of adalimumab, infliximab, and etanercept in binding
to soluble and membrane tumor necrosis factor. Clin Immunol 2009;131:308-316.
27. Cartron G, Dacheux L, Salles G et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood 2002;99:754-758.
28. Dall'Ozzo S, Tartas S, Paintaud G et al. Rituximab-Dependent Cytotoxicity by Natural Killer Cells: Influence of FCGR3A Polymorphism on the Concentration-Effect Relationship. Cancer Res 2004;64:4664-4669.
29. Bibeau F, Lopez-Crapez E, Di FF et al. Impact of Fc{gamma}RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 2009;27:1122-1129.
30. Horton HM, Bernett MJ, Pong E et al. Potent in vitro and in vivo activity of an Fc-engineered anti-CD19 monoclonal antibody against lymphoma and leukemia. Cancer Res 2008;68:8049-8057.
31. Louis EJ, Watier HE, Schreiber S et al. Polymorphism in IgG Fc receptor gene FCGR3A and response to infliximab in Crohn's disease: a subanalysis of the ACCENT I study. Pharmacogenet Genomics 2006;16:911-914.
32. Woof JM, Burton DR. Human antibody-Fc receptor interactions illuminated by crystal structures. Nat Rev Immunol 2004;4:89-99.
33. Kodelja V, Muller C, Tenorio S, Schebesch C, Orfanos CE, Goerdt S. Differences in angiogenic potential of classically vs alternatively activated macrophages. Immunobiology 1997;197:478-493.
34. Hunter MM, Wang A, Parhar KS et al. In Vitro-Derived Alternatively Activated Macrophages Reduce Colonic Inflammation in Mice. Gastroenterology 2010;138(4):1395-40.
35. Takada Y, Hisamatsu T, Kamada N et al. Monocyte Chemoattractant Protein-1 Contributes to Gut Homeostasis and Intestinal Inflammation by Composition of IL-10-Producing Regulatory Macrophage Subset. J Immunol 2010;184:2671-2676.

36. Sandborn WJ, Feagan BG, Stoinov S et al. Certolizumab Pegol for the Treatment of Crohn's Disease. N Engl J Med 2007;357:228-238.
37. Schreiber S, Khaliq-Kareemi M, Lawrance IC et al. Maintenance therapy with certolizumab pegol for Crohn's disease. N Engl J Med 2007;357:239-250.
38. Tubach F, Salmon D, Ravaud P et al. Risk of tuberculosis is higher with anti-tumor necrosis factor monoclonal antibody therapy than with soluble tumor necrosis factor receptor therapy: The three-year prospective French Research Axed on Tolerance of Biotherapies registry. Arthritis Rheum 2009;60:1884-1894.
39. Verreck FA, de BT, Langenberg DM et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. Proc Natl Acad Sci U S A 2004;101:4560-4565.
40. Kang PB, Azad AK, Torrelles JB et al. The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis. J Exp Med 2005;202:987-999.