Towards therapeutic disease control in inflammatory bowel diseases

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Chapter five

Regulatory macrophages induced by infliximab are involved in healing *in vivo* and *in vitro*

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Inflammatory Bowel Diseases, accepted for publication

Abstract

Regulatory macrophages play an important role in wound healing and gut homeostasis and have anti-inflammatory properties. Induction of this cell type ($M\phi_{ind}$) by the anti-TNF antibodies, infliximab and adalimumab, has recently been shown *in vitro*. Also, the superiority of infliximab/azathioprine combination therapy over infliximab or azathioprine monotherapy has recently been established, but the mechanism behind this remains unclear. The aim of this study is to examine the induction of regulatory macrophages in patients with and without mucosal healing in response to infliximab. In addition, we studied the effect of infliximab/azathioprine combination treatment on the differentiation and function of regulatory macrophages.

IBD patients (n=10) underwent endoscopy before and after first infliximab treatment. Immunohistochemical staining of CD68 and CD206 was performed in all patients. Mixed lymphocyte reactions (MLR) were treated with infliximab, azathioprine or both. Macrophage phenotype was evaluated by flow cytometry and inhibition of T cell proliferation was measured in a secondary MLR containing macrophages and third party lymphocytes.

A significant induction of regulatory macrophages was observed in patients with mucosal healing after treatment with infliximab, this induction was absent in patients without mucosal healing. In addition, $M\phi_{ind}$ have the ability to induce wound healing in an *in vitro* model, further suggesting a key role for infliximab induced macrophages in mucosal healing. Upon infliximab/azathioprine combination treatment, an increased number of regulatory macrophages was observed. These macrophages also displayed stronger immunosuppressive properties than macrophages induced by infliximab monotherapy.

These data show that regulatory macrophages may be involved in mucosal healing, and provide a rationale for the superiority of infliximab/azathioprine combination treatment observed in the clinic.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases (IBD) of unknown etiology resulting in loss of tolerance towards the mucosal flora. Traditionally, therapy to control IBD was aimed at reducing symptoms, but at the present time treatment is more and more focused on inducing mucosal healing and altering the disease course. The introduction of anti-TNF agents in the '90's has been an important breakthrough to accomplish this. Anti-TNF agents have been shown to induce mucosal healing, reduce steroid dependency, reduce the risk for surgery and hospitalization, and to improve the patient's quality of life. [1-5] However, not all anti-TNF agents that have been introduced in the clinic appeared effective. For instance, the TNF receptor fusion protein etanercept [6] and the IgG_4 anti-TNF antibody CDP571 [7] lacked clinical efficacy although these agents have the ability to neutralize soluble TNF. This observation raised the question why some anti-TNF agents are effective and others are not. We have recently shown that in order for an anti-TNF to inhibit T cell proliferation *in vitro*, the compound needs to bind to membrane bound TNF on activated T cells and posses an Fc region to interact with Fc receptors. [8] Upon this binding, a distinct macrophage subset is induced ($M\phi_{ind}$) with immunosuppressive capacities, including the production of anti-inflammatory cytokines and inhibition of T cell proliferation. Furthermore, it has been shown that in vitro derived regulatory macrophages reduce colonic inflammation in mice, [9] and inhibit proliferation of activated T cells. [10] On top of this, regulatory macrophages play a crucial role in wound healing, [11] and therefore the induction of this cell type may be of particular interest in the treatment of IBD and induction of mucosal healing.

Recently, a large randomized, placebo-controlled trial demonstrated the superiority of infliximab/azathioprine combination treatment over infliximab or azathioprine monotherapy in inducing and maintaining remission and mucosal healing in patients with CD. [12] The exact mechanism underlying this effect is unknown, but it likely results from the additional effect of a second immunosuppressive. Also, it is possible that azathioprine suppresses infliximab induced immunogenicity, thereby increasing infliximab efficacy. However, the specific mechanisms involved in this superiority and in mucosal healing in general are so far not completely understood.

In this study, we aimed to investigate the induction of regulatory macrophages in patients responding to infliximab therapy versus those who did not respond. Also, we examined the wound healing capacity of infliximab-induced regulatory macrophages *in vitro*. In addition, we examined the effect of infliximab/azathioprine combination treatment on the induction and function of regulatory macrophages *in vitro*.

Materials and methods

Patients

The patient material used in this study was obtained from University Hospital Gasthuisberg in Leuven (ClinicalTrials.gov number NCT00639821). All patients gave written informed consent. Ten patients with active IBD (4 UC, 6 CD), refractory to corticosteroids and/or immunosuppression, were examined. The patients underwent endoscopy with biopsies

from affected bowel (colon for UC and colonic CD, and ileum for ileal CD) within a week prior to the first infliximab infusion of 5 mg /kg body weight. Patients received a second endoscopy with biopsies at week 4 - 6. The biopsies were taken at sites of active inflammation but at a distance of ulcerations. When healing was observed at the second endoscopy, biopsies were obtained in the areas where lesions were present before therapy. The endoscopist was not blinded to treatment.
Next, biopsies were fixed in Carnoy's fixative for up to 5 hours and then dehydrated, cleared and paraffin-embedded for histological examination and/or immunohistochemistry. Haematoxylin-eosin stained slides from the paraffin block of each patient were used to score chronic intestinal inflammation using a previously reported scoring system for UC [13] and for CD. [14] The pathologists were blinded to treatment. Response to infliximab was assessed 4-6 weeks after the first infliximab treatment and classified as has been described before. [15] Briefly, for colonic CD, a response was defined as complete mucosal healing with a decrease of at least 3 points on the histological score. [14] For UC, a response consisted of a decrease to a Mayo endoscopic subscore of 0 or 1 with a decrease to grade 0 or 1 on the histological score. [13] For ileal CD (3 patients), patients with a clear improvement of the ulcerations and a decrease on the histological score [14] were considered responders. Patients who did not achieve this healing were considered non-responders although some of them showed an improvement on the histologic or endoscopic score.

Immunohistochemistry

For immunohistochemistry, Mϕ2 cells were defined as CD206+/CD68+ cells. Mϕ2 were detected using an anti-human CD206 goat polyclonal antibody (AF2534, R&D systems, Abingdon, UK) and anti-human CD68 mouse monoclonal antibody (clone KP1, M0814, Dako Belgium NV, Heverlee, Belgium). Briefly, 5 μm-thick sections were cut from the paraffin blocks of Carnoy-fixed endoscopic biopsies from the patients. After drying, deparaffinization and rehydration, epitope retrieval was performed at high pH (Dako PT Link machine, Dako Belgium NV, Heverlee, Belgium). Sections were then washed 3 times 5 min (Envision Flex wash buffer, Dako) and Envision Flex Peroxidase-Blocking Reagent (Dako) was applied for 10 min at room temperature. After a second wash step, sections were incubated with the anti-human CD206 antibody (R&D Systems, dilution 1:40) or with the anti-human CD68 antibody (Dako, dilution: 1:2000) for 30 min at room temperature. Following a third wash step, bound primary antibody was visualized by incubating the slides for 30 min with Envision Flex/HRP (Dako) and application of the Envision DAB+ Chromogen (Dako) for 10 min at room temperature. After rinsing, the slides were counterstained with haematoxylin, dehydrated, cleared and mounted. Negative controls (no application of primary antibody) were run together with the test samples. All the stains were evaluated by an experienced pathologist (GDH). Finally, the stains were analyzed using ImageJ software.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated by Ficoll Paque density-gradient centrifugation. Mφ1 macrophages were generated as described previously. [10] Briefly, monocytes were isolated by Percoll density-gradient centrifugation and cultured in RPMI 1640 containing 10% heat-inactivated FCS for 5 days. CD3 positive T cells were isolated from PBMCs using negative magnetic bead separation (Invitrogen, Paisley,

UK). Where appropriate, T cells were activated with αCD3/αCD28 beads (Invitrogen) (1 bead/5 cells).

Allogeneic mixed lymphocyte reaction (MLR)

PBMCs from two healthy donors were cultured in a 1:1 ratio in RPMI 1640 culture medium to establish a mixed lymphocyte reaction (MLR). After 48 hours of activation, cells were treated with infliximab (10 μg/ml), azathioprine (1 μM) or a combination, for 5 days. Were indicated, cells were treated with 10 μg/ml certolizumab. Finally, proliferation was measured using a ^{3}H-thymidine incorporation assay.

Isolation of infliximab-induced macrophages ($M\phi_{ind}$) and infliximab/azathioprine-induced macrophages ($M\phi_{ind/aza}$)

Infliximab induced macrophages ($M\phi_{ind}$) and infliximab/azathioprine induced macrophages ($M\phi_{ind/AZA}$) were isolated from MLR cultures from healthy volunteers. We have previously shown that upon infliximab treatment, a distinct cell population occurs on the FCS/SSC, which is strongly positive for several markers, among which CD206 (a commonly used regulatory macrophage marker) and CD14, and that these cells have immunosuppressive properties. The induction, phenotype and isolation of this cell subset have been extensively described before. [8] In brief, $M\phi_{ind}$ and $M\phi_{ind/aza}$ were isolated from the MLR based on the expression of CD14 using CD14 microbeads according to manufacturer's protocol (Miltenyi, Bergisch Gladbach, Germany). Next, cells were counted and cultured in RPMI 1640, containing 10% heat-inactivated FCS.

In vitro wound healing assay

The wound healing scratch assay was performed as previously described with a few modifications. [16] HCT116 colon epithelial cells were cultured to approximately 70% confluence in 6 wells plates under standard culture conditions. The plates were marked in order to create a reference point to identify the wound at the same place at different time points. Next, a wound was created using a plastic tip, and pictures were taken at a phase-contrast microscope. $M\phi_{ind}$ and pro-inflammatory type 1 macrophages from healthy volunteers were added to the cell culture (100.000 cells/well). To quantify the degree of wound healing, pictures were taken again after 24 hrs, and the percentage closure was calculated using Image J software.

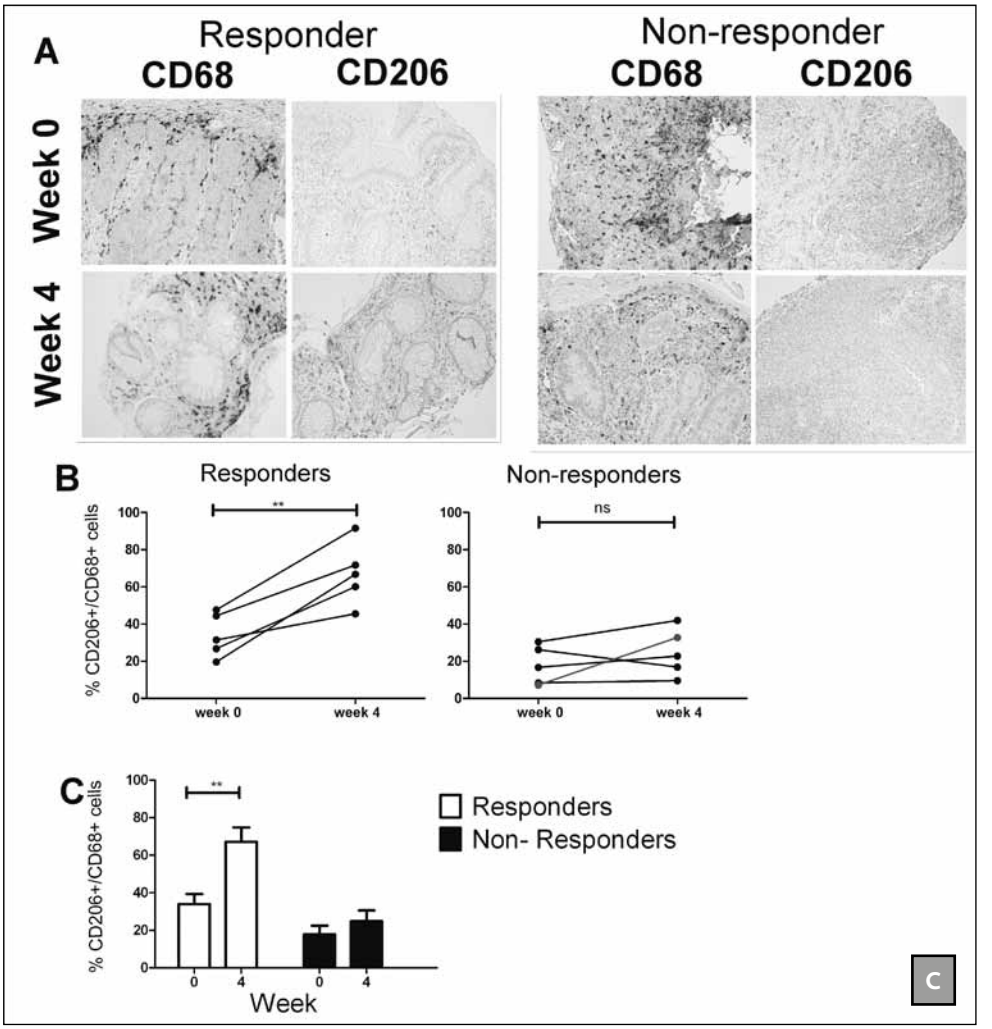
FACS analysis of regulatory macrophages

MLR were treated with infliximab, azathioprine, or a combination. After 5 days, cells were harvested, washed with FACS buffer, and stained for CD206 or isotype control. Finally, expression was analyzed by flow cytometry using a FACS Calibur (BD) and FlowJo software (Treestar Inc, Ashland, OR)

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 $p < 0.05$. Paired *t* test was used to calculate statistical significance in patients before and after infliximab therapy. Results were considered significant when $p < 0.05$

Figure 1 Infliximab induces regulatory macrophages in responders but not in non-responders.



A) Representative pictures of CD206/CD68 stainings in a patient responding to infliximab (left panel) and a non-responder (right panel). Pictures of stained biopsies before and after infliximab induction therapy are shown. B). Induction of CD206/CD68 regulatory macrophages per patient. A significant induction of CD206/CD68+ cells is observed in patients responding to infliximab (left panel), whereas this induction is absent in non-responders (right panel). The red line represents a patient with a partial response observed during endoscopy. C). Non-responders have lower amounts of CD206/CD68+ cells at baseline. ** $p \leq 0.01$ Data were analyzed using paired *t* test

Results

Infliximab induces regulatory macrophages in responders but not in non-responders

We have shown previously that anti-TNF antibodies induce regulatory macrophages *in vitro* [8] and this cell type plays an important role in wound healing. [11] Now, we aimed to investigate whether the induction of regulatory macrophages is also observed *in vivo* in patients responding to infliximab therapy. Biopsies were obtained from six CD patients (3 responders, 3 non-responders) and 4 UC patients (2 responders, 2 non-responders) before (week 0) and after (week 4 - 6) first infliximab treatment. Patient characteristics are shown in table 1. Slides from each patient before and after therapy were stained for CD68 and CD206 (also called MRC1 or MMR). CD206 is a commonly used marker for regulatory macrophages, and CD68 is a macrophage marker. Since patients with a response to infliximab usually have a decrease in the number of CD68+ cells, we used the ratio CD206+/CD68+ to identify the induction of regulatory macrophages.

A clear and significant increase was observed in the percentage of CD206+ macrophages in patients responding to infliximab induction therapy, indicating that regulatory macrophages were induced (Figure 1*A* and 1*B*). This induction was observed in each individual responding to infliximab therapy Figure 1*B*, left panel). Importantly, induction of CD206+/CD68+ cells was absent in patients not responding to infliximab therapy (Figure 1*B*, right panel). One patient (Figure 1b, right panel, red line) was considered a non-responder on

Table 1 Patient characteristics at week 0 and week 4-6.

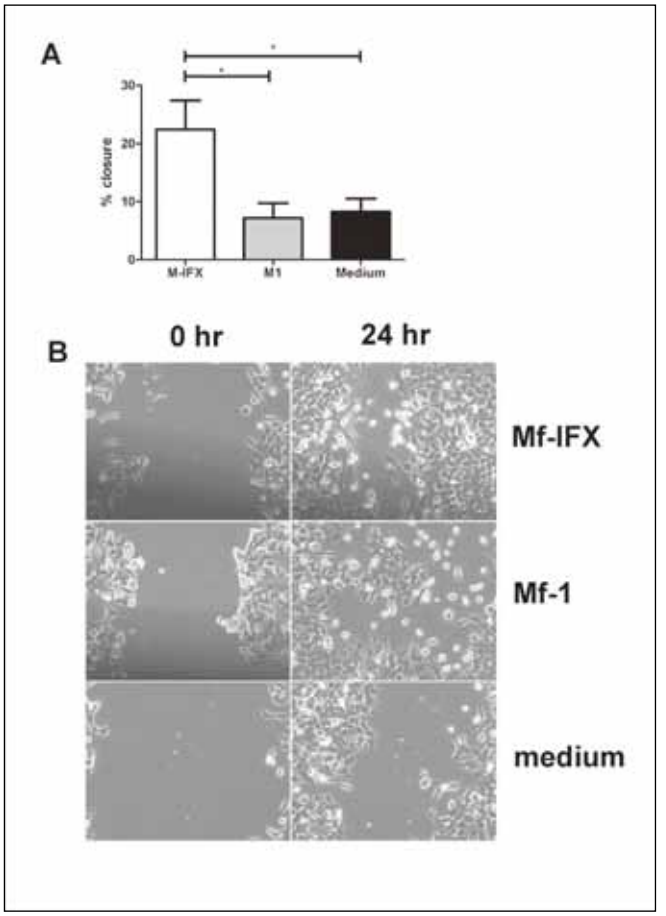
Pt nr	*UC/ CDi/ CDc*	*R/NR*	*Histological score Week 0*	*Histological score Week 4*	*Endoscopic score Week 0*	*Endoscopic score Week 4*	*%CD206/ CD68+ cells Week 0*	*% CD206/ CD68+ cells Week 4*
1	UC	R	5.4	0.3	3	1	19,7	66,8
2	UC	R	5.2	0.1	2	0	47,6	91,6
3	UC	NR	5.4	5.3	3	3	16,7	22,8
4	UC	NR	5.1	5.3	2	3	30,5	42
5	CDc	R	14/16	6/16	Active colitis	healing	26,8	60,1
6	CDc	NR	16/16	16/16	Active colitis	Active colitis	8,4	9,6
7	CDc	NR	10/16	10/16	Active colitis	Incomplete healing	7,2	32,8
8	CDi	R	10/16	3/16	Active colitis	healing	31,5	45,5
9	CDi	R	12/16	5/16	Active ileitis	Incomplete healing	44,5	71,8
10	CDi	NR	7/16	8/16	Active ileitis	Active ileitis	26,2	16,9

Biopsies were obtained before (week 0) and after (week 4 - 6) infliximab therapy. Histologic and endoscopic scores and the percentage of CD206/CD68+ cells at 0 and 4 weeks are shown. Pathologists who scored histology were blinded to treatment, endoscopists were not blinded to treatment, and the percentage CD206/CD68+ was calculated using Image J software.

UC Ulcerative colitis, CDc colonic Crohn's disease, CDi ileal Crohn's disease, R responder, NR non-responder

histological basis, but did show a partial response on endoscopy. An increase of CD206+/CD68+ cells was observed in this patient.
Taken together, these data show that regulatory macrophages similar to those induced by infliximab *in vitro*, are induced *in vivo* in patients responding to infliximab therapy.

Figure 2 $M\phi_{ind}$ induce wound healing in vitro.



A) $M\phi_{ind}$ induce wound healing *in vitro* B) representative pictures of the wound healing assay. Data are representative of at least three independent experiments. Data were analyzed using one-way ANOVA and bonferroni post-test. * $p \leq 0.05$

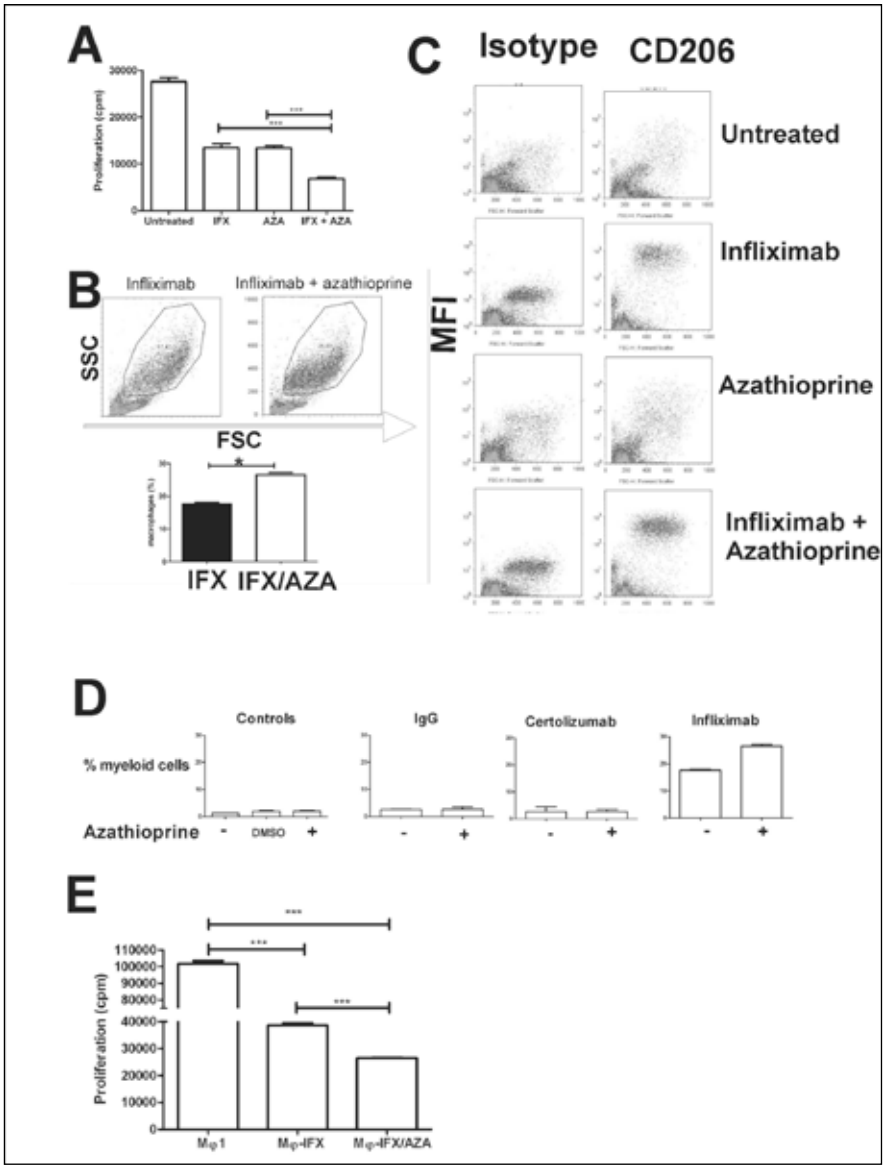
Infliximab-induced regulatory macrophages induce wound healing *in vitro*
It has been shown previously that regulatory macrophages generated *in vitro* by addition of IL-4 and IL-13 exhibit wound healing capacities. The data described above show an increased presence of macrophages displaying a regulatory phenotype after infliximab therapy. However, the presence of CD206+/CD68+ cells in patients presenting with mucosal healing does not discriminate between infliximab-mediated effects and wound healing effects induced by

another mechanism such as immunosuppression in general. Therefore, we aimed to assess specifically the wound healing capacity of macrophages induced by infliximab treatment.
To this end, macrophages were isolated from MLR cultures treated with infliximab (regulatory $M\phi_{ind}$) or generated from monocyte cultures (inflammatory Mϕ1). HCT116 colonic epithelial cells were cultured and a wound was created in the monolayer. Mϕ1, $M\phi_{ind}$ or control medium was added to the wells and images were taken at t=0 and t=24 hours. As expected, Mϕ1 did not induce wound healing above the level of control (Figure 2). In contrast, $M\phi_{ind}$ showed a clear capacity to enhance wound healing up to two-fold compared to Mϕ1 or medium alone. These data are in line with the results observed in patients responding to infliximab therapy, and further suggest that regulatory macrophages induced by infliximab induce wound healing.

Enhanced induction and function of regulatory macrophages upon infliximab/azathioprine combination treatment *in vitro*

Since the induction of $M\phi_{ind}$ correlates with response to infliximab (Figure 1), and higher response rates are observed in patients receiving infliximab/azathioprine combination treatment compared to either monotherapy, [12] we hypothesized that infliximab/azathioprine combination treatment might enhance the induction of $M\phi_{ind}$ *in vitro*. To answer this question, we treated mixed lymphocyte cultures with infliximab, azathioprine or a combination. Azathioprine and infliximab monotherapy inhibited T cell proliferation in an MLR to the same extent (Figure 3*A*). As expected, when cells in an MLR were treated with a combination of infliximab and azathioprine, T cell proliferation was further inhibited. To investigate whether this observation was a cumulative effect of a second immunosuppressive or whether another mechanism was involved, we studied the effect of combination treatment on the induction of regulatory macrophages. As shown previously, [8] anti-TNF antibodies induce a distinct subset of cells, characterized by high forward and high side scatters and expression of CD14 and CD206 when analyzed by flowcytometry. When cells were treated with azathioprine monotherapy, no induction of regulatory macrophages was observed. Strikingly, we found a significantly increased induction of this CD206+ subset upon combination treatment (Figure 3*B/C*).
We have previously shown that anti-TNF agents induce regulatory macrophages in an Fc region dependent manner, [8] and that agents lacking the Fc fragment, do not induce this cell type. Therefore, we also evaluated the combination azathioprine/certolizumab (lacking the Fc fragment) to examine whether the observed effect of enhanced $M\phi_{ind/AZA}$ was induced by infliximab in particular or a general effect of TNFα neutralization. Indeed, the effect was specific for the combination treatment of azathioprine/infliximab, since the induction was absent upon azathioprine/certolizumab or azathioprine/IgG treatment (Figure 3*D*).
To further examine the properties of regulatory macrophages induced by infliximab/azathioprine combination treatment ($M\phi_{ind/AZA}$), we aimed to investigate their immunosuppressive function. Therefore, we isolated regulatory macrophages based on CD14 expression, and co-cultured equal numbers of these cells with activated T cells from a third donor. Strikingly, $M\phi_{ind/AZA}$ displayed a stronger immunosuppressive phenotype, since $M\phi_{ind/AZA}$ showed enhanced ability to inhibit T cell proliferation compared to $M\phi_{ind}$ (Figure 3*E*).
These data show that combination treatment is superior to monotherapy *in vitro* with respect to the induction of regulatory macrophages. Not only the number of regulatory mac-

Figure 3 Enhanced induction of regulatory macrophages upon infliximab / azathioprine combination therapy.



A). Infliximab and azathioprine inhibit T cell proliferation in an MLR to the same extent, but stronger inhibition is observed upon infliximab/azathioprine combination therapy. B) Enhanced induction of regulatory macrophages in an MLR. C) Enhanced number of CD206+ cells upon infliximab/azathioprine combination therapy. This induction is absent when cells are treated with azathioprine monotreatment. D) The enhanced induction of regulatory macrophages is specific for infliximab, and is absent when co-treated with certolizumab or control IgG. E) $M\phi_{ind/AZA}$ are superior to $M\phi_{ind}$ in inhibiting T cell proliferation. 20.000 macrophages were plated and cocultured with 100.000 T cells. All figures are representative figures of at least three independent experiments. Data were analyzed using one-way ANOVA and bonferroni post-test *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

rophages is increased, but the macrophages also display a stronger immunosuppressive phenotype. These findings may provide a partial explanation for the superiority of infliximab/azathioprine combination therapy observed in patients with CD.

Discussion

The data presented here show the induction of regulatory macrophages after infliximab treatment in IBD patients. Patients responding to infliximab showed a significant induction of CD206+/CD68+ cells, whereas this induction was absent in non-responders. Since we used endoscopic and histologic healing as a definition of response, these data suggest that the induction of regulatory macrophages may be involved in the process of mucosal healing. The fact that we observe increased numbers of CD206+/CD68+ cells in patients responding to infliximab therapy does not prove directly that this effect is mediated by infliximab. However, we have previously shown induction of CD206+ macrophages by infliximab *in vitro*. [8] Additionally, we now show here that CD206+ macrophages induced by infliximab *in vitro* have wound healing capacity, similar to what has been described for IL-4/IL-13 induced regulatory macrophages previously. [17] Together these data suggests that the induction of regulatory macrophages by infliximab may contribute to mucosal healing in patients clinically responding to the treatment.

A recent clinical trial has shown that combination treatment of azathioprine and infliximab is superior to monotreatment in the induction of remission and mucosal healing in CD patients. [12] In our in vitro system, azathioprine did not induce significant numbers of macrophages. However, in combination with infliximab, azathioprine potentiated both the number and immunosuppressive capacity of CD206+ macrophages. Although the superiority of combination treatment *in vivo* probably results at least in part from the additive effects of two immunosuppresives, our data suggest an additional layer of actual synergism which may also contribute to the increased mucosal healing observed in patients.

In order to maintain tolerance, lamina propria macrophages normally display a type 2 macrophage (Mϕ2) phenotype. Mϕ2 are functionally different from type 1 macrophages (Mϕ1), since they have minor ability to respond to bacterial stimuli, and produce anti-inflammatory cytokines rather than pro-inflammatory cytokines. [18, 19] In IBD patients, lamina propria macrophages have a more Mϕ1 phenotype, as lamina propria mononuclear cells (LPMNCs) from IBD patients spontaneously produce large amounts of pro-inflammatory cytokines and are hyperresponsive to bacterial stimuli. [20-22] In line with this, lower amounts of Mϕ2 were found in mucosal biopsies from active lesions in CD patients compared to non-affected colon of the same patient, and compared to healthy controls. [9] This suggests that lamina propria macrophages from CD patients are skewed towards an Mϕ1 phenotype, thus contributing to the defect in tolerance. Since regulatory macrophages contribute to tolerance towards the mucosal flora and CD patients have decreased numbers of this cell type, the induction of Mϕ2 might be an interesting target in restoring the disturbed balance.

In conclusion, our data demonstrate the induction of regulatory macrophages in IBD patients responding to infliximab therapy. In addition, we show that infliximab induced macrophages indeed have wound healing capacity, suggesting a potential role in mucosal healing. Finally, we show that infliximab/azathioprine combination treatment potentiates

the induction of regulatory macrophages, thus providing a new rationale for the superiority of infliximab/azathioprine combination treatment observed in the clinic.

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