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Chapter 3.

Inhibition of soluble TNF-\(\alpha\) by single domain camel antibodies does not prevent experimental colitis

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Abstract

Tumour necrosis factor (TNF-α) is a pro-inflammatory cytokine, which plays an important role in the pathogenesis of inflammatory bowel diseases. Blocking of TNF-α has been demonstrated to be an effective strategy. However, most of the anti-TNF drugs have side-effects and are immunogenic. In addition, there is differential binding to the two forms of TNF-α: the transmembrane (tmTNF-α) and the soluble protein (sTNF-α). In this study we have tested a new inhibitor of sTNF-α based on the heavy chains of camel antibodies, the so-called nanobodies, in an acute and a chronic colitis mouse model. We demonstrated that these nanobodies do not ameliorate colitis: there were no differences in body weight, colon length, colon weight and histology between the treated and control group in both models. Only in the acute model nanobodies decreased the amount of cells in the caudal lymph nodes, but increased the cytokine production. The low efficacy of blocking of sTNF-α compared to a tmTNF-α blocking antibody might reflect their role in the inflammatory process.
Introduction

Crohn’s disease (CD) is characterised by chronic inflammation of the gastrointestinal tract of which the pathogenesis is unknown. Several genetic, immunological and environmental factors all contribute to the initiation and maintenance of the disease. It has been demonstrated that an exaggerated immune response against the endogenous microflora by T helper (Th) 1 and Th17 lymphocytes plays an important role in the pathogenesis of CD. This immune response is characterised by an increase of pro-inflammatory cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL)-1β, transforming growth factor-β, interferon-γ and IL-17 in the inflamed mucosa of CD patients. High concentrations of TNF-α can also be detected in the stool of CD patients. That TNF-α is a key player in the pathogenesis of CD has been shown by overexpression of TNF-α in mice, which results in the development of chronic inflammatory arthritis and Crohn’s like IBD.

TNF-α is first synthesised as a 26kDa transmembrane protein (tmTNF-α) with an intracellular tail. The metalloproteinase TNF-α converting enzyme (TACE) cleaves tmTNF-α into a soluble protein of 17kDa (sTNF-α). Many cell types, also non-immune cell types are able to produce TNF-α, however the majority of TNF-α is produced by monocytes and macrophages. TNF-α plays an important role in cell recruitment, cell proliferation, apoptosis and immune regulation via their interaction with two different TNF-α receptors. Both sTNF-α and tmTNF-α are capable to bind the 55kDa TNF receptor (TNFR)1 (CD120a) and the 75kDa TNFR2 (CD120b). However, sTNF-α has a higher affinity for TNFR1, whereas tmTNF-α prefers to bind TNFR2. Dependent on the metabolic state of the cell, receptor-mediated effects of tmTNF-α and sTNF-α result in apoptosis or nuclear factor (NF)-κB activation. TNFR1 is constitutively expressed on most cell types, whereas TNFR2 is mainly expressed on endothelial and haematopoietic cells, although during active inflammation in IBD patients and mice colitis models also epithelial cells express high levels of TNFR2. Similar to TNF-α, soluble TNF-α receptors are released by proteolytic cleavage of their transmembrane form. Both forms of soluble
TNF-α receptors are capable to function as a natural TNF-α antagonist by neutralising sTNF-α 13.

Since TNF-α seems to be a key player in a number of diseases like CD, rheumatoid arthritis (RA), sarcoïdosis, and psoriasis, strategies to neutralise TNF-α have been developed. These strategies include infliximab (chimeric IgG anti-TNF antibody), adalimumab (human IgG1 monoclonal anti-TNF antibody), certoluzimab (polyethylene Fab fragment of anti-TNF) and etanercept (TNF-α receptor 2 IgG1 invariant tail fusion protein). Although 60 to 70% of the CD patients benefit from this anti-TNF strategies 14-16, these therapies have also many (severe) side-effects including immunoreactivity and can only be administered intravenously or subcutaneously. Moreover, many patients do not respond, loose responsiveness or become intolerant to the current anti-TNF therapies. When this is the case, other anti-TNF-α based drugs can be effective 17-21. Therefore, companies are still developing alternatives to neutralise TNF-α.

We have investigated a TNF-α inhibitor that is developed by Ablynx. This anti-TNF-α is based on the discovery by the Vrije Universiteit Brussel in Belgium that camelidae produce functional antibodies that only contain heavy chains 22,23. The isolated single variable domain (VHH) of heavy-chain antibodies still harbours the full antigen binding-capacity. These so-called nanobodies have the advantage that they are oral available and they should be less immunogenic because of their size 24-26. We have tested two different nanobodies in an acute colitis model (TNBS-induced colitis) and a chronic colitis model (CD4⁺CD45RB⁺ transfer colitis). Unfortunately, both nanobodies did not prevent experimental colitis.
Materials and methods

Induction of experimental colitis

The Animal Studies Ethics Committee of the University of Amsterdam, The Netherlands, validated all experiments. 7-10 week-old BALB/c and C.B-17 severe combined immunodeficient (SCID) mice were obtained from Harlan Sprague-Dawley (Horst, The Netherlands). During the experiments, the BALB/c mice were housed under standard conditions, whereas the SCID mice were maintained in filter-top cages under specific pathogen-free conditions in our animal facility. All the mice were allowed free access to water and food. The different groups are mentioned in table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Schedule</th>
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<tr>
<td>1 CD4⁺CD45RB&lt;sup&gt;high&lt;/sup&gt; transfer</td>
<td>vehicle intragastric</td>
<td>9 daily 100µl TRIS buffer intragastric</td>
</tr>
<tr>
<td>2 CD4⁺CD45RB&lt;sup&gt;high&lt;/sup&gt; nanobody 3F-3F</td>
<td>9 daily 50µg 3F-3F intragastric in TRIS buffer</td>
<td></td>
</tr>
<tr>
<td>3 CD4⁺CD45RB&lt;sup&gt;high&lt;/sup&gt; vehicle i.p.</td>
<td>9 3x/week 100µl PBS i.p.</td>
<td></td>
</tr>
<tr>
<td>4 CD4⁺CD45RB&lt;sup&gt;high&lt;/sup&gt; nanobody 3F-3F HSA21</td>
<td>7 3x/week 50µg 3F-3F-HSA21 in PBS i.p.</td>
<td></td>
</tr>
<tr>
<td>5 CD4⁺CD45RB&lt;sup&gt;high&lt;/sup&gt; α-mouse TNF-α mAb</td>
<td>9 3x/week 50µg anti-mouse TNF-α mAb i.p.</td>
<td></td>
</tr>
<tr>
<td>6 CD4⁺CD45RB&lt;sup&gt;high&lt;/sup&gt; CD4⁺CD45RB&lt;sup&gt;low&lt;/sup&gt;</td>
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Table 1. Experimental design

Induction of CD4⁺CD45RB<sup>high</sup> transfer colitis

Chronic CD4⁺CD45RB<sup>high</sup> transfer colitis was induced as described previously 27,28. Briefly, CD4⁺ splenocytes from BALB/c mice were isolated by red cell lysis and negative selection by using rat-anti-mouse monoclonal antibodies (mAbs) against B220 (clone RA3-6B2),
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Mac-1 (clone M1/70) and CD8α (clone 53-6.7) (gift from Dr. R. Mebius, Free University Medical Centre, Amsterdam, The Netherlands). Cells that were stained with mAbs were removed in a magnetic field by using sheep-anti-rat immunoglobulin G-coated magnetic beads (Dynal, Hamburg, Germany). Finally, the resulting CD4⁺ cells were stained with cychrome-conjugated CD4 and fluorescein isothiocyanate-conjugated CD45RB mAbs (BD Biosciences, San Diego, CA) so that these cell populations could be sorted by flow cytometry (BD Biosciences, San Diego, CA). CD4⁺CD45RB^high^ T populations were 95% pure and 1-4x10⁵ of these cells were transferred to C.B-17 SCID mice as a single intraperitoneal (i.p.) injection to induce colitis. As a negative control group we used mice that were transferred with both the CD4⁺CD45RB^high^ T lymphocytes as well as the CD4⁺CD45RB^low^ T lymphocytes, since the CD4⁺CD45RB^low^ population T cells protects the mice from developing colitis.

**Induction of TNBS colitis**

TNBS colitis was induced as described previously 29. Briefly, at day 0 and day 7, 1.0 mg of 2,4,6-trinitrobenzene sulphonic acid (TNBS; Sigma Chemical Co., St. Louis, MO) dissolved in 40% ethanol (Merck, Darmstadt, Germany) was administrated rectally using a vinyl catheter positioned three cm from the anus. During this procedure, the mice were anaesthetised with isoflurane (1-chloro-2,2,2-trifluoroethyl-isoflurane-difluoromethyl-ether; Abbott Laboratories Ltd., Queenborough, Kent, England). After instillation, the mice were kept vertically for 60 seconds.

**Cell Culture and cytokine measurements**

Caudal lymph node (CLN) cells of mice with TNBS-induced colitis were isolated by passing the lymph node through a 40µm filter cell strainer (Becton/Dickson Labware, New Jersey, USA). The isolated lymphocytes were suspended in 4 ml RPMI 1640 containing L-glutamine, 10% foetal calf’s serum and antibiotics (penicillin G sodium 10000 U/ml, streptomycin sulphate 25 µg/ml, amphotericin B 25 µg/ml) (all from Gibco/BRL, Paisley, Scotland). The cells were counted and added to flat-bottom 96-well plates at 2 x 10⁵ cells
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per well in a total volume of 200μl of the same medium. The cells were cultured in the presence of immobilised α-CD3 (1:30; 145.2C11 clone) and soluble α-CD28 (1:1000; PharMingen, San Diego, CA) for 48 hours at 37°C. The supernatant was collected and used for a cytometric bead array (CBA) (BD Biosciences, San Jose, CA). A CBA was performed to determine simultaneously the production of TNF-α, IFN-γ, IL-2, IL-4 and IL-5 according to the manufactures recommendations. Two-colour flow cytometric analysis was performed using FACScan® flow cytometre (Becton Dickonson Immunocytometry Systems (BDIS), San Jose, CA). Data were acquired and analysed using Becton Dickinson CBA software.

**Parameters to assess inflammation**

The weight of the mice was recorded every day (TNBS-colitis) or twice a week (CD4⁺CD45RB<sup>high</sup> colitis). Mice had to be sacrificed if their weight loss is more than 15% compared to their initial weight or more than 25% compared to the control group without colitis. After sacrificing the mice, the CLN and the colon were collected. Through a midline incision, the colons were removed. Length and after removing the faecal material, weight of the colons (calculated for 6 cm) were measured and used as an indicator of disease-related intestinal shortening and thickening, respectively.

**Histological examination**

Longitudinally divided rolled-up parts of colons were fixed in 4% buffered formalin in PBS for 24 hours and embedded in paraffin for routine histology. Three transverse slices (5 μm), taken from each colonic sample, were stained with haematoxylin-eosine and examined by light microscopy. Colonic inflammation was evaluated microscopically in a blinded manner by an experienced pathologist. Inflammation induced by TNBS-colitis was estimated by 1) percentage of involved area, 2) the amount of follicles, 3) oedema, 4) fibrosis, 5) erosion/ulceration, 6) crypt loss and infiltration of 7) granulocytes and 8) monocytes with a maximum score of 24. Inflammation induced by CD4⁺CD45RB<sup>high</sup> colitis was estimated by the 1) percentage of involved area, 2) depletion of goblet cells, 3) epithelial hyperplasia, 4)
ulcerations, 5) crypt abscesses and 6) infiltration of granulocytes and monocytes with a maximal score of 4.

**Statistical analysis**

All data are expressed as the means ± the standard error of the mean (SEM) and were analysed using Graphpad prism 4 (Graphpad Prism v. 4 for Windows, GraphPad Software, San Diego, California USA). Differences between groups were analysed using the non-parametric Mann Whitney U test. Changes in body weight between the four groups were analysed by one-way ANOVA with a Bonferonni post-hoc test when differences between interventions were significantly different. Correlation analyses were performed using the Spearman correlation test. All statistics were performed two-tailed and values of $p<0.05$ were considered statistically significant (* $p<0.05$; ** $p<0.01$, *** $p<0.001$).
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**Results**

**Nanobodies do not ameliorate CD4\(^+\)CD45RB\(^{\text{high}}\) transfer colitis**

SCID mice that have received CD4\(^+\)CD45RB\(^{\text{high}}\) T lymphocytes (called CD4\(^+\)CD45RB\(^{\text{high}}\) transferred mice) will develop colitis in approximately four weeks characterised by wasting disease, diarrhoea and inflammation. Colitis induced by the transfer of CD4\(^+\)CD45RB\(^{\text{high}}\) T lymphocytes is accompanied by the loss of body weight (see figure 1.). All mice survived this induction of colitis, but body weight changes were significantly different between the six groups (p<0.0001). CD4\(^+\)CD45RB\(^{\text{high}}\) transferred mice treated with nanobody 3F-3F significantly developed more wasting disease compared to CD4\(^+\)CD45RB\(^{\text{high}}\) transferred mice that were treated with anti-mouse TNF-\(\alpha\) (p<0.01) and the CD4\(^+\)CD45RB\(^{\text{high}}\)CD4\(^+\)CD45RB\(^{\text{low}}\) transferred mice (p<0.001). Also CD4\(^+\)CD45RB\(^{\text{high}}\) transferred mice that received 3F-3F HSA21 developed more wasting disease compared to CD4\(^+\)CD45RB\(^{\text{high}}\) transferred mice (p<0.01), anti-mouse TNF-\(\alpha\)-treated CD4\(^+\)CD45RB\(^{\text{high}}\) transferred mice (p<0.001) and CD4\(^+\)CD45RB\(^{\text{high}}\)CD4\(^+\)CD45RB\(^{\text{low}}\) transferred mice (p<0.001). Moreover, at the day of sacrifice CD4\(^+\)CD45RB\(^{\text{high}}\) transferred mice treated with nanobody 3F-3F or 3F-3F HSA21 have lost significantly more weight than CD4\(^+\)CD45RB\(^{\text{high}}\) transferred mice treated with anti-mouse TNF-\(\alpha\) mAb (p=0.008 and p=0.01, respectively) and CD4\(^+\)CD45RB\(^{\text{high}}\)CD4\(^+\)CD45RB\(^{\text{low}}\) transferred mice (p=0.0008 and p=0.0007, respectively).

Under influence of inflammation, the muscles of the colon contract resulting in a shortening of the colon and an influx of inflammatory cells and oedema results in an increased weight of the colon. After sacrificing the mice, length and weight of the last six centimetres of the colon were determined. Although body weight changes correlated with colon length (r=0.421, p=0.002), there were no statistically significant differences in colon length between the different groups (see figure 2). Weight of the colon is negatively correlated with body weight changes (r=-0.503, p=0.0002) and with colon length (r=-0.403, p=0.0041), indicating that an increase of colon weight is associated with a loss of body weight and a decrease in colon length. The weight of the colon is significantly increased in
CD4⁺CD45RB<sup>high</sup> transferred mice that were treated with the 3F-3F nanobody (347.9 ± 38.9mg) compared to CD4⁺CD45RB<sup>high</sup> transferred mice treated with anti-mouse TNF-α mAb (214.3 ± 19.2mg; p=0.006) and CD4⁺CD45RB<sup>high</sup>CD4⁺CD45RB<sup>low</sup> transferred mice (212.2 ± 15.5mg; p=0.005). CD4⁺CD45RB<sup>high</sup> transferred mice that received the 3F-3F nanobody had significant increased colon weight compared to CD4⁺CD45RB<sup>high</sup> transferred mice treated with intragastric vehicle (347.9 ± 38.9mg vs. 245.3 ± 20.4; p=0.02). There were no significant differences in colon weight between 3F-3F HSA-21 treated CD4⁺CD45RB<sup>high</sup> transferred mice and CD4⁺CD45RB<sup>high</sup> transferred mice treated with vehicle i.p. (305.7 ± 37.2mg vs. 268.1 ± 11.5mg; p=0.6, respectively). These results indicate that both nanobodies are not able to reduce colitis in the CD4⁺CD45RB<sup>high</sup> transfer model since colon weight is not decreased, compared to CD4⁺CD45RB<sup>high</sup> transferred mice treated with vehicle.

Colonic inflammation was also analysed by histological analyses (see figure 3.). Histological scores of both the CD4⁺CD45RB<sup>high</sup> intragastric vehicle group (2.1 ± 0.2) and the CD4⁺CD45RB<sup>high</sup> i.p. vehicle group (2.0 ± 0.2) were increased compared to the CD4⁺CD45RB<sup>high</sup>CD4⁺CD45RB<sup>low</sup> transferred mice (1.2 ± 0.2; p=0.01 and p=0.02, respectively) and the CD4⁺CD45RB<sup>high</sup> transferred mice treated with anti-mouse TNF-α (1.3 ± 0.2; p=0.02 and p=0.03, respectively), so transfer of CD4⁺CD45RB<sup>low</sup> T lymphocytes and treatment of α-mouse TNF-α ameliorates colitis in CD4⁺CD45RB<sup>high</sup> transferred mice. There were no statistically significant differences in histological score between the CD4⁺CD45RB<sup>high</sup> intragastric vehicle group and the 3F-3F nanobody-treated group (2.1 ± 0.2 vs. 1.7 ± 0.2; p=0.2) and the 3F-3F HSA21 nanobody-treated group compared to the i.p. vehicle group (1.7 ± 0.2 vs.2.0 ± 0.2; p=0.4), indicating that nanobody treatment does not result in a decrease of histological parameters. Body weight changes were negatively correlated with histological scores (r=-.0336, p=0.02), indicating that loss of body weight is associated with an increase in histological scores.
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**Figure 1.** a. Bodyweight was recorded twice a week and weight changes were significant different between the six groups (p<0.0001). b. At the day of sacrifice CD4+CD45RBhigh transferred mice treated with nanobody 3F-3F or 3F-3F HSA21 lost significantly more weight than CD4+CD45RBhigh transferred mice treated with anti-mouse TNF-α mAb (p=0.008 and p=0.01, respectively) and CD4+CD45RBhighCD4+CD45RBlow transferred mice (p=0.0008 and p=0.0007, respectively). c. Colon length was not significantly different between the six groups. d. Colon weight was significantly increased in CD4+CD45RBhigh transferred mice treated with 3F-3F nanobody (■) compared to CD4+CD45RBhigh transferred mice treated with vehicle (●) (p=0.02), CD4+CD45RBhigh transferred mice treated with anti-mouse TNF-α (○) (p=0.006) and CD4+CD45RBhighCD4+CD45RBlow transferred mice (△) (p=0.005). e. Administration of soluble TNF-α inhibitors did not prevent inflammation of the colon. There are no significant differences in histological scores between CD4+CD45RBhigh mice treated with nanobody 3F-3F or 3F-3F HSA21 and the other groups. However, histological scores of both the CD4+CD45RBhigh intragastric vehicle group (■) (2.1 ± 0.2) and the CD4+CD45RBhigh i.p. vehicle group (●) (2.0 ± 0.2) were increased compared to the CD4+CD45RBhighCD4+CD45RBlow transferred mice (○) (1.2 ± 0.2; p=0.01 and p=0.02, respectively) and the CD4+CD45RBhigh transferred mice treated with anti-mouse TNF-α (△) (1.3 ± 0.2; p=0.02 and p=0.03, respectively).
Nanobodies do not prevent TNBS-induced colitis

To determine the effect of nanobodies on the development of TNBS colitis, the mice received 1mg TNBS intrarectally at day 0 and day 7, and after 9 days the mice were sacrificed. Because of the second administration of TNBS, a delayed type hypersensitivity reaction will occur and the mice develop wasting disease, diarrhoea and inflammation of the colon. Mice were treated with 3F-3F orally or i.p. The weight of the mice was recorded every day or every two days and after sacrificing the mice length and weight of the mice were measured. Bodyweight loss was not significant different between the five groups during the development of colitis and at the day of sacrifice (see figure 2.). Moreover, there are no significant differences in colon length. The weight of the colon however is significantly increased in mice treated with 3F-3F compared to mice that were treated with anti-mouse TNF-α (183.3 ± 7.4mg vs. 153.1 ± 4.4mg; p<0.001). The histological score of the 3F-3F-treated group is also significantly higher compared to the vehicle-treated group (9.8 ± 0.7 vs. 4.2 ± 0.9; p<0.0001). Besides, mice that were treated with anti-mouse TNF-α have an increased histological score compared to the control mice (8.2 ± 0.6 vs. 4.2 ± 0.9; p=0.002).
Figure 2. At day 0 and day 7, the mice received 1mg TNBS rectally (arrows in figure a.) and body weight loss was recorded daily or every two days. Body weight loss during the time of the experiment (a.), weight loss at the day of sacrifice (b.) and length of the colon (c.) were not significantly different between the five groups. d. Colon weight was significantly increased in mice treated with 3F-3F compared to mice that were treated with anti-mouse TNF-α (p=0.001). e. The histological score was significant increased in mice treated with 3F-3F compared to mice that were treated with vehicle (p<0.0001). Also mice that were treated with anti-mouse TNF-α have an increased histological score compared to the control mice (p=0.002).
Nanobodies decrease the amount of caudal lymph node lymphocytes, but increase cytokine production

To determine the effect of nanobodies on cytokine production, CLNs were collected after sacrificing the mice and the number of lymphocytes was counted. Thereafter, lymphocytes were stimulated with anti-CD3 and anti-CD28 and supernatant was collected after 24 hours to investigate the IFN-γ and TNF-α production (see figure 3). Oral administration of 3F-3F nanobody results in a significant decrease of the amount of lymphocytes in the CLN compared to the control mice (2.0 x 10⁵ vs. 4.2 x 10⁵, p=0.02) and mice treated with anti-TNF-α mAb (2.0 x 10⁵ vs. 4.1 x 10⁵, p=0.04). Also mice treated with 3F-3F HSA21 showed a decrease in lymphocyte amount compared to the control mice and anti-TNF-α mAb-treated mice, but these data were not significantly different.

![Figure 3](image.png)

Figure 3. Lymphocytes were isolated from the caudal lymph node and counted (a.). Thereafter these lymphocytes were stimulated with anti-CD3 and anti-CD28 for 24 h and supernatant was collected to measure TNF-α (b.) and IFN-γ levels (c.).

Although mice treated with 3F-3F have significantly decreased lymphocyte amounts, these lymphocytes produce significantly higher levels of TNF-α and IFN-γ compared to the control mice (678.8 ± 125.9 pg/ml vs. 82.6 ± 38.8 pg/ml, p<0.0001 and 960.0 ± 240.9 pg/ml vs. 18.5 ± 12.9 pg/ml, p<0.0001) and the anti-TNF-α mAb-treated mice (678.8 ± 125.9 pg/ml vs. 117.6 ± 34.9 pg/ml, p=0.0006 and 960.0 ± 240.9 pg/ml vs. 148.7 ± 119.4 pg/ml, p<0.004). The production of TNF-α and IFN-γ by lymphocytes isolated from mice treated with 3F-3F HSA21 is also increased, but is not significantly different compared to the control group and the anti-TNF-α mAb-treated group.
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Discussion

Although TNF inhibitors such as infliximab and adalimumab are often used in the treatment of CD, these drugs have many side-effects and can only be administered intravenously or subcutaneously. Therefore pharmaceutical companies still develop alternatives to reduce levels of TNF-α in patients with CD and other inflammatory disorders like RA. We have tested two types of nanobodies in a chronic colitis model (CD4+CD45RB\textsuperscript{high} transfer-induced colitis) and an acute colitis model (TNBS-induced colitis). Both nanobodies did not ameliorate CD4+CD45RB\textsuperscript{high} transfer colitis and TNBS-induced colitis. TNF-α is present in two forms, namely as a transmembrane and a soluble protein. Since these nanobodies block the soluble form of TNF-α only and do not decrease inflammatory processes, it seems that sTNF-α is necessary to regulate anti-inflammatory effects and/or that tmTNF-α plays an important role in pro-inflammatory processes. Probably not the soluble form of TNF-α has to be blocked, but the transmembrane form.

It has been shown earlier that infliximab and adalimumab are able to induce apoptosis of T lymphocytes and monocytes, but that the soluble TNF receptor antagonist etanercept is not 30-34. Moreover, in contrast to RA patients, IBD patients do not have clinical benefit of etanercept indicating that in IBD simply neutralising TNF-α is not enough to reduce inflammation 35. In addition, complete inhibition of TNF-α secretion in tmTNF transgenic RAG\textsuperscript{-/-} mice could not prevent or delay colitis by transfer of CD4\textsuperscript{+}CD45\textsuperscript{high} cells, although tmTNF-α seems not to be important in the induction of colitis since these mice also develop colitis if they receive TNF-deficient CD4\textsuperscript{+}CD45\textsuperscript{high} cells 36.

The soluble form of TNF-α has a higher affinity for binding to TNFR1 compared to tmTNF-α 8. It has been shown in mice that are NEMO deficient in intestinal epithelial cells, that they are more sensitive to colitis, since NEMO deficiency activates TNF-α-induced apoptosis, whereas the inactivation of TNFR1 leads to inhibition of intestinal inflammation 37. In contrast to TNFR2, TNFR1 contains a cytoplasmic death domain and
although in general activation of TNFR1 results in the induction of the NF-κB pathway, when a viral infection modifies the metabolic state of the cell the apoptotic pathway through caspase activation is initiated. Blocking of sTNF-α may lead to an impaired apoptosis in IBD patients resulting in survival of reactive T cells which can maintain inflammatory processes. Moreover, tmTNF-α has a higher affinity for TNFR2 and seems to be more involved in cell survival processes instead of cell death. Blocking of only sTNF-α may result in an increased activation of TNFR2 by tmTNF-α and consequently in an increase of cell survival of reactive T lymphocytes.

Interestingly, tmTNF-α functions not merely as a ligand for TNFRs, but it can also acts as a receptor since binding to TNFRs and TNF antagonisten may induce reverse signalling and consequently induce cell activation, cytokine suppression, or apoptosis of the tmTNF-α bearing cell. Although both etanercept and infliximab bind to tmTNF-α, only infliximab is able to induce reverse signalling resulting in apoptosis, IL-10 and TGF-β production and cell cycle G0/G1 arrest. Typically, serum levels of sTNFR1 and sTNFR2 are increased in IBD patients compared to healthy controls and especially sTNFR1 is upregulated in serum of CD patients, indicating that these increased sTNFR levels are a feedback mechanism to endeavour reduction of inflammation, but fail because of a defective pathway in these patients. This hypothesis is supported by the fact that a lack of TNFR2 expression by CD4+ lymphocytes results in an exacerbation of experimental colitis. However, another study showed that also overexpression of TNFR2 in CD4+ lymphocytes results in an exacerbation of experimental colitis, probably through an enhanced NF-κB activation via the membrane form of TNFR2. Moreover, infliximab decreases the expression of tmTNFR2 on monocytes, accordingly reducing the action of TNF-α, but increases the secretion TNFR2 by monocytes, thereby contributing to their neutralising capacity. Taken together, these results and our data demonstrate that TNF-α and its receptors may have different functions during inflammation dependent on the metabolic state of the cell and the microenvironment and that a better understanding of specific characteristics of TNF signalling will be the basis for the development of more efficient therapeutics.
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In conclusion, soluble TNF inhibitors seem not to be effective in experimental colitis, probably because sTNF-α has anti-inflammatory capacities and/ or tmTNF-α functions as pro-inflammatory ligand, which can be blocked by reverse signalling. However, more research is necessary to investigate how sTNF-α and tmTNF-α acts during inflammation and whether tmTNF-α inhibitors are more effective in the treatment of IBD than sTNF-α inhibitors.
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