Reduction of chemokine secretion in response to mycobacteria in infliximab-treated patients

Running Title: Infliximab and tuberculosis

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ABSTRACT

The use of anti-tumour necrosis factor agents as a treatment for chronic inflammatory conditions has been shown to be associated with an increased risk of developing tuberculosis.

We studied the effect of the anti-TNF antibody, infliximab, on antimycobacterial immunity in 26 patients with rheumatoid arthritis or ankylosing spondylitis using an in vitro whole blood model employing a reporter mycobacterium. Blood samples taken before, 30 minutes and 7 days after a 2-hour infliximab infusion were compared both in their ability to suppress luminescence of Mycobacterium bovis Bacille Calmette-Guérin lux and to secrete chemokines and cytokines, 24 and 96 h after infection.

No immediate effect of infliximab on mycobacterial luminescence was detected using this bioassay irrespective of whether patients were receiving their first (n=14) or maintenance (n=12) doses of infliximab. Moreover no effect on mycobacterial luminescence was detected when blood was taken 7 days after infliximab treatment (n=7). By contrast, there was a significant reduction in the chemokines implicated in cellular trafficking namely IL-8, MIP-1α, MIP-1β (24 h and 96 h) and MCP-1 (24 h) following BCG lux infection in the 30-minute post-infliximab infusion blood samples (p < 0.05). This effect was sustained by MIP-1β and MCP-1 (24 h, p < 0.05) at 7 days after infusion.

Our results suggest that development of tuberculosis in infliximab-treated patients is not directly related to the myobactericidal effects of TNF but may be due to inhibition of TNF-dependent chemokine gradients disrupting cellular migration necessary to maintain the integrity of the granuloma.

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INTRODUCTION

Anti-tumour necrosis factor (TNF) agents are increasingly used to treat a range of chronic inflammatory conditions including rheumatoid arthritis (RA), ankylosing spondylitis (AS) and Crohn’s disease (CD). One of the currently approved and widely used agents is infliximab, a chimeric monoclonal antibody (75% human, 25% mouse protein) which targets both soluble and membrane-bound forms of TNF by binding with high avidity and specificity (14). Thus infliximab neutralises the effect of this cytokine and its ability to induce other downstream biological mediators associated with chronic inflammatory diseases.

However anti-TNF therapy is also associated with an increased risk of developing opportunistic infections, particularly tuberculosis (TB) (7, 15, 18, 20, 35). This risk appears to be greater with infliximab than with other similar agents (8, 35, 36, 38) although the mechanisms remain unclear (10). TNF has pleiotropic functions in the host response to TB infection including the promotion of orderly granuloma formation and containment of disease, the induction of macrophage apoptosis as well as the stimulation and release of other cytokines, chemokines and adhesion molecules. However TNF is also believed to contribute to the necrosis, characteristic of TB (16, 37).

Infliximab-associated TB tends to occur early, often within the first 3 months of therapy (20) and in most instances appears to arise from the reactivation of latent TB infection (19). Furthermore, disease is often extrapulmonary, disseminated and associated with poor granuloma formation (9, 14, 21, 23). A number of experimental studies, both in vitro and in animals, indicate the crucial role of TNF in granuloma formation (2, 12, 30). In addition to a possible direct mycobactericidal effect, TNF is also essential for the early expression of chemokines and recruitment of leucocytes (particularly macrophages) to the site of infection (1, 2, 28). Poor
granuloma formation in TB following anti-TNF treatment may therefore be a mechanism for infliximab-associated TB, although data from humans is limited compared to animal models.

One of the recently developed models to study immune responses to mycobacteria in humans, is the *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) *lux* whole blood assay (17). This *in vitro* technique evaluates the metabolic activity (measured as luminescence) of a recombinant reporter strain of mycobacteria when exposed to whole blood (17, 31, 32). In addition, soluble mediators (chemokines and cytokines) secreted into culture supernatant can be measured simultaneously. Differences in the degree of suppression of mycobacterial luminescence have been demonstrated in this model, which relates well to the underlying sensitisation to mycobacterial antigens in healthy individuals and to clinical phenotype.

We employed this whole blood assay and chemokine and cytokine measurement to study antimycobacterial immunity in patients receiving infliximab. Our observations provide a mechanistic insight into how infliximab may predispose individuals to tuberculous infection.

**MATERIALS AND METHODS**

**Human Subjects.** Ethical permission for this study was granted by the Harrow Local Research Ethics Committee (Ethics submission No. 3126). Twenty-six adult patients aged 32-81 years (mean 60 years) with either RA (24/26) or AS (2/26) were recruited from the Rheumatology Clinic at Northwick Park Hospital, Harrow, UK. Patient information was collected on PPD Heaf grade skin status (Heaf grade 0, 16 patients; Heaf grade 1-4, 10 patients) and evidence of BCG scar/history of BCG vaccination (BCG, 8; no BCG 11; undetermined 7). Only one patient had a known past history of previously treated TB. Infliximab treatment was administered to RA and AS patients (3mg/kg and 5mg/kg respectively) at 0, 2 and 6 weeks
(loading regime) and every 8 weeks thereafter (maintenance therapy). All doses were given as a 2 h infusion, following standard protocols. Each patient served as their own internal control to avoid confounding factors such as the use of other drugs and severity of disease.

Peripheral blood (10ml) was taken from each patient prior to starting the infusion of infliximab (pre anti-TNF) and a second blood sample was taken approximately 30 minutes after completion of the infusion (post anti-TNF) i.e. 2.5 h after the start of the infusion. Seven of the 26 patients had an additional blood sample taken 7 days later (7-day post anti-TNF). Whole blood was collected in sodium heparin vacutainers (BD Biosciences) and diluted with an equal volume of RPMI 1640 (Sigma) containing L-glutamine and sodium bicarbonate supplemented with 12mM HEPES (Sigma).

**Reporter BCG.** *Mycobacterium bovis* BCG, Montreal strain, obtained from Professor Young’s group, Imperial College London, was transformed with the reporter plasmid construct, pSMT1, as previously described in detail elsewhere (31). This plasmid carries the lux AB genes from *Vibrio harveyi* under the control of a mycobacterial strong constitutive promoter (*hsp 60*). *M. bovis* BCG lux was grown to mid-log phase in Middlebrook 7H9 broth (Difco, Detroit, MI) containing 0.2 % glycerol, 0.05 % tween 80, 10% ADC (Albumin, Dextrose, Catalase, Difco, Detroit, MI) enrichment and 50 µg/ml hygromycin (Roche). Aliquots were prepared in 15 % glycerol and stored at –80 °C. The number of colony forming units (CFU) per millilitre was determined by serial dilutions on 7H11 agar (Difco, Detroit, MI) containing 0.5% glycerol, 10% OADC (Oleic acid, Albumin, Dextrose, Catalase, Difco, Detroit, MI) enrichment and 50 µg/ml hygromycin. Prior to each assay a vial of BCG lux was thawed and inoculated into 15 ml 7H9 broth and incubated at 37 °C for 48 h in a shaking incubator (150 rpm). Mycobacteria were then
diluted in phosphate buffered saline (PBS, Sigma) to obtain a luminescence reading of $1 \times 10^7$ relative light units / ml (RLU / ml). Luminescence was determined by preparing serial 10-fold dilutions in PBS (1ml total volume) and measuring for a period of 20 s in a luminometer (Berthold Autolumat LB953) following injection of 0.1ml of substrate (1% n-decyl aldehyde, Sigma). Luminescence readings are expressed as RLU. Throughout these experiments approximately three RLU corresponded to one CFU.

**BCG lux whole blood assays.** The BCG lux whole blood assay has been described in detail elsewhere (17). In brief BCG lux was inoculated in diluted whole blood (in triplicate) prepared as described above to give a final inoculum of $3 \times 10^5$ CFU/ml (1 $\times 10^6$ RLU/ml) (Multiplicity of infection 1 CFU: 1 monocyte). Luminescence was measured at the time of inoculation ($T_0$) and at 24 h and 96 h following incubation at 37 °C. Mycobacterial metabolic activity (measured as luminescence) was expressed as a Luminescence Ratio (LR) calculated as follows: 

$$LR = \frac{\text{RLU of BCG at } T_{24}}{\text{RLU of BCG at } T_0} \text{ or } \frac{\text{RLU of BCG at } T_{96}}{\text{RLU of BCG at } T_0}$$

for the 24 hr and 96 hr time-points respectively. All assays were performed double blinded.

**Cytokine and chemokine assays.** Supernatants were aspirated from BCG lux infected whole-blood samples at 0 h, 24 h and 96 h and from uninfected blood samples at 0 h (uninfected chemokine/cytokine controls). ELISA assays were performed using the commercially available DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN). The sensitivity of the assays was 5 pg/ml (Interleukin [IL]-6), 31 pg/ml (IL-10), 17 pg/ml (Interferon γ [IFN−γ]) and 96 pg/ml (IL-8). Assays for Interferon Inducible Protein of 10kDa (IP-10), Macrophage Inhibitory Protein-1α (MIP-1α), Macrophage Inhibitory Protein-1β (MIP-1β), Monocyte Chemoattractant
Protein-1 (MCP-1), Regulated on Activation Normal T-cell Expressed and Secreted (RANTES) and IL-12p40 were determined using Beadlyte Luminex multiplex assays (Upstate Ltd, Charlottesville, VA). The sensitivity of the assays was 2 pg/ml, 3 pg/ml, 8 pg/ml, 30 pg/ml, 4 pg/ml and 3 pg/ml respectively. All assays were performed following the manufacturer’s recommendations.

**Statistical analysis** All data were tested for normal Gaussian distribution but were not found to be normally distributed. Therefore the Wilcoxon signed-rank test was used to analyse non-parametric data. Statistical significance was assumed when p<0.05. All data quoted are median and interquartile range (IQR).

**RESULTS**

**Mycobacterial luminescence before and immediately after infliximab infusion.** To determine whether treatment of RA or AS patients with infliximab leads to increased growth of BCG *lux*, we simultaneously inoculated whole blood from 26 patients, taken before and approximately 30 minutes after infliximab infusion (irrespective of infliximab infusion number) with $10^5$ CFU/ml BCG *lux* mycobacteria and the luminescence ratios were compared.

There was no significant difference in the luminescence of BCG *lux* in pre- and post infliximab infusion blood samples after 24 h incubation (pre-infliximab median 0.64, IQR 0.3-1.86; post-infliximab median 0.76, IQR 0.27-2.15; p=0.43) or 96 h incubation (pre-infliximab median 1.47, IQR 0.3-3.47; post-infliximab median 1.44, IQR 0.4-4.8; p=0.68) (Fig.1).

We postulated that mycobacterial luminescence pre- and post- infusion might vary according to the number of infliximab infusions received. We therefore stratified the data by
consideration of the 14/26 pairs of samples that were taken before and after the first dose of infliximab and the 12/26 that were taken during maintenance therapy (2nd to 10th infliximab dose). No significant impairment in antimycobacterial activity was detected at 96 h in either of the sample groups (First infliximab infusion 96 h, pre-infliximab median 1.16, IQR 0.34-3.47; post-infliximab median 1.45, IQR 0.40-2.0; p=0.326.) (Maintenance therapy 96 h, pre-infliximab median 1.57, IQR 0.96-3.37; post-infliximab median 1.39, IQR 0.76-4.80; p=0.677) (Data not shown). Luminescence ratios at 24 h and 96 h after infliximab infusion were also stratified according to PPD skin test (Heaf grade 0 and Heaf grade 1-4), the presence/absence of BCG scar/history of BCG vaccination and underlying disease (RA / AS). No significant differences were noted (Data not shown). The addition of varying concentrations of infliximab or an anti-TNF antibody (R & D Systems) (18), added in vitro to whole blood from ‘healthy adults’ did not have any direct effect on BCG-lux luminescence in our whole blood assay. Data not shown.

Chemokine and cytokine production before and immediately after infliximab infusion. Supernatants harvested from BCG-lux infected cultures at 24 and 96 h and from uninfected chemokine/cytokine control cultures at 0 h were analysed for secretion of chemokines and cytokines, which are TNF-dependent and/or play a significant role in antimycobacterial immunity (IL-10 and IFN-γ production were only analysed in the 96 h supernatants). TNF was not assayed because of the confounding presence of infliximab, which may have interfered with ELISA results. There was a significant reduction in median IL-8 (24 h, 2.5 fold, p<0.0001; 96 h, 1.28 fold p=0.011), MIP-1α (24 h, 2.7 fold, p=0.0027; 96 h, 3.3 fold, p=0.0049) and MIP-1β (24 h, 1.8 fold, p=0.0002; 96 h, 2.1 fold, p=0.0379) secretion at both 24 h and 96 h in the BCG lux infected post-infliximab infusion blood samples (Fig. 2). A significant reduction was seen in
MCP-1 production in the post infliximab infusion sample at the 24 h time point only (24 h, 1.15 fold, p=0.0032). There was no significant difference in IP-10 or RANTES chemokine production (Fig. 2), or in IL-6, IL-10, IL-12p40 and IFN-γ cytokine secretion in either 24 or 96 h pre- and post infusion samples (Data not shown).

To ensure that the levels of IL-8, MIP-1α, MIP-1β and MCP-1 production and the significant differences observed at 24 and /or 96 h pre- and post- infusion were not already present at baseline (0 h) and hence were due to the effect of infection with BCG lux and the presence of infliximab, we measured these chemokines in the uninfected chemokine/cytokine control supernatants at 0 h. There was no difference in IL-8 (Pre-infliximab median 0 pg/ml, post-infliximab median 0 pg/ml, IQR 0-214.8; p=0.156) or MIP-1α (Pre-infliximab median 7.72 pg/ml, post-infliximab median 6.08 pg/ml IQR 4.80-43.54; p=0.06) production between pre- and post- infusion blood samples. Furthermore, we observed increased levels of chemokine production at both subsequent time-points (24 and 96 h) clearly demonstrating that these chemokines were induced by the infecting BCG lux organism (Figure 2).

By contrast we noted a statistically significant decrease in MIP-1β and MCP-1 production at 0 h in the post-infliximab blood samples (MIP-1β, 1.55 fold p=0.0016; MCP-1, 1.23 fold p=0.0123). However production of these chemokines was extremely low at 0 h in both pre- and post- infliximab samples (MIP-1β pre median 32 pg/ml, post median 21 pg/ml; MCP-1 pre median 103 pg/ml, post median 84 pg/ml) compared to the significantly increased levels in response to BCG lux infection at 24 h ( MIP-1β pre median 31071 pg/ml, post median 17241 pg/ml; MCP-1 pre median 18783 pg/ml, post median 16287 pg/ml) and 96 h (MIP-1 beta pre median 12638 pg/ml, post median 5958 pg/ml; MCP-1 pre median 50418 pg/ml, post median
48203 pg/ml) (Figure 2) where subsequently differences in chemokine production occurred between the pre- and post-infusion samples due to the presence of infliximab.

Mycobacterial luminescence and cytokine / chemokine production before and 7 days after infliximab infusion. Although the BCG lux infected whole blood cultures were incubated for 96 h, the actual blood sample containing infliximab was taken 30 minutes after the infusion. This sample and assay schedule may not have allowed adequate time for infliximab to exert its potential immunosuppressive action. We therefore extended our analysis in a subgroup of 7 patients receiving their first dose of infliximab and repeated the assay at 7 days after infliximab infusion, the half-life of infliximab being approximately 8.5 days. In this subset of donors there was again no significant difference in BCG lux luminescence between the pre and 7 day post-infliximab samples at 24 h and 96 h (24 h pre-infliximab median 0.47, IQR 0.3-1.36; 7-day post-infliximab median 0.93, IQR 0.45-1.46, p=0.109, 96 h pre-infiximab median 0.85, IQR 0.34-1.5; 7-day post-infliximab median 1.22, IQR 0.45-2.46, p=0.156) (Fig. 3A). However we did observe a significant reduction in both MIP-1β (24 h, 1.75 fold, p=0.0469) and MCP-1 (1.9 fold, p=0.032) in the 7 day post infliximab infusion blood samples 24 h after infection (Fig. 3B). This effect was not sustained for either chemokine at 96 h. Although there were no other statistically significant reductions in chemokine or cytokine production, a trend in reduced secretion was noted for all other chemokines at the 24 h timepoint (Fig. 3). No differences in chemokine/cytokine levels between the pre- and 7 day post-infliximab blood samples were detected in the uninfected chemokine/cytokine controls at 0 h (Data not shown).
DISCUSSION

TNF appears to play a central role in the host-immune response to Mycobacterium tuberculosis being involved in both pathogenesis and protection (12). We set out to study the effects of the anti-TNF drug, infliximab on antimycobacterial immunity in RA / AS patients using an in vitro whole blood assay employing recombinant reporter mycobacteria (BCG lux whole blood assay).

Evidence from both animal models and humans shows that anti-TNF treatment, in addition to its valuable therapeutic ability to reduce the severity of inflammatory disease, is associated with increased susceptibility to opportunistic infection, particularly TB (19) and other unusual atypical mycobacterial infections (6, 24, 26). Rapid and lethal reactivation of TB infection has been reported in TNF-deficient mice with high bacterial loads in the lungs, spleen and liver (5). Similarly TNF receptor 1 (p55 receptor) knockout (TNFR1−/−) mice and mice treated with neutralizing TNF antibody has been shown to be extremely susceptible to M. tuberculosis infection; loss of granuloma structure was a striking observation (13). These findings, emphasising the important role of TNF in antimycobacterial immunity, have also been confirmed by numerous other studies summarised in the reviews by Ehlers (9) and Tufariello et al. (34).

Our initial hypothesis was that the administration of the anti-TNF agent, infliximab would lead to increased mycobacterial luminescence in the BCG lux whole blood assay as an expression of decreased antimycobacterial immune responses. However this was not found to be the case. There was no difference in mycobacterial luminescence in whole blood assays performed both before, and 30 minutes after infliximab infusion. This was irrespective of whether patients where receiving their first or subsequent maintenance doses of infliximab. Although samples were taken relatively soon after a 2 h infliximab infusion, there was a considerable subsequent time period in
vitro (96 h) in which the effects of the anti-TNF antibody could manifest. Moreover as anti-TNF may take some time to modify overall immunological responses in vivo, and since the drug has a half-life of approximately 8.5 days (25), an additional blood sample taken 7 days after infliximab infusion also demonstrated no differences in mycobacterial luminescence in blood taken before and 7 days after infliximab infusion. These results are similar to studies by Saliu et al. (29) and Kampmann et al. (17) in which neither infliximab nor an anti-TNF antibody (R & D Systems), added to whole blood in vitro, had any effect on the control of *M. tuberculosis* or BCG lux growth respectively. For practical reasons we used the BCG lux organism in this whole blood model rather than *M. tuberculosis*. It is possible that different immunological and molecular mechanisms involved in the restriction of the growth of the pathogenic strains exist.

In addition to measuring antimycobacterial activity using the BCG lux assay, we analysed the changes in a number of pivotal chemokines and cytokines involved in anti-mycobacterial immunity. Furthermore the expression of many of these biological mediators is evident in rheumatoid joints (4, 11). Saliu et al. (29) showed that the addition of infliximab in vitro suppressed IFN-γ and IL-10 production in whole blood infected with *M. tuberculosis* culture filtrate. In addition infliximab also reduced the proportion of tuberculosis-responsive CD4 cells by 70%. In contrast a recent report demonstrated that short-term inflixmab treatment (2 to 14 weeks) in RA patients did not affect the production of IFN-γ and other pro-inflammatory (IL-1β, IL-6) and immunosuppressive cytokines (IL-10) when whole blood cultures were stimulated with heat-killed *M. tuberculosis* in vitro, although RA patients already had reduced capacity to release IFN-γ when compared to healthy controls (27). Our observations correlate with these findings in that no differences were observed in IFN-γ, IL-6, IL-10 or IL-12p40 production post infliximab infusion. However we found a highly significant reduction at both 24 h and 96 h post-infection in
the levels of chemokines IL-8 (CXCL8), MIP-1α (CCL3), MIP-1β (CCL4) and MCP-1 (CCL2) (96 h only) in the culture supernatants of blood taken 30 minutes after infliximab infusion. Similarly, macrophage production of C-X-C and C-C chemokines is reduced in response to *M. tuberculosis* infection by an anti-TNF antibody added in vitro (2, 3), delayed chemokine induction has been shown in TNF deficient mice (28) and a significant reduction in serum IL-8, MCP-1 and RANTES, occurred in patients with active RA post initial infusion of infliximab (22).

However when we examined a small subset of 7 patients whose blood was taken 7 days after infliximab infusion, the reduction was only sustained for MIP-1β and MCP-1 (24 h) although a trend of reduction in all chemokine secretion was observed post infliximab. This might be because the effect of anti-TNF on production of certain chemokine production occurs early in infection or is short-lived; alternatively it may be that this analysis was underpowered to detect these effects. The expression of chemokines appears to be partially controlled by TNF production (33). Algood et al. (2) demonstrated that chemokine production was not completely abrogated in bone marrow derived murine macrophages following neutralisation of TNF or in TNFR1−/− macrophages. Infection of TNF−/− mice with *M. tuberculosis* also resulted in an initial 2 week delay in induction of chemokine mRNA, but thereafter chemokine expression developed independently of TNF (28) suggesting that other pathways or molecules may also be involved in stimulation of chemokines. This may explain why the effect of infliximab on cytokine secretion in this assay, was not sustained in the 7 day blood sample.

C-C chemokines including MIP-1α, MIP-1β, MCP-1 and RANTES and the C-X-C chemokines, IL-8 and IP-10, produced by various cell types including neutrophils, monocytes/macrophages and lymphocytes in response to mycobacterial infection, play a potent
role in the orchestrated cellular recruitment and trafficking of inflammatory cells (reviewed by Algood et al.) (1) vital for granuloma formation and thus containment of mycobacteria (30).

In summary, our data suggest that the development of TB in infliximab treated patients is not directly related to the myobactericidal effects of TNF but may be due to inhibition of TNF-dependent chemokine gradients disrupting cellular migration necessary to maintain the integrity of the granuloma. This implies that granulomas are dynamic and active structures with considerable turnover, even in latent tuberculosis.

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**FIGURE LEGENDS**

**Figure 1.** BCG _lux_ luminescence in whole blood taken before (pre anti-TNF) and 30 minutes after (post anti-TNF) infliximab infusion from 26 adult patients with rheumatoid arthritis (24/26) or ankylosing spondylitis (2/26). The metabolic activity of BCG _lux_ was determined as a Luminescence Ratio (LR) at 24 and 96 h following infection in both the pre and post blood samples using the following formula: \( LR = \frac{\text{Relative Light Units of BCG } lux \text{ at } 96 \text{ h (or } 24 \text{ h) / \text{relative light units of BCG } lux \text{ at } 0 \text{ h}}. \) _Horizontal lines_ show median values for each group. NS - Not significant using the Wilcoxon signed-rank test.

**Figure 2.** Chemokine (IL-8, IP-10, MIP-1α, MIP-1β, MCP-1, RANTES) production was measured in BCG _lux_ infected whole blood taken before (pre anti-TNF) and 30 minutes after (post anti-TNF) infliximab infusion from 26 adult patients with RA or AS. Pre and post blood samples inoculated in triplicate were incubated for 24 and 96 h. Supernatants were harvested at each of these time-points and analysed by ELISA or Luminex Multiplex assays. _Horizontal lines_ represent the median for the group. NS - Not significant using the Wilcoxon signed-rank test.

**Figure 3.** (A) BCG _lux_ luminescence in whole blood taken from 7 patients before (pre anti-TNF) and 7 days after (7-day post anti-TNF) receiving their first infliximab infusion. Luminescence was measured after 24 h and 96 h incubation. Each point represents the median of triplicate determinations. _Horizontal lines_ represent median values for the group. NS - Not significant using the Wilcoxon signed-rank test.

(B) Chemokine (IL-8, IP-10, MIP-1α, MIP-1β, MCP-1, RANTES) production was measured in BCG _lux_ infected whole blood of 7 patients receiving their first infliximab infusion. Whole blood
was taken before (pre anti-TNF) and 7 days after (7-day post anti-TNF) infliximab infusion. Infected pre and 7-day post blood samples were incubated for 24 and 96 h. Supernatants were harvested at each of these time-points and analysed by ELISA or Luminex Multiplex assays. Horizontal lines represent median values for the group. NS - Not significant using the Wilcoxon signed-rank test.
Figure 1

[Graph showing luminescence ratio over time with pre and post anti-TNF measurements at 24 h and 96 h, with NS annotations for lack of significance.]
Figure 2

- IL-8 (ng/ml)
  - pre anti-TNF
  - post anti-TNF
  - p = 0.0001

- IP-10 (ng/ml)
  - pre anti-TNF
  - post anti-TNF
  - p = 0.011

- MIP-1 alpha (ng/ml)
  - pre anti-TNF
  - post anti-TNF
  - p = 0.0027

- MIP-1 beta (ng/ml)
  - pre anti-TNF
  - post anti-TNF
  - p = 0.0379

- MCP-1 (ng/ml)
  - pre anti-TNF
  - post anti-TNF
  - p = 0.0002

- RANTES (ng/ml)
  - pre anti-TNF
  - post anti-TNF
  - p = 0.0032
Figure 3B
Figure 1
Figure 2

[Graphs showing IL-8, IP-10, MIP-1alpha, MIP-1beta, MCP-1, and RANTES levels before and after anti-TNF treatment.]

- **IL-8 (ng/ml):**
  - Pre anti-TNF: 0.1, 1, 10, 100, 1000
  - Post anti-TNF: 0.1, 1, 10, 100, 1000
  - p < 0.0001

- **IP-10 (ng/ml):**
  - Pre anti-TNF: 0.1, 1, 10, 100, 1000
  - Post anti-TNF: 0.1, 1, 10, 100, 1000
  - p = 0.0027

- **MIP-1alpha (ng/ml):**
  - Pre anti-TNF: 0.1, 1, 10, 100, 1000
  - Post anti-TNF: 0.1, 1, 10, 100, 1000
  - p = 0.0032

- **MIP-1beta (ng/ml):**
  - Pre anti-TNF: 0.1, 1, 10, 100, 1000
  - Post anti-TNF: 0.1, 1, 10, 100, 1000
  - p = 0.0032

- **MCP-1 (ng/ml):**
  - Pre anti-TNF: 0.1, 1, 10, 100, 1000
  - Post anti-TNF: 0.1, 1, 10, 100, 1000
  - p = 0.0379

- **RANTES (ng/ml):**
  - Pre anti-TNF: 0.1, 1, 10, 100, 1000
  - Post anti-TNF: 0.1, 1, 10, 100, 1000
  - NS
Figure 3A

[Diagram showing luminescence ratio measurements over time for different conditions: Pre anti-TNF, 7 day post anti-TNF, and 96 h post anti-TNF. The y-axis represents luminescence ratio, and x-axis represents time points. The graph indicates no significant difference (NS) between Pre anti-TNF and 7 day post anti-TNF, and between 7 day post anti-TNF and 96 h post anti-TNF.]
Figure 3B