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# Anti-TNF therapy in rheumatoid arthritis: Searching for mechanisms of effect

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# 2

# *Monocyte migration to the synovium in rheumatoid arthritis patients treated with adalimumab*

*Ann Rheum Dis. 2011 Jun;70(6):1160-2.*

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## ABSTRACT

### Objectives

The mechanism of action of treatment with tumor necrosis factor (TNF) blockers in rheumatoid arthritis (RA) is still not completely understood. The aim of this study was to test if adalimumab treatment could affect the influx of monocytes into the synovium.

### Methods

We used a novel technique to analyze the migration of labelled autologous monocytes before and 14 days after initiation of adalimumab treatment using scintigraphy. CD14+ monocytes were isolated from patients with RA, using a positive selection procedure with magnetic-activated cell sorting, and labeled with $^{99m}$Tc-HMPAO. Scintigraphic scans were made 1 hour, 2 hours and 3 hours after re-infusion.

### Results

Already 14 days after the start of treatment with adalimumab a significant decrease in DAS28 was shown. Of importance, there was no significant decrease in the influx of monocytes into the joint at this time point.

### Conclusions

This study indicates that adalimumab treatment does not reduce the influx of monocytes into the synovium early after initiation of treatment. As previous studies showed a rapid decrease in macrophage infiltration after TNF- antibody therapy, which could not be explained by increased cell death, this points to an important role for enhanced efflux of inflammatory cells from the synovium.

## INTRODUCTION

Disease control has been improved by the use of tumor necrosis factor (TNF) blockade in patients with rheumatoid arthritis (RA) and other immune-mediated inflammatory diseases. However, the mechanisms by which TNF antagonists exert their effect is still not completely understood[1]. Anti-TNF antibody treatment has been shown to result in marked reduction of synovial inflammation in both RA and psoriatic arthritis[2-4]. This decrease in synovial cellularity could be observed as early as 24-48 hours after initiation of treatment[5-7].

This early reduction in synovial inflammation after TNF blockade could not be explained by apoptosis induction at the site of inflammation[6, 7], leaving either reduced cell influx or enhanced cell efflux to explain this process. In one study infliximab treatment significantly decreased the influx of $^{111}$In-labeled granulocyte migration into affected joints of RA patients [4] and in another study adalimumab significantly reduced influx of $^{99}$Tc-labeled leukocytes, whereas no decrease in influx was seen in patients treated with placebo[8].

Monocytes and macrophages are key players in the pathogenesis of RA[9]. Furthermore, the decrease in macrophage numbers in the synovium is associated with clinical improvement after effective treatment[10]. Therefore, we examined the effect of adalimumab treatment on monocyte migration towards the synovial compartment. We recently developed a procedure using a combination of immunomagnetic cell selection with CD14 coated beads, labeling with technetium-99m ($^{99m}$Tc)- hexamethylpropylene-amino-oxime (HMPAO) and scintigraphy to visualize the migratory behavior of autologous monocytes[11]. Applying this method, we showed continuous migration of monocytes into the inflamed synovial tissue of RA patients at a slow macrophage-replacement rate[12]. The slow rate of monocyte influx into the synovial compartment suggests that the rapid effect of anti-TNF therapy on macrophage infiltration cannot merely be explained by blockade of cell influx, as previously thought. Hence, we used this novel imaging technique to directly test if adalimumab treatment could affect the influx of monocytes into the synovium.

## PATIENTS & METHODS

### Patients

Eight patients with established RA according to the revised American College of Rheumatology criteria for the diagnosis of RA[13] were included. All patients had an indication for the use of anti-TNF therapy according to the guidelines of the Dutch Society for Rheumatology, which is active disease status (disease activity score evaluated in 28 joints (DAS28) ≥3.2) despite treatment with 2 conventional disease-modifying antirheumatic drugs (DMARDs). In this study all patients started with adalimumab (40 mg subcutaneously every other week) 24 hours after the baseline scans. Three patients used maximally tolerable methotrexate at a stable dosage (10-25 mg/ week). The others received adalimumab monotherapy. The use of concomitant non-steroidal anti-inflammatory drugs (NSAIDs) was permitted if stable for at least one month prior to baseline and was kept stable throughout the study. All patients provided written informed consent and approval was granted by the local medical ethics committee.

### Isolation and labeling of monocytes

Isolation and labeling of monocytes was performed as described earlier (See supplementary text).

### Scintigraphy and signal calculations

Scintigrapy and signal calculations were done as described previously (See supplementary text). This procedure was done at day -14, baseline and day 14.

### Statistical analysis

Data were analyzed using the Wilcoxon signed ranks test to determine significant changes from baseline. Correlations were calculated using the Spearman's rank correlation coefficient. Values are expressed as median and interquartile range. The calculations were performed with SPSS 16.0 for Windows

## RESULTS

### Patient characteristics

Half of the patients were IgM rheumatoid factor positive and all had erosive disease. Their median age was 53 (48.5-57) years and median disease duration was 162 (119-273) months. Individual patient characteristics are shown in Table 1.

### Monocyte influx is stable and not markedly decreased early after initiation of adalimumab treatment

Scans were made 14 days prior to the start of adalimumab treatment, at baseline and 14 days after the start of adalimumab treatment (see supplementary figure 1). The number of labeled

**Table 1** Baseline patient characteristics

| | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 | Patient 7 | Patient 8 |
|---|---|---|---|---|---|---|---|---|
| TJC of 28 joints | 4 | 6 | 25 | 16 | 12 | 23 | 4 | 17 |
| SJC of 28 joints | 3 | 6 | 4 | 12 | 6 | 20 | 15 | 7 |
| VAS general disease activity | 47 | 19 | 87 | 71 | 59 | 89 | 70 | 67 |
| ESR (mm/h) | 35 | 6 | 97 | 14 | 10 | 43 | 58 | 16 |
| C-reactive protein (mg/L) | 4.6 | 7.2 | 85.6 | 5.9 | 5.1 | 17.5 | 34.7 | 3.0 |
| DAS28 | 4.75 | 3.58 | 7.58 | 5.91 | 5.06 | 7.82 | 6.03 | 5.3 |
| Sex (0=male) | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| Age (years) | 59 | 53 | 39 | 58 | 47 | 53 | 53 | 54 |
| Disease duration (months) | 114 | 264 | 132 | 42 | 456 | 168 | 276 | 156 |
| Rheumatoid factor (1=positive) | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 |
| Erosive disease (1=yes) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

TJC = Tender joint count, SJC= Swollen joint count, VAS= Visual analogue scale, ESR= erythrocyte sedimentation rate, DAS28= disease activity score in 28 joints.

monocytes in the joint of interest per patient was comparable 1, 2 and 3 hours after re-infusion. This was true for day-14, baseline and day 14. (See supplementary table). Monocyte influx per patient at three hour post-infusion is shown in Figure 1. Of interest, there was no significant change in monocyte influx 1, 2 and 3 hours after re-infusion from day -14 to day 1, which was before the start of adalimumab treatment (p=0.33, p = 0.67, p=0.21, respectively). The heterogeneity between patients is consistent with changes in synovial macrophages that were found in serial biopsies of patients treated with placebo[14]. Importantly, the influx of monocytes did not decrease after re-infusion of monocytes comparing day 14 to baseline, indicating that adalimumab treatment did not affect the influx of monocytes early after initiation of treatment (Figure 1).

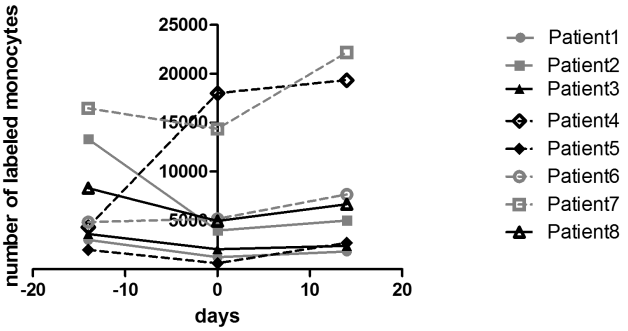


**Figure 1.** Number of monocytes in joint of interest 3 hours post re-infusion per patient at day -14, 1 and 14

### Clinical benefit of adalimumab treatment

The change in DAS28 from screening to day 84 is shown in Figure 2. The median DAS28 at baseline was 5.9 (4.8-7.2). The DAS28 did not change significantly between screening and baseline (p=0.58). There was a statistically significant decrease in median DAS28 from baseline to day 14 of 1.2 (0.72-1.8, p=0.01). At day 84 the median DAS28 had decreased to 2.8 (2.5-3.6) (Figure 2). Three patients were EULAR moderate responders to treatment and five were EULAR good responders. There was no correlation between the decrease in DAS28, ESR or CRP and the change in monocyte influx into the joints at 1 hour, 2 hours or 3 hours after re-infusion from baseline to 14 days (see supplementary figure 2).

## DISCUSSION

The results of this mechanistic study indicate that CD14+ monocyte influx into the synovium is not decreased two weeks after the start of adalimumab treatment. Earlier, TNF blockade was shown not to induce apoptosis in the synovium early after initiation of treatment, although we cannot exclude the possibility that more long-term treatment would lead to a more pro-apoptotic state, being a result of dampening of inflammation rather than its cause[1]. This suggests that the rapid decrease in synovial macrophage numbers observed after anti-TNF treatment

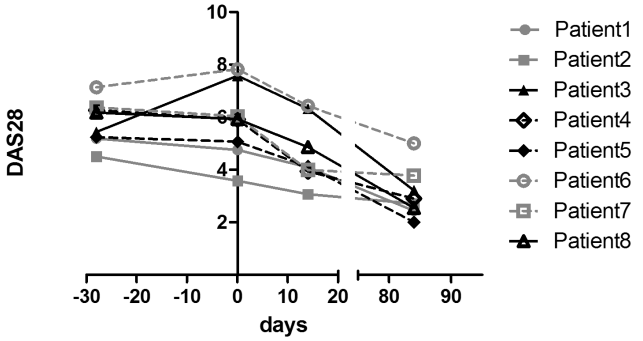


**Figure 2.** DAS28 from screening to day 84

cannot be explained by an immediate effect on monocyte influx, as previously hypothesized. These data strengthen the recent observation that monocytes migrate towards the inflamed RA synovium at a slow macrophage-replacement rate[12], which already suggested that blockade of monocyte migration would be insufficient to explain the rapid reduction of macrophage numbers found after anti-TNF treatment. Consistent with the hypothesis that TNF blockade does not interfere with high levels of monocyte influx into the synovial compartment, numbers of peripheral blood monocytes were not increased after initiation of infliximab treatment[7]. This lack of reduction in monocyte influx is in contrast with previous work showing a reduction of labeled neutrophils into affected joints after treatment with TNF-α blockers[4, 8]. Of note, there is a clear difference in replacement rate for neutrophils compared to monocytes, as large numbers of neutrophils traffic into the synovial fluid[15]. In addition, the difference might be explained in part by the differential use of adhesion molecules between neutrophils and monocytes. Very late antigen-4/ vascular cell adhesion molecule-1 (VLA-4 /VCAM-1) dependent rolling is seen mostly in monocytes whereas adhesion of neutrophils is very much dependent on lymphocyte function- associated antigen 1/inter cellular adhesion molecule-1[16]. The reduced serum levels of sICAM-1 but not sVCAM-1 (reflecting expression of adhesion molecules by endothelial cells) after administration of infliximab could perhaps explain the sustained influx of monocytes while neutrophil influx is reduced[17].

In contrast to sVCAM-1 levels in peripheral blood, VCAM-1 expression in the synovial tissue is reduced after anti-TNF treatment[3, 7]. VCAM-1 is especially highly expressed by fibroblast-like synoviocytes, and may play a crucial role in retention and survival of infiltrating monocytes/ macrophages[18]. Similarly, ICAM-1 expression is decreased in the tissue after TNF blockade. Thus, decreased expression of adhesion molecules [3] as well as chemokines [4] in the tissue may facilitate macrophage egress from the synovium, resulting in decreased macrophage numbers in the synovial tissue. In line with increased cell egress after anti-TNF treatment, we found that anti-TNF therapy results in increased lymphatic vessel formation in the synovium[19].

Taken together, these data suggest that at this early time point the anti-inflammatory effect of treatment with TNF blockers can only partly be explained by downregulation of

vascular adhesion molecules on endothelial cells and subsequent reduction in migration of inflammatory cells into the joint. Anti-TNF treatment may diminish RA disease activity by decreasing inflammatory cell retention in the synovial tissue e.g., by reducing integrins expressed by fibroblast-like synoviocytes. Accordingly, treatments aimed at integrins such as anti-LFA-1 (efatizumab) and anti-VLA-4 were shown to be effective treatment in inflammatory diseases, though serious infectious side effects were reported[20, 21].

In conclusion, this study indicates that treatment with adalimumab does not reduce the influx of monocytes into the synovium two weeks after the start of treatment. In previous work early reduction in synovial inflammation after TNF blockade could not be explained by apoptosis induction at the site of inflammation. Based on these studies, we hypothesize that efflux of inflammatory cells is a major contributor to the rapid reduction of cellularity after initiation of anti-TNF antibody therapy.

## ACKNOWLEDGEMENTS

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## SUPPLEMENTARY MATERIAL

### Isolation and labeling of monocytes

Hundred milliliters of peripheral blood was taken from each patient. CD14+ monocytes were isolated using a positive selection procedure with magnetic-activated cell sorting according to the manufacturer's protocol (MACS®Miltenyi Biotech, Bergisch Gladbach, Germany). After selection fluorescence-activated cell sorting (FACS) analysis with anti-CD-14, anti-CD3 and anti-CD66 antibodies was done to determine the purity of the sample and the recovery of CD14 positive cells. The CD14+ enriched cells were resuspended in 10 ml buffer containing 0.9% (w/v) NaCl, 20% (w/v) human serum albumin (Sanquin Blood Supply Foundation division of Plasma Products, Amsterdam, the Netherlands) and 3.8% (w/v) TNC (NVI, Bilthoven, the Netherlands) for labeling.

Exametazime (Ceretec™, RVG16226) was supplied as a ready-for-labeling kit (GE Healthcare B.V., Amersham, Cygne Centre, Eindhoven, the Netherlands). 99mTc-pertechnetate was obtained from a 99Mo-carrying Ultratechnekow® FM generator (DRN 4329, Tyco Healthcare, Mallinckrodt Medical, Petten, the Netherlands) and was eluted in accordance with the instructions of the manufacturer. Radiochemical purity control (RPC) assays were done by means of chromatography on ITLC-SG strips, using a mobile phase of 0.9% sodium chloride (NaCl). (1) Radiolabeling of cells was performed as described earlier.(2) Briefly, the cells were centrifuged and freshly prepared 99mTc-HMPAO of very high specific activity in a low volume was added to the monocyte cell pellet. After incubation the excess of 99mTc-HMPAO was diluted and subsequently removed from the cell pellet after centrifugation. The labeled monocytes were resuspended in 0.9% NaCl and re-infused into the same patient.

### Scintigraphy

An average of 20×106 monocytes labeled with 200 MBq 99mTc-HMPAO was injected intravenously within 15 minutes after radiolabeling. Whole body imaging was performed at 15 minutes and 1, 2, 3, and 20 hours post infusion using a dual head gammacamera (140 keV, window 15%, 256×1024 matrix, 10 cm/min) fitted with low energy all purpose collimators (Siemens Ecam (Siemens Healthcare, Hoffman Estates, USA) equipped with low energy high resolution collimators). Detail images of the hands (palmar) and feet (plantar) were acquired in a 256×256 matrix for 5 minutes. This procedure was repeated two weeks after the baseline scintigraphy.

### Signal Calculations

The scintigraphic scans were analyzed for signal intensity in joints and other tissues. One joint (joint of interest) was selected for more detailed quantification. The joint of interest was selected because it was a large clinically inflamed joint (ankle, knee or wrist). As these joints are large the decrease in influx of monocytes is expected to be the largest (in absolute numbers) and more sensitive to change. The signal intensity was calculated in counts per region of interest, subtracting the background signal from the joint signal. The background signal is caused by physiological distribution of Tc-99m-labelled monocytes in bone marrow, inferior to uptake in inflamed joints. For correction purposes physiological bone marrow uptake in the vicinity of the affected joint was measured. A correction was made for the number of re-infused

monocytes and the injected dose, using a standard dose source, leading to a deduction of the percentage of re-infused monocytes per ROI.



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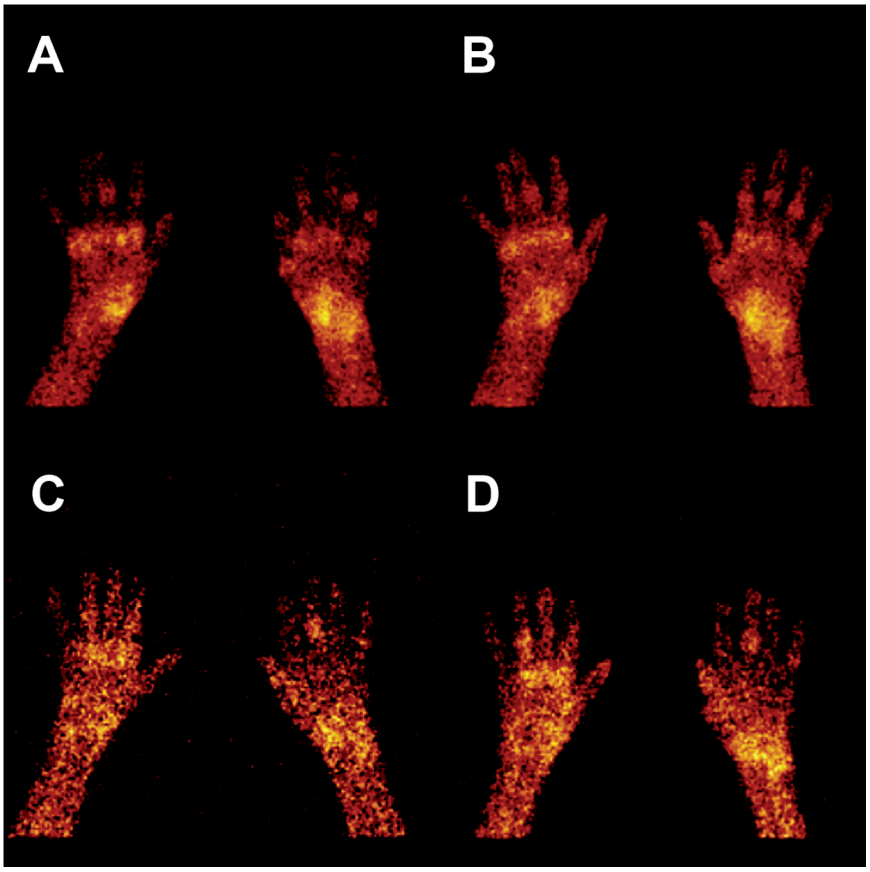
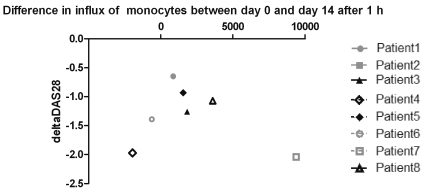
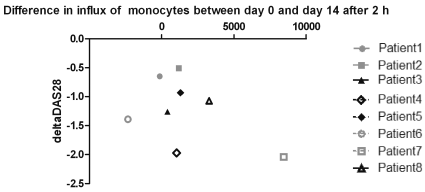
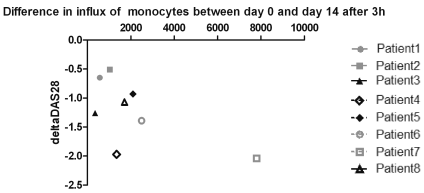
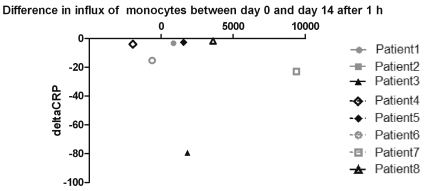


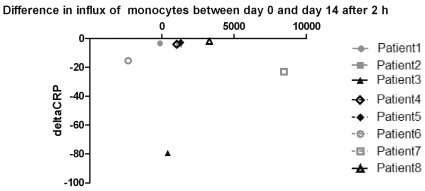

**Supplementary figure 1.** Joint of interest of patient 7 on baseline. (A) and after 14 days of adalimumab treatment (B) and joint of interest of patient 5 baseline (C) and after 14 days of adalimumab treatment (D)

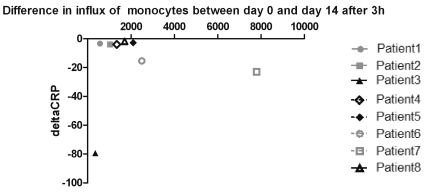


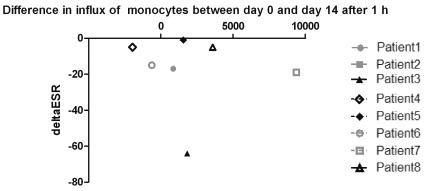



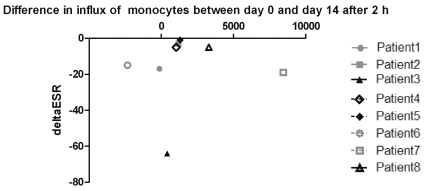



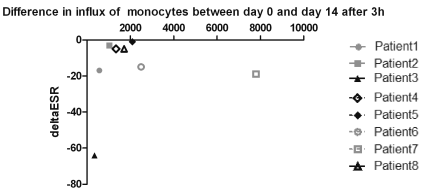




**Supplementary figure 2.** Correlations between the decrease in DAS28, ESR or CRP and the change in monocyte influx into the joints at 1 hour, 2 hours or 3 hours after re-infusion from baseline to 14 days

**Supplementary Table** Median (interquartile range) number of monocytes in joint of interest

| Hours after re-infusion | 1 | 2 | 3 |
|---|---|---|---|
| Day -14 | 3948 (2395-7279) | 5054 (3071-10416) | 4562 (3152-12071) |
| Day 1 | 3302 (874-15707) | 3935 (1590-15940) | 4458 (1448-12070) |
| Day 14 | 7661 (2484-20363) | 4316 (2242- 17113) | 5828 (2474-2474) |