Anemia of chronic disease in rheumatoid arthritis is associated with increased apoptosis of bone marrow erythroid cells. Improvement following anti-tumor necrosis factor alpha antibody therapy

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ABSTRACT

Circumstantial evidence has implicated tumor necrosis factor α (TNFα) in the pathogenesis of anemia of chronic disease (ACD) in rheumatoid arthritis (RA). We have investigated the role of TNFα in erythropoiesis of patients with active RA (n=40) and the effect of anti-TNFα antibody administration (cA2). RA patients had lower number of CD34+/CD71+ and CD36−/GlycoA+ bone marrow (BM) cells and increased proportion of apoptotic cells within the CD34+/CD71+ and CD36+/GlycoA+ cell compartments, compared to normal controls (n=24). Erythroid-burst forming units (BFU-E) obtained by BM mononuclear or purified CD34+ cells were significantly lower in RA patients compared to controls. These abnormalities were more pronounced among patients with ACD. Increased TNFα levels in patient long-term BM culture supernatants inversely correlated with BFU-E and hemoglobin and positively with the percentage of apoptotic CD34+/CD71+ and CD36+/GlycoA+ cells. Following cA2 therapy, a normalization was documented in the number of CD34+/CD71+ and CD36+/GlycoA+ cells, the number of BFU-E, and the proportion of apoptotic CD34+/CD71+ and CD36+/GlycoA+ cells, that was associated with a significant increase in hemoglobin levels compared to baseline. Recovery from anemia was more prominent in patients with ACD. The exogenous addition of an anti-TNFα antibody in the cultures increased BFU-E number in patients prior to cA2 treatment but not after treatment, further substantiating the inhibitory role of TNFα on patients’ erythropoiesis. We conclude that TNFα-mediated apoptotic depletion of BM erythroid cells may account for ACD in RA, and that cA2 administration may ameliorate ACD in these patients by down-regulating the apoptotic mechanisms involved in erythropoiesis.

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INTRODUCTION

There is substantial evidence that inflammatory cytokines subserve a crucial role in joint destruction and disease propagation in rheumatoid arthritis (RA). Among these cytokines, tumor necrosis factor alpha (TNFα) has been considered as the pivotal factor to induce and sustain tissue damage by activating the inflammatory mediator cascade, stimulating the mechanism of angiogenesis and up-regulating the vascular endothelial adhesiveness. Apart from its detection in the inflamed synovial fluid, TNFα is also found in elevated levels in patient sera, and cytokine concentration has been shown to correlate with disease activity. Furthermore, circumstantial evidence suggests that increased local TNFα production in the bone marrow (BM) may be implicated in the pathogenesis of anemia of chronic disease (ACD) seen in up to 50% of RA patients. More recently, we have shown that increased TNFα production in patients’ BM may be associated with the apoptotic depletion of hematopoietic progenitor cells in RA.

The successful effect of therapeutic blockade of TNFα in animal models of RA has highlighted the central role of the cytokine in the pathogenesis of the disease and has provided the rationale for clinical trials using a specific chimeric (human/mouse) monoclonal antibody (mAb) against TNFα known as cA2. Results from such studies have shown significant and clinically relevant improvement of active RA following treatment with cA2, alone or in combination with methotrexate. Interestingly, there is increasing evidence that, in addition to the clinical benefit and halt of progression of joint damage, cA2 therapy may also help to improve systemic manifestations of RA including ACD. Specifically, it has been shown that cA2 administration in RA patients with ACD increases significantly hemoglobin level, supporting the notion that TNFα is an important mediator in the pathogenesis of ACD. However, the exact mechanism(s) of action of the cytokine on erythropoiesis leading to ACD remain to be clarified.
The availability of cA2 and its beneficial effect in patients with RA provides the opportunity to directly address the role of TNFα in the pathogenesis of ACD. We have recently described the recovery of BM stem cell reserve and function in RA patients following cA2 therapy documenting that increased apoptosis in the stem cell compartment is, at least in part, a TNFα-mediated effect. In the present study we investigate for quantitative and functional abnormalities in BM erythroid progenitor and precursor cell populations in RA patients with or without ACD, and we examine the role and mode of action of the locally produced TNFα in their pathogenesis. In order to provide more insights into the mechanisms underlying the recovery from ACD in patients treated with cA2, we also explore the effect of the treatment in the number and functional characteristics of BM erythroid cells by comparing the pre-treatment data with those obtained after treatment.
PATIENTS, MATERIALS AND METHODS

Patients

Forty patients with RA, 25 females and 15 males, aged 18 to 74 years (median 48.5 years) were studied. All patients satisfied the 1987 revised diagnostic criteria of the American College of Rheumatology for RA\textsuperscript{12} and were eligible to receive cA2 (Remicade, Infliximab; Schering-Plough, Athens, Greece) if they had a minimum disease duration of 6 months, a history of unsuccessful treatment with at least one disease-modifying antirheumatic drugs and evidence of active disease according to established criteria.\textsuperscript{13} Patient characteristics are summarized in Table 1. Patients were assigned to receive 3 mg/kg of the antibody in a 2-hour intravenous infusion at the initiation of treatment (week 0), at weeks 2 and 6, and then every 2 months without discontinuing previous medication. As controls, 24 hematologically normal subjects undergoing surgery for lumbar fixation or healthy volunteers, age- and sex-matched with the patients were studied. Informed consent and institutional ethical committee approval according to the Helsinki Protocol, was granted prior to the study.

Peripheral blood samples

Peripheral blood samples for laboratory measurements were drawn immediately prior each cA2 infusion. Venous blood was collected into sterile EDTA tubes and analysed the same day for hemoglobin measurement. Anemia was defined as the reduction of hemoglobin below 12.0 g/dL for women and below 13.3 g/dL for men.\textsuperscript{14} Peripheral blood was also drawn into sterile tubes, allowed to clot for 30 minutes, spun at room temperature for 20 minutes at 2500 r.p.m and sera were aliquoted and stored at −70\textdegree C for erythropoietin (EPO), interleukin-6 (IL-6), and interleukin-1\beta (IL-1\beta) quantification by means of ELISA (Quantikine; R&D Systems Europe, Abingdon, UK). According to the manufacturer, the sensitivity of the assay is 0.6 mIU/mL for EPO, 0.094 pg/mL for IL-6 and 0.1 pg/mL for IL-1\beta. Patient sera were also
assayed for ferritin by means of ELISA (Abbott Laboratories; Abbott Park, Illinois). On the basis of previously reported data, serum ferritin below 60 ng/mL indicates iron deficiency anemia (IDA) whereas ferritin equal to or higher than 60 ng/mL indicates ACD.¹⁵

**Bone marrow samples**

BM samples from posterior iliac crest were aspirated at baseline and after 6 doses of cA2. BM cells were immediately diluted 1:1 in Iscove’s modified Dulbecco’s medium (IMDM; GibcoBRL, Life Technologies, Palsley, Scotland), supplemented with 100 IU/mL penicillin-streptomycin (PS; GibcoBRL, Life Technologies) and 10 IU/mL preservative-free heparin (Sigma, St Louis, MO). Diluted BM samples were centrifuged on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) (density 1.077 g/cm³) at 400g for 30 minutes at room temperature to obtain the mononuclear cells (BMMCs). Cell number and viability were assessed after staining with trypan blue.

**Purification of CD34⁺ cells**

CD34⁺ cells were isolated from BMMCs by indirect magnetic labelling (magnetic activated cell sorting; MACS isolation kit, Miltenyi Biotec GmbH, Germany) according to the manufacturer’s protocol. In each experiment, purity of CD34⁺ cells was greater than 96% as estimated by flow cytometry.

**Clonogenic progenitor cell assays**

We cultured 10⁵ BMMCs or 3x10³ CD34⁺ cells in 1 mL culture medium supplemented with 30% fetal calf serum (FCS; GibcoBRL, Life Technologies), 1% bovine serum albumin (BSA; GibcoBRL, Life Technologies), 10⁻⁴ mol/L mercaptoethanol (Sigma), 0.075% sodium bicarbonate (GibcoBRL, Life Technologies), 2 mmol/L L-glutamine (Sigma), 0.9%
methylcellulose (StemCell Technologies Inc, Vancouver, Canada), in the presence of 5 ng granulocyte-macrophage colony stimulating factor (GM-CSF; R&D Systems), 50 ng interleukin-3 (IL-3; R&D Systems) and 2 IU erythropoietin (EPO; Janssen-Ciliag Ltd, Athens, Greece). Cultures were set up in duplicate in 35-mm Petri dishes and incubated at 37°C-5%CO₂ fully humidified atmosphere. On day 14, erythroid-burst forming units (BFU-E) were identified and scored according to established criteria. When mentioned, a mouse anti-human TNFα monoclonal neutralizing antibody (R&D Systems) was added to the culture at a concentration 1.8 µg/mL. According to the manufacturer, the neutralization dose₅₀ for this antibody is 0.02-0.04 µg/mL in the presence of 0.25 ng/mL TNFα.

Long-term bone marrow cultures

Long-term BM cultures (LTBMCs) from 10⁷ BMMCs were grown according to the standard technique in 10 mL IMDM supplemented with 10% FCS, 10% horse serum (GibcoBRL, Life Technologies), 100 IU/mL PS, 2 mmol L-glutamine and 10⁻⁶ mol hydrocortisone sodium succinate (Sigma) and incubated at 33°C-5%CO₂ fully humidified atmosphere. At weekly intervals, cultures were fed by removing half of the medium and replacing it with equal volume of fresh IMDM supplemented as above. By allowing the formation of an adherent layer consisting mainly of macrophages and cells of mesenchymal origin, this culture system has been considered instrumental to evaluate the regulatory role of BM microenvironment on hematopoietic progenitor cell growth. The adherent layer is usually confluent after 3 to 4 weeks and, at that time point, cell-free supernatants may be harvested and stored at −70°C for cytokine quantification. In the present study, TNFα concentration in the supernatants was quantitated by means of a commercially available ELISA kit (Biosource International Inc, California, USA). According to the manufacturer, the sensitivity of this assay is less than 0.09 pg/mL.
Immunophenotyping and 7-amino-actinomycin D staining

Flow cytometry was used to identify the BM erythroid cells at different maturational stages. Specifically, aliquots of 100 µL BM cells were washed twice in phosphate buffer saline (PBS)-1% FCS-0.05% azide and incubated with 40 µL human γ-globulin for 10 minutes on ice. Cells were then either stained with phycoerythrin (PE)-conjugated mouse anti-human CD34 mAb (QBEND-10; Immunotech, Marseille, France) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human tranferrin receptor (CD71) mAb (YDJ1.2.2; Immunotech) or with PE-conjugated mouse anti-human glycophorine A (GlycoA) mAb (11E4B7.6; Immunotech) and FITC-conjugated mouse anti-human CD36 mAb (FA6.152; Immunotech) and incubated for 30 minutes on ice. Following two washes with PBS-1% FCS-0.05% azide, the cells were further stained with 100 µL 7-amino-actinomycin D solution (200 µg/mL) (7AAD; Calbiochem-Novabiochem, La Jolla, CA), suspended in 1 mL PBS and incubated for 20 minutes on ice protected from light as previously described. Following centrifugation, the supernatant was removed, contaminating red cells were lysed with 0.12% formic acid and samples were fixed in 0.2% parafolmadeyde using the Q-prep reagent system (Coulter, Luton, England). Fixed cells stained with the isotypic control antibodies but not with 7AAD, were used as negative controls.

Quantitative fluorescence analysis was performed in an Epics Elite model flow cytometer (Coulter Corporation, Miami, Florida) within 30 minutes of cell fixation, using five parameters: forward light scattering, 90° left-side light scattering, and triple color immunofluorescence from FITC, PE, and 7AAD. Spillover of each fluorescence into other fluorescence detectors was electronically compensated to background levels by using cells stained only with the respective-fluorescence labelled mAb or 7-AAD. List mode data were collected for 500,000 events and analysed using Epics Elite. After drawing a region around the cells with low forward and low side scatter properties where erythroid progenitor and
precursor cells are included, two scattergrams were created by combining CD34 with CD71 fluorescence and GlycoA with CD36 fluorescence gated in the above region. Finally, a scattergram was generated by combining forward light scatter with 7AAD fluorescence to quantitate 7AAD-negative (live), -dim (early apoptotic) and -bright (late apoptotic) cells in the gates of CD34+/CD71+, CD36+/GlycoA+, and CD36-/GlycoA+ erythroid cell populations. Regions were drawn around clear-cut populations, and the proportion of cells within each region was calculated excluding cell debris (Figure 1).

**Incubation of normal bone marrow cells with rhTNFα**

In a separate set of experiments, 1x10^6 BM cells from normal individuals (n=3) were suspended in 1mL IMDM-20%FCS and incubated in the absence or presence of human rhTNFα (R&D systems) at a concentration 10ng/mL and 20ng/mL. Following 48h incubation in 37°C-5%CO₂ fully humidified atmosphere, cells were washed twice with PBS-1%FCS-0.05% azide, stained and analyzed by flow cytometry as described above.

**Statistical analysis**

Numerical data were analyzed in the GraphPad Prism statistical PC program (GraphPad Software, San Diego, CA) by means of the nonparametric Mann-Whitney U test, the Student’s t-test for paired samples, and the Pearson coefficient of correlation. One-way analysis of variance (ANOVA test) was used to define differences in the percentage of apoptotic cells obtained in cultures treated with various concentrations of rhTNFα. Homogeneity of the populations studied was tested by means of the χ²-test.
RESULTS

Flow cytometric analysis of BM erythroid cells

The CD34+/CD71+ cell compartment includes the early erythroid progenitor cells whereas the CD36 cell surface marker is expressed on erythroid progenitor and early precursor erythroid cells but is lost during the subsequent erythroid differentiation.23 GlycoA is expressed on mature erythroid cells but is not present on the early progenitors.22 Accordingly, the CD36+/GlycoA+ and CD36-/GlycoA+ cells represent the early and mature precursor cells of the erythroid development, respectively.

Flow cytometric analysis of BM erythroid cells in our patients is presented in Table 2. Patients (n=21) had significantly lower number of CD34+/CD71+ cells compared to the normal controls (n=21, \( P = .0011 \)). The proportion of CD71+ cells within the CD34+ cell fraction was significantly lower in the patients (20.96 ± 11.58) compared to the controls (33.21 ± 11.11, \( P = .0027 \)) suggesting that the reduction of the CD34+/CD71+ cells in RA patients does not simply reflect the low number of total CD34+ cells previously reported in RA5 but concerns specifically the erythroid progenitors. The proportion of GlycoA+ cells was also significantly reduced in the patients (86.43 ± 4.68) compared to the controls (90.84 ± 3.90, \( P = .0020 \)). This decrease was essentially due to the lower proportion of the mature CD36-/GlycoA+ cells rather than the earlier CD36+/GlycoA+ cells in the patients (\( P = .0483 \) and \( P = .6061 \), respectively). Compared to the normal controls, both patient groups the ACD (n=11) and non-anemic (n=10), had lower number of CD34+/CD71+ cells (\( P = .0014 \) and \( P = .0398 \), respectively). In ACD patients the proportion of the mature CD36-/GlycoA+ cells but not of the early CD36+/GlycoA+ precursor cells, was significantly reduced compared to the normal controls (\( P = .0125 \) and \( P = .1267 \), respectively). In contrast, no statistically significant differences were noted between non-anemic RA patients and normal controls in the proportions of CD36-/GlycoA+ or CD36+/GlycoA+ cells (\( P = .5541 \) and \( P = .4343 \), respectively).
respectively. Finally, ACD patients had lower proportion of mature CD36^-/GlycoA^+ but not of early CD36^+/GlycoA^+ precursor cells or CD34^+/CD71^+ progenitor cells, compared to non-anemic patients ($P=.0378$, $P=.0529$ and $P=.2169$, respectively). Taken together these findings show that RA patients display low number of BM erythroid progenitor cells defined by the CD34^+/CD71^+ phenotype, low number of mature precursor cells defined by the CD36^-/GlycoA^+ phenotype, and normal number of early precursor cells defined by the CD36^+/GlycoA^+ phenotype. Interestingly, low numbers of mature CD36^-/GlycoA^+ precursor cells were seen in ACD but not in non-anemic RA patients.

**Apoptosis of the BM erythroid cells**

It has been previously shown that apoptotic control mechanisms contribute to the regulation of BM erythropoiesis.²⁴ To explore whether the decrease of BM erythroid cells in RA patients is due to increased apoptotic cell death, we evaluated the percentage of apoptotic cells within the BM erythroid cell compartments (Figure 2). We found that patient (n=21) CD34^+/CD71^+ and CD36^+/GlycoA^+ cells contained significantly higher proportion of apoptotic cells (7AAD^dim plus 7AAD^bright cells) (25.50% $\pm$ 18.44% and 42.87% $\pm$ 23.75%, respectively) compared to the normal controls (n=21) (11.36% $\pm$ 6.13% and 8.35% $\pm$ 6.76%, respectively; $P=.0053$ and $P<.0001$, respectively). In contrast, no statistically significant difference was found between patients and normal controls in the percentage of apoptotic cells detected in the CD36^-/GlycoA^+ cell compartment (3.53% $\pm$ 4.54% and 2.29% $\pm$ 0.90%, respectively; $P=.0671$). In a subset analysis we found that RA patients with ACD displayed significantly increased apoptosis within the CD36^+/GlycoA^+ cell fraction (50.88% $\pm$ 19.55%) and a trend towards higher apoptosis within the CD34^+/CD71^+ cell compartment (31.71% $\pm$ 21.72%), compared to the non-anemic patients (34.44% $\pm$ 19.11% and 19.39% $\pm$ 11.41%, respectively) ($P=.0412$ and $P=.1490$, respectively). Compared to the normal controls
However, both patient groups, the ACD and non-anemic, displayed significantly increased proportion of apoptotic cells within the CD34+/CD71+ (P=.008 and P=.047, respectively) and the CD36+/GlycoA+ (P<.0001 and P=.0002, respectively) but not the CD36-/Glyco+ (P=.0775 and P=.9490, respectively) cell compartments. Taken together, these data suggest that RA patients, particularly those with ACD, display increased apoptosis in the BM erythroid progenitor and early precursor cell compartments but not in the mature precursor cell population.

**Erythroid burst forming units**

The frequency of BFU-E in the BMMC fraction was evaluated in 26 RA patients prior to cA2 treatment and in 24 normal controls. Results are depicted in Figure 2. Of these patients, 14 had normal hemoglobin level while the remaining 12 had ACD. In the entire group of patients studied, the mean BFU-E number obtained by 10^6 BMMCs was significantly lower than the respective value obtained in the controls (200 ± 131 vs 420 ± 186, P<.0001). Compared to the controls, BFU-E numbers were significantly lower in both, the non-anemic (268 ± 148 BFU-E per 10^6 BMMCs, P=.021) and ACD (142 ± 81 BFU-E per 10^6 BMMCs, P<.0001) group of patients. Furthermore, BFU-E frequency was significantly lower in patients with ACD compared to the non-anemic patients (P=.022).

To investigate whether the decreased BFU-E colony formation in RA patients is due to the lower number of erythroid progenitor cells in the BMMC fraction or is the consequence of an intrinsic progenitor cell defect, we evaluated the clonogenic potential of immunomagnetically sorted CD34+ BM cells. We found that the number of BFU-E obtained by 5x10^3 CD34+ cells was significantly lower in the patients (147 ± 88, n=12) compared to the controls (310 ± 107, n=19; P=.0005) suggesting a defect in the clonogenic potential of patient progenitor cells possibly due to the presence of increased number of apoptotic cells.
Endogenous EPO production

EPO is the principal growth factor for maintaining erythroid progenitor cell survival. It has been demonstrated that deprivation of EPO induces apoptosis of immature erythroid colony forming cells through down-regulation of Bcl-X(L) anti-apoptotic protein. To investigate whether increased apoptosis of patient BM erythroid cells might be due to decreased endogenous EPO production, we evaluated EPO levels in 14 ACD or non-anemic RA patients. Results were compared to serum EPO levels of a reference group (55 normal individuals or subjects with iron deficiency anemia). There was no statistically significant difference in hemoglobin levels between patients (mean 11.70 ± 1.67 g/dL, range 8.1-14.2 g/dL) and control subjects (mean 12.54 ± 2.25 g/dL, range 6.0-16.3 g/dL; P=196). We found that EPO levels did not differ statistically between the RA group (mean 20.75 ± 19.28 mIU/mL, range 5.15-80.0 mIU/mL) and the reference group (mean 21.63 ± 30.76 mIU/mL, range 2.70-140 mIU/mL, P=.0688). EPO levels inversely correlated with hemoglobin values in both, the RA group (r = -0.808, P<.001) and the reference group (r = -0.895, P<.0001) (Figure 3). Furthermore, to characterize EPO production as appropriate or inappropriate for a given hemoglobin value in our patients, we defined the observed/predicted ln(EPO) ratio (O/P ratio) for each sample as previously reported. The mean O/P ratio in the patients (0.99 ± 0.12) was within the 95% confidence limits (0.95-1.08) of the reference group (mean O/P ratio 1.01 ± 0.24, P=.687) suggesting an adequate EPO production in our patients and indicating that mechanism(s) independent of EPO suppression are probably implicated in the apoptotic depletion of patient BM erythroid cells.

TNFα in LTBMCs and its effect on apoptotic depletion of BM erythroid cells

The levels of TNFα in LTBMC supernatants from 26 of the patients have been presented elsewhere. We have reported that cytokine levels are significantly higher in RA patients
(17.87 ± 12.01 pg/mL) compared to the normal controls (6.28 ± 2.40 pg/mL, n=11; P=.0005).

In the present study by further analyzing the data on the basis of patient hemoglobin levels, we found that TNFα concentration was significantly higher in the supernatants of patients with ACD (n=14) (22.68 ± 14.96 pg/mL) compared to the non-anemic patients (n=12) (13.14 ± 5.82 pg/mL, P=.0373). Both patient groups, ACD and non-anemic, displayed significantly higher cytokine levels compared to the control subjects (P = .0009 and P = .0051). Individual TNFα values inversely correlated with the values of hemoglobin (r = -0.436, P = .007) and the number of BFU-E (r = -0.530, P = .0009) (Figures 4A and 4B, respectively) and positively with the percentage of apoptotic CD34+/CD71+ (r = .708, P < .0001) and CD36+/GlycoA+ (r = 0.664, P = .0001) cells in the entire group of subjects studied (Figures 4C and 4D, respectively). In contrast, TNFα levels did not correlate with the percentage of apoptotic CD36-/GlycoA+ cells (r = 0.084, P = .678). These data suggest that the increased local TNFα production by BM stromal cells probably accounts for the apoptotic depletion of patient erythroid progenitor and early precursor cells and is possibly involved in the pathogenesis of ACD in RA.

To further investigate this hypothesis we incubated normal BM cells (n=3) in the absence or presence of human rhTNFα as described above. Following 48h incubation, a significant, concentration-dependent increase in the percentage of apoptotic cells was obtained in the CD34+/CD71+ and CD36+/GlycoA+ cell compartments in the cultures treated with rhTNFα compared to the untreated cultures (F=161.31>F^2_6 , P<.001 and F=259.43>F^2_6 , P<.001; respectively) (Figure 5). In contrast, no statistically significant difference was found between treated and untreated with rhTNFα cultures in the percentage of apoptotic cells detected in the CD36+/GlycoA+ cell compartment (F=1.032<F^2_6 , P>.05). These data, coupled with the lack of correlation between TNFα and apoptotic CD36+/GlycoA+ cells, suggest that TNFα induces apoptosis in the erythroid progenitor and early precursor cell compartments but does
not affect the mature erythroid precursor cells.

**Effect of anti-TNF α treatment on the BM erythroid cells**

Having demonstrating the negative effect of the locally produced TNFα on the BM erythroid cell homeostasis, we next examined the effect of anti-TNFα treatment on the quantitative and functional characteristics of BM erythroid progenitor and precursor cells in RA patients (n=12) following 6 doses of cA2. Results were compared to pre-treatment values (Table 3). Changes in TNFα concentration in LTBMC supernatants of these patients have been previously reported. We have shown that the levels of the cytokine decrease dramatically following treatment (P=.0072). In the present study, we found that the proportion of CD34+/CD71+ and CD36+/GlycoA+ cells increased significantly following treatment (P=.0006 and P=.022, respectively) and this increase was associated with a significant reduction in the percentage of apoptotic cells within the CD34+/CD71+ and CD36+/GlycoA+ compartments (P=.0011 and P=.0008, respectively). We also found a significant increase in the number of BFU-E obtained by BMMCs of the patients post-treatment (402 ± 147 BFU-E per 10^6 BMMCs) compared to pre-treatment values (220 ± 153 BFU-E per 10^6 BMMCs, P=.0049). These data further corroborate the assumption of a TNFα-induced apoptotic depletion of BM erythroid cells in RA patients.

To further support these *ex vivo* findings, we next tested the effect of the exogenous addition of an anti-TNFα neutralizing antibody on the BFU-E colony formation by patient BMMCs pre- and post-cA2 therapy. If increased local TNFα production accounts for the impaired erythropoiesis in RA, then the inclusion of this inhibitor in the culture should significantly enhance erythropoiesis in patients prior to treatment but not after treatment. We used the antibody at a concentration 100-fold higher than the highest TNFα value of 55 pg/mL found in patient LTBMC supernatants. In patients studied before therapy with cA2
(n=4), the exogenous addition of anti-TNFα in the culture increased significantly the number of BFU-E (260 ± 81 per 10^6 BMMCs) in comparison to their untreated cultures (223 ± 78 per 10^6 BMMCs, P=.004). In contrast, in patients studied post cA2 therapy (n=3), no statistically significant difference was found between untreated and treated with anti-TNFα cultures, in the number of BFU-E obtained (363 ± 370 per 10^6 BMMCs vs 340 ± 313 per 10^6 BMMCs, respectively; P>.05) (Figure 5). These data further support the inhibitory effect of TNFα on erythropoiesis in RA patients.

Effect of anti-TNFα treatment on hemoglobin levels

To examine whether decreased apoptosis in the BM erythroid cell compartments following anti-TNFα treatment may influence the systemic erythroid cell homeostasis, we examined the effect of 6 doses of cA2 treatment on hemoglobin levels. Patient hemoglobin values at baseline (n=40) are shown in Table 1. Twenty-three (57.6%) of the patients had anemia (mean hemoglobin value 11.28 ± 1.12 g/dL). Among anemic patients, 15 had ACD and 8 IDA according to the criteria described above. There was a female predominance in the non-anemic and IDA group compared to ACD patients (P<.05 and P<.01, respectively) however, to our knowledge, this difference does not affect any of the parameters investigated. The percentage of hemoglobin changes in patient groups following cA2 treatment are depicted in Figure 7. Overall, patients’ hemoglobin increased significantly following treatment (mean 13.01 ± 1.30 g/dL, P=.0004). As anticipated, there was a reduction in EPO levels (14.64 ± 8.65 mIU/mL) compared to baseline (21.38 ± 20.84 mIU/mL) in 12 patients studied, however the difference obtained was not statistically significant (P=.285). More specifically, a significant increase of hemoglobin was observed in the total group of anemic patients (mean 12.57 ± 1.40 g/dL) compared to their baseline values (P=.00039). This increase was mainly due to the significant increase obtained in the group of ACD patients (mean 12.88 ± 1.45
g/dL, $P=.0032$). A modest improvement of anemia was also noted in RA patients with IDA (mean 11.99 ± 1.16 g/dL) although the difference obtained from the respective baseline values was not statistically significant ($P=.0705$). These data confirm and extend previous observations suggesting an improvement of anemia in RA patients following treatment with cA2.11

**Effect of anti-TNFα treatment on peripheral cytokine and EPO levels**

In addition to TNFα, other inflammatory cytokines such as IL-1β and IL-6 may play a role in the pathogenesis of ACD.27 By evaluating the changes of serum cytokine levels in patients after treatment (n=12), we found a significant reduction in IL-6 values (10.17 ± 12.76 pg/mL) compared to pre-treatment values (18.22 ± 16.43 pg/mL, $P=.0066$). Serum IL-1β levels displayed also a reduction following treatment (0.82 ± 0.79 pg/mL), although the difference obtained was not statistically significant compared to pre-treatment levels (1.41 ± 1.56 pg/mL, $P=.223$).
DISCUSSION

Anemia associated with RA has been considered as the prototype of ACD. Its pathogenesis is multifactorial but inflammatory cytokines, particularly TNFα, appear to play a prominent role. Animals treated with TNFα develop anemia with ACD characteristics. TNFα has been reported to cause ACD by modulating macrophage iron metabolism. More recent evidence, however, has suggested that TNFα inhibits BM erythropoiesis by signaling a direct negative effect on erythroid progenitor cell growth and/or by stimulating the production of inhibitory cytokines by BM accessory cells. Moreover, in vitro studies have demonstrated the inhibitory effect of the increased, local or systemic, TNFα production on the BM erythroid colony formation in RA patients with ACD and clinical reports have suggested that serum cytokine levels strongly correlate with the degree of anemia. More direct evidence for the role of TNFα in the pathogenesis of ACD in RA has recently become available from clinical trials using in vivo TNFα blockade that demonstrate a significant improvement of ACD following treatment.

The data of the present study confirm the beneficial effect of cA2 administration on anemia of RA patients. Approximately 60% of our patients were anemic and among them 65% had ACD characteristics, highlighting the increased frequency of this condition in RA as previously reported. After 6 doses of cA2, a significant improvement of hemoglobin levels was observed in the total study group of patients compared to their baseline values. As expected, the most prominent increase was obtained in the group of ACD patients that was elevated to 14%. Based on previous reports suggesting that mechanisms independent of erythropoietin production and probably related to BM erythropoiesis underlie the recovery from ACD following cA2 treatment, we investigated the effect of such treatment in the quantitative and functional characteristics of BM erythroid cells in RA.

At baseline, patients displayed a significant defect in the BM erythroid cell reserve and
function indicated by the low frequency of erythroid progenitor and precursor cells, the low BFU-E colony formation by BM progenitor cells and the increased apoptosis in the BM erythroid progenitor and precursor cell compartments. These abnormalities were associated to a markedly increased local TNFα production by patient BM stromal cells, as was demonstrated by evaluating the cytokine levels in LTBMC supernatants. The observed BM abnormalities were more prominent in the group of patients with ACD. Specifically, we found that compared to the control subjects, RA patients, particularly those with ACD, displayed a significant reduction in the CD34+/CD71+ cell compartment, normally comprising the early erythroid progenitors. A similar reduction was observed in the proportion of CD36/GlycoA+ late precursor but not the CD36+/GlycoA+ early precursor cells. Furthermore, our patients, especially those with ACD, displayed increased apoptosis within the CD34+/CD71+ and CD36+/GlycoA+ but not within the CD36/GlycoA+ cell compartments. According to the current aspect of BM homeostasis, the rate of apoptotic cell death is balanced by the rate of cell proliferation; when the balance tilts toward cell death, increased proliferation occurs to maintain homeostasis. Accordingly, one could postulate that the presence of an apoptotic stimulus in patients’ BM may increase the proliferation of the CD36+/GlycoA+ cells thus maintaining their overall number, but finally leads to the reduction of the non-proliferating CD36-/GlycoA+ mature cells.

Apoptotic mechanisms control erythropoiesis in normal and pathologic conditions and adequate EPO production has been demonstrated to protect erythroid progenitor cells from apoptosis. In keeping with previous reports suggesting normal EPO response to anemia in RA, our patients displayed normal endogenous EPO production indicating that mechanism(s) other than EPO suppression affects BM erythroid cell survival. On the basis of our recent findings that increased local TNFα production is related to the apoptotic depletion of BM progenitor cells in RA, we examined whether TNFα might play a causal
role in patients’ impaired erythropoiesis. We found that increased TNFα production was most pronounced in patients with ACD and that cytokine concentration inversely correlated with the number of BFU-E and the values of hemoglobin, and positively with the percentage of apoptotic erythroid cells, suggesting the involvement of TNFα in the pathogenesis of anemia in our patients and highlighting the key importance of apoptosis in causation of ACD by TNFα. The enhanced erythroid cell apoptosis obtained by incubating normal BM cells with rhTNFα further corroborates this suggestion. The increased BFU-E colony numbers obtained by incubating patient BMMCs with anti-TNFα neutralizing antibody prior to the in vivo anti-TNFα treatment but not post treatment, further emphasizes the role of TNFα in inhibiting erythropoiesis in RA patients. Our data do not allow to discern a direct from an indirect effect of TNFα on apoptotic depletion of patient erythroid cells since experiments were performed using BMMCs. Other investigators, however, using single-cell cloning experiments have shown that TNFα-inhibition of erythroid colony formation is mainly directly mediated.30

We next investigated the changes in BM homeostasis in the patients following cA2 administration. On the basis of our hypothesis for a TNFα-mediated apoptotic depletion of patient BM erythroid cells, we predicted that effective TNFα neutralization in vivo using cA2 might improve anemia by reducing apoptosis in the erythroid progenitor and precursor cell compartments. Indeed, in addition to an increase in hemoglobin found in all patient groups, a significant reduction was observed in the percentage of apoptotic cells within the CD34+/CD71+ and CD36+/GlycoA+ cell compartments that was paralleled with a significant increase, compared to baseline, in the proportion of CD34+/CD71+ and CD36+/GlycoA+ BM cells and the number of BFU-E. These findings, in association with the significant decrease in TNFα production by patient BM stromal cells following treatment, further support the concept of a TNFα-mediated suppression of erythropoiesis in RA and indicate that a major effect of cA2 administration in correcting ACD is the blockade of the TNFα-induced
apoptotic depletion of BM erythroid cells. The decrease of EPO levels following treatment, probably due to the correction of anemia, corroborates further the assumption that increased apoptosis of BM erythroid cells in RA is not related to EPO suppression.

In agreement with previous reports, we also found a significant reduction in circulating IL-6 but not IL-1β levels following treatment with cA2 compared to baseline values, that might have an additional effect in improvement of anemia. However, the role of IL-6 in the pathogenesis of ACD has been reported to be related to abnormal regulation of iron metabolism or to a hemodilutional effect rather than to a direct negative effect on erythroid development.

The long-term exposure to the myelosuppressive agent methotrexate might be a contributing factor affecting the BM erythroid cell reserve and function in RA patients since treatment with methotrexate has been reported to affect the late stage of hematopoietic development. The fact, however, that patients’ BM erythropoiesis was improved following anti-TNFα treatment without discontinuing methotrexate indicates that TNFα is the major factor affecting the erythroid development in RA.

In conclusion, this study suggests that patients with RA exhibit low frequency and increased apoptosis of BM erythroid progenitor and precursor cells due to increased local production of TNFα. We have also provided in vitro and ex vivo evidence that TNFα-induced accelerated apoptosis of BM erythroid cells, largely contributes to the pathogenesis of ACD in RA. TNFα blockade using cA2, improves ACD in RA patients and the beneficial effect of the treatment is mediated, at least in part, by down-regulating the TNFα-induced apoptotic mechanisms in the BM. This resource of findings in RA patients and the better understanding of the mechanism of action of this novel therapeutic intervention may facilitate the design of anti-TNFα therapeutic approaches for the clinical management of TNFα-related diseases or TNFα-mediated disease manifestations. Data from this study may have implications in the
understanding of mechanism(s) of anemia associated not only with RA but also with other chronic inflammatory diseases.
ACKNOWLEDGMENTS

We would like to thank Mrs. Claudia Gemetzi, Ms. Athina Damianaki and Mrs. Helen Koutala for their technical assistance. We also thank Dr. Helen Kouroumali, Dr. Niki Lydataki and Mrs Maria Kasapaki for providing BM samples and data from RA patients.
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48 Fries JF: The hierarchy of quality-of-life assessment, the Health Assessment Questionnaire (HAQ), and issues mandating development of a toxicity index. Control Clin Trials. 1991;12:106S-117S.
FIGURE LEGENDS

**Fig 1. Flow cytometric analysis of BM erythroid cells.** (A) Scattergram of FSC vs SSC, to allow gating on cells with low FSC and low SSC properties where BM erythroid cells are included (R1). (B) Scattergram of anti-CD34 vs anti-CD71 fluorescence gated on R1, to allow gating on the CD34+/CD71+ early erythroid progenitor cells (R2). (C) Scattergram of anti-GlycoA vs anti-CD36 fluorescence gated on R1, to allow gating on the CD36+/GlycoA+ cells (R3) and CD36-/GlycoA+ cells (R4) representing the early and late erythroid precursor cells respectively. (D) Scattergram of FSC vs 7AAD fluorescence gated on R2, showing 7AADbright (late apoptotic), 7AADdim (early apoptotic), and 7AAD- (live) cells. Similar scattergrams were gated on R3 and R4 regions. FSC = forward light scatter; SSC = right angle light scatter; GlycoA = GlycophorinA; 7AAD = 7-amino-actomycin D.

**Fig 2. Percentage of apoptotic cells in the BM erythroid cell compartments and BFU-E number in RA patients and normal controls.** The left bars represent the mean percentage (± SE) of apoptotic cells detected by flow cytometry within the BM erythroid progenitor and precursor cell compartments in the total group of RA patients, the RA patients with anemia of chronic disease (ACD), the non-anemic RA patients and the control subjects. The right bars represent the mean BFU-E colony values (± SE) obtained in the clonogenic assay by 10^6 BMMCs in the above study groups. Comparison between patient and control values was performed by means of the nonparametric Mann-Whitney U test. (*) P<.05. (**) P<.01. (***) P<.001
Fig 3. Relationship between serum Epo levels and hemoglobin concentration. Linear regression analysis for the correlation between serum Epo levels and hemoglobin in reference subjects (upper diagram) and RA patients (lower diagram). Regression lines are shown as solid lines and the 95% confidence limits as dotted lines. Regression equations, coefficient of correlation (r) and degree of significance (P) are indicated. (●) Non-anemic subjects; (○) anemic subjects.

Fig 4. Correlation between the levels of TNFα in LTBMC supernatants and the values of Hgb, the numbers of BFU-E and the proportions of apoptotic BM erythroid cells. Diagrams show the linear regression analysis for the correlation between the values of TNFα in LTBMC supernatants and the levels of Hgb (Diagram A), the numbers of BFU-E obtained by BMMCs in the clonogenic assay (Diagram B), and the proportions of apoptotic CD34+/CD71+ progenitor (Diagram C) and CD36+/GlycoA+ precursor cells (Diagram D) in the entire group of subjects studied. Coefficient of correlation (r) and degree of significance (P) are indicated. Regression lines are shown as solid lines and the 95% confidence limits as dotted lines. (●) RA patients; (○) normal controls.
**Fig 5. Effect of rhTNFα on apoptotic depletion of normal BM erythroid cells.** We incubated $1 \times 10^6$ BM cells from normal subjects ($n=3$) in the absence or presence of human rhTNFα at concentrations 10ng/mL and 20ng/mL for 48h. The percentage of apoptotic cells in the early progenitor ($\Diamond$) CD34+/CD71+, the early precursor ($\Box$) CD36+/GlycoA+ and late precursor ($\bigcirc$) CD36-/GlycoA+ cell compartments were evaluated using flow cytometry and 7-amino-actomycin D (7AAD). Each point in the diagram represents the mean ($\pm$ SEM) of the three experiments. Comparison between treated and untreated with rhTNFα cultures in each cell compartment was performed using the one-way analysis of variance (ANOVA test).

**Fig 6. Effect of anti-TNFα neutralizing antibody on BFU-E colony formation.** Exogenously added mouse anti-human TNFα neutralizing antibody in short-term culture of BMMCs at a dose of 1.8 $\mu$g/mL increased significantly the number of BFU-E in RA patients studied prior to cA2 treatment but not in the patients studied post treatment. Bars represent the mean BFU-E number ($\pm$ SEM) obtained by $10^6$ BMMCs from RA patients pre- ($n=4$) or post- ($n=3$) cA2 treatment. Comparison between treated and untreated with neutralizing antibody cultures was performed using the Student’s t-test for paired samples.
Fig 7. Percentage of changes of Hgb levels following cA2 treatment compared to baseline (pre-treatment) levels. Bars represent the mean (± SEM) percentage of change of Hgb compared to baseline levels in the total group of patients, the group of total anemic patients, and the groups of ACD (anemia of chronic disease) and IDA (iron deficiency anemia) patients. Differences from the respective baseline values were evaluated using the Student’s t-test for paired samples.
Table 1. Baseline characteristics of RA patients treated with cA2

<table>
<thead>
<tr>
<th>Clinical Data</th>
<th>Non-anemic (n=17)</th>
<th>ACD patients (n=15)</th>
<th>IDA Patients (n=8)</th>
<th>All patients (n=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median, range)</td>
<td>44 (30-61)</td>
<td>48 (26-74)</td>
<td>48 (18-67)</td>
<td>48 (18-74)</td>
</tr>
<tr>
<td>Sex (f/m)</td>
<td>14/3</td>
<td>6/9</td>
<td>8/0</td>
<td>28/12</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>141±112*</td>
<td>125±73</td>
<td>118±54</td>
<td>131±88</td>
</tr>
<tr>
<td>Number of swollen joints</td>
<td>42±14</td>
<td>28±9</td>
<td>43±10</td>
<td>37±14</td>
</tr>
<tr>
<td>HAQ score</td>
<td>1.27±0.57</td>
<td>0.96±0.48</td>
<td>0.87±0.05</td>
<td>1.07±0.57</td>
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<table>
<thead>
<tr>
<th>Laboratory variables</th>
<th>Non-anemic (n=17)</th>
<th>ACD patients (n=15)</th>
<th>IDA Patients (n=8)</th>
<th>All patients (n=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin, g/dL</td>
<td>13.24±0.71</td>
<td>11.42±1.31</td>
<td>11.02±0.6</td>
<td>12.09±1.34</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>88.03±5.16</td>
<td>81.75±9.46</td>
<td>72.77±9.74</td>
<td>82.63±9.64</td>
</tr>
<tr>
<td>Serum iron, µmol/L</td>
<td>71.6±36.6</td>
<td>30.8±12.3</td>
<td>39.1±23.6</td>
<td>49.8±32.8</td>
</tr>
<tr>
<td>Ferritin, ng/mL</td>
<td>47.82±26.41</td>
<td>109.88±98.0</td>
<td>15.15±10.11</td>
<td>62.71±73.16</td>
</tr>
<tr>
<td>ESR, mm/h</td>
<td>39±23</td>
<td>51±18</td>
<td>63±18</td>
<td>48±22</td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td>1.38±1.56*</td>
<td>3.57±4.12</td>
<td>2.12±2.12</td>
<td>2.35±2.98</td>
</tr>
<tr>
<td>RF (positive)</td>
<td>9</td>
<td>8</td>
<td>6</td>
<td>23</td>
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</table>

<table>
<thead>
<tr>
<th>Medication</th>
<th>Non-anemic (n=17)</th>
<th>ACD patients (n=15)</th>
<th>IDA Patients (n=8)</th>
<th>All patients (n=40)</th>
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</thead>
<tbody>
<tr>
<td>Methotrexate†</td>
<td>17</td>
<td>15</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>Corticosteroids‡</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>

(*) Values are expressed as means ± 1 standard deviation (SD).
(†) 15-20 mg/weekly; (‡) 5-10 mg/daily.

Abbreviations: ACD, Anemia of chronic disease; IDA, Iron deficiency anemia; HAQ, Health Assessment Questionnaire⁴⁸; MCV, Mean Corpuscular Volume; ESR, Erythrocyte Sedimentation Rate; CRP, C-Reactive Protein; RF, Reumatoid Factor
Table 2. Flow-cytometric analysis of BM cells

<table>
<thead>
<tr>
<th></th>
<th>Non-anemic RA patients (n=10)</th>
<th>ACD RA patients† (n=11)</th>
<th>Total RA patients‡ (n=21)</th>
<th>Normal controls (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD34⁺/CD71⁺ cells</td>
<td>0.39 ± 0.29 #</td>
<td>0.26 ± 0.18</td>
<td>0.32 ± 0.24</td>
<td>0.60 ± 0.33</td>
</tr>
<tr>
<td>median (range)</td>
<td>0.3 (0.1-1.1)</td>
<td>0.2 (0.1-0.6)</td>
<td>0.3 (0.1-1.1)</td>
<td>0.6 (0.1-1.4)</td>
</tr>
<tr>
<td>P value*</td>
<td>0.0398</td>
<td>0.0014</td>
<td>0.0011</td>
<td></td>
</tr>
<tr>
<td>P value**</td>
<td>0.2169</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CD36⁺/GlycoA⁺ cells</td>
<td>22.18 ± 8.05</td>
<td>27.91 ± 6.66</td>
<td>25.28 ± 7.59</td>
<td>23.98 ± 6.35</td>
</tr>
<tr>
<td>median (range)</td>
<td>23.2 (11.1-38.6)</td>
<td>28.3 (19.3-42.3)</td>
<td>23.7 (11.1-42.3)</td>
<td>23.5 (13.1-38.2)</td>
</tr>
<tr>
<td>P value*</td>
<td>0.4343</td>
<td>0.1267</td>
<td>0.6061</td>
<td></td>
</tr>
<tr>
<td>P value**</td>
<td>0.0529</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CD36⁻/GlycoA⁺ cells</td>
<td>65.61 ± 9.60</td>
<td>58.43 ± 8.03</td>
<td>61.73 ± 9.16</td>
<td>66.77 ± 9.09</td>
</tr>
<tr>
<td>median (range)</td>
<td>64.40 (46.5-78.5)</td>
<td>58.70 (42.0-71.2)</td>
<td>62.3 (42.0-78.5)</td>
<td>69.1 (43.9-78.8)</td>
</tr>
<tr>
<td>P value*</td>
<td>0.5541</td>
<td>0.0125</td>
<td>0.0483</td>
<td></td>
</tr>
<tr>
<td>P value**</td>
<td>0.0378</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(†) ACD: Anemia of chronic disease. (‡) Non-anemic and ACD RA patients. (#) Values are expressed as mean ± 1SD.

NOTE: Comparison (*) between patients in group and normal controls; (**) between patients with ACD and non-anemic patients. Statistical analysis was performed using the nonparametric Mann-Whitney U test. P value ≤ 0.05 was considered as statistically significant.
### Table 3. Flow-cytometric data in RA patients after anti-TNFα treatment

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment (n=12)</th>
<th>Post-treatment (n=12)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total BM cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CD34⁺/CD71⁺ cells</td>
<td>0.35 ± 0.19⁺</td>
<td>1.15 ± 0.57</td>
<td>0.0006</td>
</tr>
<tr>
<td>median (range)</td>
<td>0.32 (0.1-0.6)</td>
<td>1.25 (0.1-1.9)</td>
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</tr>
<tr>
<td>% CD36⁺/GlycoA⁺ cells</td>
<td>29.06 ± 6.80</td>
<td>24.29 ± 7.26</td>
<td>0.101</td>
</tr>
<tr>
<td>median (range)</td>
<td>28.98 (21.4-42.3)</td>
<td>22.8 (15.9-36.0)</td>
<td></td>
</tr>
<tr>
<td>% CD36⁻/GlycoA⁺ cells</td>
<td>56.17 ± 7.09</td>
<td>64.09 ± 9.12</td>
<td>0.0220</td>
</tr>
<tr>
<td>median (range)</td>
<td>57.10 (42.0-67.9)</td>
<td>67.45 (47.6-73.3)</td>
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<tr>
<td><strong>CD34⁺/CD71⁺ cell fraction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 7AAD⁺⁺⁺ cells</td>
<td>33.11 ± 11.82</td>
<td>19.20 ± 10.60</td>
<td>0.0011</td>
</tr>
<tr>
<td>median (range)</td>
<td>37.0 (6.9-45.3)</td>
<td>17.30 (6.9-42.6)</td>
<td></td>
</tr>
<tr>
<td><strong>CD36⁺/GlycoA⁺ cell fraction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 7AAD⁺⁺⁺ cells</td>
<td>42.89 ± 22.27</td>
<td>19.34 ± 16.08</td>
<td>0.0008</td>
</tr>
<tr>
<td>median (range)</td>
<td>40.44 (6.9-75.49)</td>
<td>14.9 (4.6-53.6)</td>
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</tr>
<tr>
<td><strong>CD36⁻/GlycoA⁺ cell fraction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 7AAD⁺⁺⁺ cells</td>
<td>2.24 ± 1.71</td>
<td>2.84 ± 1.25</td>
<td>0.378</td>
</tr>
<tr>
<td>median (range)</td>
<td>1.55 (1.0-6.49)</td>
<td>2.7 (1.0-5.5)</td>
<td></td>
</tr>
</tbody>
</table>

(⁺) All values are expressed as means ± SD. Statistical analysis was performed using the Student’s t-test for paired samples. P value ≤ 0.05 was considered as statistically significant.
Figure 1
Figure 2
Figure 3

\[ \ln y = -0.399x + 7.433 \]
\[ r = 0.894, p < 0.0001 \]

\[ \ln y = -0.402x + 7.460 \]
\[ r = 0.808, p < 0.001 \]
Figure 4
Figure 5
Figure 6

![](Image)
Figure 7

% Hgb change from baseline

- TOTAL pts
- ANEMIC pts: P<0.001
- ACD pts: P<0.005
- IDA pts: N.S.
Anemia of chronic disease in rheumatoid arthritis is associated with increased apoptosis of bone marrow erythroid cells. Improvement following anti-tumor necrosis factor alpha antibody therapy

Helen A Papadaki, Heraklis D Kritikos, Vasilis Valatas, Dimitrios T Boumpas and George D Eliopoulos