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Depletion of synovial macrophages in rheumatoid arthritis by an anti-FcγRI-Calicheamicin immunoconjugate

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Running title: FcγRI-directed immunoconjugate depletes macrophages in RA

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Key words: Calicheamicin, CD64, immunoconjugate, monocytes/macrophages, Th1
Abstract

**Background:** Monocytes/macrophages are indicated to have an important and versatile role in joint inflammation and destruction in rheumatoid arthritis (RA).

**Objective:** To determine the efficiency of monocyte/macrophage elimination by a novel drug-conjugated antibody (CD64-Calicheamicin, CD64-CaMi) directed to the high affinity receptor for IgG (FcγRI).

**Methods:** Mononuclear cells from peripheral blood and synovial fluid of RA patients were cultured in the presence of CD64-CaMi. Cell death of monocytes/macrophages was measured by analysis of phenotypic changes (light scatter patterns, CD14- and FcγRI expression) and nuclear DNA fragmentation. The selectivity of CD64-CaMi was checked by using FcγRI-deficient and FcγRI-transfected cell lines. In addition, the indirect effect of CD64-CaMi-induced macrophage cell death on arthritic T(h1) cell activity was determined.

**Results:** Inflammatory macrophages from RA synovial fluid, expressing increased FcγRI levels, were efficiently killed by CD64-CaMi via induction of DNA fragmentation. CD64-CaMi-induced cell death of monocytes/macrophages from peripheral blood of RA patients proved less efficient. Induction of synovial macrophage death by CD64-CaMi was accompanied by an efficient inhibition of proinflammatory T(h1) cytokine production.

**Conclusion:** Together, the presented data suggest elimination of macrophages through a novel FcγRI-directed CD64-CaMi to be feasible. Since monocytes from the peripheral blood are also eliminated by this immunoconjugate, additional experimental studies should validate its potential for local (intra-articular) application in the treatment of RA.
Introduction
Patients with rheumatoid arthritis (RA) are characterised by chronic inflammation and destruction of their joints (1). Macrophages in the inflamed joints of RA patients are multitalented cells that are postulated to contribute to joint inflammation by inducing a multitude of biological responses, including the secretion of a wide range of pro-inflammatory and tissue-destructive mediators, and presentation of antigen, resulting in T cell and (indirectly) B cell activation (2;3). A number of approaches to counter macrophage activity resulted in substantial improvements in the treatment of RA and have increased our understanding of the mechanisms that contribute to joint inflammation (4;5). However, a considerable number of RA patients do not benefit from these therapies and the effects are transient. It has been recognized that blockade of one or some of the multitude of macrophage effector functions may not be sufficient to control the inflammatory response in RA (6-8). Recently we put forward a novel concept to completely block macrophage function by causing selective macrophage elimination exploiting the high-affinity receptor for IgG, FcγRI (CD64) (9;10).

Monocytes and macrophages from patients with RA and healthy controls constitutively express substantial levels of FcγRI (11-13). Antibodies binding to FcγRI, not only via their Fc-tail, but also experimentally generated antibodies that selectively bind to distinct parts of FcγRI (14) are endocytosed very efficiently (15). This characteristic of FcγRI was exploited previously to eliminate macrophages with enhanced FcγRI expression by using an anti-FcγRI (CD64) antibody to which the plant toxin ricin A (RTA) was chemically linked (CD64-RTA). Importantly, activated macrophages from RA joints were selectively killed compared to peripheral blood monocytes (10), which was related to increased FcγRI levels on synovial macrophages compared to peripheral blood monocytes. This suggested that in RA FcγRI might represent a target for other CD64-conjugated drugs to induce a specific elimination of inflammatory macrophages.

To improve the potency of CD64-immunoconjugates we considered another potent drug to target to FcγRI. Members of the enediyne family of antibiotics are among the most toxic compounds described to date (16). Calicheamicin (CaMi) is a highly potent drug of this family that binds to DNA in a sequence-specific way, thereby causing breaks in double-stranded DNA, which represents an initial step leading to cell death (17).

Methods
Patients. Peripheral blood (PB) and synovial fluid (SF) samples from 12 patients with active RA visiting our outpatient clinic were used. All patients fulfilled the 1987 revised American College for Rheumatology (ACR) criteria for RA (18). Nine patients were rheumatoid factor positive. Patients ranged in age from 46 to 80 years (mean 61 ± 11 years), 8 patients were treated with methotrexate, 3 took sulfasalazine and 3 received prednisone. The patients ranged in disease duration from 2 to 42 years (mean 18 ± 7 years).

Cell cultures and reagents. PB and SF were diluted 1:1 with RPMI 1640 medium (Gibco, NY, USA) containing glutamine (2mM), penicillin (100 U/ml) and streptomycin sulfate (100 µg/ml; RPMI*). Subsequently mononuclear cells (MC) were isolated by density centrifugation using Ficoll-Paque (Pharmacia, Uppsala, Sweden). Viability of cells, checked by trypan blue exclusion, always exceeded the 95%. Isolated PBMC or SFMC (5.10⁶ cells/ml) were cultured in RPMI* supplemented with 10% pooled human male AB serum (Red Cross Blood Transfusion Centre, Utrecht, The Netherlands) in 200 µl round-bottomed 96-well plates under standardised conditions at 37°C. Culture periods and concentrations of additions such as immunoconjugate are indicated where relevant.
**Immunocjugate.** The immunoconjugate CD64-Calicheamicin (CD64-CaMi) was constructed exactly as described for the calicheamicin-conjugated CD33 antibody (19) and was kindly provided by Wyeth Research (Pearl river, NJ). The hydrolysable chemical conjugate allows the CaMi to be released intracellular from the CD64 monoclonal antibody (H22, Medarex, Annandale, NJ, ref (20)) via a reducible linker. To make a comparison with other CD64 (H22)-conjugated antibodies possible, CD64-CaMi concentrations were calculated from the concentrations of antibody used. The conjugated antibody molecules that were produced contained 4 CaMi molecules per CD64 (H22) molecule.

**Fc\(\gamma\)RI expression.** To analyse Fc\(\gamma\)RI expression on monocytes and macrophages, SFMC (5.10^5 cells) were double stained with FITC-labelled anti-Fc\(\gamma\)RI monoclonal antibody (clone 32.2, Medarex) and PE-labelled CD14 (clone Tuk4, DAKO, Glostrup, Denmark) at 4 °C for 15 minutes. The Fc\(\gamma\)RI detection antibody (clone 32.2) recognises an Fc\(\gamma\)RI epitope, distinct of the epitope recognised by the CaMi-coupled antibody H22 (CD64-CaMi) and is not hindered by binding of CD64-CaMi (21). After staining, cells were resuspended in PBS containing 0.5% BSA and 0.1% azide and directly analysed by flow cytometry (FACScan, BD) using WinMDI software (BD).

**Determination of cell death by light scatter changes and CD14 expression.** Light scatter changes (forward scatter, FSC and side scatter, SSC), detected by FACS analyses (FACScan, BD) are characteristic of cell death. Upon apoptosis induction these light scatter changes most sensitively measure cell death and strongly correlate with trypan blue staining, DNA strand breaks and Annexin V binding (22;23). Since loss of CD14 expression was recently shown to be an early effector mechanism by which cell death of monocytes is mediated (e.g. induced by IL-4 or Fas cross-linking) (22;24), CD14 expression levels were also studied as a measure of cell death.

**Apoptosis.** To assess macrophage apoptosis, nuclear DNA fragmentation was determined using propidium-iodide (PI) staining as described previously (25). Briefly, after culture MC were fixed by adding 500 µl of cold 70% ethanol for at least 2 hours at -20°C. The cells were then washed with PBS and incubated for 20 minutes at 37 °C with buffer comprising 0.05 M Na\(_2\)HPO\(_4\), 0.0025 M citric acid and 0.1% Triton X-100 to extract small intracellular DNA fragments. During the same period macrophages were stained by FITC-labelled CD68 (clone EBM11, DAKO, Glostrup, Denmark). CD68 was used since apoptotic monocytes/macrophages were shown to loose CD14 expression. CD68 proved to be an intracellular marker with a persistent expression and served to identify apoptotic (and intact) macrophages. After this period, propidium iodide (Sigma, St Louis, MO, 20 µg/ml) was added to stain the (remaining, subdiploid) nuclear DNA. Fluorescence was subsequently measured by flow cytometry.

**Proliferation assays.** To study the efficacy and Fc\(\gamma\)RI-mediated selectivity of CD64-CaMi the inhibition of IIA1.6 cell proliferation was assessed. These cells lack Fc\(\gamma\)R expression and are derived from the murine A20 B cell lymphoma and have been shown to belong to a distinct subset of CD5+ B cell/macrophage cells (26). IIA1.6 cells transfected with the FcR \(\gamma\)-chain cDNA were additionally transfected with either Fc\(\gamma\)RI cDNA, or (as a control) Fc\(\alpha\)RI cDNA (26;27). Transfected IIA1.6 cells (2.10^5 cells/ml) were cultured for 3 days in 96-well round-bottomed plates in RPMI supplemented with 10% fetal bovine serum, 50 µg/ml gentamycin, 2mM L-glutamine, 1mM sodium pyruvate and 5 µM methotrexate. Cells were cultured in the presence or absence of different concentrations of CD64-CaMi (10^{-12} M to 10^{-8} M).
Proliferation was measured by incorporation of methyl \(^3\)H-Thymidine (NEN, Boston, MA) during the last 18 hours of culture.

**Cytokine measurements.** To assess the effect of CD64-CaMi-induced macrophage death on Th1 cytokine production, SFMC (1.10^6 cells/ml) were cultured in 24-well flat-bottomed plates for 3 days in the presence or absence of CD64-CaMi. To detect modulation of Th1 cell activity which may be due to indirect T cell death or loss of T cell activity, T cell cytokine production after this culture period was assessed upon specific T cell stimulation by soluble CD3/CD28 monoclonal antibodies (1\(\mu\)g/ml, CLB-T3/4E and CLB-CD28, CLB Reagentia, Amsterdam, The Netherlands) for 24 hours (28). Previously we have shown that cytokine production (IFN\(\gamma\), TNF\(\alpha\)) of MC upon CD3/CD28 stimulation is for more than 90% T cell derived (29) and does not require cross-linking by Fc\(\gamma\) receptors on antigen presenting cells (29;30). After this T cell stimulation, media were harvested and freed of cellular material by centrifugation (5 min, 900 g), frozen in liquid nitrogen and stored at -20 °C. IFN\(\gamma\) and TNF\(\alpha\) levels in the culture supernatants were determined by ELISA according to manufacturer's instructions (Biosource Europe, Nivelles, Belgium).

**Statistical analyses.** Wilcoxon, and Mann-whitney U tests were used for non-paired, and paired observations, respectively. Spearman statistical tests were used for correlation analyses. Data were considered statistically significant at p<0.05.

**Results**

**Induction of Fc\(\gamma\)RI-dependent cell death by CD64-CaMi.** To test whether CD64-CaMi can trigger selective Fc\(\gamma\)RI-dependent cell death, the effect of immunoconjugate was evaluated on the proliferation of the IIA1.6 B cell/macrophage lymphoma cell-line which lacks Fc\(\gamma\)Rs (26). IIA1.6 cells, stably transfected with Fc\(\gamma\)RI (and the Fc\(\gamma\)R \(\gamma\)-chain) were efficiently killed by CD64-CaMi as observed by a strong reduction in proliferation, at concentrations of 10^{-9} and 10^{-8} M (Figure 1). IIA1.6 cells expressing a transfected control Fc receptor, Fc\(\alpha\)RI (and the Fc\(\gamma\)R \(\gamma\)-chain) were not significantly affected by CD64-CaMi, indicating CD64-CaMi to induce a selective Fc\(\gamma\)RI-dependent killing.

**CD64-CaMi immunoconjugate induces death of monocytes/macrophages from RA patients.** As shown in previous studies (10;12;13), Fc\(\gamma\)RI was expressed at substantial levels on monocytes/macrophages from both peripheral blood (PB) and synovial fluid (SF) from RA patients (Figure 2A). The Fc\(\gamma\)RI expression level was significantly higher on SF than on PB monocytes/macrophages (p<0.05, data not shown).

On the basis of the forward - side light scatter (FSC-SSC) characteristics of mononuclear cell subpopulations can be distinguished that consist mainly of lymphocytes or monocytes/macrophages. Based on decreases in FSC and SSC, characteristic of cell death, incubation with CD64-CaMi for 24 hours resulted in cell death of monocytes/macrophages (data not shown). Macrophage death assessed by light scatter changes strongly correlated with more specific markers of macrophage death (10), such as CD14 expression (Figure 2) and nuclear DNA fragmentation of CD68\(^+\) cells (Figure 3).

Down-regulation of CD14 expression on monocytes/macrophages by several reagents represents an early event in the apoptotic cascade of these cells and correlates with increased DNA fragmentation (10;22-24;31). CD64-CaMi-induced monocyte/macrophage cell death, measured by CD14 reduction and consequently a reduced number of CD14\(^+\) cells (representative analysis is shown in Figure 2A), was observed to be significant at concentrations of 10^{-9} and 10^{-8} M (Figure 2B). At a concentration of 10^{-9} M CD64-CaMi the
decrease in viable SF CD14+ cells was significantly higher than that of PB CD14+ cells (p<0.05).

The reduction of SF CD14+ cells by CD64-CaMi was associated by FcγRI down-regulation. The average of the mean fluorescence intensity ± SEM of FcγRI at control 174 ± 31 was significantly (p<0.05) reduced to 104 ± 19 at 10^{-9} M (at 10^{-8} M the cell number to analyse was too low to permit a reliable calculation of FcγRI expression levels). The effects on FcγRI expression levels of PB CD14+ cells (average MFI ± SEM at control 101 ± 15 to 48 ± 5 at 10^{-9} M, p<0.05) were in the same order. Although FcγRI expression levels on SF macrophages were higher than on monocytes and CD64-CaMi-induced killing was more efficient for SF macrophages than for PB monocytes, we could not detect a significant correlation between these two parameters.

Monocyte/macrophage death induced by CD64-CaMi is mediated by DNA fragmentation. Nuclear DNA fragmentation represents a hallmark of the apoptotic process and was measured by propidium iodide staining of nuclear DNA upon extraction of intracellular fragmented DNA (25). A representative experiment showing reduction in DNA content of CD68+ cells with and without immunoconjugate treatment is shown in Fig.3A. Incubation for 24 h with CD64-CaMi resulted in a strong increase in dead CD68+ cells with reduced levels of nuclear DNA (figure 3B; on average from 4.2 ± 0.2 to 57.5 ± 18.5% at 10^{-8} M, p<0.05). In contrast to these effects, no significant cell death of CD68+ lymphocytes was observed (from 8.2 ± 4.0 to 13.0 ± 8.9 at 10^{-8} M). In line with these results, only minimal effects were observed when cell death of lymphocytes was measured by reduction in cell size (data not shown), which is characteristic of cell death (10;22) and detected by light scatter changes using flow cytometry.

Macrophage death is followed by abrogation of lymphocyte activation. Macrophages in particular play a crucial role in the persistent T cell activity in RA by cell-cell contact and the production of soluble mediators (3). Therefore, it was tested whether CD64-CaMi-induced macrophage death could lead to prevention of Th1 cell activity. After 3 days of culture in the absence of the immunoconjugate, T cells, upon restimulation with CD3/CD28, produced considerable amounts of IFNγ and TNFα. Previously we have demonstrated that this production is for more than 90% T cell derived (29). T cell cytokine production was strongly prevented when macrophages had been deleted by CD64-CaMi (Figure 4). IL-4 production at all times was below detection limit (data not shown).

Discussion
The present study shows an efficient and selective depletion of monocytes/macrophages by using an FcγRI-directed immunoconjugate (CD64-CaMi). Cell death of activated macrophages was associated with DNA fragmentation, and was followed in time by a reduction in Th1 cell activity.

Induction of cell death by CD64-CaMi seems to be more potent than CD64-RTA (10). At an optimal concentration (10^{-8} M) the induction of cell death (measured by CD14 reduction) by CD64-CaMi for both PB monocytes and SF macrophages is stronger (83 and 97%, respectively) than cell death induced by CD64-RTA, which we have previously described (18 and 54% cell death, respectively) (10). The finding that both synovial macrophages and monocytes from the circulation were efficiently killed by CD64-CaMi differs from CD64-RTA. This latter immunoconjugate was shown to induce death of synovial macrophages more efficiently than monocytes at a similar concentration of CD64 antibody as used in the present study (10). This difference in killing of monocytes may be due to different mechanisms of action between CaMi and RTA. Whereas RTA inhibits protein synthesis and induces caspase activity that finally leads to DNA damage and fragmentation (32), CaMi
translocates to the nucleus where it binds DNA and directly causes DNA strand breaks (17).

RA synovial macrophages differ greatly from monocytes in their activation status and protein synthesis. This could contribute to the different killing efficiencies in macrophages and monocytes by RTA compared to CaMi. Cell death of monocytic cells induced by CaMi may consequently be less dependent on the activation status and FcγRI expression level. This could explain why CD64-CaMi-induced cell death does not correlate tightly with FcγRI expression levels in contrast to CD64-RTA-induced cell death (10).

Activated T cells are present in large numbers in RA joints and are suggested to strongly contribute to joint inflammation by the activation of macrophages and B cells (3;33;34). Previously it was shown that CD64-RTA-induced cell death of synovial macrophages cocultured with lymphocytes is followed in time by the abrogation of Th1-driven (35;36) antigen-induced (mycobacterial hsp60) lymphocyte responses (10). The present study supports the assumption that elimination of monocytic cells by a CD64-immunoconjugate can lead to an almost complete prevention of Th1 cell responses (IFNγ and TNFα production), due to a decreased costimulatory function of the monocytic cells. In line with these data, T cell resolution in cutaneous inflammation was observed in mice following local elimination of dermal macrophages by CD64-RTA (9). Together these data suggest that CD64-immunoconjugates could lead to resolution of inflammation not only by removing inflammatory monocytic cells but indirectly also by preventing lymphocytic responses.

Previously CD64-RTA was demonstrated to selectively eliminate activated synovial macrophages whereas only a small percentage of circulating monocytic cells was affected. This suggested that such an immunoconjugate might be safe in systemic treatment of RA patients. In support of this it was found that systemic administration of CD64-RTA successfully inhibited experimental arthritis in a human FcγRI-transgenic rat model and was well tolerated without significant side effects (manuscript submitted for publication). The present work shows effective killing of monocytic cells from the site of inflammation and from the circulation. Since monocytes also play a role in the defense against pathogens this suggests that systemic administration of CD64-CaMi could lead to an undesired immune compromising state. However, in case of persistently inflamed joints, local (intra-articular) elimination of both macrophages and monocytes by CD64-CaMi may be desirable. This may not only lead to elimination of persistent inflammatory macrophages but also of recently infiltrated monocytes from peripheral blood that could start to contribute to the inflammatory process. The resolution of skin inflammation after elimination of FcγRI+ cutaneous inflammatory macrophages upon local administration indicates the efficiency and safety of this approach (9;37). Further studies should reveal the effectiveness and potential risks of CD64-CaMi in arthritis upon intra-articular administration.

The present study indicates FcγRI on synovial macrophages to represent a suitable target for immunoconjugate-mediated depletion of these versatile cells that play an important proinflammatory role in RA, as well as other joint diseases. Future studies should however validate the potential of FcγRI-directed immunoconjugates as therapeutic agents for treatment of RA.

Legends to the figures

**Figure 1.** CD64-CaMi induces FcγRI-dependent cell death of FcγRI-transfected, but not FcαRI-transfected IIA1.6 cells. Proliferation of IIA1.6 cells (2.10^5/ml) was measured by \[^3\]H-Thymidine incorporation (proliferation) after 3 days of culture (n=3). The effects of CD64-CaMi were expressed as percentages of control cultures in the absence of immunoconjugate. Statistically significant differences of p<0.05 are indicated (*).

**Figure 2.** Flow cytometric analysis of the effect of CD64-CaMi on peripheral blood (PB) monocytes and synovial fluid (SF) macrophages from RA patients. A) Representative FcγRI/CD14 staining of mononuclear cells from PB and SF cultured for 24 hours with or without CD64-CaMi. CD64-CaMi-induced death of monocytic cells was associated with a reduction in CD14^+ monocytic cells from 22.9% to 14.0% (40% reduction). B) Average of CD64-CaMi-induced monocyte/macrophage cell death from PB and SF (both n=6) are expressed as a decrease in viable cells versus control, measured as a reduction in CD14 expression after 24 h of culture. Asterisks indicate significant differences of CD64-CaMi-induced cell-death compared to control cultures (* p<0.05). At 10^{-9} M CD64-CaMi, cell death of macrophages from SF, measured by reduction in CD14^+ cells (on average 57 ± 7%) was significantly higher than in PB (19 ± 3%, p<0.05, indicated by #).

**Figure 3.** Induction of nuclear DNA fragmentation of CD68^+ cells by CD64-CaMi. DNA content of CD68^+ lymphocytic and CD68^+ monocytic cells was stained with propidium iodide and measured by flow cytometry (FACS). A) Representative analysis showing that incubation of SFMC with CD64-CaMi (10^{-8} M for 24 hours) results in a strong increase in apoptotic CD68^+ cells with reduced nuclear DNA content due to DNA fragmentation (from 3.5% to 92.1 % of CD68^+ cells). B) On average (n=3, 10^{-8}M for 24 hours) a change from 4.2 ± 0.2% apoptotic macrophages in control culture to 57.5 ± 18.5% upon culture with CD64-CaMi (p<0.05) was observed. Apoptotic cell death of CD68^+ lymphocytic cells was not significantly changed (8.2 ± 4.0% to 13.1 ± 8.9%). Significant cell death of CD68^+ cells is indicated (*, p<0.05).

**Figure 4.** CD64-CaMi-induced macrophage apoptosis in time leads to prevention of CD3/CD28-induced T cell cytokine secretion (n=3). SFMC were cultured with (10^{-8} M and 10^{-9} M) or without CD64-CaMi for 3 days. After this T cells were stimulated by CD3/CD28 co-stimulation for 24 hours, and IFN\(\gamma\) and TNF\(\alpha\) levels were measured. Statistically significant inhibitions of T cell activity upon (pre-) treatment with CD64-CaMi compared to control cultures are indicated (*, p<0.05).
Reference List


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Figure 1

The graph shows the concentration of CD64-CaMi in M (M) on the x-axis ranging from 0 to 120 on the y-axis. The concentration is plotted against the percentage of H-thym. inc (% vs control) for both FcγRI and FcαRI.

- FcγRI shows a decrease in H-thym. inc (% vs control) as the concentration of CD64-CaMi increases.
- FcαRI also shows a decrease in H-thym. inc (% vs control) with increasing concentration of CD64-CaMi.

Significant differences are indicated by an asterisk (*) on the graph.
Figure 2

A

B

CD64-CaMi

Viable CD14+ cells (%)

Con

CD14

FcγRI

0

0

10^{-12}

10^{-11}

10^{-10}

10^{-9}

10^{-8}

CD64-CaMi (M)

PB

SF

* #

22.9 %

3.4 %

14.0 %

14.7 %
Figure 3

A

CD68⁻ CD68⁺

Control

CD64-CaMi

DNA content

apoptotic cells (%)

CD68⁺ cells
CD68⁻ cells

B

DNA content

Events

CD64-CaMi (M)

con 10⁻¹² 10⁻¹¹ 10⁻¹⁰ 10⁻⁹ 10⁻⁸

*
Figure 4

The figure shows a bar graph comparing the levels of IFNγ and TNFα (pg/ml) in the presence of different concentrations of CD64-CaMi (M). The x-axis represents the concentration of CD64-CaMi (M), ranging from 0 to 10^-8 M, while the y-axis represents the levels of IFNγ and TNFα in pg/ml, ranging from 0 to 5000 pg/ml.

- At 0 M CD64-CaMi, the levels of IFNγ and TNFα are baseline.
- As the concentration of CD64-CaMi increases to 10^-9 M and 10^-8 M, there is a significant increase in the levels of IFNγ and TNFα, as indicated by the asterisks (*) on the bars.