CD64-Directed Immunotoxin Inhibits Arthritis in a Novel CD64 Transgenic Rat Model

Anneke J. van Vuuren, Joel A. G. van Roon, Vanessa Walraven, Ilonka Stuij, Martin C. Harmsen, Pamela M. J. McLaughlin, Jan G. J. van de Winkel and Theo Thepen

*J Immunol* 2006; 176:5833-5838; doi: 10.4049/jimmunol.176.10.5833

http://www.jimmunol.org/content/176/10/5833

### References

This article cites 28 articles, 3 of which you can access for free at: http://www.jimmunol.org/content/176/10/5833.full#ref-list-1

### Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

### Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

### Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Macrophages are known to play a key role during inflammation in rheumatoid arthritis (RA). Inflammatory macrophages have increased expression of CD64, the high-affinity receptor for IgG. Targeting this receptor through a CD64-directed immunotoxin, composed of an Ab against CD64 and Ricin A, results in effective killing of inflammatory macrophages. In this study, we show elevated levels of CD64 on synovial macrophages in both synovial lining and synovial fluid in RA patients. The CD64-directed immunotoxin efficiently eliminates activated synovial macrophages in vitro, while leaving quiescent, low CD64-expressing macrophages unaffected. To examine whether killing of CD64 macrophages results in therapeutic effects in vivo, we established an adjuvant arthritis (AA) model in newly generated human CD64 (hCD64) transgenic rats. We demonstrate that hCD64 regulation in this transgenic rat model is similar as in humans. After AA induction, treatment with CD64-directed immunotoxin results in significant inhibition of disease activity. There is a direct correlation between immunotoxin treatment and decreased macrophage numbers, followed by diminished inflammation and bone erosion in paws of these hCD64 transgenic rats. These data support synovial macrophages to play a crucial role in joint inflammation in AA in rats and in human RA. Selective elimination of inflammatory macrophages through a CD64-directed immunotoxin may provide a novel approach for treatment of RA. The Journal of Immunology, 2006, 176: 5833–5838.

Rheumatoid arthritis (RA) is characterized by an inflammatory process in synovium resulting in progressive destruction of cartilage and bone in affected joints. The abundance and activation of synovial macrophages in inflamed synovium correlates with severity and chronicity of RA (1, 2). Macrophages are very versatile cells and exert a multitude of biological functions contributing to maintenance of inflammation and bone destruction. For instance, they can secrete a range of proinflammatory cytokines, chemokines, and metalloproteinases, which can directly trigger tissue damage (3, 4). Macrophages also regulate T cell and dendritic cell functions, and serve as APCs, in which capacity they might be involved in epitope spreading. In this capacity, macrophages can directly trigger tissue damage (3, 4). Macrophages also regulate T cell and dendritic cell functions, and serve as APCs, in which capacity they might be involved in epitope spreading. In contrast, macrophage functioning is subjected to control from the local milieu, like IFN-γ produced by inflammatory T cells. The interaction between macrophages and their environment may lead to a “vicious” circle, which maintains inflammation without external stimuli, resulting in a chronic phase.

At present, blockade of proinflammatory cytokines represents one approach to treat RA. Especially TNF-α, produced by macrophages, proved to be a key cytokine in destructive arthritis. Anti-TNF-α therapy results in impressive protection against joint inflammation and joint damage in RA patients (5, 6), although significant numbers of patients do not respond to this anti-TNF-α therapy. In these nonresponding patients, blockade of a single macrophage effector function is probably not sufficient enough to control disease. Therefore, counteracting complete macrophage activity, rather than inhibition of individual inflammatory mediators, may prove more efficacious. This idea is confirmed by experiments in which macrophages were eliminated through intraarticular injections of chlodronate containing liposomes relying upon strong phagocytic capacity of macrophages (7).

One hallmark of inflammatory macrophages is strongly enhanced expression of CD64 (FcγRI), the high-affinity receptor for IgG (8, 9). CD64 expression is limited to cells from the myeloid lineage and can be up-regulated by several cytokines like IL-10 and IFN-γ, both of which are enhanced in inflammatory RA joints. In addition, CD64 expression can be induced on neutrophils in humans (10). Endocytosis and phagocytosis have proven to be very efficient through the CD64 receptor which qualifies this receptor as a potential avenue to target macrophages (11).

In this study, we targeted inflammatory macrophages through CD64, aiming to selectively eliminate the activated inflammatory macrophages from arthritic joints. Therefore, we constructed an anti-CD64 Ab, chemically linked to the plant toxin Ricin A (RiA). Ricin is a well-defined catalytic inhibitor of protein synthesis at the level of the 60S ribosome. The structure of Ricin consists of a very potent ribosome-inactivating A chain linked by a disulfide bond to a galactose-specific lectin (B chain or binding chain). The A and B chains were separated, and after purification, the A chain was

---

**CD64-Directed Immunotoxin Inhibits Arthritis in a Novel CD64 Transgenic Rat Model**

Anneke J. van Vuuren, Joel A. G. van Roon, Vanessa Walraven, Ilonka Stuij, Martin C. Harmsen, Pamela M. J. McLaughlin, Jan G. J. van de Winkel, and Theo Thepen

Macrophages are known to play a key role during inflammation in rheumatoid arthritis (RA). Inflammatory macrophages have increased expression of CD64, the high-affinity receptor for IgG. Targeting this receptor through a CD64-directed immunotoxin, composed of an Ab against CD64 and Ricin A, results in effective killing of inflammatory macrophages. In this study, we show elevated levels of CD64 on synovial macrophages in both synovial lining and synovial fluid in RA patients. The CD64-directed immunotoxin efficiently eliminates activated synovial macrophages in vitro, while leaving quiescent, low CD64-expressing macrophages unaffected. To examine whether killing of CD64 macrophages results in therapeutic effects in vivo, we established an adjuvant arthritis (AA) model in newly generated human CD64 (hCD64) transgenic rats. We demonstrate that hCD64 regulation in this transgenic rat model is similar as in humans. After AA induction, treatment with CD64-directed immunotoxin results in significant inhibition of disease activity. There is a direct correlation between immunotoxin treatment and decreased macrophage numbers, followed by diminished inflammation and bone erosion in paws of these hCD64 transgenic rats. These data support synovial macrophages to play a crucial role in joint inflammation in AA in rats and in human RA. Selective elimination of inflammatory macrophages through a CD64-directed immunotoxin may provide a novel approach for treatment of RA. The Journal of Immunology, 2006, 176: 5833–5838.
coupled to CD64 Abs to generate a very specific cell-reactive conjugate. Recently, we have shown that this CD64-directed immunotoxin (CD64-RiA) is very efficient in a chronic inflammatory skin model in human CD64 (hCD64) transgenic mice (12). Also successful antitumor activity using RiA-conjugated Abs, directed against several tumors, have been reported in cancer patients (13, 14).

In the present study, we determined levels of CD64 on activated macrophages in both synovial lining as in synovial fluid from RA patients, and examined the in vitro susceptibility of these cells for CD64-RiA-mediated killing.

To test whether killing of CD64 macrophages results in therapeutical effects in vivo, we established an adjuvant arthritis (AA) model in rats which bears a very close pathological resemblance to RA in patients (15, 16). For this purpose, we generated hCD64 transgenic rats. First, we characterize these hCD64 transgenic rats, by determining hCD64 expression, regulation of this hCD64 expression using the cytokines IFN-γ and G-CSF, as well as functionality of this hCD64 receptor in transgenic rats. After successful induction of AA in these hCD64 transgenic rats, we performed studies in which activated macrophages were eliminated in vivo through CD64-RiA. Furthermore, front paws of these transgenic rats were extensively analyzed for inflammation, and bone erosion using macrophage markers ED1, ED2, and receptor activator of NF-κB ligand (RANKL). RANKL is produced by activated T cells (17), and mediates differentiation and activation of osteoclasts involved in bone erosion, a key event in arthritis. In addition, RANKL regulates lymphocyte development, and augments T cell/ dendritic cell cooperative interactions (17, 18). We document effective elimination of activated macrophages accompanied by diminished inflammation and bone erosion at the histological level resulting in significant inhibition of disease activity. The data presented here indicate the employability of CD64 targeted immunotoxins for the treatment of RA.

**Materials and Methods**

**Immunotoxins**

Immunotoxin CD64-RiA was prepared by Medarex. Humanized CD64 mAb (H22) was chemically conjugated to two molecules of low-glycosylated RiA using a cleavable cross-linker N-succinimidyl-3-(2-piryldyldi-thio) propionate. All procedures were performed under good laboratory practice conditions according to the manufacturer’s instructions. Conjugated CD64-RiA was purified using size exclusion chromatography and purity was checked on SDS-PAGE (12).

**Cell depletion experiments**

Mononuclear cells (MC) were isolated from synovial fluid of RA patients as previously described (19). Viable synovial fluid macrophages (5 × 10⁶ cells/ml) were cultured for 24 h in the absence or presence of CD64-RiA (2 × 10⁻¹⁰ to 2 × 10⁻⁸ M RiA). The cytotoxic effect was measured by determining apoptosis.

**Apoptosis**

To assess macrophage apoptosis, nuclear DNA fragmentation, which is a hallmark of the apoptosis process, was determined using propidium iodide staining (20). Because apoptotic MC lost CD14 expression (21), CD68⁺CD14⁻ was used to detect apoptotic CD68⁺ cells. During this time, macrophages were stained with CD68⁺Ab (clone EB111; DAKO). Afterward propidium iodide (10 µg/ml; Sigma-Aldrich) was added to stain nuclear DNA, and fluorescence was analyzed by flow cytometry.

**Generation CD64 transgenic rats**

An 18-kb linear genomic DNA fragment encoding the entire 9.4-kb coding region of the human FcγRIIA gene as well as its own promoter and regulatory elements was microinjected into fertilized oocytes of 4-wk-old Wistar rats (Harlan). The same construct was previously used in transgenic mice and extensively studied (11). Injected oocytes were transferred to pseudopregnant Wistar foster mothers (22). One transgenic founder was mated with Wistar rats, and hemizygous transgenic offspring was identified by Southern blot analysis and genomic PCR. Offspring was routinely checked for CD64 expression, for which 25 µl of blood was incubated with 10.1 FcγR, followed by lysis of erythrocytes, fixation of white blood cells in FACs Lysis Solution (BD Biosciences), and flow cytometry. Transgenic offspring were crossed to Lewis (University of Maastricht, Maastricht, The Netherlands) background for induction of AA. The F₂ and F₃ generations were used in the studies presented in this manuscript. Rats were maintained at the Central Laboratory Animal Institute (Utrecht University) and all experiments were approved by the Utrecht University animal ethics committee.

**Antibodies**

To detect hCD64 expression, we used FITC-conjugated mAb 10,1 (Sero- tec) or PE-conjugated CD64 mAb 22 (BD Biosciences). To identify specific rat cells EDrB as a monocyte marker, His48 or RP-1 (BD Biosciences) as neutrophil marker, Ox33PE as B cell marker, and Ox19PE as T cell marker were used. EDrB and ED1Fv (Sero-tec) were used to study macrophages and monocytes in histochemistry analyses, and RANKL (Santa Cruz Biotechnology) measured activated T cells. Streptavi- din-H₂A was used to detect biotinylated mAb. In phagocytosis experiments, we used the bispecific Ab (m22 × rabbit anti-Candida albicans IgG) (23), and in Ab-dependent cellular cytotoxicity (ADCC) experiments the bispecific Ab MDX-H210 (18). Ed9, His48, Ox33, and Ox19 were gifts from E. Dopp (Free University, Amsterdam, The Netherlands).

**Cytokine regulation of CD64 in transgenic rats**

To investigate regulation of hCD64 expression by cytokines, transgenic rats were injected i.v. with recombinant rat IFN-γ (1 × 10⁶ Urat) (pro- vided by Dr. P. van der Meide, U-Cytech, Utrecht, The Netherlands). hCD64 expression was measured 24 h later. Recombinant human pegylated G-CSF (75 µg/200 µl saline) (provided by Dr. J. Andresen, Amsterdam, Thor- sand Oaks, CA) was injected i.p. in transgenic rats, and blood collected 3 days later. Erythrocytes were lysed from blood samples, and remaining leukocytes were analyzed for hCD64 expression by flow cytometry using mAb 10.1FITC.

**Phagocytosis**

*C. albicans* (American Type Culture Collection (ATCC), 448585) phagocytosis was performed as previously described (23). *C. albicans* was cultured overnight at 37°C in Sabouraud maltose broth (Difco), then centrifuged, washed three times with PBS, and counted. *C. albicans* was FITC labeled at concentration of 0.1 mg/ml FITC (Sigma-Aldrich) in 0.1 M sodium phosphate buffer (pH 9.6) for 30 min at room temperature, washed three times with PBS, aliquoted, and stored at −20°C until use. For phagocytosis 1 × 10⁵ peg-G-CSF primed rat neutrophils were incubated with 4 × 10⁶ FITC-labeled cells *C. albicans* in RPMI 1640 medium (Invitrogen Life Technologies) in the absence or presence of bispecific Ab (m22 × α-Can) (10 µg/ml). Yeast binding to neutrophils was quantified by flow cytometry. In addition, phagocytosis was studied in cytokin preparations by light microscopy.

**ADCC assay**

The killing capacity of rat neutrophils was investigated in ⁵¹Cr-release assays (26). ⁵¹Cr-labeled SKRB-3 cells (human breast carcinoma, HTB-30; ATCC) (5 × 10⁶ cells/well) were plated in RPMI 1640 medium, peg-G-CSF primed rat neutrophils (4 × 10⁶ cells/well) were then added in the presence or absence of different concentrations of bispecific Ab (BsAb) MDX-H210 (0.1 µg/ml, 0.4 µg/ml, and 2.0 µg/ml). BsAb MDX-H210 recognizes the proto-oncogene product HER-2/neu (24). After incubation at 37°C for 4 h, ⁵¹Cr release was measured in supernatants. These experiments were performed without serum complement.

**Induction and clinical assessment of AA**

Experimental arthritis was induced by an intradermal injection of *Myco- bacterium tuberculosis* (strain H37Ra) (1 mg/100 µl IFA; Difco) at the tail base. Rats were examined daily for developing clinical signs of arthritis in a blinded fashion. Severity of arthritis was determined by scoring of each
paw on a scale of 0–4 based on the degree of swelling, erythema, and deformation of joints (maximum score = 16) (15, 16). When individual rats achieved a total score of 4, they were i.v. injected at days 0, 1, 2, 4, 6, and 8 with CD64-RiA (750 µL, 10^{-6} M RiA) or with saline (placebo treated). At day 9 after treatment, rats were killed and paws removed for immunohistochemical analyses.

**Immunohistochemistry**

From all experimental animals we dissected the front paws, as well as tissue samples from spleen, kidney, and liver. Tissues were frozen in liquid nitrogen, and stored at −80°C before use. Sections (6 µm) were cut on a freezing microtome and mounted on coated slides. Nondecalcified front paws were cut using a carbide-tipped knife and attached to adhesive plastic tape (25). Human synovial tissue samples were preincubated with 10% normal human serum for 20 min before CD64 (10.1FITC) or isotype (IgG1FITC) staining in PBS containing 1% normal human serum for 45 min. All rat tissue samples were preincubated with 10% normal rat serum, before slides were stained for CD64. Tissue macrophages were stained with ED2FITC, and monocytes/macrophages with ED1FITC. Alkaline phosphatase-conjugated sheep α-FITC (Boehringer Mannheim) was used for detection as described (12). Activated T cells were stained with RANKL. Goat α-rabbit Biotin, and streptavidin peroxidase were used for detection. Peroxidase activity was assayed with H_{2}O_{2} as substrate and diaminobenzidine (Sigma-Aldrich) as chromogen. All slides were counterstained with hematoxylin. Processed tissues were evaluated and scored by two independent observers in a blinded fashion. Amount of staining per evaluated section was expressed from no staining (−), through few cells staining (+), to very abundant staining (+++), refer to Fig. 3d. An independent, pathologist validated randomly selected evaluated slides.

**Statistical analyses**

The Student t test was used to compare arthritis scores over time of the CD64-RiA-treated group, and nontreated group. Data were considered significant at p < 0.05.

**Results**

**Targeting CD64 expressing macrophages in RA**

To investigate whether CD64 expression levels were enhanced in chronic inflammation, we first studied CD64 expression in synovial tissues obtained from RA patients by immunohistochemistry. Synovial biopsies of RA patients, obtained from knee joint replacement surgery, consistently showed abundant staining for CD64 in lining, sublining, perivasculair area, and stroma (Fig. 1, a and b). The staining pattern was congruent to CD68 staining, which is a human macrophage marker (data not shown). In contrast, control synovial biopsies from individuals, which do not suffer from RA, showed occasional CD64 expression on lining cells only (data not shown). Macrophages isolated from synovial fluid of RA patients also showed high levels of CD64 expression compared with monocytes isolated from peripheral blood by FACS analyses (21).

Apoptosis is a controlled form of cell death, associated with normal physiology, as opposed to necrosis, which is associated with acute injury to cells. We investigated whether synovial fluid macrophages, identified by CD68 expression, from RA patients could be killed by CD64-RiA via apoptosis. Addition of CD64-RiA (2 × 10^{-8} M RiA) for 24 h to synovial fluid macrophage cultures resulted in strongly increased numbers of apoptotic CD68^{+} cells with reduced DNA content (on average 55.4 ± 13.5%, p < 0.01, n = 5) (Fig. 1c), indicating apoptosis induction.

To test whether killing of CD64 macrophages would have therapeutic effects in vivo, we established an AA model in hCD64 transgenic rats.

**Characterization of hCD64 transgenic rats**

To generate hCD64 transgenic rats, we used a genomic DNA fragment encompassing the entire human FcγRI gene (11). Eight copies of the transgene were incorporated, as determined by quantitative southern analyses (data not shown), in a stable transgenic Wistar line.

Peripheral blood samples from nontransgenic littermates showed no hCD64 expression (Fig. 2a) in contrast to blood samples from transgenic rats, in which hCD64 expression (mean fluorescence intensity (MFI) = 370 ± 45, n = 12) was determined (Fig. 2b). No hCD64 expression was observed on transgenic B cells (data not shown), nor on transgenic T cells (Fig. 2c). However, neutrophils (data not shown), and monocytes (Fig. 2d) expressed hCD64.

To investigate regulation of hCD64 expression in transgenic rats, we used the cytokines pegylated G-CSF and IFN-γ. Growth factor G-CSF increased hCD64 expression (MFI = 1350 ± 47, n = 6) and in addition, the number of neutrophils (44 vs 8% in non-G-CSF-treated transgenic littermates) (Fig. 2e). IFN-γ also increased hCD64 expression levels (MFI = 1049 ± 53, n = 4) (Fig. 2f) in blood of transgenic rats, compared with blood samples of non-IFN-γ-treated transgenic littermates (MFI = 370 ± 45).

Next, functionality of hCD64 expression in transgenic rats, we used the cytokines pegylated G-CSF and ADCC was studied. Hereto, a BsAb (m22 (m22 × αCan), recognizing both hCD64 and C. albicans, was used. In the presence of this hCD64BsAb, 69.9 ± 4.9% of transgenic neutrophils showed binding to C. albicans, vs 15.3 ± 4.7% of nontransgenic neutrophils (n = 6, p < 0.005). Phagocytosis of C. albicans was demonstrated by light microscopy in cytospin preparations (Fig. 2, g and h). Furthermore, ADCC experiments were performed in which human breast carcinoma cells (SKBR-3 cells) were lysed by G-CSF-primed transgenic rat neutrophils via a BsAb (MDXH210) in 51Cr-release assays (26). Transgenic neutrophils efficiently killed SKBR-3 cells (73.5 ± 5%), in contrast to nontransgenic.
neutrophils (6.4 ± 2.6%, n = 3). These data demonstrated expression of hCD64 to be regulated similarly as in man, and confirmed functionality of hCD64 in this novel transgenic rat model.

**Effect of CD64-RiA on AA**

To study the in vivo effect of CD64-RiA, AA was induced in hCD64 transgenic rats by s.c. injection of *M. tuberculosis* in IFA. AA represents an experimental model that closely resembles arthritis pathology in humans (15, 16). Animals were monitored daily for development of clinical signs. Each paw was scored for joint swelling and erythema. In transgenic as well as in nontransgenic animals, arthritis developed with similar kinetics and to the same extent, excluding an influence of the hCD64 gene in the transgenic animals (data not shown). When individual animals reached a total score of four, they were injected with CD64-RiA i.v., or saline as control (total of six injections). CD64-RiA treatment showed significant inhibition of arthritis progression (n = 7), compared with control animals (n = 8), in which arthritis scores reached maximum levels (p < 0.005) (Fig. 3a). A total of four experiments were performed and showed reproducible data.

In addition to the clinical scoring, extensive immunohistochemical analyses of front paws of all experimental animals were performed. These analyses confirmed inflammation in all non-treated animals, manifested by severe infiltration with lymphocytes and mononuclear cells, including CD64-expressing monocytes and macrophages (Table I). Infiltrates were observed both intra- and periarticularly, reaching out into the upper dermal layers. These were accompanied by cartilage destruction and mild to severe erosion of tarsal and metatarsal bones (Fig. 3b). In CD64-RiA-treated animals, however, no or only mild inflammation was observed, primarily detectable as small, well delineated periarticular infiltrates, while only very minor cartilage or bone destruction was observed (Table I, Fig. 3c). The nontreated animals also showed increased new bone formation, resulting in deformation of affected joints (Fig. 3, a and b).

**FIGURE 3.** CD64-RiA treatment inhibits arthritis progression in CD64-transgenic rats. The median arthritis score of one representative experiment of four is shown. Seven transgenic rats were injected with CD64-RiA, and eight controls were injected with saline on days 0, 1, 2, 4, 6, and 8 (p < 0.005) (a). Immunohistochemical analyses of front paws (×10 objective). Rats were killed at day 9 after treatment started, paws were isolated, and sections were stained for CD64. Positive CD64 staining and bone erosion observed in a placebo-treated rat (b). Less CD64 staining and no bone erosion was present in CD64-RiA-treated animals (c). CD64-positive cells were present in upper dermis layer of placebo-treated animals (d), but not in CD64-RiA-treated rats (e).
Table I. Immunohistochemical staining of left front paws of hCD64 transgenic rats

<table>
<thead>
<tr>
<th>Marker</th>
<th>Placebo Treated</th>
<th>CD64-RiA</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IA</td>
<td>EA</td>
<td>IA</td>
</tr>
<tr>
<td>ED1</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>ED2</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD64</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>RANKL</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*After induction of arthritis, and subsequent treatment for 9 days with either CD64-RiA (n = 7) or saline (placebo) (n = 8), animals were killed, and paws were removed. Transgenic control animals (n = 6) had no arthritis induced and were not treated. IA, Intra-articular; EA, Extra-articular; NT, not tested.

In synovial lining, ED2 staining, representing tissue macrophages, showed expression patterns comparable to CD64. In infiltrates, however, far less ED2 expression was observed compared with CD64. Staining for ED1, a broader marker than ED2, that stains both monocytes, macrophages, and dendritic cells (25), showed a staining pattern comparable to CD64 (Table I). Osteoclasts are involved in bone erosion, therefore we determined RANKL, which differentiates and activates these osteoclasts. RANKL staining of front paws was decreased in CD64-RiA-treated animals, compared with nontreated animals (Table I). This corresponded with the observed mild bone erosion in CD64-RiA-treated rats compared with nontreated animals.

Discussion

Addition of hCD64-RiA to macrophage cultures isolated from synovial fluid of RA patients showed apoptosis of CD68+ cells. We previously showed that only IFN-γ stimulated monocytic U937 cells were effectively killed by CD64-RiA, whereas nonstimulated U937 cells, which do have baseline CD64 expression, remained unaffected (12). This indicates that apart from CD64 expression, activation of target cells may be a prerequisite for the cytotoxic effect of CD64-RiA. Recent work indicates that insufficient apoptosis of inflammatory cells in RA joints might contribute to pathogenesis (27, 28). Besides selective elimination, a strong inhibition of proinflammatory cytokines like TNF-α and IL-1β was observed (21). Due to the versatile role of macrophages in both inflammation and bone destruction, we hypothesize that reduction of the number of activated macrophages in RA may be more beneficial than blockade of single cytokines like TNF-α, which is frequently used in the clinic at the moment. In addition, the new strategy to eliminate activated macrophages only via targeting CD64 may further clarify the role of these macrophages in development and progression of RA.

Analysis of the hCD64 transgenic rats documented CD64 expression on monocytes and neutrophils, and showed expression to be increased by IFN-γ and G-CSF. Human neutrophils do not continuously express CD64, although they can express CD64 under inflammatory conditions (8). The higher hCD64 expression levels on both monocytes and neutrophils in transgenic rats, compared with humans, may be attributable to the number of gene copies into the genome, which has also been observed in a murine hCD64 transgenic line (11). However, phagocytosis and ADCC experiments established functionality of hCD64 in transgenic rats. In conclusion, this hCD64 transgenic rat represents a novel animal model, in which CD64 expression, regulation of expression, and receptor functioning is similar to that in humans.

AA has been demonstrated to be a rat model, that closely resembles RA in humans. Therefore, the in vivo effect of CD64-RiA was studied in these CD64 transgenic rats, after induction of AA. Injection of CD64-RiA in arthritic animals induced a significant inhibition of disease progression compared with control animals, injected with saline. In all experiments however, a small number of rats did not respond to CD64-RiA treatment, which might be attributable to the mixed genetic Wistar/Lewis background of the transgenic animals.

Immunohistochemical staining of front paws showed extensive staining for CD64 and ED1 in inflammatory infiltrates in nontreated animals, while ED2, a tissue macrophage marker, showed far less staining, suggesting specific elimination of activated macrophages in CD64-RiA-treated animals. These data indicate that increased CD64 expression is already induced in the very early stages of differentiation from monocyte to inflammatory macrophage. RANKL staining was also decreased in CD64-RiA-treated animals, and corresponded to an observed mild bone erosion in these rats. This indicates that elimination of activated macrophages may result in decreased numbers of activated T cells, subsequently leading to diminished bone erosion and joint damage. This establishes a direct correlation between CD64-RiA treatment, and decreased macrophages numbers observed in vivo, followed by diminished inflammation and bone erosion. It can, however, not be excluded that CD64-expressing neutrophils, which may also be affected by CD64-RiA treatment, play a role in the pathophysiology of arthritis. Part of the observed effects in our experiments could, therefore, be attributed to depletion of neutrophils. Previous in vitro studies using the same CD64-RiA immunotoxin on human synovial fluid cells (17), however, clearly showed anti-inflammatory effects due to elimination of macrophages, rather than neutrophils.

Overall, a direct correlation between microscopic observations and macroscopic arthritis scores was found in the in vivo experiments. Histological examination of liver, spleen, and kidney sections from all animals showed no abnormalities. Adjuvant arthritis represents an animal model in which it is notorious difficult to intervene as the inflammatory processes involving cytokine production, cell recruitment, and activation are already ongoing, thereby closely resembling the situation in RA patients. Despite this, CD64-RiA treatment was found to significantly inhibit arthritis progression in the transgenic animals. In addition, we observed the presence of activated CD64-positive macrophages, in both synovial lining and fluid of RA patients, as well as efficient killing of these cells with CD64-RiA. Taken together, these data indicate that elimination of CD64-positive activated macrophages through a CD64-immunotoxin may provide a novel approach for treatment of RA.

Acknowledgments

We thank Prof. Bijlsma for providing human RA material, Prof. Slootweg for evaluation of the immunohistochemical analyses, and employees of the Central Animal Laboratory for excellent animal care.

Disclosures

The authors have no financial conflict of interest.

References

8. van de Winkel, J. G. J., and P. J. Capel. 1993. Human IgG Fc receptor hetero-