Targeting the Hemoglobin Scavenger receptor CD163 in Macrophages Highly Increases the Anti-inflammatory Potency of Dexamethasone

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Abstract

Synthetic glucocorticoids are potent anti-inflammatory drugs but serious side effects such as bone mobilization, muscle mass loss, immunosuppression, and metabolic alterations make glucocorticoid therapy a difficult balance. The therapeutic anti-inflammatory effect of glucocorticoids relies largely on the suppressed release of tumor-necrosis factor-α and other cytokines by macrophages at the sites of inflammation. We have now developed a new biodegradable anti-CD163 antibody-drug conjugate that specifically targets the glucocorticoid, dexamethasone to the hemoglobin scavenger receptor CD163 in macrophages. The conjugate, that in average contains four dexamethasone molecules per antibody, exhibits retained high functional affinity for CD163. In vitro studies in rat macrophages and in vivo studies of Lewis rats showed a strong anti-inflammatory effect of the conjugate measured as reduced lipopolysaccharide-induced secretion of tumor-necrosis factor-α. The in vivo potency of conjugated dexamethasone was about 50-fold that of nonconjugated dexamethasone. In contrast to a strong systemic effect of nonconjugated dexamethasone, the equipotent dose of the conjugate had no such effect, measured as thymus lymphocytes apoptosis, body weight loss, and suppression of endogenous cortisol levels. In conclusion, the study shows antibody-drug conjugates as a future approach in anti-inflammatory macrophage-directed therapy. Furthermore, the data demonstrate CD163 as an excellent macrophage target for anti-inflammatory drug delivery.

Received 24 December 2011; accepted 19 April 2012; advance online publication 29 May 2012. doi:10.1038/mt.2012.103
This study now demonstrates the first design, construction and characterization of an anti-inflammatory macrophage-targeting ADC generated by linking GC to an anti-CD163 mAb. The anti-inflammatory potential was studied by analysing the in vitro and in vivo efficacy in suppression of lipopolysaccharide (LPS)-induced inflammation.

RESULTS

Design and synthesis of anti-CD163-dexamethasone conjugate

Figure 1 shows a schematic structure of the anti-CD163-dexamethasone-hemisuccinate-NHS esters to the primary amino groups of the mouse anti-rat CD163 monoclonal antibody Ed-2 (anti-CD163), which binds specifically to macrophages in rat tissues. In average, four dexamethasone molecules were conjugated per antibody with less than 1% remaining as free dexamethasone in the final preparations. Gel electrophoresis and size exclusion chromatography showed that the dexamethasone conjugation of anti-CD163 did not lead to the degradation or the formation of aggregates (Supplementary Figure S1 online). In a similar way, we synthesized anti-CD163 conjugated with the alternative GC analogues, prednisolone, and fluocinolone acetonide, which both have accessible hydroxyl-groups for hemisuccinate linkage (results not shown).

CD163-mediated binding of anti-CD163-dexamethasone conjugate

Western blot analysis (Figure 2a) and flow cytometric binding analysis (Figure 2b) of Chinese hamster ovary (CHO) cells expressing rat recombinant CD163 (Figure 2a) showed maintained reactivity and specificity of anti-CD163 after dexamethasone conjugation. Accordingly, flow cytometry of rat spleen cells demonstrated specific binding of the antibody conjugate to CD172a and CD4 positive cells, i.e., monocytes/macrophages (Figure 2c).

CD163-mediated uptake of anti-CD163-dexamethasone conjugate

Analysis of transfected CHO cells expressing rat CD163 showed an efficient uptake of 125I-labeled anti-CD163, whereas no uptake was seen in nontransfected control CHO-cells (Figure 3). Degradation of the antibody measured as increase in the appearance of free 125I in the medium increased slightly after 2 hours compared with nontransfected cells. The uptake and cellular localization of anti-CD163-dexamethasone in the CHO cells as monitored by confocal microscopy showed uptake and intracellular colocalization of anti-CD163 and dexamethasone after 30 minutes (Figure 4a). After 2 hours, only partial colocalization of dexamethasone and the antibody was observed, thus indicating separation of the drug from the conjugate. Similar data were obtained for macrophage-like cells from rat spleen, whereas other nonmacrophage spleen cells did not exhibit measurable uptake of anti-CD163 or dexamethasone (Figure 4b).

In vitro analysis of the anti-inflammatory effect of anti-CD163-dexamethasone conjugate

The anti-inflammatory effect of anti-CD163-dexamethasone was examined in vitro by measuring LPS-mediated TNF-α production in rat macrophages incubated with free dexamethasone, anti-CD163-dexamethasone, control conjugate (IgG-dexamethasone), or nonconjugated anti-CD163 for 15 minutes (Figure 5) and then challenged with LPS after 18 hours. Anti-CD163-dexamethasone, control IgG-dexamethasone conjugates, and free dexamethasone (1 μg/ml) prevented LPS-mediated stimulation of rat macrophages, whereas anti-CD163 or vehicle alone had no effect on TNF-α secretion (data not shown). At dosing interval 10−5–10−6 mg/ml, the effect of anti-CD163-dexamethasone was significantly higher than that of free dexamethasone (P = 0.046) and IgG-dexamethasone (P = 0.028), and the effect of dexamethasone was higher than that of IgG-dexamethasone. The observed superior effect of anti-CD163 conjugated dexamethasone on the suppression of TNF-α secretion relative to free dexamethasone was only observed for shorter incubation times. When incubation was prolonged to 1 hour, the difference in effect between the two was abrogated (not shown). This probably reflects that passive diffusion of glucocorticoid in this system is slightly slower than the delivery via the CD163-mediated endocytosis.

The in vivo anti-inflammatory effect and pharmacokinetics of anti-CD163-dexamethasone conjugate

The anti-inflammatory effect of anti-CD163-dexamethasone in vivo was examined in rats injected with LPS that induces endotoxemia and acute inflammation. Nineteen hours before the challenge with LPS, rats were i.v.-injected with dexamethasone, anti-CD163-dexamethasone, control-IgG-dexamethasone, or vehicle control. The inflammatory response to the LPS challenge was monitored by measuring TNF-α and IL-1 in the rat serum (Figure 6a). Dexamethasone was tested in two doses, a high dose (1 mg/kg) and a low dose (0.02 mg/kg). The high dose corresponds to the dose for maximal efficacy in other rat studies. The anti-CD163-dexamethasone conjugate was tested in two doses, where the dexamethasone content corresponded to the low dexamethasone content (0.02 mg/kg) and a fivefold lower dose (0.004 mg/kg). As compared with vehicle, a significantly lower TNF-α response was measured in animals treated with free high doses dexamethasone, whereas no effect was seen with the low dose. However, in the anti-CD163-dexamethasone form, the effect of the low dose (0.02 mg/kg) was comparable with the effect of the 1 mg/kg free dexamethasone dose. Similar
data were obtained for serum IL-1, where the low doses of anti-
CD163-dexamethasone almost completely abrogated the LPS-
induced increase in IL-1. The inhibition of IL-1 induction by
anti-CD163-dexamethasone was more efficient than that of free
dexamethasone, even when this was at a 50-fold higher concen-
tration (Figure 6b) (P < 0.02).

Figure 7 shows the plasma disappearance of the therapeu-
tic dose of anti-CD163-dexamethasone (2.4 mg protein/kg) in
rats. Fifty percent of the conjugate disappeared from plasma in
20 minutes. The clearance curves of the anti-CD163 and dexam-
ethasone moieties of the conjugate were virtually identical
indicating a high stability of the conjugate in plasma (Figure 7).
Furthermore, nonconjugated anti-CD163 showed a similar
clearance profile as compared with anti-CD163-dexamethasone administered in the same doses (data not shown). However,
the clearance of $^{125}$I-labeled anti-CD163 at a 50-fold lower dose
(0.05 mg/kg) showed a half-life of 4 minutes due to uptake in
mainly spleen and liver (Supplementary Figure S2 online).
This indicates that the CD163 system is saturable.

**Systemic nonmacrophage effects of anti-CD163-
dexamethasone conjugate**

Comparison of the effect of the conjugate and high dose dexam-
ethasone showed that two injections of 1 mg/kg free dexametha-
sone for acute therapy virtually erased cortisol from circulation
after 2 days, whereas the equipotent dose (in terms of anti-
inflammatory effect) of the conjugate had no significant effect
on the cortisol levels (Figure 8a). Furthermore, we measured
the effect on the weight of thymus, spleen, and whole body. The
weight of the thymus after the administration of dexametha-
sone is a well-known parameter that reflects the corticosteroid-
mediated apoptosis of lymphocytes, which are the dominating
cells in the thymus.27–29 Accordingly, two injections of 1 mg/kg
free dexamethasone led to about a twofold reduction in thy-
mus weight after 2 days as compared with the other groups
(ANOVA test, P < 0.0001) (Figure 8b). No effect on thymus
weight was observed for any of the other groups compared with
vehicle-treated rats. Similar results were found for the spleen,
which also undergoes a slight weight dexamethasone-induced
reduction due to lymphocyte loss (Figure 8b) (ANOVA test, \(P < 0.0001\)). High dose weight dexamethasone injections (1 mg/kg) also reduce overall body (reduction of muscle, bone, and connective tissue) and a significant weight loss was seen compared with vehicle or dexamethasone conjugate injections (48 hours post first injection) (Figure 8d) (ANOVA, \(P = 0.0015\)).

A prolonged study of systemic effects (Supplementary Figure S3 online) running for 9 days with four injections revealed a similar picture, but in this case with far more pronounced effect of the high dexamethasone dose on the weight of thymus (>75% reduction), spleen, and body. Endogenous cortisol was completely suppressed as in the short term study. The low concentrations of free dexamethasone exhibited significant reductions in the weight of thymus (\(P < 0.0001\)) and spleen (\(P < 0.002\)) as compared with vehicle. Conjugated dexamethasone at 0.02 mg/kg exhibited a small but significant reduction in thymus weight (\(P < 0.0001\)). Interestingly, low dose free dexamethasone has a significantly higher effect on thymus and spleen weight than the corresponding conjugated CD163 targeted dose (\(P = 0.013\) and 0.039, respectively), thus indicating that not only the low concentration of dexamethasone, but also its targeting to macrophages reduces corticosteroid-mediated apoptosis of lymphocytes.

**DISCUSSION**

The present study describes a rat anti-CD163 mAb-dexamethasone conjugate as a novel type of anti-inflammatory ADC. This anti-CD163-dexamethasone ADC has conserved binding affinity for CD163 and an \textit{in vivo} potency about 50-fold higher than that of nonconjugated dexamethasone measured as reduction in LPS-induced TNF-\(\alpha\) secretion. In contrast to a strong systemic effect of nonconjugated dexamethasone, the equipotent (in terms of effect on TNF-\(\alpha\) secretion) dose of the ADC had no or low effect measured as thymus, spleen, and body weight reduction. According to the allometric conversion factor recommended by the Federal Drug Administration guidelines,\textsuperscript{30} the high dexamethasone dose used in this study corresponds to the upper end of dosing in human therapy and the low dose dexamethasone present in the conjugate represent the other end.\textsuperscript{31,32}

As GSs represent the most potent class of anti-inflammatory drugs, it has been a long wish to develop GC analogues with a preferential anti-inflammatory effect compared with other effects that cause the severe adverse effects making prolonged GCs therapy hazardous. Whereas the regulation of GC potency and the reduction of unwanted mineralocorticoid effects of GCs have proved successful by changing side groups in the cyclopentanophenanthrene structure, it has not been possible to develop steroid variants with a specific anti-inflammatory effect avoiding the systemic GC effect.
The ADC approach for directing an anti-inflammatory drug to nosylated albumin has been studied. These studies have shown development, but to our knowledge this is the first study of using cytostatic drugs to cancer cells and several drugs are in clinical sites of inflammation.

The reason is probably that the cytosolic GC receptor cannot mediate differentiated signals upon ligand binding. Our approach has therefore been to develop a low dose GC therapy with a specific high dose effect on macrophages including the macrophages at the sites of inflammation. Therefore been to develop a low dose GC therapy with a specific high dose effect on macrophages including the macrophages at the sites of inflammation. The GC-conjugates may also be used in the general proteolytic milieu in the endocytic pathway, dexamethasone may be released in the lysosome and reach the cytosol by passive diffusion. This could suggest a longer response time but our in vitro efficacy study in rat macrophages indicated in fact a faster uptake of dexamethasone by CD163-mediated endocytosis (Figure 5) when it was conjugated to a high affinity anti-CD163 antibody.

The overall gain in efficacy of the conjugated dexamethasone compared with free dexamethasone was far higher in the in vivo rodent system compared with the in vitro system with CD163-expressing rat macrophages in culture. Diffusion of the nonconjugated drug, but not the conjugated drug, into the entire pool of body cells (mostly CD163-negative) in the in vivo settings may explain this difference.

The present data on a model of acute inflammation also opens exciting avenues for potential new treatment regimens using macrophage-targeted dexamethasone (or other GCs) in inflammatory diseases (e.g., rheumatoid arthritis and Mb. Crohn) where TNF-α has been validated as a target for biological drugs. It is tempting to speculate that macrophage-targeted dexamethasone may have an even more pronounced anti-inflammatory effect than anti-TNF-α biologicals because the macrophage-release of other pro-inflammatory cytokines also will be reduced as demonstrated for IL-1 in this study (Figure 6). Furthermore, the drug will be more effective in reducing paracrine TNF-α signalling at the local sites of inflammation. The GC-conjugates may also be used in

Figure 5 Anti-CD163-dexamethasone suppression of LPS-mediated TNF-α stimulation in vitro. Rat spleen cells were cultured and incubated with serial dilutions of anti-CD163-dexamethasone conjugate, mouse IgG-dexamethasone, and free dexamethasone for 15 minutes, washed and incubated overnight. Cell supernatants were analyzed for TNF-α after 4 hours of LPS stimulation. The graph shows the normalized mean values ± 1 SD from six independent studies using six different rats for splenocyte preparation (range of maximum TNF-α levels was 100–200 pg/ml). Anti-CD163-dexamethasone was consistently significantly different from free dexamethasone (P < 0.05) for all individual studies. SD, standard deviation; TNF-α, tumor-necrosis factor-α.

Figure 6 Increased anti-inflammatory efficacy of anti-CD163-dexamethasone. Female Lewis rats were pretreated with anti-CD163-dexamethasone, free dexamethasone, mouse IgG-dexamethasone or vehicle (n = 6 per group) 19 hours before stimulation with LPS. The concentration of (a) TNF-α and (b) IL-1 in serum samples were determined 2 hours post-LPS injection in sandwich ELISA assays. Values are mean ± 1 SD. ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; TNF-α, tumor-necrosis factor-α.
indications such as sepsis and acute hepatitis (e.g., acute alcoholic hepatitis) where high dose GC treatment normally is complicated by the risk of stimulating progression of sepsis.

The high monocyte/macrophage-restricted CD163 expression in macrophages and the high endocytic activity of CD163 suggests that the receptor is an ideal target candidate for directing GC drugs to macrophages. Furthermore, the use of CD163 for GC entry is supported by the positive regulation of the CD163 expression and the high CD163 expression in macrophages at sites of inflammation, such as arthritis and atherosclerosis. In addition to the direct targeting of macrophages in inflamed areas, it is also possible that GCs may prime circulating CD163-expressing monocytes to be recruited to sites of inflammation.

The use of CD163-mediated targeting of macrophages may not be restricted to inflammation only, but may also be a potential strategy in other indications where macrophages play a pivotal role. In certain infectious diseases, such as tuberculosis and HIV, the pathogen uses the macrophages as host cell. Furthermore, some myelomonocytic-derived cancers, for instance histiocytic sarcomas, express CD163, and targeting cytotoxic drugs seems highly relevant to investigate. Finally, the rare lysosomal storage disease, Gaucher’s disease, of which certain patients can be treated by injections of the missing functional enzyme glycosylceramidase in a mannosylated form, may be regarded as potential indication. These Gaucher patients have multiorgan accumulation of large abnormal lipid-loaded macrophages (Gaucher cells) with a very high CD163 expression. It seems therefore likely that the targeting of the enzyme to CD163 that immediately will direct it to the lysosome would greatly lower the cost of therapy, which at present is hampered by the need of high and expensive doses of enzyme.

In conclusion, antibody-mediated drug-targeting to the hemoglobin scavenger receptor CD163 is a new potential

Figure 7 Time course of injected anti-CD163-dexamethasone. Anti-CD163-dexamethasone was administrated intravenously and blood samples were collected at serial time-points post injection (n = 4). Serum concentrations of anti-CD163 (open circle) and dexamethasone (filled square) were determined in sandwich ELISA assays. Values are mean ± 1 SD. ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

Figure 8 Systemic nonmacrophage effect of anti-CD163-dexamethasone in rats. Systemic effect was measured as suppression of (a) serum cortisol concentration and reduction organ ((b) thymus and (c) spleen) and (d) body weight. The cortisol concentration and the weight of the thymus, spleen, and whole body were measured upon killing the rats 2 days after injection of anti-CD163-dexamethasone, mouse control IgG-dexamethasone, free dexamethasone or vehicle at day 0 and 1 (n = 4 per group). Values are mean ± 1 SD. SD, standard deviation.
approach for a safe therapy of acute and perhaps also chronic inflammatory diseases as well as it serves as new direction for development of improved treatment of other classes of disease.

**MATERIALS AND METHODS**

**Synthesis of dexamethasone-hemisuccinate-NHS ester rat anti-CD163 mAb conjugate.** Dexamethasone (dexa) (Sigma-Aldrich, Broendby, Denmark) (1.00 g, 2.55 mmol) and succinic anhydride (Sigma-Aldrich) (1.27 g, 12.69 mmol) were stirred overnight at room temperature (RT) in 15 ml pyridine. The solution was poured into a mixture of 50 ml ice and 20 ml HCl (4 mol/l), filtered and the obtained precipitate was washed twice with 20 ml ice cold HCl (4 mol/l). The precipitate was dissolved in tetrahydroduran and transferred to a round bottom flask, evaporated three times with toluene. Finally, the solid was dried on high vacuum overnight. Dexamethasone-hemisuccinate (1.25 g, 100%) appeared as a white solid. Dexamethasone-hemisuccinate (500 mg, 0.988 mol) was dissolved in 20 ml dry THF. N-hydroxysuccinimide (Sigma-Aldrich) (171 mg, 1.48 mmol) and N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide (200 mg, 1.04 mmol) were added and the reaction was stirred overnight at RT. The solvent was removed in vacuo and the product was purified by flash chromatography (pentane-EtOAc; 2:8) giving dexamethasone-hemisuccinate-NHS (376 mg, 63%) as a white solid. Dexamethasone-hemisuccinate-NHS ester (dexamethasone-hemisuccinate-NHS) was stored at −20 °C. In order to conjugate mAb with dexamethasone, 50 µl of a 1 mg/ml dexamethasone-hemisuccinate-NHS solution in DMSO were added slowly to 1 ml of a 1 mg/ml solution of the rat CD163 antibody Ed-2 (ABD Serotec, Düsseldorf, Germany) (anti-CD163) or control murine IgG (Mouse gamma globulin, Jackson ImmunoResearch, Suffolk, UK) in PBS pH 8.3 while gently agitating. Reaction mix was then incubated for 15 minutes at 25 °C while gently agitating. The conjugation was terminated by adding 100 µl 1 mol/l glycine in phosphate-buffered saline (PBS) pH 8.3 per ml reaction mix and incubated at 25 °C for further 30 minutes with gentle agitation. The reaction mixture was subsequently dialyzed using spin filters (Amicon-Ultra, 30K, Millipore, Copenhagen, Denmark) into PBS pH 7.4 (Invitrogen, Taastrup, Denmark). Conjugates were sterile filtered and analyzed for protein concentration and the amount of free and total (free + conjugated) GC. Samples were diluted to the desired concentration in PBS, pH 7.4 and stored either at 4 °C or in liquid nitrogen.

To test for potential aggregation or fragmentation equal amounts (50 µg) of anti-CD163 and anti-CD163-dexamethasone conjugate were compared either by SDS-PAGE (NuPAGE Novex 4-12% Bis-Tris polyacrylamide gels (Invitrogen A/S, Taastrup, Denmark)) in 1 × MOPS running buffer (Invitrogen) or compared by analytical high-pressure size exclusion chromatography coupled to a multi-angle light scattering instrument (Wyatt Technology Europe GmbH, Dernbach, Germany) using a TSKgel G4000PW × 1 column with PBS as running buffer, elution was monitored at 210 nm. For measuring the total dexamethasone content in the conjugate preparations, 20 µl conjugate samples were hydrolyzed by incubation with 50 µl of 0.1 mol/l hydrochloric acid. Samples were filtered through 0.45 µm RC membrane syringe filters (Pierce, Allerod, Denmark) and volumes from 1 to 50 µl were loaded on an C18 column (Hyperclone C18, 3 µm, 150 × 460, Phenomenex) in an Shimadzu 10 A HPLC System (Shimadzu, Kyoto, Japan) and eluted isocratically with 40% acetonitrile (HPLC-grade, Scharlau, Barcelona, Spain) in 50 mmol/l potassium-acetate buffer, pH 5.0 at 0.5 ml/min. Elution was monitored by measuring 240 nm absorption by a Shimadzu SPD 10A VP detector. The column heater was set to 30 °C and autosampler/vial holder to 4 °C. To measure the level of free dexamethasone or dexamethasone-hemisuccinate the sample was loaded directly onto the column without hydrolysis. The amount of dexamethasone or dexamethasone-hemisuccinate in the injected sample was calculated by integration of peak areas, a 2 µg/ml dexamethasone solution in 10% ethanol (Dexamathasone for HPLC; Sigma-Aldrich), respectively dexamethasone-hemisuccinate solution were used to obtain linear calibration curves. The protein concentration was determined using the Quick Start Bradford Dye Reagent (BioRad, Copenhagen, Denmark) according to the instructions of the manufacturer and bovine gamma globulin (Pierce; Thermo Scientific, Copenhagen, Denmark) as reference. The Bradford assay was calibrated by a total amino acid analysis determination of protein in an anti-CD163-dexamethasone sample.

**Rat CD163-expressing CHO cells.** Rat CD163 cDNA was obtained by gene synthesis using the GenPept Accession No. NP_001101357 as template (GenScript, Piscataway, NJ). Rat C163 cDNA was subsequently subcloned into the pcDNA5/FRT plasmid (Invitrogen) using KpnI and HindIII restriction enzymes (Fermentas GmbH, St. Leon Rot, Germany) and T4 DNA ligase (Invitrogen). Stably transfected CHO cells expressing rat CD163 were established by transfecting the rat CD163 cDNA containing plasmid into FlpIn CHO cells using the FlpIn system (Invitrogen) and subsequently maintained in Hyclone CCM5 (Thermo Fischer Scientific, Copenhagen, Denmark) supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin, and 300 µg/ml Hygromycin (Invitrogen).

**Western blot analysis of binding to rat spleen lysate.** Approximately 1 × 10⁵ transfected CHO cells expressing rat CD163 or the corresponding mock cell line were lysed using 1 ml PBS buffer, pH 7.4, containing 1% Triton X-100 (Merck KGaA, Darmstadt, Germany), and “complete mini protease inhibitor cocktail” (Roche, Hvidovre, Denmark). Thirty microliter of the lysate was loaded and analyzed on an SDS-PAGE gel as described. The SDS-PAGE–resolved proteins were transferred onto a polyvinylidene difluoride membrane using the iBlot gel transfer device (Invitrogen), and blocked with a buffer (2 mmol/l CaCl₂, 1 mmol/l MgCl₂, 10 mmol/l HEPES, and 140 mmol/l NaCl; pH 7.8) supplemented with 0.05% Tween-20 and 5% nonfat milk, and subsequently incubated with anti-CD163 or anti-CD163-dexamethasone. After washing, the membranes were incubated with HRP-conjugated anti-mouse IgG antibody (1:20,000 dilution; Sigma-Aldrich), and immunoreactive bands were visualized with enhanced chemiluminescence western blotting substrate.

**Uptake of anti-CD163 in rat CD163 expressing CHO cells.** ¹²⁵I-labeling of mouse anti rat CD163 mAb (Ed-2) and endocytosis in rat CD163-transfected CHO cells and mock-transfected CHO cells were carried as previously described for labeling of anti-human CD163 mAb and endocytosis of the radiolabel in human CD163 CHO cells.⁶

**Flow cytometric analyses of CD163-expressing CHO cells and rat splenocytes.** The cell suspensions were washed in PBS pH 7.4 (0.1% NaN₃) and the cell density adjusted to 5 × 10⁵/ml. CD163 expressing CHO cells were incubated at 0.5 × 10⁶/ml with Ed-2 or anti-CD163-dexamethasone (0.1 µg) in 100 µl PBS pH 7.4 (0.1% NaN₃, and 2% fetal bovine serum (FBS) (PAA Laboratories, Cölbe, Germany)) for 45 minutes at 4 °C in the dark. Subsequently, the cells were washed in PBS (0.1% NaN₃) and incubated with anti-mouse IgG-FITC for 30 minutes at 4 °C. Cell suspensions of rat splenocytes were incubated at 0.5 × 10⁶/ml with anti-CD163-dexamethasone labeled with Alexa Fluor 488 using the Zenon Mouse IgG labelling kit (Invitrogen), anti-dexamethasone (Abcam, Cambridge, UK) labeled with PerCP using the Lighting LINK PerCP Conjugation Kit (Innova Bioscience, Cambridge, UK), anti-rat CD4–APC (Invitrogen), and anti-rat CD172a–PE (ED-9) (ABD Serotec) (0.2–0.5 µg) in 100 µl PBS pH 7.4 (0.1% NaN₃ and 2% FBS) for 45 minutes at 4 °C. Negative/isotype control staining of the rat splenocytes was performed with mouse IgG1–Alexa Fluor 488 (ABD Serotec) and rabbit IgG PerCP (Santa Cruz Biotechnology, Santa Cruz, CA). The stained cells were washed twice in PBS pH 7.4 (0.1% NaN₃ and 2% FBS) by centrifugation at 225g for 5 minutes, 4 °C before analysis on a FACSCalibur flow cytometer (BD Biosciences, Copenhagen, Denmark). The data were further analyzed using the FlowJo software package (Tri Star, Origon).
The rat in vitro LPS model. Rat spleen cell suspensions were prepared from female Lewis rats (Harlan, Blackthorn, UK). The purified cells were suspended in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and 2 mmol/l L-glutamine and cultured (2 × 10^6/well) overnight in 96-well flat bottomed plates (125µl/well) at 37°C and 5% CO_2. Anti-CD163-dexamethasone, control IgG-dexamethasone, free dexamethasone (Sigma-Aldrich), nonconjugated anti-CD163 or vehicle (PBS pH 7.4) were serially diluted in supplemented RPMI 1640 medium and added (100 µl) to the overnight cell cultures at final concentrations ranging from 1 to 10^{-7} µg dexamethasone/ml. After incubation for 15 minutes, the cells were washed twice in RPMI medium by centrifugation at 1,200 rpm for 5 minutes and careful aspiration of supernatant before addition of supplemented RPMI medium to the wells and further incubation overnight. Each incubation condition was tested in duplicates or triplicates in each experiment. After 18 hours, the cells were challenged with LPS (Sigma-Aldrich) (250ng/ml) for 4 hours before supernatant was aspirated from each well and frozen at −20°C. The supernatants were analyzed for the presence of TNF-α using the rat CytoSet Antibody Pairs kit (Invitrogen) in a sandwich enzyme-linked immunosorbent assay according to the manufacturer’s instructions.

Confocal microscopy of CHO cells and rat splenocytes. A mixture of mock-transfected CHO cells and CHO cells expressing CD163 or rat spleen cell suspensions were cultured overnight in Hyclone CCMM supplemented with 300 µg/ml Hygromycin or RPMI 1640 medium supplemented with 10% FBS and 2 mmol/l L-glutamine in chambered cover glass (Nunc A/S, Roskilde, Denmark) coated with Poly-D-Lysine (Sigma-Aldrich). Adhered cells were incubated with anti-CD163-dexamethasone for 30 minutes at 37°C and 5% CO_2, and rinsed three times with PBS containing 10% FBS (also used for later rinses) and further incubated for 2 hours. Following incubation, the cells were rinsed three times before fixation in 4% paraformaldehyde for 10 minutes at RT. After two rinses in BD Perm/Wash buffer (BD Bioscience), the cells were permeabilized with Perm/Wash buffer containing FBS and saponin for 15 minutes at RT. Next, the cells were rinsed two times in Perm/Wash buffer and incubated 1 hour at RT with anti-mouse IgG-Alexa Fluor 488 (Invitrogen) and anti-dexamethasone (Abcam) labeled with PerCP using the LIGHTING Link PerCP Conjugation Kit (Innova Bioscience), diluted in Perm/Wash buffer. Following three rinses in Perm/Wash buffer, the cells were fixed in 4% paraformaldehyde for 10 minutes at RT. Finally, the cells were incubated with Hoechst 33342 (Sigma-Aldrich) before visualization using a Zeiss LSM-710 confocal microscope system (Carl Zeiss Microlmaging GmbH, Jena, Germany).

Animals. Female Lewis rats, 9–11 weeks of age, were obtained from Harlan Laboratories, United States. All animal experiments were performed after 1–3 weeks acclimation on standard diet. The animals were maintained under controlled temperature (20 ± 2°C) and light (lights on 08:00–20:00 hours). The experimental protocols were approved by The Danish Experimental Animal Inspectorate.

Pharmacokinetics of anti-CD163-dexamethasone in rats. The rats were placed and adjusted in a restrainer (Agnthos, Linoing, Sweden) to allow for intravenous injection without anesthesia. Anti-CD163-dexamethasone was administrated to the rats intravenously via the tail vein (2.4 mg Ab/kg, n = 4). To examine anti-CD163-dexamethasone pharmacokinetics, blood samples were collected from the sublingual vein of the tongue at multiple time-points post anti-CD163-dexamethasone injection. Plasma concentration of protein bound of anti-CD163 (n = 3). Blood samples were collected from the sublingual vein of the tongue at multiple time-points post anti-CD163-dexamethasone injection. Plasma concentration of protein bound of anti-CD163 (n = 3).

The rat in vivo LPS model. Female Lewis rats (9–11 weeks) were injected intravenously with either anti-CD163-dexamethasone, control IgG-dexamethasone, free dexamethasone-phosphate (Sigma-Aldrich), or vehicle (PBS pH 7.4) (0.9mg/kg) was injected intravenously in the tail vein. Blood samples were collected at the following time points: before glucocorticoid injection, 2 hours post-LPS injection, and again after 24 hours. Serum samples were analyzed for TNF-α and IL1β using the rat TNF-α ELISA kit (Invitrogen) and the rat IL1β kit (Invitrogen) according to the manufacturer's instructions. Two days post-LPS challenge, the animals were anaesthetized using isoflurane, euthanized by cervical dislocation and thymus and spleen were removed and weighed.

Analysis of systemic effects of anti-CD163-dexamethasone conjugate in rats. Female Lewis rats (9–11 weeks) were injected intravenously twice with either anti-CD163-dexamethasone, control IgG-dexamethasone, free dexamethasone 21-phosphate (Sigma-Aldrich), or vehicle (PBS pH 7.4) (group size 4 rats). Blood samples were collected at the following time points: before drug injection, 24 hours post first drug injection, and again 24 hours after the second injection. The animals were anaesthetized using isoflurane, euthanized by cervical dislocation and thymus and spleen were removed and weighed. Body weights were monitored daily. The plasma cortisol concentration was measured using the Cortisol assay from Roche Diagnostics (Roche Diagnostics, Hvidovre, Denmark) on a Cobas-6000 analyzer according to the manufacturer's instructions. In a second similar study, rats were injected four times at day 0, 2, 5, and 7 and killed on day 8 (n = 4).

SUPPLEMENTARY MATERIAL.
Figure S1. Analysis of structural integrity after dexamethasone conjugation of anti-CD163.
Figure S2. Plasma clearance of low dose anti-CD163 in rats.
Figure S3. A 9-day study of systemic nonmacrophage effect of anti-CD163-dexamethasone in rats measured as suppression of plasma cortisol concentration and reduction of organ (thymus and spleen) and body weight.

ACKNOWLEDGMENTS
This study has been financially supported by ERC grant 233312 for the TROJA project (Targeting Receptors Of jointly Assembled Drug-Ligand Complexes) and by Cytoguide Aps. We thank Kit Husted and Dorte Hemansen for technical assistance with the animal studies. J.H.G., H.J.M., and S.K.M. are minority shareholders of Cytoguide Aps.
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