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Chimeric Anti-CD14 IGG2/4 Hybrid Antibodies for Therapeutic Intervention in Pig and Human Models of Inflammation

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CD14 is a key recognition molecule of innate immune responses, interacting with several TLRs. TLR signaling cross-talks extensively with the complement system, and combined CD14 and complement inhibition has been proved effective in attenuating inflammatory responses. Pig models of human diseases have emerged as valuable tools to study therapeutic intervention, but suitable neutralizing Abs are rare. Undesired Fc-mediated functions, such as platelet activation and IL-8 release induced by the porcine CD14-specific clone Mil2, limit further studies. Therefore, an inert human IgG2/IgG4 hybrid C region was chosen for an rMil2. As revealed in ex vivo and in vivo pig experiments, rMil2 inhibited the CD14-mediated proinflammatory cytokine response similar to the original clone, but lacked the undesired Fc-effects, and inflammation was attenuated further by simultaneous complement inhibition. Moreover, rMil2 bound porcine FcRn, a regulator of half-life and biodistribution. Thus, rMil2, particularly combined with complement inhibitors, should be well suited for in vivo studies using porcine models of diseases, such as sepsis and ischemia-reperfusion injury. Similarly, the recombinant anti-human CD14 IgG2/4 Ab, r18D11, was generated with greatly reduced Fc-mediated effects and preserved inhibitory function ex vivo. Such Abs might be drug candidates for the treatment of innate immunity-mediated human diseases. The Journal of Immunology, 2013, 191: 4769–4777.

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We demonstrate that such novel Abs are unique tools for future human CD14 Abs endowed with the IgG2/IgG4 hybrid Fc region. To this end, we have generated recombinant anti-porcine and anti-human CD14 Abs endowed with the IgG2/IgG4 hybrid Fc region. We have previously used porcine and human porcine whole blood in vivo models of gram-negative bacteria–induced inflammation and observed marked inhibitory effects of anti-CD14, particularly in combination with inhibition of the complement system, on a broad spectrum of inflammatory mediators (22–27). Because the pig is emerging as a nonprimate mammal of choice for preclinical studies of human diseases, including those involving innate immunity and spectrum of inflammatory mediators (22–27), there is an urgent need for anti-porcine CD14 Abs that do not induce undesired effector functions.

The aim of this study was to construct and characterize such Abs with minimal ability to activate complement and bind to FcRs. To this end, we have generated recombinant anti-porcine and anti-human CD14 Abs endowed with the IgG2/IgG4 hybrid Fc region. We demonstrate that such novel Abs are unique tools for future studies of CD14 inhibition using porcine in vivo models, and pave the way for human therapy with CD14 inhibition, preferentially in combination with complement inhibition.

Materials and Methods

Abs and inhibitors

Commercial anti-CD14 Abs and isotype controls were purchased at Diatec Monoclonals AS (Oslo, Norway) and AbD Serotec (Kidlington, U.K.) as follows: mouse anti-human CD14 IgG1 clone 18D11 (Diatec), 18D11 IgG1 Fab5 (Diatec), mouse anti-porcine CD14 IgG2b clone Mi2 (Serotec), and FITC-conjugated Mi2 (Serotec), isotype controls mouse IgG1 (Diatec), mouse IgG1 Fab5 (Diatec), and FITC-conjugated mouse IgG2b (Serotec). Furthermore, we used the fully humanized anti-CS IgG2/4 Ab eculizumab (Soliris), purchased from Alexion Pharmaceuticals (Cheshire, CT), as isotype control for recombinant IgG2/4 and humanized anti-CD20 IgG1 Ab rituximab (MabThera) from Roche (Welwyn Garden City, U.K.) as control for ELISA-based binding studies. The rMi2 Ab preparation used in the experiments shown in Figs. 3 and 6 was produced by ExcellGene SA (Monthey, Switzerland). This Ab consists of the same amino acid sequence presented in Supplemental Table I, but was expressed in a CHO cell-expression system. It was pure and free of single chains, as confirmed by SDS-PAGE (not shown).

Goat-anti human IgG κ pooled antisera, HRP-conjugated goat-anti human IgG Fc pooled antisera, HRP-conjugated goat anti-mouse Ig Ab, and PE-conjugated anti-mouse IgG were purchased from Southern Biotech (Birmingham, AL). Mouse monoclonal anti-human IgG2 Ab (clone 3C7) was purchased from Hytest (Turku, Finland). Endotoxin-free recombinant bacterial OmcC1 (also known as coxvirus) (29), a 16.8-kDa protein, was provided by Varleigh Immuno Pharmaceuticals (Jersey, Channel Islands). OmcC1 has been shown to inhibit complement activation effectively in pigs (30).

Variable gene retrieval and cloning of recombinant anti-CD14 Abs

Original hybridoma cell clones were generated in the laboratories of the coauthors T.E. (18D11) and C.R.S. (Mi2). After brief culture, the cells were harvested and total RNA was extracted using mirVana (Life Technologies, Ambion, Austin, TX). Variable genes were specifically reverse transcribed from 500 ng total RNA using SuperScript II reverse transcriptase and oligonucleotide primers, which were designed to bind downstream of the variable genes in conserved sequences encoding the constant regions of heavy and light chains. After removal of input RNA from the sample by RNaseH digestion (New England Biolabs, Bedford, U.K.), poly dCTP 3′-tailing of the cDNA was performed using terminal transferase (Roche Diagnostics, Mannheim, Germany), and fragments containing the variable gene segments were amplified by nested PCR using Phusion DNA polymerase (Finnzymes, Vantaa, Finland) and new sets of primers containing RgII and MgII restriction sites. The amplicons were inserted in cloning vectors before sequencing analyses. All primers were synthesized by Sigma-Aldrich (Steinheim, Germany; Supplemental Table II).

A well-established protocol (31) was used to subclone the mature variable heavy and variable L chain genes into pLNOH2 and pLNOK expression vectors, respectively. The sequence encoding the human IgG2/4 hybrid constant H chain was consistent with the literature (14) and inserted into pLNOH2. All genes were synthesized by GenScript (Piscataway, NJ). The control Ab specific for a hapten (4-hydroxy-3-iodo-5-nitrophenylacetic acid [NIP]) was also expressed from pLNOH2 and pLNOK (31). Thus, two plasmids were generated for the expression of each of the three recombinant Abs, which target human CD14 (recombinant 18D11, r18D11), porcine CD14 (recombinant Mi2, rMi2, or NIP [recombinant anti-NIP; rNIP]). For transfection, plasmid DNA was purified using EndoFree Plasmid Maxi or Mega Kit from Qiagen (Hilden, Germany). Amino acid sequences of the recombinant Abs and related IMGT accession numbers are displayed in Supplemental Table I.

Cell culture

Adherent HEK293-E16NA cells were subcultured at 5% CO2 and 37°C using DMEM containing 4.5 g/l L-glucose (Lonza, Verviers, Belgium) and supplemented with 10% FBS and 5% anti-FBS (PAAS Laboratories, Pasching, Austria), 4 mM L-glutamine (Lonza), 10,000 U/ml penicillin, and 10,000 μg/ml streptomycin (Lonza). The day before transfection, 4 × 10⁶ cells were seeded in a 75-cm² tissue culture flask (Techno Plastic Products, Trasadingen, Switzerland) and grown for an additional 24 h to reach 90% confluence. Cotransfection with light and H chain encoding plasmid DNA in an equimolar amount was performed using OptiMEM (Life Technologies, Invitrogen Carlsbad, CA) following the manufactures instructions. Transient expression was performed in a fed-batch procedure with harvest of cell culture supernatant every third day over 12 d. The cells were detached and centrifuged for 5 min at 230 × g followed by careful aspiration of the supernatant and immediate resuspension and reseeding of the cells in 12 ml fresh OptiMEM. The supernatant was stored at −20°C until Ab purification.

Counts Automated Cell Counter (Life Technologies, Invitrogen). IgG purification

Concentrators, spin columns, and kits for purification of recombinant Abs and subsequent buffer exchange were purchased from Thermo Scientific, Pierce Endotoxin, Rockford, Ill. The combined supernatants of each expression culture were centrifuged for 10 min at 1500 × g and subsequently filtrated using a sterile vacuum filter system with a 0.22-μm cellulose acetate membrane (Corning Glass Works, Corning, NY). Then, solutions were concentrated using Pierce’s concentrators with a 20-kDa m. cutoff (MWCO), and OptiMEM was exchanged to sterile PBS using 10 ml Zeba Desalt Spin Columns with a 7-kDa MWCO. The recombinant Abs were purified using an NAB Protein A Plus Spin Kit with a binding capacity of 7 mg IgG per 0.2 ml resin. Ab-containing fractions were combined before buffer exchange to sterile PBS using 2 ml Zeba Desalt Spin Columns with a 7-kDa MWCO and optional upconcentration to 0.5–1 mg/ml using Amicon Ultra 0.5-mI spin columns with a 50-kDa MWCO (Millipore, Carrigtwohill, Ireland). Endotoxin levels in the final batch preparations were less than 0.04 EU/ml, determined using QCL-1000 (Lonza, Walkersville, MD).

In vivo expression was monitored for the IgG1-specific goat-anti human IgG pooled antisera diluted to 1 μg/ml in carbonate buffer as capture and the HRP-conjugated, Fc-specific goat-anti human IgG pooled antisera diluted 1:8000 in PBS for detection (see above).

SDS-PAGE and Western blot

All materials were purchased from Bio-Rad Laboratories AB (Hercules, CA), except where indicated different materials were purchased using SDS-PAGE on a Mini-PROTEAN Tetra Cell using Mini-PROTEAN Precast Gels (4–15%) and Tris Glycin SDS buffer. Samples were diluted in 2x Laemmli buffer with or without 5% β-mercaptoethanol. Gels were stained with BioSafe Coomassie G250 following the manufacturer’s instructions. Alternatively, proteins were blotted onto a Hybrid ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). After blocking with 5% nonfat dry milk, the membrane was incubated with primary mouse anti-human IgG2 Ab (clone 3C7; 1 μg/ml) and secondary HRP-conjugated goat anti-mouse Ig Ab. Detection of specific bands by ECL was performed using SuperSignal West Dura (Pierce Biotechnology). Images were taken with a ChemiDoc XRS+ system.

CD14 binding in flow cytometry

Fresh human or porcine whole blood was drawn into tubes containing the anticoagulants lepirudin (Refldan; Pharmion, Copenhagen, Denmark) or EDTA, respectively. Blood samples were preincubated for 10 min with Ab, ≤20 μg/ml anti-human CD14 Abs r18D11, 18D11 Fab5 (batch 2068, lot 1383), or isotype controls rNIP and mIgG1 Fab5 (lot 1501), or 100 μg/ml anti-porcine CD14 Abs rMi2, Mi2 (lot 1106), or the isotype controls.
for IgG2a and mlgG2b, eculizumab (Soliris) and mlgG2b (lot 1631), respectively. Endotoxin-free Dulbecco phosphate buffered saline. PBS (Sigma-Aldrich), was used for diluting the Abs. Subsequently, blood samples were incubated for another 15 min in the presence of detection Ab, 10 μg/ml 18D11 (batch 719, lot 3110) or 150 μg/ml FITC-conjugated Mi12 (batch 1107). Their binding was detected either by using a secondary PE-conjugated anti-mouse IgG or through direct FITC fluorescence. Erythrocytes were lysed using FACS Lysing solution (BD Biosciences, Franklin Lakes, NJ; human blood) or a solution of 0.16 M ammonium chloride, 0.13 M sodium bicarbonate, 0.12 mM EDTA (TrisTiptop III) and 0.04% (v/v) paraformaldehyde (porcine blood). In subsequent flow cytometry analyses, human monocyte and porcine granulocyte populations were selected based on the forward scatter–side scatter dot plot, and CD14 binding was recorded as mean or median fluorescence intensity, respectively. In contrast to humans, porcine CD14 is constitutively expressed on mature granulocytes (32, 33). Fluorescence intensities in the presence of the fluorescein-labeled Abs only were set to 100%. Flow cytometry analyses on human samples were performed using an LSRII and FACS Diva software version 5.0.3; porcine samples were analyzed on a FACSCalibur using Cell Quest Pro version 5.2.1 for data acquisition (all from BD Biosciences).

Construction, production, and purification of recombinant soluble FeRn and FcRy variants

The vector containing a truncated version of human FeRn (hFeRn) H chain cDNA encoding the three ectodomains (encoded by rat FeRn variant) was synthesized by Genscript and subcloned into pcDNA3-oriP. The vector also contains a cDNA encoding human β2-microglobulin and the EBV oriP. A truncated cDNA segment encoding the extracellular domains of porcine FeRn (pFeRn) was synthesized by Genscript and subcloned into the pcDNA3-GST-hFeRn contains a cDNA encoding human β2-microglobulin and the EBV oriP. A truncated cDNA segment encoding the extracellular domains of porcine FeRn (pFeRn) was synthesized by Genscript and subcloned into the pcDNA3-GST-hFeRn-GST-β2m- containing replication (oriP) also contains a cDNA encoding human β2-microglobulin and the EBV oriP. A truncated cDNA segment encoding the extracellular domains of porcine FeRn (pFeRn) was synthesized by Genscript and subcloned into pcDNA3- oriP vector using the restriction sites Sall and XhoI. The vector containing the ectodomain of human FeRn (H chain encoding the Schistosoma japonicum GST) has been described elsewhere (34). The vector-denoted pCDNA3-hFeRn-GST-β2m- of replication (oriP) also contains a cDNA encoding human β2-microglobulin and the EBV oriP. A truncated cDNA segment encoding the extracellular domains of porcine FeRn (pFeRn) was synthesized by Genscript and subcloned into the pcDNA3-GST-hFeRn-GST-β2m-oriP vector using the restriction sites Sall and XhoI. The vector containing the ectodomain of human FeRn (H chain encoding the Schistosoma japonicum GST) has been described elsewhere (34). All recombinant soluble FeRn receptors were produced by transfection of HEK293-EBNA cells, and secreted receptors were purified using a GSTrap column as described previously (34).

ELISA for 14g, FcYR, and FeRn binding

Sixty-nine–well plates (Nunc, Roskilde, Denmark) were coated with serial dilution of the Abs (6.0–0.09 μg/ml) and incubated overnight at 4°C followed by washing three times with PBS/Tween (pH 7.4). The wells were blocked with 4% skimmed milk (Neogen Europe, Auche in Ruhr, U.K.) for 1 h at room temperature and then washed in PBS/Tween (pH 6.0). Purified hFeRn-GST or pFeRn-GST (1 μg/ml) were diluted in 4% skimmed milk in PBS/Tween (pH 6.0) and preincubated with an HRP-conjugated anti-Abs (Ab) (GE Healthcare U.K., Buckinghamshire, U.K.) diluted 1:5000 and added to the coated plates. The plates were incubated for 1 h at room temperature and washed with PBS/Tween (pH 6.0). Bound receptor was detected by adding 100 μl of 3,3',5,5'-tetramethyl benzidine substrate (Calbiochem-Novabiochem, Nottingham, U.K.). The absorbance was measured at 450 nm using a Sunrise TECAN spectrophotometer. The assay described above was also performed using PBS/Tween (pH 7.4) in all steps. The same setup was used with GST-fused versions of hFcYRIIb, hFcYRIIa (allotype His131), hFcYRIIIb (alleotype Val158), and hFcYRIIIa (1 μg/ml each). In addition, a biotinylated human CD14 (hCD14, 4 μg/ml) preparation was incubated with the Abs and detected using ALP-conjugated streptavidin (GE Healthcare). Absorbance was measured at 405 nm.

Whole blood ex vivo model of inflammation

The whole blood model has been described in detail previously (36). Fresh human venous or porcine arterial blood was drawn directly into tubes containing the anticoagulant lepirudin (Pharmion) at a final concentration of 50 μg/ml. In 1.8-ml Cryo Tube vials (Nunc, Roskilde, Denmark), the blood was preincubated with up to 20 μg/ml anti-human CD14 (r18D11 or 18D11), or 50 μg/ml anti-porcine CD14 (Mi12 or Mi2) at 37°C for 10 min prior to an additional 2-h incubation in the presence of 100 ng/ml ultrapure LPS from Escherichia coli (O111:B4 (InvivoGen, San Diego, CA) for human blood or 1 × 10^11/ml heat-inactivated E. coli (strain LE392, ATCC33572) for porcine blood. As negative controls, PBS with MgCl2 and CaCl2 (Sigma-Aldrich) and isotype controls were used. Adverse effects were tested using nonactivated whole blood samples. After the addition of 10 mM (human) or 20 mM (porcine) EDTA, plasma was gained by 15 min centrifugation at 3220 × g and 4°C. Levels of TNF, IL-6, and IL-1β in human plasma were determined using Bioplex technology (Bio-Rad Laboratories AB). Levels of TNF, IL-1β, and IL-8 in porcine plasma were determined using ELISA (Quantikine, R&D Systems, Minneapolis, MN). Platelet count was quantified by impedance using a CELL-DYN Sapphire hematological analyzer (Abbott Laboratories, Abbott Park, IL).

Effect of Mi12 combined with the complement C5 inhibitor OmCl on cytokine production in porcine whole blood ex vivo

Whole blood was incubated with 1 × 10^6 E. coli per milliliter for 2 h at 37°C in the absence or presence of inhibitors and controls. TNF, IL-1β, and IL-8 blood was analyzed as described above.

Effect of Mi12 combined with the complement C5 inhibitor OmCl on leukocyte tissue factor expression

For analysis of leukocyte expression of tissue factor (TF), porcine whole blood was incubated with 5 × 10^6 E. coli per milliliter in the absence or presence of inhibitors and controls. After incubation, the tubes were put on ice, citrate was added to stop the activation, and the samples further analyzed by flow cytometry. One sample was split into two tubes and stained with sheep anti-human TF (Affinity Biologicals, Ancaster, Canada) and control sheep IgG (Sigma-Aldrich, Saint Louis, MO), respectively. All samples were incubated for 30 min at 4°C, and red cells were lysed and centrifuged at 300 × g for 5 min at 4°C. The cells were washed with PBS (0.1% BSA, BioTest, Dreieich, Germany). Samples were further stained with rabbit anti-sheep IgG-PE conjugate (Santa Cruz Biotechnology, Dallas, TX) and with an additional 0.16 M ammonium chloride, 10 mM EDTA, pH 6.8, and from 10 above. The cells were resuspended in PBS (0.1% BSA) before they were run at the flow cytometer (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ). Granulocytes were gated in a forward scatter–side scatter dot plot, and TF expression was given as median fluorescence intensity.

In vivo application of anti-porcine CD14 Abs

Norwegian domestic pigs (Sus scrofa domesticus, outbred stock) with a weight of 2.2 kg were isolated at the day of intervention. Anesthesia was induced with 5% sevoflurane in a mixture of air and oxygen until sleep. After establishment of an i.v. line, the piglets received fentanyl (15–20 μg/kg) which was tracheotomized in the supine position, and a microcuffed endotracheal tube from Kimberly-Clark (Roswell, GA) with inner diameter of 4 mm was inserted. Maintenance anesthesia was provided with an infusion of fentanyl (50 μg/kg/h) and isoflurane 1–2% in oxygen-enriched air administered from a Leon plus ventilator from Heinen and Loewenstein (Bed Ems, Germany). An artery line was inserted in the right or left carotid artery for blood sampling during the experiments and for continuous measurement of mean artery pressure. The piglets were monitored with electrocardiography and pulse oximetry. Ventilator settings were adjusted to maintain 7.40 pH and oxygen saturation above 96%. Hemodynamic parameters were collected using ICU/pilot software, CMA Microdialysis (CMA Microdialysis AB, Solna, Sweden) and CMA Monitor 30. To compensate for dehydration needs, the animals received a background infusion of isotonic sodium chloride solution, Salidx (Braun Medical A/S, Vestskogen, Norway) at 10 ml/kg/h.

To compare the effect of the Mi12 and the Mi12 on healthy piglets, increasing amounts of a stock solution of 1 mg/ml Mi12 or Mi2 (batch 1106) were injected i.v. into two piglets at indicated times to a maximum dose of 5.36 mg/kg. Arterial blood samples were collected, in tubes containing the anti-clotting EDTA or citrate. To investigate the biological effect of Mi12 in the inflammatory response, two piglets underwent the E. coli sepsis regimen as described previously (17). One control piglet was given saline, and one piglet was given a bolus dose of 5 mg/kg Mi12 before infusion of the bacteria.

Data presentation and statistics

All graphs were generated and statistical analyses were performed using GraphPad Prism version 5.03 from GraphPad Software (San Diego, CA). If not indicated differently, arithmetic mean values and SEM are displayed. Statistical significance was calculated using ANOVA and Tukey, Dunnett, or Bonferroni posttest analysis for subgroup comparison as indicated in the figure legends. Student t test was used to compare combined inhibition of anti-CD14 and OmCl compared with the two single inhibitions.

Ethics

The study was approved by the Norwegian Government Regional Committee for Medical Research and by the Norwegian Animal Research Authority.

Results

Cloning and expression of recombinant anti-CD14 Abs

Recombinant anti-porcine CD14 (rMi12) and anti-human CD14 (r18D11) Abs were generated, as both mouse human chimeras with
murine variable and human constant regions (Supplemental Table I). For both region, the H chain C region (CH) was chosen such that the CH1 and hinge regions were from IgG2, whereas the CH2 and CH3 domains were from IgG4. The variable genes encoding the Ab specificities are identical to those of the original murine clones 18D11 and Mil2 (Supplemental Table I). raNIP with the same C region was also generated and included as isotype control in further studies.

All Abs were readily expressed in adherent HEK293-EBNA cells after transient transfection, although at different levels. During expression under serum-free conditions, \( \sim 20 \) mg/ml rMil2 was produced, while r18D11 and raNIP were produced at 4-8 mg/ml (Fig. 1A-C). For rMil2, production reached a maximum of 15 pg/cell/d between days 6 and 9 (Fig. 1A). The recombinant Abs were purified from the cell culture supernatant, subjected to re-

![Figure 1](http://www.jimmunol.org/)

**FIGURE 2.** Functional characterization of anti-porcine CD14 Ab rMil2. (A) Whole porcine blood was incubated with 150 \( \mu \)g/ml FITC-conjugated Mil2 (FITC-Mil2) and increasing concentrations of unlabeled Mil2 (○), control IgG2/4 (eculizumab) (○), the original clone Mil2 (●) or mlgG2b isotype control (■). Remaining FITC-Mil2 binding to the granulocyte population was recorded as median fluorescence intensity of using flow cytometry. Fluorescence intensity in presence of FITC-Mil2 alone was set to 100%. (B and C) Release of the proinflammatory cytokines IL-1β (B) and TNF (C) from porcine whole blood induced with 1 \( \times \) 10^7/ml E. coli strain LE392 in presence of increasing concentrations of rMil2 (○), Mil2 (●), control IgG2/4 (○), or mlgG2b (■). After 120 min incubation, plasma cytokine levels were determined with ELISA, and those measured in absence of any exogenous Ab were set to 100%. (D) Porcine whole blood was incubated with increasing concentrations of rMil2 or Mil2, or ctrl IgG2/4 or mlgG2b isotype controls (up to 71.4 \( \mu \)g/ml). Platelet counts were determined by flow cytometry in routine analysis. Significance for differences in values for rMil2 (○) and Mil2 (●) was calculated by two-way ANOVA and Bonferroni post test (**p < 0.001). (E) Blood slides from samples containing 71.4 \( \mu \)g/ml rMil2 or Mil2 were stained with nuclear stain and investigated by light microscopy. Pictures were taken at a 1000-fold magnification. WBCs, RBCs, and platelets (thrombocytes [TRCs) are indicated by arrows. The single asterisk in the picture for Mil2 indicates a platelet aggregate with surrounding leukocytes that was not observed in rMil2-treated blood. (F) Porcine whole blood was incubated with 50 \( \mu \)g/ml rMil2 (○), Mil2 (●), control IgG2/4 (○) or mlgG2b (■). After 120 min, plasma levels of IL-8 were measured using ELISA. Data are given as mean and SEM for (A, D, and F) (all \( n = 3 \)) and as mean and range for (B) and (C) (\( n = 2 \)). Significance for difference in values for Mil2 and rMil2 (**p < 0.001) was calculated by one-way ANOVA and Tukey post tests. n.s., not significant.
ducing and nonreducing SDS-PAGE, and compared with commercially available batches of their original murine clones (Fig. 1D). The recombinant Abs were also detected by an anti-human IgG2 hinge Ab after Western blotting (Fig. 1E).

**Functional characterization of the recombinant anti-porcine CD14 Ab rMil2**

Whole blood from healthy pigs was used to study Ag-binding and CD14-blocking effects of the recombinant anti-porcine CD14 Ab rMil2. rMil2 effectively bound to and displaced the original clone, Mil2, from CD14+ granulocytes (Fig. 2A). Both rMil2 and Mil2 competed equally well with FITC-conjugated Mil2 in binding to CD14, and they blocked nearly 50% of the binding sites at 15 μg/ml. At this concentration, direct binding of rMil2 to porcine granulocytes was saturated (not shown).

Furthermore, rMil2 effectively inhibited the proinflammatory cytokine response in whole blood induced by 1 × 10⁷ cells/ml heat-inactivated *E. coli* (Fig. 2B, 2C). Therefore, it was as effective as Mil2 in the block of IL-1β release, and slightly less inhibitory on TNF release. In the presence of 10 μg/ml and 50 μg/ml of either Ab, IL-1β and TNF plasma levels were reduced by at least 75% and 50%, respectively.

Next, we tested for unwanted IgG-Fc mediated effects of rMil2 and Mil2 in the absence of inflammatory stimuli (Fig. 2D–F). We observed a dose-dependent drop in platelet counts for Mil2 (Fig. 2D). This highly significant drop was most likely the result of platelet activation and aggregation, and platelet aggregates surrounded by leukocytes were observed in blood slides from the same samples (Fig. 2E). In addition, Mil2 induced a strong spontaneous IL-8 release (Fig. 2F). Neither IL-8 secretion nor platelet drop nor aggregation were observed in the presence of rMil2. None of the Abs induced significant complement activation, measured as terminal C5b-9 complement complex formation (not shown).

**Effect of rMil2 in combination with the complement C5 inhibitor OmCI on the inflammatory response**

Based on recent promising data supporting a combined inhibition of CD14 and the complement system as a therapeutic approach for inflammatory conditions (37), we investigated the effect of the combination of OmCI and the two Mil2 Abs, or an isotype control Ab. Plasma was analyzed by flow cytometry and expressed as median fluorescence intensity (MFI). Results are shown as mean ± SEM of four to eight experiments. 

**Statistical significance between combined inhibition versus the two single inhibitions (for detailed values, see Results).**

Statistical significance between combined inhibition versus the two single inhibitions (for detailed values, see Results).

*Statistical significance as compared with *E. coli* alone (left column). *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 3.** Effect of rMil2 in combination with C5-inhibitor OmCI on the inflammatory response in porcine blood in vitro. Porcine whole blood was incubated with *E. coli* in the presence of PBS (*E. coli*), the complement C5 inhibitor OmCI (0.32 μM), original anti-CD14 Ab Mil2 (25 μg/ml), recombinant anti-CD14 Ab rMil2 (25 μg/ml), the combination of OmCI and the two Mil2 Abs, or an isotype control Ab. Plasma was analyzed for cytokines. (A) TNF. (B) IL-1β. (C) IL-8. (D) TF expression on granulocytes was measured by flow cytometry and expressed as median fluorescence intensity (MFI). Results are shown as mean ± SEM of four to eight experiments.

The recombinant anti-human CD14 Ab r18D11, was tested with respect to Ag binding and inhibition of CD14-mediated cytokine release. It dose-dependently outcompeted the binding of the original clone, 18D11, to CD14-positive sites on human monocytes (Fig. 4A). The same was observed with a F(ab)’2 fragment of the original clone. This indicates that the Abs bind to the same epitope, as expected. Equimolar amounts of r18D11 (10 μg/ml) displaced 50% of 18D11 from its binding sites. The lower competitive activity of 10 μg/ml r18D11 compared with 10 μg/ml of the F(ab)’2 fragment of the murine clone is due to difference in molarities. Neither rNIP nor a control F(ab)’2 fragment bound human CD14 (Fig. 4A).

Furthermore, both 18D11 and r18D11 inhibited *E. coli* ultrapure LPS-induced release of the proinflammatory cytokines IL-1β, TNF, and IL-6 in human whole blood, in a dose-dependent manner.
Activation, whereas the F(ab)\textsubscript{r18D11}, and raNIP induced the same low level of complement
pathway and Fc receptors binding of the recombinant IgG2/4 Abs to C1q
Rs was measured by ELISA (Fig. 5). Importantly, none of them bound to C1q, whereas the positive control, a human IgG1 (rituximab), did so in a dose-dependent manner (Fig. 5A). The same was observed for all tested FcγRs, except for FcγRIIa allotype His131 (Fig. 5C). Here, the recombinant Abs bound to the receptor, though less than human IgG1.

Furthermore, we tested for binding to the FcRn, which plays a crucial role in IgG t½ regulation and biodistribution. Recombinant IgG2/4 Abs bound both human and porcine FcγRn receptors in vitro with dose responses comparable to those for the positive controls, human IgG1, or a porcine IgG pool (Figs. 5G–K). In accordance with the reported pH dependency, both the human and porcine FcγRn bound their IgG ligands at an acidic pH (pH 6.0), whereas binding of IgG at physiologic pH (∼pH 7.4) was negligible. Binding occurs at the CH2 and CH3 domains of the Fc region, with amino acid 435 (His435 in IgG2) being a key contact residue (12).

In vivo adverse effects induced by anti-porcine CD14 Ab Mil2 are not observed after rMil2 injection in a pig

Intravenous bolus injections of Mil2 to pigs have been observed to disturb the porcine hemodynamics by causing severe peripheral vasodilatation, increase in heart rate, drop in systemic arterial pressure and loss of platelets, together interpreted as reactions that appeared to be anaphylaxis (E.B. Thorgersen, A. Barratt-Due, E.W. Nielsen, and T.E. Mollnes, unpublished observations). Mil2 and rMil2 were therefore compared for induction of these adverse effects in vivo using Norwegian domestic piglets (Fig. 6A–D). In two piglets, a total of 5.36 mg/kg Mil2 or rMil2 were injected as increasing dose over a period of 45 min (Fig. 6A). In vivo binding of Mil2 and rMil2 to porcine CD14 was demonstrated by the blocked binding of FITC-conjugated Mil2 to CD14-positive granulocytes in blood samples collected during infusion. FITC-conjugated Mil2 was added to the blood samples immediately preceding flow cytometry analyses (Fig. 6B). At a total dose of 1.12 mg/kg, which was reached after 20 min, more than 50% of the available cell-bound CD14 was saturated.

Hemodynamic readouts were recorded. Injection of Mil2, but not rMil2, caused an increase in heart rate after 10 min, at which time a total of 0.32 mg/kg Ab had been given (Fig. 6C). For Mil2, the heart rate reached its maximum of ∼300 beats/min during the next 5 min and then slowly fell to baseline. Mil2 injection also caused a reversible drop in mean arterial blood pressure, which again was not seen for rMil2 (not shown). Finally, Mil2 injection induced a gradual depletion of platelets, whereas rMil2 did not affect platelet counts (Fig. 6D). The loss of free platelets in the presence of Mil2 could also be visualized on blood slides from the same blood samples (not shown). Thus, none of the adverse effects observed in vivo with Mil2 were observed with rMil2.

In vivo cytokine response induced by E. coli was abolished by rMil2

The biologic effect of rMil2 was then investigated. rMil2 was given as a bolus dose to a piglet, and the leukocyte expression of CD14 before and after this bolus was measured using fluorescence-labeled
A reduction in CD14 expression by 94% was observed after the bolus of rMil2 was given, consistent with a virtually complete saturation of CD14 by rMil2 in vivo (Fig. 6E). Furthermore, rMil2 virtually abolished the E. coli–induced cytokine response (Fig. 6F–I). TNF, IL-1β, IL-6, and IL-8 were reduced by 71%, 89%, 88%, and 100%, respectively (area under the curve).

Discussion
In this study, we have generated and intensively tested a recombinant anti-porcine CD14 IgG2/4 Ab (rMil2), which showed to be functional with respect to neutralization of LPS-induced cytokine production and free of undesired Fc-mediated effects. Our data demonstrate that rMil2 can be used for in vivo therapeutic intervention in pig models of inflammation and, thus, constitutes a valuable tool for future studies of in vivo CD14 function, in particular of its crosstalk with complement, since we showed a substantial additional effect by combining rMil2 with the C5 inhibitor, as compared with their single effects.

Blocking human CD14 with the mouse monoclonal anti-CD14 Ab clone 18D11 (F(ab)92 fragment has been a powerful inter-

![FIGURE 5. In vitro binding of recombinant IgG2/4 hybrid Abs to complement and Fc-receptors. Increasing concentrations of rMil2 (○), r18D11 (△) or raNIP (□) were incubated with (A) immobilized human C1q, (B) the human Fc receptors FcγRI, (C) FcγRIIa (allotype His131), (D) FcγRIIb, (E) FcγRIIIa (allotype Val158), (F) FcγRIIIb, and (G, H) human (hFcRn) or (I, J) porcine FcRn (pFcRn) at acidic (pH 6.0) and neutral pH (pH 7.4). Human IgG1 (○) (A–H) or porcine IgG (pIgG; ♦) (I, J) served as positive controls. Binding was measured as absorbance at 450 nm. Data are given as mean and SD (n = 3 independent experiments).

![FIGURE 6. In vivo application of anti-porcine CD14 Abs Mil2 and rMil2. Healthy newborn piglets (A–D) were infused i.v. with increasing amounts of the original clone Mil2 (n = 1) or rMil2 (n = 1) and observed for 50 min. (A) After initial small doses within the first 10 min, Mil2 or rMil2 were given every 5 min for an additional 35 min. After 45 min, a maximum final concentration of 5.36 mg/kg was reached. (B) Saturation of endogenous CD14 binding sites as a function of rMil2 (○) or Mil2 (●) concentration was measured by flow cytometry. Blood samples were incubated with 150 μg/ml FITC-conjugated Mil2, the porcine granulocyte population was selected, and the median fluorescence intensity for bound FITC-Mil2 was recorded. Fluorescence intensity in the absence of nonconjugated anti-CD14 (time point zero) was set to 100%. (C) The heart rate (HR) was recorded in real time throughout the experiments. (D) Blood platelet counts are given as a function of rMil2 (○) or Mil2 (●) concentration. They were corrected for actual hematocrit levels that reflect blood dilution caused by sampling and reconstitution. Levels in the absence of anti-CD14 Ab were set to 100%. Two piglets underwent E. coli sepsis (E–I). One piglet was injected with a bolus dose of rMil2 before i.v. infusion with bacteria and one received saline as control. Expression of leukocyte CD14 was measured before and after rMil2 injection (Fig. 6E). Cytokine release was measured during the 4-h observation period (Fig. 6F–I) comparing saline (●) with rMil2 (○). Anti-CD14, Fluorescence-labeled rMIL2; Isotype ctr, the isotype control for rMIL2.
vention in ex vivo inflammatory models using exogenous danger ligands such as gram-negative bacteria or endogenous danger ligands like meconium (22, 23, 26, 27, 38, 39). In the current study, we have generated a recombinant anti-human CD14 IgG2/4 Ab (r18D11) that blocked CD14-mediated inflammatory responses in a human whole blood model of inflammation, was virtually inert with respect to Fc-mediated binding to complement and FcγRs, and induced no oxidative burst (Figs. 4 and 5). Thus, r18D11 constitutes a highly promising lead for future anti-inflammatory drug engineering and therapeutic intervention.

To study the many roles of CD14 in vivo, pigs are emerging as a valuable test model system. We provide a recombinant anti-porcine CD14 IgG2/4 Ab—rMil2. The original clone Mil2 from which rMil2 is derived has already been used as intervention in porcine sepsis (17). Despite the fact that the application of Mil2 was efficient in reducing the inflammatory response, its bolus application was hampered by the induction of an initial reaction appearing to be anaphylaxis and had a clear limitation for further study. In this study, we show that Mil2 induces unwanted IL-8 release in vitro (Fig. 2) and platelet activation both in vitro and in vivo (Figs. 2 and 6). The latter was accompanied with hemodynamic changes, including decreased arterial blood pressure and increased heart rate. Importantly, we document that none of these effects were induced when rMil2 was used instead, indicating a major step forward with respect to CD14 inhibition in porcine models of relevance to human disease. Finally, we showed that the biologic activity of rMil2 was preserved, as compared with the original Mil2, by blocking leukocyte CD14 and by abolishing *E. coli*–induced cytokine production in vivo. The effect of anti-DC14 on the growth of *E. coli* was not studied. We have, however, shown previously that Mil2 does not affect *E. coli* survival in whole pig blood, in contrast to a complement inhibitor, that increased bacterial survival (25).

IgG infusion-related in vivo reactions that appear to be anaphylaxis, as well as FcγR–Ab-dependent cell cytotoxicity or complement-dependent mediated cytotoxicity, are unwanted events in anti-CD14 based inflammatory therapeutic strategy, where maintenance of homeostasis is the main concern. Now, recombinant anti-CD14 IgG2/4 Abs with minimum Fc-mediated effector functions are available. Of the human IgG subclasses, IgG2 and IgG4 exert the least FcγR binding and complement fixation activities, respectively (21, 40, 41). As expected, the combination of the two subclasses in a human IgG2/4 subclass hybrid abolished binding to complement and any all FcγRs, except FcγRIIa (alloype His131), a low-affinity activating FcγR (Fig. 5). All conventional FcγRs bind their Fc ligands at a site that involves the lower hinge region and the two CH2 domains (42, 43). The recombinant IgG2/4 CH hybrid Abs carry sequences from the human IgG2 subclass in the lower hinge, and all FcγR contact residues in the CH2 domain that were derived from IgG4 are identical to those found in IgG2 (43). Thus, our data are consistent with the fact that FcγRIIa (alloype His131) is the only FcγR that binds IgG2, and thus the IgG2/4 subclass hybrid, with reasonable affinity (21). Importantly, pigs are not known to express FcγRIIa, and the homology between other human and pig FcγRs is more than 60% (44–46). For example, the most important residues in FcγRIIa for IgG binding, Trp87 and Trp110, are conserved between the pig and human receptors. FcγRIIa has been shown to play a key role in IgG mediated anaphylaxis (20), but is bound only weakly by human IgG2 (21). It is, therefore, not surprising that rMil2 with its IgG2/4 hybrid C region does not induce an anaphylactic reaction in pigs.

Human IgG is both readily bound and taken up by pig cells expressing porcine FcRn (47). FcRn regulates the serum t1/2 of Abs by a recycling mechanism that requires pH-dependent binding (48). In this study, we provide evidence that the human IgG2/4 subclass hybrid Abs bind porcine FcRn in such a pH-dependent manner. This evidence will contribute further to the successful use of Abs with human constant regions in pig.

The exact binding sites of 18D11 and Mil2, and thus of r18D11 and rMil2, on human and porcine CD14 are still not defined. However, as they block CD14 function, it is likely that they bind to either the N-terminal LPS-binding pocket of CD14 or parts of the LPS-signaling motif. Importantly, the hydrophobic binding pocket can also accommodate other acylated endogenous and exogenous ligands of CD14 and TLRs (49–51). Therefore anti-CD14 Abs, like r18D11 and rMil2, may affect pattern recognition signaling upon a wide range of threats, being more efficient than, for example, LPS mimics.

Recently, intensive cross talk has been described for TLR signaling and the complement system, which itself is associated with a plethora of acute and chronic inflammatory disorders (52, 53). This functional interplay has been recognized as an important regulatory mechanism to control both innate and adaptive immune responses (reviewed in Refs. 54–56), and combined inhibition of CD14 and complement has been suggested as an effective therapeutic approach in conditions associated with detrimental activation of the innate immune system (57, 58). We recently showed that combined inhibition on CD14, using the original clone Mil2, and the complement inhibitor OmC1, reduces inflammation, hemostatic disturbances and improved hemodynamics in a porcine model of *E. coli* sepsis (37). These results were obtained despite the adverse effects seen with the original clone Mil2. In this study, we show that rMil2 combined with the complement C5 inhibitor OmC1 efficiently attenuates the *E. coli*–induced cytokine response and TF expression in porcine whole blood without any adverse effects (Fig. 3).

The anti-CD14 Abs reported in this study would be particularly valuable tools for future in vivo studies to explore the combined inhibition of CD14 and complement as a therapeutic approach for inflammatory diseases in the future.

**Acknowledgments**

We thank the routine laboratories of the medical chemistry department at Oslo University Hospital Rikshospitalet for hematology analyses and Margareta Nilsson for performing porcine cytokine analyses.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


**Supplementary Table 1.** Protein sequences of the murine variable and human constant regions of the recombinant mouse human chimeric IgG2/4 hybrid antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Chain</th>
<th>Amino acid sequence (N→C)</th>
<th>IMGT ¹</th>
</tr>
</thead>
</table>
| r18D11   | Variable light      | MGWSCLLFLVATATGVHSVNNH1VTQSPLVSALAVSLQQRATISCR
ASESVDSYGNMHPMWHQQPKPQPKLLYVASQNLNSGQVPARFS
GSQSRTDPTLPIDTPVADVDVATTVYQQNQNDIFYFPGGTKE1
|          | Variable heavy      | MGWSCLLFLVATATGVHSVQVLESOGGLMPPKSLKCAAA
SGFTFKTYALNVRQAPGFTLEKVARISKSNNTTYYADSVK
DRPTISRDSQNNMLYPLMNLALEDTAMYYCYRVPQGTSFPAYW
GQGTTLVTVSA[A] |
| rMIL2    | Variable light      | MGWSCLLFLVATATGVHSVDDIVMTSSQKFMSFTSVGDRVSVTCK
ASQVGYTANVWQQPKQPQSPKALISASRTCSGVFDRTNGS
GTDFMTLISVQSSDLDAYFCQYYTFFGATTKLEL[A] |
|          | Variable heavy      | MGWSCLLFLVATATGVHSVQVLRQQPGAEILVRPSASVSLCSAKA
SGYTPTTNYMNWVKQPMEGLNGWIRIDEPSTHYNQNFKD
AILTVKSDSSSTAMQLSSLTLTYEEDAVYCTRKE1QWAGYFDY
GQGTTTLTVSA[A] |
| All      | Constant light      | TVAAASVPFIFPPSQDEQLSGTASVVCLNNPFRAYQKQKVVD
NALSQGNSQGVTEQD5KSDSTLSSTLTKADYRXXHYAC
ETVQGLSSLSPVTSPNRCG- |
|          | Constant heavy      | STKGPSVFLAPAFSRSSTTSRSTAAALCGLKDYPPFRPVTVSNSSG
ALTSQVHTTAPOAVLSQVLSQQVTPSSPGTQTYTNCNCH
KPSNKTVPKNCRRKVCCPCPAPPVAGPSVFL/ |
|          | IgG2/IgG4           | J00230/K01316 |

¹ IMGT accession number. Nucleotide sequence alignment with the IMGT database revealed at least 90 % identity of the variable genes with the listed entries.

[...], amino acid derived from splicing site, connecting coding sequences of the variable and constant genes in light chains (R, arginine) and heavy chains (A, alanine).

Underlined, leader sequence encoded by the expression vectors; bold underlined, residues PSVFLF are identical in IgG2 and IgG4.
**Supplementary Table 2.** Primers used for V-gene retrieval

<table>
<thead>
<tr>
<th>Binding site</th>
<th>Primer ID</th>
<th>Primer sequence (5'→3')</th>
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<tbody>
<tr>
<td>Murine constant heavy γ1*</td>
<td>IgG1CH1_rev</td>
<td>GTTTGCAGCAGATCCAGGG</td>
</tr>
<tr>
<td></td>
<td>IgG1CH1_Mlu1_rev2</td>
<td>ATACGCGTGTTTGCAGCAGATCCAGGG</td>
</tr>
<tr>
<td>Murine constant heavy γ2b*</td>
<td>IgG2bCH1_rev</td>
<td>GAGTTCCAAGTCACAGTCACTG</td>
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<tr>
<td></td>
<td>IgG2bCH1_Mlu1_rev</td>
<td>ATACGCGTAGTTGTATCTCCACACCCAGG</td>
</tr>
<tr>
<td>Murine constant light kappa*</td>
<td>IgGKC_rev</td>
<td>GCCATCAATCTTCCAACCTTGACA</td>
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<tr>
<td></td>
<td>IgGKC_Mlu1_rev</td>
<td>ATACGCGTACTGAGGCACCTCCAGATG</td>
</tr>
<tr>
<td>Poly-dC tail</td>
<td>PolyG_BglIII_fwd</td>
<td>ATATAGATCTGGGGGGGGGGGGGGGGGG</td>
</tr>
</tbody>
</table>

* reverse complementary binding

Underlined, restriction sites for MluI and BglII, respectively