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Selective Blockade of CD28-Mediated T Cell Costimulation Protects Rhesus Monkeys against Acute Fatal Experimental Autoimmune Encephalomyelitis

Krista G. Haanstra,* Karin Dijkman,* Noun Bashir,* Jan Bauer,† Caroline Mary,‡ Nicolas Poirier,‡ Paul Baker,§ Linda Scobie,§ Bert A. ’t Hart,*§ and Bernard Vanhove‡,∥

Costimulatory and coinhibitory receptor–ligand pairs on T cells and APC control the immune response. We have investigated whether selective blockade of CD28–CD80/86 costimulatory interactions, which preserves the coinhibitory CTLA4–CD80/86 interactions and the function of regulatory T (Treg) cells, abrogates the induction of experimental autoimmune encephalomyelitis (EAE) in rhesus monkeys. EAE was induced by intracranial immunization with recombinant human myelin oligodendrocyte glycoprotein (rhMOG) in CFA on day 0. FR104 is a monovalent, PEGylated-humanized Fab’ Ab fragment against human CD28, cross-reactive with rhesus monkey CD28. FR104 or placebo was administered on days 0, 7, 14, and 21. FR104 levels remained high until the end of the study (day 42). Placebo-treated animals all developed clinical EAE between days 12 and 27. FR104-treated animals did not develop clinical EAE and were sacrificed at the end of the study resulting in a significantly prolonged survival. FR104 treatment diminished T and B cell responses against rhMOG, significantly reduced CNS inflammation and prevented demyelination. The inflammatory profile in the cerebrospinal fluid and brain material was also strongly reduced. Recrudescence of latent virus was investigated in blood, spleen, and brain. No differences between groups were observed for the B-herpesvirus CMV and the polyomaviruses SV40 and SA12. Cross-sectional measurement of lymphocryptovirus, the rhesus monkey EBV, demonstrated elevated levels in the blood of FR104-treated animals. Blocking rhesus monkey CD28 with FR104 mitigated autoreactive T and B cell activation and prevented CNS pathology in the rhMOG/CFA EAE model in rhesus monkeys. The Journal of Immunology, 2015, 194: 000–000.

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Abbreviations used in this article: ADA, antidrug Ab; ADEM, acute disseminated encephalomyelitis; ALN, axillar lymph node; AU, arbitrary unit; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; HEV, hepatitis E virus; IL-1RA, IL-1R antagonist; LCV, lymphocryptovirus; LFB, luxol fast blue; MNC, mononuclear cell; MS, multiple sclerosis; PAS, periodic acid–Schiff; rhMOG, recombinant human myelin oligodendrocyte glycoprotein; SI, stimulation index; Treg, regulatory T.

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This model is characterized by an early disease onset (between 11 and 40 d) and a very rapid disease progression from first clinical signs until ethical endpoints indicate euthanasia (between a few hours to 1–2 d) (14–16). The disease incidence is 100%, and the model has been validated with the anti-αβ3 mAb natalizumab (Tysabri) (15). We report that FR104 has a profound suppressive effect on the induction of EAE in six treated animals as compared with placebo-treated animals at the level of T and B cell activation, resulting in greatly diminished CNS pathology. Under conditions of immune suppression or indeed altered immune status, latent viral activation can lead to significant complications for the patient, if not treated prophylactically, in particular for the herpesviruses and polyomaviruses (17–19). We investigated whether reactivation of existing latent endogenous viral infections could be detected in blood and tissues of both placebo and FR104-treated animals. We report possible exacerbation only of macaque lymphocryptovirus (LCV), a gammaherpesvirus related to EBV.

Materials and Methods

Abs and in vitro evaluation

FR104 is a monovalent, FcGlylated, humanized Fab’ Ab fragment that antagonizes binding of CD28 to CD80 and CD86. Because of its monovalent nature and its target epitope lying outside the C’D loop seen by superagonist anti-CD28 Abs (20), FR104 cannot induce T cell activation (11).

To assess whether FR104 abrogates the function of naturally occurring primate Treg cells, PBMC were isolated from heparinized venous blood from healthy rhesus monkeys (n = 4) using density gradient centrifugation. CD4+CD25+ and CD4+CD25− T cells were isolated and cultured as previously described (21), with minor modifications. In brief, CD4+CD25+ T cells were separated from CD4+CD25− cells using the Treg isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany). CD4+CD25+ cells were stimulated with plate-bound anti-CD3 (1 μg/ml) in the presence or absence of CD4+CD25− Treg cells. FR104 (10 μg/ml) was added to evaluate its effect on the inhibitory effect of CD4+CD25+ Tregs on CD4+CD25− proliferating effector T cells. Proliferation was assessed by 3H-thymidine incorporation during the last 18 h of a 5-d culture.

To establish whether the rhesus monkey EAE model was suitable to investigate the effect of FR104, we first tested whether CD28 blocking is effective to suppress rhMOG-activated cells ex vivo. To test this, we used stored PBMC, spleen cells, or inguinal lymph node cells from monkeys with EAE, which had an established positive response against rhMOG, as described previously (15, 22). Mononuclear cells (MNC) were cultured in quadruplicate (100,000/well) in culture medium with or without rhMOG (5 μg/ml), in the presence or absence of FR104 (10 μg/ml). Inhibition of CD28 interaction with CD80/86 by CTLA4-Ig (10 mAb) antagonizes binding of CD28 to CD80 and CD86. Because of its monovalent binding of FR104 was detected using a mouse anti-human mAb (catalog no. NaM76-5F3; Effinimmune), diluted to 1/10,000 at 50°C. As a control, cells were treated with the anti-CD28 Abs (20), FR104 cannot induce T cell activation (11).

Animals

Naive, adult rhesus monkeys (Macaca mulatta) were randomly selected from the purpose-bred colony of the Biomedical Primate Research Centre and housed under conventional conditions. Animals were only included after a complete physical, hematological, and biochemical checkup had been performed, and monkeys were declared healthy. During the study, monkeys were pair-housed and remained under intensive veterinary care. The daily diet consisted of commercial food pellets for nonhuman primates (Sniff, Soest, Germany), supplemented with rice, raisins, peanuts, and fresh fruit. Drinking water was provided ad libitum.

The experimental design, all study protocols and experimental procedures were approved and reviewed by the Biomedical Primate Research Centre’s Ethics Committee, in accordance with Dutch law on animal experimentation.

Cerebrospinal fluid (CSF) samples were collected via the cisterna magna prior to euthanasia from sedated monkeys and were processed as described previously (15).

EAE induction and monitoring

EAE was induced with rhMOG, a recombinant protein produced in Escherichia coli that represents the extracellular domain of human MOG, residues 1–125; production and purification was as described previously (24). Animals were immunized on day 0 with 300 μg rhMOG dissolved in 300 μl PBS and emulsified with an equal volume of CFA (Difco Laboratories, Detroit MI). The total volume of 600 μl was injected into the dorsal skin at six sites, targeting both the inguinal and the axillary regions. Clinical signs were scored daily by observers blinded to the treatment using a previously described semiquantitative scale (14, 25, 26): 0, no clinical signs; 0.5, loss of appetite and vomiting; 1, substantial reduction of general condition; 2, ataxia, sensory loss and/or visual problems; 2.5, incomplete paralysis of one (hemiparesis) or two sides (paraparesis); 3, complete paralysis of one (hemiplegia) or two sides (paraplegia); 4, complete paralysis (quadriplegia); and 5, moribund. The rhMOG-induced EAE model in rhesus monkeys is characterized by acute onset and rapid disease progression, reaching a moribund state within 24 h. To avoid suffering, monkeys were euthanized at EAE score ≥ 2.5 or at score 2 when the animal was not expected to survive until the next day.

Pharmacokinetics assessment

Concentrations of FR104 in serum samples from rhesus monkeys were quantified by ELISA. In brief, 96-well microtiter plates were coated with CD28Fc (catalog no. 342-CD-200; R&D Systems) at 2 μg/ml in carbonate buffer (pH 9) at 50 μl/well overnight at 4°C. After washing, free binding sites were blocked using PBS/0.1% Tween/1% BSA) at 100 μl/well for 2 h at 37°C. Sera were diluted 1/1,000, 1/3,000, 1/9,000, and 1/27,000 and incubated at 50 μl/well for 2 h at 37°C. After washing with PBS/Tween binding of FR104 was detected using a mouse anti-human κ mAb (catalog no. NaM76-5F3; Effinimmune), diluted to 1/10,000 at 50 μl/well for 1 h at 37°C. Detection of bound Abs was enhanced using a peroxidase-labeled donkey anti-mouse IgG (catalog no. 715-036-151; Jackson ImmunoResearch Laboratories) diluted at 1/2000 at 50 μl/well for 1 h at 37°C. Tetrathiomethoxamine substrate was added at 50 μl/well, and color development was allowed for 10 min at room temperature. The reaction was stopped with 50 μl/well H2SO4, and absorbance was read at 450 nm.

Concentrations were calculated using a two-parameter fit of the linear part of a titration curve with known concentrations of FR104.

Antidrug Abs monitoring

Antidrug Abs (ADA) serum levels were evaluated by ELISA. FR104 was coated on 96-well microtiter plates at 5 μg/ml in borate buffer (pH 9.5) 50 μl/well overnight at 4°C. After washing, plates were blocked with PBS/0.1% Tween/0.25% gelatin at 100 μl/well for 2 h at 37°C. Sera were diluted 1/15 at 50 μl/well for 2 h at 37°C. IgM ADA were detected using rabbit anti-human IgM (catalog no. F2020; DakoCytomation), and IgG ADA were detected using rabbit anti-human IgG (catalog no. F2020; DakoCytomation), both diluted to 1/200 and incubated at 50 μl/well for 1 h at 37°C. Detection was enhanced by incubation with peroxidase-labeled goat anti-rabbit IgG (catalog no. 111-035-144; Jackson ImmunoResearch Laboratories) diluted at 1/2000 and incubated at 50 μl/well for 1 h at 37°C. ABTS substrate was added at 50 μl/well for 15 min at room temperature. The reaction was stopped with 50 μl/well H2SO4 and absorbance was read at 405 nm.

Cellular immune responses against rhMOG

PBMC were isolated before and once weekly after EAE induction and at the time of necropsy. At necropsy, MNC were also isolated from the spleen, and axillary lymph nodes (ALN). Isolated MNC were dispensed in quadruplicate at 1 × 105 cells/well in 96-well round-bottom microtiter plates with or without 0.5 μg/ml rhMOG. MNC proliferation was assayed by the incorporation of 3H-thymidine (0.5 μCi/well) during the final 18 h of a 5-d culture. Cells were harvested for beta scintillation counting (Topcount NXT, Packard, Ramsey, NJ). The results obtained from the different culture conditions were expressed as stimulation index (SI): (cpm of MNC proliferation with rhMOG)/(cpm of MNC proliferation without rhMOG). An SI ≥ 3 was considered positive.

Ab responses against rhMOG

Serum samples were collected prior to EAE induction, one weekly thereafter, and at necropsy. CSF samples were obtained at necropsy. CSF
and sera were tested for the presence of Abs against rhMOG by ELISA in 96-well microtiter plates. Plates were coated with rhMOG (5 μg/ml) and incubated overnight at 4°C. After washing and blocking with PBS/1% BSA, the wells were incubated in duplicate with 1:100 or 1:1000 diluted sera or CSF. Bound rhesus monkey Abs were detected with alkaline phosphatase–labeled goat-anti-human IgG (1:2,000; catalog no. AH11305; Invitrogen) or alkaline phosphatase–labeled goat-anti-human IgM (1:10,000; catalog no. A9794; Sigma-Aldrich, Zwijndrecht, the Netherlands). Conju- gate binding was quantified with p-nitrophenyl phosphate (Sigma-Aldrich). OD values were converted to arbitrary units (AU) using the same positive control on all plates as reference.

**Quantification of demyelination and cell infiltration**

Left hemispheres were fixed with 4% formaldehyde and subsequently processed for histopathology as described previously (15, 27). Briefly, three samples were excised from standardized regions of each brain and em- bedded in paraffin. Sections were deparaffinized and processed for H&E staining to quantify inflammation and luxol fast blue/priodic acid–Schiff (LFB/PAS) staining for demyelination. In addition, immunohistochemical stainings for CD3 and MRPl4 were performed. LFB/PAS-stained sections were scanned and analyzed with ImageJ (version 1.44p, a public domain image processing and analysis program developed by W. Rasband at the National Institutes of Health). White and gray matter areas and the demyelinated areas of all sections were selected with the selective tools of the software and quantified. Demyelination is expressed relative to total white and gray matter surface. Inflammation in each section (three sections per animal) was scored on an arbitrary scale: 0, no inflammation; 0.5, some inflam- mation (some perivascular infiltrates); 1.0, moderate inflammation (many small perivascular infiltrates); and 2.0, strong inflammation (many small and large perivascular infiltrates, deep infiltration of T cells in the paren- chyma). Afterward, for each animal, the average score of the three sections was calculated.

**Quantitative RT-PCR**

The corpus callosum was sampled for quantitative RT-PCR analysis. Tis- sues were stored at −80°C. RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), according to the instructions of the manufacturer. RNA concentrations were determined using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA was prepared from mRNA using the RevertAid First-Strand cDNA synthesis kit (Thermo Scientific).

Intron spanning primer-probe pairs were designed with the Universal Probe Library Assay Design Center (Roche, Basel, Switzerland). Primers were purchased from Invitrogen Life Technologies (Wilmington, DE). RT-PCRs were performed using TaqMan supermix and CFX96 Real-Time system (Bio-Rad, Hercules, CA). Levels of mRNA for each gene of interest were expressed as relative concentrations compared with ABL or GAPDH, taking PCR efficiencies into account. When mRNA levels of investigated genes in particular samples were below the detection level, the relative concentra- tions to ABL or GAPDH were set to 0. This occurred in three samples only (IL-6: animals P6 and F2; CCR7: animal F4).

**Cytokine/chemokine analysis of the CSF**

The supernatants obtained after centrifugation of the CSF samples were analyzed with Luminex technology. A monkey-specific cytokine/ chemokine 29-plex panel (Novex; Life Technologies) was used accord- ing to the instructions of the manufacturer. Included cytokines/chemokines are listed in Table II. Briefly, 50 μl CSF was incubated in duplicate for 2 h with anticytokine/chemokine Ab–coated beads. All CSF samples were incubated on the same plate at room temperature on an orbital shaker at 750 rpm. The cytokine/chemokine standard curve provided with the kit was included on the plate. After washing, biotinylated Abs supplied with the kit were added to the beads and incubated for 1 h and after another washing step, streptavidin–R-PE was added and incubated for 30 min. After removal of the streptavidin–R-PE and washing, the beads were resuspended in wash solution, after which the plate was read on a Bio-Plex 200 system (Bio-Rad, Veenendaal, the Netherlands). Concentrations of the cytokines and chemokines in the CSF were calculated using the Bio-Plex Manager Software 4.1 program (Bio-Rad). Results are given as median and range (minimum – maximum).

**Assessment of endogenous viral reactivation**

DNA was isolated from blood, spleen, and brain using the DNeasy blood and tissue kit (Qiagen) and viral RNA from sera using the QiAamp viral RNA minikit (Qiagen), according to the manufacturer’s instructions. Samples were taken, at set intervals, before and after treatment with FR104, and quantitative PCR was used to test the levels of virus. All assays used the TaqMan Gene Expression system (Applied Biosystems) in a final volume of 25 μl using the ViiA 7 real time (Applied Biosystems). For CMV, pri- mers at a concentration of 0.5 μM, 5’-GTATGGACCGCCGATCTGT-3’ (forward) and 5’-GTATCCGGTTCACAGCA-3’ (reverse), and probe 6Fam-TCCACCTCATAGGCGGAAGG-TAMRA were used at a concen- tration of 0.25 μM, were cycled in a 25-μl volume for 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 53°C for 30 s and 60°C for 60 s. For LCV, primers were as follows: 5’-GGACCTGCA- CTAACAGAGA-3’ (forward) and 5’-GAACTCYTGACCGACTACAT-3’ (reverse) and the probe 6Fam-TCGCCCTTGTGGCGAGG-GC-TAMRA were also used at a concentration of 0.5 and 0.25 μM, respectively, and cycled in a 25-μl volume for 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 30°C for 30 s, and 60°C for 60 s. The hepatitis E virus (HEV) levels were measured using a HepatitisE@ceeram Tools kit by Ceeram (La Chapelle sur Erdre, France), according to the manufacturer’s instructions. Samples were quantified against the World Health Organiza- tion HEV standard (28).

For polyomavirus SA12, amplification was as previously described by Cantalupo et al. (29). Standard plasmid control pUC19-SA12 was provided by Prof. P. Cantalupo (University of Pittsburgh, Pittsburgh, PA).

For SV40 amplification, the primers were as follows: 5’-GTGTCTGC- GATTCACCTATG-3’ (forward) and 5’-CAGCACTGGCCCTC-3’ (reverse) and the probe 6Fam-TGATGAATGGGACGACTGGAGGA-TAMRA. These were at a concentration of 0.5 and 0.125 μM, respectively. The total reaction volume was 25 μl, and the cycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For graphical presentation on a log scale, LCV DNA levels below the limit of detection or quantification were set to 1.

**Statistical analysis**

Statistical analysis was performed using Prism 6 for Mac OS X (GraphPad, San Diego, CA). Survival curves were compared using the log-rank test (Mantel–Cox). Average group data are given as mean ± SEM or as median and range (minimum – maximum). Significance of differences between groups was calculated using the nonparametric t test (Mann–Whitney U test). DNA levels were measured using a HepatitisE@ceeram Tools kit by Ceeram (La Chapelle sur Erdre, France), according to the manufacturer’s instructions. Samples were quantified against the World Health Organiza-

**Results**

FR104 blocks an in vitro recall response and does not interfere with Treg cells

We have previously reported that CD28 blockade enhanced Treg function in vitro in humans and in vivo in allograft rejection models in baboons and cynomolgus macaques (30, 31). To examine whether the same principle can be observed in rhesus monkeys we tested the effect of FR104 on rhesus monkey Tregs in a previously published assay (21). CD4+CD25+ cells stimulated with plate- bound anti-CD3 proliferated vigorously. In this experiment the average response was 33,276 ± 11,459 cpm. Addition of natu- rally occurring CD4+CD25+ Treg cells reduced the anti-CD3 stimulated proliferation to 2.2 ± 1.1% of the response without added Tregs (Fig. 1A). The effect of FR104 on the natural Tregs was investigated as well. The response of CD4+CD25+ cells in itself was inhibited by FR104 down to 43 ± 11% of the response without FR104 (data not shown). However, CD4+CD25+ cells inhibited this proliferation even further, to similar levels as in the absence of FR104 (Fig. 1A), indicating that suppression by the natural Treg cells were not affected by FR104. Control cultures containing the double amount of CD25− cells, thus equaling the amount of cells in the CD4+CD25− and CD4+CD25+ coinubations, was not inhibited (Fig. 1A), indicating that the inhibition observed in the CD25−/CD25+ coculture was not due to exhaustion of the medium. In conclusion, as previously demonstrated for human, baboon, and cynomolgus monkey Treg cells (30, 31), FR104 does not interfere with rhesus monkey natural Treg cell function.

Next, we tested the effect of FR104 on the ex vivo recall response against rhMOG of MNC from rhMOG-sensitized rhesus monkeys.

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Stored cells from monkeys with EAE, which had an established positive response toward rhMOG, were stimulated with rhMOG ex vivo in the presence or absence of FR104 or CTLA4-Ig. Fig. 1B shows that MNC proliferation was inhibited by FR104 to the same extent as by CTLA4-Ig (Fig. 1B). These results underline the important role of CD28 in secondary T cell responses against rhMOG.

Pharmacokinetics and immunogenicity of FR104 in rhesus monkeys

We tested FR104 at a dose of 10 mg/kg FR104 administered on post immunization days 0, 7, 14, and 21 based on data from extensive pharmacokinetic and pharmacodynamic monitoring in baboons (12) and cynomolgous monkeys (11). FR104 through levels were homogenous and consistently >50 μg/ml in all animals (Fig. 2A).

Only in animal F3 we observed higher levels than in the others. Interestingly, primate IgG ADA levels were detected only in this same animal after day 11, but this was not observed in any of the other animals (Fig. 2B). IgM ADA levels were below the detection limit (data not shown).

FR104 prevents clinical signs of EAE

All placebo-treated animals developed the typical clinical signs of EAE and were euthanized between days 12 and 27 (Fig. 3A, Table I), which is in line with previously reported data in this model (14, 22). None of the FR104-treated animals displayed any signs of EAE, not even after the last placebo animal had been euthanized; resulting in significantly prolonged survival (p = 0.0005) (see Fig. 3A). It was decided to euthanize the FR104-treated animals at around day 42, which was 3 wk after the last FR104 injection. Serum samples collected after the last FR104 dosing contained gradually declining levels of FR104, but these were still between 16 and 82 μg/ml on the day of sacrifice (see below).

Immune responses to rhMOG

Data obtained in an equivalent nonhuman primate EAE model, the rhMOG-induced model in marmosets, show that the core pathogenic T cells are derived from effector memory cells present in the natural repertoire (32). Thus, we tested cellular and humoral immune responses against rhMOG to determine whether blockade of T cell costimulation via CD28 with FR104 might affect the generation of pathogenic cells.

Mononuclear cells. We monitored anti-rhMOG proliferative responses of PBMCs longitudinally at a weekly basis (data not shown) and at necropsy, together with responses by MNC isolated from spleen and axillar lymph nodes. MNC proliferation against rhMOG was observed in ALN (Fig. 3B) in five of six vehicle control–treated animals. Only in monkey P5 we could not detect a positive anti-rhMOG response. Positive responses in PBMC collected longitudinally (data not shown) and at necropsy (Fig. 3B) and in splenic MNC (Fig. 3B) were less frequently observed. In FR104-treated animals MNC responses against rhMOG were observed in both ALN (Fig. 3B) and splenic MNC (data not shown).
rhMOG were below the threshold value (SI > 3) in all analyzed compartments and all time points, except for monkey F3, which had some low positive rhMOG responses in PBMC from day 21 onward (data not shown) and in PBMC, spleen, and ALN at necropsy (Fig. 3B). In the ALN the stimulation indices were significantly (p = 0.0108) lower in the FR104 group (2.42 ± 0.595) as compared with the placebo group (10.4 ± 2.32). Abs. High serum levels of anti-rhMOG IgM and IgG were detected in all placebo animals (Fig. 3C, 3D). Already on day 11 the IgM and IgG serum levels in FR104-treated monkeys were significantly (both p = 0.0022) lower (126.1 ± 48.85 and 14.02 ± 5.799 AU, respectively) than in the placebo group (1814 ± 396.9 and 1773 ± 352.1 AU, respectively). Despite a moderate increase, suppression of IgM and IgG Ab production by FR104 persisted throughout the whole observation period.

**Effects on cerebral inflammation and demyelination**

Formalin-fixed hemispheres were processed for histological examination. Three tissue blocks were excised from corresponding regions of each hemisphere. Representative sections were prepared

![Figure 3](http://www.jimmunol.org/)
FIGURE 4. Effects of FR104 on cerebral inflammation and demyelination. Brain sections from placebo and FR104-treated animals were stained for myelin (left panels, LFB/PAS), T cells (middle panels, CD3 staining), and recently immigrated macrophages (right panels, MRP14). From both groups, two animals are presented, one with the least and one with the most severe pathology. (A–C) White matter area of animal P1 (least). (Figure legend continues)
and examined for the presence and intensity of demyelination (Fig. 4, LFB/PAS panels) and inflammation.

In the placebo group, all animals showed inflammation in all areas investigated. Inflammation ranged from moderate numbers of perivascular infiltrates to many large perivascular infiltrates with additional infiltration deep in the parenchyma. These lesions consisted mainly of T cells and infiltrating macrophages (Fig. 4, CD3 and MRP14 panels). In the FR104 group, five of six animals did not reveal inflammatory infiltrates. The other animal of this group only showed some small perivascular infiltrates.

FIGURE 5. Treatment with FR104 modulates the expression in the brain of mRNA for inflammation markers, as analyzed with quantitative PCR. Expression levels of mRNA was normalized against the household gene ABL. Relative concentrations of mRNA levels of investigated genes that were below the detection limit (IL-6: animals P6 and F2; CCR7: animal F4) were set to 0.

LFB/PAS staining shows some perivascular demyelination but no confluent plaque. Staining for CD3 and MRP14 show moderate numbers of T cells and macrophages. Rectangles indicate areas enlarged in the insert. (D–F) White matter area of animal P5 (most severely affected). The depicted section contains large demyelinating (necrotic) lesions. Stainings for CD3 show moderate numbers of T cells and large numbers of MRP14+ myeloid cells representing macrophages and granulocytes that are abundant in the lesions. The inset shows an area (blood vessel) at the border of the lesion with MRP14+ macrophages. (G–I) White matter of animal F3 (least affected). This animal showed no demyelination and no infiltration of T cells and macrophages. The inserts shows a small area next to a blood vessel without any infiltrating cells. (J–L) White matter of animal F1 (most severely affected). This animal showed some infiltration of T cells but absence of demyelination and infiltrating macrophages. The inserts show some infiltrating T cells but the absence of infiltrating macrophages. Scale bar, 500 μm [in (A), (D), (G), and (J)]. Arbitrary scores for infiltration (M) and percentage demyelination of the total gray and white matter areas (N) per group. Only infiltration is significantly less in the FR104 group (p = 0.0022) whereas demyelination is not (p = 0.0606). Arrows marked with an asterisk (A and D) indicate demyelinated areas. Areas marked with GM (A, B, G, and H) indicated gray matter areas in the white matter of the lower part of the corpus callosum. In rhesus monkeys, unlike in rodents, gray matter areas are embedded within the white matter tracts of the tempolateral corpus callosum.
The severity of inflammation was significantly less in FR104-treated monkeys than in the placebo-treated group ($p = 0.0022$; Fig. 5M). Demyelination was observed in four of six animals from the placebo group. Demyelination in this placebo group ranged from small to moderate demyelinating rims around perivascular infiltrates (three animals) to the presence of large demyelinating (necrotic) plaques as seen in one animal. These large demyelinating/necrotic plaques besides many macrophages also contained large numbers of polymorphonuclear granulocytes. No demyelination was observed in any of the FR104-treated animals, but because of the absence of demyelination in the analyzed sections of the placebo animals P2 and P6, this is only trending toward significance ($p = 0.0606$; Fig. 4N).

Only small-sized perivascular cuffs of inflammatory cells were detected in the spinal cords of only two animals (P2 and P2), and in

**FIGURE 6.** Parallel analysis of CSF and serum collected at necropsy. (A) Absolute leukocyte counts in the CSF. In the same CSF samples, anti-rhMOG IgM (B) and IgG (C) levels were recorded. All parameters illustrate the profound suppressive effect of FR104. (D) Cytokine and chemokine levels were measured in the serum (CSF see Table II). Serum of placebo animals contained significantly less IL-1β, CCL2, CCL11, and CCL22 as compared with FR104-treated animals. For CCL2, this difference is due to a significant decrease in necropsy sera in placebo animals. For CCL11, this is due to a significant increase in FR104-treated animals.
none of the animals spinal cord demyelination was found (data not shown).

**Quantitative PCR of brain specimens**

The corpus callosum was sampled for RT-PCR analysis as in the rhesus monkey EAE model lesions are commonly detected in this region. Expression levels of mRNA for CD3 (T cells), CD19 (B cells), CD63 (neutrophils) and a series of T cell homing and activation markers (CD28, CTLA4, CD25, and CCR7) and cytokines (IL-6, IL-8, and IFN-γ) were assessed and expressed relative to the expression of ABL mRNA for all animals (Fig. 5). Expression levels relative to GAPDH gave similar results (data not shown). Similar expression levels of CD3 and CD19 mRNA were detected in both groups, but, consistent with histological data, the relative expression of CD63 as a marker for neutrophils was significantly higher in placebo-treated animals. Expression levels of mRNA for a number of

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<th>Table II. Luminex analysis of cytokines and chemokines in necropsy CSF samples</th>
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<td>Placebo Group Median (Min – Max)</td>
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<td>Median placebo – median FR104 ≥ 25 pg/ml; p ≤ 0.05</td>
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<td>IL-12</td>
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<tr>
<td>IL-15</td>
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<tr>
<td>IFN-γ</td>
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<tr>
<td>IL-1RA</td>
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<tr>
<td>G-CSF</td>
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<tr>
<td>MIF</td>
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<tr>
<td>CCL2 (MCP-1)</td>
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<tr>
<td>CXCL10 (IP-10)</td>
</tr>
<tr>
<td>CXCL11 (I-TAC)</td>
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<tr>
<td>Median placebo – median FR104 &lt; 25 pg/ml; p ≤ 0.05</td>
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<tr>
<td>IL-1β</td>
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<tr>
<td>IL-4</td>
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<tr>
<td>CD19 (MIP-1α)</td>
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<tr>
<td>CCL3 (MIP-1b)</td>
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<tr>
<td>CCL4 (MIP-1b)</td>
</tr>
<tr>
<td>CCL5 (RANTES)</td>
</tr>
<tr>
<td>CCL11 (Eotaxin)</td>
</tr>
<tr>
<td>CCL22 (MDC)</td>
</tr>
<tr>
<td>CXCL9 (MIG)</td>
</tr>
<tr>
<td>Not significantly different (p &gt; 0.05)</td>
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<tr>
<td>IL-17</td>
</tr>
<tr>
<td>TNF-α</td>
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<tr>
<td>EGF</td>
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<tr>
<td>HGF</td>
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<tr>
<td>CCL3 (MIP-1a)</td>
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<td>CCL4 (MIP-1β)</td>
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<td>CCL22 (MDC)</td>
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<tr>
<td>CXCL9 (MIG)</td>
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*Above detection range.

Viral copy number values are reported as copies per microgram DNA (± SD) in each column. Assays were carried out in triplicate over a minimum of three experiments. BLD, below limit of detection or limit of quantification of the assay.
FR104 mitigates the inflammatory profile in the CSF

CSF samples from rhesus monkeys developing EAE contain elevated leukocyte numbers (15). The absence of leukocytes in the CSF of animals treated with FR104 supports the conclusion that neuroinflammation was suppressed in these animals (Fig. 6A).

After centrifugation for removal of cells, CSF samples were analyzed for the presence of anti-rhMOG IgM and IgG Abs. These were clearly detectable in the placebo-treated monkeys, but they were significantly reduced in monkeys treated with FR104 (Fig. 6B, 6C). IgG Abs were found in all placebo animals (range, 31.803–249.0 AU), whereas these were absent in normal CSF (data not shown). IgG Abs were undetectable in the FR104-treated animals (range, 0.000–2.100 AU; p = 0.0022). IgM titres are, because of some background staining, not always negative in normal CSF, but elevated levels were found in some but not all placebo animals (range, 8.500–310.9 AU). In the FR104 group, IgM levels were significantly (p = 0.0238) reduced (range, 0.000–20.90 AU).

The CSF was also analyzed by Luminex multiplex technology for the presence of cytokines and chemokines. An inflammatory profile was evident in all placebo animals. Of the 29 tested cytokines and chemokines, 19 were significantly elevated in placebo-treated animals compared with monkeys treated with FR104 (Table II). Of these 19 significantly elevated cytokines/chemokines, some had only a low difference between medians and the biological relevance of these significant, but low-level differences is questionable. Using an arbitrarily chosen cutoff difference between medians of ≥25 pg/ml, we found higher levels in placebo-treated than FR104-treated monkeys for 11 cytokines and chemokines: IL-2, IL-6, IL-12, IL-15, IFN-γ, IL-1R antagonist (IL-1RA), G-CSF, MIF, CCL2, CXCL10, and CXCL11. Interestingly, IL-17 was detected in only two of the six placebo-treated animals, and TNF-α was not detected in any of them nor were IL-17 and TNF-α detected in any of the FR104-treated animals (Table II). CSF samples taken prior to EAE induction were not available from the monkeys in the current study. However, in four pre-EAE induction CSF samples available from a previously reported study (15), the two most prominently expressed cytokines/chemokines IL-6 and CCL2 could not be detected (data not shown), further indicating that the high levels of cytokines and chemokines found in the CSF of the animals with EAE, were not already present before EAE induction, but are truly a result of the inflammation in the brain.

FR104 affects cytokine and chemokine levels in the blood

Cytokines and chemokines were also determined in sera collected prior to EAE induction and at necropsy. This enabled us to calculate serum/CSF ratios. From the 11 cytokines and chemokines in the CSF that were higher (difference between medians of ≥25 pg/ml) in placebo animals, IL-2, IL-6, IL-12, IL-15, CCL2, and CXCL10 were significantly higher in the CSF than in the serum (not shown), indicating that these factors might be locally produced in the brain and had not leaked into the CSF from the blood. IL-10, FGF-basic and VEGF were also significantly higher in the CSF, as compared with the serum.

A comparison of the cytokine and chemokine levels in sera collected at necropsy from all animals, revealed significant differences between the two groups for IL-1β, CCL2, CCL11, and CCL22 (Fig. 6D). Interestingly, the levels of all these cytokines were lower in the placebo animals as compared with the FR104-treated animals. Comparison with pre-EAE induction sera indicated that for CCL2 this was due to decreased levels in the necropsy sera of EAE animals compared with preinduction levels. For CCL11, this was due to an increase in necropsy sera of FR104-treated animals as compared with pre-EAE induction sera.

FR104 and reactivation of existing endogenous viral infections in treated animals

A potential adverse effect of strong immunosuppression is the recrudescence of latent virus infection. To test whether the treatment with FR104 impairs the capacity of the animals to control chronic latent virus infection, we examined by quantitative PCR blood, spleen, and brain for expression of a number of viruses relevant to human infections/complications to see whether reactivation was induced by the FR104 treatment. CMV, LCV, SA12, SV40, and HEV were all assayed in blood at day −7 and at days 7 and 14 and up to day 21 post-EAE induction and at euthanasia as well as in spleen and brain tissue sampled at euthanasia.

The polyomavirus SA12 was detected in the blood of F5 only at euthanasia, albeit at very low levels (871 ± 302 mean copies/μg DNA). SA12 was also detected in the spleen tissue at necropsy but not in brain (Table III). HEV was not detected in blood or feces in any of the animals in either group (data not shown).

CMV virus levels in the blood were at the limit of detection and therefore considered insignificant in both the treated and control groups. However, virus was detected in the spleens of animals in both groups confirming presence of the virus and that lack of reactivation was not due to the lack of prior exposure (Table III). No CMV DNA was detected in the brain tissue.
DNA from the polyomavirus SV40 was detectable in brain tissue from three of six placebo-treated animals (P3, P4, and P5) and three of six FR104-treated animals (F2, F4, and F5) but the mean copy number between the groups was found not to be significant (Table III).

The gammaherpesvirus macaque LCV, the rhesus monkey representative of EBV, was detected in the blood of animals F4 and P3 at day −7 (Fig. 7). In the placebo-treated group, low levels of viral DNA were detected continuously, until euthanasia (Fig. 7A). Virus levels for the FR104-treated group appeared to increase after day 7 peaking at day 14 then decreasing at day 21. In animals F1, F2, F3, and F4, levels increased again after the fourth injection of FR104 at day 21 post-EAE induction. Levels of virus remained low in animals F5 and F6. LCV was detectable in the spleen also (Table III). Interestingly, animal F3 had elevated levels in the tissues for CMV, LCV, and SA12.

Discussion
Despite demonstrated effects in RA and promising results in MS, abatacept has the theoretical disadvantage to block both interactions of CD80/CD86 with CD28 and with CTLA4, thereby potentially abrogating downmodulatory effects of CTLA-4. In contrast, CD28 blockade by specific mAbs will abrogate immune activation by interfering with the interaction of CD80/CD86 with CD28 but will not affect the interaction of CD80/CD86 with CTLA4. However, anti-CD28 mAbs have been notorious for their potential (super)agonistic effects on T cells (for review, see Ref. 9). Modified versions without cross-linking activity may be useful antagonists of T cell–APC interaction without T cell stimulation (9). In this paper, we report the results of a preclinical efficacy study of FR104, a novel monovalent, PEGylated mAb against human CD28, which presents no agonistic and superagonistic properties in vitro (12) and in vivo (11).

PEGylated FR104 did not bind marmoset CD28 but bound rhesus monkey CD28 with equivalent affinity as human CD28 (data not shown). We report in this paper that FR104 did not affect the function of rhesus monkey naturally occurring Tregs (Fig. 1A) and that it inhibited a rhMOG-induced ex vivo recall response (Fig. 1B). These data formed a solid basis for subsequent efficacy testing of FR104 in vivo in the rhesus monkey EAE model. This model recapitulates aspects of the human neuroinflammatory disease MS but is also used as a generic autoimmune disease model. This implies that promising results obtained in the EAE model may have broader relevance than only for MS.

The validity of CD28 signaling as target of immunotherapy in the EAE model has been firmly established in the mouse EAE model because CD28 knockout mice are either resistant to EAE induction or the disease is less severe (4–6, 33). Moreover, blocking of CD28 signaling by antagonists of CD80/CD86 (7, 33) or CD28 directly (8) prevented EAE induction. Our studies in the nonhuman primate EAE model revealed an essential difference between the EAE model in inbred/SPF laboratory mice and outbred nonspecific pathogen-resistant primates, namely that the encephalitogenic T cells in the latter in inbred/SPF laboratory mice and outbred nonspecific pathogen-resistant primates, namely that the encephalitogenic T cells in the latter in the former model (14, 15) and in the rhesus monkey (16). The cause of this discrepancy is not known but could be due to the sampling procedure because tissue for mRNA isolation was collected from the right hemisphere, or it could be due to the absence of demyelination in the case of rebound disease.

It was demonstrated in rodents that CD28 plays a role in the induction of EAE but that primed cells can promote EAE in the absence of CD28-mediated signaling (5). Therefore, treatment with FR104 was initiated on day 0 to avoid that treatment was initiated at the stage where the pathogenic process could not be reverted by FR104 and thereby missing a possible effect of FR104. None of the FR104-treated animals developed EAE, leading to significantly prolonged disease-free survival in this group (p = 0.0005; Fig. 3A). Treatment with FR104 was stopped after the last animal in the placebo group had been euthanized. We chose to euthanize FR104-treated animals before FR104 levels had waned completely (Fig. 2A) because we wanted to examine the effect of FR104 treatment on the absence of CNS pathology, which may have disappeared in the case of rebound disease.

FR104 treatment suppressed peripheral T cell activation against rhMOG in vivo (Fig. 3B). Although anti-rhMOG IgG serum levels were detectable, these were substantially lower than those observed in placebo animals. Anti-rhMOG IgM Abs were not detected at all. Furthermore, levels of IgM-ADA were negative, and IgG-ADA were very low in five of six animals. Only monkey F3 had high IgG-ADA. These combined observations hint at a possible inhibitory effect of FR104 on CD28-mediated T cell help for Ig isotype switching, as observed in mouse models (38, 39) and as class-switching was also affected by abatacept (40).

In comparison with our previous study (15), we found less demyelination. The reason for this is not an on average lower percentage demyelinated area but merely results from inclusion of both white and gray matter in this study as compared with focusing on white matter only in our previous study. Probably because of the absence of demyelination in two of the placebo-treated animals, the difference between the groups for demyelination shows only a trend toward significance (p = 0.0606).

Interestingly, immunohistochemistry analysis revealed presence of CD3 + cell infiltration in all placebo animals but only in one monkey of the FR104-treated group (F1, Fig. 4K, 4M). However, CD3 mRNA expression in the brain was found in all FR104 animals, and the relative levels did not differ between the groups (Fig. 5, upper left panel). The cause of this discrepancy is not known but could be due to the sampling procedure because tissue for mRNA isolation was collected from the right hemisphere and tissue for histopathology from the left hemisphere, or it could be due to regulation on the translational level. However, more consistent with the clinical data, we observed that mRNA levels for markers for T cells (CD28) and of T cell activation (CTLA4 and CD25) for the T cell secondary lymphoid organ homing marker CCR7 and the Th1 cytokine IFN-γ were significantly lower in FR104-treated animals as compared with the placebo group (Fig. 5).

Expression of CD19 mRNA was, like CD3 mRNA, also not different between the groups. B cell numbers were not quantified by histopathology because these were previously not found in lesions of untreated controls (15), which seems in contrast with the presence of CD19 mRNA in the brains of placebo and FR104 animals. The strikingly elevated expression of mRNA for CD6,
a marker of neutrophils, and of mRNA for CXCL8 (IL-8), a neutrophil chemokine, is consistent with the abundant presence of this cell type in brain lesions in the rhesus monkey rhMOG/CFA EAE model. Because FR104 prevented inflammation in the brain, mRNA for these markers remained significantly lower in the treated animals as compared with placebo-treated animals. Interestingly, although IL-6 mRNA levels were significantly higher in the placebo-treated group, the levels were very low, to even being undetectable in animals P6 and F2. However, this was the cytokine most abundantly present in the CSF.

The mRNA levels of indicated immune markers were also assayed in the lymphoid compartment (i.e., spleen, ALN, and blood). The significantly higher CD63 mRNA level in blood of placebo animals is consistent with the observed elevated blood neutrophil counts at necropsy in animals with EAE in this study (data not shown) and unpublished results from other rhesus monkey EAE studies by our group (15, 22) as well as published results in the myelin/CFA EAE model in rhesus monkeys (41). CD63 mRNA was also elevated in ALN, although only trending toward significance, but not in the spleen. Neutrophils play a role in certain rodent EAE models as well (42–44) but their role in MS has not yet been thoroughly investigated. Elevated MNC numbers were detected in the CSF of placebo-treated animals. This is reminiscent of observations in patients with acute disseminated encephalomyelitis (ADEM) (45, 46). Analysis of the cytokine and chemokine levels in CSF revealed very high amounts of IL-6, IL-1RA, CCL2, and CXCL10 of the placebo-treated animals, whereas levels were very low or absent in the CSF of the FR104-treated animals. The presence of these cytokines and chemokines has been observed in MS patients, as well as in patients with ADEM (47, 48).

Biologics have been documented to activate viruses of concern (17–19), and viral reactivation after immunosuppression has also been documented in nonhuman primates (49–51). To determine whether FR104 led to reactivation of latent viral pathogens, animals were assayed for the presence of herpesviruses, polyomaviruses, and HEV. Overall, no significant reactivation was observed for rhesus CMV or the polyomaviruses, although the viruses were detected in the spleen. HEV was undetectable. However, some evidence of LCV reactivation was seen in animals of both groups with a higher viremia observed in those treated with FR104. EBV, the human representative within the genus LCV, is a known predictor of malignancy development (52) and was shown to be associated with acute encephalitis during treatment with abatacept (19). In this study, even though viral levels increased in the blood, there was no evidence of virus in the brain tissue, and high levels of LCV in spleen tissue were only observed in one animal. Given the short duration of these experiments and because the levels in blood were extremely variable between animals, it is not possible to convincingly demonstrate whether LCV is indeed truly reactivated in this model and poses a risk for use of FR104.

In conclusion, we report that i.v. administration of FR104 is well tolerated and shows promising clinical effects in the rhMOG-induced rhesus monkey EAE model. The results confirm the central pathogenic role of T cells in the model. Moreover, the preclinical data are promising in view of a future therapy for patients with autoimmune neuroinflammatory disease. Regarding the clinical perspectives, these observations are encouraging for future treatment of ADEM patients and possibly also for MS, although the effect on on-going chronic inflammation needs to be established. However, the observation that FR104 has a similar suppressive effect as CTLA4-Ig on a recall immune response against rhMOG in vitro (Fig. 1B) offers an encouraging perspective for further exploring the immunosuppressive properties of FR104 in MS patients. A point of caution is the possible exacerbation of macaque LCV, which indicates that it may be wise to monitor patients treated with FR104 for recrudescence of latent EBV.

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Disclosures
C.M., N.P., and B.V. are current employees of and own stock or equity from EffImmune.

References


Corrections


The eighth author’s name was omitted from the article. The corrected author and affiliation lines are shown below.

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