The Journal of Immunology

First-in-Human Study in Healthy Subjects with FR104, a Pegylated Monoclonal Antibody Fragment Antagonist of CD28

Nicolas Poirier, Gilles Blancho, Maryvonne Hiance, Caroline Mary, Tim Van Assche, Jos Lempoels, Steven Ramael, Weirong Wang, Virginie Thepenier, Cecile Braudeau, Nina Salabert, Regis Josien, Ian Anderson, Ian Gourley, Jean-Paul Soulillou, Didier Coquoz and Bernard Vanhove

J Immunol published online 14 November 2016
http://www.jimmunol.org/content/early/2016/11/12/jimmunol.1601538

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Published November 14, 2016, doi:10.4049/jimmunol.1601538

First-in-Human Study in Healthy Subjects with FR104, a Pegylated Monoclonal Antibody Fragment Antagonist of CD28

Nicolas Poirier,*,†,‡ Gilles Blancho,*,†,§ Maryvonne Hiance,‡ Caroline Mary,*,†,‡ Tim Van Assche,¶ Jos Lempoels,¶ Steven Ramael,¶ Weirong Wang,‖ Virginie Thepenier,*,†,‡ Cecile Braudeau,*,†,# Nina Salabert,*,†,§,# Regis Josien,*,†,§,# Ian Anderson,‖ Ian Gourley,‖ Jean-Paul Soulillou,*,† Didier Coquoz, and Bernard Vanhove*,†,‡,#**

FR104 is a monovalent pegylated Fab′ Ab, antagonist of CD28, under development for treatment of transplant rejection and autoimmune diseases. In contrast to CD80/86 antagonists (CTLA4-Ig), FR104 selectively blunts CD28 costimulation while sparing CTLA-4 and PD-L1 coinhibitory signals. In the present work, FR104 has been evaluated in a first-in-human study to evaluate the safety, pharmacokinetics, pharmacodynamics, and potency of i.v. administrations in healthy subjects. Sixty-four subjects were randomly assigned to four single ascending dose groups, two double dose groups and four single ascending dose groups challenged with keyhole limpet hemocyanin. Subjects were followed up over a maximum of 113 d. Overall, the pharmacokinetics of FR104 after a single and double infusions was approximately linear at doses ≥0.200 mg/kg. CD28 receptor occupancy by FR104 was saturated at the first sampling time point (0.5 h) at doses above 0.02 mg/kg and returned to 50% in a dose-dependent manner, by day 15 (0.020 mg/kg) to 85 (1.500 mg/kg). FR104 was well tolerated, with no evidence of cytokine-release syndrome and no impact on blood lymphocyte subsets. Inhibition of anti-keyhole limpet hemocyanin Ab response was dose-dependent in FR104 recipients and was already apparent at a dose of 0.02 mg/kg. Abs to FR104 were detected in 22/46 (48%) of FR104 recipients and only 1/46 (2.2%) was detected during drug exposure. In conclusion, selective blockade of CD28 with FR104 was safe and well tolerated at the doses tested. The observed immunosuppressive activity indicated that FR104 has potential to show clinical activity in the treatment of immune-mediated diseases. ***The Journal of Immunology*****, 2016, 197: 000–000.**

The centrality of the CD28:CD80/CD86 (CD28:B7) costimulatory pathway in T cell function makes it an attractive therapeutic target for modulating pathogenic and protective T cell-mediated immune responses. The blockade of this pathway with CTLA4-Ig drugs (Orencia and Nulojix) has proven efficacy in ameliorating symptoms in rheumatoid arthritis (1), juvenile idiopathic arthritis (2, 3), and in the prevention of kidney transplant rejection (4). However, experimental and emerging clinical data in transplant models support the theoretical concern that, because they are directed at B7, CTLA4-Ig drugs interfere with CTLA-4:B7-mediated signals that are important immune checkpoints (5–9) and are crucial to the development and function of regulatory T cells (Tregs) (10–12). Inhibition of anti-inflammatory and tolerogenic CTLA-4–driven pathways in T cells may explain why targeting B7 molecules has failed to lead to uniform transplant tolerance in rigorous primate models, in man (13, 14), and in selected rodent models (15, 16).

As predicted, based on the opposing effects of engagement of CD28 and CTLA-4 by B7 family ligands on adaptive immunity, we and others have shown that blocking the CD28:CD80/CD86 (CD28:B7) costimulatory pathway by selectively targeting CD28 instead of B7 is highly effective to modulate pathogenic T cell responses (17). Non-activating antagonist monovalent Ab fragments against CD28 prevented allograft rejection in mice (18) as well as in non-human primates with a higher efficacy than with CTLA4-Ig reagents (5, 19–21). Besides transplantation, preclinical proofs of concept have also been obtained in non-human primate models of multiple sclerosis (22), rheumatoid arthritis (23), and psoriasis (24). Mechanistically, selective CD28 antagonists have demonstrated their potential to induce long-term Ag-specific unresponsiveness in a cutaneous delayed type hypersensitivity model (25) and after kidney transplantation in non-human primates (19). In this latter case, hypo-responsiveness was associated with an increased infiltration of the kidney graft by Tregs.

*Institut National de la Santé et de la Recherche Médicale UMR 1064, Nantes F44093, France; †Institut de Transplantation Urologie Néphrologie, Centre Hospitalier Universitaire, Université de Nantes, Nantes F44000, France; ‡OSE Immunotherapeutics S.A., Nantes F44200, France; §Centre Hospitalier Universitaire Nantes, Laboratoire d'Immunologie, Centre d'immunomonitorage Nantes-Atlantique, Nantes, F44000, France; ¶SGS Life Science Services, Clinical Pharmacology Unit Antwerp, Antwerp 2060, Belgium; ‖Janssen Research & Development, LLC, Spring House, PA 19477; #LabEx ImmunoGraft Oncology, Nantes F44000, Nantes, France; and **Copexis S.A., Pully CH-1009, Switzerland

ORCIDs: 0000-0002-8958-1117 (T.V.A.); 0000-0001-7900-7413 (R.J.); 0000-0002-8676-5690 (I.G.); 0000-0001-8270-3831 (D.C.).

Received for publication September 7, 2016. Accepted for publication October 13, 2016.

The authors received funding for some analytical developments from the European project TRIAD (EU-FP7-Health program EC-GA No281493).

N.P., W.W., B.V., R.J., C.B., N.S., G.B., and J.-P.S. analyzed data. C.M., T.V.A., J.L., V.T., and S.R. conducted the clinical trial. D.C., M.H., B.V., I.A., and I.G. established the protocol. B.V., D.C., and N.P. wrote the manuscript.

Address correspondence and reprint requests to Dr. Bernard Vanhove, OSE Immunotherapeutics S.A., 22 Bd Benoni Goullin, 44200 Nantes, France. E-mail: bernard.vanhove@ose-immuno.com

The online version of this article contains supplemental material.

Abbreviations used in this article: ADA, anti-drug Ab; AE, adverse event; CV, coefficient of variation; KLH, keyhole limpet hemocyanin; MAD, multiple ascending dose; PD, pharmcodynamic; PK, pharmacokinetic; QTcF, Friderica's corrected QT; RO, receptor occupancy; SAD, single ascending dose; SEB, *Staphylococcus aureus* enterotoxin-B; TEAE, treatment-emergent adverse event; Treg, regulatory T cell.

The development of compounds inhibiting the CD28:B7 costimulatory pathway by targeting CD28 has been challenging. After the dramatic administration to human volunteers of the TGN1412 superagonist anti-CD28 mAb (26), it became clear that human T cells are more reactive to CD28 stimulation than their murine or monkey counterparts (27), and that any new drug candidate targeted at T cells must present an absolute antagonist activity. The reason TGN1412 induced polyclonal T cell signaling has been mainly assigned to the target epitope lying in the C''D domain of CD28, which after cross-linking with an Ab results in a receptor clusterization associated with a non-physiological T cell activation (28). However, even though anti-CD28 Abs recognizing epitopes lying outside the C''D domain cannot be superagonist, they still deliver residual agonist signals related to receptor cross-linking, leading to the release of inflammatory cytokines (29). We previously demonstrated that the key property of an anti-CD28 mAb in being agonist or antagonist is related to the valency, rather than to the potential cross-linking of this Ab by FcR-expressing accessory cells through its Ig Fc domain (30). Therefore, we developed FR104, a monovalent Ab fragment originating from the CD28.3 mAb (31), pegylated to improve its pharmacokinetic properties (20).

The FR104 binding epitope on CD28 is composed of a conformational epitope formed by the CDEF loops of CD28 (19). FR104 was shown devoid of human T cell agonist properties in vitro, even in the presence of anti-CD3 Abs or when cross-linked with secondary Abs. It was also evaluated *trans*-vivo in humanized mice (20), as well as in baboons, a species where memory CD4+ T cells express similar levels of CD28 to humans (27). No evidence of T cell agonism was found in these preclinical models.

Here, we reported on the first-in-human administration in healthy volunteers to explore the safety and tolerability of single and multiple ascending i.v. doses of FR104 to characterize the pharmacokinetics and pharmacodynamics aspects, immunological changes and to gain access to preliminary efficacy in controlling an Ab response to a keyhole limpet hemocyanin (KLH) Ag challenge.

Materials and Methods

Study design

This study is a first-in-human, phase I, randomized, double-blind, placebo-controlled, single center study evaluating single and multiple ascending i.v. doses of FR104 in healthy subjects. It was approved by the Ziekenhuisnetwerk Antwerpen Independent Ethics Committee (Protocol Number FR104-CT01; EudraCT Number 2015-000302-19; Clinical-Trials.gov identifier: NCT02800811). The study was conducted at SGS Life Science Services, Clinical Pharmacology Unit Antwerp, Antwerp, Belgium, in compliance with the Good Clinical Practice guidelines and the principles of the Declaration of Helsinki.

Participants

Eligible male and female subjects gave written informed consent. Key inclusion criteria were general good health, 18–60 y of age, weight of ≥50 kg and no more than 100 kg with a BMI of 18–30 kg/m^2. Exclusion criteria were any significant past medical history or abnormal laboratory tests.

Interventions

The test drug was FR104 (supplied by Effimune, now OSE Immunotherapeutics SA., Nantes, France), whereas the comparator drug (matching placebo) was Ringer's lactate solution. FR104 was supplied as 5 ml solution containing FR104 100 mg (20 mg/ml) and stored at 2–8°C (36–46°F) protected from light. Syringes with the appropriate dilution or placebo were prepared by SGS Pharmacy and provided to the investigator in a double-blind manner.

The doses were initially selected based on population pharmacokinetic-pharmcodynamic (PK-PD) modeling of non-human primate data (with allometric adaptation) and human data were later included after the interim analysis. The initial dose (minimal anticipated biological effect level, 0.005 mg/kg) corresponded to a modeled CD28 receptor occupancy (RO) at Cmax of ∼20%.

For dose levels ≤0.05 mg/kg, FR104 was administered by i.v. infusion of 10 ml for 30 min, after dilution to the correct concentration in Ringer's lactate solution. For other dose levels, FR104 was administered by i.v. infusion of 100 ml for 30 min, after dilution to the correct concentration in Ringer's lactate solution.

Treatment regimens

In Part 1, 50 subjects were selected [single ascending dose (SAD): two cohorts of 22 (Cohort A) and 28 (Cohort B) subjects] and 14 subjects in Part 2 [multiple ascending dose (MAD); Table I]. During each treatment period, subjects were housed at the study center from the day before dosing (day -1) until day 5. A staggered dose approach was applied, i.e., one or two subjects were dosed (day 1) followed by one or two subjects 48 h later (day 3), and the remaining subjects of the dose-level group were dosed 48 h later. The total duration of the study was 36 wk, including an 11 wk follow-up after the last repeat dose. An interval of at least 14 d (last to first administration) was applied between all dose levels. Individual subjects on a same day of dosing were dosed at least 60 min apart.

Part 1: single ascending dose. Cohort A enrolled 22 subjects in one of the four dose-level groups: 0.005 mg/kg in group 1, 0.050 mg/kg in group 2, 0.200 mg/kg in group 3, and 0.500 mg/kg in group 4. In each of groups 1 and 2, four subjects were randomized to either FR104 or placebo in a 3:1 ratio so that three subjects received FR104 and one received placebo. In each of group 3 and 4, seven subjects were randomized to either FR104 or placebo in a 5:2 ratio so that five received FR104 and two received placebo. The randomization ensured that in the first group of two subjects, one received FR104 and one received placebo. A predefined hold of ∼12 wk was made after dosing in group 4, for interim evaluation of PK, PD and safety, on which decisions for the next dose levels were based. A total of 28 subjects were enrolled in one of the four dose-level groups (seven in each group) in Cohort B: 0.500 mg/kg in group 7, 0.200 mg/kg in group 8, 1.5 mg/kg in group 9, and 0.020 mg/kg in group 9bis. The subjects were randomized to either FR104 or placebo in a 5:2 ratio. In addition to the common assessments, subjects in this cohort received a KLH challenge on the day of FR104 injection.

Part 2: multiple ascending dose. In Part 2, 14 subjects were enrolled in one of the two dose level groups (seven in each group): 0.2 mg/kg in group 10 and 0.5 mg/kg in group 11. Each subject received two administrations of FR104 or placebo separated by an interval of 28 d.

Cytokine assessment

Blood samples were collected pre- and postdose at hours 1, 2, 4, 8, 24 (day 2). Samples were also collected on day 15, 43, 57, 85, and 113. Cytokines IFN-γ, ILs 1 β, 2, 4, 6, 8, 10, 12p70 (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, and IL-12p70), and TNF-α were assayed in serum using a validated electrochemiluminescence immunosorbent assay method using the commercial kit Pro Inflammatory Panel I V-Plex assay (# K15049G from Meso Scale Discovery, Rockville, MA). MULTI-SPOT plates were precoated with capture Abs. Samples were loaded on to wells with a solution containing detection Abs conjugated with electro-chemiluminescent labels (MSD SULFO-TAG). Analytes in the sample bound to capture Abs immobilized on the working electrode surface; recruitment of the detection Abs by the bound analyte completed the sandwich. After addition of MSD Reading Buffer, a voltage was applied to the plate electrodes, and a light emission occured if significant SULFO-TAG labeled Abs had bound to the plate. The instrument measured the intensity of emitted light to provide a quantitative measure of the analyte in the sample.

Pharmacokinetic analysis

Serial blood samples for PK assessment were collected at the following time points relative to infusion start time: pre- and postdose at hours 0.5, 0.75, 1, 2, 4, 8, 24 (day 2), 48 (day 3), and day 5, 8, 15, 29, 43, 57, 85, and 113 (>0.5 mg/kg dose groups only). Concentrations of FR104 in serum were determined using a validated method involving quantitative electrochemiluminescence immunosorbent assay on an MSD Sector Imager 6000 (MSD, Gaithersburg, MD). Pharmacokinetic calculations were performed by SGS-LSS using Phoenix WinNonlin 6.2 or higher (Pharsight Corporation, Palo Alto, CA). The lower and upper limits of quantification for the assay were 100 and 2000 ng/ml, respectively. The overall precision and accuracy of the quality controls and standards were ≤20% and within ± 20%, respectively. A standard curve was constructed, enabling sample concentrations to be estimated by interpolation from the fitted curve.

Pharmacodynamic analysis

Blood samples were collected at the same time points as PK analyses for CD28 receptor occupancy (CD28 RO), using a validated method involving

cytofluorometry. Each blood sample to be tested was split in half and one was mixed with an excess of FR104. Upon RBC lysis, FR104 binding to CD28 was investigated by flow cytometry, on lymphocytes stained with a FITC conjugated anti-CD3 Ab, successively using an anti-PEG rabbit mAb and an Alexa fluor 405-conjugated anti rabbit secondary Ab. Thus, RO was calculated by performing the ratio between the mean fluorescence intensity (MFI) of the well without excess of FR104 and the well with an excess of FR104. The sensitivity of the method was 0.25 μg/ml of FR104. Intradonor replicates precision ranged between 1 and 9% (coefficient of variation [CV]) and interdonor precision ranged between 16 and 22% (CV). CD28 expression level was measured by the MFI of FR104 in excess conditions.

Peripheral T lymphocyte subpopulations and activation status were assessed by flow cytometry on stabilized whole blood using TransFix/EDTA vacuum blood collection tubes (Cytomark, Buckingham, U.K.). CD45, CD3, CD28, CD45RO, CD4, CD8, CD25, CD127, CD69, and CCR7 markers were measured to define the following subpopulations: naive T cells, activated T cells, memory T cells, central memory T cells, effector memory T cells, and resting effector memory T cells, in the CD4+ and CD8+ compartments. Natural Tregs were also recorded.

All other laboratory testing, including EBV PCR and EBV IgG and IgM Abs, have been performed by the clinical biology laboratory of the investigational center, using standard procedures.

The potential effect of FR104 on immune responsiveness of blood cells was investigated ex-vivo. Blood samples were collected by venipuncture or via indwelling cannula in the forearm into TruCulture blood collection tubes (Myriad RBM, Austin, TX) containing *Staphylococcus aureus* enterotoxin-B (SEB) + LPS stimuli and into control tubes, for each blood draw. Tubes were maintained at 37°C for 24 h before plasma was mechanically extracted and frozen until analysis of IL-2, IFNg and IL-8 cytokines. Cytokines were analyzed with the same ELISA method used to assess cytokines in the serum.

Immunogenicity assessment

Blood samples for anti-FR104 Ab detection were collected at screening and on day 1 (predose), day 15, 29, 57, 85, and/or 113. The titration of anti-FR104 Abs (anti-drug Ab, or ADA) in serum was performed using a validated electrochemiluminescence, bridging immunogenicity assay. The method used an acidic treatment of the serum samples to allow, when necessary, dissociation of FR104/ADA followed by a single-step assay bridging format whereby ADA are captured in solution by a combination of biotinylated and sulfo-TAG labeled forms of FR104. Complex formation is subsequently detected by ECL on the MSD platform. Using an ADA generated in rabbits as a positive control, the sensitivity of the assay was 3.46 ng/ml (with a CV of 31%) and the mean drug tolerance was 179 μg/ml (with a CV of 26%).

KLH immunization

Blood samples for anti-KLH Ab detection were collected at screening and on day 15, 29, 57, 85 and/or 113. Anti-KLH IgG Abs were measured by a qualified ELISA in serum using commercial kit Human Anti-KLH IgG (# 700-140-KLG from α Diagnostic International, San Antonio, TX).

Statistical methods

The sample size was determined for this study based on a precedent set by other phase 1 studies similar in design and after consultation with the Medicine and Health Care Regulatory Agency (U.K.), the Paul Ehrlich Institute (Germany), the Federal Agency for Medicines and Health Products (Belgium) and the European Medicines Agency. A sample size of 64 healthy volunteers was deemed sufficient to meet the objectives of the protocol. All statistical calculations were performed using the SAS (version 9.2) software for statistical computations, and SAS for graphical purposes.

Results

Participant flow

The study was conducted at one clinical center from March 27, 2015, to February 19, 2016. A total number of 65 subjects was divided over two study parts. In Part 1, 37 subjects were administered a single i.v. dose of FR104 (ranging from 0.005 to 1.5 mg/kg) and 14 subjects were administered a single i.v. dose of placebo. In Part 2, 10 subjects were administered two i.v. doses of FR104 (0.2 or 0.5 mg/kg) and four subjects were administered two i.v. doses of placebo, separated by an interval of 28 d. All but one subject (in Part 1, Cohort A, group 2) received study drug as planned (see above). All subjects were randomized and treated. They all completed the study and were included in the safety analysis. One subject (Part 1, Cohort A, group 2) was excluded from the PK and PD analyses due to a protocol deviation related to a dysfunction of the infusion pump. Due to this incorrect dose administration, one additional subject was included in group 2. One subject in Part 2 did not receive the second dose of FR104 at 0.500 mg/kg due to receiving prohibited concomitant medications following a treatment-unrelated adverse event (AE). The subject completed all visits as per protocol. They were included in the PK and PD population, but data taken after day 29 were excluded from the analysis. There were no subjects for whom the blinding code was broken by the investigator or the sponsor (Table I).

Baseline data

Subject demographics are shown in Table II. Most subjects in all treatment groups were white. Their age, weight and BMI fell within the inclusion criteria defined in the protocol. The flow of assessments for all subjects at screening and day -1 is shown in Supplemental Table I.

Pharmacokinetics

Fig. 1A illustrates the FR104 concentration-time data for all dose groups. In Cohort A (groups 1–4, no KLH immunization, FR104 0.005 to 0.500 mg/kg doses), the pharmacokinetics of FR104 were approximately linear from the 0.200 mg/kg dose with a $t_{1/2}$ ranging from 146 (0.200 mg/kg) to 182 h (0.500 mg/kg). Lower doses of FR104 at 0.005, 0.020 and 0.050 mg/kg resulted in insufficient measurable concentrations for reliable PK parameter estimation, although the clearance of FR104 was estimated to be higher and the $t_{1/2}$ was estimated to be shorter. In Cohort B (groups 7–9bis, KLH immunization) as in Cohort A, the PK of FR104 were approximately linear from the 0.200 mg/kg dose with a $t_{1/2,}$ ranging from 150 (0.200 mg/kg) to 210 h (1.500 mg/kg). Mean FR104 Cmax ranged from 117 to 37,700 ng/ml following doses 0.004–1.5 mg/kg and area under the curve ad infinite ranged from 0.705 to 7.010 mg/ml following doses 0.2–1.5 mg/kg.

The PK of FR104 were also evaluated after two infusions of FR104 at 0.200 and 0.500 mg/kg given 28 d apart (Part 2). The pharmacokinetics were approximately linear with a $t_{1/2}$ ranging from 169 to 203 h. The PK parameters were similar after FR104 0.200 mg/kg infusion at day 1 and 29. Accumulation of serum concentrations was observed after infusion of FR104 at 0.500 mg/kg, with an increase of ∼20% in dose-normalized area under the curve from T0 to day 28 (AUC0-28d) and area under the curve ad infinite.

Table I. Treatment regimens

	Dose (mg/kg)	FR104 (46 Subjects)	Placebo (18 Subjects)
Part 1 cohort A: single ascending dose			
Group 1	0.005	3	1
Group 2	0.05	3	1
Group 3	0.2	5	2
Group 4	0.5	5	2
Approximately 12 wk hold for PK, PD and safety evaluation			
Part 1 cohort B: single ascending dose, KLH challenge			
Group 7	0.5	5	2
Group 8	0.2	5	2
Group 9	0.02	5	2
Group 9 bis	1.5	5	2
Part 2: multiple ascending dose			
Group 10	0.2	5	2
Group 11	0.5	5	2

Table II. Subject demographics at baseline

	Placebo	Total FR104
Safety population	18	47
Gender, *n* (%)		
Male	12 (67)	26 (55)
Female	6	21
Age (years)		
Mean (SD)	52.7 (9.28)	52.1 (7.88)
Median	56.7	55.5
Range	20; 60	22; 60
Race, *n* (%)		
White	17 (94)	46 (98)
Asian	1 (6)	0
Black/African American	0	1 (2)
BMI (kg/m^2)		
Mean (SD)	25.04 (2.25)	25.64 (2.196)
Median	24.88	25.78
Range	20.2; 28.8	19.9; 29.7

Pharmacodynamics

After infusion of FR104, a dose-dependent CD28 RO on the T cells in peripheral blood was observed (Fig. 1B). The CD28 receptors were saturated at the first sampling time point (0.5 h) after infusion of FR104 at doses of 0.020 mg/kg and higher. At day 29, RO was still above 50% after infusion of FR104 at 0.500 and 1.500 mg/kg doses. The CD28 RO returned to 50% in a dose dependent manner, from day 15 (0.020 mg/kg) to day 85 (1.500 mg/kg). The CD28 RO remained above 50% when the FR104 serum concentrations were just above the lower limit of quantification (<100 ng/ml), and above 80% when the FR104 serum concentrations were above 200 ng/ml. This suggests that FR104 has a high affinity to its receptor in vivo and that even low concentrations of FR104 may be pharmacologically active. After infusion of the second dose at 0.200 mg/kg, receptors became saturated again, which resulted in a saturation above 50% lasting for more than 60 d. Two doses of 0.500 mg/kg given 28 d apart resulted in 100% saturation of the receptors for 60 d.

There was no change considered significant in the total lymphocyte count and lymphocyte subsets, including naive T cells, memory T cells, and natural Treg cells, caused by FR104 at any dose and any dose regimen, and the levels of these subsets stayed in their pretreatment range in all groups during the study. The expression level of CD28 by lymphocyte subsets assessed by measuring MFI was also unaffected (Fig. 2).

Owing to the cytokine release that previously occurred after administration of superagonist or divalent antagonist anti-CD28 mAbs in humans (26, 29), potential cytokine release has been closely followed up. No elevation of cytokines was observed in the serum of any volunteer. Only background levels were recorded, which are considered non-clinically relevant (Fig. 3).

To evaluate the impact of FR104 on T cell reactivity, blood cells of each subject were challenged with a standardized stimulus (SEB + LPS) in whole-blood cultures that had been developed specifically for use in a clinical trial situation (TruCulture) (32). After ex-vivo stimulation with SEB + LPS, IFN-γ, IL-2, and IL-8 cytokines strongly increased in pretreatment (T0) whole-blood cultures. A fraction of subjects, however, showed an absence or moderate induction of IL-2, IFN-γ, and IL-8 synthesis after stimulation at baseline (Fig. 4A). Owing to this variability, the assay only picked up a significant inhibition of inducible IL-2 synthesis after administration of FR104 at doses above 0.100 mg/kg on the two and 96 h time points, but not at other time points. SEB + LPS-induced synthesis of IFN-γ and IL-8, cytokines also secreted by non-T cells, was not modulated by treatment with FR104 in any group (Fig. 4B).

Control of anti-KLH Ab response in humans

The effect of FR104 on the response to KLH challenge was evaluated by measuring anti-KLH IgG Abs. Both the time to formation of anti-KLH Abs and the level of Abs were inhibited by FR104 in a dose-responsive manner. After a single dose of FR104 at 0.020 mg/kg, a reduction in the production of anti-KLH Abs was already visible on day 15 post-KLH immunization, which reached an average of 50% of the placebo group on day 29. The anti-KLH response was maintained at that level in comparison with the placebo group until the last day of observation (day 85). After the single dose of 0.200 mg/kg, the average reduction in the production of anti-KLH Abs was ~85% on day 15 and 29, and 70 and 60% on day 57 and 85, respectively, as compared with the response of the placebo group. After the single dose of 0.500 mg/kg, the average reduction in the production of anti-KLH Abs was >90% on day 15 and 29, and ~80 and 75% on day 57 and 85, respectively. At the highest dose level (FR104 1.500 mg/kg),

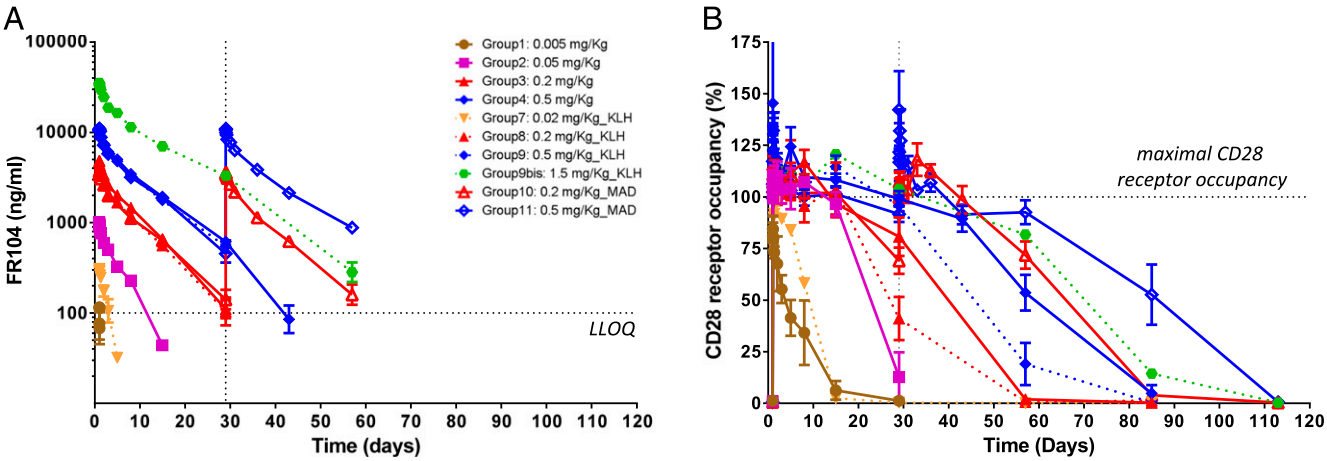

FIGURE 1. Pharmacokinetic and pharmacodynamic data. (**A**) Mean serum FR104 concentrations ± SEM (semilog scale) from predose to end of study. Treatment groups shown are: single ascending doses (black symbols), single ascending doses + KLH immunization (empty symbols), multiple ascending doses (MAD, shaded symbols). *n* = 3–5 subjects per treatment group. (**B**) Mean CD28 receptor occupancy ± SEM across the FR104 treatment groups indicated in A from predose to end of study. *n* = 3–5 subjects per treatment group. Assessment of receptor occupancy in subjects dosed with placebo resulted in a background signal ranging between 0 and 5%. LLOQ, lower limit of quantification.

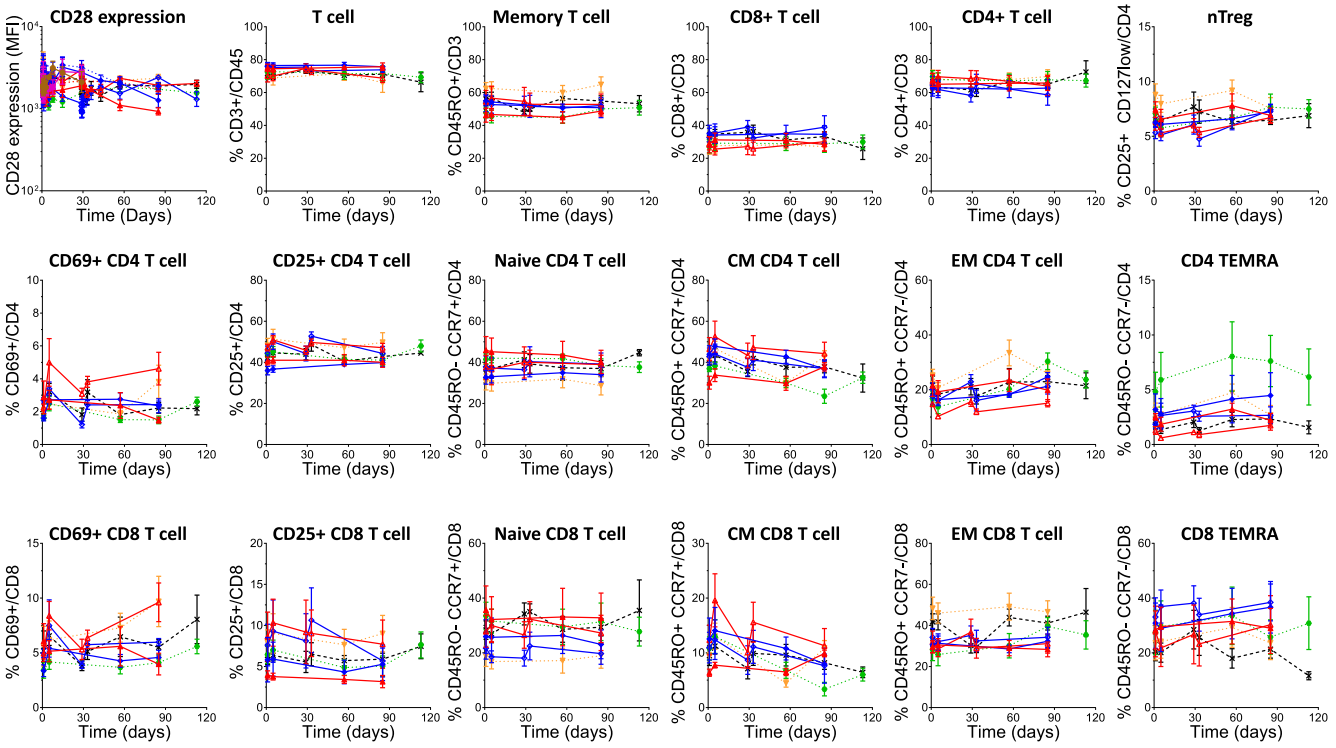

FIGURE 2. Flow cytometry analysis of CD28 expression level, T lymphocyte subpopulations frequency and activated T cell frequency on stabilized whole blood samples. T cell subpopulations were defined using the following gating strategy in CD45+ CD3+ cells: natural Treg (nTreg): CD25+ CD127 low CD4+; activated: CD69+ or CD25+; memory T cells: CD45RO+; naive: CD45RO- CCR7+; central memory (CM): CD45RO+ CCR7+; effector memory (EM): CD45RO+ CCR7−; TEMRA: CD45RO− CCR7−. Symbols refer to the groups defined in Fig. 1A, plus × for pooled placebo subjects (n = 12). Data are means ± SEM.

the formation of anti-KLH Abs was essentially suppressed until day 57. On day 85 and 113, however, some response (~75% reduction) was recorded. Due to variabilities in the control group (some control subjects showed a low anti-KLH response), statistical significance was only reached for FR104 doses of 0.5 and 1.5 mg/kg (Fig. 5).

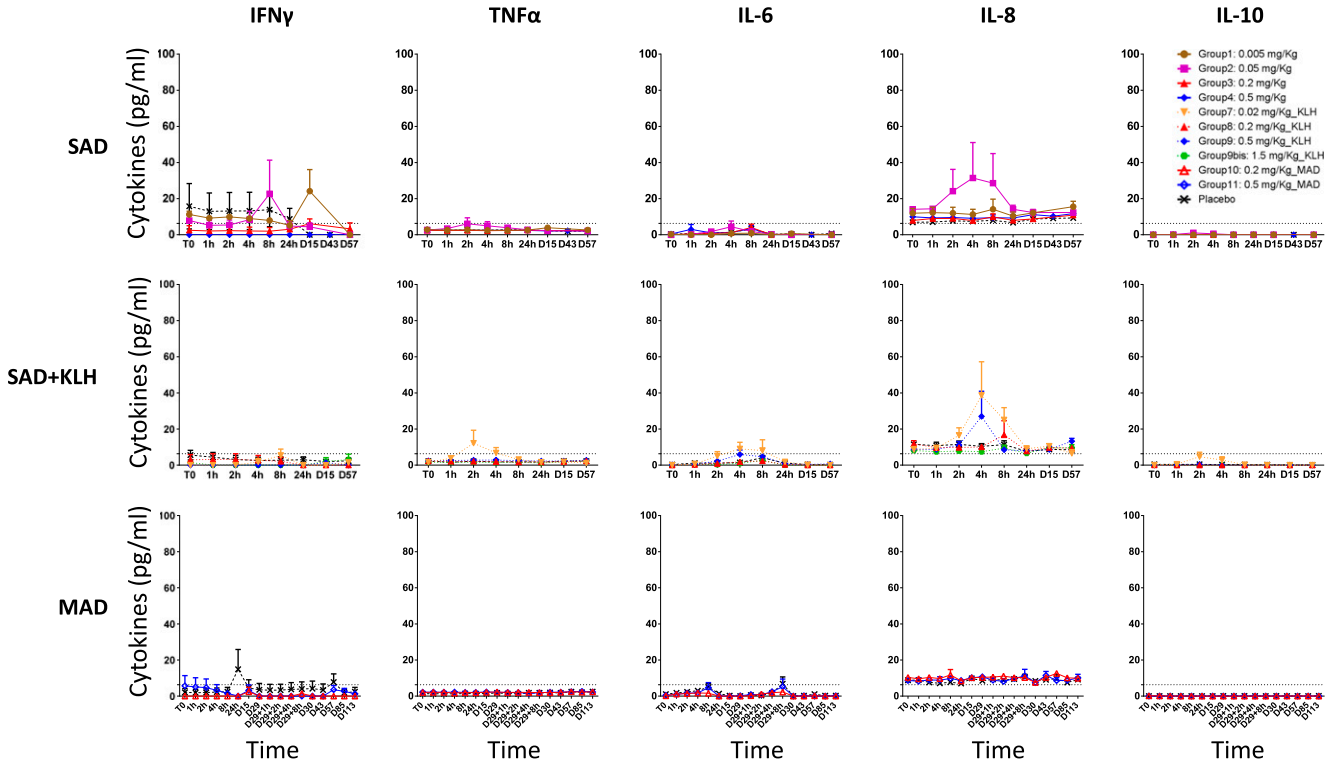

FIGURE 3. IFN-γ, TNF-α, IL-6, IL-8, IL-10 cytokines in FR104 and placebo recipients from predose to the end of the study. Dotted bar: lower limit of quantification. Data are means ± SEM. SAD + KLH, single ascending dosed subjects immunized with KLH. Symbols refer to the groups defined in Fig. 1A, plus ×-for placebo subjects in SAD (n = 6), SAD + KLH (n = 8) and MAD (n = 4) groups. Other cytokines assessed (IL 12p70 IL-1β, IL-2, IL-4) were found negative for all samplings (not shown). SAD; single ascending doses; MAD, multiple ascending doses.

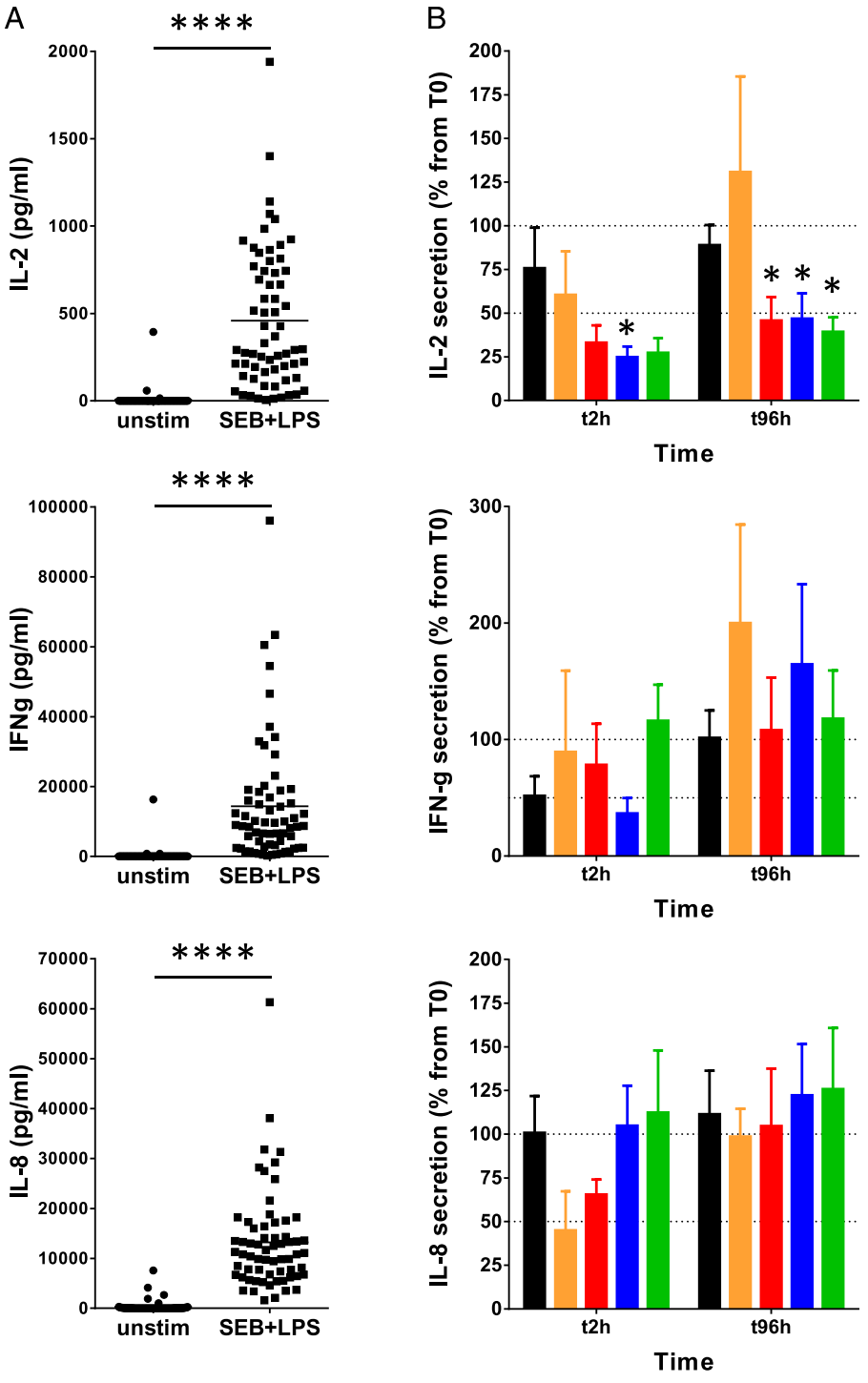

FIGURE 4. Ex-vivo stimulation of blood cells with SEB + LPS in whole-blood cultures (TruCulture). (**A**) Blood samples at baseline were not stimulated or stimulated with SEB + LPS and maintained for 24 h in culture. Cytokines in the plasma were then assessed by ELISA. Dots represent individual subjects. (**B**) Measurement of IL-2, IFN-γ and IL-8 secretion by SEB + LPS stimulated blood samples drawn at the indicated time points in single ascending dose groups (with or without KLH stimulation) receiving FR104 at the indicated dose level. Only cultures of the donors tested in this study showing sufficient responses to the stimuli at baseline (>100 pg/ml) have been included in the analysis: Placebo (n = 9), 0.02 mg/kg (n = 5), 0.20 mg/kg (n = 9), 0.50 mg/kg (n = 9) and 1.50 mg/kg (n = 3). Data are mean cytokine concentrations normalized to T0 time points, ± SEM. *$p < 0.05$, ****$p < 0.0001$.

Immunogenicity

In Part 1 Cohort A (no KLH immunization), zero (0%), two (50%), three (60.0%) and one (20.0%) subject were reported as positive with Abs against FR104 (ADAs) at the last sample (day 85) following an infusion of FR104 at 0.005, 0.050, 0.200 and 0.500 mg/kg, respectively. In Part I Cohort B (KLH immunization), one (20%), three (60.0%), four (80.0%) and two (40.0%) subjects had ADAs at the last sample after 0.020, 0.200, 0.500 mg/kg (day 85), and 1.500 mg/kg (day 113) FR104 infusion, respectively. In Part 2 (repeat doses), three subjects (60.0%) had ADAs after both FR104 0.200 and 0.500 mg/kg infusion at the last sample (day 113). The earliest ADAs were detected from day 29 in Part 1 (Cohorts A and B) and from day 57 in Part 2. Approximately half of the ADA+ subjects (10/22) were of relativelfy low titers (<30). The development of ADA did not induce any AEs nor modify cytokine release. Given that ADAs when present appeared only after the disappearance of FR104 in the blood of all subjects but one, no conclusion can be made on their potential impact on PK.

Safety

No death or related serious AEs occurred during the study. One subject was reported with the serious AEs nephrolithiasis that was considered not related to the study drug by the investigator after FR104 0.500 mg/kg infusion and KLH challenge. None of the subjects discontinued the study drug or the study because of a treatment-emergent adverse event (TEAE). One subject in Part 2

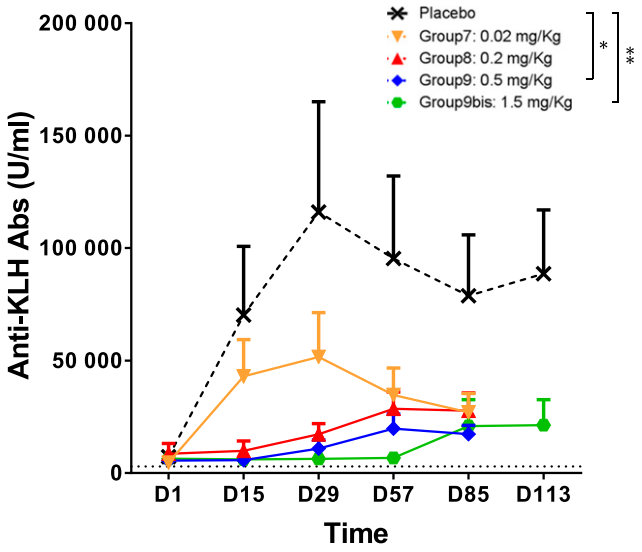

FIGURE 5. Anti-KLH IgG (ng/ml) levels over time by treatment group. Serum samples were drawn at the indicated time points and assessed by anti-KLH ELISA. Data are means ± SEM. Dotted line: lower limit of quantification. *$p < 0.05$, **$p < 0.01$.

did not receive the second dose of FR104 0.500 mg/kg on day 29 due to receiving prohibited medication (ibuprofen, paracetamol, and tramadol) for the AE myalgia. The subject was not withdrawn from the study and completed all study visits as per protocol. All reported TEAEs were of mild or moderate severity (Table III). TEAEs considered to be at least possibly related to the study drug by the investigators were reported in five of the 10 subjects who received FR104 infusion, and in none of the subjects after placebo infusion. By preferred term, the most frequently reported TEAEs after FR104 infusion were back pain, headache, and vomiting. Possible treatment-related events after FR104 infusion were headache, vomiting, aphthous stomatitis, oral herpes, dry mouth, nausea, nasopharyngitis, fatigue, gingivitis, dysgeusia, diarrhea, vision blurred and influenza-like illness.

No clinically relevant or consistent changes in median values for laboratory, hematology, virology, vital signs, oxygen saturation, and electrocardiogram parameters were observed after administration of the study drug in any volunteer.

Postbaseline EBV viral load results were negative or weak positive in all except one subject, and postbaseline EBV Capsid IgM Ab results were negative or equivocal for all subjects in the study (Fig. 6). EBV capsid and nuclear Ag IgG Abs were positive in all subjects (a requirement for entry into the study) and remained unchanged from their baseline level throughout the study (data not shown). One subject receiving two IV doses of placebo had a positive EBV viral load result on day 15 with a viral load of log 3.83 IU/m. At the next time point on day 29, the test was negative. Later assessments were negative on day 43, positive with viral load of 2.7 log IU/ml on day 57, negative on day 85 and positive on day 113 with viral load <2.5 log IU/ml. This subject remained asymptomatic.

During the study, a Friderica's corrected QT (QTcF) interval >450 ms and a QTcF change from baseline of >30 ms were reported in one subject in Cohort B after placebo infusion and KLH challenge. In Part 2, a QTcF interval >450 ms was reported in one subject after FR104 0.200 mg/kg infusion, a QTcF change from baseline of >60 ms in one subject after FR104 0.200 mg/kg and a QTcF change from baseline of >30 ms was reported in one and three subjects after FR104 0.200 mg/kg and FR104 0.500 mg/kg infusion, respectively. None of these changes were considered clinically significant. No clinically significant vital signs or pulse oximetry changes were observed in Part 1 (Cohort A and B) or Part 2.

Two subjects in Cohort A and three subjects in Part 2 were reported to have abnormalities on physical examination. For these subjects, the abnormality was considered clinically significant and the observation was reported as TEAE: nasopharyngitis and conjunctivitis for the subjects in Cohort A and upper respiratory tract infection, myalgia, and gout for the subjects in Part 2.

Discussion

To our knowledge, this trial represents the first administration to humans of FR104, a humanized pegylated Fab′ Ab fragment antagonist of CD28. FR104 was well tolerated at all doses tested, with the most common TEAE being headache. FR104 administration was not associated with cytokine release syndrome or activation of T cells. In previous attempts to target CD28 using anti-CD28 monoclonal Abs [TGN1412 (26), FK734 (29)] clinical development was stopped and/or complicated by induced cytokine release. As FR104 was not associated with such complications, targeting CD28 with monovalent antagonist Abs remains an attractive therapeutic strategy for costimulation blockade.

In the years following the catastrophic administration in man of the TGN1412 anti-CD28 mAb (26), the mechanism responsible for the massive cytokine release observed at the time has been uncovered: immunologically, human effector memory CD4+ and CD8+ T cells are CD28-positive and poised for immediate release of IFN-γ, TNF-α and IL-2 cytokines after stimulation. Two structural features made TGN1412 a so-called superagonist Ab. First, it was directed at the C''D loop of CD28, which is mandatory for inducing Ag-independent CD28-mediated signaling. Abs binding outside the C''D loop cannot activate T cells in an Ag-independent manner (28). Second, the IgG format was required to cross-link CD28 with an appropriate divalent stoichiometry (CD28 being itself homodimeric) resulting in the formation of CD28 clusters on the plasma membrane associated with T cell signaling (30). FR104 has been conceived to be antagonist of the CD28-CD80/86 interactions with a target epitope overlapping with the MYPPPY motif involved in this interaction, thereby lying outside the C''D epitope of the superagonist Abs (19). In addition, its monovalent Fab′ format prevents formation of CD28-drug-CD28 complexes associated with intracellular signaling events, as well as absence of Fc domain that could engage with Fc receptors and induce some activation of target T cells (29). Interestingly, aggregated monovalent anti-CD28 Abs or addition of secondary Abs to monovalent anti-CD28 Abs were shown to not recapitulate the agonistic properties of IgG anti-CD28 Abs (30). Before the initiation of the current phase I study in healthy volunteers, the antagonistic properties of FR104 had been assessed thoroughly in vitro, as previously recommended (33, 34) as well as in vivo using humanized mice (20) and baboons (27). Indeed, baboon effector memory CD4+ and CD8+ T cells have been shown to express CD28 in a similar manner to humans and are responsive to superagonist or agonist anti-CD28 Abs. As a matter of comparison, preclinical assessment in macaques failed to predict that TGN1412 would induce a massive cytokine release in man most probably because macaque effector memory CD4+ T cells lose CD28 expression during their differentiation (35). The design of the trial also mitigated the risk of being exposed to agonist/superagonist Abs.

Cytokine assessment in serum in man demonstrated a complete absence of agonist or superagonist activity of FR104 at any dose,

Table III. A summary of TEAEs per treatment in Part 1 (Cohort A and B) and Part 2

Part 1 (SAD), Cohort A						
FR104 dose	Placebo	Group 1 (0.005 mg/kg)	Group 2 (0.05 mg/kg)	Group 3 (0.2 mg/kg)	Group 4 (0.5 mg/kg)	All Subjects
Number of subjects with at least one TEAE	2 (33.3)	1 (33.3)	3 (75.0)	4 (80.0)	2 (40.0)	10 (58.8)
Severe AE	0	0	0	0	0	0
TEAE leading to death	0	0	0	0	0	0
Mild TEAE (worst severity)	1 (16.7)	0	3 (75.0)	1 (20.0)	0	4 (23.5)
Moderate TEAE (worst severity)	1 (16.7)	1 (33.3)	0	3 (60.0)	2 (40.0)	6 (35.3)
Severe TEAE (worst severity)	0	0	0	0	0	0
TEAE for which the study drug was discontinued	0	0	0	0	0	0
At least possibly treatment-related TEAE	0	0	1 (25.0)	3 (60.0)	1 (20.0)	5 (29.4)

Part 1 (SAD), Cohort B (KLH Challenge)						
FR104 dose	Placebo	Group 7 (0.02 mg/kg)	Group 8 (0.2 mg/kg)	Group 9 (0.5 mg/kg)	Group 9 bis (1.5 mg/kg)	All Subjects
Number of subjects with at least one TEAE	7 (87.5)	3 (60.0)	3 (60.0)	3 (60.0)	1 (20.0)	10 (50.0)
Severe AE	0	0	0	1 (20.0)	0	1 (5.0)
TEAE leading to death	0	0	0	0	0	0
Mild TEAE (worst severity)	6 (75.0)	2 (40.0)	2 (40.0)	1 (20.0)	1 (20.0)	6 (30.0)
Moderate TEAE (worst severity)	1 (12.5)	1 (20.0)	1 (20.0)	2 (40.0)	0	4 (20.0)
Severe TEAE (worst severity)	0	0	0	0	0	0
TEAE for which the study drug was discontinued	0	0	0	0	0	0
At least possibly treatment-related TEAE	2 (25.0)	1 (20.0)	1 (20.0)	1 (20.0)	0	3 (15.0)

Part 2 (MAD)				
FR104 dose	Placebo	Group 10 (0.2 mg/kg)	Group 11 (0.5 mg/kg)	All Subjects
Number of subjects with at least one TEAE	3 (75.0)	5 (100.0)	4 (80.0)	9 (90.0)
Severe AE	0	0	0	0
TEAE leading to death	0	0	0	0
Mild TEAE (worst severity)	2 (50.0)	2 (40.0)	3 (60.0)	5 (50.0)
Moderate TEAE (worst severity)	1 (25.0)	3 (60.0)	1 (20.0)	4 (40.0)
Severe TEAE (worst severity)	0	0	0	0
TEAE for which the study drug was discontinued	0	0	0	0
At least possibly treatment-related TEAE	1 (25.0)	2 (40.0)	3 (60.0)	5 (50.0)

even after repeat dose administration. Background or close to background levels of IFN-γ, TNF-α and IL-8 in some subjects recorded at baseline or after FR104 administration (Fig. 3) are far below those measured after administration of TGN1412, which peaked at 5000 pg/ml for these three cytokines (26). When present, ADA became apparent in most subjects only after FR104 being eliminated from the plasma. Therefore, it is difficult to estimate the effect of ADA on PK and PD parameters and especially on cytokines. In one subject, however, (Part 2, dose 0.2 mg/kg MAD), ADA became apparent while CD28 RO was still measurable. In this subject, there was no cytokine release either, consistent with the preclinical observation that FR104 in the presence of ADA that would bridge the monovalent FR104 does not recapitulate the agonistic properties of anti-CD28 Abs in their IgG format. Of note, the relatively high incidence of immunogenicity for FR104 is in a similar range to what has been reported for Cimzia (certolizumab pegol), a pegylated Fab′ Ab fragment (CIMZIA clinical pharmacology BLA 125160/0) and some other biologics such as Remicade and Humira (36). FR104 did not alter total lymphocyte counts or lymphocyte subsets. T cell phenotype was also unmodified. Given that CD28 may also regulate migration of primed T cells to target tissue (37), these data together with absence of cytokine release and absence of target T cell activation reinforce the purely antagonist activity of FR104.

The PK of FR104 was approximately linear at doses ≥0.200 mg/kg. Similar $t_{1/2}$, area under the curve ad infinite and related PK parameters were observed between groups (SAD and MAD) for the same dose level.

As with other immunosuppressive molecules, administration of FR104 to human subjects might be expected to increase susceptibility to infections. Kidney transplant recipients treated with belatacept (mostly combined with other immunosuppressive treatments, however) and who were EBV-seronegative at baseline had an increased occurrence of post-transplant lymphoproliferative diseases, particularly involving the CNS (38). Because the mechanism of action of FR104 overlaps belatacept, a lymphoproliferation elicited by de novo EBV infection has been considered a possible AE in the current study. Therefore, only subjects that were EBV-seropositive at baseline have been included here. The postdose assessment of EBV viral load indicated absence of treatment-associated viral reactivation and the overall safety profile did not point out increased susceptibility to infection, indicating that over the short period of time tested here, selective CD28 blockade with FR104 did not compromise the overall immune surveillance.

Assessing the immunosuppressive potential of a drug candidate in healthy volunteers has been made possible by the availability of the Immucothel (Biosyn, Carlsbad, CA) vaccine, containing KLH. It has been used previously to measure the immunosuppression of

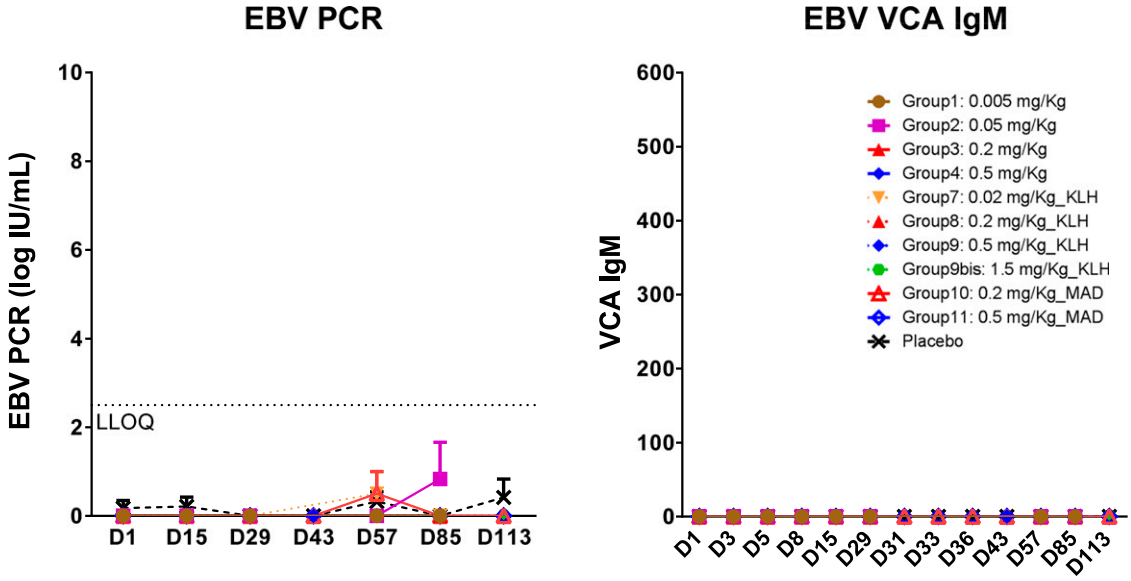

FIGURE 6. EBV status from predose to end of study. EBV viral load was assessed in blood by PCR at the indicated time points. IgM Abs against EBV capsid Ag (VCA) were also assessed in serum to measure anti-EBV immune reactivation. Data are means ± SEM. LLOQ, lower limit of quantification.

fingolimod (39). Six weeks after vaccination with Immucothel, volunteers receiving high doses of fingolimod presented a mean reduction of their anti-KLH IgG responses of ∼80%. Comparatively, at the same time point, high-dose FR104 administration (1.5 mg/kg) resulted in complete control of IgG responses. At later time points (day 113), some KLH responses were observed; however, that might be due to the persistence of the Ag and an ongoing stimulation of the immune system, at a time point when CD28 desaturation with FR104 occurs.

In conclusion, to our knowledge this is the first study to evaluate the safety, PK and PD of FR104 in healthy subjects. The data provide initial evidence that the CD28-CD80/86 interactions can be blocked with a monovalent Ab targeting CD28 without inducing serious AEs. The findings support further clinical development of FR104, which will establish the role and optimal dosage regimen for FR104 in autoimmunity and transplantation.

Acknowledgments

We thank Sabrina Pengam (OSE Immunotherapeutics), Dr. Catherine Ruiz (OSE Immunotherapeutics) and Dr. Peter Tjeerdsma (Janssen Biotech) for assistance in the management of the CT-001 clinical trial.

Disclosures

B.V., D.C., M.H., J.-P.S., C.M., and N.P. are shareholders of OSE Immunotherapeutics, a company owning CD28 antagonists. W.W., I.A., and I.G. are employees of Janssen Research & Development, LLC, and own stocks of Johnson & Johnson. The other authors have no financial conflicts of interest.

References

1. Genovese, M. C., J. C. Becker, M. Schiff, M. Luggen, Y. Sherrer, J. Kremer, C. Birbara, J. Box, K. Natarajan, I. Nuamah, et al. 2005. Abatacept for rheumatoid arthritis refractory to tumor necrosis factor alpha inhibition. *N. Engl. J. Med.* 353: 1114–1123.
2. Ruperto, N., D. J. Lovell, P. Quartier, E. Paz, N. Rubio-Pérez, C. A. Silva, C. Abud-Mendoza, R. Burgos-Vargas, V. Gerloni, J. A. Melo-Gomes, et al; Paediatric Rheumatology INternational Trials Organization; Pediatric Rheumatology Collaborative Study Group. 2008. Abatacept in children with juvenile idiopathic arthritis: a randomised, double-blind, placebo-controlled withdrawal trial. *Lancet* 372: 383–391.
3. Ruperto, N., D. J. Lovell, P. Quartier, E. Paz, N. Rubio-Pérez, C. A. Silva, C. Abud-Mendoza, R. Burgos-Vargas, V. Gerloni, J. A. Melo-Gomes, et al; Paediatric Rheumatology International Trials Organization and the Pediatric Rheumatology Collaborative Study Group. 2010. Long-term safety and efficacy of abatacept in children with juvenile idiopathic arthritis. *Arthritis Rheum.* 62: 1792–1802.
4. Vincenti, F., G. Blancho, A. Durrbach, P. Friend, J. Grinyo, P. F. Halloran, J. Klempnauer, P. Lang, C. P. Larsen, F. Mühlbacher, et al. 2010. Five-year safety and efficacy of belatacept in renal transplantation. *J. Am. Soc. Nephrol.* 21: 1587–1596.
5. Ville, S., N. Poirier, J. Branchereau, V. Charpy, S. Pengam, V. Nerriere-Daguin, S. Le Bas-Bernardet, F. Coulon, C. Mary, A. Chenouard, et al. 2016. Anti-CD28 antibody and belatacept exert differential effects on mechanisms of renal allograft rejection. *J. Am. Soc. Nephrol.* DOI: 10.1681/ASN.2015070774.
6. Poirier, N., G. Blancho, and B. Vanhove. 2011. A more selective costimulatory blockade of the CD28-B7 pathway. *Transpl. Int.* 24: 2–11.
7. Poirier, N., G. Blancho, and B. Vanhove. 2012. CD28-specific immunomodulating antibodies: what can be learned from experimental models? *Am. J. Transplant.* 12: 1682–1690.
8. Ville, S., N. Poirier, G. Blancho, and B. Vanhove. 2015. Co-stimulatory blockade of the CD28/CD80-86/CTLA-4 balance in transplantation: impact on memory T cells? *Front. Immunol.* 6: 411.
9. Poirier, N., and B. Vanhove. 2016. Anti-CD28 therapeutics for the treatment of autoimmune disorders. *Drugs Future* 41: 229–235.
10. Charbonnier, L. M., B. Vokaer, P. H. Lemaître, K. A. Field, O. Leo, and A. Le Moine. 2012. CTLA4-Ig restores rejection of MHC class-II mismatched allografts by disabling IL-2-expanded regulatory T cells. *Am. J. Transplant.* 12: 2313–2321.
11. Dilek, N., N. Poirier, P. Hulin, F. Coulon, C. Mary, S. Ville, H. Vie, B. Clémenceau, G. Blancho, and B. Vanhove. 2013. Targeting CD28, CTLA-4 and PD-L1 costimulation differentially controls immune synapses and function of human regulatory and conventional T-cells. *PLoS One* 8: e83139.
12. Levitsky, J., J. Miller, X. Huang, D. Chandrasekaran, L. Chen, and J. M. Mathew. 2013. Inhibitory effects of belatacept on allospecific regulatory T-cell generation in humans. *Transplantation* 96: 689–696.
13. Larsen, C. P., T. C. Pearson, A. B. Adams, P. Tso, N. Shirasugi, E. Strobert, D. Anderson, S. Cowan, K. Price, J. Naemura, et al. 2005. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am. J. Transplant.* 5: 443–453.
14. Haanstra, K. G., J. Ringers, E. A. Sick, S. Ramdien-Murli, E. M. Kuhn, L. Boon, and M. Jonker. 2003. Prevention of kidney allograft rejection using anti-CD40 and anti-CD86 in primates. *Transplantation* 75: 637–643.
15. Zheng, X. X., T. G. Markees, W. W. Hancock, Y. Li, D. L. Greiner, X. C. Li, J. P. Mordes, M. H. Sayegh, A. A. Rossini, and T. B. Strom. 1999. CTLA4 signals are required to optimally induce allograft tolerance with combined donor-specific transfusion and anti-CD154 monoclonal antibody treatment. *J. Immunol.* 162: 4983–4990.
16. Lin, H., J. C. Rathmell, G. S. Gray, C. B. Thompson, J. M. Leiden, and M. L. Alegre. 1998. Cytotoxic T lymphocyte antigen 4 (CTLA4) blockade accelerates the acute rejection of cardiac allografts in CD28-deficient mice: CTLA4 can function independently of CD28. *J. Exp. Med.* 188: 199–204.
17. Vanhove, B., G. Laflamme, F. Coulon, M. Mougin, P. Vusio, F. Haspot, J. Tiollier, and J. P. Soulillou. 2003. Selective blockade of CD28 and not CTLA-4 with a single-chain Fv-alpha1-antitrypsin fusion antibody. *Blood* 102: 564–570.
18. Zhang, T., S. Fresnay, E. Welty, N. Sangrampurkar, E. Rybak, H. Zhou, X. F. Cheng, Q. Feng, C. Avon, A. Laaris, et al. 2011. Selective CD28 blockade attenuates acute and chronic rejection of murine cardiac allografts in a CTLA-4-dependent manner. *Am. J. Transplant.* 11: 1599–1609.
19. Poirier, N., A. M. Azimzadeh, T. Zhang, N. Dilek, C. Mary, B. Nguyen, X. Tillou, G. Wu, K. Reneaudin, J. Hervouet, et al. 2010. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. *Sci. Transl. Med.* 2: 17ra10.

20. Poirier, N., C. Mary, N. Dilek, J. Hervouet, D. Minault, G. Blancho, and B. Vanhove. 2012. Preclinical efficacy and immunological safety of FR104, an antagonist anti-CD28 monovalent Fab′ antibody. *Am. J. Transplant.* 12: 2630–2640.
21. Poirier, N., N. Dilek, C. Mary, S. Ville, F. Coulon, J. Branchereau, X. Tillou, V. Charpy, S. Pengam, V. Nerriere-Daguin, et al. 2015. FR104, an antagonist anti-CD28 monovalent fab′ antibody, prevents alloimmunization and allows calcineurin inhibitor minimization in nonhuman primate renal allograft. *Am. J. Transplant.* 15: 88–100.
22. Haanstra, K. G., K. Dijkman, N. Bashir, J. Bauer, C. Mary, N. Poirier, P. Baker, C. L. Crossan, L. Scobie, B. A. 't Hart, and B. Vanhove. 2015. Selective blockade of CD28-mediated T cell costimulation protects rhesus monkeys against acute fatal experimental autoimmune encephalomyelitis. *J. Immunol.* 194: 1454–1466.
23. Vierboom, M. P., E. Breedveld, Y. S. Kap, C. Mary, N. Poirier, B. A. 't Hart, and B. Vanhove. 2016. Clinical efficacy of a new CD28-targeting antagonist of T cell co-stimulation in a non-human primate model of collagen-induced arthritis. *Clin. Exp. Immunol.* 183: 405–418.
24. Poirier, N., M. Chevalier, C. Mary, J. Hervouet, D. Minault, S. Le Bas-Bernardet, L. Belarif, V. Daguin, E. Cassagnau, B. Vanhove, and G. Blancho. 2016. Selective CD28 antagonist prevents Aldara-induced skin inflammation in non-human primates. *Exp. Dermatol.* 25: 233–234.
25. Poirier, N., M. Chevalier, C. Mary, J. Hervouet, D. Minault, P. Baker, S. Ville, S. Le Bas-Bernardet, N. Dilek, L. Belarif, et al. 2016. Selective CD28 antagonist blunts memory immune responses and promotes long-term control of skin inflammation in nonhuman primates. *J. Immunol.* 196: 274–283.
26. Suntharalingam, G., M. R. Perry, S. Ward, S. J. Brett, A. Castello-Cortes, M. D. Brunner, and N. Panoskaltsis. 2006. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N. Engl. J. Med.* 355: 1018–1028.
27. Poirier, N., C. Mary, S. Le Bas-Bernardet, V. Daguin, L. Belarif, M. Chevalier, J. Hervouet, D. Minault, S. Ville, V. Charpy, et al. 2014. Advantages of Papio anubis for preclinical testing of immunotoxicity of candidate therapeutic antagonist antibodies targeting CD28. *MAbs* 6: 697–707.
28. Lühder, F., Y. Huang, K. M. Dennehy, C. Guntermann, I. Müller, E. Winkler, T. Kerkau, S. Ikemizu, S. J. Davis, T. Hanke, and T. Hünig. 2003. Topological requirements and signaling properties of T cell-activating, anti-CD28 antibody superagonists. *J. Exp. Med.* 197: 955–966.
29. Shiao, S. L., J. M. McNiff, T. Masunaga, K. Tamura, K. Kubo, and J. S. Pober. 2007. Immunomodulatory properties of FK734, a humanized anti-CD28 monoclonal antibody with agonistic and antagonistic activities. *Transplantation* 83: 304–313.
30. Mary, C., F. Coulon, N. Poirier, N. Dilek, B. Martinet, G. Blancho, and B. Vanhove. 2013. Antagonist properties of monoclonal antibodies targeting human CD28: role of valency and the heavy-chain constant domain. *MAbs* 5: 47–55.
31. Nunès, J., S. Klasen, M. Ragueneau, C. Pavon, D. Couez, C. Mawas, M. Bagnasco, and D. Olive. 1993. CD28 mAbs with distinct binding properties differ in their ability to induce T cell activation: analysis of early and late activation events. *Int. Immunol.* 5: 311–315.
32. Duffy, D., V. Rouilly, V. Libri, M. Hasan, B. Beitz, M. David, A. Urrutia, A. Bisiaux, S. T. Labrie, A. Dubois, et al; Milieu Intérieur Consortium. 2014. Functional analysis via standardized whole-blood stimulation systems defines the boundaries of a healthy immune response to complex stimuli. *Immunity* 40: 436–450.
33. Stebbings, R., L. Findlay, C. Edwards, D. Eastwood, C. Bird, D. North, Y. Mistry, P. Dilger, E. Liefooghe, I. Cludts, et al. 2007. "Cytokine storm" in the phase I trial of monoclonal antibody TGN1412: better understanding the causes to improve preclinical testing of immunotherapeutics. *J. Immunol.* 179: 3325–3331.
34. Findlay, L., D. Eastwood, R. Stebbings, G. Sharp, Y. Mistry, C. Ball, J. Hood, R. Thorpe, and S. Poole. 2010. Improved in vitro methods to predict the in vivo toxicity in man of therapeutic monoclonal antibodies including TGN1412. *J. Immunol. Methods* 352: 1–12.
35. Eastwood, D., L. Findlay, S. Poole, C. Bird, M. Wadhwa, M. Moore, C. Burns, R. Thorpe, and R. Stebbings. 2010. Monoclonal antibody TGN1412 trial failure explained by species differences in CD28 expression on CD4+ effector memory T-cells. *Br. J. Pharmacol.* 161: 512–526.
36. Bito, T., R. Nishikawa, M. Hatakeyama, A. Kikusawa, H. Kanki, H. Nagai, Y. Sarayama, T. Ikeda, H. Yoshizaki, H. Seto, et al. 2014. Influence of neutralizing antibodies to adalimumab and infliximab on the treatment of psoriasis. *Br. J. Dermatol.* 170: 922–929.
37. Mirenda, V., S. J. Jarmin, R. David, J. Dyson, D. Scott, Y. Gu, R. I. Lechler, K. Okkenhaug, and F. M. Marelli-Berg. 2007. Physiologic and aberrant regulation of memory T-cell trafficking by the costimulatory molecule CD28. *Blood* 109: 2968–2977.
38. Grinyó, J., B. Charpentier, J. M. Pestana, Y. Vanrenterghem, F. Vincenti, R. Reyes-Acevedo, A. M. Apanovitch, S. Gujrathi, M. Agarwal, D. Thomas, and C. P. Larsen. 2010. An integrated safety profile analysis of belatacept in kidney transplant recipients. *Transplantation* 90: 1521–1527.
39. Boulton, C., K. Meiser, O. J. David, and R. Schmouder. 2012. Pharmacodynamic effects of steady-state fingolimod on antibody response in healthy volunteers: a 4-week, randomized, placebo-controlled, parallel-group, multiple-dose study. *J. Clin. Pharmacol.* 52: 1879–1890.