

# Advantages of *Papio anubis* for preclinical testing of immunotoxicity of candidate therapeutic antagonist antibodies targeting CD28

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Antagonist anti-CD28 antibodies prevent T-cell costimulation and are functionally different from CTLA4Ig since they cannot block CTLA-4 and PDL-1 co-inhibitory signals. They demonstrated preclinical efficacy in suppressing effector T cells while enhancing immunoregulatory mechanisms. Because a severe cytokine release syndrome was observed during the Phase 1 study with the superagonist anti-CD28 TGN1412, development of other anti-CD28 antibodies requires careful preclinical evaluation to exclude any potential immunotoxicity side-effects. The failure to identify immunological toxicity of TGN1412 using macaques led us to investigate more relevant preclinical models.

We report here that contrary to macaques, and like in man, all baboon CD4-positive T lymphocytes express CD28 in their effector memory cells compartment, a lymphocyte subtype that is the most prone to releasing cytokines after reactivation. Baboon lymphocytes are able to release pro-inflammatory cytokines in vitro in response to agonist or superagonist anti-CD28 antibodies. Furthermore, we compared the reactivity of human and baboon lymphocytes after transfer into non obese diabetic/severe combined immunodeficiency (NOD/SCID) interleukin-2 $\gamma$  knockout mice and confirmed that both cell types could release inflammatory cytokines in situ after injection of agonistic anti-CD28 antibodies. In contrast, FR104, a monovalent antagonistic anti-CD28 antibody, did not elicit T cell activation in these assays, even in the presence of anti-drug antibodies. Infusion to baboons also resulted in an absence of cytokine release.

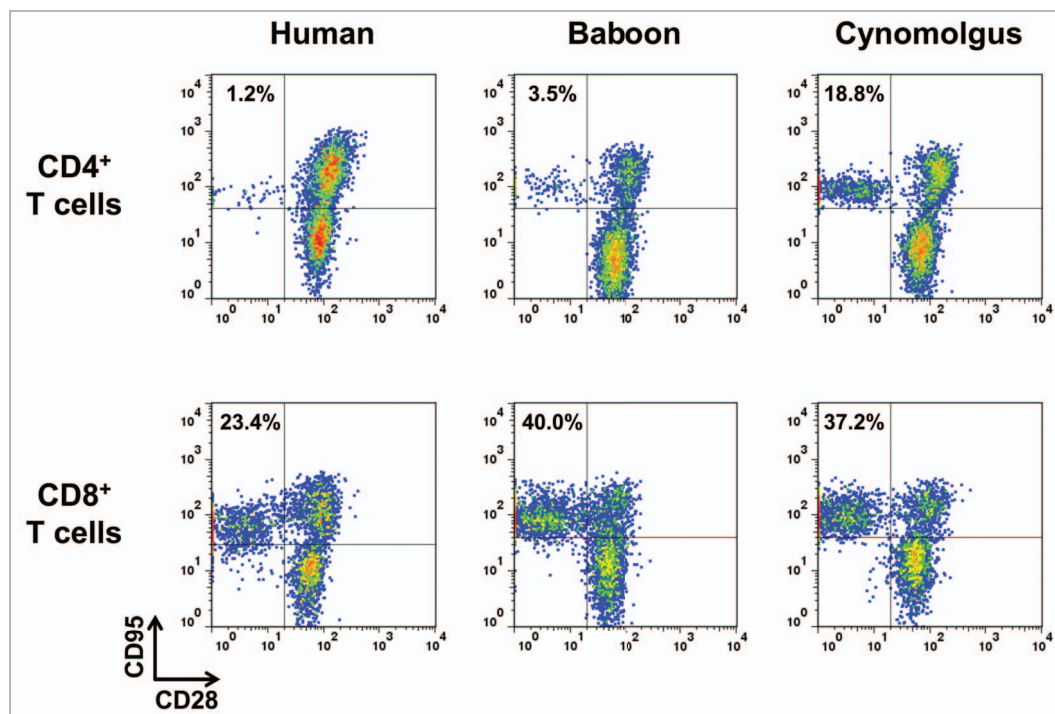
In conclusion, the baboon represents a suitable species for preclinical immunotoxicity evaluation of anti-CD28 antibodies because their effector memory T cells do express CD28 and because cytokine release can be assessed in vitro and trans vivo.

## Introduction

Immunotherapies with monoclonal antibodies (mAbs) or other recombinant proteins targeting receptors directly expressed on immune cells became a success story and a flourishing field of development to modulate immune responses in diverse indications such as oncology, inflammation, autoimmunity, transplantation, neuroscience and infectious diseases.<sup>1,2</sup> Among these immune cells, T lymphocytes represent a major therapeutic target, especially the costimulatory molecules they express, which regulate differentiation into either pathogenic effector T cells (Teff) or anti-inflammatory regulatory T cells (Treg). The CD28-CD80/86-CTLA-4 costimulatory system functions like a molecular rheostat, where CD28-CD80/86 engagement induce activation, proliferation and survival of Teff, as well as dampen Treg function, while CTLA-4-CD80/86 interaction is essential for the suppressive function of Treg, delivers anti-proliferative signals to Teff and confers a sub-immunogenic

function to antigen-presenting cells (APC).<sup>3</sup> This central immune checkpoint pathway was hence the subject of intense research and development. CD80/86 antagonists have proved immunosuppressive efficacy and were approved for marketing as treatments for rheumatoid arthritis<sup>4</sup> (abatacept, Orencia<sup>®</sup>; Bristol-Myers Squibb) and renal transplantation<sup>5</sup> (belatacept, Nulojix<sup>®</sup>; Bristol-Myers Squibb). However, because these compounds also inhibit CTLA-4 inhibitory signals, we and others have suggested that selectively targeting CD28 might present advantages over CD80/86 blockade as it would prevent engagement of CD80/86 with CD28, but not with CTLA4.<sup>3,6-12</sup> The theoretical advantage of selective CD28 blockade compared with CD80/86 blockade was further reinforced by two recent discoveries in the field of costimulation: (1) PD-L1 was identified as an additional ligand of CD80 capable of inhibiting T cell responses,<sup>13,14</sup> and (2) ICOSL (B7-H2) interacts with CD28 to induce T lymphocytes proliferation, cytokines secretion and survival signals.<sup>15</sup>

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**Figure 1.** CD28 expression by human, baboon and cynomolgus macaque memory T cells. One representative immunophenotyping analysis of CD28 in CD4<sup>+</sup> (upper panel) and CD8<sup>+</sup> (lower panel) memory (CD95<sup>+</sup>) and naïve (CD95<sup>-</sup>) T lymphocytes of human, baboon and cynomolgus macaque PBMC. Data are representative of at least three independent analyses.

The clinical translation of compounds aimed at CD28, however, has been hampered by the poor evaluation of their potential immunotoxicology at the pre-clinical level. Eight years ago, administration of TGN1412, a superagonist anti-CD28 mAb, to healthy volunteers caused a dramatic accident in a Phase I trial due to an acute and severe cytokine release syndrome (CRS), which had not been predicted by current preclinical animal models.<sup>16</sup> Indeed, the target epitope of TGN1412 was the C<sup>2</sup>D basolateral domain of CD28,<sup>17</sup> which, after antibody-mediated cross-linking, induces a non-physiological and antigen-independent polyclonal activation of T lymphocytes in rodent and human T cells. In addition, anti-CD28 mAbs in their IgG form present agonist properties even when binding outside the C<sup>2</sup>D loop, resulting from receptor cross-linking and T lymphocytes costimulation in synergy with T-cell receptor (TCR) signals.<sup>10</sup> Interaction with Fc receptors does not seem to be a dominant mechanism driving the agonist properties of anti-CD28 mAbs because ‘silenced’ (with a mutated Fc domain preventing interaction with Fc receptors) divalent anti-CD28 mAbs still costimulate T cells.<sup>18</sup> Therefore, to avoid any agonistic or superagonistic anti-CD28 mAbs activities, they must target an epitope other than C<sup>2</sup>D and must be monovalent.<sup>19</sup> One of the reasons preclinical assessment failed to predict that TGN1412 would induce cytokine release in man is most probably that in macaques, the species used for this assessment, the effector memory subset of T lymphocytes (T<sub>EM</sub>) have lost CD28 expression in the CD4<sup>+</sup> compartment.<sup>20–22</sup> Yet T<sub>EM</sub> cells are the most prone cell population to rapidly release inflammatory cytokines after

activation and represent the likely source of pro-inflammatory cytokines released after TGN1412 infusion.

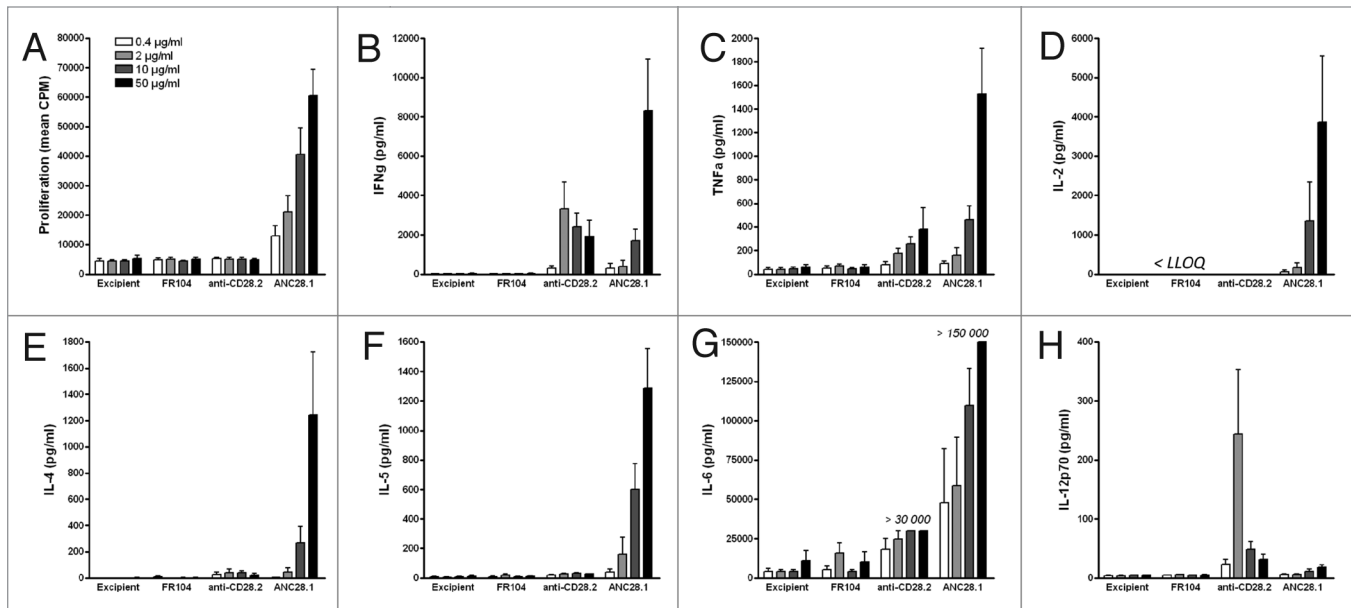
Here, we report that in baboons (*Papio anubis*), in contrast with macaques, CD28<sup>+</sup>CD4<sup>+</sup> T lymphocytes are barely detectable in peripheral blood and that, in baboons, like in man, T<sub>EM</sub> cells are CD28<sup>+</sup>. We therefore postulated that the baboon might be a more relevant species for predicting potential immunotoxicity of mAbs targeting CD28 at the preclinical level.

We previously developed FR104, a humanized monovalent pegylated anti-CD28 Fab’ antibody that demonstrated specific inhibition of CD28 interaction with CD80/86 and ICOSL.<sup>7</sup> In accordance with the recommendations published after the TGN1412 accident,<sup>23,24</sup> we evaluated FR104 in vitro on human peripheral blood mononuclear cells (PBMC), and trans vivo in a humanized mouse model,<sup>7</sup> and also measured the absence of immunotoxicity in vivo in baboons. The data presented here adds to the relevance of this model to evaluate the absence of immunotoxicity by showing that baboon memory CD4<sup>+</sup> T lymphocytes express CD28 similarly to human and are able to proliferate and secrete pro-inflammatory cytokines in vitro and trans vivo in the presence of agonist or superagonist anti-CD28 mAbs.

## Results

### CD28 expression by memory T lymphocytes of different primate species

We first evaluated CD28 expression by peripheral blood CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes from humans, baboons and cynomolgus macaques. We observed that the majority of baboon CD4<sup>+</sup> T lymphocytes expressed CD28 (98.1 ± 1.2%, n = 7) similarly to human, whereas, as previously described,<sup>20</sup> a considerable frequency (ranging from 5 to 25%, n = 3) of CD4<sup>+</sup> T lymphocytes did not express CD28 in cynomolgus (data not shown). We confirmed that these CD28-negative CD4<sup>+</sup> T lymphocytes, as well as CD28-negative CD8<sup>+</sup> T cells were of the memory phenotype since they expressed Fas receptor (CD95) in the three species (Fig. 1). Therefore, baboon CD4<sup>+</sup> T lymphocytes express CD28 in their naïve as well as in their memory subsets, similarly to humans.



**Figure 2.** Proliferation and cytokine release of human PBMC co-cultured with human endothelial cells in response to anti-CD28 mAbs. (A) Proliferation at 72 h, (B) IFN $\gamma$ , (C) TNF, (D) IL-2, (E) IL-4, (F) IL-5, (G) IL-6 and (H) IL-12p70 release at 48 h after addition of 0.4, 2, 10 or 50  $\mu$ g/ml of humanized pegylated Fab' fragment FR104, divalent agonist anti-CD28.2 mAb and superagonist anti-CD28 ANC28.1 mAb or of an equivalent volume of excipient. Histogram bars are means  $\pm$  SEM from data obtained with four different blood donors. LLOQ, Lower limit of quantification.

### In vitro comparison of human vs baboon T cells responses to anti-CD28 mAbs

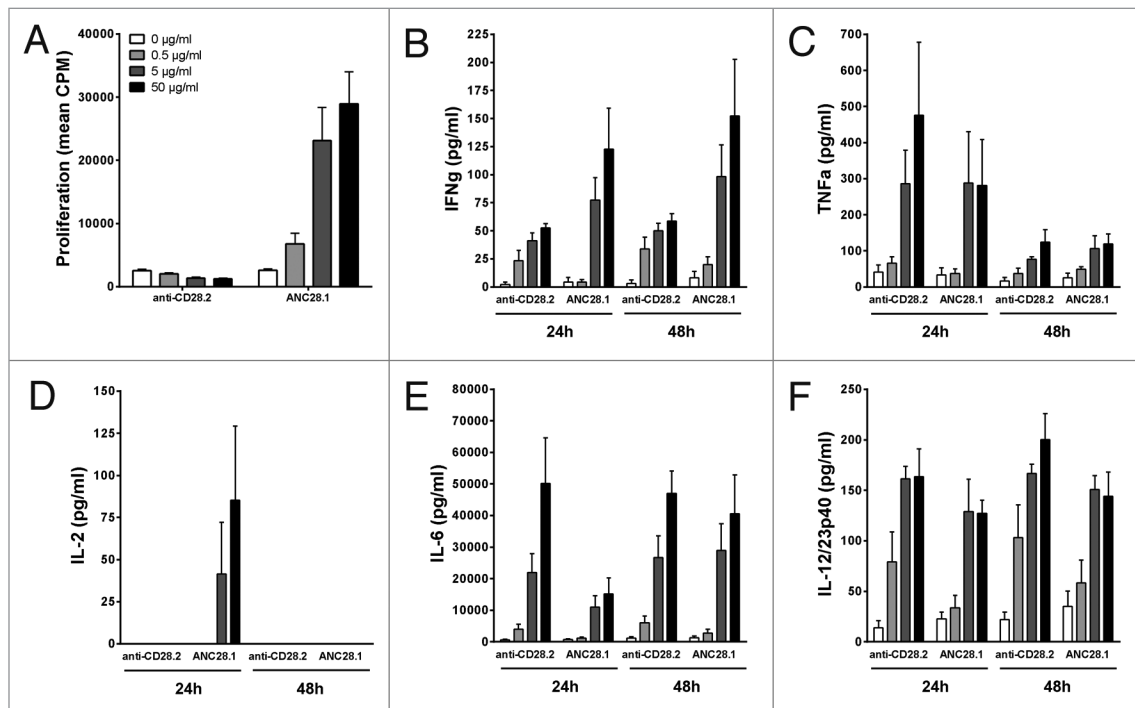
The TGN1412 accident prompted the development, evaluation and comparison of novel in vitro assays for mimicking in vivo cytokine release and lymphocytes proliferation.<sup>25,26</sup> The development of co-culture systems, such as human endothelial cell monolayers with human PBMC, although not fully mimicking the cytokine profile observed in vivo, has been considered as a reasonable surrogate assay to obtain cytokine responses predictive of in vivo conditions.<sup>27,28</sup> We therefore used this assay to evaluate capacities of baboon PBMC to respond to agonistic or superagonist anti-CD28 stimulation and perform comparison with humans. First, we confirmed that human PBMC cultured over human umbilical vein endothelial cell (HUVEC) monolayers vigorously proliferated and secreted diverse pro-inflammatory cytokines, including interferon (IFN) $\gamma$ , tumor necrosis factor (TNF), interleukin (IL)-2, IL-4, IL-5, and IL-6, in a dose-response manner in response to superagonist (clone ANC28.1) anti-CD28 mAbs (Fig. 2). In contrast, conventional agonist (clone CD28.2) anti-CD28 mAbs induced moderate cytokine secretion in this assay and did not induce lymphocytes proliferation. Surprisingly, anti-CD28.2 mAbs induced IL-12p70 (p35 + p40 subunits) secretion mainly at lower concentration, while superagonist anti-CD28 mAbs did not. This induction was not the result of possible endotoxin contamination in the antibody preparation, which was similar to the background of the culture medium. As expected, antagonist Fab' anti-CD28 fragments (FR104) did not elicit any cytokines release or T lymphocytes proliferation (Fig. 2).

We then evaluated the response of baboon PBMC in the same HUVEC monolayers co-culture assay. Baboon PBMC responded

very similarly to human cells in regard to lymphoproliferation, since superagonist anti-CD28 mAbs induced important proliferation in a dose-response manner, while agonistic anti-CD28 mAbs did not (Fig. 3). However, although both agonistic and superagonist anti-CD28 mAbs induced measurable cytokine (IFN $\gamma$ , TNF, IL-6 and IL-12 or IL-12/IL-23p40) secretion at 24 or 48 h of co-culture in a dose-response manner, the magnitude of the release with baboon PBMC was overall weaker than with human cells. As observed with human PBMC, agonistic anti-CD28 antibody did not elicit measurable IL-2 release with baboon PBMC.

### Trans vivo comparison of human vs baboon T cells responses to anti-CD28 mAbs

We recently described that humanized mice (immunodeficient mice reconstituted by human PBMC injection: HuPBL mice) models enable evaluation and detection of superagonist anti-CD28 mAbs activity by measuring T lymphocytes activation markers and pro-inflammatory cytokines release in vivo.<sup>7</sup> To confirm in vivo that baboon PBMC still respond to superagonist anti-CD28 mAbs, we conducted similar experiments in immunodeficient non-obese diabetic/severe combined immunodeficiency (NOD/SCID) IL-2 $\gamma$  knockout (NSG) mice reconstituted with freshly isolated baboon PBMC. We observed that baboon PBMC engrafted with a lower efficiency than human PBMC in NSG mice. Indeed, two weeks after infusion of  $50 \times 10^6$  human PBMC, human T cell engraftment reached  $75.2 \pm 7.7\%$  in the blood (Fig. 4A). In contrast, although some mice reconstituted with baboon PBMC showed significant (> 10%) T lymphocytes engraftment one week after infusion, engraftment at two weeks stayed moderate ( $17.7 \pm 11.1\%$ ) (Fig. 4B). Considering the low number of blood leukocytes in naïve NSG



**Figure 3.** Proliferation and cytokine release of baboon PBMC co-cultured with human endothelial cells in response to anti-CD28 mAbs. (A) Proliferation at 72 h, (B) IFN $\gamma$ , (C) TNF, (D) IL-2, (E) IL-6 and (F) IL-12/23p40 release at either 24 and 48 h after addition of excipient (0), 0.5, 5 or 50  $\mu$ g/ml of divalent agonist anti-CD28.2 and superagonist anti-CD28 ANC28.1 mAbs. Data are mean  $\pm$  SEM of 4 different blood donors.

mice ( $120 \pm 20 \times 10^3$  cells/ml,  $n = 57$ , data not shown), we calculated that baboon blood T lymphocytes count was 14 times lower ( $26 \pm 15 \times 10^3$  cells/ml) compared with human T cell count ( $364 \pm 10 \times 10^3$  cells/ml) two weeks after reconstitution.

Two weeks after PBMC injection, these mice were randomly assigned to a treatment group consisting of a single injection of either superagonist anti-CD28 mAbs at 1 mg/kg or FR104 at 5 mg/kg or an equivalent volume of excipient. Injection of a superagonist anti-CD28 mAbs, but not of FR104, significantly increased the frequency of CD69 $^+$  and CD25 $^+$  activated T lymphocytes in the spleen or blood of NSG mice reconstituted by human or baboon PBMC (Fig. 4). Similarly, whereas no difference could be observed in term of cytokines secretion between mice treated with FR104 or excipient, administration of a superagonist anti-CD28 mAbs resulted in a rapid (one to four hours after injection) and significant release of pro-inflammatory cytokines measured in the plasma of NSG mice reconstituted by human or baboon PBMC (Fig. 4). However, the level of baboon cytokines was significantly lower than human cytokines (even at T0 before administration of treatment), in accordance with the lower engraftment of baboon T cells in these mice.

#### In vivo immunotoxicity evaluation of FR104 in baboon

Three baboons were injected once intravenously with FR104 at 20 mg/kg and immunotoxicity parameters were compared with two baboons that received an equal volume of excipient. The pharmacokinetics and pharmacodynamics analysis showed that C-max concentration in sera reached between 160 to 542  $\mu$ g/ml within one hour. The  $T\beta_{1/2}$  elimination half-life ranged between 8.6 and 9.9 d (mean: 9.3 d) and receptor occupancy in

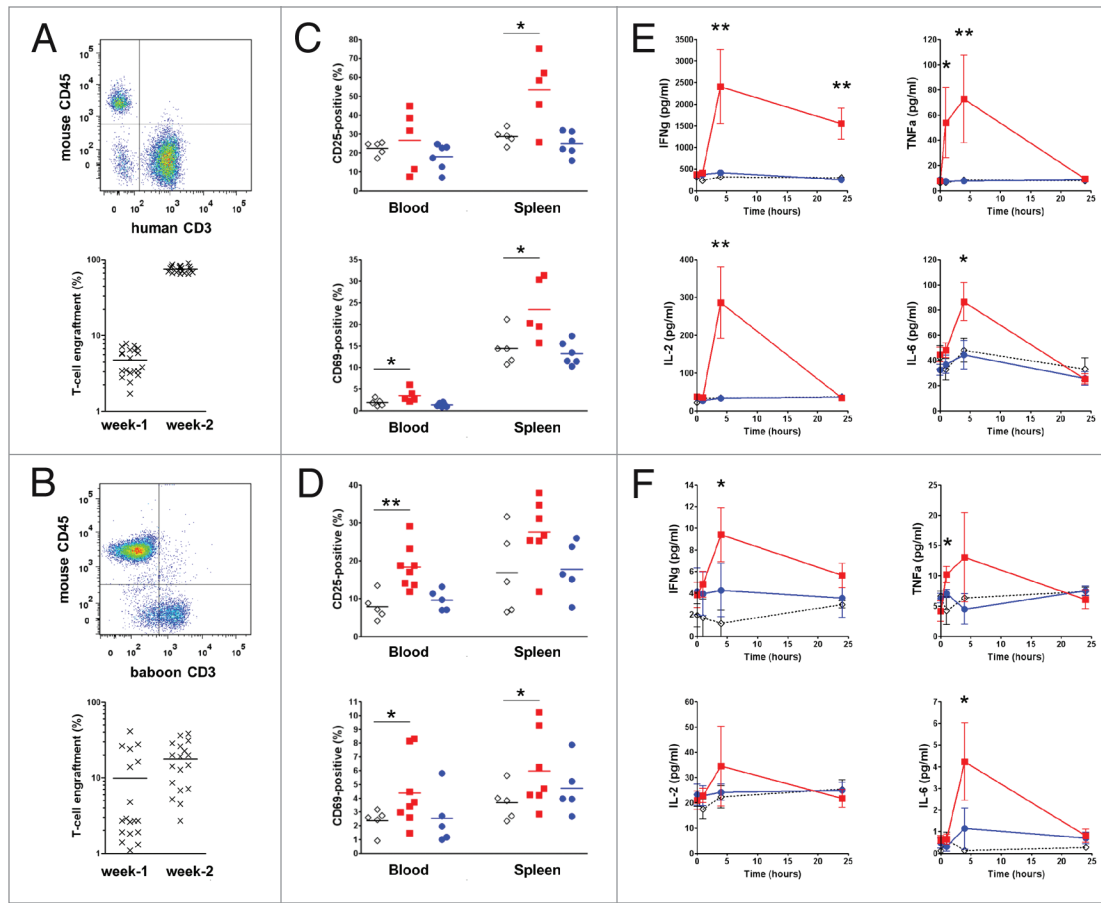
periphery was maintained at 100% over two months, as long as through levels were above 1  $\mu$ g/ml (Fig. 5A).

Proinflammatory cytokines (IFN- $\gamma$ , TNF, IL-2, IL-4, IL-5 and IL-6) remained undetectable (under the limit of detection: 13.2 pg/ml for IFN- $\gamma$ , 1.6 pg/ml for TNF, 14.4 pg/ml for IL-2, 3.6 pg/ml for IL-4, 1.2 pg/ml for IL-5) in all recipient baboons that received FR104 or excipient (Fig. 5B), with the exception of IL-6, which was detected at low level in the sera of all animals four hours after intravenous (IV) injections (levels ranging from 1.1 to 84.7 pg/ml), and therefore probably related to the procedure, including anesthesia.

Finally, we did not observe clinical symptoms of cytokine release syndrome based on hematology (Fig. 5C-F), body temperature, blood pressure, cardiac frequency and oxygen saturation (Fig. 5G-J) parameters, which all stayed within physiological ranges for both FR104 and excipient treated baboons. It should be noted that for two baboons (one treated with FR104, one with excipient), we experienced a fault in a warming electric blanket (used for the first two hours of general anesthesia to avoid hypothermia), explaining a significant drop in body temperature during this period.

#### In vitro immunotoxicity assessment in the presence of anti-drug antibodies

Due to overall concerns related to mAbs immunogenicity,<sup>29</sup> we investigated if creating immune complexes and cross-linking monovalent anti-CD28 mAbs with anti-drug antibodies (ADA) would modify monovalent-related antagonist properties and induce proliferation or cytokines release of human PBMC. We purified IgG from several ADA-positive sera from two baboons



**Figure 4.** In vivo stimulation of human or baboon PBMC with anti-CD28 mAbs in NSG mice. One representative flow cytometry analysis (upper panel) and quantification of engraftment (low panel) of (A) human (n = 20) and (B) baboon (n = 18) cells in NSG mice two weeks after PBMC transfer. Each mark represents an individual mouse. (C, D) Percentage of activated human and baboon T lymphocytes expressing CD25 and CD69 among CD3<sup>+</sup> cells in recipient blood and spleen 48 h after drug injection. Two weeks after PBMC transfer, mice received either 50  $\mu$ g i.p. of superagonist anti-CD28 ANC28.1 mAb (red square symbols; n = 5 for human and n = 7 for baboon), 150  $\mu$ g of humanized pegylated Fab' fragment FR104 (blue dots; n = 6 for human and n = 5 for baboon) or an equivalent volume of excipient (open diamond; n = 5 for human and baboon). Each symbol represents an individual mouse. Horizontal bars indicate the mean. (E) Human and (F) baboon cytokine release after treatment in same experiments as in C and D; data are means  $\pm$  SEM. \* $P$  < 0.05 and \*\* $P$  < 0.01 compared with control conditions. Cumulative data were obtained from three independent series of experiments using three different human and three baboon blood donors.

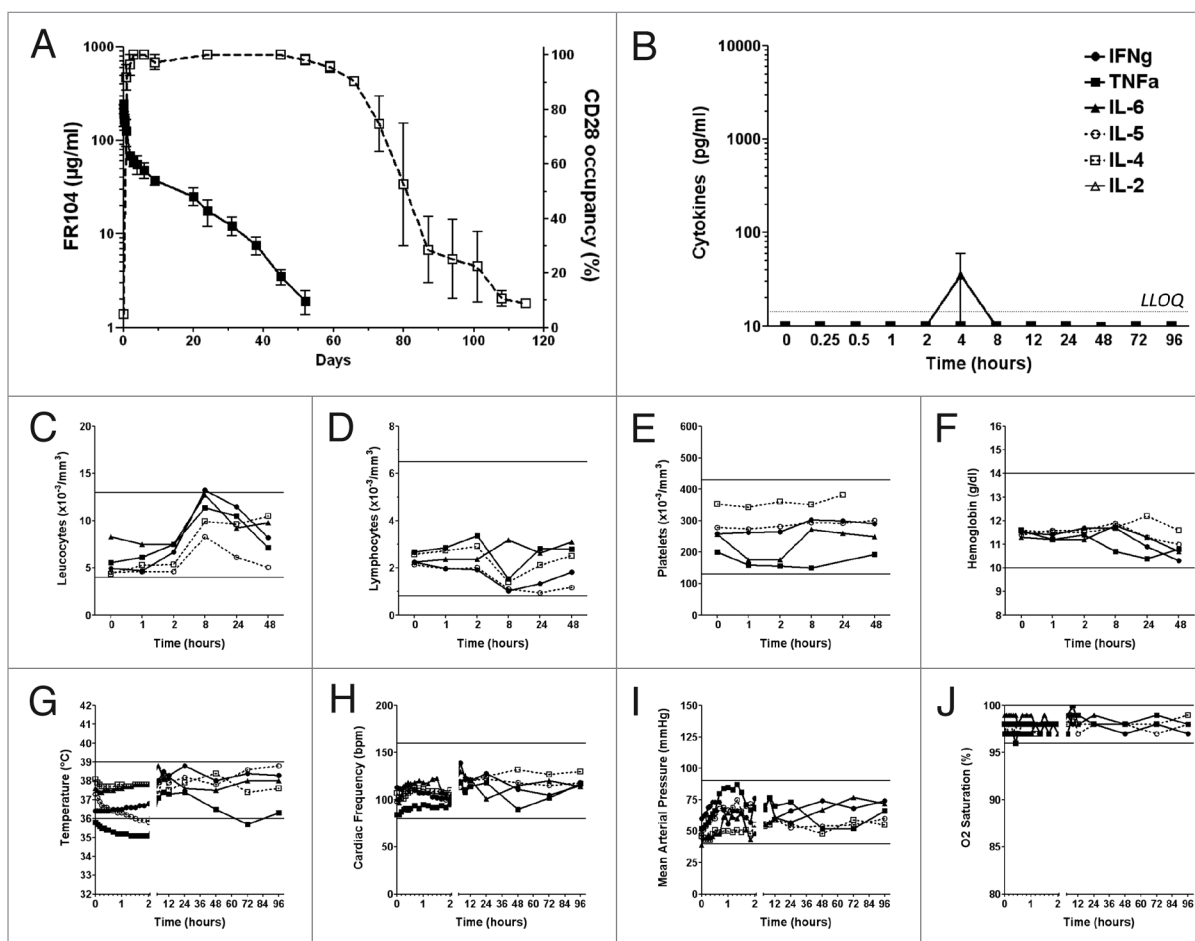
(titers determined by ELISA were 1/800 for ADA#1+ and 1/50 for ADA#2+ sera; data not shown) previously exposed to FR104. In parallel, we purified IgG from a pool of sera derived from up to 10 naive baboons as negative control (ADA-). Importantly, we verified that ADA IgG had conserved their anti-FR104 activity after purification (data not shown).

To assess potential agonist properties monovalent anti-CD28 mAbs could acquire in the presence of ADA, we added antibodies to anti-CD3 stimulated human PBMC cultured in polypropylene plate that we previously described as the most stringent assay to detect agonist properties.<sup>7</sup> As expected, agonist as well as superagonist anti-CD28 mAbs increased lymphoproliferation and cytokines release, more particularly IFN $\gamma$  for agonist mAbs (Fig. 6A). In contrast, addition of FR104 had no effect even in the presence of purified IgG ADA+ at a concentration up to 1.3 mg/ml (Fig. 6B). Notably, at very high concentration (1.3 mg/ml) polyclonal immunoglobulins exercised immunoregulatory function, observed also in the absence of FR104 (data not shown).

## Discussion

Selective CD28 antagonists have demonstrated superior efficacy over CD80/86 antagonists in experimental preclinical models of transplantation and autoimmune diseases.<sup>3,8</sup> However, such selective antagonists have not yet been formerly evaluated in the clinic due to the difficulty of assessing the innocuity of antagonist anti-CD28 mAbs, in relation to the deleterious effects of superagonists anti-CD28 mAbs in humans.

A series of sensitive immunotoxicity assays, including mAb immobilization, culture of T cells at high density, evaluation in whole blood or co-culture assays with endothelial cells, have been developed and characterized to avoid a repeat of the TGN1412 incident with new therapeutics.<sup>25,26</sup> Following these recommendations, evaluation of a humanized monovalent pegylated anti-CD28 Fab' fragment (FR104) did not show induction of T lymphocytes proliferation or cytokines release, even in the presence of polyclonal activation (ex. anti-CD3).<sup>7,19</sup>



**Figure 5.** Immunotoxicity evaluation of FR104 after intravenous injection in baboon. (A) Serum concentration (black square; left y-axis) of FR104 and receptor occupancy (open square; right y-axis) on blood T lymphocytes measured by flow cytometry after a single intravenous administration at 20 mg/kg in three different baboons. Data are mean  $\pm$  SEM (B) Serum concentration of indicated cytokines measured after FR104 injection in the three animals described in (A). Data are mean  $\pm$  SEM. Dotted line represents the highest lower limit of quantification (LLOQ). (C) leukocytes count, (D) lymphocytes count, (E) platelets count, (F) hemoglobin count, (G) body temperature, (H) cardiac frequency, (I) mean arterial pressure and (J) oxygen saturation recorded after FR104 injection in the three animals described in (A; black symbols and solid lines) as well as in two other excipient-treated baboons (open symbols and dotted lines). Each animal is represented by a different symbol. The horizontal lines indicate the range of normal values in baboons.<sup>39</sup>

Using the co-culture assay with endothelial cell, Findlay et al.<sup>28</sup> described that CD28.2 (an agonist anti-CD28 mAb that has no superagonist activity) did not induce T-cell proliferation or cytokine release. In our study, we confirmed the absence of induced proliferation with CD28.2, but recorded significant levels of IFN $\gamma$ , TNF, IL-6 and IL-12 release. Otherwise, whereas the severity of the adverse response of TGN1412 was correlated with the level of IL-2 release,<sup>30</sup> we observed that level of IFN $\gamma$  release could be more predictive of agonist properties of divalent anti-CD28 mAbs.

In spite of these sensitive assays, current non-human primate models used for preclinical toxicological evaluation are not predictive of clinical outcome in man, in part because they use monkeys, which do not express CD28 on a significant part of their T<sub>EM</sub> cells, which are cells that rapidly secrete cytokines after stimulation. For that purpose, we developed a trans vivo model where severe immunodeficient mice (NSG) were infused with human PBMC.<sup>7</sup> NSG mice reconstitution by human PBMC,

although incomplete, allowed pertinent mAbs immunotoxicity evaluation in vivo on human cells, since early engraftment was predominantly composed of human CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes as well as natural killer (NK) cells. Furthermore, in contrast to conventional in vivo models in naïve animals, in this humanized mice model, human T lymphocytes undergo proliferation and are activated due to homeostasis and xenoreactivity leading to a florid graft-vs.-host-disease.<sup>7</sup> Indeed, besides superagonist anti-CD28 mAbs, other therapeutics known to induce CRS in some patients, such as alemtuzumab (anti-CD52), rituximab (anti-CD20) and muromonab-CD3 (OKT3), were described to stimulate cytokine release from NK cells and T lymphocytes.<sup>20,25</sup> In this trans vivo model, the humanized monovalent pegylated anti-CD28 Fab' fragment (FR104) remained antagonist, while superagonist or agonist divalent anti-CD28 mAbs were able to induce both T lymphocytes activation and cytokine release.

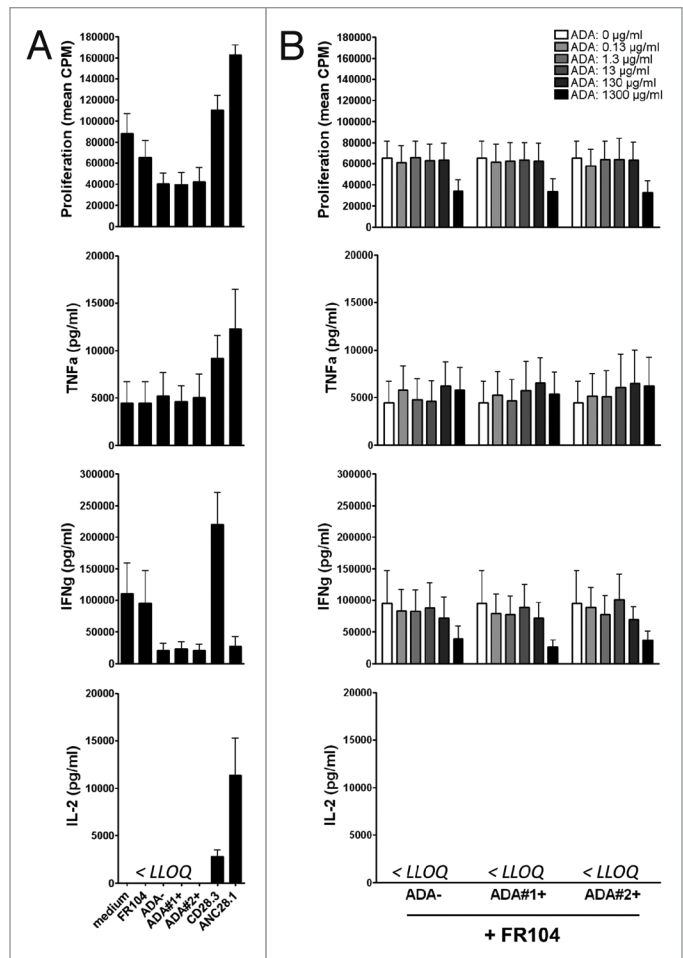
Even though in vitro assays and humanized rodents are helpful, in vivo immunotoxicity evaluation in a relevant

species remains most certainly the best predictive assessment.<sup>31</sup> Concerning TGN1412, whereas binding affinity for CD28 was similar between cynomolgus macaques and humans,<sup>21,32,33</sup> PBMC from macaques were not stimulated in vitro by immobilized TGN1412, contrary to results with human cells.<sup>24,34</sup> It was first suggested that the differences could be due to the loss of Siglecs inhibitory molecules during human evolution,<sup>35</sup> but Richard Stebbings group later reported that CD4<sup>+</sup> effector memory T cells were the predominant source of cytokine release after TGN1412 infusion, and that macaque CD4<sup>+</sup> T cells, but not human counterparts, lose CD28 expression during their differentiation into memory cells.<sup>20</sup> Whereas it is now obvious that cynomolgus or rhesus macaques cannot constitute relevant species for preclinical immunotoxicity evaluation of emerging drugs targeting CD28, these animals still continue to be used for that purpose.<sup>36</sup>

In this study, we found that the majority of baboon CD4<sup>+</sup> memory T cells expressed CD28, similar to humans. Furthermore, we observed in vitro and trans vivo that baboon PBMC were activated, proliferated and secreted pro-inflammatory cytokines in response to agonist or superagonist anti-CD28 mAb stimulation. Baboon T lymphocytes, however, quantitatively released less cytokines compared with human cells in the same co-culture assay. This could first be explained by altered molecular interactions between baboons PBMC and xenogeneic human endothelial cells, important for anti-CD28-mediated T cells activation, such as ICOS/ICOSL.<sup>37</sup> Second, an inhibitory Siglecs molecule lost during human evolution<sup>35</sup> could probably explain why human PBMC seem more prone to produce cytokines in response to anti-CD28 mAb stimulation. In spite of a lower response than in the human system, our study illustrated that assays with baboon cells can be predictive of potential immunological toxicity of anti-CD28 mAbs, and increases the relevance of data showing the presence or absence of cytokines released in the serum of baboons after infusion of an anti-CD28 mAb, since it is difficult due to ethical considerations to consider ANC28.1 or TGN1412 injection to baboon as positive controls. Of note, administration of FR104, a drug candidate antagonist of CD28, to baboons did not induce detectable cytokine release and no clinical signs of CRS.

Immunogenicity and development of ADA is another safety concern when developing monovalent antagonists. To circumvent the lack of representativity of anti-PEG or anti-Vk antibodies that have been recently used to address that problem,<sup>36</sup> we purified IgG from baboons immunized with FR104 and containing ADA and added these antibodies to human cells activated with anti-CD3 and FR104. In these experiments, adding ADA-positive or ADA-negative IgG at either low (0.13 µg/ml), moderate or very high concentration (1.3 mg/ml) did not modify antagonist properties of FR104. This suggested that creating immune complexes of monovalent Fab' fragments with ADA does not reproduce the agonist effect of dimeric IgG anti-CD28 antibodies on T cells, an effect that might be of conformational origin.<sup>19</sup>

In conclusion, we reported that *Papio anubis* can be considered an elective species for the preclinical assessment of immunotoxicity of therapeutic anti-CD28 mAbs. Moreover,



**Figure 6.** In vitro response of human PBMC to antagonist anti-CD28 mAbs and ADA. Human PBMC were stimulated with anti-CD3 antibodies and different types of anti-CD28 mAbs plus indicated concentrations of IgG containing ADA. (A) Proliferation at 72 h and cytokine release at 48 h in the presence of excipient, FR104 (10 µg/ml), divalent agonist anti-CD28.3 mAbs (10 µg/ml), superagonist anti-CD28 ANC28.1 mAbs (10 µg/ml), ADA-negative purified IgG (1.3 mg/ml), or ADA-positive purified IgG from two different baboons (1.3 mg/ml). (B) Same experiments as in (A) where human PBMC were cultured with 10 µg/ml of FR104 and increasing concentrations of ADA-negative or ADA-positive purified IgG (ranging from 0 to 1300 µg/ml as indicated in the upper panel). Data are means ± SEM of 3 different human blood donors. LLOQ, Lower limit of quantification.

immunodeficient mice reconstituted by human PBMC provide a suitable complementary model by allowing evaluation in vivo, directly on human target-expressing cells, of agonist or superagonist properties of anti-CD28 mAbs. Any translation of a novel CD28 antagonist into clinical application should first take these models into consideration.

## Materials and Methods

### Flow cytometry

Fluorescent mAbs against mouse CD45 (30-F11) and human CD3 (SP34-2), CD4 (L200), CD8 (RPA-T8), CD25 (M-A251), CD28 (28.6), CD69 (FN50) and CD95 (DX2) were from BD

Biosciences. FR104 staining was performed with a polyethylene glycol (PEG) rabbit mAb (Epitomics) followed by a fluorescent goat anti-rabbit IgG (Invitrogen). CD28 receptor occupancy by FR104 on T lymphocytes was determined by performing the ratio of median fluorescent intensity (MFI) of FR104 staining between an unmodified blood sample and a blood sample incubated for 30 min at room temperature with a saturating concentration of FR104 (5  $\mu\text{g/ml}$ ). Samples were acquired on a BD FACS CANTO™ flow cytometer (BD Bioscience) and analyzed with FlowJo software.

#### Endothelial cell co-culture assay

Human umbilical vein endothelial cell (HUVEC), used between the second and fifth passage, were originally obtained from Lonza and cultured in Endothelial Cell Basal Medium (Promocell) supplemented with 10% heat-inactivated human AB sera, 0.4% endothelial cell growth supplement/heparin (Promocell), hydrocortisone (1  $\mu\text{g/ml}$ , Promocell), human basic fibroblast growth factor (1 ng/ml, Promocell), human epidermal growth factor (0.1 ng/ml, Promocell), 100 U/ml penicillin (Life Technologies) and 0.1 mg/ml streptomycin (Life Technologies) at 37 °C in a 5% CO<sub>2</sub> humidified air incubator, as previously described.<sup>38</sup> HUVEC were seeded at  $20 \times 10^3$  cells/well in 96-well flat-bottomed microtiter plates (Nunc) precoated with 1% gelatin (Sigma-Aldrich). After 24 h of HUVEC culture, supernatants were aspirated and  $125 \times 10^3$  PBMC (from healthy human or baboon donors) isolated by density gradient centrifugation, were added to the HUVEC monolayers and cultured in complete medium (RPMI-1640 medium supplemented with L-glutamine, sodium pyruvate, Hepes, antibiotics, all from Gibco) supplemented with either 2% heat-inactivated human AB sera or 2% heat-inactivated baboon sera. Superagonist anti-CD28 mAb (ANC28.1, Calbiochem), divalent IgG anti-CD28 mAb (CD28.2, BD Biosciences), monovalent anti-CD28 Fab' fragment (FR104, from Effimune as previously described<sup>7</sup>) or its excipient were added from day 0 of the co-culture. Fifty  $\mu\text{l}$  supernatant were collected from each triplicate at 24 and 48 h and then analyzed for cytokines concentration using ProcartaPlex™ multiplex immunoassays (Affymetrix) specific for human or non-human primate cytokines. At day 3 of co-culture, cells were pulsed with 1  $\mu\text{Ci}$  of <sup>3</sup>H-thymidine during the final eight hours of culture and proliferation was evaluated in a scintillation counter.

#### Anti-CD3 biological assay

$10^5$  human PBMC from healthy volunteers were cultured in 96-well U-bottomed microtiter plates (Nunc) precoated with 1  $\mu\text{g/ml}$  of anti-CD3 antibody (OKT3) in complete medium (described above) supplemented with 2% heat-inactivated human AB sera, in the presence of 10  $\mu\text{g/ml}$  of superagonist anti-CD28 mAb (ANC28.1, Calbiochem), divalent IgG anti-CD28 mAb (CD28.2, BD Biosciences), monovalent anti-CD28 Fab' fragment (FR104, Effimune) or 1300  $\mu\text{g/ml}$  of Protein G purified IgG from control baboons or from baboon sera previously immunized against FR104. To study the possibility of Fab' fragment crosslinking by ADA, the same purified baboon IgG preparations were added at either 1300, 130, 13, 1.3 or 0.13  $\mu\text{g/ml}$  in wells already containing FR104 at 10  $\mu\text{g/ml}$ . 25  $\mu\text{l}$

supernatant was collected at 48 h of culture to measure IFN $\gamma$ , TNF and IL-2 concentration with human BD OptEIA Elisa Kits (BD Biosciences). At day 3 of co-culture, cells were pulsed with 1  $\mu\text{Ci}$  of <sup>3</sup>H-thymidine during the final 8 h of culture and proliferation was evaluated in a scintillation counter.

#### Animals and treatments

Seven to ten week old IL-2 $\gamma$  knockout mice (Charles River) were irradiated (2 Gy) and infused intraperitoneally (i.p.) with  $50 \times 10^6$  freshly isolated human or baboon PBMC from healthy donors, as previously described.<sup>7</sup> Mice were then maintained in aseptic conditions and were monitored every week for T-lymphocytes engraftment in the blood. Two weeks after PBMC injection, mice were treated once i.p. with 50  $\mu\text{g}$  of superagonist anti-CD28 (ANC28.1), 150  $\mu\text{g}$  of anti-CD28 Fab' fragment (FR104), or equivalent volume of excipient. Retro-orbital blood samples were performed before, 1, 4 and 24 h after drug injection and human or baboon cytokines concentration in mouse sera was determined by CBA Flex (BD Bioscience). T-lymphocytes activation was analyzed by flow cytometry on blood and spleen cells harvested 48 h after antibody injection. Baboons (*Papio anubis*, from the CNRS Primatology Center, Rousset, France), were housed at the large animal facility of our laboratory. All experiments were performed under general anesthesia with Zoletil (Virbac, Carros, France) followed when necessary by ventilation with a mixture of nitrous oxide, oxygen and isoflurane (Forène, Abbot). Three animals received a single IV injection of FR104 at 20 mg/kg and two others animals received an equivalent volume of excipient. Blood samples were drawn at different time point for receptor occupancy, FR104 concentration, hematological and biochemical analyses, as well as cytokines concentration measured by BD CBA Non-human Primate Th1/Th2 Cytokine Kit (BD Biosciences). All experiments in mice and non-human primates were performed in accordance with the recommendations of the Institutional Ethical Guidelines of the "Institut National de la Santé Et de la Recherche Médicale" (France).

#### Statistical analyses

All variables were expressed as mean  $\pm$  SEM and were compared when appropriated using the Mann-Whitney non parametric test. P values less than 0.05 were considered statistically significant. All statistical analyses were performed with GraphPad Prism (GraphPad Software).

#### Disclosure of Potential Conflicts of Interest

NP, CM and BV are shareholders and employees of Effimune, a company developing CD28 antagonists.

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## Author contributions

NP designed, organized and performed in vitro and in vivo experiments, analyzed and interpreted the data and prepared the manuscript. CM and SLB performed in vitro experiments on human cells. VD and LB performed in vivo experiments on

mice. MC performed flow cytometry experiments. JH and DM assisted with baboon experiments. SV and VC performed in vivo experiments on baboons. GB organized and supervised baboon experiments. BV funded the research, designed experiments, analyzed and interpreted the data and edited the manuscript.

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