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## OPEN

# Homogeneous Expansion of Human T-Regulatory Cells Via Tumor Necrosis Factor Receptor 2

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T-regulatory cells ( $T_{\text{regs}}$ ) are a rare lymphocyte subtype that shows promise for treating infectious disease, allergy, graft-versus-host disease, autoimmunity, and asthma. Clinical applications of  $T_{\text{regs}}$  have not been fully realized because standard methods of expansion *ex vivo* produce heterogeneous progeny consisting of mixed populations of CD4<sup>+</sup> T cells. Heterogeneous progeny are risky for human clinical trials and face significant regulatory hurdles. With the goal of producing homogeneous  $T_{\text{regs}}$ , we developed a novel expansion protocol targeting tumor necrosis factor receptors (TNFR) on  $T_{\text{regs}}$ . In *in vitro* studies, a TNFR2 agonist was found superior to standard methods in proliferating human  $T_{\text{regs}}$  into a phenotypically homogeneous population consisting of 14 cell surface markers. The TNFR2 agonist-expanded  $T_{\text{regs}}$  also were functionally superior in suppressing a key  $T_{\text{reg}}$  target cell, cytotoxic T-lymphocytes. Targeting the TNFR2 receptor during *ex vivo* expansion is a new means for producing homogeneous and potent human  $T_{\text{regs}}$  for clinical opportunities.

T-regulatory cells ( $T_{\text{regs}}$ ) are a small subset of T-lymphocytes with diverse clinical applications in transplantation, allergy, infectious diseases, GVHD, autoimmunity, cancer, among others<sup>1–10</sup>. One fundamental problem stymieing their clinical development is their relative paucity: naturally occurring  $T_{\text{regs}}$  constitute only 1–5% of total CD4<sup>+</sup> T cells in blood, and remain largely dormant until activated. Their expansion is therefore crucial for harvesting sufficient quantities to investigate their roles in basic biology and clinical medicine<sup>11,12</sup>. Standard methods of  $T_{\text{reg}}$  expansion<sup>13–29</sup> are conducted *ex vivo* for reinfusion into patients because the expansion agents are too toxic for *in vivo* administration. Four expansion agents are commonly used alone or in various combinations: IL-2, anti-CD3, anti-CD3 plus anti-CD28, and rapamycin. However, those standard agents are problematic because they produce heterogeneous progeny consisting of phenotypically and functionally mixed populations of CD4<sup>+</sup> T cells. Heterogeneous CD4<sup>+</sup> T cell populations hold risk because they are capable of releasing pro-inflammatory cytokines, and they possess cells with diverse, sometimes antagonistic functions. Heterogeneous populations are also deemed by regulatory agencies as impure and irreproducible, impeding the advance of human clinical trials. Thus a major research goal has been to find new ligands to selectively expand  $T_{\text{regs}}$  into homogeneous progeny.

In humans,  $T_{\text{regs}}$  are defined by co-expression of CD4<sup>+</sup> and high expression of the interleukin-2 (IL-2) receptor alpha chain CD25<sup>hi</sup>.  $T_{\text{regs}}$  also feature inducible levels of intracellular transcription factor forkhead box P3 (FOXP3)<sup>30,31</sup>. Here we chose to focus on TNF and its receptors on  $T_{\text{regs}}$ . While animal studies indicate that TNF induces proliferation of  $T_{\text{regs}}$ , the evidence in humans, both *in vivo* and *in vitro*, is conflicting and the topic for review articles attempting to explain the contradictory literature<sup>32,33</sup>. It has been speculated that the conflicting findings could be due in part to the manufacturing problems with TNF and its tendency in culture to aggregate into different complexes, perhaps with different signaling consequences<sup>34</sup>. Also TNF has two receptors, TNFR1 and TNFR2, each of which controls different signaling pathways. Unlike TNFR1, which has ubiquitous cellular expression, TNFR2 is expressed in a more limited manner, restricted primarily to subpopulations of T cells, endothelial cells, and neurons<sup>35</sup>. Research in primates suggests that TNFR2-specific ligands are likely to have minimal systemic toxicity because of TNFR2's restricted cellular distribution<sup>36</sup>. Naturally occurring  $T_{\text{regs}}$  appear to express TNFR2 at a higher density than TNFR1<sup>37–39</sup>. These features might make human TNFR2 an advantageous molecular target on  $T_{\text{regs}}$ .

We began by examining whether soluble TNF could expand human  $T_{\text{regs}}$  *in vitro*. Once we determined that TNF could successfully expand them, we then used monoclonal antibodies to tease apart the roles of TNFR1 and



TNFR2. We studied the newly screened antibodies' effects on  $T_{reg}$  proliferation, signaling, phenotype, and function by multiple *in vitro* assays using isolated fresh human CD4 T cells from over 500 donors. In these experiments, our purpose was to compare performance of our TNF antibodies against standard methods of  $T_{reg}$  expansion. Once we found that one TNFR2 monoclonal antibody could expand  $T_{regs}$  into a homogeneous population with potent functional capacity, we sought to extend our findings to humans with a small randomized, controlled clinical trial with the TNF-inducer Bacillus Calmette-Guerin (BCG), an approved drug.

## Results

**Functional effects of TNF and TNFR monoclonal antibodies on  $T_{regs}$ .** The purpose of this study was to use four standard methods for  $T_{reg}$  expansion and examine the effects of adding TNF or TNF receptor antibodies to the culture. We sought to improve the purity and potency of the expanded  $T_{regs}$ , perhaps creating a preparation of cells more suitable for human trials. The four expansion protocols used as a screening tool for monoclonal antibodies were tests with human T cells with a) IL-2 expansion, b) anti-CD3 expansion, c) anti-CD3 and anti-CD28 expansion, and d) anti-CD3 and anti-CD28 expansion with rapamycin, all with the absence or addition of TNF and newly screened or applied TNFR receptor monoclonal antibodies.

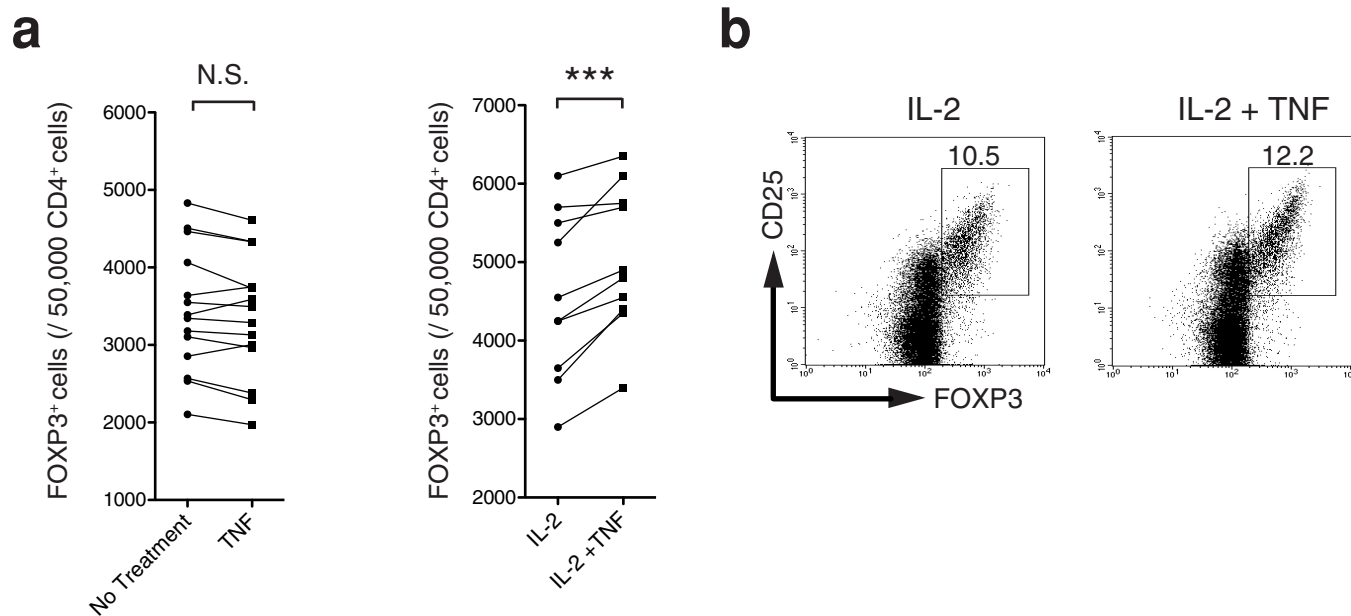
IL-2 is important for  $T_{reg}$  induction and maintenance in mice<sup>4</sup>. To gain some early appreciation of the human version of this cytokine on human cultured T lymphocytes, we first cultured freshly isolated human CD4+ cells from 14 human subjects only with TNF or IL-2 for 16 hours (Fig. 1). While finding no induction of  $T_{regs}$ , assessed by inducible FOXP3, we observed a significant increase in  $T_{regs}$  after adding IL-2 with TNF. This percentage increase in the numbers of  $T_{regs}$  was due to greater numbers of  $T_{regs}$ . Co-incubation of TNF and IL-2 produced a significant increase in  $T_{regs}$  over IL-2 alone (Fig. 1a). By flow cytometry, TNF and IL-2 co-incubation also increased the number of CD4 + CD25<sup>hi</sup> FOXP3 cells in cultured human cells from blood (Fig. 1b).

We first explored whether both TNF receptors were needed for the TNF effect. Because TNF signals through two receptors, we studied

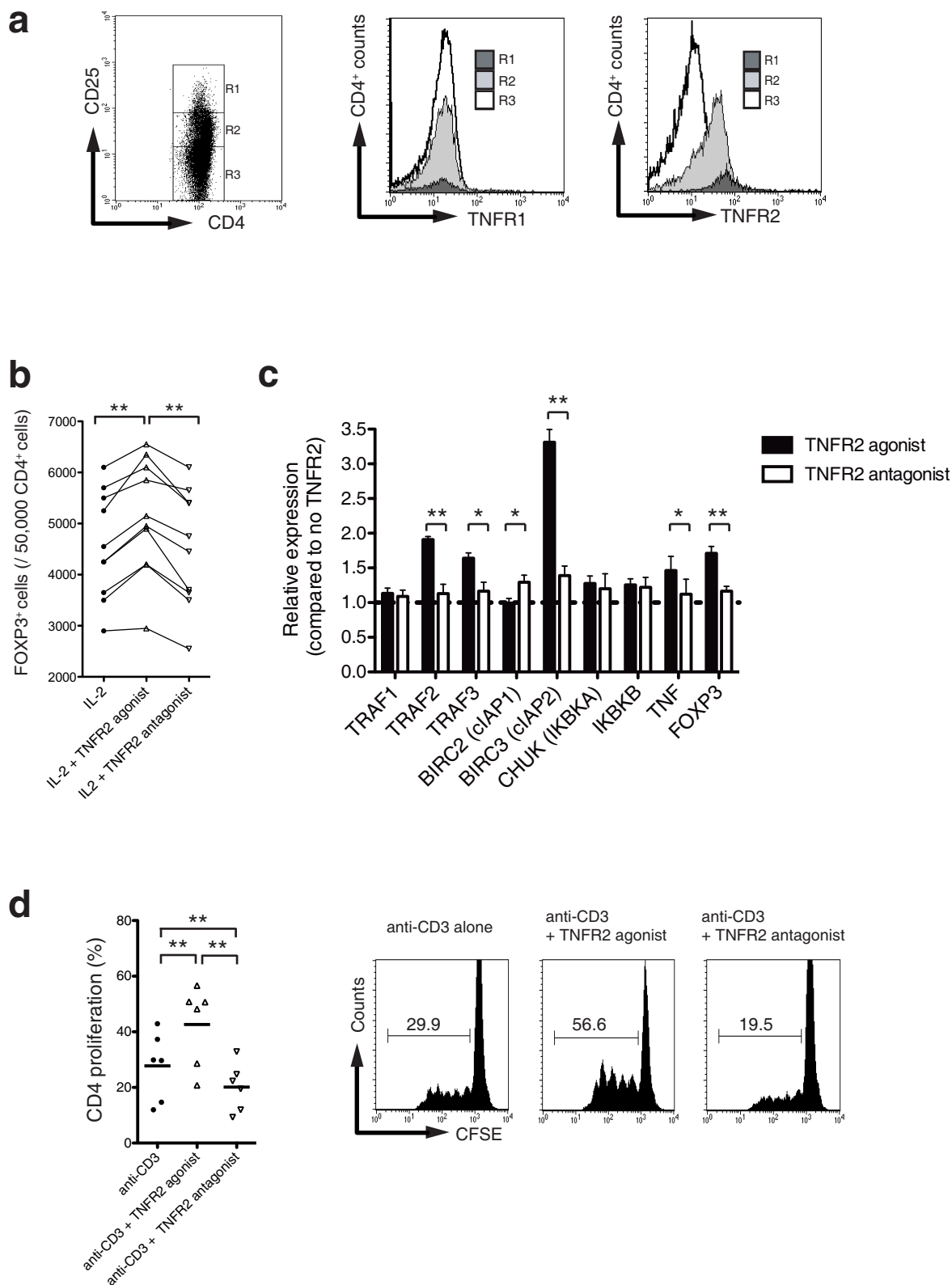
each TNFR receptor in isolation using newly produced and commercially available monoclonal antibody ligands to TNFR1 and TNFR2. Unlike cytokines that can be difficult to manufacture and have varying solubility, antibodies have excellent solubility. In freshly isolated CD4+ cells, we examined expression levels of each TNFR in relation to CD25+ expression. TNFR1 expression on CD4+ cells, regardless of CD25+ expression levels, was unchanged using flow cytometry (Fig. 2a, middle panel), whereas TNFR2 preferentially expressed CD4+CD25<sup>hi</sup>  $T_{regs}$  by nearly a factor of 10 (Fig. 2a, right panel). This confirms earlier studies that TNFR2 is more densely expressed on  $T_{regs}$ <sup>37–39</sup>.

Screening all available TNFR1 and TNFR2 monoclonal antibodies (mAbs) on isolated CD4+ cells sampled from fresh human blood enabled selective study of each TNF receptor, unlike studying TNF, which acts through both receptors and can have manufacturing and solubility problems stemming from the use of *E. Coli* and yeast systems. Although most of the newly produced and commercially available TNFR1 or TNFR2 mAbs failed to induce or suppress FOXP3+  $T_{regs}$  after stimulation by presence of IL-2 for 16 hours (Supplementary Figs. 1, 2), we eventually identified two TNFR2 mAbs with significant, yet opposing, effects on FOXP3 induction (Fig. 2b). Studying freshly cultured cells from 10 subjects, one TNFR2 antibody significantly induced FOXP3 expression in CD4+ human T cells and expanded these cells in culture (which we designated the “TNFR2 agonist”), whereas the other TNFR2 monoclonal had no benefit in augmenting intracellular FOXP3 expression and with these culture conditions prevented CD4 T cell proliferation (which we designated the “TNFR2 antagonist”) (Fig. 2b). The reason for the increases in FOXP3 with the newly identified TNFR2 agonist was almost entirely secondary to expansion of absolute  $T_{reg}$  cell numbers. There was no statistical difference between IL-2 alone and IL-2 plus TNFR2 antagonist in  $T_{reg}$  cell numbers.

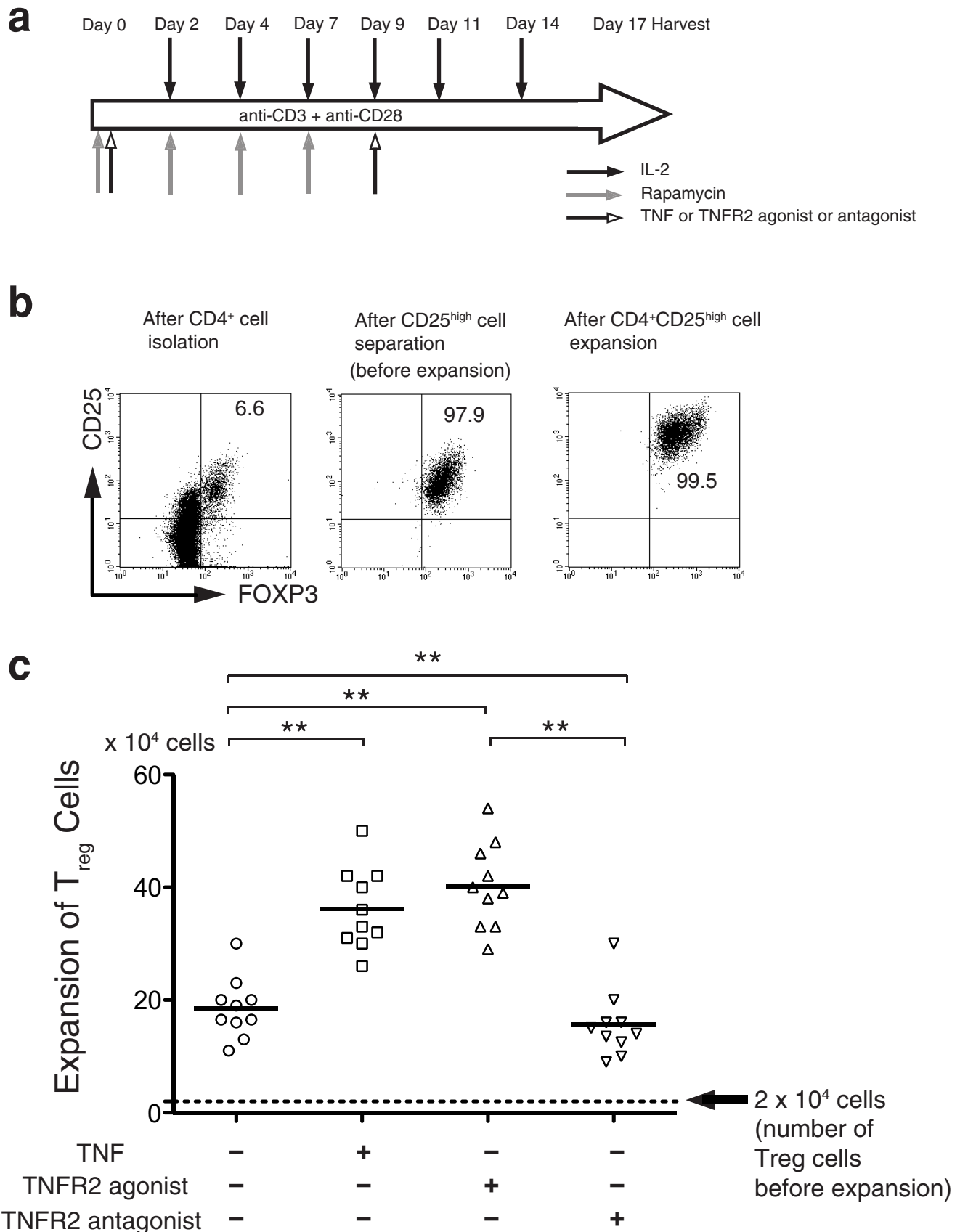
Having identified two functionally opposing TNFR2 mAbs, we measured their effects on isolated CD4+ T cells by examining downstream mRNA expression in signaling proteins specific to TNF receptor activation. After either 30 minutes or 24 hour stimulation



**Figure 1 | Human CD4+ T cells cultured with TNF and/or IL-2 and measured for FOXP3 expression.** (a) In freshly isolated CD4+ cells from human blood, TNF alone does not induce FOXP3 in culture, but does induce it to higher levels when co-incubated with IL-2, compared to IL-2 alone. (b) A representative flow histogram confirms greater intracellular induction of FOXP3 in CD4 + CD25<sup>hi</sup>  $T_{regs}$  after co-incubation with TNF and IL-2 than with IL-2 alone. The data in (a) are from 14 subjects (left panel) and 10 subjects (right panel). Figures in flow diagrams are %. (\*\*\*)  $P < 0.001$ , by paired t-test).



**Figure 2 | TNFR2 agonist- versus antagonist-treated  $T_{reg}$ s and opposing effects *in vitro*.** (a) TNFR2 is preferentially expressed on  $CD4 + CD25^{hi}$  T cells (b) one TNFR2 antibody induced the numbers of FOXP3, acting as an agonist, and the other TNFR2 antibody suppressed the numbers of FOXP3, acting as antagonist. (c) In a signaling pathway assay, purified  $CD4^{+}$  cells, incubated with IL-2, the TNFR2 agonist and antagonist trigger differences in relative downstream expression of mRNA, especially in signaling proteins TRAF2, TRAF3 and apoptosis inhibitor cIAP2 that are preferentially induced by TNFR2 agonism. Data represented are means  $\pm$  SEM from 4 subjects. (d) TNFR2 agonist triggers greater % increase in proliferation in samples from 6 subjects measured by flow cytometry (left panel) and with CFSE measurements (right panels), and representative results from a typical experiment are presented with CFSE measurements (right panels). The numbers in a bar represent the percentage of cells that enter division. The TNFR2 antagonist suppresses  $CD4^{+}$  proliferation (left panel) and inhibits expansion measured by CFSE dilution (right panel). (\* $P < 0.05$  or \*\* $P < 0.01$ , by paired t-test).



**Figure 3 | TNFR2 agonist versus antagonist on expansion of T<sub>reg</sub> population.** (a) Protocol for purifying CD4 + CD25<sup>hi</sup> cells from CD4<sup>+</sup> cells from fresh blood and expanding for 16 days by incubation in 96 well round-bottom plate ( $2 \times 10^4$  cells/well) with anti-CD3 and anti-CD28 antibodies, human IL-2, and rapamycin. (b) Representative CD25 and FOXP3 flow diagrams of CD4<sup>+</sup> cells before versus after CD25<sup>hi</sup> purification and expansion, indicating purity of populations. (c) Cell counts of purified T<sub>regs</sub>, by treatment group, reveal that TNFR2 agonist induced more expansion than any other group. (\*\* $P < 0.01$ , by paired t-test.) The TNFR2 antagonist suppresses expansion versus standard treatment. Data in (c) are samples from 10 subjects.



by the TNFR2 agonist or antagonist, relative mRNA expression was significantly different. The TNFR2 agonist stimulated expression of TNF, TRAF2, TRAF3, BIRC3 (cIAP2) and FOXP3 (24 hour data shown). In contrast, the TNFR2 antagonist stimulated expression of cIAP1, but not TRAF2, TRAF3 or FOXP3 (Fig. 2c). This was not only confirmed in four human subjects (Fig. 2c), but also with 32 additional human subjects with the TNFR2 agonist and an additional 8 subjects with the TNFR2 antagonist. Distinct intracellular signaling was triggered by the newly identified antibody agonist and the antibody antagonist to TNFR2 (Fig. S2b).

The effects of the TNFR2 agonist and antagonist were also studied on purified human CD4<sup>+</sup> T cells co-cultured with anti-CD3. When CD4<sup>+</sup> proliferation was studied with anti-CD3 combined with the TNFR2 agonist, the highest degree of proliferation occurred. This absolute expansion and proliferation was almost exclusively (85%) due to the expansion of T<sub>regs</sub>. In contrast, the TNFR2 antagonist suppressed CD4<sup>+</sup> proliferation relative to the control, anti-CD3 alone, and relative to anti-CD3 + TNFR2 antagonist (Fig. 2d, left-most panel). The same findings were observed after 4 days by directly measuring CD4<sup>+</sup> proliferation by flow cytometry and measuring CD4<sup>+</sup> proliferation by CFSE dilution (Fig. 2d, three right-most panels).

Despite high expression of TNFR2 on T<sub>regs</sub>, some TNFR2 expression is also observable on CD4<sup>+</sup> T cells that are not true T<sub>regs</sub> because they only express intermediate levels of CD25, i.e., CD4<sup>+</sup>CD25<sup>mid</sup> cells. We therefore studied the impact of overnight incubation on CD25<sup>mid</sup> cell subpopulations of IL-2 alone, IL-2 and TNF, or IL-2 and TNFR2 agonist, or IL-2 and TNFR2 antagonist. We found a rise in the proportion of CD25<sup>hi</sup>FOXP3<sup>-</sup> cells similar to effector cells with IL-2 and TNF stimulation alone, or IL-2 and TNFR2 agonist alone (data not shown). However, we observed suppression with IL-2 and TNFR2 antagonist relative to the other three groups. Therefore the TNFR2 agonist and antagonist, studied by the same assay, showed opposing effects on the same CD25<sup>+</sup>FOXP3<sup>-</sup> cell population.

We separated fresh human blood to obtain pure CD4<sup>+</sup> and CD25<sup>hi</sup> co-expressing T<sub>regs</sub> (Fig. 3). We purified and expanded these T<sub>regs</sub> *in vitro* using another standard method of T<sub>reg</sub> expansion, anti-CD3 plus anti-CD28 plus IL-2 for 16 days with or without TNF, TNFR2 antagonist, or TNFR2 agonist (Fig. 3a), then rested them overnight before counting. We added rapamycin (until day 7) because it selectively expands the highest number of T<sub>regs</sub> with greatest capacity for suppressing CD8<sup>+</sup> cells<sup>40–43</sup>. This process successfully produced CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub> (Fig. 3b). We assessed T<sub>reg</sub> expansion by treatment group: standard treatment, treatment with TNF, TNFR2 agonist, or TNFR2 antagonist. The TNFR2 agonist outperformed every other group, expanding T<sub>reg</sub> numbers at least twofold higher than standard treatment or antagonist treatment (Fig. 3c). Because rapamycin is known to inhibit proliferation, we examined the effects of treatment without rapamycin, yet found similarly opposing effects between agonist versus antagonist treatment, albeit at smaller mean absolute values (Supplementary Fig. 3). The yields of expanded cells tended to be less without rapamycin, but the agonist still expanded T<sub>regs</sub>.

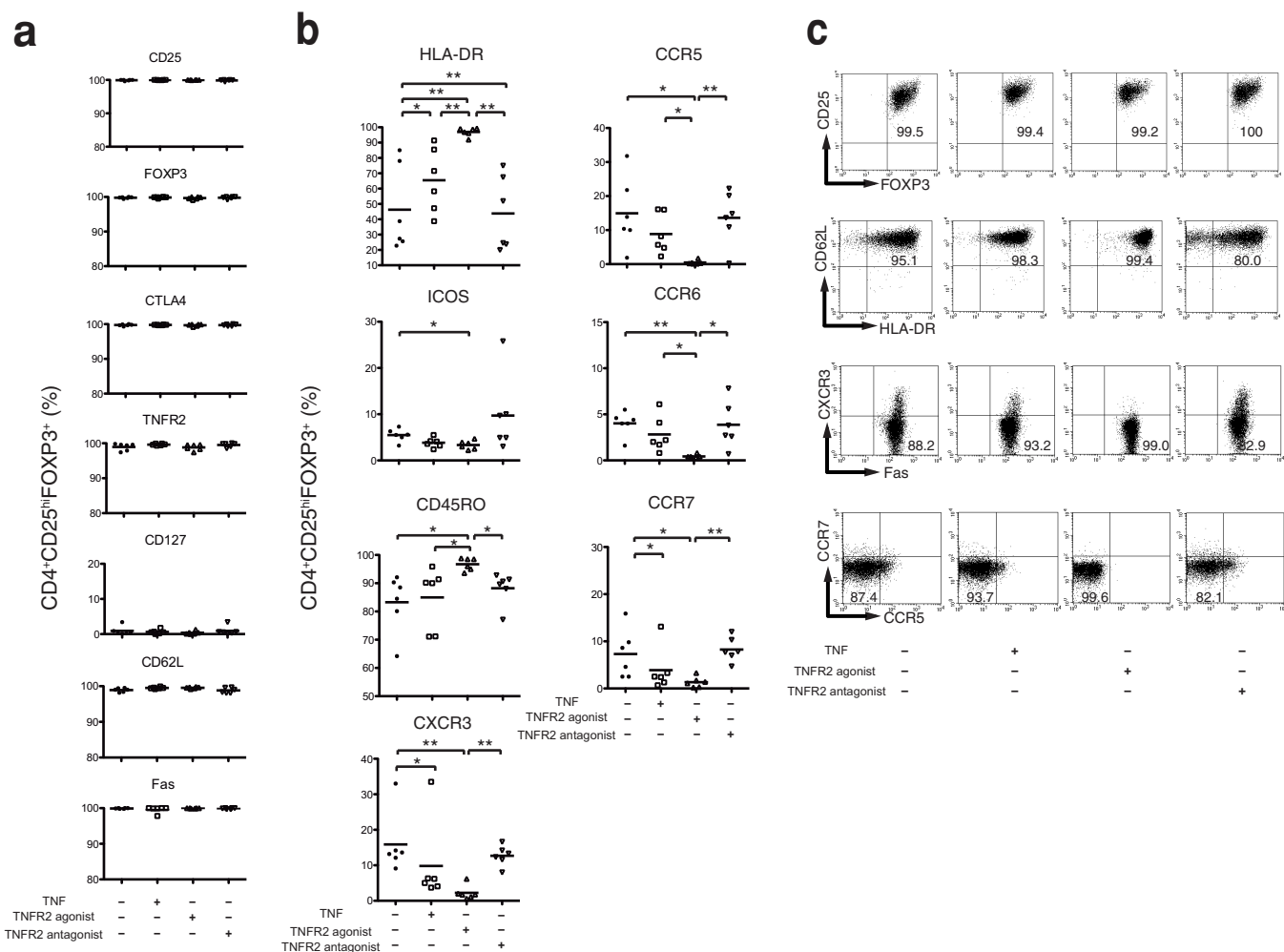
**TNFR2 agonist expansion and homogeneity of T<sub>regs</sub>.** We next investigated whether T<sub>regs</sub>, treated by TNFR2 agonist *in vitro*, possessed more homogeneous T<sub>reg</sub> cell surface markers than those given standard treatment or antagonist treatment. Comparing at first the phenotypes for 7 cell surface markers, all treatment groups highly expressed T<sub>reg</sub> signature markers FOXP3 and CD25 (Fig. 4a). The expression levels of FOXP3 were similar to levels before treatment (Supplementary Fig. 4, 5). Nearly 100% of expanded CD25<sup>hi</sup> T<sub>regs</sub> in each group were positive for CTLA4, TNFR2, CD62L, Fas, and negative for CD127 (Fig. 4a). Comparing the surface phenotypes for the next 7 cell surface markers, the TNFR2 agonist produced the most homogeneous cells for such markers as HLA-DR, ICOS,

CD45RO, CXCR3, CCR4, CCR6 and CCR7 (Fig. 4b). Surface markers, such as HLA-DR, ICOS, CD45RO and chemokine receptors, were differentially expressed between the agonist vs. antagonist treatment (Fig. 4b,c). Similar results were observed in T<sub>regs</sub> expanded without rapamycin (Supplementary Fig. 4). T<sub>regs</sub> expanded by TNFR2 agonist—relative to most other comparator groups, especially the TNFR2 antagonist—yielded a surprisingly homogeneous population of cells with this phenotype: CD4<sup>+</sup>CD25<sup>hi</sup>FOXP3<sup>+</sup>CTLA4<sup>+</sup>TNFR2<sup>+</sup>CD127<sup>-</sup>CD62L<sup>+</sup>Fas<sup>+</sup>HLA-DR<sup>+</sup>CD45RO<sup>+</sup>CCR5<sup>-</sup>CCR6<sup>-</sup>CCR7<sup>-</sup>CXCR3<sup>-</sup>ICOS<sup>-</sup>. The mean fluorescence intensity (MFI), a direct measure of the average density of the protein per cell, similarly revealed that for most surface markers, the TNFR2-agonist treated cells were more homogeneous than those produced by standard treatment or TNFR2 antagonist treatment (Supplementary Fig. 6). Further investigation should determine whether these expanded cells maintain phenotypic homogeneity over time. Before treatment, T<sub>reg</sub> markers were more heterogeneous (Supplementary Fig. 5). *In vitro* studies of mixed T<sub>reg</sub> populations, which include CD45RO<sup>+</sup>FOXP3<sup>low</sup> T cells, produce pro-inflammatory cytokines. This particular phenotype is found in up to 50% of FOXP3<sup>+</sup> T cells.

One of the most upregulated markers by TNFR2 agonist-treated T<sub>regs</sub> was HLA-DR, which is reported to have higher suppressive activity against CD8<sup>+</sup> T<sup>+</sup> cells, suggestive of an effector T<sub>reg</sub><sup>44</sup>. In contrast to HLA-DR, all four chemokine receptors were strongly down-regulated. Although the lack of chemokine receptors might result in failure to migrate to the site of inflammation, another homing receptor, CD62L, which was highly expressed in all treatment groups (Fig. 4), is crucial for entering the site of pathogenic T cell presence in acute GVHD. The fact that agonist-treated T<sub>regs</sub> were CD45RO<sup>+</sup> and CCR7<sup>-</sup> and displayed significantly higher expression levels of Fas, measured by MFI (Supplementary Fig. 6), contributes to the view that they are activated effector T<sub>regs</sub>.

**TNFR2 agonist-treated T<sub>regs</sub> and CD8<sup>+</sup> suppression.** One key function of T<sub>regs</sub>, especially in autoimmunity, is to suppress the function of autoreactive cytotoxic CD8<sup>+</sup> T cells. We assessed this functional capacity by mixing T<sub>regs</sub> from each treatment group with CFSE-stained autologous PBMC, after having stimulated them with anti-CD3mAb and IL-2 for 4 days. Autologous CD8<sup>+</sup> T cells, the responder cells, were tested for suppression by observing the ratios of responders to T<sub>regs</sub>. Ratios of dilution enable study of dose-dependence. All groups of T<sub>regs</sub> displayed suppressive function on CD8<sup>+</sup> T cells, but the degree varied by treatment group (Fig. 5a, left panel). TNFR2 agonist-treated T<sub>regs</sub>, for example, showed the strongest suppressive capacity at 1:1 ratio (by leaving the fewest number of CD8<sup>+</sup> cells) and then became progressively weaker at higher ratios. However, the antagonist-treated cells displayed weaker suppressive capacity that was essentially no different from that of standard treatment. At a suppression index of 2:1, the TNFR2 agonist-treated group showed greater suppression than did the antagonist and standard treatment groups (Fig. 5a, right panel). Similar results were observed with T<sub>regs</sub> treated without rapamycin (Supplementary Fig. 7a). The results are consistent with known phenotypes and expansion capacity of functional T<sub>regs</sub>.

**TNFR2 agonist-treated T<sub>regs</sub> and cytokine production.** We found that all treatment groups had relatively limited ability to produce intracellular IFN $\gamma$  and IL-10 after PMA and ionomycin stimulation. But TNFR2 agonist and the TNF only-treated T<sub>regs</sub> produced the lowest percentages of IFN $\gamma$ <sup>+</sup> cells (Fig. 5b, lowest left panel). The antagonist-treated T<sub>regs</sub> showed significantly higher IFN $\gamma$  production than the agonist (Fig. 5b, lower left panel). In similar experiments without rapamycin, the TNFR2 agonist-treated group not only produced lower IFN $\gamma$ , but also lower IL-10 and TNF production relative to TNF or standard treatment, respectively (Supplementary Fig. 7b). Agonist-treated T<sub>regs</sub> also showed the



**Figure 4 | Homogeneity of cell surface markers on T<sub>regs</sub>, by treatment type.** (a) All treatment groups are highly positive for T<sub>reg</sub> markers such as CD25, FOXP3, CTLA4, TNFR2, CD62L, and Fas and negative for CD127. (b) TNFR2 agonist-treated T<sub>regs</sub> almost uniformly express HLA-DR<sup>+</sup>, CD45RO<sup>+</sup> and almost uniformly lack markers such as ICOS<sup>-</sup>, CXCR3<sup>-</sup>, CCR5<sup>-</sup>, CCR6<sup>-</sup> and CCR7<sup>-</sup>, CXCR3<sup>-</sup>. (c) Representative flow diagrams show that TNFR2 agonist-treated T<sub>regs</sub> have greater uniformity of T<sub>reg</sub> markers than do other groups (\* P < 0.05, \*\* P < 0.01 by t-test).

fewest number of TH1 Transcription Factor (T-bet) + T<sub>regs</sub> (Fig. 5c), which is consistent with lower IFN $\gamma$  production (Fig. 5c). One of the reasons for these T<sub>regs</sub> showing high suppression capacity over CD8 T cells may be due to T<sub>regs</sub> lacking the ability to produce IFN $\gamma$ .

**Clinical trial induction of human T<sub>regs</sub>.** We sought to determine in a human clinical trial whether TNF triggers T<sub>reg</sub> expansion. This trial was important because the human literature holds contradictory evidence<sup>33</sup>. Because an FDA-approved version of TNF does not exist and manufacturing of a stable human form of TNF is difficult, we administered a well-known, strong inducer of naturally secreted TNF, *Bacillus Calmette Guérin* (BCG). BCG is a generic vaccine already on the market for decades for treating tuberculosis and bladder cancer. In diverse animal models of autoimmunity, BCG or CFA (the equivalent of BCG but not manufactured under cGMP conditions) can induce T<sub>regs</sub> and can eliminate autoimmunity. TNF induction is solely responsible for these beneficial effects, because they can be eliminated by anti-TNF antibodies<sup>40,41</sup>.

The small proof-of-principle, double-blind placebo-controlled clinical trial enrolled two human subjects. One subject received repeat BCG injections ( $1.6\text{--}3.2 \times 10^6$  cfu/injection) and one placebo subject received repeat saline, twice, 4-weeks apart. Both were monitored weekly for 20-weeks to study the pharmacokinetics of TNF and T<sub>reg</sub> induction. After each BCG injection, TNF induced T<sub>regs</sub> in a bi-modal fashion with slightly delayed kinetics (Fig. 6a, left panels).

After 20 weeks of observation, saline injections induced neither TNF nor T<sub>regs</sub> (Fig. 6b, center panels). The total CD4<sup>+</sup> cell counts did not change in the BCG and placebo patients other than in the percentages of CD4<sup>+</sup> CD25<sup>hi</sup>FOXP3<sup>+</sup> cells (data not shown). Because TNF is not the only cytokine stimulated by BCG injection, we also sought to determine whether TNF was necessary and sufficient for induction of T<sub>regs</sub>. During routine care, a rheumatoid arthritis subject on Enbrel (etanercept), an anti-TNF antibody, was administered a BCG vaccine for protection from tuberculosis. The same tight weekly TNF and T<sub>reg</sub> monitoring revealed that Enbrel blocked both endogenous secretion of TNF and induction of T<sub>regs</sub> (Fig. 6c, right panels). This *in vivo* evidence supports endogenous TNF as being obligatory for T<sub>reg</sub> induction in humans.

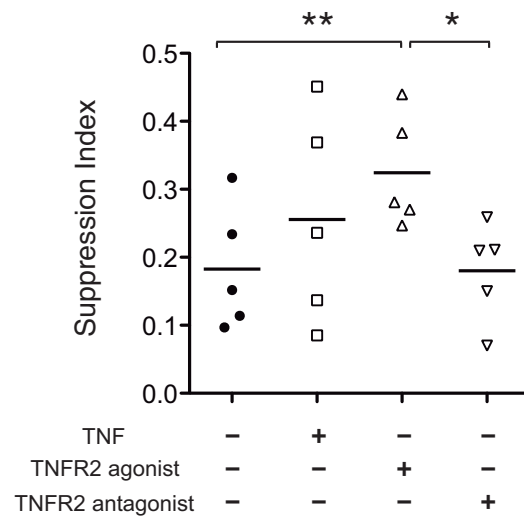
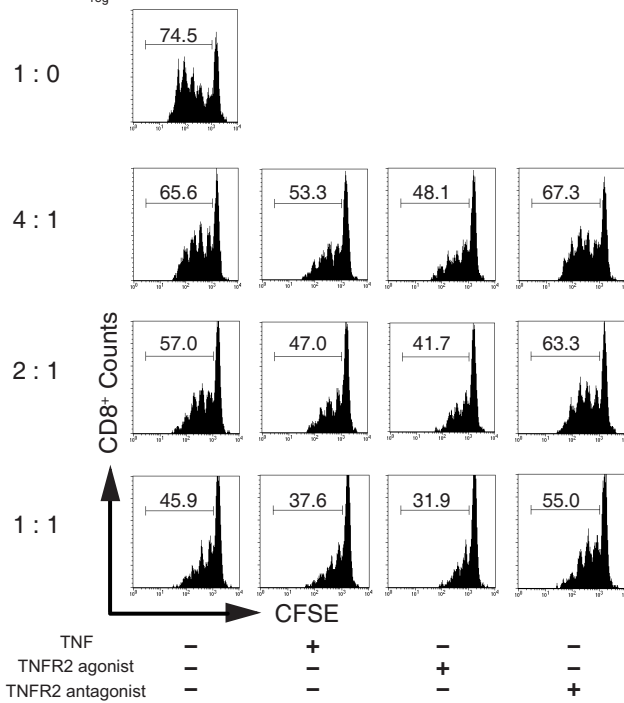
## Discussion

We have developed a new method, acting through the TNF and especially the TNFR2 receptor, to obtain *ex vivo* an expanded and homogeneous populations of human T<sub>regs</sub>. We identified a novel TNFR2 agonist that, combined with four standard T<sub>reg</sub> expansion protocols, IL-2, anti-CD3, anti-CD3 and anti-CD28 (with or without rapamycin), produces phenotypically homogeneous T<sub>regs</sub>. These homogeneous T<sub>regs</sub> also have greater capacity to suppress cytotoxic T lymphocytes than does standard treatment. The expanded T<sub>regs</sub> bore the near homogeneous phenotype CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup>

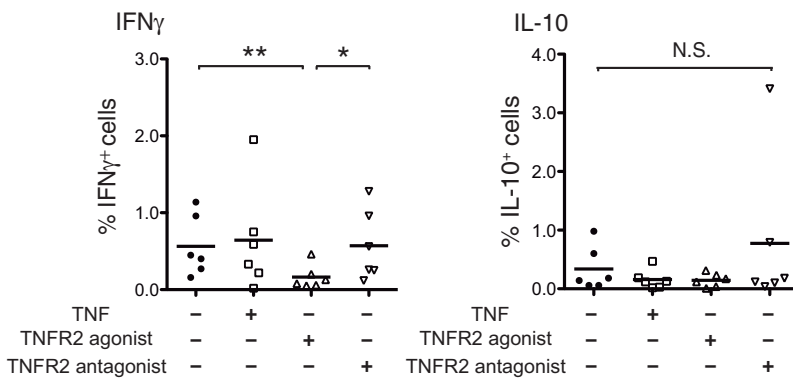
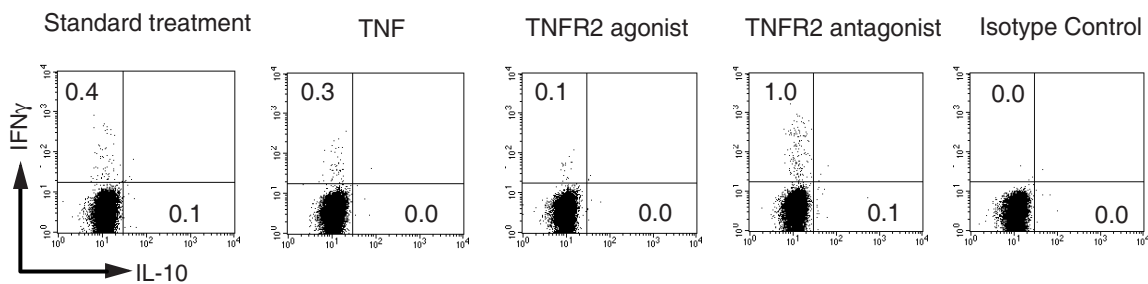


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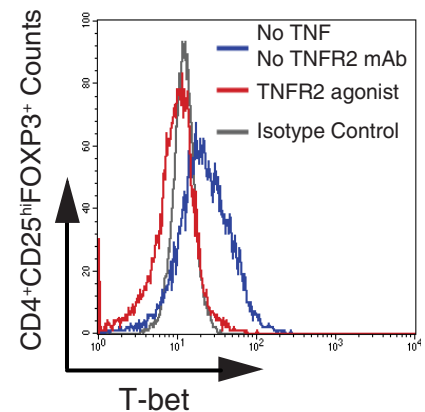
Responder : T<sub>reg</sub>



**b**

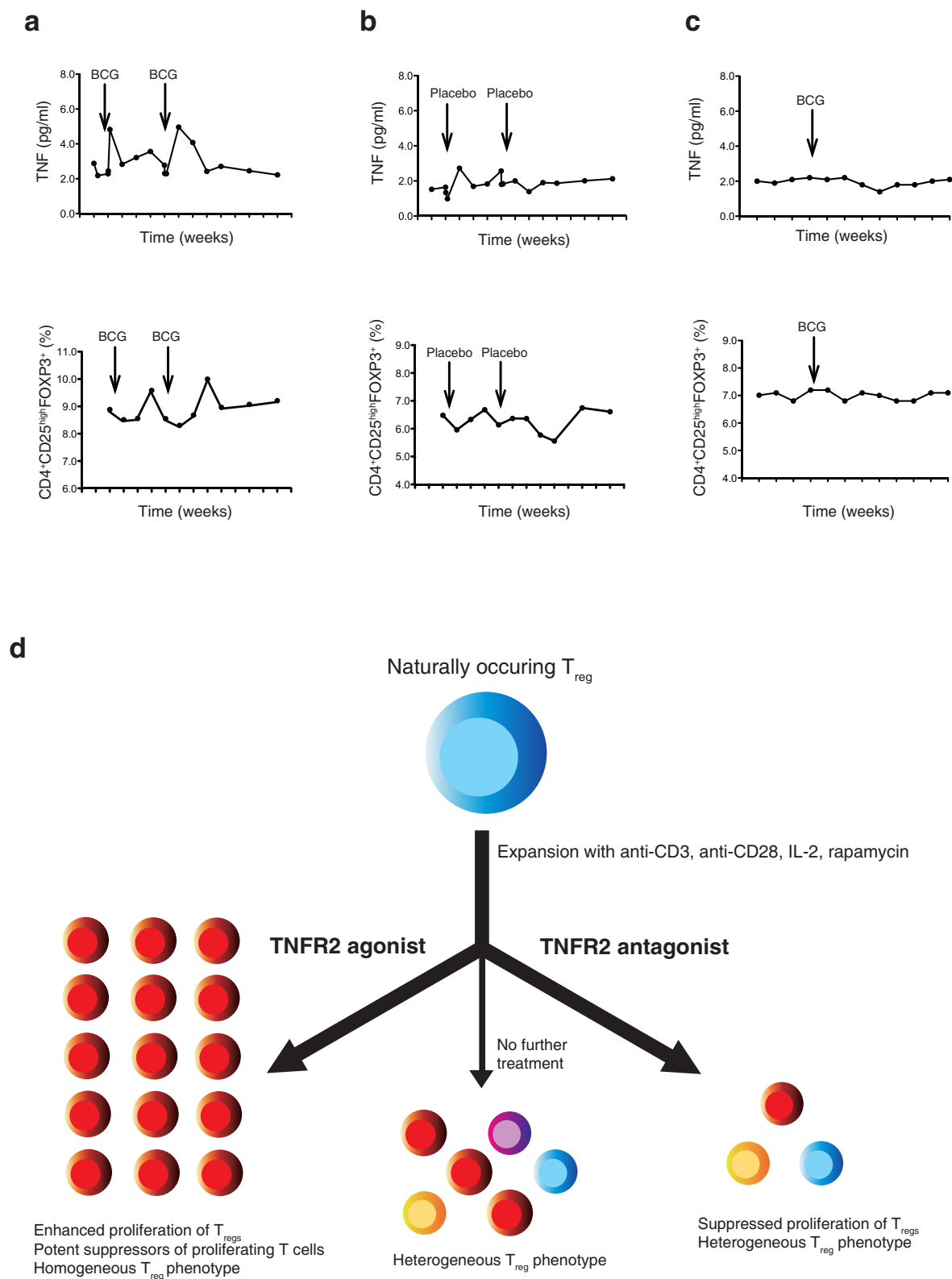


**c** T-bet



**Figure 5 | Functional assays of T<sub>regs</sub> expanded under four treatment conditions.** (a) In a representative case, TNFR2 agonist-treated T<sub>regs</sub> exerted stronger and dose-dependent suppression of CD8+ cell numbers, compared to other groups, at all dilutions or suppression ratios (left panel, third column). (\* P < 0.05, \*\* P < 0.01 by t-test.) Using a suppression index of 2 : 1 (CD8+ Responders to T<sub>regs</sub>), TNFR2 agonist suppression of CD8+ cells is greater than standard treatment and TNFR2 antagonist treatment (right panel). (b) TNFR2 agonist-treated T<sub>regs</sub> produce a lower percentage of IFN $\gamma$ + cells and (c) lower numbers of T-bet+ cells after stimulation with PMA and ionomycin for 24 hours. Data in (a, right panel) are samples from 5 subjects and (b, lower panel) are from 8 subjects.





**Figure 6** | TNF induction of  $T_{regs}$  in humans and summary finding of TNFR2 agonist and antagonist activity. (a) In a small double-blinded, placebo-controlled trial of human subjects, BCG treatment induces TNF and shortly thereafter a surge of  $T_{regs}$  appears in the treated subject. (b) In contrast, in the same double-blinded trial, placebo treatment induces neither TNF nor  $T_{regs}$  in the circulation. (c). BCG treatment of a human subject taking chronic Enbrel (etanercept), an anti-TNF antibody, reveals neither TNF nor  $T_{reg}$  induction. This finding supports endogenous TNF as being necessary for  $T_{reg}$  expansion in humans. (d) After expansion, the TNFR2 agonist is better than TNFR2 antagonist at proliferating and yielding more phenotypically homogeneous  $T_{regs}$  ( $CD4^+ CD25^{hi} FOXP3^+ CTLA4^+ TNFR2^+ CD45RO^+ CD62L^+ CD127^-$ ,  $HLA-DR^{hi} CCR5^- CCR7^- CXCR3^- ICOS^-$ ), with higher suppression capacity for  $CD8^+$  cells, and lower cytokine-producing capability. The TNF2 antagonist was able to suppress  $T_{reg}$  expansion.





**Cell proliferation and suppression assays.** For CD4 proliferation experiments, CD4+ cells were stained with 1  $\mu$ M carboxyfluorescein diacetate succinimidyl ester (CFSE). Cells were plated at the density of  $2 \times 10^5$  cells/well in 96-well plate with anti-CD3mAbs. Four days later, cells were collected and analyzed.

For T<sub>reg</sub> suppression assay, autologous PBMCs were used as responders. PBMC were collected using Ficoll-Paque, cryopreserved at  $-80^\circ\text{C}$ , and thawed at the day before mixed with T<sub>regs</sub> and rested overnight in RPMI 1640 and 10 U/ml IL-2. The next day, responder cells were stained with CFSE (1  $\mu$ M). Responder cells ( $5 \times 10^4$  cells) and expanded T<sub>regs</sub> were mixed at various ratios, and stimulated with anti-CD3mAb and IL-2. After 4 days, cells were collected and analyzed.

**Statistical analysis.** All data analyses were performed by the paired Student *t* test using GraphPad Prism-5 software (GraphPad Software, La Jolla, CA). We considered two-sided *p* value 0.05 as significant.

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## Author contributions

Y.O. performed, developed the methods and analyzed all experiments in this paper except the clinical trial, T.M. performed the experiments on human donors looking for Treg intracellular signaling after TNFR2 agonism or antagonism, L.W. was involved in the overall clinical trial for this paper, D.L.F. designed the clinical trial and worked with Y.O. on the design and interpretation of the Treg experiments in this paper.

## Additional information

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