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Anti-CD3 therapy promotes tolerance by selectively depleting pathogenic cells while preserving regulatory T cells¹

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Abstract

Monoclonal anti-CD3 antibodies (mAbs) have been used clinically for two decades to reverse steroid-resistant acute graft rejection. In autoimmune diabetes, short course treatment with FcR non-binding (FNB) anti-CD3 mAb in mice with recent onset of diabetes induces long-term disease remission. Induction of tolerogenic regulatory T cells (Tregs) has been implicated to be one of the mechanisms of action by FNB anti-CD3 mAb in these settings. In this study, we examined the effect of FNB anti-CD3 mAb treatment on the homeostasis of naïve, effector and regulatory T cells *in vivo*. Anti-CD3 treatment induced a transient systemic rise in the percentage but not absolute number of CD4⁺Foxp3⁺ Tregs due to selective depletion of CD4⁺Foxp3[−] conventional T cells. T cell depletion induced by FNB anti-CD3 mAb was independent of the pro-apoptotic proteins Fas, caspase 3, and Bim and was not inhibited by over-expression of the anti-apoptotic protein, Bcl-2. Tregs were not preferentially expanded and we found no evidence of conversion of conventional T cells into Tregs suggesting that the pre-existing Tregs are resistant to anti-CD3-induced cell death. Interestingly, expression of the transcription factor Helios, which is expressed by thymus-derived natural Tregs, was increased in Tregs after FNB anti-CD3 mAb treatment suggesting that the anti-CD3 treatment can alter, and potentially stabilize, Treg function. Taken together, the results suggest that FNB anti-CD3 therapy promotes tolerance by restoring the balance between pathogenic and regulatory T cells.

INTRODUCTION

Immunotherapy targeting CD3 molecules has shown tremendous promise for the treatment of autoimmune diseases and the prevention of allograft rejection in humans and in murine pre-clinical models (1). In patients, the anti-CD3 monoclonal antibody (mAb) OKT3 was described as an efficient treatment to prevent acute renal allograft rejection more than twenty years ago (2). However, the development of OKT3 and other anti-CD3 mAb therapeutics were hampered by serious side-effects associated with a “cytokine storm” released as a consequence of generalized T cell activation (3, 4). T cell activation by anti-CD3 mAb depends on immobilization of the mAb on antigen-presenting cells via Fc receptors (FcR) and crosslinking of TCR/CD3 complexes at the interface of T cells and antigen presenting cells (5). Thus, various forms of FcR-non-binding (FNB) anti-CD3 mAb were developed to avoid cytokine storm elicited by multivalent crosslinking of TCR/CD3. Remarkably, these FcR-non-binding (FNB) anti-CD3 mAbs retained their immunosuppressive properties in pre-clinical models *in vivo* without the toxicity associated

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with the parental anti-CD3 mAb and they demonstrated clinical efficacy in the treatment of acute renal allograft rejection (6–9).

Despite the great promises of FNB anti-CD3 mAb treatment in autoimmunity and transplantation, the mechanisms of action of these reagents *in vivo* are still not clearly defined. Previous data from our laboratory has shown that FNB anti-CD3 mAbs are not passive blockers of TCR:MHC interactions but instead induce partial phosphorylation of the CD3 ζ and CD3 ϵ chains and inefficient recruitment and phosphorylation of CD3-associated kinase ZAP70 (10). As a consequence, downstream activation of PLC γ , calcium flux and NFATc nuclear translocation, and MAP kinase phosphorylation were reduced when compared to that induced by multivalent anti-CD3 mAb engagement (10, 11). These *in vitro* observations correlated well with experiences in human patients treated with FNB anti-CD3. While both FcR-binding and FcR non-binding anti-CD3 mAb-induced transient T cell depletion in NOD mice and patients (12), the depletion was more complete with FcR-binding anti-CD3 mAb (13).

CD4⁺Foxp3⁺ regulatory T cells (Tregs) can suppress effector T cell (Teff) responses *in vitro* and *in vivo* and they are crucial for the maintenance of peripheral tolerance and the prevention of autoimmunity (14, 15). Thus, it is not surprising that recent studies have suggested that rather than altering Teffs, FNB anti-CD3 mAb treatment results in the generation of Tregs (16–18). However, these conclusions have been controversial (19, 20) as potential changes in the relative frequency of Teff and Tregs following FNB anti-CD3 mAb treatment, as well as the potential for T cell migration out of the blood and secondary lymphoid tissues have made it difficult to evaluate the direct effect of the therapy on each cell subset.

In this study, we examined the influence of FNB anti-CD3 mAb on conventional versus regulatory T cells. We demonstrate that although FNB anti-CD3 mAb induced an increase in the relative percentage of Tregs, this process was not due to *de novo* generation or expansion of Tregs. Instead, the increased Treg to Teff ratio was due to preferential depletion of conventional T cells *in vivo* through Fas- and caspase 3-independent pathways. Furthermore, FNB anti-CD3 mAb treatment led to increased expression of Helios in Tregs, suggesting stabilization of Tregs, which may account for the protracted efficacy of the drug.

MATERIALS AND METHODS

Mice

BALB/c and C57BL/6 (B6) mice were purchased from Charles River (Wilmington, MA), NOD mice were purchased from Taconic (Germantown, NY) and B6.Caspase 3-deficient mice were purchased from Jackson Laboratories (Bar Harbor, ME). DO11.10 TCR-Tg mice, B6.Bcl-2 transgenic mice (a gift from Marisa Alegre), B6.Bim-deficient mice, Fas-deficient BALB/c.lpr/lpr mice, BALB/c.FasL-deficient gld/gld mice, NOD.Foxp3.GFP-Cre and NOD.Foxp3.GFP-Cre $\times$ Rosa26.flox.stop.YFP were bred at our facility. All mice were housed in a specific pathogen-free facility at The University of California at San Francisco. All experiments complied with the Animal Welfare Act and the National Institutes of Health guidelines for the ethical care and use of animals in biomedical research and were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Antibodies and other reagents

FNB mouse-specific anti-CD3 mAb, 145-2C11- γ 3 (2C11-IgG3) was produced in our laboratory. Another FNB mouse-specific anti-CD3 mAb-producing cell line, 145-2C11-IgG2a-Ala-Ala, was provided as a gift from Centocor/Johnson & Johnson and the antibody

was produced in our laboratory. Anti-Fc receptor mAb, 2.4G2 (UCSF cell culture facility, San Francisco, CA), mitogenic hamster anti-CD3 mAb, 145-2C11 (BioLegend); anti-CD4 mAb, RM4-5 (eBiosciences); anti-CD8 mAb, 53-6.7 (Southern Biotechnologies); anti-CD25 mAb, PC61 (eBiosciences); anti-FoxP3 mAb, FJK-16 (eBiosciences); anti-Thy1.1 mAb, OX-7 (BioLegend), anti-PD-1 mAb, J43 (eBiosciences); anti-Neuropilin-1 polyclonal antibodies (R&D); and anti-Helios mAb, 22F6 (BioLegend) were purchased. CFSE was purchased from Molecular Probes Inc. (Eugene, OR). FTY720 provided as a gift by Novartis Pharmaceuticals (St. Louis, MO) was administered daily i.p. at a dose of 20µg/day. EasySep mouse CD4 T cell enrichment kit was purchased from StemCell Technologies (Vancouver, BC, Canada). Mouse IgG whole molecule from Rockland Immunochemicals for Research (Gilbertsville, PA), a gift from Amplimmune Inc. (Rockville, MD), was used as a control.

Flow cytometry and cell sorting

Single-cell suspensions were prepared from the spleen and LN of indicated mice using standard procedures. For flow cytometry, cells were stained for 20–30 min on ice in staining buffer (2% FCS and 0.01% sodium azide in PBS). For cell sorting, cells were stained and washed in medium containing 2% FCS, and sorted on a Mo-Flo cytometer™ (Beckman Coulter Inc, Miami, FL) to greater than 95% purity. Flow cytometric analyses were performed on a BD LSRII flow cytometer with Diva software (BD/PharMingen, San Jose, CA).

Adoptive transfer experiments and in vivo treatments

Unless otherwise indicated, WT NOD or BALB/c mice between 8–20 weeks of age were treated i.v. with 10µg FNB anti-CD3 mAb daily for five consecutive days and control mice received whole mouse Ig using the same regimen. For adoptive transfer experiments, $9\text{--}10 \times 10^6$ sorted CD4⁺Foxp3-GFP[−], 12×10^6 cells enriched CD4⁺ CFSE-labeled T cells or $0.4\text{--}0.5 \times 10^6$ sorted PD-1[−]Nrp1[−] (Helios^{lo/−}) or PD-1⁺Nrp1⁺ (Helios^{hi}) Tregs were transferred into syngeneic recipients via retro-orbital injection on day 0. On days 1–5, recipients were treated with FNB anti-CD3 mAb or control Ig as described above. Eight days after adoptive transfer, T cell proliferation was examined by flow cytometry. For analysis of transferred Helios^{lo/−} or Helios^{hi} Tregs Thy1.1⁺ cells were enriched prior to flow cytometric analysis: cells from all lymph nodes and spleen were harvested and incubated with 2µg/ml Thy1.1-APC in 200µl for 30 minutes at 4°C. Cells were washed, resuspended in 200µl and incubated with 50µl anti-APC magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 minutes at 4°C. Cells were washed and cell suspension was passed through a MACS Separation LS column (Miltenyi Biotec) to obtain a Thy1.1-enriched fraction, which was used for flow cytometric analysis.

Peripheral blood lymphocyte counts were obtained using a Hemavet 950 instrument (Drew Scientific, Dallas, TX). To determine trafficking into peripheral tissues, mice were treated for 5 consecutive days with 20µg FNB anti-CD3 mAb and harvested one day after treatment. Mice were perfused using PBS before harvesting spleen, peripheral lymph nodes, bone marrow, lungs, liver, small intestine and colon. Bone marrow isolated from femurs and tibias was dissociated using mechanical disruption by passing through 23-gauge needles repeatedly. Lung tissues were digested with a mixture collagenase and DNase. Livers were initially minced into single cell suspensions using syringe plungers, and leukocytes were enriched using percoll density gradient centrifugation. The intestinal tracts were washed extensively with PBS to remove fecal content and mucus coating and the tissue was then cut into small pieces and digested using a mixture collagenase and DNase to make single cell suspensions. The total cell counts from each organ and percentages of CD4⁺ and CD8⁺ T

cells were then used to calculate the numbers of CD4⁺ and CD8⁺ T cells present in that organ.

Cell death assays

DMEM-glutamax medium (UCSF cell culture facility, San Francisco, CA) supplemented with 5% heat-inactivated FCS (Summit Biotechnology, Ft. Collins, CO), 100 U/ml penicillin, 100 U/ml streptomycin, non-essential amino acids, 10mM HEPES (UCSF cell culture facility, San Francisco, CA) and 50μM β-mercaptoethanol (Biorad, Hercules, CA) was used for cell culture. To prepare Th1 effector T cells, single-cell suspensions were prepared from the spleen and LN of DO11.10 mice. DO11.10 cells were stimulated with 0.1μg/ml ova peptide in the presence of 400 ng/ml IFNγ and 25μg/ml anti-IL-4 for 7 to 10 days, and medium supplemented with 20 U/ml recombinant human IL-2 on day 4 to 5 after the initiation of the culture. The cells were restimulated with 0.1μg/ml ova peptide without added IFNγ and anti-IL-4 (clone 11B11). The DO11.10 cells were harvested one week later and adoptively transferred or restimulated with FNB anti-CD3-IgG3 overnight and the numbers of viable cells were determined using flow cytometry after anti-Thy-1 and PI staining.

For measurement of cell death after *in vivo* treatment, mice were treated with two 10μg doses of FNB anti-CD3 mAb or control Ig 24 hrs apart and harvested 38 hrs after the first injections. Single cell suspensions from lymph nodes were incubated *in vitro* in media only, or in the presence of 1ng/ml rIL-2 (eBiosciences, San Diego, CA) or 10ng/ml rIL-7 (Peprotech, Rock Hill, NJ). Cultures were harvest at 26 or 48 hrs and cell death was assessed by DAPI (Invitrogen, Carlsbad, CA) inclusion by flow cytometry.

Statistical analysis

The statistical significance of differences between groups in Figure 1 was determined by the two-tailed student t-test using Excel software. The statistical significance of differences between groups in all other figures was determined by the Mann-Whitney test using Prism software.

RESULTS

FNB anti-CD3 mAb preferentially induces cell death of CD4⁺Foxp3⁻ cells *in vivo*

In agreement with previous reports (13), 10μg/day × 5 days FNB anti-CD3 mAb treatment induced a transient decrease in the percentage of CD4⁺ T cells and an increase in the percentage of CD4⁺Foxp3⁺ Tregs in secondary lymphoid organs (Figure 1A and 1B). While the absolute CD4⁺ cell numbers decreased (Figure 1C), mirroring the percentage decrease, the absolute CD4⁺Foxp3⁺ cell numbers decreased (Figure 1D), in contrast to the percentage increase. The decrease in CD4⁺Foxp3⁺ Treg cell number was not as pronounced as that observed for the total CD4⁺ T cell population. These results suggest that the FNB anti-CD3 mAb treatment may not induce the expansion of endogenous Tregs, but transiently altered the proportion of Tregs due to a selective loss of CD4⁺Foxp3⁻ cells in secondary lymphoid organs.

Previous reports have demonstrated that FNB-anti-CD3 treatment is particularly effective at controlling autoreactive T cells at the time of diabetes onset in the NOD mice (13, 18). In that setting, many autoreactive T cells are activated effector T cells. To determine the relative sensitivity of recently activated effector versus naïve T cells to FNB anti-CD3 mAb induced depletion *in vivo*, we compared the rate of depletion of endogenous naïve T cells and adoptively transferred activated Teffs after FNB anti-CD3 mAb treatment. The Th1 Teffs were generated *in vitro* by activating T cells from DO11.10 TCR transgenic mice with

their cognate peptide under Th1 skewing conditions. FNB anti-CD3 treatment induced 12–37% reduction in the total number of endogenous splenic T cells (Table 1). In the same animals, depletion of transferred activated Th1 Teffs was consistently greater (40–66%) than that of the endogenous naïve cells (Table I). Thus, taking all of our data together, T cells showed a hierarchy in their sensitivity to FNB anti-CD3 mAb induced depletion: recently activated Teffs are the most sensitive, followed by naïve T cells, while Tregs are the most resistant.

The loss of CD4⁺ cells after FNB anti-CD3 mAb treatment could result from preferential migration out of lymph nodes or depletion of these cells. First, analysis of T cell counts in other tissues such as bone marrow, intestine, liver and lung in mice treated with FNB anti-CD3 mAb revealed no significant increase in CD4⁺ or CD8⁺ T cells that could account for the loss of these cells from the secondary lymphoid organs (data not shown). To examine the possibility of T cell migration more directly, mice were treated with FNB anti-CD3 mAb under the cover of FTY720 to block exit of T cells from LNs (21) and the number of CD4⁺ T cells and percentage of Tregs were examined in LNs. FTY720 treatment led to a significant drop in the lymphocyte count in the peripheral blood demonstrating that LN exit was blocked (data not shown). FNB anti-CD3 mAb induced a similar drop in the CD4⁺ T cell count and an increase in the percentage of Tregs in the FTY720 and control treated mice (Figure 2A and data not shown). Thus, the selective loss of CD4⁺Foxp3[−] cells after FNB anti-CD3 mAb treatment was not due to their preferential trafficking out of LNs, suggesting that the relative increase of CD4⁺Foxp3⁺ compared to CD4⁺Foxp3[−] was due to preferentially depletion of CD4⁺Foxp3[−] cells.

Mechanism of FNB anti-CD3 mAb-mediated cell death

In order to measure cell death caused by *in vivo* treatment with FNB anti-CD3 mAb, cells harvested from mice that had been treated with FNB anti-CD3 mAb *in vivo* were incubated *in vitro*. Cells harvested from FNB anti-CD3 mAb-treated mice showed increased cell death, as measured by DAPI uptake, compared to mice treated with control antibody (Figure 2B and 2C). Cell death was not prevented by addition of IL-2 or IL-7 (Figure 2D and 2E) suggesting that FNB anti-CD3 mAb-induced cell death was not a consequence of cytokine deprivation *in vitro* and resulted from an apoptotic program that was initiated by FNB anti-CD3 mAb *in vivo*.

Typically, T cell death can be induced by either cell surface death receptors such as Fas and TNF α RI or mitochondrial cell death effector molecules such as Bim (22, 23). T cells deficient in Fas ligand (gld) or Fas (lpr) were resistant to FNB anti-CD3 mAb induced cell death *in vitro* (Figure 3A and data not shown). However, lpr mice treated with FNB anti-CD3 mAb showed a similar level of T cell depletion as in WT mice (Figure 3B) suggesting that the *in vivo* mechanism of cell death was distinct from that seen *in vitro*.

Previous studies by our group and others showed that *in vivo* depletion of naïve T cells by the classical anti-CD3 mAb (clone 145-2C11) or by superantigen is mediated by the pro-apoptotic protein Bim and blocked by over-expression of the anti-apoptotic proteins Bcl-2 or Bcl-x_L (24, 25). We investigated whether depletion of T cells *in vivo* by FNB anti-CD3 mAb was mediated by a similar mechanism. CD4⁺ T cells were depleted to a similar extent in Bim-deficient and WT mice (Figure 3C). This depletion resulted in a similar rise in the percentages of Tregs as observed in earlier experiments (data not shown). It was possible that other Bim-related mitochondrial pro-apoptotic proteins, such as Bad and Bax, mediated FNB anti-CD3 mAb-induced T cell death. Cell death mediated by these proteins is blocked by over expression of the anti-apoptotic protein Bcl-2 (26). To determine potential roles of other mitochondrial pro-apoptotic factors in FNB anti-CD3 mAb-induced T cell depletion, T cell numbers in WT and Bcl-2 transgenic mice were compared after FNB anti-CD3 mAb

treatment. The results showed that CD4⁺ T cell depletion was similar in WT and Bcl-2 transgenic mice (Figure 3D). Finally, we analyzed the role of caspase 3, which is the downstream caspase activated by both intrinsic and extrinsic apoptosis pathways and shown to be required for anti-CD3 mAb-induced death of T cells *in vitro* (27). Treatment of caspase 3 deficient mice with FNB anti-CD3 mAb resulted in depletion of CD4⁺ T cells in the LNs similar to that seen in WT mice (Figure 3E).

FcR non-binding anti-CD3 mAb does not promote the conversion or expansion of Tregs

Our results show that one of the effects of FNB anti-CD3 treatment is T cell depletion. We next examined the effects of FNB anti-CD3 mAb treatment on the homeostasis of Tregs. To first determine if FNB anti-CD3 treatment promoted the conversion of naïve Foxp3⁻ T cells into Foxp3⁺ Tregs, CD4⁺Foxp3⁻ cells from Foxp3-GFP reporter mice were sorted and transferred to lymph-replete recipient mice that were then treated with FNB anti-CD3 mAb. Analysis after 7 days showed that the percentage of Foxp3⁺ cells within the transferred population was not statistically different between control and FNB anti-CD3 mAb-treated mice (Figure 4A) suggesting that stable Foxp3 expression was not induced by FNB anti-CD3 mAb. To confirm the lack of transient Foxp3 induction following FNB anti-CD3 treatment, NOD.Foxp3.GFP-Cre × Rosa26.flox.stop.YFP mice (28) were treated and the proportions of YFP⁺GFP⁺ “bona fide” Tregs and YFP⁺GFP⁻ “transient” Foxp3 expressing cells were analyzed on day 14 after treatment (Figure 4B). Although the percentage of CD4⁺ cells that expressed Foxp3 at any time (total YFP⁺) was increased in FNB anti-CD3 treated mice, which can be attributed to a loss of Foxp3⁻ cells (Figure 4C), the percentage of “bona fide” Tregs (YFP⁺GFP⁺) did not change (Figure 4D). Thus, there was no evidence that FNB anti-CD3 mAb promoted generation of adaptive Tregs or transient expression of Foxp3 *in vivo*.

The previous experiments clearly demonstrated that FNB anti-CD3 mAb treatment does not induce Treg conversion. However, these data could not distinguish whether the increased frequency of Tregs was due solely to preferential loss of Foxp3⁻ cells or involved the concomitant expansion of Foxp3⁺ Tregs (16–18). In order to determine the effects of FNB anti-CD3 mAb on the proliferation of Foxp3⁻ vs Foxp3⁺ CD4⁺ T cells, total CD4⁺ cells, marked congenically, were CFSE-labeled and transferred to recipient mice that were either treated with control antibody or FNB anti-CD3 mAb (Figure 5A). As expected, the percentage of Foxp3⁺ cells within the transferred population increased, as a result of preferential depletion of Foxp3⁻ cells (Figure 5A). The therapy resulted in a decrease in the ratio of Foxp3⁻ to Foxp3⁺ cells in FNB anti-CD3 mAb-treated mice (Figure 5B). Interestingly, FNB anti-CD3 mAb treatment led to a decrease in the percentage of undivided Foxp3⁻ cells (Figure 5C). Importantly, there was no difference in the percentage of Foxp3⁺ cells that had not divided (Figure 5D) demonstrating that FNB anti-CD3 mAb does not induce proliferation of existing Foxp3⁺ Tregs.

FNB anti-CD3 mAb treatment alters the balance of Helios-expressing Tregs

Although the preferential depletion of conventional T cells following anti-CD3 mAb therapy in NOD mice could account for short-term remission of diabetes, it remained possible, if not likely, that qualitative changes in Tregs (other than expansion or induction) may play a role in long-term stable remission. It was recently reported that the transcription factor Helios is expressed by thymus-derived natural Tregs but reduced or absent in adaptive Tregs induced *in vitro* by TGFβ (29). Furthermore, Helios^{lo/-} Tregs produce more IFNγ and IL-2 than Helios^{hi} Tregs (29), suggesting that the Helios^{lo/-} population may be more “plastic” or unstable. We hypothesized that FNB anti-CD3 mAb may alter the relative proportion of Helios^{hi} to Helios^{lo/-} Tregs thereby increasing the proportion of stable/suppressive Tregs. The treatment of mice with FNB anti-CD3 mAb led to an increase in the percentage and

absolute number of Helios^{hi} Tregs (Figure 6A and B). This increase could be due to proliferation or preferential survival of Helios^{hi} Tregs or induced expression of Helios in Helios^{lo/-} cells. As shown in Figure 5 above, FNB anti-CD3 mAb did not induce proliferation of Tregs. Therefore, it is unlikely that proliferation of Helios^{hi} cells was responsible for the increased percentage of this Treg. To obtain Helios^{hi} and Helios^{lo/-} Tregs, we used the surface markers PD-1 and Neuropilin-1, which are preferentially expressed by Helios^{hi} Tregs (Yadav, Bluestone, et al., manuscript submitted). Sorted CD25⁺PD-1⁻Nrp1⁻ cells, of which 44–55% were Helios^{lo/-}, and CD25⁺PD-1⁺Nrp1⁺ cells, of which 69–70% were Helios^{hi} were transferred to lympho-replete recipients that were then treated with FNB anti-CD3 mAb. An average of 24% of transferred Helios^{lo/-} Tregs expressed Helios after control Ig treatment, while 68% of Helios^{hi} Tregs expressed high levels of Helios consistent with a stable phenotype *in vivo* (Figure 6C). FNB anti-CD3 mAb treatment resulted in an increased percentage of Helios expressing Tregs in both the sorted Helios^{lo/-} (68%) and Helios^{hi} (82%) populations. Our transfer experiments could not rule out preferential survival of Helios^{hi} Tregs but nonetheless our results show that the treatment induces Helios expression in Tregs and causes previously unrecognized qualitative changes in this cell population.

DISCUSSION

FcR non-binding anti-CD3 mAbs effectively control autoimmunity and transplant rejection in pre-clinical murine models and clinical trials in patients (1). The mechanisms underlying the tolerogenic outcome of FNB anti-CD3 mAb therapy are still ill-defined. In particular, effects of FNB anti-CD3 mAbs on conventional T cells versus Tregs are controversial. In this study, we addressed this issue directly by comparing the impact of FNB anti-CD3 mAbs on conventional CD4⁺Foxp3⁻ T cells CD4⁺ Foxp3⁺ Tregs. Our data showed that although both T cell subsets underwent partial depletion following FNB anti-CD3 mAb treatment, there was a relative enrichment of Tregs. While killing of T cells induced by FNB anti-CD3 mAb was dependent on the Fas pathway *in vitro*, it occurred independently of Fas, Bim and caspase 3 *in vivo*. Importantly, the increase in the frequency of Tregs was not associated with induction or expansion of adaptive Tregs. In addition, FNB anti-CD3 mAb treatment increased the proportion of Helios-expressing Tregs. Thus, our study suggests that FNB anti-CD3 mAb therapy promotes tolerance and prevents autoimmunity by restoring the balance between pathogenic autoreactive Teffs and suppressive Tregs and potentially increases the stability of the Treg population.

Current theories on the underlying mechanisms of the pre-clinical and clinical efficacy of FNB anti-CD3 mAb therapy often involve the generation of Tregs in addition to its effects on Teffs. The notion of FNB anti-CD3 induction of Tregs was supported by increases of CD4⁺CD25⁺ Treg frequencies observed after FNB anti-CD3 mAb treatment in several murine models, but absolute numbers of Tregs were not reported in these studies (16–18). Our study demonstrates that the relative enrichment of Tregs following FNB anti-CD3 mAb treatment was due to differential sensitivity of conventional and regulatory T cells to cell death, such that activated Teffs are most sensitive and Tregs are least sensitive. We found no evidence of Treg induction or expansion after FNB anti-CD3 treatment. Similar to our results, other studies have not observed increased Treg percentages after FNB anti-CD3 mAb treatment *in vivo* (19, 20, 30). Considering the therapeutic applications of FNB anti-CD3 mAbs, this finding has important implications and clarifies the mode of action of these reagents. For instance, the preferential effects of the FNB anti-CD3 mAbs on Teffs help to explain why anti-CD3 treatment is most effective at the time of peak disease activity (13, 18).

The mechanism responsible for cell death after FNB anti-CD3 mAb treatment remains to be elucidated. Single genetic deficiency of Fas, Bim and caspase 3 or overexpression of Bcl-2 did not prevent cell depletion. Thus, these data suggest FNB anti-CD3 mAb may activate multiple apoptotic pathways *in vivo* such that absence of one does not prevent cell death or that one main pathway is activated, but in its absence, there is compensation by the other pathways. Alternatively, it is possible that FNB anti-CD3 mAb activates cell death pathways that are independent of caspases such as necrosis or autophagy. In addition, it remains unclear why conventional T cells are more susceptible to FNB anti-CD3 induced death than Tregs. A possible hypothesis is that TCR signaling is altered in Tregs compared to non-Tregs such that different downstream signals are activated in each cell type. Indeed, it was shown that overexpression of Foxp3 results in decreased PLC γ 1, ERK1/2 and S6 phosphorylation after TCR engagement (31). Alternatively, there may be differential expression of pro- or anti-apoptotic factors in Tregs versus conventional T cells that are responsible for the preferential death of conventional T cells. Conversely, if expression of these proteins is similar, post-translational modifications that cause their sequestration and affect their activity may be different (32). Interestingly, Tregs have also been shown to be less susceptible to cell death caused by low levels of whole body irradiation (33), suggesting that this mechanism may also be relevant in protecting Tregs from other signals that induce cell death.

The recent report of Helios expression as a marker for thymus-derived natural Tregs (29), led us to ask whether FNB anti-CD3 mAb had preferential effects on natural versus adaptive Tregs. Our data shows increased expression of Helios after FNB anti-CD3 mAb treatment, suggesting that Helios expression can be induced on a previously Helios^{lo/-} population although we cannot rule out the possibility that Helios^{lo/-} cells are preferentially depleted by the treatment. Helios expression does not seem to directly affect Treg function (29); however, data from our laboratory suggests that Helios^{lo/-} adaptive Tregs are not able to control autoimmune responses *in vivo* (Yadav, Bluestone, et al., manuscript submitted). Our laboratory also showed that at the time of disease onset, the Tregs in the pancreas of NOD mice express lower levels of Foxp3 and CD25, likely making them unstable and dysfunctional, and that treatment with IL-2/anti-IL-2 complexes can prevent disease by augmenting the percentage of Tregs in the pancreas (34). Finally, a greater proportion of Helios^{lo/-} Tregs produce cytokines (35); thus, we suggest that Helios expression is a marker of stable and functional Tregs. Therefore, we hypothesize that in addition to restoring the balance between effector and regulatory T cells, FNB anti-CD3 mAb also affects the balance between stable/functional and unstable/nonfunctional Tregs.

Finally, whether these results can be extended to the human setting is still controversial. Interestingly, a population of CD8⁺Foxp3⁺ cells arises in human patients treated with OKT3- γ 1(Ala-Ala2) (36), which we do not observe in mice treated with FNB anti-CD3 mAb. Unlike murine T cells, human T cells transiently express Foxp3 protein upon activation (37); however, Foxp3 expression does necessarily not confer suppressive function. Therefore, in humans, Foxp3 expression may not be a reliable marker of Tregs as it is in mice. In addition, the effect of FNB anti-CD3 mAb on human cells has been mostly studied *in vitro*; however it is possible that *in vitro* effects differ from *in vivo* effects, as we show that *in vitro* T cell death is Fas mediated but genetic depletion of Fas has no effect on T cell depletion *in vivo*.

In conclusion, our results suggest that FNB anti-CD3 mAb treatment reverses autoimmunity by deleting autoreactive pathogenic T cells and restoring the balance of pathogenic versus regulatory T cells towards increased regulation. This outcome is achieved by the selective sensitivity of Teffs and naïve T cells versus Tregs to FNB anti-CD3 mAb-induced cell death but not by *de novo* induction of adaptive Tregs or expansion of natural Tregs. Our results

have important implications for the design of FNB anti-CD3 mAb-based immunotherapies in autoimmune diseases and suggest the pertinence of developing combination therapies that would strengthen FNB anti-CD3 mAb modes of action. For instance, although counter-intuitive, anti-CD3 immunotherapy might be best when coupled with antigen immunization that drives the differentiation of conventional cells into an activated state, which will be more sensitive to anti-CD3 mAb induced cell death.

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Abbreviations used in this article

FNB	Fc Receptor Non-Binding
mAb	monoclonal antibody
Tregs	regulatory T cells
Teffs	effector T cells
LN	lymph node

Figure 1. FNB anti-CD3 mAb increases the percentage but not the absolute number of CD4⁺Foxp3⁺ T cells

Mice were treated with FNB anti-CD3 mAb (filled squares) or control Ig (empty circles) and peripheral LNs were harvested at the indicated times. Total cell numbers were determined and T cells subsets were analyzed by flow cytometry. Data shown is normalized to the Ig-treated age-matched mice that were harvested on the same day to control for changes in cell number due to age. (A) Normalized percentage of CD4⁺ cells within lymphocyte gate. (B) Normalized percentage of Foxp3⁺ cells within the CD4⁺ gate. (C) Normalized total CD4⁺ cell number. (D) Normalized total CD4⁺Foxp3⁺ cell number. Each data point represents an individual mouse. Data is representative of two independent experiments.

Figure 2. Depletion of T cells induced by FNB anti-CD3 mAb is due to cell death *in vivo*
 (A) Mice were treated with FTY720 daily on days 1–8 to block lymphocytes from exiting LNs and with FNB anti-CD3 mAb on days 2–6. Percentage of CD4⁺ T cells in LN of saline and FTY720 treated NOD mice were analyzed on day 9. Individual mice from two independent experiments are shown. (B–E) Mice were treated twice with FNB anti-CD3 mAb 24 hrs apart. 38hrs after first injection cells from LNs were harvested and cultured *in vitro* for the times specified. (B) Representative plots gated on CD4⁺ cells showing viability measured by DAPI uptake at the indicated times after incubation in media only. (C) Viability of cells cultured in media only, or media supplemented with (D) 1ng/ml IL-2 or (E) 10ng/ml IL-7. Data shown represents average of 5–6 mice pooled from at least two independent experiments. Bars represent standard deviation between samples.

Figure 3. FNB anti-CD3 mAb induces T cell death via distinct mechanisms *in vitro* and *in vivo* (A) Th1 cell lines generated from WT or Fas ligand-deficient gld DO11.10 TCR transgenic mice were exposed to a titrated dose of FNB anti-CD3 mAb *in vitro* and the number of viable cells, after overnight culture, was determined by flow cytometry by counting CD4⁺Annexin⁻PI⁻ cells. (B–D) WT, lpr, Bim-deficient or Bcl-2 transgenic mice were treated with FNB anti-CD3 mAb or PBS as control and the relative number or percentage of CD4⁺ cells in the LNs was determined one week after the initiation of the treatment. (E) Caspase-3 deficient mice were treated with control Ig or FNB anti-CD3 and the percentage of CD4⁺ cells in LNs was determined by flow cytometry on day 7. Data shown are a summary of at least two independent experiments.

Figure 4. FNB anti-CD3 mAb does not promote the conversion or expansion of Tregs

(A) CD4⁺GFP⁺ cells, genetically marked by the Thy 1 congenic marker, were sorted from Fxp3-GFP reporter mice and transferred to WT recipients that were treated with FNB anti-CD3 mAb. On day 7, LNs were harvested and expression of Fxp3 protein was analyzed on the transferred cells by flow cytometry. Representative dot plots and summary of two independent experiments are shown. (B) Fxp3.GFP-Cre × Rosa26.flox.stop.YFP mice were treated with FNB anti-CD3 mAb and LNs were analyzed on day 14 by flow cytometry. Representative FACS plots gated on CD4⁺ T cells are shown. (C) Percentage of YFP⁺ cells within the CD4⁺ population (sum of upper and lower right quadrants of plots shown in B). (D) Percentage of GFP⁺ cells within the YFP⁺ population (upper right quadrant divided by

sum of upper and lower right quadrants of plots shown in B). Summary of two independent experiments is shown.

Figure 5. FNB anti-CD3 mAb preferentially depletes CD4⁺Foxp3⁻ T cells

Purified CD4⁺ T cells, marked by the Thy 1 congenic marker, were labeled with CFSE and transferred to WT recipient mice that were treated with FNB anti-CD3. LNs were harvested on day 7 analyzed by flow cytometry. (A) Representative FACS plots and pooled data showing CFSE dilution and Foxp3 expression gated on transferred CD4⁺ T cells. (B) Ratio of Foxp3⁻ to Foxp3⁺ cells. (C) Representative histograms and pooled data showing the percentage of Foxp3⁻ cells that remained undivided. (D) Percentage of Foxp3⁺ cells that remained undivided. Each dot represents an individual mouse and data shown is pooled from two independent experiments.

Figure 6. FNB anti-CD3 mAb alters the balance of Helios-expressing Tregs

(A–B) NOD mice were treated with FNB anti-CD3 mAb. On day 7 all LNs and spleen were harvested and the percentage and total number of CD4⁺Foxp3⁺Helios^{hi} were calculated. Data shown are a summary of three independent experiments. (C) Thy1.1⁺ CD25⁺PD-1[−]Nrp1[−] (Helios^{lo/-}) and CD25⁺PD-1⁺Nrp1⁺ (Helios^{hi}) Tregs were sorted and transferred to Thy1.2⁺ mice. Recipients were treated with FNB anti-CD3 mAb. All LNs and spleen were harvested and Thy1.1 cells were enriched as described in the methods. Helios expression on transferred Foxp3⁺ cells was analyzed by flow cytometry. Representative histograms are shown on top and pooled data from two independent experiments are shown on bottom.

Table IRate of FNB anti-CD3 mAb depletion of naïve and Th1 effector CD4 cells *in vivo*^I

	Rate of depletion (%)	
	Endogenous naïve CD4	Transferred Th1
Exp1	36.8	65.5
Exp2	36.8	66.3
Exp3	12.1	40.0
Exp4	35.5	66.4

^ITh1 cell clones were differentiated as described in the materials and methods section and transferred to lympho-replete hosts that were then treated with FNB anti-CD3. The rate of depletion was calculated by comparing the percent of endogenous naïve (CD4⁺CD62L⁺CD25) and transferred cells in spleens of control vs treated mice.