



# Modulation of the Human T Cell Response by a Novel Non-Mitogenic Anti-CD3 Antibody

Hirokazu Shiheido, Chen Chen, Masaki Hikida, Takeshi Watanabe, Jun Shimizu\*

Center for Innovation in Immunoregulative Technology and Therapeutics, Graduate School of Medicine, Kyoto University, Kyoto, Japan

## Abstract

The agonistic anti-human CD3 $\epsilon$  antibody (Ab), OKT3, has been used to control acute transplant rejection. The *in vivo* administration of OKT3 was previously shown to induce the partial depletion of T cells and unresponsiveness (anergy) in the remaining CD4 $^+$  T cells. However, this therapy is also associated with the systemic release of several cytokines, which leads to a series of adverse side effects. We established a novel anti-human CD3 $\epsilon$  Ab, 20-2b2, which recognized a close, but different determinant on the CD3 $\epsilon$  molecule from that recognized by OKT3. 20-2b2 was non-mitogenic for human CD4 $^+$  T cells, could inhibit the activation of T cells *in vitro*, and induced T cell anergy in *in vivo* experiments using humanized mice. Cytokine release in humanized mice induced by the administration of 20-2b2 was significantly less than that induced by OKT3. Our results indicated that the CD3 $\epsilon$  molecule is still an attractive, effective, and useful target for the modulation of T cell responses. The establishment of other Abs that recognize CD3 $\epsilon$ , even though the determinant recognized by those Abs may be close to or different from that recognized by OKT3, may represent a novel approach for the development of safer Ab therapies using anti-CD3 Abs, in addition to the modification of OKT3 in terms of the induction of cytokine production.

**Citation:** Shiheido H, Chen C, Hikida M, Watanabe T, Shimizu J (2014) Modulation of the Human T Cell Response by a Novel Non-Mitogenic Anti-CD3 Antibody. PLoS ONE 9(4): e94324. doi:10.1371/journal.pone.0094324

**Editor:** Mauricio Martins Rodrigues, Federal University of São Paulo, Brazil

**Received:** January 16, 2014; **Accepted:** March 15, 2014; **Published:** April 7, 2014

**Copyright:** © 2014 Shiheido et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by the Special Coordination Funds by the Ministry of Education, Culture, Sports, Science and Technology of Japan and Astellas Pharma Inc. in the Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of this manuscript. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

**Competing Interests:** The authors declare no financial conflict of interest.

\* E-mail: jun@ak.med.kyoto-u.ac.jp

## Introduction

T cells become fully activated when they recognize an antigen and receive signals through co-stimulatory molecules. The activation of T cells is also known to be accompanied by the temporary down-modulation of the T cell receptor (TCR)/CD3 complex on the cell surface [1–3]. The manipulation of these events in the early stages of T cell activation, for example, by modifying antigenic determinants and/or by blocking the interaction between co-stimulatory molecules and ligands, has been shown to induce T cell unresponsiveness (anergy) [4–7]. We previously demonstrated that inducing the down-modulation of the TCR/CD3 complex without stimulating T cells resulted in the modulation of T cell responses [8].

In our previous study [9], we reported and characterized an Ab (Dow2) against mouse CD4 $^+$  T cells that was established based on its ability to induce the down-modulation of the TCR/CD3 complex and simultaneously not stimulate CD4 $^+$  T cells. Dow2 (rat IgG $_{2a}$ ) recognized mouse CD3 $\epsilon$ , induced T cell anergy *in vivo*, and was more effective than the well-known agonistic anti-mouse CD3 $\epsilon$  Ab, 145-2C11, in terms of the induction of an immunosuppressive state. Although undesired cytokine production needs to be considered when the anti-CD3 Ab is used *in vivo*, Dow2 was significantly less effective at inducing cytokine production *in vivo* than 145-2C11 [9].

In the present study, we attempted to establish an anti-human monoclonal Ab that could induce the down-modulation of the TCR/CD3 complex, but not the activation of T cells as well as the

anti-mouse Ab, Dow2, as described above. 20-2b2, the Ab established based on these criteria, was characterized in *in vitro* experiments and *in vivo* systems using humanized mice. 20-2b2 also recognized human CD3 $\epsilon$ . However, the mode of recognition by 20-2b2 differed from that of the well-studied agonistic anti-human CD3 $\epsilon$  Ab, OKT3. 20-2b2 could induce human CD4 $^+$  T cell anergy *in vivo* and was significantly less harmful in terms of cytokine induction *in vivo*.

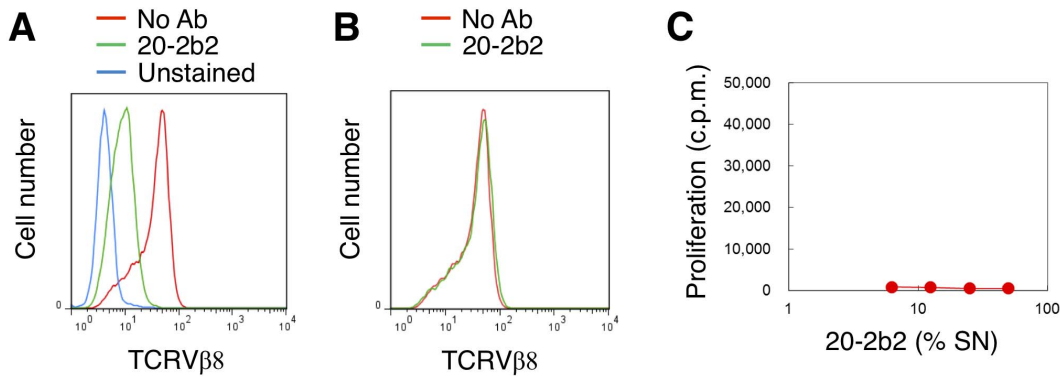
## Materials and Methods

### Antibodies and reagents

Antibodies against CD3 $\epsilon$  (OKT3 and M-20), TCRV $\beta$ 8, rat IgG, and mouse IgG were purchased from eBiosciences, Santa Cruz, and BD Biosciences. Phytohemagglutinin (PHA) and pokeweed mitogen (PWM) were purchased from Sigma. All FACS data were acquired on an FACSCanto II flow cytometer (BD Biosciences) using FACSDiva software. Data were analyzed using Flowjo software (Treestar). We analyzed cytokine contents using a Bio-Plex kit (Bio-Rad) following the manufacturer's instructions.

### Ethics statement

The study protocol was approved by the Review Board for human studies in Kyoto University. Peripheral blood was obtained from consenting healthy adult donors. All donors provided written informed consent. All mice were maintained in a specific pathogen-free animal facility. The experimental procedures and housing conditions for animals were approved by the Animal



**Figure 1. Non-stimulatory 20-2b2 induced the down-modulation of TCR expression.** (A) Jurkat cells were cultured in the presence (green) or absence (red) of 20-2b2 (50% SN) at 37°C. Cells were washed and stained with the anti-TCRV $\beta$ 8 Ab two hours later. Unstained Jurkat cells are shown as a blue line. (B) Jurkat cells were cultured in the presence (green) or absence (red) of 20-2b2 (50% SN) at 4°C for 20 min, and then stained with anti-TCRV $\beta$ 8 Ab conjugated with FITC. (C) The PBMCs of healthy donors were cultured with the titrated amount of 20-2b2. Cell proliferation was measured two days later. Data are expressed as the mean  $\pm$  SD of triplicate cultures. The proliferation of PBMCs without a stimulus was measured as  $718 \pm 346$  cpm. Results are representative of three independent experiments (A–C). doi:10.1371/journal.pone.0094324.g001

Experimental Committee at Kyoto University School of Medicine, and all animals were cared for and treated humanely in accordance with the Institutional Guidelines for Experiments using Animals.

### Cell preparation

Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood using a Ficoll-Paque PLUS (GE Amersham) gradient. CD4<sup>+</sup> T cells were isolated using magnetic beads conjugated with the anti-CD4 Ab and a magnetic column (Miltenyi Biotec). Jurkat cells [10] and J.RT3-T3.5 [11,12] were purchased from the American Type Culture Collection (ATCC).

### Preparation of monoclonal antibodies

Wistar rats (2 months old, purchased from Japan SLC) were intraperitoneally immunized three times every 2 wks with CD4<sup>+</sup> T cells ( $2 \times 10^6$ ) prepared from fresh healthy human PBMC, and were intravenously injected with human CD4<sup>+</sup> T cells ( $1 \times 10^7$ ) one month later. Spleen cells were fused with P3X63Ag8.653 myeloma cells (from ATCC) 3 days after the final immunization. Supernatants (SNs) from the resulting hybridomas were screened for their ability to induce the down-modulation of the TCR/CD3 complex. Selected hybridomas in terms of stable-inducing activity were subjected to subsequent cloning. One clone, 20-2b2 (rat IgG<sub>2a</sub>), was established.

### Cell culture

Human T cells ( $1 \times 10^4$ /w) were stimulated with anti-CD3/CD28-coated beads ( $2 \times 10^4$ /w, Dynabeads, Invitrogen). Cells were maintained at 37°C with 5% CO<sub>2</sub> in RPMI supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) unless otherwise stated. The proliferation of T cells was assessed by measuring the incorporation of [<sup>3</sup>H]TdR (37 kBq/well) for the final 4 hr of a 2- to 3-day culture.

### Immunoblotting

Cells were washed twice with PBS, and lysed for 1 hr at 4°C in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100) supplemented with protease and phosphatase inhibitors (both purchased from Nacalai Tesque, Japan). Cell lysates were then separated from the debris by

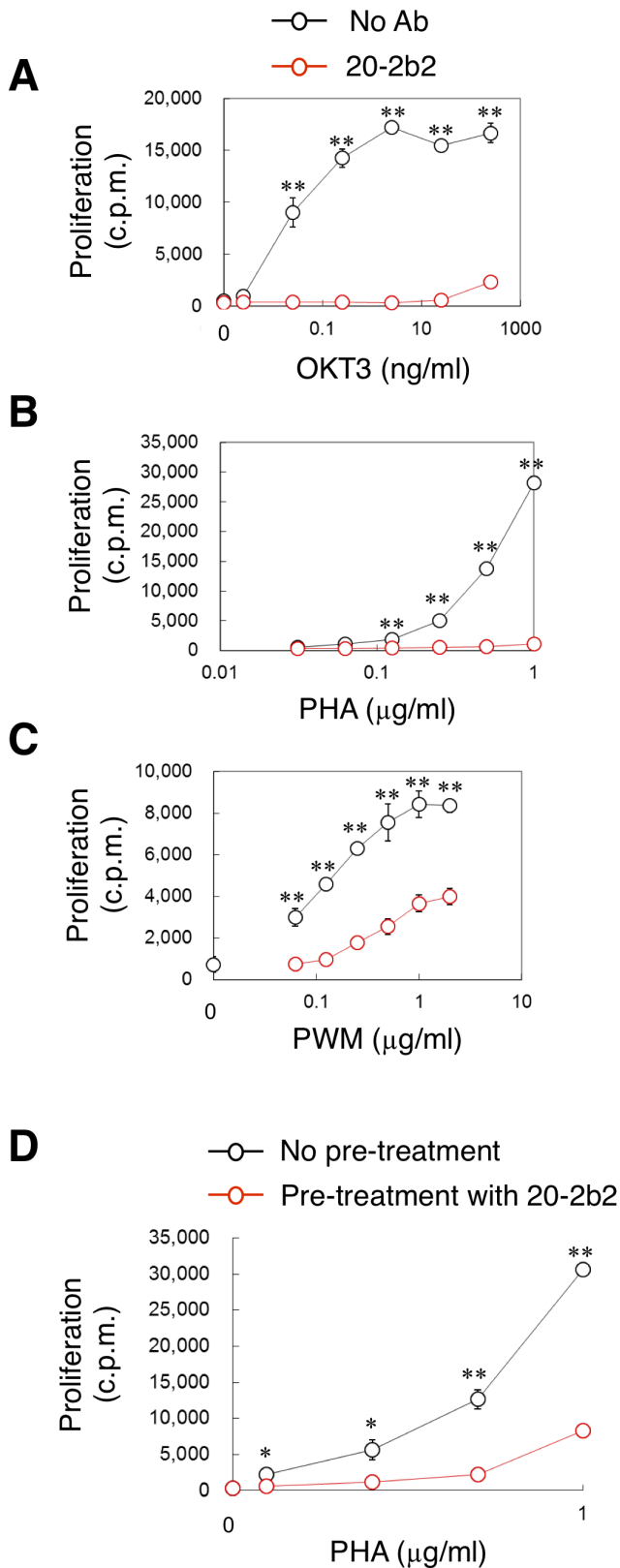
centrifugation at 20,000 *g* for 15 min at 4°C. Protein concentrations were determined by the BCA protein assay (Thermo), 5–20  $\mu$ g cell lysates were separated by SDS-PAGE under reduced conditions, and proteins were electrotransferred onto PVDF membranes (Millipore). After blocking with blocking-one (Nacalai Tesque) in Tris-Buffered saline containing 0.1% Tween 20, the membrane was incubated overnight at 4°C with the indicated primary antibody, washed, and subjected to chemiluminescence detection with the HRP-conjugated secondary antibody with ECL (Millipore). In some experiments, cell lysates (500–1000  $\mu$ g) were incubated with the indicated primary antibody for 2 hr at 4°C. Immunocomplexes were precipitated with protein A-Sepharose (Sigma) for 1 hr at 4°C. Immunoprecipitates were washed four times with ice-cold wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% Triton X-100). Immunoprecipitated proteins were eluted with sample buffer containing 100 mM DTT and heated for 10 min at 96°C.

### Plasmid preparation and transfection

Total RNA was extracted from  $3 \times 10^6$  Jurkat cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, followed by reverse transcription with the Superscript III first-strand synthesis system for RT-PCR (Invitrogen) using oligo(dT)<sub>20</sub>. The resultant cDNA was used as a template for PCR using 5'-AAGCGGCCGACCATGTCAGTCGGGCACTCA-3' and 5'-AAGGATCCACCTCAGATGCGTCTCTGATTCAGG-3' as forward and reverse primers, respectively, to obtain the full-length of the human CD3 $\epsilon$  (hCD3 $\epsilon$ ) gene. The PCR product was cloned into the NotI/BamHI site of the pQCXIX-derived pQCXIXGFP vector that encoded the GFP gene downstream of the IRES site, resulting in the pQC-hCD3 $\epsilon$ GFP expression vector. We transfected Yac-1 cells with the human CD3 $\epsilon$  expression vector, pQC-hCD3 $\epsilon$ GFP, using Lipofectamine LTX and Plus Reagent (Invitrogen) according to the manufacturer's instructions. GFP<sup>+</sup> cells were sorted and expanded. This cycle was repeated, and the resulting stable line was used as hCD3 $\epsilon$ -Yac-1 cells.

### Humanized mice

Six-week-old female NOD/shi-scid/ $\gamma$ c<sup>null</sup> (NOG) mice were obtained from the Central Institute for Experimental Animals (CIEA). Mice were irradiated with 2 Gy, and  $1 \times 10^5$  cord blood CD34<sup>+</sup> cells, which were obtained from Lonza Walkersville Inc.,



**Figure 2. 20-2b2 inhibited the activation of T cells *in vitro*.** (A–C) The PBMCs of healthy donors were cultured with an anti-CD3 Ab (OKT3) (A), PHA (B), or PWM (C) in the presence (red symbols) or absence (black) of 20-2b2 (50% SN). (D) PBMCs were pre-treated with medium (black) or 20-2b2 (red) at 37°C. Cells were washed three times and cultured with PHA two hours later. Cell proliferation was measured two

days later. Values are shown as the mean  $\pm$  SD of triplicate cultures. Data are representative of more than three (A–C) and three (D) independent experiments. \* $p$ <0.01, \*\* $p$ <0.001 by the Student's *t*-test. doi:10.1371/journal.pone.0094324.g002

were transferred into these mice by an intravenous injection. Mice were then used as humanized NOG (Hu-NOG) mice 20–30 weeks later.

## Results

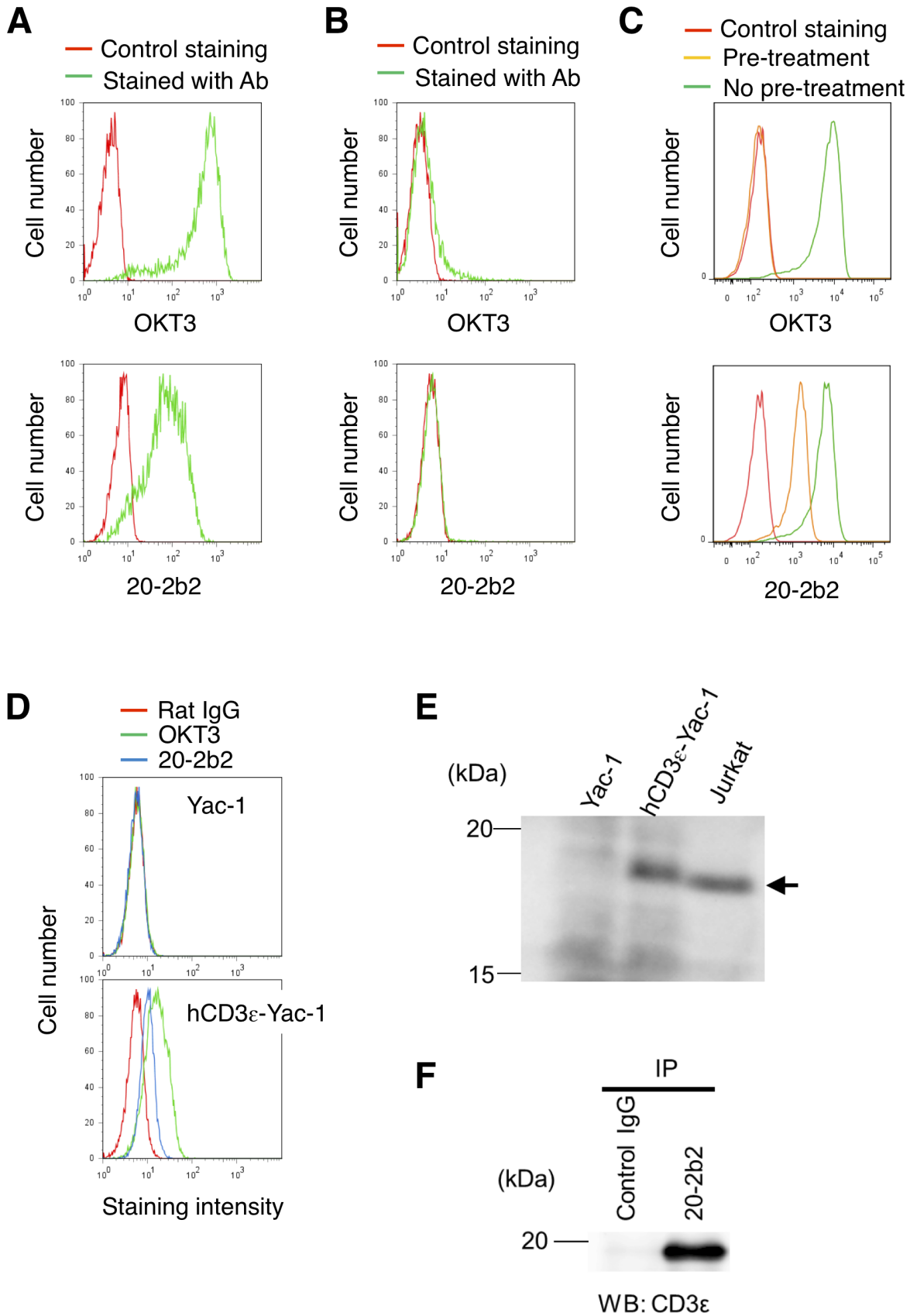
### 20-2b2 inhibited CD4<sup>+</sup> T cell activation

Rats were immunized with human CD4<sup>+</sup> T cells prepared from PBMCs, spleen cells from immunized rats were fused with myeloma cells, and the resulting hybridomas were selected based on their ability to induce the down-modulation of the TCR/CD3 complex in the human T cell line, Jurkat, which used TCRV $\beta$ 8 [13]. The selected hybridomas were further screened for their ability to have no stimulatory activity on T cells in PBMCs. We established the monoclonal Ab, 20-2b2 (rat IgG<sub>2a</sub>) from this two step screening. As shown in Figure 1A, pre-treating Jurkat cells with 20-2b2 at 37°C for two hours resulted in diminished staining with the anti-TCRV $\beta$ 8 Ab. In contrast, pre-treating Jurkat cells with 20-2b2 at 4°C had no influence on staining with the anti-TCRV $\beta$ 8 Ab (Figure 1B), which demonstrated that neither 20-2b2 nor the anti-TCRV $\beta$ 8 Ab were competitive in staining (Figure 1B), and that pre-treating Jurkat cells with 20-2b2 at 37°C resulted in the down-modulation of TCR (Figure 1A). Whole PBMCs were then cultured with a titrated amount of 20-2b2. However, 20-2b2 did not induce any proliferation (Figure 1C). PBMCs cultured with an agonistic anti-CD3 Ab (OKT3 at 1 ng/ml) as a positive control led to the marked proliferation (36,602 $\pm$ 2,228 cpm in the same experiment shown as Figure 1C) and production of IL-2 (16 U/ml). In contrast, stimulating PBMCs with 20-2b2 resulted in less than 1 U/ml of IL-2 being produced. These results demonstrated that 20-2b2 had the ability to induce the down-modulation of the CD3/TCR complex (Figure 1A) without activating T cells (Figure 1C).

We further investigated the biological activities of 20-2b2 *in vitro*. Whole PBMCs were cultured with various stimuli (agonistic anti-CD3 $\epsilon$  Ab, OKT3; T cell-mitogen, PHA; and T cell- and B cell-mitogen, PWM) in the presence or absence of 20-2b2. 20-2b2 strongly inhibited the *in vitro* activation of T cells against any stimuli (Figure 2A–C). Furthermore, the pre-treatment of cells with 20-2b2 resulted in the inhibition of T cell activation (Figure 2D). These results demonstrated that 20-2b2 was not only a non-stimulatory (Figure 1C), but also inhibitory Ab for T cells (Figure 2A–C), and suggested that 20-2b2 was unlikely to be toxic for T cells *in vitro* (Figure 2D).

### 20-2b2 recognized CD3 $\epsilon$

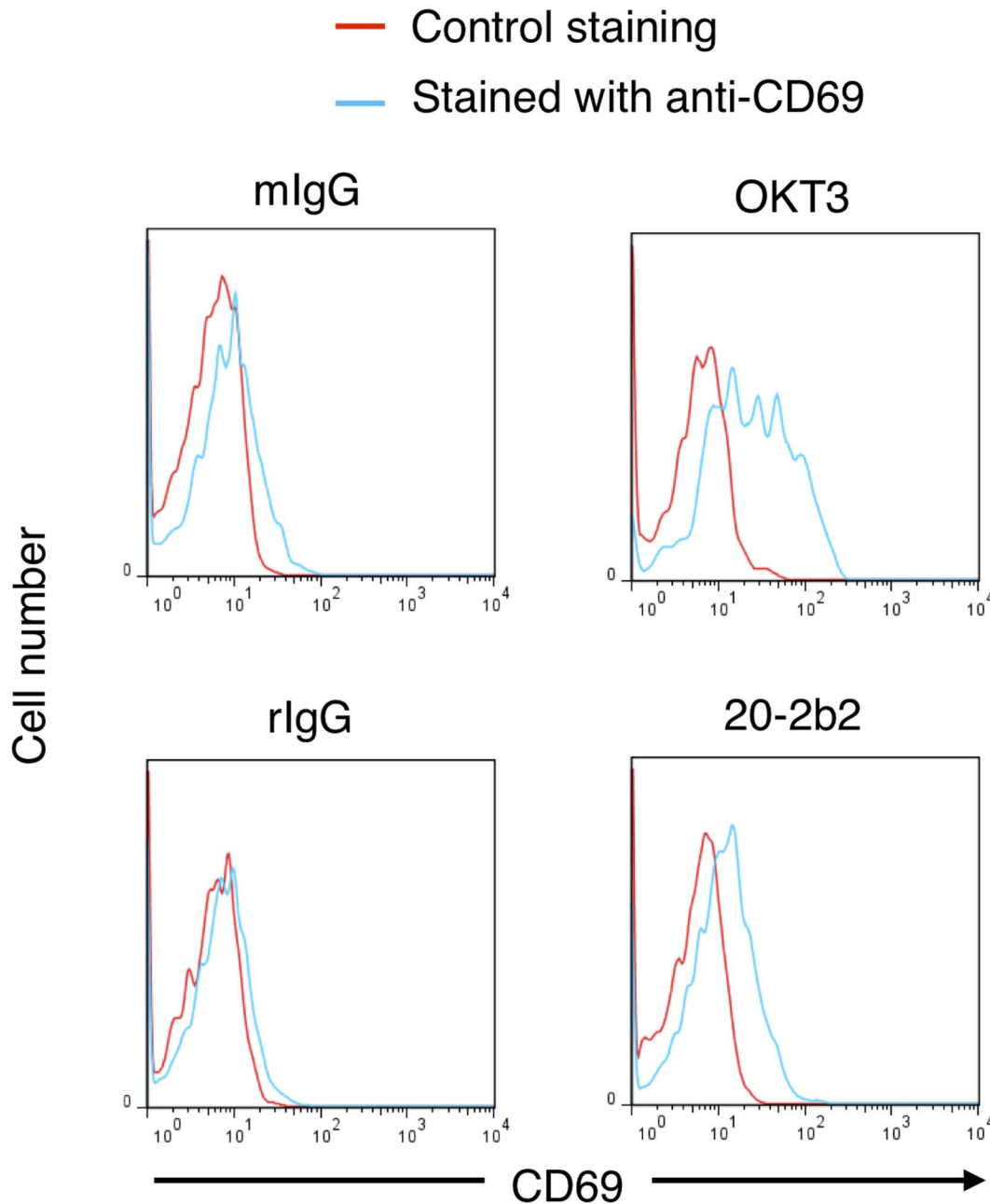
We then attempted to identify the target molecule recognized by 20-2b2. 20-2b2 could stain Jurkat cells as well as the anti-human CD3 $\epsilon$  Ab, OKT3 (Figure 3A). On the other hand, the mutant cell line derived from Jurkat cells, J.RT-3-T3.5 [11,12], which do not express either CD3 or TCR on their surface, was not stained with OKT3 or 20-2b2 (Figure 3B). Furthermore, the pre-treatment of Jurkat cells with 20-2b2 resulted in unstaining with OKT3 (Figure 3C, top panel), while pre-treating with OKT3 led to diminished staining with 20-2b2 (Figure 3C, bottom panel). Taken together, these results prompted us to assume that 20-2b2 may recognize a component of the CD3/TCR complex, possibly the determinant on CD3 $\epsilon$  molecules. To confirm this, the mouse T cell line, Yac-1, was transfected with human CD3 $\epsilon$  (hCD3 $\epsilon$ ), and a stable line was established (see Materials and Methods).



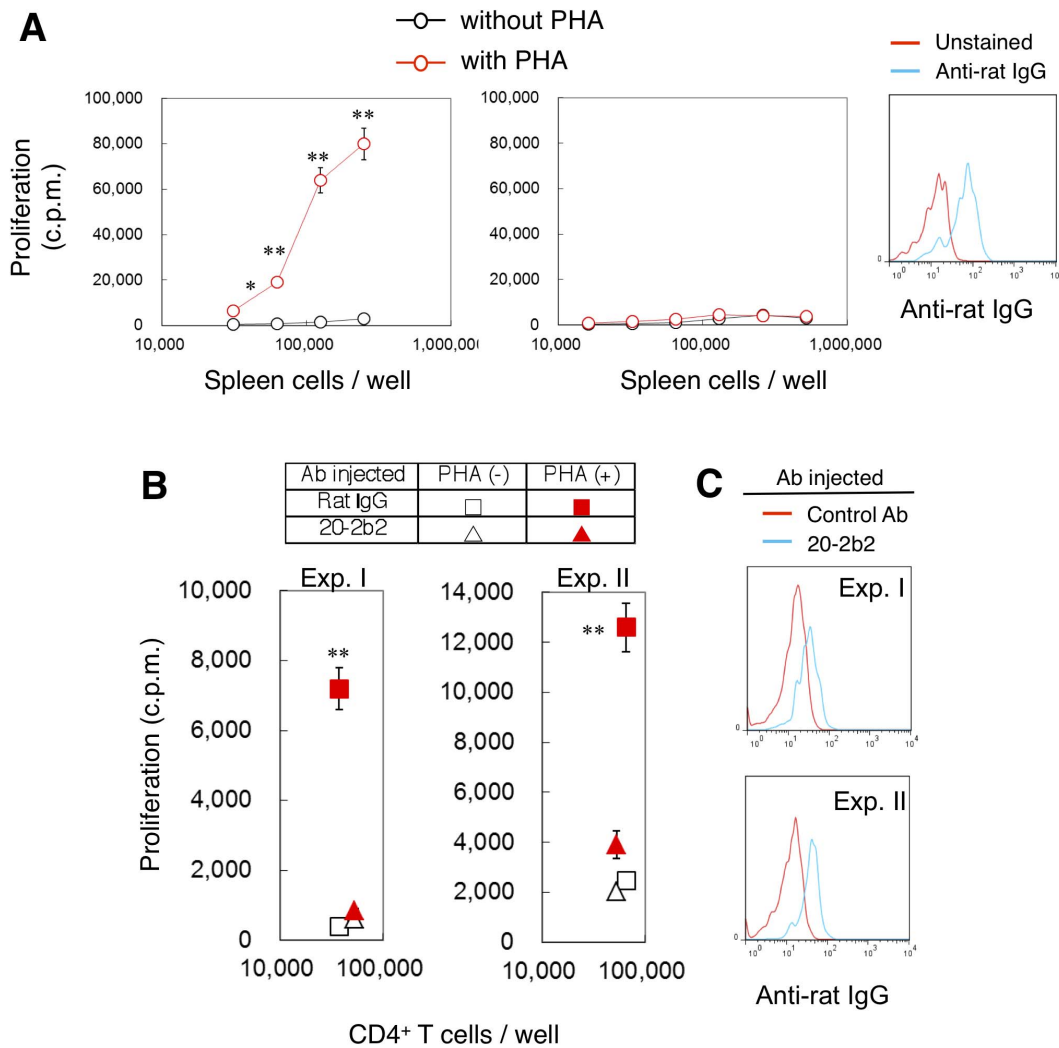
**Figure 3. 20-2b2 recognized CD3ε.** (A, B) Jurkat (A) or J.RT3-T3.5 cells (B) were stained with anti-CD3ε Ab (OKT3) or 20-2b2 (green lines). Unstained cells are shown as red lines. (C) Jurkat cells were pre-treated with 20-2b2 (top panel) or OKT3 (bottom panel), and were then stained with the indicated Ab (yellow lines). Jurkat cells stained without the pre-treatment are shown as green lines. Negative control staining is shown as red lines. (D) Yac-1 or Yac-1 cells transfected with human CD3ε (hCD3ε-Yac-1) were stained with the anti-CD3ε Ab (OKT3) (green) or 20-2b2 (blue). Cells stained with rat IgG as a negative control are shown as red lines. (E) Cell lysates prepared from the indicated cells were separated in SDS-PAGE and blotted with 20-2b2. Arrow: the target molecule recognized by 20-2b2. (F) The lysate from Jurkat cells was immunoprecipitated (IP) with 20-2b2 or control rat IgG. Immunoprecipitates were separated by SDS-PAGE. The blot was probed (WB) with the anti-CD3ε Ab (M-20). Results are representative of three independent experiments (A-F).  
doi:10.1371/journal.pone.0094324.g003

Mouse CD3ε in Yac-1 cells was expected to be partly substituted by hCD3ε in the resulting transfected stable line (hCD3ε-Yac-1). The anti-hCD3ε Ab (OKT3) could stain hCD3ε-Yac-1

(Figure 3D). 20-2b2 could also stain hCD3ε-Yac-1, but with less intensity. We performed more biochemical experiments. Lysates prepared from Yac-1, hCD3ε-Yac-1, and Jurkat cells were



**Figure 4. 20-2b2 induced the expression of CD69 on CD4<sup>+</sup> T cells *in vivo*.** Hu-NOG mice were intravenously inoculated with one of the following Abs (20 μg/mouse); mouse IgG<sub>2a</sub> (mIgG), rat IgG (rIgG), OKT3, or 20-2b2. Spleen cells were stained with the anti-CD4 Ab and a control Ab or anti-CD69 Ab six hours later. Cells stained with a control Ab (red) or anti-CD69 Ab (blue) in CD4<sup>+</sup> T cells were shown as a histogram. Results are representative of three individual Hu-NOG mice.  
doi:10.1371/journal.pone.0094324.g004



**Figure 5. 20-2b2 induced CD4<sup>+</sup> T cell anergy *in vivo*.** (A) Hu-NOG mice were intravenously injected with one of the following Abs (100  $\mu$ g/mouse); control Ab (rat IgG) (left panel) or 20-2b2 (middle panel). Whole spleen cells were prepared and cultured in the presence (red) or absence (black) of PHA (1  $\mu$ g/ml) twenty-four hours later. Cell proliferation was measured two days later. Values are shown as means  $\pm$  SD of triplicate cultures. Aliquots of spleen cells from 20-2b2-injected Hu-NOG mice were stained with the anti-CD4 Ab with or without the anti-rat IgG Ab. Staining with the anti-rat IgG Ab on CD4<sup>+</sup> T cells is shown as blue line. CD4<sup>+</sup> T cells without anti-rat IgG Ab staining are shown as red lines. Data are representative of more than five experiments. (B) Two NOG mice (2 Gy irradiated) as recipients were inoculated with spleen cells from one Hu-NOG mouse. One NOG mouse as a recipient was injected with the control Ab (square, 20  $\mu$ g/mouse) and another NOG mouse with 20-2b2 (triangle, 20  $\mu$ g/mouse). CD4<sup>+</sup> T cells were prepared from these NOG mice seven days later and cultured in the presence (closed red) or absence (open black) of PHA (1  $\mu$ g/ml). Cell proliferation was measured three days later. Values are shown as the mean  $\pm$  SD of triplicate cultures. (C) Aliquots of CD4<sup>+</sup> T cells from control Ab-injected (red lines) or 20-2b2-injected NOG mice (blue lines) in (B) were stained with anti-rat IgG. Data from two individual experiments (Exp. I and Exp. II) are shown. \* $p$ <0.01, \*\* $p$ <0.001 by the Student's *t*-test, cell proliferation induced by the PHA stimulation with the control Ab versus the 20-2b2 injection.

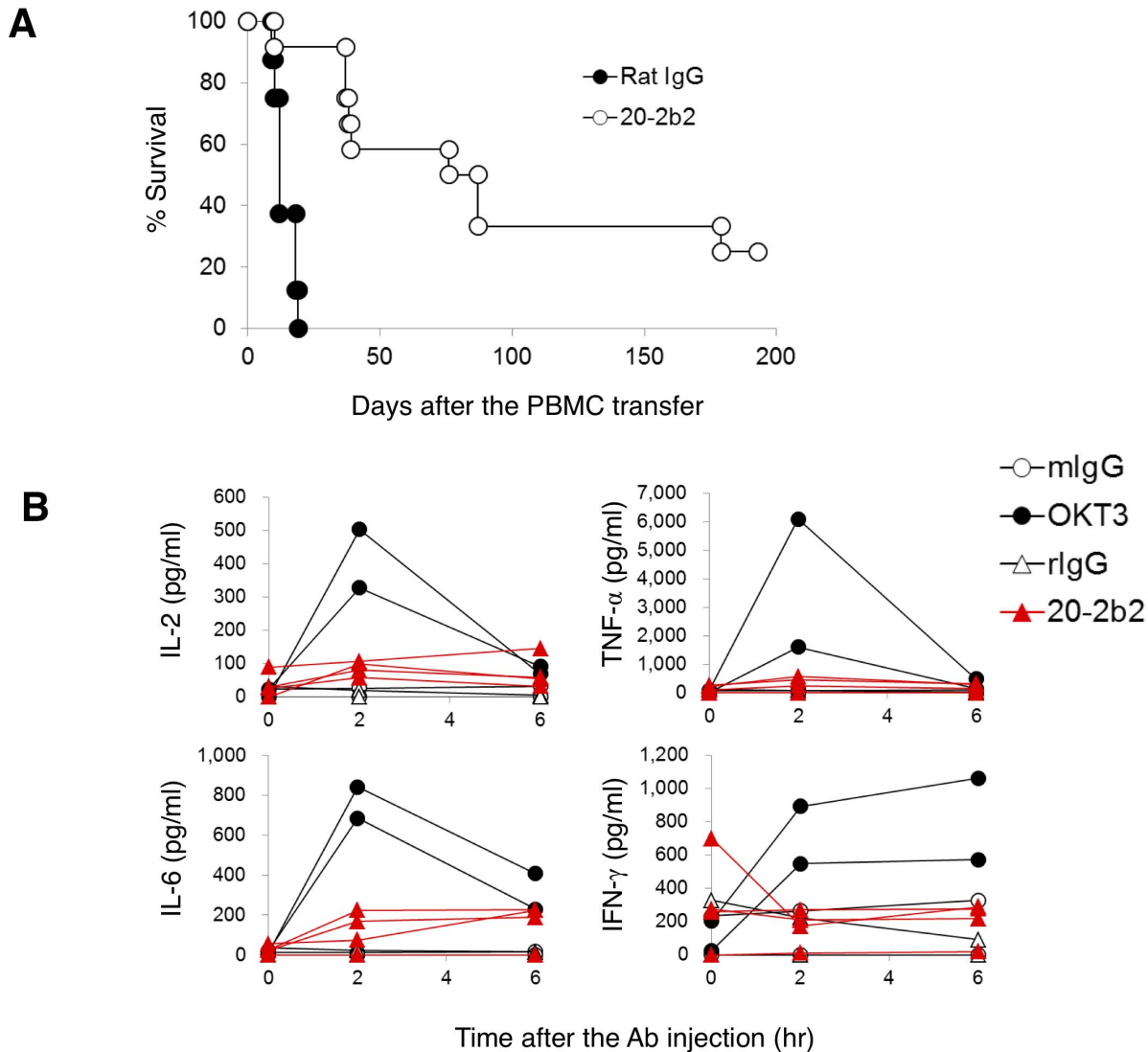
doi:10.1371/journal.pone.0094324.g005

subjected to SDS-PAGE under non-reducing conditions, transferred onto a membrane, and blotted with 20-2b2 (Figure 3E). The resulting specific band with a molecular weight of 18 kDa was detected in both lysates from the hCD3 $\epsilon$ -positive cells, hCD3 $\epsilon$ -Yac-1, and Jurkat cells, but not from the hCD3 $\epsilon$ -negative cells, Yac-1. Moreover, we confirmed that the immunoprecipitated protein from Jurkat cell lysates with 20-2b2 was detected with the anti-CD3 $\epsilon$  Ab (Figure 3F). Taken together, these results demonstrated that 20-2b2 recognized human CD3 $\epsilon$ , whereas the determinant recognized by 20-2b2 may be close to, but different from that recognized by OKT3 (Figure 3C). In addition, 20-2b2 may have lower affinity against CD3 $\epsilon$  molecules than that of

OKT3 because weaker staining with 20-2b2 was observed, as shown in Figure 3A and D.

### 20-2b2 induced CD4<sup>+</sup> T cell anergy *in vivo*

The *in vivo* biological activities of 20-2b2 were then examined. 20-2b2 (20  $\mu$ g/mouse) was intravenously injected into humanized NOG (Hu-NOG) mice (Figure 4). We observed that almost all CD4<sup>+</sup> T cells in the peripheral blood were depleted 6 hr after the injection of 20-2b2 (% CD4<sup>+</sup> T cells in the peripheral blood at 0 hr: mean  $\pm$  SD = 17.0  $\pm$  3.0,  $n$  = 3; at 6 hr: 1.2  $\pm$  0.3,  $n$  = 3;  $p$  < 0.001 by the Student's *t*-test). We also obtained similar results by injecting the agonistic anti-CD3 Ab (OKT3, 20  $\mu$ g/mouse, % CD4<sup>+</sup> T cells in the peripheral blood: 15.3% at 0 hr to 1.2% at



**Figure 6. Immunosuppressive activity of 20-2b2 *in vivo*.** (A) Irradiated (2 Gy) NOG mice were intravenously injected with whole PBMCs ( $2 \times 10^6$ /mouse) from a healthy donor on day 0. Mice were intravenously injected with the control Ab (closed symbols,  $n = 8$ ) or 20-2b2 (open symbols,  $n = 12$ ) on days 2, 9, and 16 (20  $\mu\text{g}/\text{mouse}/\text{injection}$ ). The survival of mice was monitored. Data from two independent experiments were pooled.  $p < 0.001$  in rat IgG versus 20-2b2, by the F-test. (B) Hu-NOG mice were intravenously injected with one of the following Abs (20  $\mu\text{g}/\text{mouse}$ ); control mouse IgG<sub>2a</sub> (mlgG), rat IgG (rlgG), OKT3, or 20-2b2. Serum samples were obtained 0, 2, and 6 hours after the injection. Cytokine levels were measured. Data from the same Hu-NOG mouse are linked with a line. Data from two experiments are shown. Standard deviations were less than 10% of the mean. doi:10.1371/journal.pone.0094324.g006

6 hr in a representative experiment). In contrast, a significant number of CD4<sup>+</sup> T cells existed in the spleen at the same time point. Furthermore, the slightly increased expression of CD69 (the earliest inducible activation marker of T cells) was observed on CD4<sup>+</sup> T cells from 20-2b2-injected Hu-NOG mice, although this expression level was lower than that induced by OKT3 (Figure 4, the increase in staining = (the mean fluorescence intensity (MFI) by anti-CD69 Ab staining) – (MFI by control staining): mean  $\pm$  SD =  $22.6 \pm 8.38$  in OKT3-injected Hu-NOG mice,  $n = 3$ ;  $10.4 \pm 4.84$  in 20-2b2-injected Hu-NOG mice,  $n = 3$ ;  $p < 0.05$  by the Student's *t*-test). These results demonstrated that 20-2b2 was partly depletive *in vivo* and actively functioned against CD4<sup>+</sup> T cells *in vivo*.

Whole spleen cells were prepared 24 hr after Hu-NOG mice were inoculated with 20-2b2. Spleen cells from control Ab-injected Hu-NOG mice mounted cell proliferation against the T cell

mitogen, PHA (Figure 5A, left panel). On the other hand, cell proliferation was not observed in spleen cells from 20-2b2-injected Hu-NOG mice (Figure 5A, middle panel); we confirmed the existence of T cells in these spleen cells (for example, in the same experiment, as shown in Figure 5A middle panel, 32.3% CD4<sup>+</sup> T cells and 16.9% CD8<sup>+</sup> T cells were contained in human CD45<sup>+</sup> cells). We also observed the staining of CD4<sup>+</sup> T cells from 20-2b2-injected Hu-NOG mice with the anti-rat IgG Ab (Figure 5A, right panel), which indicated that the injected 20-2b2 was binding and remained on the T cell surface.

We further investigated the *in vivo* function of 20-2b2. Spleen cells from unmanipulated Hu-NOG mice were transferred into two other NOG mice on day 0, which were then injected with the control Ab or 20-2b2 on the same day. CD4<sup>+</sup> T cells (>80% purity) were prepared from these mice on day 7, and cultured with PHA for three days. On day 7 after the 20-2b2 injection, we still

observed the low intensity staining of CD4<sup>+</sup> T cells from 20-2b2-injected NOG mice with the anti-rat IgG Ab (Figure 5C), which indicated that injected 20-2b2 still slightly remained on the cell surface. As shown in Figure 5B, whereas CD4<sup>+</sup> T cells from control Ab-injected mice could proliferate following the PHA stimulation, CD4<sup>+</sup> T cells from 20-2b2-injected mice exhibited T cell anergy. Taken together, these results indicated that 20-2b2 possessed the ability to induce anergy in T cells *in vivo*. Furthermore, the injection of 20-2b2 alone without the simultaneous injection of any antigen resulted in the induction of T cell anergy, which demonstrated that the induction of T cell anergy by 20-2b2 did not require an antigen stimulation.

We used a xenogenic graft versus host disease (GVHD) model to examine the ability of 20-2b2 to suppress immune responses *in vivo* (Figure 6A). Irradiated NOG mice were transferred with PBMCs from a healthy donor on day 0 and then injected with a control Ab or 20-2b2 on days 2, 9, and 16. All NOG mice injected with the control Ab died within 19 days (median graft survival time (MST) = 13.75 days). In contrast, 20-2b2 significantly prolonged the survival of recipient mice (MST = 51.38 days). We examined whether human CD4<sup>+</sup> T cells existed in the peripheral blood of NOG mice transferred with human PBMCs and treated with 20-2b2. In a representative experiment, CD4<sup>+</sup> T cells ranging from 12.5 to 34.9% were detected on day 29 in the peripheral blood of NOG mice (n = 5) treated with 20-2b2. On day 40, 85-89% of CD4<sup>+</sup> T cells were observed. Taken together, these results demonstrated that the inoculation with 20-2b2 induced a temporary decrease in CD4<sup>+</sup> T cells in the peripheral blood, as described above, that 20-2b2 was a strong immunosuppressive agent against human cells even *in vivo*, and that this prolongation was not simply due to the depletion of T cells. The injection of 20-2b2 starting from day 2 was effective even after the transfer of PBMCs into NOG mice on day 0, which suggested that 20-2b2 was immunosuppressive even for primed T cells and/or T cells being primed.

The possibility of the induction of cytokine release has to be considered when the anti-CD3 Ab is used *in vivo* [14–16]. Therefore, Hu-NOG mice were injected with the control Abs, OKT3 or 20-2b2, and serum was collected 2 and 6 hours later. As shown in Figure 6B, OKT3 strongly induced the release of all cytokines examined. In contrast, 20-2b2 weakly induced the release of cytokines. These results demonstrated that 20-2b2 was an ideal and safer Ab in terms of cytokine release for inducing an immunosuppressive state *in vivo*.

## Discussion

The anti-human CD3ε Ab, OKT3, was the first monoclonal Ab to be used clinically [17–20]. OKT3 exhibited strong immuno-

suppressive potency in its first clinical trials. However, because of its lymphocytic mitogenic activity, OKT3 was found to be a potent inducer of cytokines *in vivo*, including IL-2, IFN-γ, and, especially, TNF-α [21,22]. The production of these cytokines resulted in various adverse side effects [21,23]. To solve these problems, a modification to the Fc region in the Ab and/or the humanization of Abs were introduced [24–27], and engineered Abs were characterized using *in vivo* models [27–31]. Although these Abs exhibited significantly diminished mitogenic activity (although not completely non-mitogenic), the mild release of cytokines has still been reported [28,29,32,33]. These studies were performed by focusing on limited anti-CD3ε Abs, including OKT3 or other particular anti-CD3ε Abs. Abs against the same molecule, but a different determinant, are known to exhibit different biological activities and effectivities [8,34]. Therefore, this may represent one approach to establish Abs against novel determinants on the CD3ε molecule for the development of and improvements in Ab therapies focused on the CD3ε molecule. In the case of 20-2b2 reported in this study, a modification in the Fc region of 20-2b2 may improve its less, but not null, adverse effects (Figure 6B). Furthermore, as shown in this study, establishing new Abs with the ability to down-modulate the TCR/CD3 complex, but not to stimulate T cells, that recognize the determinant on CD3ε, which is close to or different from that recognized by well-characterized anti-CD3ε Abs, such as OKT3, may provide a new tool for inducing immunosuppression.

We demonstrated that 20-2b2 was not simply a binding Ab, but actively functioned against CD4<sup>+</sup> T cells *in vivo*; e.g. the induction of the activation marker, CD69 (Figure 4). However, its efficiency in inducing the expression of CD69 was lower than that of OKT3. This low efficiency or incompleteness may be important in inducing T cell anergy. Previous studies demonstrated that manipulations/modifications in the early stage of T cell activation led to the induction of T cell anergy [4,5,7]. Therefore, the slight induction of the activation marker by 20-2b2 was attributed to an incomplete activation process in the early stage of T cell activation, and may result in the induction of T cell anergy (Figure 5). The molecular mechanisms by which 20-2b2 induces T cell anergy need to be investigated in more detail.

## Acknowledgments

We thank Kozue Urata for the excellent technical assistance.

## Author Contributions

Conceived and designed the experiments: HS JS. Performed the experiments: HS JS CC. Analyzed the data: HS JS. Contributed reagents/materials/analysis tools: MH TW. Wrote the paper: JS.

## References

- Cantrell DA, Davies AA, Crumpton MJ (1985) Activators of protein kinase C down-regulate and phosphorylate the T3/T-cell antigen receptor complex of human T lymphocytes. *Proc Natl Acad Sci U S A* 82: 8158–8162.
- Dietrich J, Hou X, Wegener AM, Geisler C (1994) CD3 gamma contains a phosphoserine-dependent di-leucine motif involved in down-regulation of the T cell receptor. *EMBO J* 13: 2156–2166.
- Luton F, Buferne M, Davoust J, Schmitt-Verhulst AM, Boyer C (1994) Evidence for protein tyrosine kinase involvement in ligand-induced TCR/CD3 internalization and surface redistribution. *J Immunol* 153: 63–72.
- Janeway CA Jr, Bottomly K (1994) Signals and signs for lymphocyte responses. *Cell* 76: 275–285.
- Schwartz RH (1996) Models of T cell anergy: is there a common molecular mechanism? *J Exp Med* 184: 1–8.
- Grakoui A, VanDyk LF, Dowdy SE, Allen PM (1998) Molecular basis for the lack of T cell proliferation induced by an altered peptide ligand. *Int Immunol* 10: 969–979.
- Sloan-Lancaster J, Shaw AS, Rothbard JB, Allen PM (1994) Partial T cell signaling: altered phospho-zeta and lack of zap70 recruitment in APL-induced T cell anergy. *Cell* 79: 913–922.
- Nishida E, Chen C, Morita A, Shimizu J (2011) Inhibition of T cell activation through down-regulation of TCR-CD3 expression mediated by an anti-CD90 Ab. *Immunol Lett* 136: 163–170.
- Shiheido H, Aoyama T, Takahashi H, Hanaoka K, Abe T, et al. (2014) A novel CD3-specific antibody induces immunosuppression via impaired phosphorylation of LAT and PLCγ1 following T-cell stimulation. *Eur J Immunol* DOI: 10.1002/eji.201344146
- Abraham RT, Weiss A (2004) Jurkat T cells and development of the T-cell receptor signalling paradigm. *Nat Rev Immunol* 4: 301–308.
- Ohashi PS, Mak TW, Van den Elsen P, Yanagi Y, Yoshikai Y, et al. (1985) Reconstitution of an active surface T3/T-cell antigen receptor by DNA transfer. *Nature* 316: 606–609.
- Weiss A, Stobo JD (1984) Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J Exp Med* 160: 1284–1299.



13. Fleischer B, Necker A, Leget C, Malissen B, Romagne F (1996) Reactivity of mouse T-cell hybridomas expressing human V $\beta$  gene segments with staphylococcal and streptococcal superantigens. *Infect Immun* 64: 987–994.
14. Kaufman A, Herold KC (2009) Anti-CD3 mAbs for treatment of type 1 diabetes. *Diabetes Metab Res Rev* 25: 302–306.
15. Chatenoud L (2004) Anti-CD3 antibodies: towards clinical antigen-specific immunomodulation. *Curr Opin Pharmacol* 4: 403–407.
16. Martin A, Tisch RM, Getts DR (2013) Manipulating T cell-mediated pathology: targets and functions of monoclonal antibody immunotherapy. *Clin Immunol* 148: 136–147.
17. Cosimi AB, Burton RC, Colvin RB, Goldstein G, Delmonico FL, et al. (1981) Treatment of acute renal allograft rejection with OKT3 monoclonal antibody. *Transplantation* 32: 535–539.
18. Cosimi AB, Colvin RB, Burton RC, Rubin RH, Goldstein G, et al. (1981) Use of monoclonal antibodies to T-cell subsets for immunologic monitoring and treatment in recipients of renal allografts. *N Engl J Med* 305: 308–314.
19. (1985) A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. Ortho Multicenter Transplant Study Group. *N Engl J Med* 313: 337–342.
20. Vigerel P, Chkoff N, Chatenoud L, Campos H, Lacombe M, et al. (1986) Prophylactic use of OKT3 monoclonal antibody in cadaver kidney recipients. Utilization of OKT3 as the sole immunosuppressive agent. *Transplantation* 41: 730–733.
21. Abramowicz D, Schandene L, Goldman M, Crusiaux A, Vereerstraeten P, et al. (1989) Release of tumor necrosis factor, interleukin-2, and gamma-interferon in serum after injection of OKT3 monoclonal antibody in kidney transplant recipients. *Transplantation* 47: 606–608.
22. Chatenoud L, Ferran C, Legendre C, Thouard I, Merite S, et al. (1990) In vivo cell activation following OKT3 administration. Systemic cytokine release and modulation by corticosteroids. *Transplantation* 49: 697–702.
23. Hirsch R, Gress RE, Pluznik DH, Eckhaus M, Bluestone JA (1989) Effects of in vivo administration of anti-CD3 monoclonal antibody on T cell function in mice. II. In vivo activation of T cells. *J Immunol* 142: 737–743.
24. Cole MS, Stellrecht KE, Shi JD, Homola M, Hsu DH, et al. (1999) HuM291, a humanized anti-CD3 antibody, is immunosuppressive to T cells while exhibiting reduced mitogenicity in vitro. *Transplantation* 68: 563–571.
25. Friend PJ, Hale G, Chatenoud L, Rebello P, Bradley J, et al. (1999) Phase I study of an engineered aglycosylated humanized CD3 antibody in renal transplant rejection. *Transplantation* 68: 1632–1637.
26. Xu D, Alegre ML, Varga SS, Rothermel AL, Collins AM, et al. (2000) In vitro characterization of five humanized OKT3 effector function variant antibodies. *Cell Immunol* 200: 16–26.
27. Bolt S, Routledge E, Lloyd I, Chatenoud L, Pope H, et al. (1993) The generation of a humanized, non-mitogenic CD3 monoclonal antibody which retains in vitro immunosuppressive properties. *Eur J Immunol* 23: 403–411.
28. Herold KC, Burton JB, Francois F, Poumian-Ruiz E, Glandt M, et al. (2003) Activation of human T cells by FcR nonbinding anti-CD3 mAb, hOKT3 $\gamma$ ma1(Ala-Ala). *J Clin Invest* 111: 409–418.
29. Bisikirska B, Colgan J, Luban J, Bluestone JA, Herold KC (2005) TCR stimulation with modified anti-CD3 mAb expands CD8 $^{+}$  T cell population and induces CD8 $^{+}$ CD25 $^{+}$  Tregs. *J Clin Invest* 115: 2904–2913.
30. Alegre ML, Peterson LJ, Xu D, Sattar HA, Jeyarajah DR, et al. (1994) A non-activating "humanized" anti-CD3 monoclonal antibody retains immunosuppressive properties in vivo. *Transplantation* 57: 1537–1543.
31. Kuhn C, You S, Valette F, Hale G, van Endert P, et al. (2011) Human CD3 transgenic mice: preclinical testing of antibodies promoting immune tolerance. *Sci Transl Med* 3: 68ra10.
32. Popma SH, Griswold DE, Li L (2005) Anti-CD3 antibodies OKT3 and hOKT3 $\gamma$ ma1(Ala-Ala) induce proliferation of T cells but impair expansion of alloreactive T cells; aspecific T cell proliferation induced by anti-CD3 antibodies correlates with impaired expansion of alloreactive T cells. *Int Immunopharmacol* 5: 155–162.
33. Li J, Davis J, Bracht M, Carton J, Armstrong J, et al. (2006) Modulation of antigen-specific T cell response by a non-mitogenic anti-CD3 antibody. *Int Immunopharmacol* 6: 880–891.
34. Nishioka T, Nishida E, Iida R, Morita A, Shimizu J (2008) In vivo expansion of CD4 $^{+}$ Foxp3 $^{+}$  regulatory T cells mediated by GITR molecules. *Immunol Lett* 121: 97–104.