doi: 10.1111/j.1365-3083.2007.01994.x

Agonistic Anti-4-1BB Antibody Promotes the Expansion of Natural Regulatory T Cells While Maintaining Foxp3 Expression

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Received 17 January 2007; Accepted in revised form 19 May 2007

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Abstract

The engagement of the 4-1BB (CD137) co-stimulatory pathway promotes the activation and proliferation of conventional $CD4^+$ T and $CD8^+$ T cells, but the role of 4-1BB co-stimulation in $CD4^+$ $CD25^+$ regulatory T cells (Treg) is less clear. In particular, whether 4-1BB stimulation affects the expression of Foxp3, a master gene for Treg, is unknown. This study demonstrates that co-stimulation of 4-1BB engaged by an agonistic antibody promotes the proliferation of Treg in a dependent manner of low-concentration interleukin-2 *in vitro*. The 4-1BB-expanded Treg maintain Foxp3 expression and their ability to suppress conventional $CD4^+$ T cells and their feature to produce no interleukin-2. However, the 4-1BB-expanded Treg produce increased levels of interferon-γ, whose significance is unknown. Thus, 4-1BB co-stimulation plays a role in the expansion of functional $CD4^+$ $CD25^+$ Treg cells without adversely affecting their suppressive activity.

Introduction

4-1BB (CD137), a member of the tumour necrosis factor receptor superfamily, is expressed on several types of cells, including activated T cells [1], dendritic cells [2, 3], monocytes [4], natural killer cells [5], neutrophils [6] and osteoblasts [7]. Unlike CD28, which is important for the activation of naïve T cells, 4-1BB co-stimulates activated T cells. A number of studies have demonstrated that the interaction of 4-1BB with its natural ligand or agonistic monoclonal antibody (MoAb) plays a role in $CD4^+$ T-cell responses both *in vitro* and *in vivo* [8, 9]. The 4-1BB co-stimulatory pathway is able to stimulate both T-cell proliferation and the production of interleukin-2 (IL-2) when T-cell receptor (TCR) signals are provided simultaneously [10]. 4-1BB may preferentially co-stimulate $CD8^+$ T cells [11]. Moreover, 4-1BB-mediated signalling plays a critical role in preventing activation-induced cell death, promoting the rejection of cardiac allograft and skin transplants, increasing T-cell cytolytic potential [11], and eradicating established tumours [12]. $CD4^+$ $CD25^+$ regulatory T cells (Treg) are the most important subset of suppressor cells and play a critical role in the maintenance of immunological tolerance [13]. TCR engagement is required for the suppressive activity of $CD4^+$ $CD25^+$ Treg; however, the role of co-stimulation in the Treg is largely unknown.

Foxp3, which encodes a transcription factor that is genetically defective in an autoimmune and inflammatory syndrome in human and mice, is specifically expressed by naturally arising $CD4^+$ Treg [14]. Foxp3 is absolutely required for the development of $CD4^+$ $CD25^+$ Treg, but the molecules and/or factors that control the expression of Foxp3 on Treg are less clear.

In this study, we find that stimulation of 4-1BB by an agonistic antibody promotes the proliferation of Treg in a dependent manner of low-concentration interleukin-2 *in vitro*. Significantly, the anti-4-1BB-expanded $CD4^+$ $CD25^+$ Treg maintain the features of Treg.

Materials and methods

Mice. Female BALB/*c* mice (5–6 weeks) were used in all experiments. Mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). Mice were bred and maintained under specific pathogen-free conditions in the animal facilities of Shandong University.

Reagents and antibodies. Anti-CD3 MoAb (clone 145-2C11), phycoerythrin (PE)-conjugated anti-CD25 (clone

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PC61.5), fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (clone GK1.5) MoAb, and FITC-conjugated anti-mouse/rat Foxp3 (clone FJK-16s) were purchased from eBiosciences (San Diego, CA, USA). A mouse $CD4^+$ $CD25^+$ T isolation Kit was obtained from Miltenyi Biotec (Auburn, CA, USA). Mouse anti-4-1BB MoAb (clone 158,321) was purchased from R&D systems. Rat IgG2a isotype control MoAb was obtained from eBiosciences. Anti-IL-2 antibody (clone S4B6) was purchased from BD Biosciences (San Jose, CA, USA).

Cell preparation. Murine spleens were gently minced in RPMI-1640 containing 10% fetal bovine serum (FBS) (GIBCO-BRL, Carlsbad, CA, USA). $CD4^+$ T cells were purified from spleens using a MACS Separation Column System (Miltenyi Biotec) and then $CD4^+$ $CD25^+$ T and $CD4^+$ $CD25^-$ T cells were separated using the magnetic cell separator according to the manufacturer's protocols. The $CD4^+$ $CD25^-$ T and $CD4^+$ $CD25^+$ T subpopulations were routinely 95% and 85–90% pure respectively.

Cell proliferation. Spleen cells were incubated with 50 μg/ml of mitomycin C at 37 °C for 20 min and washed three times with RPMI-1640 media containing 10% FBS and then used as antigen-presenting cell (APC) in proliferation assays. Purified spleen $CD4^+$ $CD25^-$ T and $CD4^+$ $CD25^+$ T cells were stimulated with 0.5 μg/ml of soluble anti-CD3 and APC in the presence of 10 μg/ml of anti-4-1BB MoAb or rat IgG. In another group, the purified cells were stimulated by 2 μg/ml of coated anti-CD3 and 10 μg/ml of soluble anti-CD28 or anti-4-1BB MoAb without APC. After 48 h, 100 μl of supernatants were collected for cytokine detection and fresh media were added. The cells were labelled with 0.5 μCi/well [^{3}H]-thymidine for the final 16 h, and then harvested and counted in a liquid scintillation counter (model SN 6930; Shanghai Hesuo Rihuan Photoelectric Instrument Co. Ltd, Shanghai, China). The data were shown as mean counts per minute (cpm) in triplicate wells.

Anti-IL-2 antibody blocking. Purified spleen $CD4^+$ $CD25^+$ T cells were stimulated with 0.5 μg/ml of soluble anti-CD3, APC, and 10 μg/ml of anti-4-1BB MoAb in the presence or absence of 20 μg/ml of anti-IL-2 antibody. Cells were cultured at 37°C in 5% CO_2 for 72 h. The cells were labelled with 0.5 μCi/well [^{3}H]-thymidine for the final 16 h and then harvested and counted in a liquid scintillation counter as described above.

Co-culture of $CD4^+$ $CD25^+$ T and $CD4^+$ $CD25^-$ T cells. Purified spleen $CD4^+$ $CD25^+$ T cells were stimulated with 0.5 μg/ml of soluble anti-CD3 and APC in the presence of anti-4-1BB or rat IgG for 72 h. The expanded $CD4^+$ $CD25^+$ T cells were then washed three times with phosphate-buffered saline (PBS). Fresh separated spleen $CD4^+$ $CD25^-$ T cells were stimulated with 0.5 μg/ml of soluble anti-CD3 and APC in the presence or absence of the 4-1BB-expanded $CD4^+$ $CD25^+$ T cells at 37°C in 5% CO_2 condition for 72 h. The cells were labelled with 0.5 μCi/well [^{3}H]-thymidine for the final 16 h and then harvested and counted in a liquid scintillation counter as described above.

Real-time PCR. Total cellular RNA was extracted from purified $CD4^+$ $CD25^+$ cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed to cDNA using Reverse Transcription system (Promega, Madison, WI, USA) according to the manufacturer's protocol. The expression levels of Foxp3 were measured by real-time PCR using Platinum SYBR Green qPCR Supermix UDG kit (Invitrogen). The primers used for real-time PCR are as follows: Foxp3 primers: forward 5′-CCCA GGAAAGACAGCAACCTT-3′ and reverse 5′-TTCT CACAACCAGGCCACTTG-3′; cyclophilin A primers: forward 5′-AGGGTGGTGACTTTACACGC-3′ and reverse 5′-ATCCAGCCATTCAGTCTTGG-3′. SYBR green qPCR was performed using the ABI PRISM 7000 Sequence Detection System (ABI, Foster City, CA, USA). The conditions comprised an initial holding at 50 °C for 2 min and a subsequent holding at 95 °C for 10 min, which was followed by a two-step PCR programme at 95 °C for 15 s and 60 °C for 60 s for 40 cycles.

Cytokine induction and determination. Cells were stimulated with anti-CD3 MoAb and APC in the presence or absence of anti-4-1BB MoAb for 48 h. Cell supernatants (100 μl) were collected and IL-2 and interferon-γ (IFN-γ) production was measured with ELISA kits (Jingmei Biotech Co, Ltd, China). The IL-2 detection kit has a minimum detection of 4 pg/ml and variation is less than 10%. The INF-γ detection kit has a minimum detection of 15 pg/ml and variation is less than 8.5%.

Flow cytometry analysis. Cells (1×10^6) were resuspended in PBS containing 0.5% BSA (washing buffer). For the staining of surface molecule, the cells were incubated with FITC-, PE-conjugated MoAb or their negative control antibodies as indicated for 30 min on ice. After washing twice with washing buffer, intracellular staining of Foxp3 was performed using a mouse Foxp3 staining kit (eBiosciences) according to the manufacturer's instructions. Cells were analysed using the Beckman Coulter Cytomics^{TM} FC 500 (Beckman Coulter Inc., Fullerton, CA, USA).

Statistical analysis. Data analysis was done using the Student's *t*-test to assess differences between the different study groups. A value of $P < 0.05$ was considered statistically significant.

Results

The expansion of $CD4^+$ $CD25^+$ Treg cells by agonistic anti-4-1BB antibody needs low-concentration IL-2 *in vitro*

To examine the effect of 4-1BB co-stimulation on Treg, freshly isolated $CD4^+$ $CD25^+$ Treg cells and $CD4^+$ $CD25^-$ T cells were cultured with anti-CD3

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MoAb in the presence or absence of agonistic anti-4-1BB MoAb respectively. T-cell proliferation was measured by ^{3}H-TdR incorporation assay. The stimulation by anti-CD3 MoAb alone or in the presence of anti-4-1BB MoAb failed to induce $CD4^+$ $CD25^+$ T-cell proliferation (Fig. 1A). By contrast, the presence of anti-4-1BB MoAb resulted in a significant increase in $CD4^+$ $CD25^-$ T-cell proliferation, which was comparable with that in the presence of CD28 MoAb (Fig. 1A).

To determine whether the non-responsiveness of $CD4^+$ $CD25^+$ Treg cells to anti-CD3 MoAb and anti-4-1BB MoAb is due to a lack of IL-2, we examined the proliferation of Treg cells in the presence of recombinant IL-2 (rIL-2) cytokine or APC respectively. Consistent with previous findings [15], there was a significant increase in the proliferation of $CD4^+$ $CD25^+$ Treg cells stimulated by anti-CD3 MoAb and anti-4-1BB MoAb in the presence of 20 U/ml of rIL-2 (Fig. 1B). Similarly, the presence of APC also resulted in an increase in $CD4^+$ $CD25^+$ Treg cells proliferation, synchronously stimulated by anti-CD3 and anti-4-1BB MoAb (Fig. 1C). A similar effect was observed in $CD4^+$ $CD25^-$ Treg cells stimulated by anti-4-1BB antibody. Furthermore, the proliferation of $CD4^+$ $CD25^+$ Treg stimulated by anti-4-1BB MoAb in the presence of anti-CD3 MoAb and APC could be blocked by anti-IL-2 MoAb (Fig 1D). Thus, IL-2 is necessary for the expansion of $CD4^+$ $CD25^+$ Treg cells mediated by agonistic anti-4-1BB MoAb.

4-1BB MoAb-expanded $CD4^+$ $CD25^+$ Treg cells produce less IL-2 but more IFN-γ

T-cell receptor stimulation and 4-1BB co-stimulation, independent of the CD28 co-stimulatory pathway, are capable of inducing IL-2 production in the conventional T-cell populations [10, 16]. However, it has been shown that the $CD4^+$ $CD25^+$ Treg lack IL-2 production upon TCR stimulation [15]. To determine whether 4-1BB co-stimulation induce IL-2 production in $CD4^+$ $CD25^+$ Treg, freshly purified $CD4^+$ $CD25^+$ Treg cells from

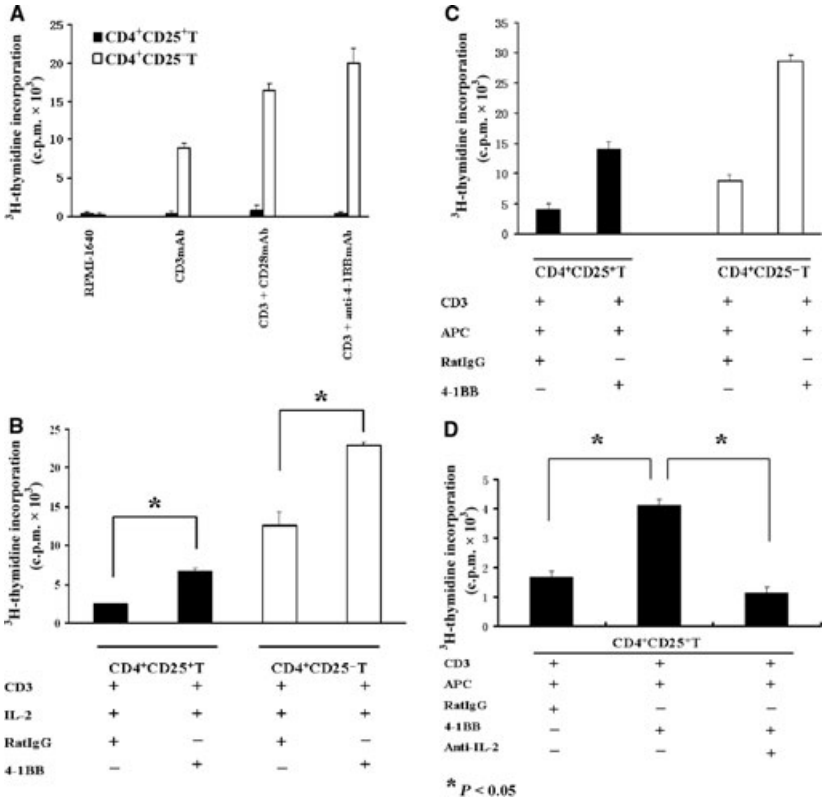

Figure 1 Agonistic anti-4-1BB MoAb promotes the proliferation of $CD4^+$ $CD25^+$ T cells in the dependent manner of low-concentration IL-2. (A) Freshly separated $CD4^+$ $CD25^-$ T and $CD4^+$ $CD25^+$ T cells from BALB/*c* mice were activated with 2 μg/ml of coated anti-CD3 MoAb in the presence or absence of 10 μg/ml of anti-CD28 or anti-4-1BB MoAb for 72 h. (B) Freshly purified $CD4^+$ $CD25^-$ T and $CD4^+$ $CD25^+$ T cells were activated with 0.5 μg/ml of anti-CD3 MoAb and exogenous IL-2 (20 U/ml) in the presence of 10 μg/ml of anti-4-1BB MoAb or rat IgG2a for 72 h. (C) Freshly purified $CD4^+$ $CD25^-$ T and $CD4^+$ $CD25^+$ T cells were activated with 0.5 μg/ml of anti-CD3 MoAb and APC in the presence of 10 μg/ml of anti-4-1BB MoAb or rat IgG2a (control MoAb) for 72 h. (D) The proliferation of $CD4^+$ $CD25^+$T cells stimulated by 0.5 μg/ml of anti-CD3 MoAb, APC (1×10^6 cells/well) and 10 μg/ml of anti-4-1BB MoAb was blocked by anti-IL-2 antibody (20 μg/ml). All samples were labelled with 0.5 μCi [^{3}H]-thymidine/well for the last 16 h and all conditions were performed in triplicate and reported as mean ± SD. The shown results are representative of five independent experiments.

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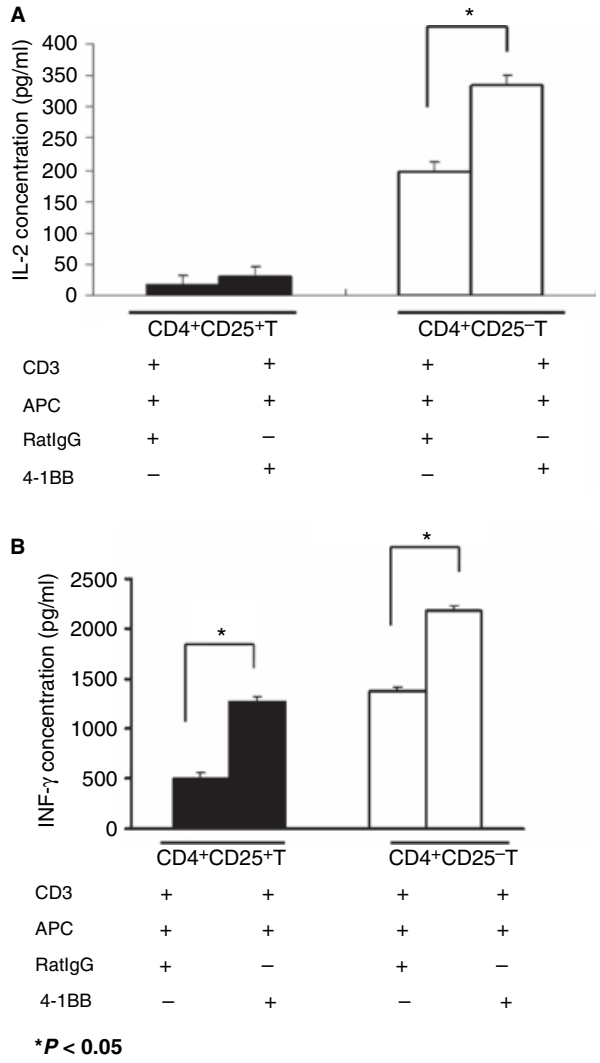

Figure 2 4-1BB-expanded $CD4^+$ $CD25^+$ Treg cells produce less IL-2 but more IFN-γ. $CD4^+$ $CD25^-$ T and $CD4^+$ $CD25^+$ T cells were activated with 0.5 μg/ml of anti-CD3 MoAb and APC (1×10^6/well) in the presence of 10 μg/ml of anti-4-1BB MoAb or rat IgG. After 48 h, culture supernatant was collected and levels of IL-2 (A) and INF-γ (B) were analysed by an ELISA. All conditions were performed in triplicate and reported as mean ± SD. All results are representative of three independent experiments.

BALB/*c* mice were stimulated by anti-CD3 MoAb and APC in the presence of anti-4-1BB MoAb or isotype control antibody. Forty-eight hours later, the levels of IL-2 in the supernatant were analysed by enzyme-linked immunoadsorbent assay (ELISA). As shown in Fig. 2A, although co-stimulation of 4-1BB significantly induced $CD4^+$ $CD25^-$ Treg cells to secrete high levels of IL-2 cytokine, it failed to upregulate the level of IL-2 secreted by $CD4^+$ $CD25^+$ Treg. Unexpectedly, anti-4-1BB MoAb dramatically increased IFN-γ secretion in $CD4^+$ $CD25^+$ Treg (Fig. 2B). These results indicated that the 4-1BB co-stimulatory pathway could stimulate $CD4^+$ $CD25^+$ Treg to produce more IFN-γ, but less IL-2 cytokine.

4-1BB MoAb-expanded $CD4^+$ $CD25^+$ Treg cells maintain Foxp3 expression

The transcription factor Foxp3 is associated with the development of Treg [17]. To determine whether Foxp3 is involved in the proliferate response of $CD4^+$ $CD25^+$ Treg cells to 4-1BB co-stimulation, the expression of Foxp3 in 4-1BB-expanded $CD4^+$ $CD25^+$ Treg was analysed by real-time PCR at RNA levels and by flow cytometry at protein levels. Although the expression of Foxp3 mRNA was slightly higher in anti-4-1BB-expanded $CD4^+$ $CD25^+$ Treg than that in isotype antibody-treated $CD4^+$ $CD25^+$ Treg or fresh purified $CD4^+$ $CD25^+$ Treg (Fig. 3), there was no statistical significance among the three groups ($P > 0.05$). The result of Foxp3 protein expression in anti-4-1BB-expanded $CD4^+$ $CD25^+$ Treg was similar to that of gene expression. These data demonstrate that the 4-1BB co-stimulatory signal does not decrease at least the expression of Foxp3 when it leads to the expansion of $CD4^+$ $CD25^+$ Treg.

4-1BB MoAb-expanded $CD4^+$ $CD25^+$ Treg cells retain the ability to suppress $CD4^+$ $CD25^-$ T cells

To determine whether the $CD4^+$ $CD25^+$ Treg cells expanded by agonistic anti-4-1BB MoAb retained suppressive activity, the expanded $CD4^+$ $CD25^+$ Treg cells were co-cultured with freshly isolated $CD4^+$ $CD25^-$ T-responder cells in the presence of anti-CD3 MoAb and APC for 72 h. The results showed that 4-1BB-expanded $CD4^+$ $CD25^+$ Treg cells as well as fresh purified or control antibody-treated Treg cells were able to suppress $CD4^+$ $CD25^-$ T-cell proliferation (Fig. 4). Thus,

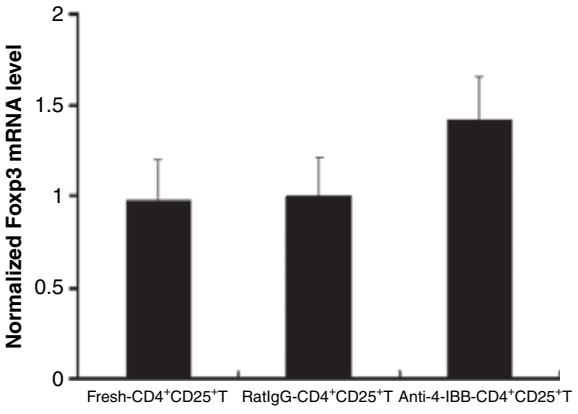

Figure 3 4-1BB-expanded $CD4^+$ $CD25^+$ Treg cells maintain Foxp3 expression. Freshly purified $CD4^+$ $CD25^+$ T cells were activated with 0.5 μg/ml of anti-CD3 MoAb and APC (1×10^6/well) in the presence of 10 μg/ml of anti-4-1BB MoAb or rat IgG. The levels of Foxp3 expression were analysed by real-time PCR. All conditions were performed in triplicate and reported as mean ± SD. All results are representative of three independent experiments.

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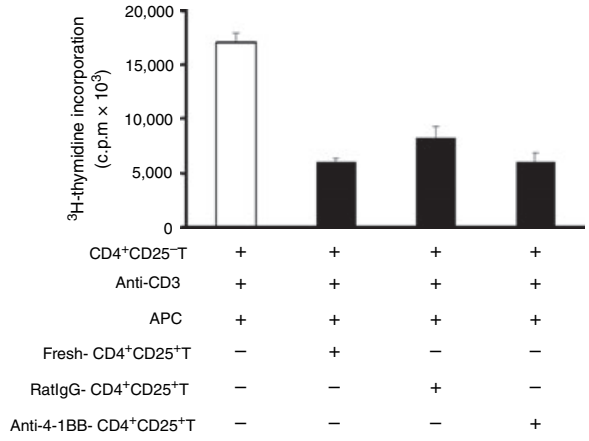

Figure 4 4-1BB-expanded $CD4^+$ $CD25^+$ T cells retain the ability to suppress $CD4^+$ $CD25^-$ T cells. Freshly isolated $CD4^+$ $CD25^-$ T cells were co-cultured with fresh purified or rat IgG-treated or anti-4-BB-expanded $CD4^+$ $CD25^+$ Treg cells in the presence of 0.5 μg/ml of anti-CD3 MoAb and APC stimulation for 72 h. All samples were labelled with 0.5 μCi [^{3}H]-thymidine/well for the last 16 h. All conditions were performed in triplicate and reported as mean ± SD. All results are representative of five independent experiments.

4-1BB-expanded $CD4^+$ $CD25^+$ Treg cells retain their suppressive activity.

Discussion

4-1BB is a molecule expressed on $CD4^+$ $CD25^-$ responder T cells as well as $CD4^+$ $CD25^+$ Treg [18, 19]. The ligation of 4-1BB has been consistently shown to enhance TCR-driven proliferation and survival of conventional responder $CD4^+$ $CD25^-$ T cells [8, 9]. However, the role of 4-1BB in $CD4^+$ $CD25^+$ Treg remains controversial. In this regard, one report showed that the stimulation of 4-1BB with its natural ligand (4-1BB-Fc) promoted Treg proliferation *in vitro* and *in vivo* [20], whereas the other indicated that anti-4-1BB MoAb failed to expand $CD4^+$ $CD25^+$ Treg [21].

In this study, we find that agonistic anti-4-1BB MoAb indeed promote $CD4^+$ $CD25^+$ Treg proliferation, which requires exogenous IL-2 cytokine. As shown in Fig. 1A–C, anti-4-1BB MoAb could not induce the proliferation of $CD4^+$ $CD25^+$ Treg in the absence of IL-2 but could do that in the presence of IL-2 or APC. Furthermore, $CD4^+$ $CD25^+$ Treg proliferation stimulated by anti-4-1BB MoAb in the presence of APC could be blocked by anti-IL-2 MoAb (Fig. 1D). As IL-2 is needed for the effect of 4-1BB co-stimulation in $CD4^+$ $CD25^+$ Treg proliferation, at least two possibilities exist for 4-1BB signalling. One would be that 4-1BB signalling interacts with the IL-2 pathway to increase the responsiveness and/or sensitivity of Treg to IL-2. The other scenario would be that 4-1BB might deliver a signal that antagonizes the negative signalling to release the 'brake' for Treg. Secondly, 4-1BB signalling enhances IFN-γ but not IL-2 production in $CD4^+$ $CD25^+$ Treg. This finding reveals a differential pathway for IL-2 and IFN-γ production in Treg, in which 4-1BB signalling affects the latter, not the former. In recent years, many researchers have noticed that IFN-γ can have paradoxical functions, eliciting inflammatory T helper 1 (Th1)-driven immune responses in some circumstances and enabling induced Treg to control immune responses in others [22, 23]. This observation may help explain the observation that 4-1BB signalling enhances anti-tumour immunity despite its promotion of growth of Treg [24]. The molecular pathway by which 4-1BB regulates IFN-γ secretion remains to be determined. Thirdly, our data demonstrate that the 4-1BB co-stimulatory signal neither affects the expression of Foxp3 in anti-4-1BB MoAb-expanded $CD4^+$ $CD25^+$ Treg cells, nor does it cancel their suppressive activity to the $CD4^+$ $CD25^-$ T-responder cells (see Figs. 3 and 4). Thus, the stimulation of 4-1BB by an agonistic antibody promotes the proliferation of $CD4^+$ $CD25^+$ Treg in a dependent manner of low-concentration interleukin-2 *in vitro*. Significantly, the 4-1BB MoAb-expanded $CD4^+$ $CD25^+$ Treg maintain the features of Treg.

Acknowledgments

The author thanks Dr Wanjun Chen at NIDCR for critical reading of the manuscript. This work was supported by grants from the Natural Science Foundation of China (No. 30,628,015) and "973" Program (No. 2006CB-503,803).

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