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Constitutive expression of 4-1BB on T cells enhances $CD4^+$ T cell responses

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Abbreviations: CHS, contact hypersensitivity; DNFB, 1-fluoro-2,4,dinitrobenzene; TG, transgenic; WT, wild type

Abstract

4-1BB, a transmembrane molecule, member of the tumor necrosis factor receptor superfamily, is an important costimulatory molecule in the immune response, plays a key role in the clonal expansion and survival of $CD8^+$ T cells. In this study, we investigated 4-1BB regulation of $CD4^+$ T cell responses using 4-1BB transgenic (TG) mice that constitutively expressed 4-1BB on mature T cells. We first showed that $CD4^+$ T cells of 4-1BB TG mice had more sustained proliferative capacity in response to TCR/4-1BB stimulation *in vitro* compared to WT mice. Secondly, 4-1BB TG mice exhibited a more elevated contact hypersensitivity (CHS) response mediated by $CD4^+$ Th1 cells due to more vigorous expansion of and apoptotic inhibition of $CD4^+$ T cells. Finally, $CD4^+$ T cells of 4-1BB TG mice had a heightened capacity for T cell priming. Overall, our results demonstrate the involvement of 4-1BB in $CD4^+$ Th1 cell responses by regulating the clonal expansion and survival of $CD4^+$ T cells as seen in $CD8^+$ T cells.

Keywords: antigens, CD4; antigens, differentiation; cytokines; T lymphocyte; T lymphocyte, hyper-inducer; T lymphocyte subsets

Introduction

Optimal immune responses are initiated by the engagement of the T-cell receptor (TCR) on T cells with MHC/peptide complexes on an antigen (Ag)-presenting cell (APC) and a second costimulatory signal (Waldman, 1999). To date, several classes of molecules have been known to function as costimulatory molecules, including cell adhesion molecules, CD28/ICOS family, and TNF receptor superfamily members (Watts and DeBenedette, 1999). Even though it is not clear that all costimulatory molecules are essential for T cell responses, it seems that each molecule serves different functions with some redundancy and synergy to each other. For example, stimulation of CD28 has a much larger proliferative effect on $CD4^+$ T cells than $CD8^+$ T cells (Watts and DeBenedette, 1999). Like CD40 and OX40 (Kopt *et al*., 1999; Whimire *et al*., 1999), CD28 is required for generating antiviral $CD4^+$ effector T cells, but is dispensable for $CD8^+$ effector T cells (Watts and DeBenedette, 1999). In contrast, 4-1BB signals are important for the generation of antiviral $CD8^+$ effector T cells (DeBeneddette *et al*., 1999; Tan *et al*., 1999; Kwon *et al*., 2002b). In particular, 4-1BB stimulation induces a drastic promotion and prolongation of $CD8^+$ T cell proliferation and survival *in vivo* and *in vitro* (Takahashi *et al*., 1999; Cooper *et al*., 2002; Maus *et al*., 2002).

There is accumulating evidence that 4-1BB is implicated in immune responses mediated by $CD4^+$ T cells, including alloimmune responses (DeBeneddette *et al*., 1999; Blazar *et al*., 2001; Nozawa *et al*., 2001) and inflammation (Seko *et al*., 2001; Sun *et al*., 2002). Although there is a controversy regarding the mechanism by which 4-1BB regulates $CD4^+$ T cell-mediated responses, as with $CD8^+$ T cells, signaling through 4-1BB appears to promote cell proliferation and survival *in vitro* (Gramaglia *et al*., 2000; Cannons *et al*., 2001; Wen *et al*., 2002). However, two *in vivo* observations (Mittler *et al*., 1999; Sun *et al*., 2002) indicate that there are different mechanisms of 4-1BB

costimulation in $CD4^+$ T cells from that in $CD8^+$ T cells. Sun *et al*. (2002) demonstrated that despite an early clonal expansion of Ag-specific $CD4^+$ T cells after 4-1BB stimulation, the activated $CD4^+$ T cells rapidly undergo activation-induced cell death (AICD). The difference might be largely due to intrinsic property of the two T-cell subsets in terms of their proliferative and apoptotic responses to TCR or TCR/4-1BB signals (Fouls *et al*., 2002; Sun *et al*., 2002). A finding reported by Mittler *et al*. (1999) suggests a novel mechanism by which stimulation of 4-1BB can abrogate humoral immune responses against a T-cell-dependent Ag through the induction of helper T cell anergy, in which process $CD8^+$ T cells do not seem to be required. In this case, the mechanism for 4-1BB regulation of $CD4^+$ T-cell responses might be *via* 4-1BB signal augmentation of the immunosuppressive activity of dendritic cells (DCs), which in turn results in suppression of helper T-cell function leading to antibody (Ab) production (Mittler *et al*., 1999).

In the present study, we assessed $CD4^+$ T cell responses using 4-1BB TG mice *in vivo* and show that constitutive expression on $CD4^+$ T cells leads to heightened $CD4^+$ T cell-mediated responses. Our data indicate that this is due to sustained expansion of $CD4^+$ T cells and inhibition of apoptosis of the subsets. Thus, like $CD8^+$ T cells, 4-1BB regulates the ability of $CD4^+$ T cells to proceed through cell division without extensive apoptosis and is therefore required for maximal clonal expansion in the primary $CD4^+$ T cell responses.

Materials and Methods

Reagents

Hyridomas producing anti-4-1BB mAbs (3E1 or 3H3, rat IgG2a) were described previously (Shuford *et al*., 1997). 3E1 was conjugated with FITC according to the standard protocols and 3E1-FITC was used to examine cell surface expression of 4-1BB. 3H3 was used to stimulate T cells. Anti-CD3 (2C11), anti-CD3-FITC, anti-CD4-PE (GK1.5), anti-CD8-PerCP (53-6.7), and appropriate isotpe-matched mAbs were from BD PharMingen (San Diego, CA).

4-1BB transgenic mice

A full length mouse 4-1BB DNA containing *Bam*HI sites at both 5' and 3' ends was generated by PCR, using a 4-1BB cDNA as a template, and inserted into the *Bam*HI sites of the plasmid p1017 which contains the lck proximal promoter, the human growth hormone gene genomic sequence and a poly $(A)^+$ site (Figure 1A; Chaffin *et al*., 1990; Shimizu *et al*., 2001). A fragment containing lck promoter/4-1BB/human growth hormone/poly $(A)^+$ site was excised by *Not*I and used for subsequent microinjection. 4-1BB transgenic founder mice were generated by injecting the fragment into FvB eggs. Southern blot analysis of tail DNA was used to screen for transgenic animals. One line generated from founders expressed high levels of 4-1BB mRNA and protein (Figure 1A and B). This line was backcrossed to the C57BL/6 strain more than three generations.

Proliferation assay

1×10^5 cells/well were cultured in 96-well plates (Costar, Cornimg, NY) in triplicate and cell division was assessed by the addition of 1 μCi [^{3}H] thymidine (ICN Biomedicals, Irvine, CA) to 0.2 ml cultures for the last 18 h of culture. Cells used for the proliferation assay were thymocytes, total splenocytes or purified $CD4^+$ T cells or $CD8^+$ T cells. $CD4^+$ or $CD8^+$ T cells were purified using anti-CD4 or anti-CD8-conjugated magnetic beads (MACS) from Mitenyi Biotech (Auburn, CA). Purity was confirmed to be over 90% by FACScan flow cytometry.

Contact hypersensitivity (CHS) responses

For 1-fluoro-2,4-dinitrobenzene (DNFB)-induced CHS, wild type (WT) and transgenic (TG) mice were sensitized epicutaneously with 0.1% DNFB in acetone:olive oil (4:1) on days 0 and 1 and then challenged epicutaneously on day 5 with 0.25% DNFB. Ear thickness was measured with a low pressure spring-loaded engineer's micrometer (Mitutoyo, Tokyo, Japan) before the allergen challenge (0 h) and 24 and 48 h after the allergen challenge. In some cases, lymphocytes were isolated from the draiming lymph node (DLNs) and stainded with anti-CD4-PE or anti-CD8-PerCP plus annexin V-FITC. The stained cells were analyzed on FACScan flow cytometry.

In vivo T cell priming and recall responses stimulated with KLH

WT and TG mice were immunized with 50 μg of KLH together with CFA in a total volume of 50 μl into each hind footpad. On day 9 after immunization, $CD4^+$ T cells were purified from the DLNs. 5×10^4 of the purified $CD4^+$ T cells were added into each well of a 96-well plate and stimulated with the indicated concentration of KLH in the presence of 2.5×10^5 APCs for 3 days. Purity was confirmed to be $>90\%$. The APCs used were isolated from WT spleens and irradiated (2000 rad). Proliferation was assessed as described above.

In vivo production of KLH-specific Ab

Fifty μg of KLH was used to immunize WT and TG mice as describe above. For the primary Ab determination, serum was collected on day 7 for IgM and day 14 for the IgG3 to examine the concentration of anti-KLH-specific Abs.

Assay for anti-KLH-specific Ab production

Each well of an ELISA microplate was coated with 10 μg/ml of KLH in carbonate buffer (pH 9.0) by overnight incubation at 4°C. Plates were washed and subsequently blocked with 1% BSA in PBS for 1 h at 37°C. Sera from WT or TG mice, diluted in PBS (from 1:10 to 1:10,000), were added to the wells and incubated for 1 h at room temperature. The plates were then washed, and bound Abs were diluted by incubation with goat anti-mouse IgM or IgG3 conjugated with horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL). After an additional 1-h incubation, color reactions were performed using 1-step slow TMB-ELISA substrate (Pierce, Rockford, IL). The reaction was stopped by 2 M H_2SO_4, and the OD readings at 405 nm were evaluated.

Results

Generation of 4-1BB TG mice in the T cell lineage

4-1BB is not expressed on resting T cells and is upregulated by activation (Vinay and Kwon, 1999; Kwon *et al*., 2000; Kwon *et al*., 2002a; Kwon *et al*., 2003). To investigate the role of 4-1BB in T cells *in vivo*, we generated TG mice that constitutively express the 4-1BB protein under the control of the proximal lck promoter (Figure 1A), which gives rise to a T cell lineage-specific expression of 4-1BB (Chaffin *et al*., 1990; Shimizu *et al*., 2001). By Southern blot using a full-length 4-1BB cDNA probe, two transgene-positive founders were identified and bred (Figure 1B). Northern blot analysis showed that one line expressed abundant 4-1BB mRNA in thymus, and less in lymph nodes and spleen (Figure 1C). Other organs did not express 4-1BB. Consistent with the Northern blot result, a similar expression pattern of the 4-1BB protein were found in thymus, lymph node, and spleen (Figure 1D). Using FACS, high levels of 4-1BB expression were also observed on thymocytes of TG mice. Approximately 90% of $CD4^+$ or $CD8^+$ thymocytes expressed 4-1BB, indicating that 4-1BB began to be expressed from the double positive ($CD4^+CD8^+$) stage of thymocyte development (Figure 2A). As expected, 4-1BB was expressed on essentially all mature $CD4^+$ and $CD8^+$ T cells of peripheral lymphoid tissues, including spleen, lymph node, and peripheral blood (Figure 1B-D).

4-1BB TG mice were normal in gross appearance, size, body weight, and reproductive activities. There was no abnormality in the development of lymphoid organs in TG mice: The lymphoid organs contained similar profiles of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and macrophages to those of littermate control (WT) mice (data not shown). The TG mice also did not display splenomegaly and B cell depletion with aging as seen in 4-1BB ligand (4-1BBL) TG mice which were engineered to overexpress 4-1BBL on APCs (Zhu *et al*., 2001). However, the thymocytes of TG mice had defects in the proliferative response to TCR or TCR/4-1BB stimulation and they underwent a

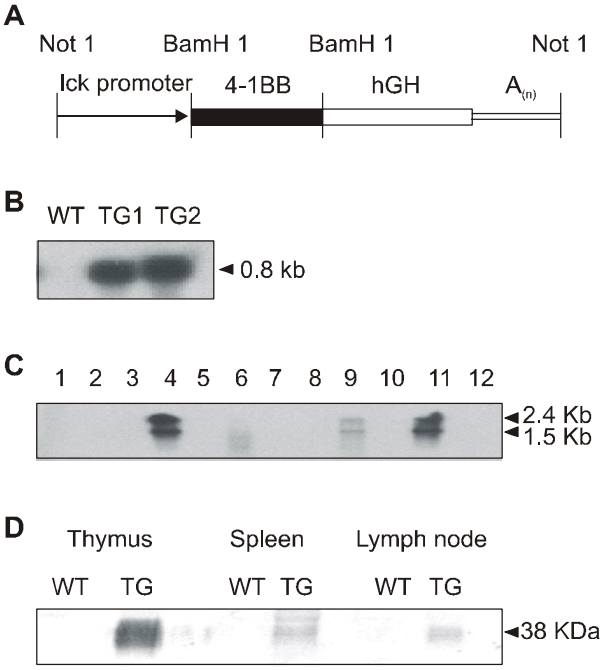

Figure 1. Construct of 4-1BB transgene and its expression. (A) A schematic map of the 4-1BB transgene construct. The cDNA of murine 4-1BB was inserted into the p1071 plasmid between the lck proximal promoter and human growth hormone genomic sequence. *Not*I restriction digest was then used to isolate the transgene cassette from the plasmid. (B) Southern blot of isolated mouse genomic DNA. Nontransgenic (WT) and two lines of transgenic (TG) mice were compared. Southern analysis of *Bam*HI-digested mouse genomic DNA was performed using radiolabeled 4-1BB cDNA as a probe. (C) Northern blot analysis of various organs from TG (otherwise indicated). Fifteen μg of total RNA was used for hybridization. 1, brain; 2, salivary gland; 3, heart; 4, thymus; 5, thymus (WT); 6, lung; 7, liver; 8, pancreas; 9, spleen; 10, kidney; 11, lymph node; 12, lymph node (WT). (D) Immunoprecipitation-Western blot analysis of thymus, lymph node, and spleen. A single cell suspension was prepared from TG thymus, lymph node, or spleen, and 1×10^6 cells/ml were biotinylated according to the manufacturer (Pierce, Rockford, IL). Cell lysate was then immunoprecipitated using 3 μg of anti-4-1BB (1AH2) or isotype IgG. Western blotting was performed with streptavidin coupled with alkaline phosphatase.

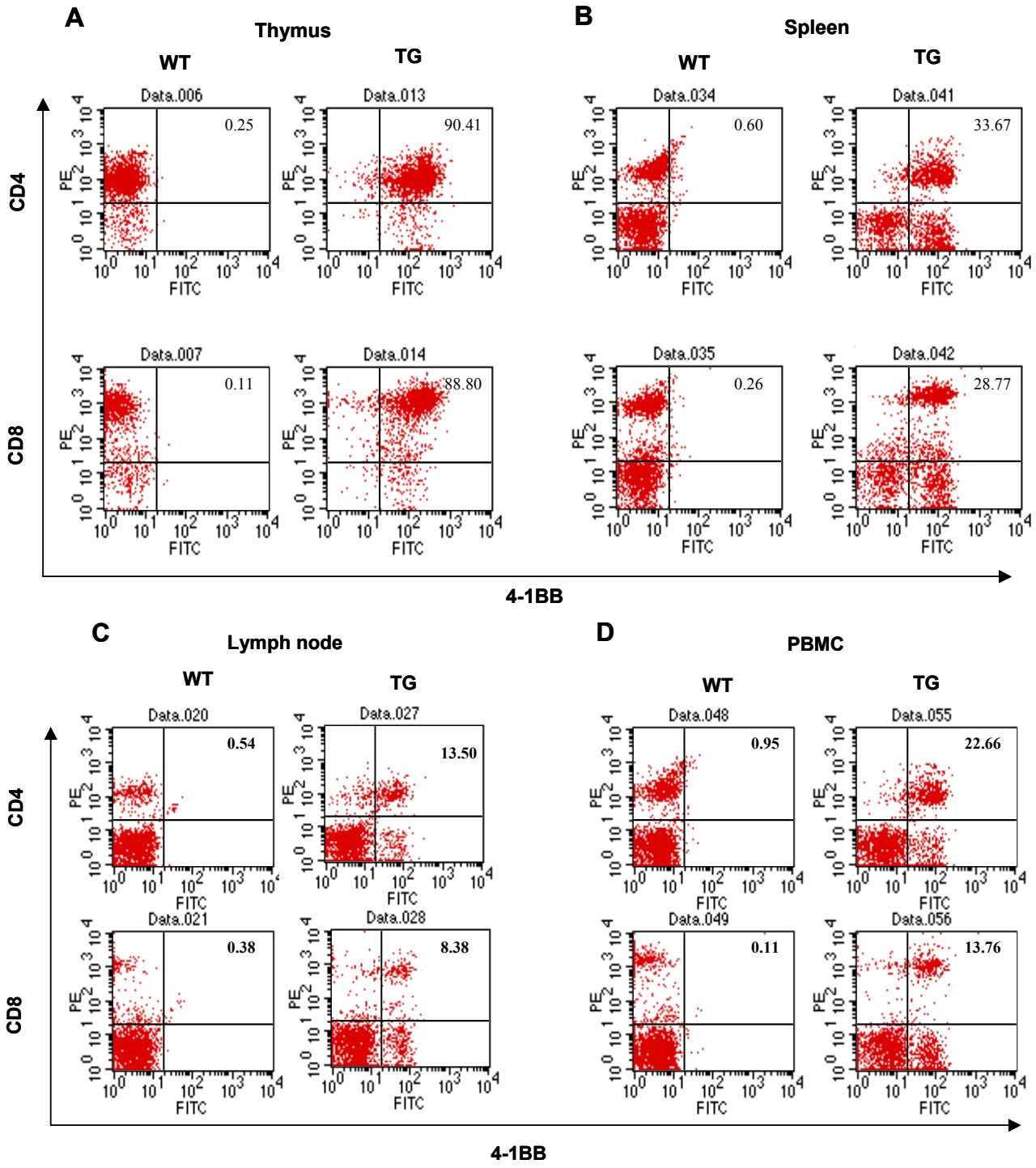

Figure 2. 4-1BB expression on T cells of 4-1BB TG mice. T cells were isolated from thymus (A), spleen (B), lymph nodes (C), or peripheral blood (D), double stained with anti-CD4-PE or anti-CD8-PerCP and anti-4-1BB-FITC mAbs, and analyzed using FACScan.

significantly higher percentage of apoptosis during *in vitro* culture for 24 h compared to WT mice (manuscript in preparation).

A heightened and sustained CD4$^+$ T cell proliferative response of 4-1BB TG vs WT mice

To directly evaluate the role of constitutively expressed 4-1BB in regulating the response of T cells, total

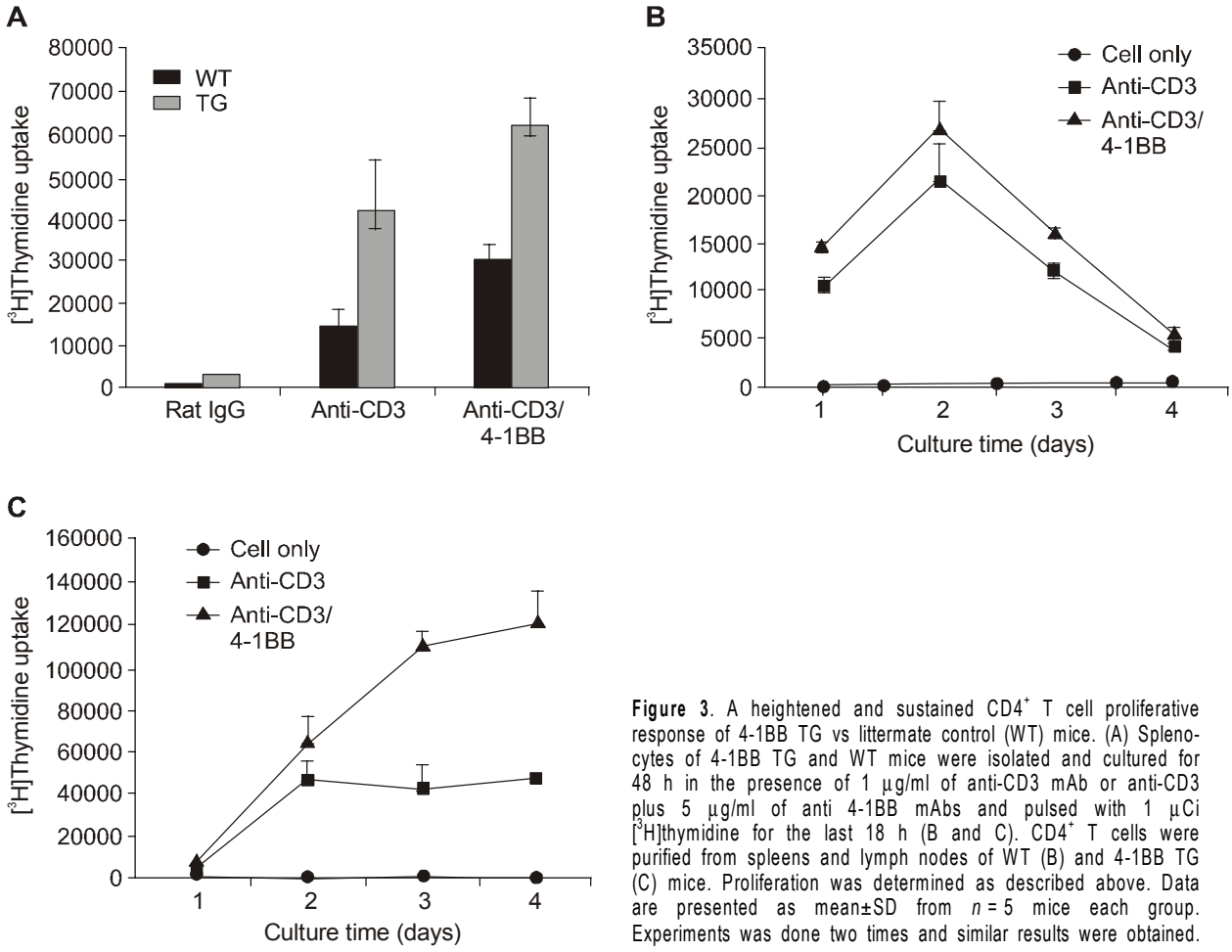

Figure 3. A heightened and sustained CD4+ T cell proliferative response of 4-1BB TG vs littermate control (WT) mice. (A) Splenocytes of 4-1BB TG and WT mice were isolated and cultured for 48 h in the presence of 1 μg/ml of anti-CD3 mAb or anti-CD3 plus 5 μg/ml of anti 4-1BB mAbs and pulsed with 1 μCi [3H]thymidine for the last 18 h (B and C). CD4+ T cells were purified from spleens and lymph nodes of WT (B) and 4-1BB TG (C) mice. Proliferation was determined as described above. Data are presented as mean±SD from $n = 5$ mice each group. Experiments was done two times and similar results were obtained.

splenocytes of WT and TG mice were used to test their ability to respond to an optimal dose of anti-CD3 (for TCR stimulation) or anti-CD3/4-1BB. As shown in Figure 3A, TG splenocytes exhibited a significantly higher proliferation in response to TCR or TCR/4-1BB stimulation than did WT splenocyted. When we examined the proliferative response of purified CD4+ T cells to TCR or TCR/4-1BB stimulation on a daily base, CD4+ T cells of TG vs WT mice showed different proliferation patterns (Figure 3B and C). WT CD4+ T cells had a maximal level of proliferative responses to TCR or TCR/4-1BB stimulation on day 2 and their proliferation declined thereafter (Figure 3B). In contrast, TG CD4+ T cells maintained their proliferative capcacity in response to TCR stimulation after day 2 (Figure 3C). When simultaneously stimulated by anti-CD3 and anti-4-1BB, the proliferative response of TG CD4+ T cells kept on increasing until day 4. A similar pattern of proliferative responses of TG CD8+ T cells was observed as seen in CD4+ T cells. These data suggest that enforced overexpression of 4-1BB in T cells heightens and sustains the proliferative capacity of T cells.

Heightened contact hypersensitivity responses in 4-1BB TG vs WT mice

Contact hypersensitivity (CHS) is believed to be mediated by CD4+ T cells. CHS responses in TG was examined vs WT mice. For this study, the Th1 predominant allergen dinitro-flourobenzene (DNFB) was used as the allergen (Simon *et al*., 1994). As shown in Figure 4A, 4-1BB TG mice exhibited significantly increased ear swelling when measured at 24 h and 48 h after DNFB challenge. Since allergen-specific CD4+ T cells are expanded after the allergen challenge and their pool determines the extent of CHS responses, we examined T cell population of DLNs. Reflecting the CHS result, the DLNs of TG mice contained a significantly higher percentage of CD4+ T cells than did those of WT mice (Figure 4B). In contrast, a similar percentage of CD8+ T cells existed in the DLNs of both groups of mice (not shown), indicating that DNFB specifically induced expansion of

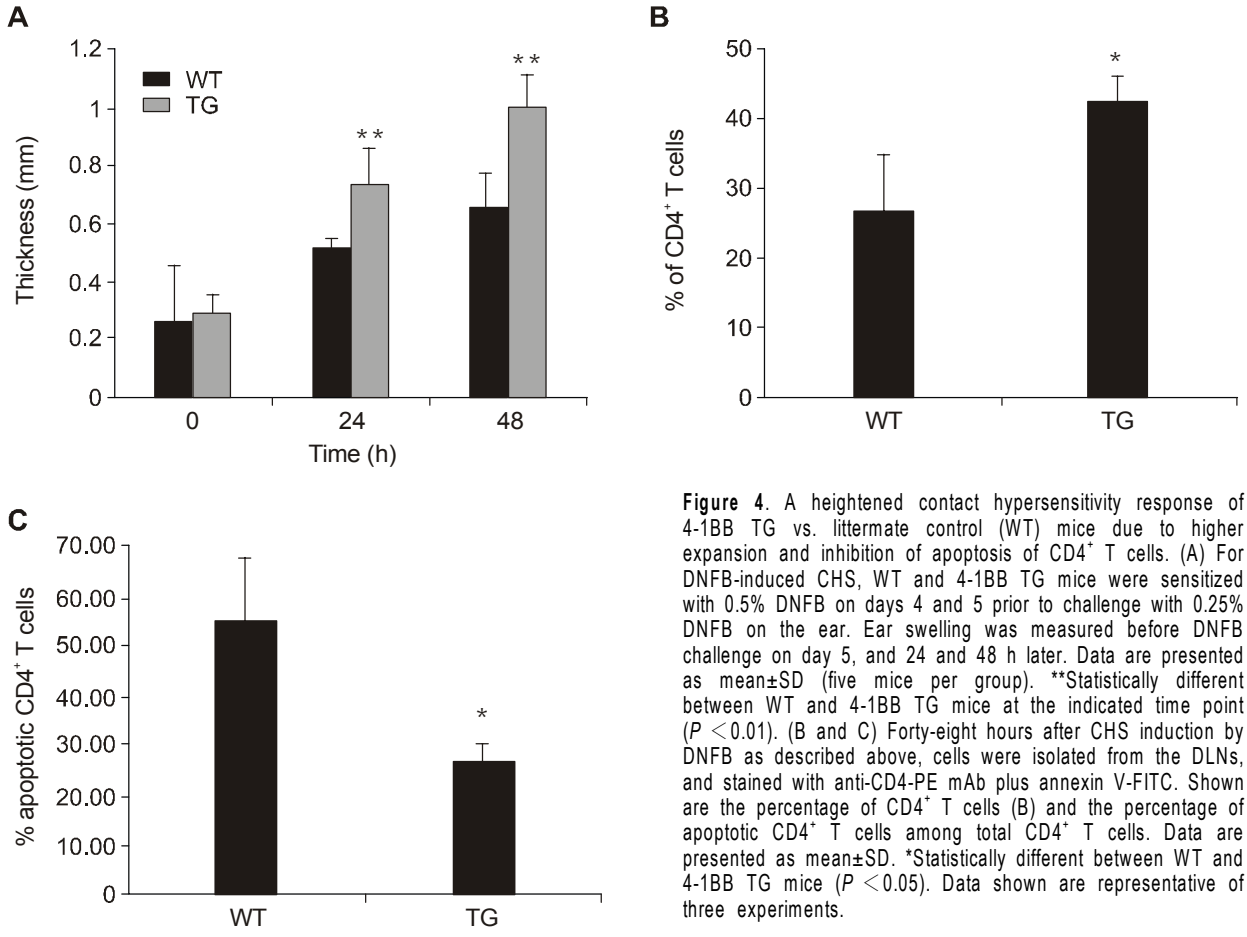

Figure 4. A heightened contact hypersensitivity response of 4-1BB TG vs. littermate control (WT) mice due to higher expansion and inhibition of apoptosis of $CD4^+$ T cells. (A) For DNFB-induced CHS, WT and 4-1BB TG mice were sensitized with 0.5% DNFB on days 4 and 5 prior to challenge with 0.25% DNFB on the ear. Ear swelling was measured before DNFB challenge on day 5, and 24 and 48 h later. Data are presented as mean±SD (five mice per group). **Statistically different between WT and 4-1BB TG mice at the indicated time point ($P < 0.01$). (B and C) Forty-eight hours after CHS induction by DNFB as described above, cells were isolated from the DLNs, and stained with anti-CD4-PE mAb plus annexin V-FITC. Shown are the percentage of $CD4^+$ T cells (B) and the percentage of apoptotic $CD4^+$ T cells among total $CD4^+$ T cells. Data are presented as mean±SD. *Statistically different between WT and 4-1BB TG mice ($P < 0.05$). Data shown are representative of three experiments.

$CD4^+$ T cells. Interestingly, we consistently observed a significantly lower percentage of Annexin V-positive $CD4^+$ T cells in the DLNs of TG mice (Figure 4C). There was a small population of $CD8^+$ T cells undergoing apoptosis in the DLNs of both groups of mice, which might correspond to apoptotic $CD8^+$ T cells for maintaining homeostasis (data not shown). Taken together, these data suggest that constitutive expression of 4-1BB on T cells greatly promotes expansion and survival of Ag-specific $CD4^+$ T cells.

Enhancement of T cell priming and Ag-specific Ab production in 4-1BB TG mice

To further examine the *in vivo* effect of constitutively-expressed 4-1BB on Ag-specific T cell activation, TG or WT mice was immunized with keyhole limpet hemocyanin (KLH) and tested their *in vitro* T cell recall proliferative response. Recall proliferative assays were performed using purified $CD4^+$ T cells from immunized mice and APCs derived from unimmunized WT mice. A significantly enhanced response was observed in TG mice, and the Ag dose-response curve was shifted by several orders of magnitude (Figure 5A). These results suggest that constitutive expression of 4-1BB on T cells greatly contributes to enhancement of Ag-specific T cell priming.

The role of 4-1BB was next investigated during the course of primary humoral immune responses against KLH, a well-known T cell-dependent Ag. Sera from TG mice exhibited no difference from those from WT mice in KLH-specific IgM Ab production (Figure 5B). This was expected because IgM is known to be T cell-independent. In contrast, production of higher levels of T cell-dependent IgG3 Abs was observed in TG vs WT mice. Increased production of IFN-γ in TG mice may explain the increase in IgG3 because IFN-γ is known to drive IgG3 switching (Finkelman *et al.*, 1998).

Discussion

In this study, an insight into 4-1BB regulation of $CD4^+$ T cell responses was explored by comparing $CD4^+$ T cell-mediated immune responses in WT and those

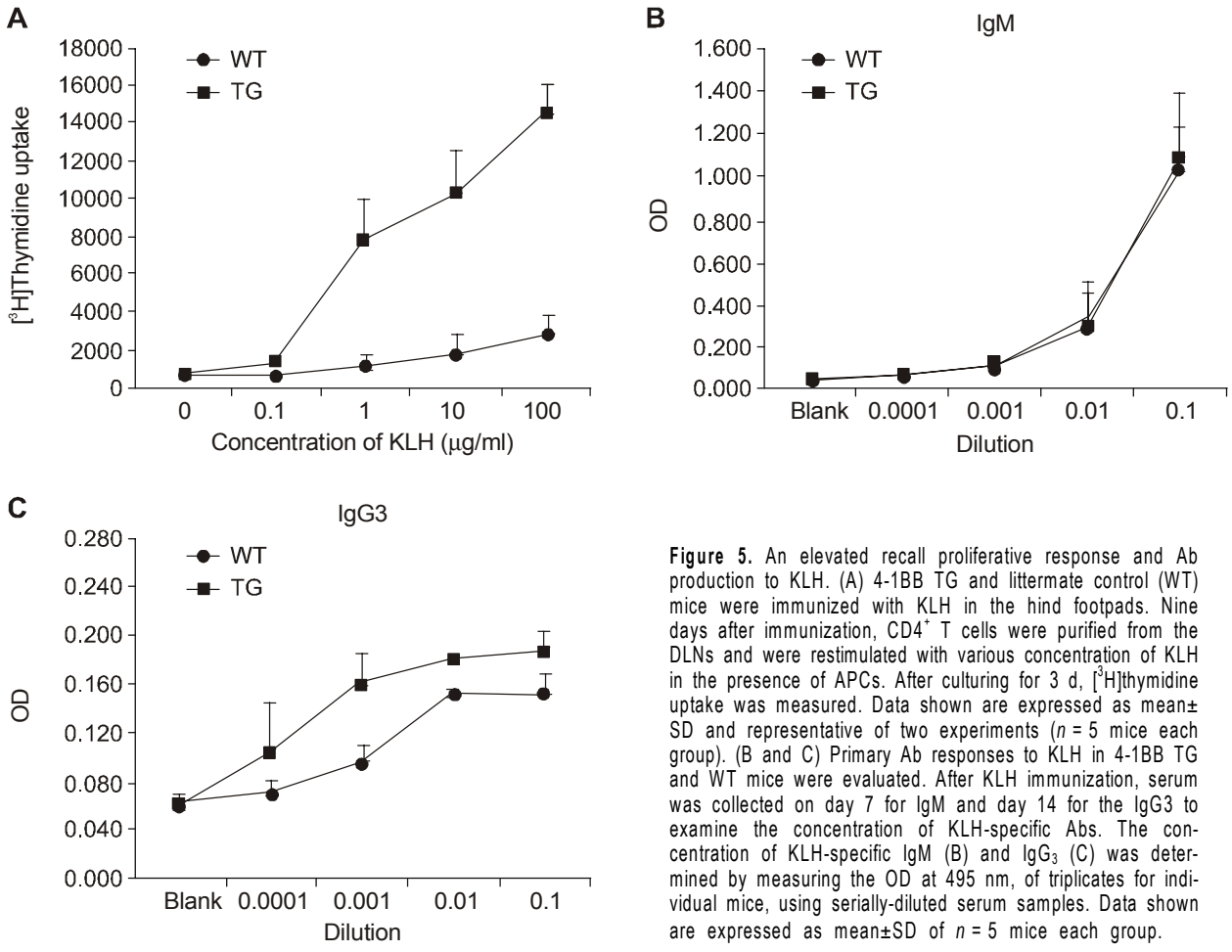

Figure 5. An elevated recall proliferative response and Ab production to KLH. (A) 4-1BB TG and littermate control (WT) mice were immunized with KLH in the hind footpads. Nine days after immunization, CD4$^+$ T cells were purified from the DLNs and were restimulated with various concentration of KLH in the presence of APCs. After culturing for 3 d, [^{3}H]thymidine uptake was measured. Data shown are expressed as mean± SD and representative of two experiments (n = 5 mice each group). (B and C) Primary Ab responses to KLH in 4-1BB TG and WT mice were evaluated. After KLH immunization, serum was collected on day 7 for IgM and day 14 for the IgG3 to examine the concentration of KLH-specific Abs. The concentration of KLH-specific IgM (B) and IgG_3 (C) was determined by measuring the OD at 495 nm, of triplicates for individual mice, using serially-diluted serum samples. Data shown are expressed as mean±SD of n = 5 mice each group.

in 4-1BB TG mice that were engineered to constitutively express 4-1BB on mature T cells. Recently, the costimulatory mechanism regulated by 4-1BB has been revealed in CD8$^+$ T cells. Remarkable growth and survival of CD8$^+$ T cells can be achieved by TCR/CD28/4-1BB stimulation compared with TCR/CD28 stimulation (Maus *et al*., 2002). Since 4-1BB expression is induced by TCR or TCR/CD28 signals, these findings suggest the need for 4-1BB signals to sustain the growth and survival of CD8$^+$ T cells (Watts and DeBenedette, 1999). This idea is also supported by the observation that naive CD8$^+$ T cells initially activated via TCR/CD28 signals become refractory to repeated TCR/CD28 stimulation (Kim *et al*., 1998). The promotion and prolongation of CD8$^+$ T cell proliferation and survival by 4-1BB costimulation appears to be mediated, at least in part, through increased production of IL-2 and expression of Bcl-xL (Lee *et al*., 2002; Maus *et al*., 2002). These *in vitro* results were confirmed by *in vivo* findings indicating that 4-1BB costimulatory signals play a crucial role in the generation of effector and memory cytotoxic T cells (CTLs) by increasing the number of CTLs cells (DeBeneddette *et al*., 1999; Tan *et al*., 1999; Cooper *et al*., 2000; Bertram *et al*., 2002; Kwon *et al*., 2002b).

Two *in vitro* observations have provided evidence that similarly to CD8$^+$ T cells, 4-1BB signals can support cell proliferation and survival of CD4$^+$ T cells (Cannons *et al*., 2001; Wen *et al*., 2002). In this study, the sustained proliferative response of TG CD4$^+$ T cells was demonstrated after *in vitro* TCR/4-1BB stimulation, which was not seen in that of WT CD4$^+$ T cells. These results indicate that constitutive expression on 4-1BB on CD4$^+$ T cells might make a critical contribution to maintaining the proliferative capacity. These data also indicate that the sustained proliferative response of TG CD4$^+$ T cells could take place without CD28 signals that are required for the induction of 4-1BB expression on WT T cells together with TCR signals. Even though the exact number of allergen-specific CD4$^+$ T cells in CHS responses was not measured, the DLNs of TG mice showed existence of a significantly higher percentage of CD4$^+$ T cells after the allergen challenge, which may reflect

the poulation size of the clonally expanded allergen-specific $CD4^+$ T cells. This idea was further supported by two observations. One is that the proliferative response of KLH-specific $CD4^+$ T cells was greater in TG mice than was in WT mice. Another is that a similar percentage of $CD8^+$ T cells presumably representing those that were not specific for the allergen was contained in the DLNs of TG and WT mice after the allergen challenge. Moreover, $CD4^+$ T cells of TG mice exhibited a remarkedly reduced apoptosis after the allergen challenge. Taken together, these *in vivo* data are consistent with the *in vitro* results showing that 4-1BB signals induce progression of cell division and sustaining survival of $CD4^+$ T cells.

Analysis of TG mice demonstrated enhanced IgG3 production to KLH compared with WT mice. Since KLH is known to be a T cell-dependent Ag on Ab production, the enhanced Ab responses observed may be due to enhanced T cell priming. As indicated in CHS responses, 4-1BB signals play a critical role in the induction of inflammatory $CD4^+$ Th1 responses. Similarly, 4-1BB signals may evoke $CD4^+$ Th1 IFN-γ expression and switch B cells to make IFN-γ-dependent Abs such as IgG2a and IgG3. This idea can be supported by the observations that 4-1BB-deficient mice had reduced production of IgG2a and IgG3 to KLH than did WT mice (Kwon *et al*., 2002b) and also by the observations that blockade of the 4-1BB costimulatory signals resulted in enhanced Th2-dependent IgE and anti-double standed DNA (dsDNA) IgG1 autoantibody production possibly *via* increased production of IL-4 and decreased production of IFN-γ in chronic graft-versus-host disease (GVHD) (Nozawa *et al*., 2001). Reversely, stimulation of 4-1BB totally abrogates Th2-dependent Ab production (Kim J and Kwon B, unpublished data). These data together highlight the involvement of 4-1BB costimulatory signals in $CD4^+$ Th1-mediated immune responses. However, we could not exclude the possibility that 4-1BB is involved in $CD4^+$ Th2 responses. Currently, studies are under way to reveal this aspect of 4-1BB function.

In summary, we have shown that 4-1BB represents an important costimulatory molecule in the $CD4^+$ Th1 T cell response. The primary mechanism of action appears to be to regulate proliferation and cell survival. Thus overall 4-1BB acts to determine the number of $CD4^+$ T cells that are generated in the primary immune responses. Further studies will be needed to define the involvement of 4-1BB in Th1 vs Th2 responses.

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