EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 41, No. 12, 896-911, December 2009

# Expression of 4-1BB and 4-1BBL in thymocytes during thymus regeneration

**Young-Mi Kim[1]*, Hye Kyung Kim[2,3,4]*, Hyo Jin Kim[2,3], Hee-Woo Lee[5], Seong-A Ju[6], Beom K. Choi[7], Byoung S. Kwon[7], Bong-Seon Kim[2], Jae-Bong Kim[2], Young-Tak Lim[1,8] and Sik Yoon[2,3,4,8,9]**

[1]Department of Pediatrics
[2]Department of Anatomy
[3]Medical Research Center for Ischemic Tissue Regeneration
[4]Pusan National University Medical Science
Education Center (BK21 Program)
Pusan National University School of Medicine
Yangsan 626-870, Korea
[5]Research Institute for Veterinary Science
College of Veterinary Medicine
Seoul National University
Seoul 151-742, Korea
[6]University of Ulsan Biomedical Research Center
Ulsan 680-749, Korea
[7]Division of Cell and Immunobiology and
R&D Center for Cancer Therapeutics
National Cancer Center
Goyang 410-769, Korea
[8]Medical Research Institute
Pusan National University School of Medicine
Yangsan 626-870, Korea
[9]Corresponding author: Tel, 82-51-510-8044;
Fax, 82-51-510-8049; E-mail, sikyoon@pusan.ac.kr
*These authors contributed equally to this work.
DOI 10.3858/emm.2009.41.12.095

Accepted 10 August 2009

Abbreviations: ATCC, American type culture collection; DC, dendritic cells; DN, double-negative; dNTP, deoxynucleotide triphosphate; DP, double-positive; SP, single-positive; NGFR, nerve growth factor receptor; RAG, recombination-activating gene; TBS, Tris-buffered saline; TNFR, tumor necrosis factor receptor; TSA-1, thymic shared antigen-1; FITC, fluorescein isothiocyanate

## Abstract

**4-1BB, a member of the tumor necrosis factor receptor (TNFR) superfamily, is a major costimulatory receptor that is rapidly expressed on the surface of $CD4^+$ and $CD8^+$ T cells after antigen- or mitogen-induced activation. The interaction of 4-1BB with 4-1BBL regulates immunity and promotes the survival and expansion of activated T cells. In this study, the expression of 4-1BB and 4-1BBL was examined during regeneration of the murine thymus following acute cyclophosphamide-induced involution. Four-color flow cytometry showed that 4-1BB and 4-1BBL were present in the normal thymus and were preferentially expressed in the regenerating thymus, mainly in $CD4^+CD8^+$ double-positive (DP) thymocytes. Furthermore, the $CD4^{lo}CD8^{lo}$, $CD4^+CD8^{lo}$ and $CD4^{lo}CD8^+$ thymocyte subsets, representing stages of thymocyte differentiation intermediate between DP and single-positive (SP) thymocytes, also expressed 4-1BB and 4-1BBL during thymus regeneration but to a lesser degree. Interestingly, the 4-1BB and 4-1BBL positive cells among the $CD4^+CD8^+$ DP thymocytes present during thymus regeneration were $TCR^{hi}$ and $CD69^+$ unlike the corresponding controls. Moreover, the 4-1BB and 4-1BBL positive cells among the intermediate subsets present during thymus regeneration also exhibited $TCR^{hi/int}$ and $CD69^{+/int}$ phenotypes, indicating that 4-1BB and 4-1BBL are predominantly expressed by the positively selected population of the $CD4^+CD8^+$ DP and the intermediate thymocytes during thymus regeneration. RT-PCR and Western blot analyses confirmed the presence and elevated levels of 4-1BB and 4-1BBL mRNA and protein in thymocytes during thymus regeneration. We also found that the interaction of 4-1BB with 4-1BBL promoted thymocyte adhesion to thymic epithelial cells. Our results suggest that 4-1BB and 4-1BBL participate in T lymphopoiesis associated with positive selection during recovery from acute thymic involution.**

**Keywords:** 4-1BB ligand; antigens, CD137; cell differentiation; thymus gland; T-lymphocytes

## Introduction

The 4-1BB receptor (also referred to as CD137, ILA and TNFRSF9), is a member of the low affinity nerve growth factor receptor/tumor necrosis factor receptor (NGFR/TNFR) family of integral type I membrane protein. It is a potent T cell costimulatory receptor induced primarily by activated T cells (Kwon and Weissman, 1989; Pollok *et al.,* 1993). A high affinity ligand for 4-1BB (4-1BBL; also called CD137L and TNFSF9), a type II membrane protein

of the TNF superfamily, has been found to be mainly expressed on activated antigen-presenting cells such as dendritic cells (DCs), B cells, and macrophages (Goodwin *et al.,* 1993; Laderach *et al.,* 2003; Schwarz, 2005).

The costimulatory ability of 4-1BB is of importance in that 4-1BB on its own can provide a costimulatory signal that activates resting T cells independently of other costimulatory molecules such as the CD28, even though signaling through CD28 has been shown to be critical for activation of T cells, and CD28 is widely considered the primary T cell receptor delivering costimulatory signals to resting T cells (Lenschow *et al.,* 1996; Saoulli *et al.,* 1998; Sica and Chen, 2000; Croft, 2003; Watts, 2005). Costimulation through 4-1BB by either 4-1BBL or agonistic monoclonal antibodies (mAbs) enhances T cell activity (Pollok *et al.,* 1993; Hurtado *et al.,* 1997), promotes $CD8^+$ T cell survival (Takahashi *et al.,* 1999), eradicates established tumors (Melero *et al.,* 1997; Ye *et al.,* 2002), promotes rejection of cardiac and skin allografts (Cho *et al.,* 2004), broadens primary antiviral $CD8^+$ T cell responses (Halstead *et al.,* 2002), enhances the memory pool of antigen-specific $CD8^+$ T cells (Bertram *et al.,* 2002), and increases T cell cytolytic potential (Shuford *et al.,* 1997). In addition, 4-1BB-mediated signals suppress $CD4^+$ T cell responses (Mittler *et al.,* 1999) and ameliorate both antigen-induced organ-specific autoimmune diseases such as experimental autoimmune encephalomyelitis (Sun *et al.,* 2002b) and experimental autoimmune rheumatoid arthritis (Seo *et al.,* 2004), and spontaneous systemic autoimmune diseases such as systemic lupus eythematosus (Sun *et al.,* 2002a; Foell *et al.,* 2003). The mechanisms underlying these immunosuppressive effects are not yet fully understood, but it has been shown that 4-1BB-mediated suppression of rheumatoid arthritis is caused by antigen-dependent induction of $CD11c^+CD8^+$ T cells that produce IFN-$\gamma$, which suppresses antigen-specific $CD4^+$ T cells by an indoleamine 2,3-dioxygenase-dependent mechanism (Seo *et al.,* 2004). Importantly, the 4-1BB receptor/ligand system, like other members of the TNF-$\alpha$ systems, can signal in a bidirectional manner. Therefore, activation signals may be initiated not only in the cells that express the receptor but also in the cells bearing the 4-1BBL, e.g., the DCs (Tan *et al.,* 1999; May *et al.,* 2002; Lippert *et al.,* 2008). These findings establish an important role for 4-1BB/4-1BBL in the immune regulation.

Despite these facts that 4-1BB and 4-1BBL play critical roles in T cell function, little is known about the characteristics and function of 4-1BB and

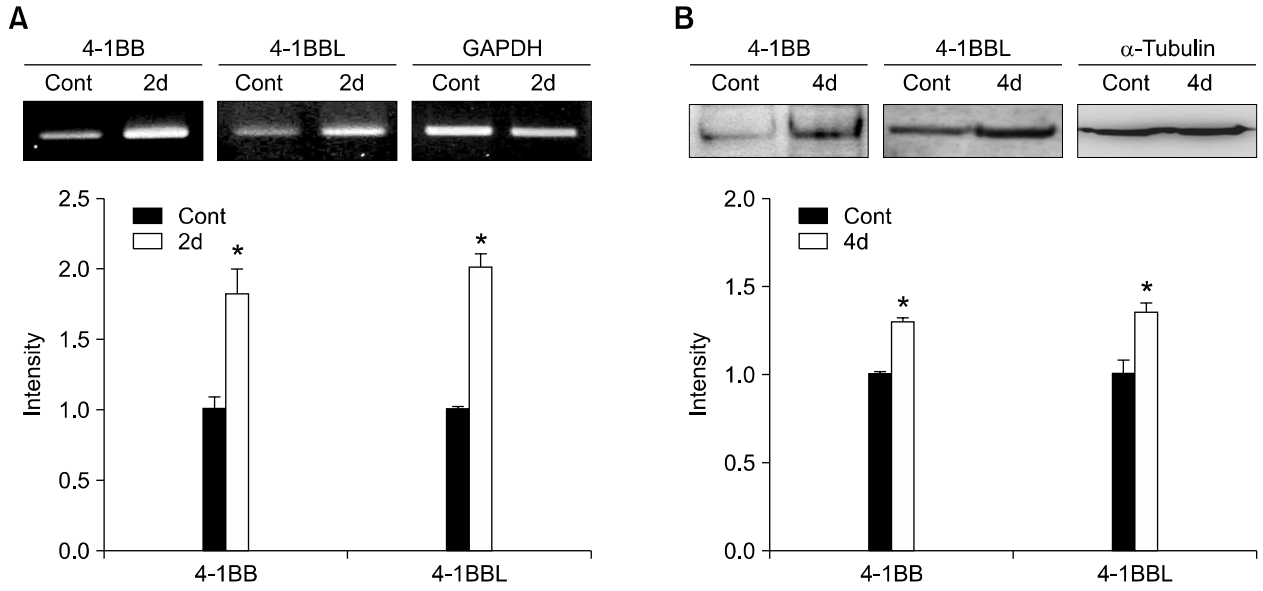


**Figure 1.** Expression of 4-1BB and 4-1BBL mRNA and protein in mouse thymocytes during thymus regeneration. B6 mice (8-10-week-old) were given a single intraperitoneal dose of cyclophosphamide (400 mg/kg body weight), and were killed in groups of four after 2 or 4 days. (A) RT-PCR analysis of 4-1BB, 4-1BBL and GAPDH in thymocytes from control mice (Cont) and 2 days after cyclophosphamide treatment (2d). Gene expression levels were determined after normalization to GAPDH. (B) Western blot analysis of 4-1BB, 4-1BBL and $\alpha$-tubulin in thymocytes from control (Cont) and 4 days after cyclophosphamide treatment (4d). Data are expressed as ratios of 4-1BB and 4-1BBL protein normalized to $\alpha$-tubulin protein. Band densities were measured by scanning densitometry and expressed as means + SD. Data are representative of three independent experiments with four or more animals in each group.

4-1BBL in the thymus, the central lymphoid organ for the development of bone marrow-derived precursor cells into mature T cells. Thus, the aim of the present study was to investigate the expression of 4-1BB and 4-1BBL using a mouse thymus regeneration model in which mature T cells are actively produced from bone marrow-derived precursor cells through the orchestrated processes of T cell development, and to shed light on the role of 4-1BB and 4-1BBL in the thymus.

## Results

### Presence of 4-1BB and 4-1BBL in the thymocytes and their up-regulation during thymus regeneration

We first tested for expression of 4-1BB and 4-1BBL in control mouse thymocytes by RT-PCR and Western blotting, and detected a low level of expression of both components. Interestingly, their expression was strongly up-regulated during thymus regeneration after cyclophosphamide-induced acute thymic involution (Figures 1A and 1B). In addition, flow cytometric analysis of 4-1BB and 4-1BBL expression demonstrated that their expression during regeneration was regulated in a time-dependent manner (Figures 2A and 2B). The proportion of 4-1BB-expressing thymocytes peaked on day 5 (Figure 2A), and similar kinetics were observed for 4-1BBL-expression (Figure 2B). A significant decrease in the total number of thymocytes was also observed in cyclophosphamide-treated mice during thymus regeneration (Table 1).

**Table 1.** Total thymocyte number during thymus regeneration

| | Total thymocyte number ($\times 10^5$) |
|---|---|
| Control | 1550.0 ± 212.1 |
| 1d | 162.0 ± 67.9 |
| 2d | 18.3 ± 0.4 |
| 3d | 6.3 ± 3.6 |
| 4d | 5.2 ± 0.1 |
| 5d | 5.7 ± 1.8 |
| 6d | 20.1 ± 5.6 |
| 7d | 28.1 ± 8.1 |
| 10d | 430.0 ± 56.6 |

### Expression of 4-1BB and 4-1BBL is strongly up-regulated in $CD4^+CD8^+$ DP thymocytes during thymus regeneration

To identify the cell types which express 4-1BB and 4-1BBL, we explored the expression of 4-1BB and 4-1BBL in the thymocyte subsets from control and regenerating thymus by flow cytometry. We first investigated the conventional four thymocyte subsets. In the normal mouse thymus, $CD4^-CD8^-$ double-negative (DN) and $CD4^+CD8^+$ double-positive (DP) thymocytes barely expressed 4-1BB, whereas $CD4^+$ single-positive (SP) and $CD8^+$ SP thymocytes expressed 4-1BB weakly (Figure 3A). Similarly, in the normal mouse thymus, $CD4^-CD8^-$ DN, $CD4^+CD8^+$ DP and $CD4^+$ SP thymocytes barely expressed 4-1BBL, whereas and $CD8^+$ SP

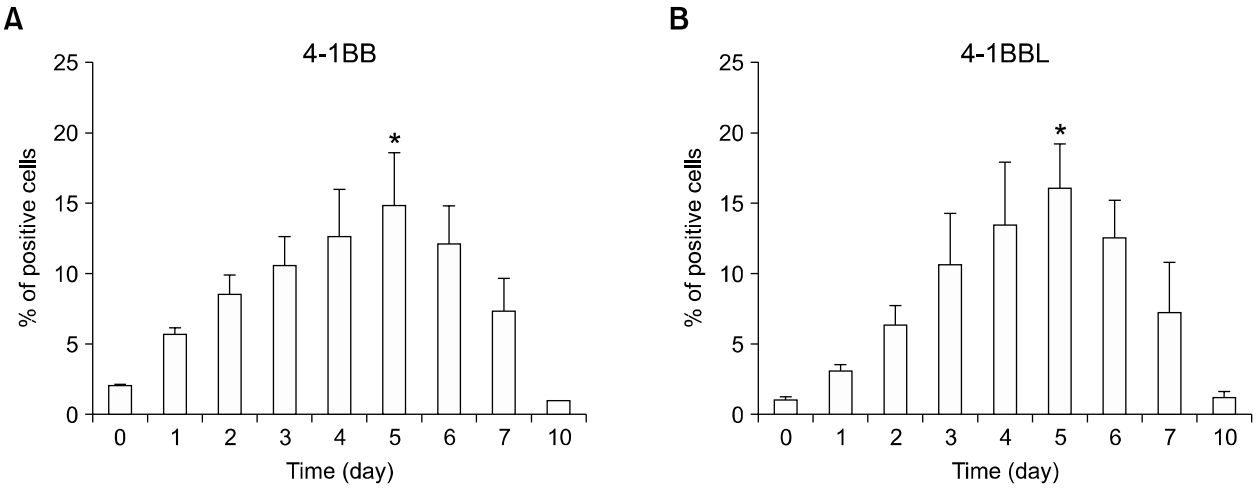


**Figure 2.** Flow cytometric analysis of the expression of 4-1BB and 4-1BBL in total thymocytes during thymus regeneration. B6 mice were killed in groups of four at 1, 2, 3, 4, 5, 6, 7 and 10 days after injection of cyclophosphamide, and thymocytes were stained with biotinylated anti-4-1BB and biotinylated anti-4-1BBL, followed by PE-streptavidin. Percentages of 4-1BB (A) and 4-1BBL (B) positive cells among total thymocytes were calculated. *$P < 0.05$, by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Data are means + SD of more than three independent experiments with four or more animals in each group.

thymocytes expressed 4-1BBL weakly (Figure 3B). Interestingly, we observed marked up-regulation of 4-1BB and 4-1BBL expression in the DP thymocytes during thymus regeneration, peaking around

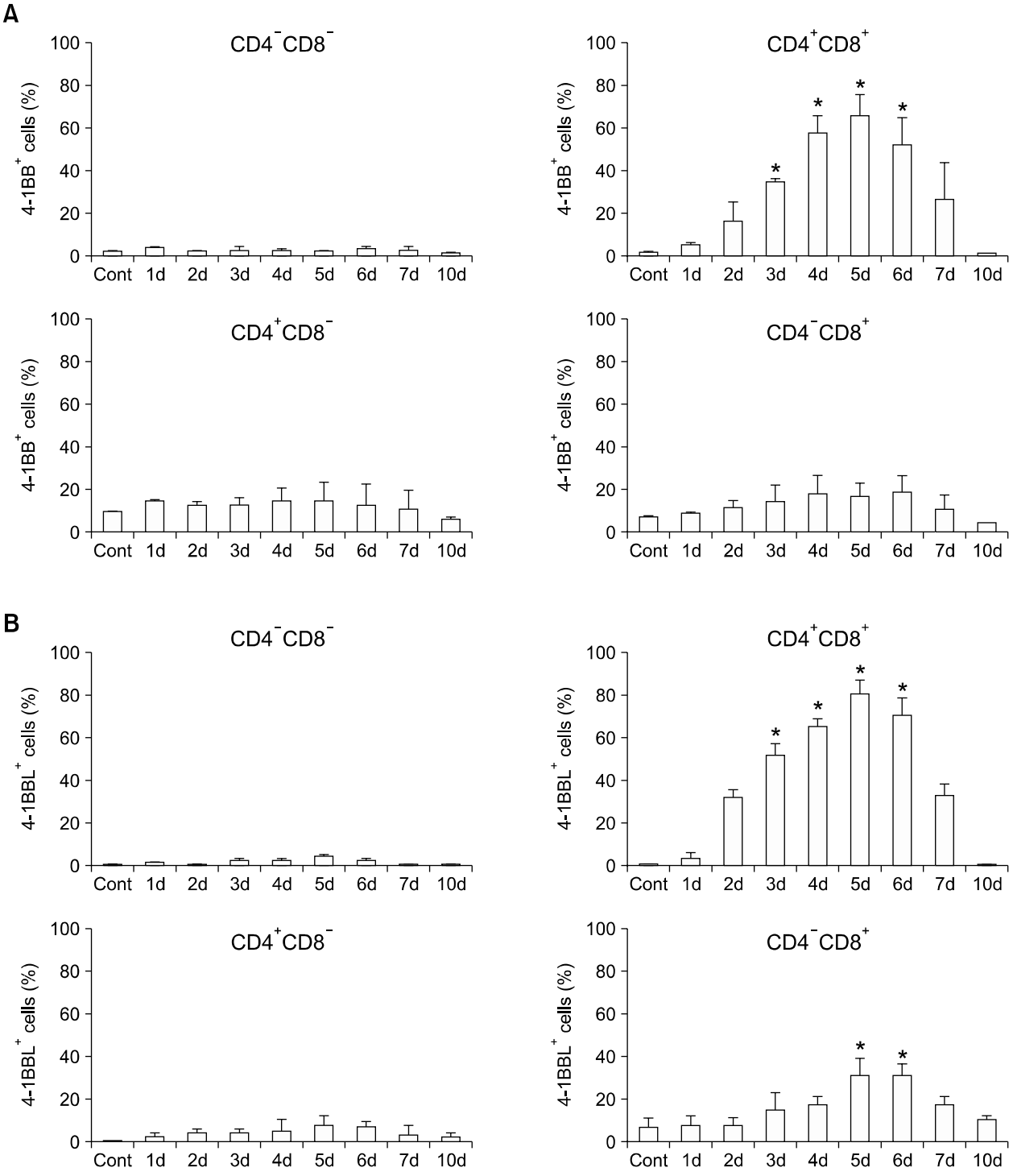


**Figure 3.** Expression of 4-1BB (A) and 4-1BBL (B) in the four major thymocyte subsets during thymus regeneration. (C) The phenotypic distribution of the four thymocyte subsets during thymus regeneration. Groups of four B6 mice were killed 1, 2, 3, 4, 5, 6, 7 and 10 days after injection of cyclophosphamide. Thymocytes were stained with Pacific Blue-anti-CD4, APC-Cy7-anti-CD8, biotin-anti-4-1BB, biotin-anti-4-1BBL and PE-streptavidin. Data are means + SD of at least three independent experiments with four or more animals per group. *$P < 0.05$ by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

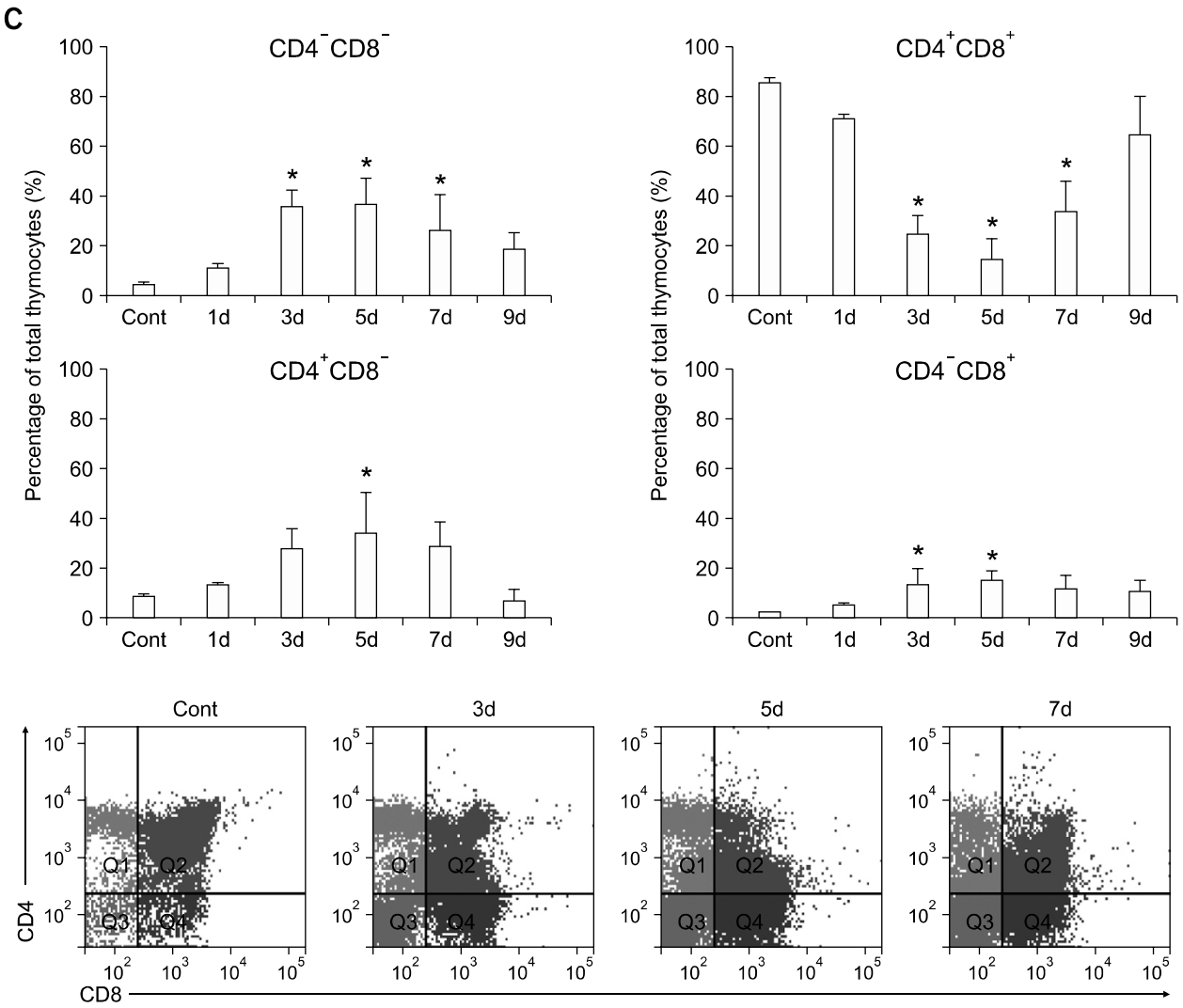


**Figure 3.** Continued.

day 5 (Figures 3A and 3B). $CD8^+$ SP thymocytes also showed significantly enhanced expression of 4- 1BBL during thymus regeneration, but to a much lesser degree (Figure 3B). The $CD4^+$ SP thymocytes expressed a slightly higher level of 4-1BB than of 4-1BBL throughout the entire period of thymus regeneration but their expression did not change during regeneration (Figures 3A and 3B). The $CD4^-CD8^-$ DN thymocytes barely expressed 4-1BB or 4-1BBL during regeneration (Figures 3A and 3B). In addition, we noted that cyclophosphamide-treated mice during thymus regeneration exhibited a significant decrease in the number of DP thymocytes but showed a significant increase in the number of DN, $CD4^+$ SP and $CD8^+$ SP thymocytes relative to control mice (Figure 3C).

### 4-1BB and 4-1BBL are expressed predominantly in $CD4^+CD8^+TCR^{hi}$, $CD4^{lo}CD8^{lo}TCR^{int}$, $CD4^+CD8^{lo}TCR^{hi/int}$ and $CD4^{lo}CD8^+TCR^{hi/int}$ thymocyte subsets during thymus regeneration

To further characterize these cell populations which express 4-1BB and 4-1BBL, the thymocytes were divided into nine subpopulations on the basis of CD4 and CD8 fluorescence intensities, classified as -, lo and + (Figure 4). There were thus nine categories: $CD4^-CD8^-$, $CD4^{lo}CD8^-$, $CD4^-CD8^{lo}$, $CD4^+CD8^+$, $CD4^{lo}CD8^{lo}$, $CD4^+CD8^{lo}$, $CD4^{lo}CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes (Figure 4A). In addition, to define their maturational status, we assessed TCR expression in these subsets. In the normal thymus, this classification yielded the following subsets: $CD4^-CD8^-TCR^-$, $CD4^{lo}CD8^-TCR^-$, $CD4^-CD8^{lo}TCR^{-/int}$, $CD4^+CD8^+TCR^{-/int}$, $CD4^{lo}CD8^{lo}$

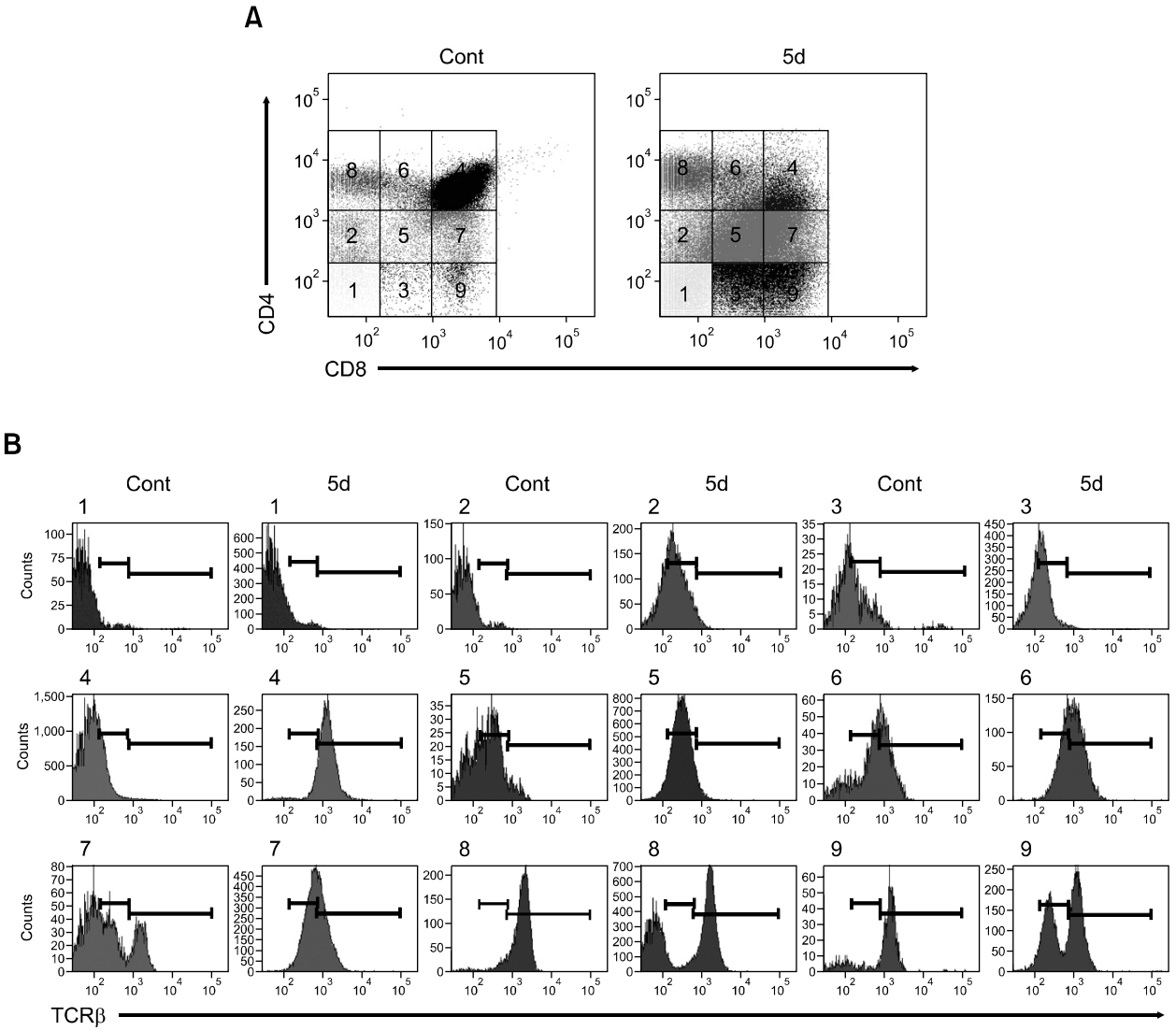


**Figure 4.** Upregulation of TCR expression in the $CD4^+CD8^+$ DP and intermediate thymocyte subsets during thymus regeneration. Thymocytes were stained with Pacific Blue-anti-CD4, APC-Cy7-anti-CD8, and FITC-anti-TCRβ. (A) Nine thymocyte subsets were defined on the basis of CD4 and CD8 fluorescence intensities: $CD4^-CD8^-$, $CD4^{lo}CD8^-$, $CD4^-CD8^{lo}$, $CD4^+CD8^+$, $CD4^{lo}CD8^{lo}$, $CD4^+CD8^{lo}$, $CD4^{lo}CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ thymocytes. (B) Expression of TCRβ was analyzed by flow cytometry in the nine thymocyte subsets during thymus regeneration. Levels of TCRβ expression in thymocytes of control B6 mice (Cont) and of mice 5 days after cyclophosphamide treatment (5d) are shown. Data are representative of more than three independent experiments with four or more animals in each group.

$TCR^{-/int}$, $CD4^+CD8^{lo}TCR^{-/int/hi}$, $CD4^{lo}CD8^+TCR^{-/int/hi}$, $CD4^+CD8^-TCR^{hi}$ and $CD4^-CD8^+TCR^{hi}$ (Figure 4B). Of interest, TCR expression increased significantly in some of these subpopulations during thymus regeneration, peaking around day 5 after cyclophosphamide treatment. On day 5, these nine subsets exhibited the following phenotypes: $CD4^-CD8^-TCR^-$, $CD4^{lo}CD8^-TCR^{-/int}$, $CD4^-CD8^{lo}TCR^{-/int}$, $CD4^+CD8^+TCR^{hi}$, $CD4^{lo}CD8^{lo}TCR^{int}$, $CD4^+CD8^{lo}TCR^{int/hi}$, $CD4^{lo}CD8^+TCR^{int/hi}$, $CD4^+CD8^-TCR^{-/int/hi}$ and $CD4^-CD8^+TCR^{-/int/hi}$ (Figure 4B). The level of TCR expression in the DP thymocyte subpopulation, in particular, increased markedly (Figure 4B). The $CD4^{lo}CD8^{lo}$, $CD4^+CD8^{lo}$ and $CD4^{lo}CD8^+$ thymocyte subsets, belonging to the intermediate stage of thymocyte differentiation from DP to SP thymocytes, also displayed intermediate to high levels of TCR expression but to a lesser degree than the $CD4^+CD8^+$ DP population (Figure 4B).

We also assessed TCR expression in gated 4-1BB and 4-1BBL positive DP, $CD4^{lo}CD8^{lo}$, $CD4^+CD8^{lo}$ and $CD4^{lo}CD8^+$ thymocytes during thymus regeneration. The level of TCR expression was high in the DP thymocytes, intermediate to

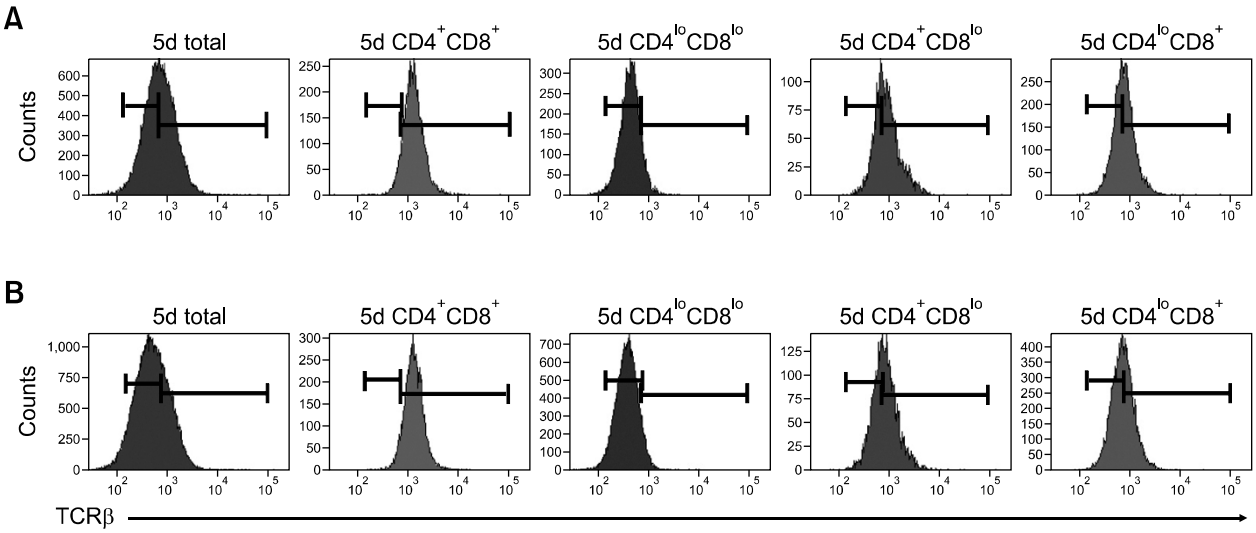


**Figure 5.** TCR expression in 4-1BB and 4-1BBL positive $CD4^+CD8^+$ DP and intermediate thymocytes during thymus regeneration. Thymocytes were stained with Pacific Blue-anti-CD4, APC-Cy7-anti-CD8, and FITC-anti-TCRβ. The expression of TCRβ was analyzed by flow cytometry in the $CD4^+CD8^+$ thymocytes (Cont $CD4^+CD8^+$) from normal B6 mice, and in gated 4-1BB (A) and 4-1BBL (B) positive cells in the total (5d total), $CD4^+CD8^+$ (5d $CD4^+CD8^+$), $CD4^{lo}CD8^{lo}$ (5d $CD4^{lo}CD8^{lo}$), $CD4^+CD8^{lo}$ (5d $CD4^+CD8^{lo}$), and $CD4^{lo}CD8^+$ (5d $CD4^{lo}CD8^+$) thymocytes from mice 5 days after cyclophosphamide treatment. Data are representative of at least three independent experiments with four or more animals per group.

high in $CD4^+CD8^{lo}$ and $CD4^{lo}CD8^+$ thymocytes, and intermediate in $CD4^{lo}CD8^{lo}$ thymocytes (Figure 5A). A similar pattern was seen for 4-1BBL (Figure 5B).

Next, we examined the expression of 4-1BB and 4-1BBL in the nine subpopulations by flow cytometry. The majority of thymocytes in the normal mouse thymus barely expressed 4-1BB and 4-1BBL (Figures 6A and 6B). The proportion of 4-1BB positive cells in $CD4^+CD8^+$ DP thymocytes increased markedly during thymus regeneration and there were also smaller increase in the intermediate thymocyte subsets (Figure 6A). The expression of 4-1BBL in these nine thymocyte subpopulations exhibited essentially the same pattern (Figure 6B). However, there was no significant change in the number of $CD8^+$ SP thymocytes that expressed 4-1BBL. This contrasted with the situation in the $CD8^+$ SP thymocytes in the original thymocyte subsets, and thus indicated that there was no real increase in 4-1BBL expression in $CD8^+$ SP thymocytes during thymus regeneration (Figures 3B and 6B).

### 4-1BB and 4-1BBL are preferentially expressed by positively selected thymocytes in the $CD4^+CD8^+$, $CD4^{lo}CD8^+$, $CD4^+CD8^{lo}$ and $CD4^{lo}CD8^{lo}$ thymocyte subsets during thymus regeneration

The above findings indicate that 4-1BB and 4-1BBL are preferentially expressed in $CD4^+CD8^+$ DP and the intermediate thymocytes during thymus regeneration, and that these thymocytes show a high level of TCR expression during thymus regeneration. This raised the question as to whether 4-1BB and 4-1BBL expression in these thymocyte populations is related to positive selection. To address this issue, we observed CD69 expression on these thymocytes during thymus regeneration. Only a small percentage of DP thymocytes expressed CD69 in the normal thymus but this number increased dramatically during thymus regeneration (Figure 7A). Next, in order to see whether pre- and post-selected thymocytes differ in the expression of 4-1BB and 4-1BBL, we compared CD69 expression in the 4-1BB and 4-1BBL positive and negative thymocytes during thymus regeneration (Figures 7B and 7C). We indeed found a strong correlation between 4-1BB and 4-1BBL expression and CD69 expression (Figures 7B and 7C). There was also a good correlation between 4-1BB and 4-1BBL expression and CD69 expression in the intermediate thymocytes (Figures 7B and 7C).

### 4-1BBL up-regulates expression of 4-1BB and 4-1BBL in mouse thymocytes

To investigate the function of 4-1BBL, we tested its ability to stimulate 4-1BB expression in mouse thymocytes. Flow cytometric analysis revealed greatly increased expression of 4-1BB on mouse thymocytes grown for 48 h after exposure to 100 ng/ml 4-1BBL, compared to control thymocytes

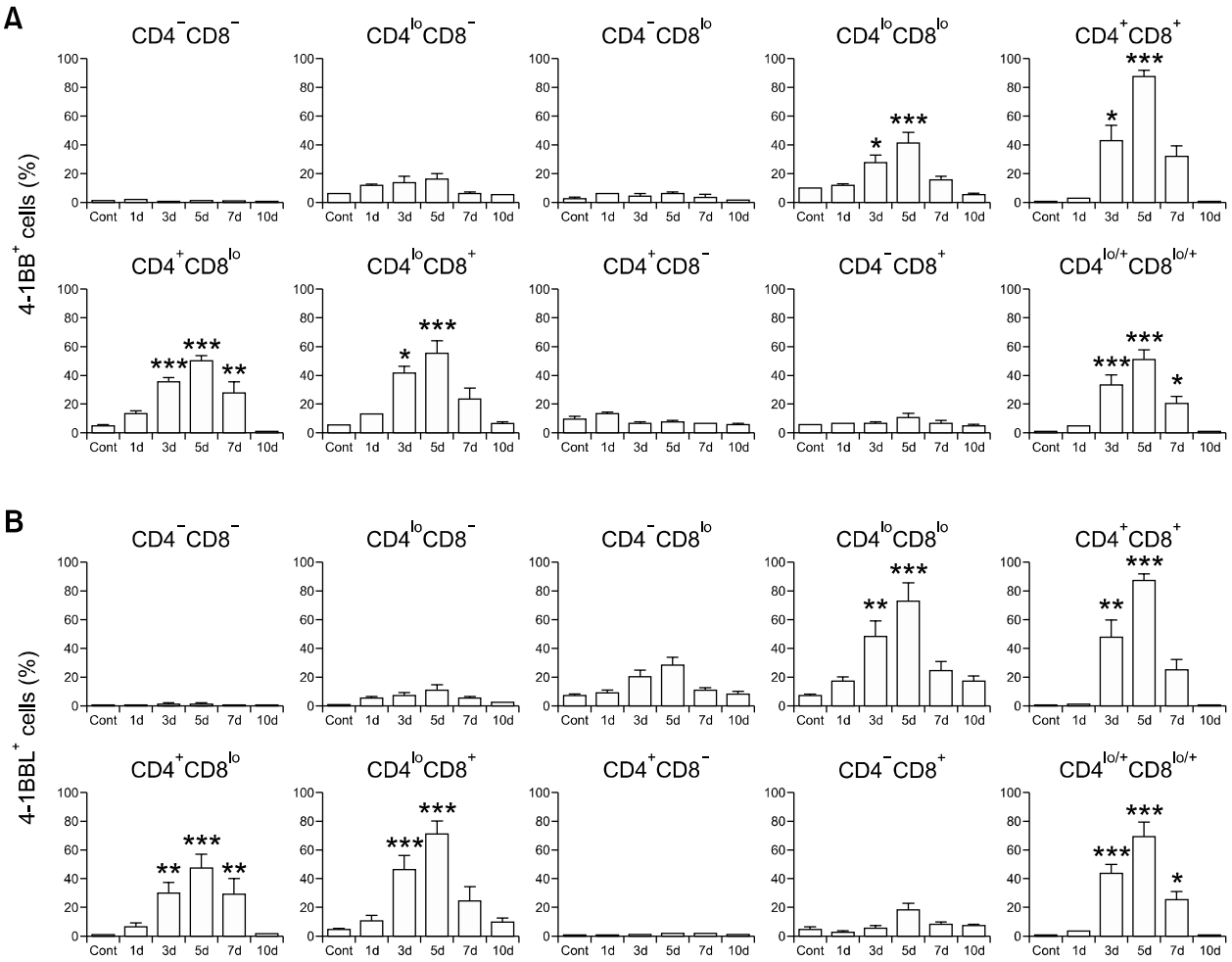


**Figure 6.** Upregulated expression of 4-1BB and 4-1BBL in the $CD4^+CD8^+$ DP and intermediate thymocytes during thymus regeneration. B6 mice were killed in groups of four at 1, 3, 5, 7 and 10 days after injection of cyclophosphamide. They were stained with Pacific Blue-anti-CD4, APC-Cy7-anti-CD8, biotin-anti-4-1BB, biotin-anti-4-1BBL, and PE-streptavidin, and expression of 4-1BB (A) and 4-1BBL (B) was analyzed by flow cytometry in the nine thymocyte subsets. Data are the means + SD of at least three independent experiments with four or more animals in each group. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

cultured for 48 h and freshly isolated thymocytes; only slight up-regulation of 4-1BBL expression was observed 12 h after 4-1BBL treatment (Figures 8A and 8B). In contrast to 4-1BB expression, expression of 4-1BBL was significantly higher in control thymocytes cultured for 48 h than in freshly isolated thymocytes (Figures 8A and 8B).

### 4-1BBL enhances thymocyte adhesion to mouse thymic epithelial cells

To assess whether signaling through 4-1BB affects thymocyte adhesion to thymic epithelial cells we performed a cell adhesion assay. We first checked the expression of 4-1BB in mouse thymic epithelial cells by RT-PCR and Western blotting, and detected their expression of both 4-1BB mRNA and protein. Interestingly, it was found that a significant increase in the number of thymocytes adhering to the thymic epithelial cells after treatment with 4-1BBL (Figure 9), indicating that 4-1BBL evidently promotes thymocyte adhesion to thymic epithelial cells.

## Discussion

We have shown in the present study that 4-1BB and 4-1BBL are present on the thymocytes of normal and regenerating thymus, and that they are markedly up-regulated - predominantly in the $CD4^+CD8^+$ DP, and the $CD4^{lo}CD8^{lo}$, $CD4^+CD8^{lo}$ and $CD4^{lo}CD8^+$ intermediate thymocyte subsets- during thymic regeneration after the acute thymic

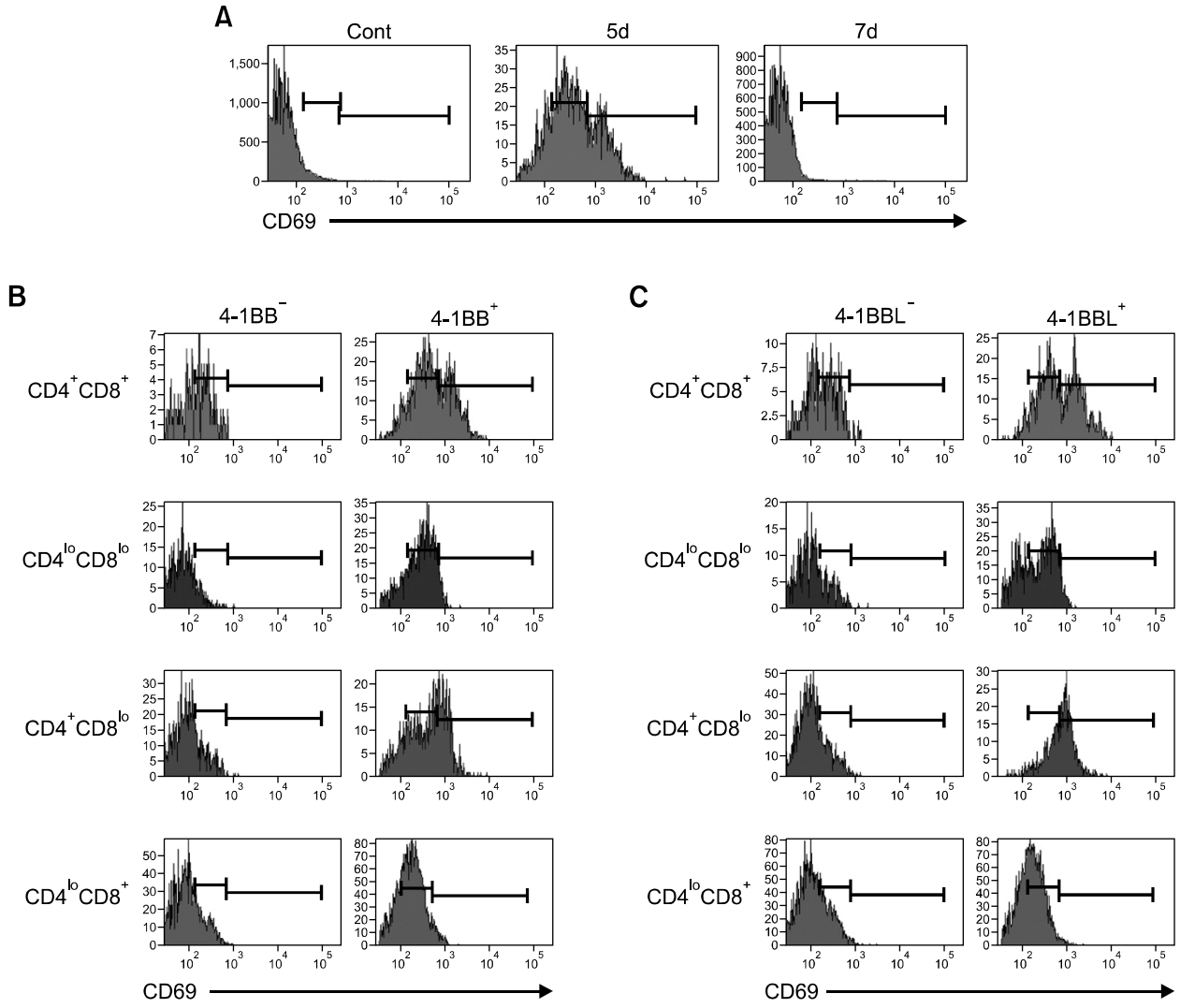


**Figure 7.** CD69 expression in the 4-1BB and 4-1BBL positive $CD4^+CD8^+$ DP and intermediate thymocytes during thymus regeneration. Thymocytes were stained with Pacific Blue-anti-CD4, APC-Cy7-anti-CD8, and FITC-anti-CD69. (A) Expression of CD69 on the $CD4^+CD8^+$ DP thymocytes from control B6 mice (Cont), and mice 5 (5d) and 7 days (7d) after cyclophosphamide treatment. The histograms show expression of CD69 in gated 4-1BB (A) and 4-1BBL (B) positive $CD4^+CD8^+$, $CD4^{lo}CD8^{lo}$, $CD4^+CD8^{lo}$, and $CD4^{lo}CD8^+$ thymocytes from mice 5 days after cyclophosphamide treatment. Data are representative of three independent experiments with four or more animals in each group.

involution induced by cyclophosphamide treatment of mice.

The expression of 4-1BBL in the nine thymocyte subpopulations during thymus regeneration exhibited essentially the same pattern as that of 4-1BB, indicating that 4-1BB and 4-1BBL are expressed in the same compartment of the thymus associated with the developmental stages of the $CD4^+CD8^+$ DP, and the intermediate thymocyte subsets, where interactions between 4-1BB and 4-1BBL take place to maintain the normal physiological functions of the thymus. Expression of 4-1BB and 4-1BBL during thymus regeneration in the four major thymocyte subsets was essentially the same as their expression in the nine thymocyte subpopulations except for the 4-1BBL expression in $CD8^+$ SP thymocytes during thymus regeneration. However, we were subsequently able to show that the discrepancy between the expression of 4-1BBL in the $CD8^+$ SP thymocytes analyzed in the four major thymocyte subsets and that analyzed in the nine thymocyte subsets was due to the fact that the $CD8^+$ SP thymocytes examined in the four thymocyte subsets contained 4-1BBL positive $CD4^{lo}CD8^+$ thymocytes .

TCR expression was also strikingly enhanced in the $CD4^+CD8^+$ DP, and intermediate thymocyte subsets during thymic regeneration, indicating that

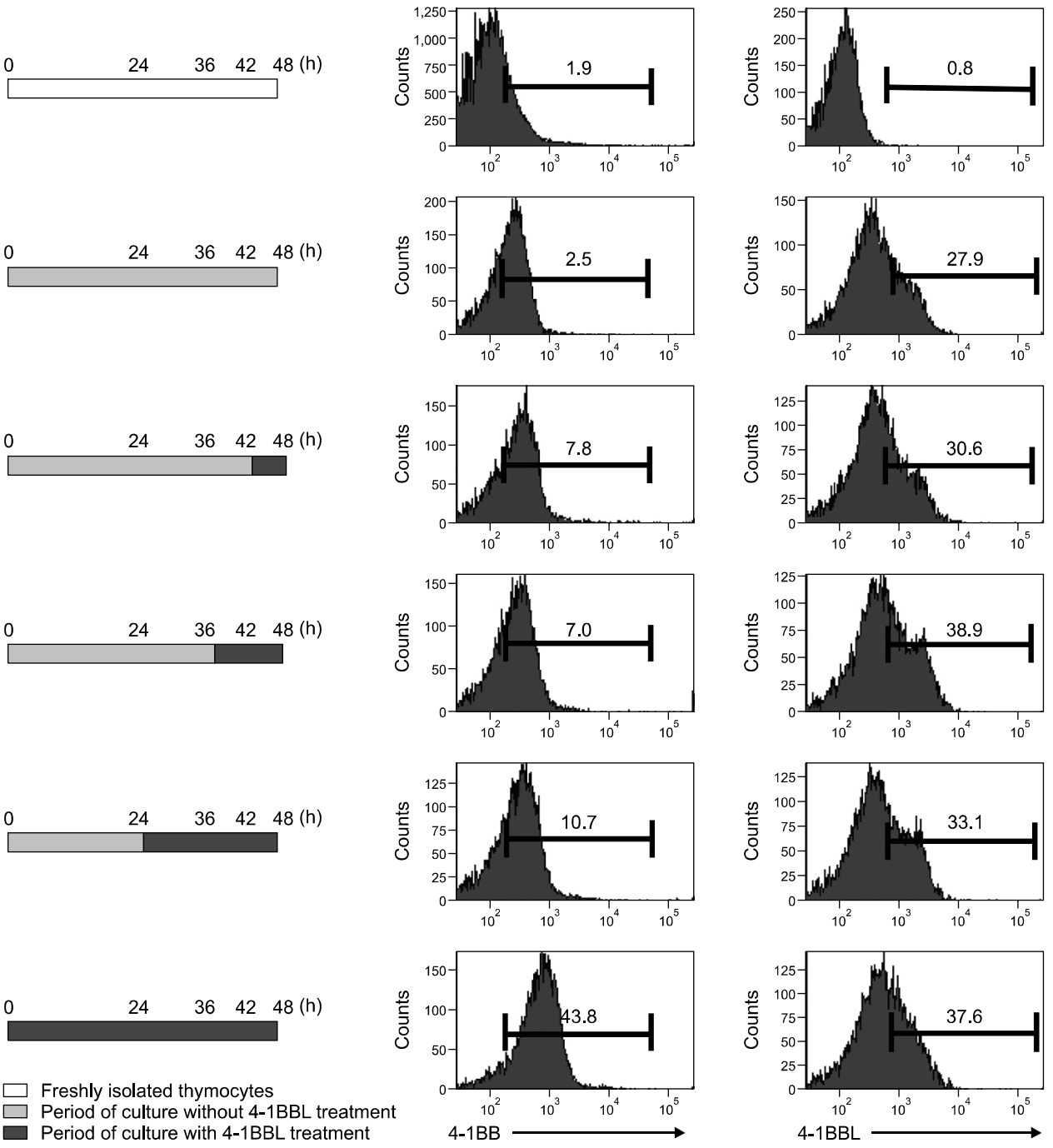


**Figure 8.** Flow cytometric analysis of the expression of 4-1BB and 4-1BBL in the mouse thymocytes. Freshly isolated thymocytes were exposed to 4-1BBL (100 ng/ml), and incubated for 6, 12, 24 and 48 h. All the cells, except the freshly isolated thymocytes, were cultured in RPMI 1640 medium for the same length of time (48 h) regardless of the duration of treatment with 4-1BBL, and stained with biotin-anti-4-1BB or biotin-anti-4-1BBL followed by PE-streptavidin. The histograms show the fluorescence intensities of 4-1BB and 4-1BBL. Data are representative of three independent experiments with similar results.

the expression of 4-1BB and 4-1BBL during thymic regeneration is associated with thymocyte maturation. TCR complexes are not only vital for the functioning of mature T lymphocytes, but are also responsible for directing the maturation of immature T cell precursors in the thymus. It has been demonstrated that different TCR sequences are associated with different maturation pathways

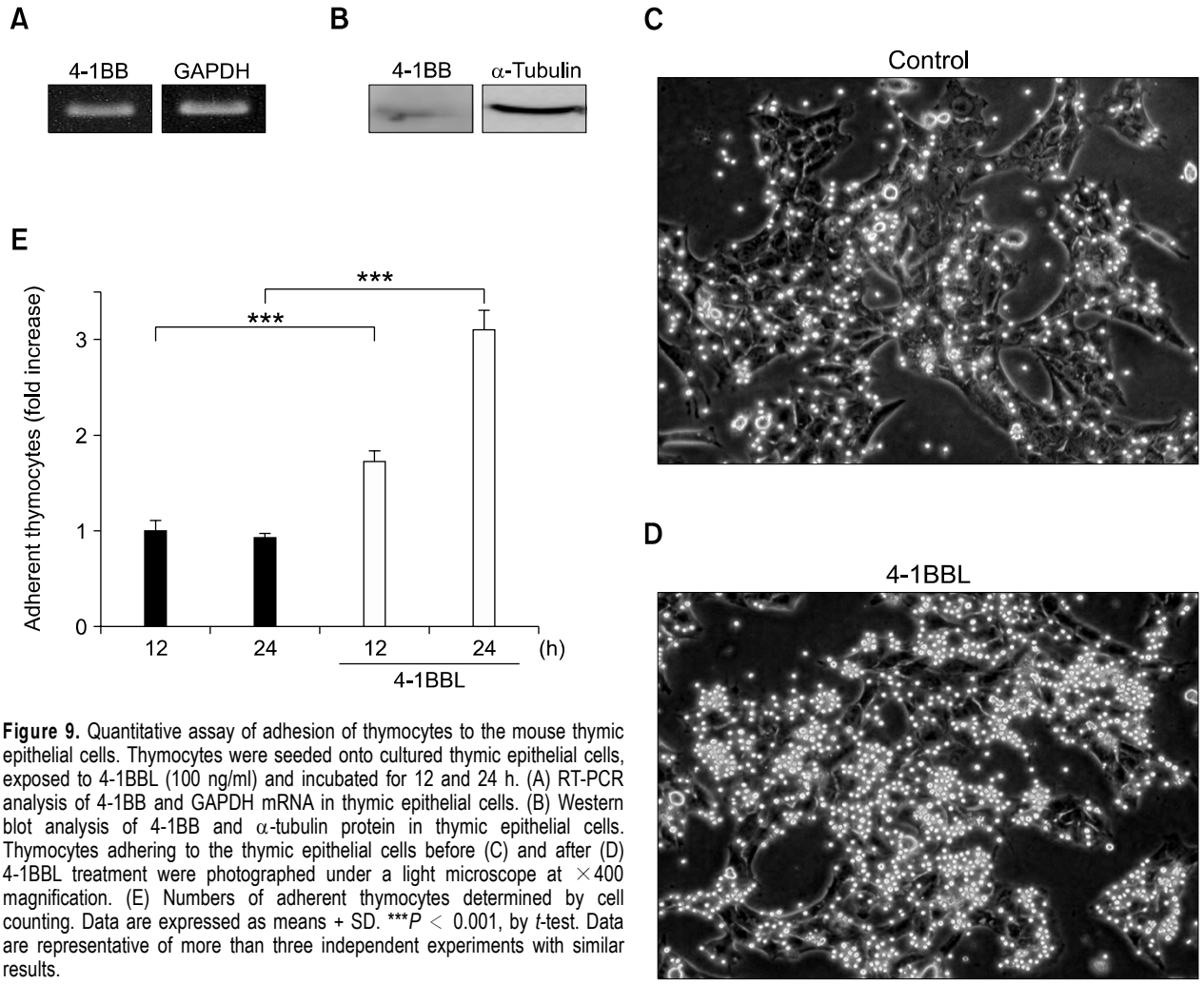


**Figure 9.** Quantitative assay of adhesion of thymocytes to the mouse thymic epithelial cells. Thymocytes were seeded onto cultured thymic epithelial cells, exposed to 4-1BBL (100 ng/ml) and incubated for 12 and 24 h. (A) RT-PCR analysis of 4-1BB and GAPDH mRNA in thymic epithelial cells. (B) Western blot analysis of 4-1BB and α-tubulin protein in thymic epithelial cells. Thymocytes adhering to the thymic epithelial cells before (C) and after (D) 4-1BBL treatment were photographed under a light microscope at ×400 magnification. (E) Numbers of adherent thymocytes determined by cell counting. Data are expressed as means + SD. ***$P < 0.001$, by *t*-test. Data are representative of more than three independent experiments with similar results.

(Correia-Neves *et al.,* 2001), and several lines of evidence point to two major sequences of CD4/CD8 T cell differentiation: from $CD4^+CD8^+TCR^{lo}CD69^-$ to $CD4^{lo}CD8^{lo/+}TCR^{int}CD69^+$ and finally to $CD4^+CD8^{lo}TCR^{int/hi}CD69^+$ (to mature $CD4^+CD8^-TCR^{hi}$) or $CD4^{lo}CD8^{lo/+}TCR^{hi}CD69^+$ (to mature $CD4^-CD8^+TCR^{hi}$) (Lucas and Germain, 1996; Anderson *et al.,* 1999). 4-1BB and 4-1BBL expression on developing T cells during thymus regeneration agreed with these sequences, suggesting that 4-1BB and 4-1BBL may be involved in the differentiation of the thymocytes.

Importantly we found that 4-1BB and 4-1BBL were preferentially expressed in the positively selected thymocytes among the $CD4^+CD8^+$ DP and the intermediate thymocytes, suggesting a potential role of 4-1BB and 4-1BBL in positive selection or in regulating certain activities of post-selected thymocytes during the transition from $CD4^+CD8^+$ DP to intermediate DP thymocytes. Positive selection is a key step in T cell development because it leads to the survival of $CD4^+CD8^+$ DP thymocytes already pre-programmed to undergo differentiation, and also provides essential differentiation-inducing signals (Anderson *et al.,* 1997, 1999). It is well documented that TCR-mediated signals induce many phenotypic changes during the positive selection, including up-regulation of TCR expression on DP thymocytes, termination of the activities of the recombination-activating genes (RAG-1 and RAG-2), expression of CD69, and down-regulation of either CD4 or CD8 (Turka *et al.,* 1991; Borgulya *et al.,* 1992; Brändle *et al.,* 1992, 1994; Bendelac *et al.,* 1992; Swat *et al.,* 1993; Yamashita *et al.,* 1993; Anderson *et al.,* 1997, 1999; Correia-Neves *et al.,* 2001). In agreement with the two-signal hypothesis for T cell activation it has been shown that interactions between the TCR and MHC-peptide complexes are not enough to induce complete

maturation of $CD4^+CD8^+$ DP thymocytes, suggesting that additional accessory signals are required (Groves *et al.,* 1997; Anderson *et al.,* 1999). In particular, the costimulatory molecules that participate in the initial phases of positive selection have not yet been identified, although some molecules expressed by $CD4^+CD8^+$ DP thymocytes, such as CD2, CD5, CD24, CD28, CD49d, CD81 and thymic shared antigen 1 (TSA-1), have been shown to possess costimulatory activity *in vitro* in the absence of thymic epithelium, suggesting that these accessory molecules are involved in positive selection (Cibotti *et al.,* 1997; Anderson *et al.,* 1999).

Furthermore, there is growing evidence for a role of 4-1BB and 4-1BBL in hematopoiesis. 4-1BBL is expressed on hematopoietic stem cells, differentiating common myeloid progenitors and granulocyte-macrophage progenitors; in addition, 4-1BB is inducible on activated myeloid progenitors, and both components are implicated in the development of DCs (Kim *et al.,* 2002; Lee *et al.,* 2008b). It has been also shown that 4-1BB and 4-1BBL are expressed by bone marrow and CD34 cells, respectively¸ and that reverse signaling through 4-1BBL enhances the proliferation of $CD34^+$ cells and their differentiation to myeloid cells, especially macrophages (Jiang *et al.,* 2008a, 2008b). Mice exposed to anti-4-1BB mAb displayed evidence of dysregulated hemopoiesis, and developed lymphopenia, thrombocytopenia, and anemia (Niu *et al.,* 2007). These data point to a novel function of 4-1BB and 4-1BBL in the growth and differentiation of hematopoietic progenitor cells.

It is well known that thymocyte adhesion to thymic epithelial cells - i.e., the interaction between thymocytes and to thymic epithelial cells - constitutes key events in T cell development. In this context, our data that 4-1BBL promoted thymocyte adhesion to thymic epithelial cells suggest that 4-1BBL and 4-1BB play a role in the postnatal T cell development. Previous studies have shown that cell interactions between thymocytes and thymic epithelial cells through cell adhesion molecules such as ICAM-1 and VCAM-1 are indispensable for immature thymocytes to develop into mature T cells in the thymus (Fine and Kruisbeek, 1991; Salomon *et al.,* 1994, 1997; Wada *et al.,* 1996). Interestingly, preliminary results of our ongoing studies show some evidence that 4-1BBL upregulates the expression of ICAM-1 and VCAM-1 on the thymic epithelial cells, suggesting that 4-1BBL/4-1BB signaling in the thymic epithelial cells augments thymocyte-thymic epithelial cell interactions through stimulation of the expression of these adhesion molecules (unpublished results). However, the precise roles of 4-1BB and 4-1BBL in thymus function during thymus regeneration remain to be clarified.

Taken together, our findings suggest that 4-1BBL signaling via the 4-1BB receptor on thymocytes, especially the $CD4^+CD8^+$ DP and intermediate thymocyte subsets, is involved in the processes of positive selection and regeneration in the mouse thymus leading to the production of new functional T cells during thymic regeneration.

## Methods

### Cell lines and cell culture

The generation, maintenance, and functional characterization of the mouse thymic subcapsular cortex or thymic nurse epithelial cells (427.1) have been described by Faas *et al.* (1993). These cells were cultured in DMEM (Gibco BRL, Grand Island, NY) containing 10% FBS (Gibco BRL) and 2 mM glutamine (Sigma, St. Louis, MO).

### Experimental model of acute thymic involution and regeneration

Adult male, specific pathogen-free, C57BL/6 mice were purchased from Dae Han Bio Link (Seoul, Korea). They were housed three to four per cage and maintained under a 12 h light/dark cycle at 24°C in a specific pathogen-free and humidity-controlled facility and were provided with standard sterile food and water *ad libitum*. They were allowed to adjust to their environment for 1 week, and were used at 8-10 weeks of age. Since cyclophosphamide, a DNA alkylating agent commonly used in chemotherapy, is a long-established method of investigating thymic regeneration (Milićević *et al.,* 1984; Yoon *et al.,* 1997, 2003; Lee *et al.,* 2005, 2007, 2008a), the animals were given a single intraperitoneal dose of cyclophosphamide (400 mg/kg body weight, Sigma) in normal saline, and were killed in groups of four or more at 1, 2, 3, 4, 5, 6, 7 and 10 days after injection. Mice given the same amount of normal saline were used as controls. Animal care and all experimental procedures were conducted in accordance with the "Guide for Animal Experiments" produced by the Korean Academy of Medical Sciences.

### Antibodies and reagents

The following fluorochrome-conjugated mAbs were purchased from BD Biosciences (San Jose, CA): Pacific Blue-conjugated anti-CD4 (RM4-5), allophycocyanin (APC)-Cy7-conjugated anti-CD8 (53-6.7), fluorescein isothiocyanate (FITC)-conjugated anti-TCRβ (H57-597), and FITC-anti-CD69 (H1.2F3). Biotinylated anti-4-1BB and 4-1BBL antibodies were purchased from BioLegend (San Diego, CA) and goat polyclonal anti-4-1BB and anti-4-1BBL antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PE-streptavidin was from BD Biosciences and the 2.4G2 hybridoma (anti- FcγRII/III) was from the American Type Culture Collection (ATCC;

Manassas, VA). Recombinant murine 4-1BBL was obtained from Peprotech (Rocky Hill, NJ).

## Thymocyte isolation and flow cytometry

Immunophenotypic analysis of cells was performed by two-, three- or four-color analysis on a FACSCanto II (BD Biosciences) with FACSDiva software (BD Biosciences). Single cell suspensions were prepared from the thymus by pressing the thymus in a 70-μm nylon mesh cell strainer (BD Falcon, BD Biosciences), and depleted of red blood cells in ACK lysis solution. The number of cells isolated from the thymus was determined by counting live cells detected by trypan blue exclusion.

For flow cytometry, cells were first incubated with 2.4G2 culture supernatant to block nonspecific antibody binding and then stained with a combination of the indicated fluorochrome-conjugated or biotinylated mAbs. When biotinylated-mAbs were used, the cells were subsequently incubated with PE-streptavidin. Background fluorescence was determined on cells stained with fluorochrome-labeled isotype-matched nonreactive mAbs. An electronic gate was set on the thymocytes using forward and side scatter characteristics. In all experiments, cells were gated on forward and side scatter to eliminate dead/dying cells and debris.

## Quantitation of thymocyte adhesion to thymic epithelial cells

The assay of adhesion of thymocytes to thymic epithelial cells was based on that described by Barda-Saad *et al.* (1996). Briefly, the mouse thymic epithelial cells were seeded in 60 mm culture dishes at a density of $3 \times 10^5$ cells per dish in DMEM containing 10% FBS, and incubated for 12 and 24 h after treatment with 4-1BBL (100 ng/ml). Freshly isolated thymocytes were seeded onto layers of the thymic epithelial cells at a density of $8 \times 10^6$ cells per dish, and incubated for 3 h. After removing non-adherent thymocytes by gentle washing, the adherent thymocytes were examined with an Olympus BX50 microscope, and photomicrographs were captured digitally at 1,360 × 1,024 pixel resolution with an Olympus DP70 digital camera. In addition, the adherent thymocytes were collected by gentle pipetting and counted using a hemocytometer after trypan blue staining.

## Western blot analysis

Thymic proteins were isolated using a protein extraction solution (PRO-PREP Protein Extraction Solution, Intron, Seoul, Korea). The lysates were centrifuged at 13,000 rpm for 15 min at 4°C. Protein concentrations were determined with the Bradford protein assay reagent (Bio-Rad, Hercules, CA). Equal amounts of protein samples were heated for 10 min at 95°C in sample buffer and separated by 10% SDS-PAGE, using a Mini-Protean III system (Bio-Rad, Melville, NY). The proteins were transferred onto a PVDF membrane (Bio-Rad) by semi-dry transfer (Bio-Rad), and the membrane was incubated overnight at 4°C with anti-4-1BB (sc-11811, Santa Cruz Biotechnology) and anti-4-1BBL (sc-11819, Santa Cruz Biotechnology) at a dilution of 1:200 in Tris-buffered saline (TBS, 20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 2% skim milk. After three washes with TBS-T (TBS containing 0.1% Tween 20) containing 1% skim milk, the membrane was incubated for 2 h at room temperature with secondary antibody (anti-goat IgG peroxidase conjugate, sc-2768, Santa Cruz Biotechnology) diluted 1:1000, and washed three times with TBS-T. Immunoreactivity was detected by enhanced chemiluminescence (ECL, SuperSignal West Pico Chemiluminescent Substrate kit, Pierce, Rockford, IL) according to the manufacturer's instructions, and images were captured and quantified with a LAS-3000 imaging system (Fujifilm, Tokyo, Japan). Data were expressed as ratios of 4-1BB and 4-1BBL normalized to α-tubulin to correct for any error in spectrophotometric protein quantification or in pipetting.

## RT-PCR analysis

Total RNA was isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA); briefly, samples were transferred to tubes containing 1 ml of the RNA extraction solution. The homogenates were chloroform extracted, isopropanol precipitated, ethanol washed, and resuspended in 30 μl of distilled water. RNA concentrations and purity were determined by absorbance at 260 and 280 nm. Samples exhibiting an absorbance ratio (260/280) greater than or equal to 1.7 were used. First strand cDNA was obtained by reverse transcription (RT) using 2 μg of mouse thymocyte RNA. The reaction was conducted in 25 μl of buffer containing 0.5 μg of oligo (dT)12-18 primer (Promega), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 40 mM DTT, 0.5 mM deoxynucleotide triphosphate (dNTP) mixture (Promega), 10 units RNase inhibitor (Promega), and 200 units of MMLV reverse transcriptase (Promega). After incubation at 42°C for 60 min, the reaction was stopped by heating at 70°C for 15 min and the cDNA was used as a template for PCR amplification using gene-specific primers for mouse 4-1BB and 4-1BBL. Specific primers were designed for each gene (Bioneer, Cheongwon-Kun, Chungbuk, Korea), and the primers for mouse 4-1BB consisted of forward primer (5'-GGGAAACAACTGTTACCACG-3'), corresponding to nucleotides 115-134, and reverse primer (5'-AGACCTTCCGTCTAGAGAGC-3') complementary to nucleotides 547-566 in the mouse 4-1BB gene sequence [GenBank:NM_011612]. The primers for mouse 4-1BBL consisted of forward primer (5'-CTCTCCTGTGTTCGCCAAGC-3'), corresponding to nucleotides 399-418, and reverse primer (5'-CCAGCCTTCAGGAGCAACAG-3') complementary to nucleotides 825-844 in the mouse 4-1BBL gene sequence [GenBank:NM_009404]. These were used to amplify 452 bp and 366 bp fragments, respectively, and to detect the mouse 4-1BB and 4-1BBL transcripts. The cDNA was amplified with an automated thermal cycler (TECHNE, Teddington, UK) in a final volume of 25 μl containing 2 μl of cDNA solution, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 mM dNTP mixture (Promega), 0.4 pmol of each primer and 5 units of Taq DNA polymerase (Promega). The amplification procedure consisted of an initial denaturation at 94°C for 5 min followed by 28 cycles of denaturation at 94°C for 30 s, primer annealing at 57°C for 30 s, and

extension at 72°C for 30 s, with a final extension at 72°C for 10 min, and ending with a 4°C hold cycle. The amplified products were analyzed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and visualized under UV light. Band intensities of the PCR products were measured using an image analysis program (MetaMorph, Universal Imaging Corporation, Downingtown, PA). Data were expressed as ratios of 4-1BB and 4-1BBL mRNA normalized to GAPDH mRNA amplified from the same cDNA samples.

### Statistical analysis

Data are expressed as means + SD. For comparisons of multiple groups, we used one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. In addition, to compare pairs of groups we used Student's two-tailed *t*-test. Statistical significance was set at $P < 0.05$.

### Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2006-311-E00150) and by the MRC program of MOST/KOSEF (R13-2005-009). Seong-A Ju was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2007-412-J00302).

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