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RANKL stimulates proliferation, adhesion and IL-7 expression of thymic epithelial cells

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Abbreviations: CY, cyclophosphamide; ICAM-1, intercellular adhesion molecule-1; OPG, osteoprotegerin; RANKL, receptor activator of NF-κB ligand; VCAM-1, vascular cell adhesion molecule-1

Abstract

In many clinical situations which cause thymic involution and thereby result in immune deficiency, T cells are the most often affected, leading to a prolonged deficiency of T cells. Since only the thymic-dependent T cell production pathway secures stable regeneration of fully mature T cells, seeking strategies to enhance thymic regeneration should be a key step in developing therapeutic methods for the treatment of these significant clinical problems. This study clearly shows that receptor activator of NF-κB ligand (RANKL) stimulates mouse thymic epithelial cell activities including cell proliferation, thymocyte adhesion to thymic epithelial cells, and the expression of cell death regulatory genes favoring cell survival, cell adhesion molecules such as ICAM-1 and VCAM-1, and thymopoietic factors including IL-7. Importantly, RANKL exhibited a significant capability to facilitate thymic regeneration in mice. In addition, this study demonstrates that RANKL acts directly on the thymus to activate thymus regeneration regardless of its potential influences on thymic regeneration through an indirect or systemic effect. In light of this, the present study provides a greater insight into the development of novel therapeutic strategies for effective thymus repopulation using RANKL in the design of therapies for many clinical conditions in which immune reconstitution is required.

Keywords: cell adhesion molecules; RANK ligand; regeneration; thymopoietins; thymus gland

Introduction

The thymus, the central lymphoid organ, is a complex epithelial organ in which thymocyte development is dependent upon the sequential contribution of morphologically and phenotypically distinct stromal cell compartments that comprise the thymic microenvironment. The unique combination of cellular interactions, cytokines, and chemokines within this microenvironment induces thymocyte precursors to undergo a differentiation program that leads to the generation of functional T cells. Thymic epithelial cells, which constitute a major component of the stromal cells of the thymus, provide a crucial signal for intrathymic T cell development and selection.

It has been well documented that acute thymic involution induced in experimental animals is followed by intensive regeneration after removal of the causative stimuli (Milicevic *et al.,* 1984; Yoon *et al.,* 1997, 2003; Lee *et al.,* 2005, 2007). Cyclophosphamide (CY) exhibits a selective suppressive action on various populations and subpopula-

tions of lymphoid cells (Turk and Poulter, 1972; Ozer *et al.,* 1982; Zhu *et al.,* 1987). Thus, the application of CY represents a long-established method to induce thymic involution and investigate thymic regeneration (Milicevic *et al.,* 1984; Yoon *et al.,* 1997, 2003; Milicevic and Milicevic, 2004; Lee *et al.,* 2005, 2007). Moreover, CY as well as a number of other agents that induce the acute thymic involution, can be successfully used not only to study the process of regeneration, but also to shed light on the normal thymocytopoiesis, because the changes that occur during the former always mirror the latter process and enable its better understanding (Milicevic and Milicevic, 2004). Of importance, it has been suggested that thymic epithelial cells may play a critical role in thymic regeneration (Milicevic *et al.,* 1984; Yoon *et al.,* 1997, 2003; Milicevic and Milicevic, 2004; Lee *et al.,* 2005, 2007). However, the molecular mechanisms underlying thymus regeneration are still largely unknown. Thus, it is of considerable clinical importance to understand the mechanism governing thymus regeneration and develop methods of normalizing or improving host immunity when immune function is depressed due to thymic involution. Therefore, to achieve this goal, an important approach is to identify thymotrophic factors that can promote thymus regeneration.

The receptor activator of NF-κB ligand (RANKL) is a type II membrane protein of 316 amino acids with a predicted molecular mass of 35 kDa, and a member of the TNF family (Yasuda *et al.,* 1998). In addition to the pivotal role of RANKL in bone metabolism (Simonet *et al.,* 1997), RANKL has also been shown to participate in various immune system functions as in the case of other members of the TNF ligand and TNF receptor superfamilies (Choi *et al.,* 2005; Park *et al.,* 2005). RANKL was identified as a crucial factor in lymphocyte development and lymph node organogenesis (Anderson *et al.,* 1997; Kong *et al.,* 1999a, b). Furthermore, the expression of RANKL was detected on early thymocyte precursors (Anderson *et al.,* 1997), and RANKL-deficient mice showed defects in the early differentiation of T lymphocytes, suggesting that RANKL is a novel regulator of early thymocyte development at the stage of pre-TCR expression (Kong *et al.,* 1999a, b). Interestingly, in a previous study, we found that RANKL expression was strongly upregulated in thymic epithelial cells during thymic regeneration implying that RANKL could play a role in the development of T cells during thymic regeneration (Lee *et al.,* 2005). Importantly, it was also recently revealed that RANKL-RANK signals from $CD4^+CD3^-$ inducer cells regulate development of Aire-expressing thymic epithelial cells in the thymic medulla, and that RANK deficiency in the thymic epithelial cells promote onset of autoimmunity, indicating RANK as a key regulator of central tolerance (Rossi *et al.,* 2007).

Thus, the aim of the present study was to elucidate the effect of RANKL on the thymic epithelial cells and to shed light on its role in thymus regeneration.

Materials and 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; SNEC), deep cortex or cortical reticular epithelial cells (1308.1; CREC), and medullary epithelial cells (6.1.7; MEC) have been described by Faas *et al*. (1993). These cell lines were cultured in DMEM (Gibco BRL, Grand Island, NY) containing 10% FBS (Gibco BRL) and 2 mM glutamine (Sigma, St. Louis, MO).

Animals, experimental thymic regeneration model, and *in vivo* treatment of RANKL

Male C57BL/6 mice were purchased from Dae Han Bio Link (Seoul, Korea) and maintained under a 12 h light/dark cycle at 24°C in a specific pathogen-free and humidity-controlled facility. The mice were used at 8-10 weeks of age and they were given a single intraperitoneal dose of CY (450 mg/kg, Sigma) as described in previous studies (Yoon *et al.,* 1997, 2003; Lee *et al.,* 2005, 2007). The animals were given RANKL intraperitoneally at a dose of 10 μg per mice. Treatment of RANKL began 8 h after CY treatment and continued daily for 3 days. The mice were sacrificed at 24 h after the last injection of RANKL.

Preparation of recombinant RANKL and OPG proteins

Recombinant RANKL and OPG proteins were prepared using the pET-32b Expression System (Novagen, Madison, WI). RT-PCR was performed using total RNA isolated from mouse thymus to amplify each fragment encoding the extracellular domain of RANKL and the full-length open reading frame (ORF) of OPG with the following primers: RANKL, sense, 5'-AAGCTTATGTTCCATGTTTCTTTTAGAT-3', and antisense, 5'-CTCGAGTTTATACTGCCCTTCAAAATT-3'; OPG, sense, 5'-CTCGAGTTATAAGCAGCTTATTTTCACG-3', and antisense, 5'-CTCGAGTTATAAGCAGCTTATTTTCACG-

3'. The amplified PCR products were cloned into the pGEM T easy vector (Promega, Madison, WI). The DNA fragments of RANKL and OPG digested with *Hin*dIII and *Xho*I were inserted into the pET-32b expression vector and transformed into *E. coli* strain BL21 (DE3). Overexpression of RANKL and OPG proteins were induced by the addition of 1 mM IPTG and purified by affinity chromatography with Ni-NTA agarose resin (Qiagen, Valencia, CA).

Cell proliferation assay

The thymic epithelial cell lines were plated into 96-well microplates at 2×10^3 cells/well in DMEM containing 10% FBS, then transferred to medium containing 1% FBS. These cell lines were incubated for 1, 2, 3, 4 and 6 days after treatment with thioredoxin (0.5 μg/ml), RANKL (0.5 μg/ml), and a mixture of RANKL (0.5 μg/ml) and OPG (2 μg/ml), respectively. A soluble OPG protein was used as a decoy receptor inhibiting the effect of RANKL on the proliferation of the thymic epithelial cells. Cell proliferation was assayed using the Cell Proliferation Reagent WST-1 (Roche, Mannheim, Germany).

Thymocyte isolation

After small cuts were made into the thymic capsules, and thymi were gently agitated in RPMI-1640 (Gibco BRL) using a magnetic stirrer at 4°C for 40 min. The supernatant was passed three times through a 70-μm mesh and centrifuged. After the cell pellet was resuspended in ACK lysis solution, the thymocyte suspension was washed with Hanks' balanced salt solution (Gibco BRL) buffer, and viable cells were counted using a hemocytometer after trypan blue staining.

Quantitation of thymocyte adhesion to thymic epithelial cells

An assay of adherence of thymocytes to thymic epithelial cells was based on that described by Barda-Saad *et al.* (1996). Briefly, freshly isolated thymocytes were seeded onto a layer of thymic epithelial cell lines in 6-well microplates at 1.5×10^7 cells/well in DMEM containing 10% FBS, and then incubated for 4 h after treatment with RANKL (0.5 μg/ml), and a mixture of RANKL (0.5 μg/ml) and OPG (2 μg/ml). After removing the non-adherent thymocytes by gentle washing, the adherent thymocytes to the thymic epithelial cell lines were collected by treatment with 0.5 mM EDTA (Sigma) in PBS and were counted using a hemocytometer after trypan blue staining.

Table 1. Primer pairs used in template synthesis for RT-PCR analysis.

Target gene	Gene bank (Accession no.)	Sequences of primer	Product size
VCAM-1	NM011693	S: 5'-CCCAAGGATCCAGAGATTCA-3' A: 5'-ACGTCAGAACAACCGAATCC-3'	658 bp
ICAM-1	NM010493	S: 5'-GAGAGTGGACCCAACTGGAA-3' A: 5'-CTTTGGGATGGTAGCTGGAA-3'	459 bp
Bcl-2	NM009741	S: 5'-AGTCGGGACTTGAAGTGCCA-3' A: 5'-GGTACATCATTGATAATGCA-3'	851 bp
Bax	NM007527	S: 5'-GGTTTCATCCAGGATCGAGCAGG-3' A: 5'-ACAAAGATGGTCACGGTCTGCC-3'	446 bp
Bcl-xL	X83574	S: 5'-TTGGACAATGGACTGGTTGA-3' A: 5'-CTGCTCAAAGCTCTGATACG-3'	414 bp
IL-7	NM008371	S: 5'-GCCCTGTCACATCATCTGAGTGCC-3' A: 5'-CAGGAGGCATCCAGGAACTTCTG-3'	496 bp
GM-CSF	X03019	S: 5'-GTCACCCGGCCTTGGAAGCAT-3' A: 5'-ACAGTCCGTTTCCGGAGTTGG-3'	368 bp
RANK	AF019046	S: 5'-AAACCTTGGACCAACTGCAC-3' A: 5'-ACCATCTTCTCCTCCCGAGT-3'	377 bp
GAPDH	NM001001303	S: 5'-CAACTCCCTCAAGATTGTCAGC-3' A: 5'-GGGAGTTGCTGTTGAAGTCACA3'	449 bp

All primers were designed to have an optimum annealing temperature at 57°C.
Abbreviations: S, sense; A, antisense

RT-PCR analysis

Total RNA was isolated from each sample using Easy-Blue RNA Extraction Reagent (Intron, Seoul, Korea). First strand cDNA was obtained by a reverse transcription system (Gibco BRL) and the cDNA was used as a template for PCR amplification using gene-specific primers (Table 1). PCR amplification was performed in 25 or 30 cycles of 94°C for 60 s, 57°C for 60 s and 72°C for 60 s, and the amplified products were analyzed by electrophoresis. Band intensities of the PCR products were measured using an image analysis program (MetaMorph, Universal Imaging Corp., Downingtown, PA).

Immunocytochemistry

For immunofluorescence, the mouse thymic epithelial cell lines were cultured on glass coverslips (Paul Marienfeld, Lauda-Königshofen, Germany) with a diameter of 12 mm coated with 50 μg/ml of poly-L-lysine (Sigma) and were fixed with cold 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min. Subsequently, the fixative was removed by washing three times for 5 min with cold PBS and then the cells were incubated with 0.5% Triton X-100 in PBS for 5 min. The cells were washed again with cold PBS and they were incubated with 2% BSA (Sigma) for 60 min at room temperature. The excess solution was shaken off and the cells were incubated for 16-18 h at 4°C with a goat polyclonal anti-intercellular adhesion molecule-1 (ICAM-1) antibody, diluted 1 : 100 (sc-1511, Santa Cruz Biotechnology, Santa Cruz, CA) or a goat polyclonal anti-vascular cell adhesion molecule-1 (VCAM-1) antibody, diluted 1 : 100 (sc-1504, Santa Cruz Biotechnology). Following incubation with the primary antibody, the cells were washed three times for 5 min with cold PBS and incubated with $F(ab')_2$ fragments of an affinity-purified donkey anti-goat FITC-conjugated antibody, diluted 1:100 (Jackson ImmunoResearch Laboratories, West Grove, PA). They were then rinsed in cold PBS and were mounted onto glass slides using Vectashield (Vector Laboratories, Burlingame, CA). Controls for the staining procedure included the following: (1) Omission of the primary antibody from the reaction sequence and its replacement with non-immune donkey serum, (2) Omission of the secondary antibody from the reaction sequence. The labeled cells were examined with an Olympus BX50 microscope equipped with fluorescent epi-illumination. Photomicrographs were captured digitally at 1,360 × 1,024 pixel resolution with an Olympus DP70 digital camera.

Adult thymic organ culture (ATOC)

Thymi were obtained from the mice 3 days after CY treatment. Thymi were cut into small pieces and moistened with a solution of HEPES-buffered RPMI 1640 plus 10% FBS. The thymic fragments were cultured on a 0.45 μm filter (Millipore, Bedford, MA) placed on the top of Gelfoam sponges (Upjohn, Kalamazoo, MI). Each Gelfoam sponge was hydrated overnight in a six well plate containing HEPES-buffered DMEM with sodium pyruvate supplemented with 2 mM glutamine, 1× nonessential amino acids, 5×10^{-5} M 2β-mercaptoethanol, and 20% heat-inactivated FBS. The thymic fragments were cultured in 6-well plates in a humidified incubator with an atmosphere of 5% CO_2 in air at 37°C. Cultures were fed on day 7 with supplemented HEPES-buffered DMEM and maintained for a maximum of 14 days. After the cultured thymus fragments were cut on a Reichert cryostat (5 μm thick), the slides were stained with H&E.

Statistical analysis

Data are expressed as the mean ± SEM for each condition. The results were statistically analyzed by the two-tailed Student's *t*-test (*t*-test). Statistical significance was achieved when $P < 0.05$.

Results

RANKL stimulates mouse thymic epithelial cell proliferation

Prior to beginning studies to investigate the effects of RANKL on the activity of thymic epithelial cells, the presence of RANKL and RANK transcripts was confirmed by RT-PCR analysis in the three types of mouse thymic epithelial cell lines used in the present study (Figure 1). RANKL significantly stimulated the proliferation of SNEC, CREC and MEC (Figure 2). This promoting effect of RANKL on the proliferation of these thymic epithelial cells was totally abolished by simultaneous incubation of RANKL with OPG (Figure 2). RANKL exerted its effects on the thymic epithelial cell proliferation in a dose- and time-dependent manner (data not shown). The thioredoxin control exhibited no effect on the proliferation of the thymic epithelial cells as in the untreated control (Figure 2).

RANKL upregulates Bcl-2 and Bcl-xL expression in mouse thymic epithelial cells but downregulates Bax expression

To investigate the effect of RANKL on the ex-

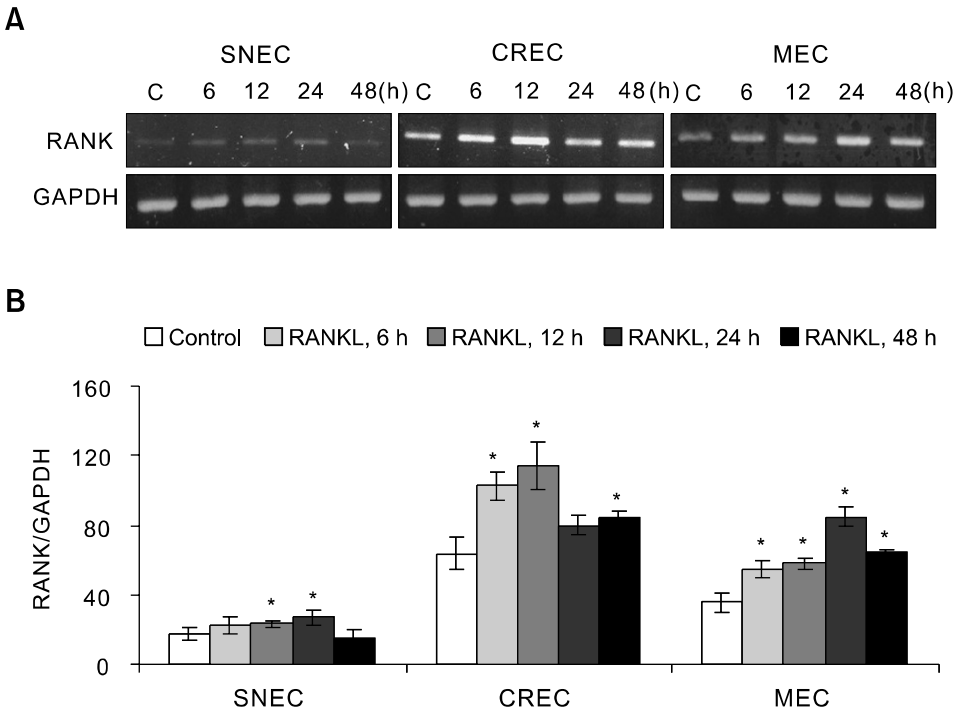

Figure 1. Analysis of RANK mRNA expression in different types of mouse thymic epithelial cell lines. The three types of mouse thymic epithelial cells (SNEC, CREC and MEC) were incubated for 6, 12, 24 and 48 h after treatment with RANKL (0.5 μg/ml). (A) RT-PCR analysis for gene expression of RANK and GAPDH was performed. (B) The density of each band was measured by a scanning densitometry and then expressed as the mean ± SD. Data are expressed as ratios of RANK mRNA normalized to GAPDH mRNA. $*P < 0.05$ compared with the corresponding control value as determined by *t*-test.

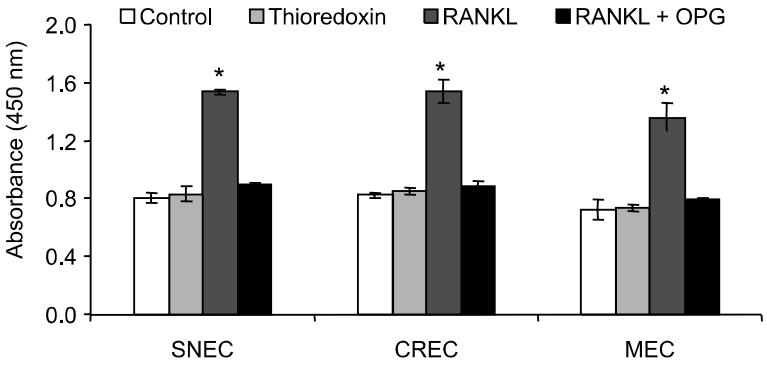

Figure 2. Effect of RANKL on proliferation of different types of mouse thymic epithelial cell lines. The three types of mouse epithelial cells (SNEC, CREC and MEC) were incubated for 4 days after treatment with thioredoxin (0.5 μg/ml), RANKL (0.5 μg/ml), and a mixture of RANKL (0.5 μg/ml) and OPG (2 μg/ml), respectively. The rate of cell proliferation was assessed by WST-1 assay. Data are expressed as the mean ± SD. $*P < 0.01$ compared with the corresponding control value as determined by *t*-test.

pression of Bcl-2, Bcl-xL and Bax mRNA in the three types of the mouse thymic epithelial cell lines, these cells were incubated for 1 or 2 day(s) following treatment with RANKL after induction of apoptosis by H_2O_2 treatment. As a result of RT-PCR analysis, the expression of Bcl-2 and Bcl-xL mRNA after treatment of the thymic epithelial cells with RANKL was shown to be significantly upregulated in all three cell lines on both day 1 and 2 whereas that of Bax mRNA was significantly decreased under the same conditions (Figure 3).

RANKL enhances thymocyte adhesion to mouse thymic epithelial cells and increases the gene expression of ICAM-1 and VCAM-1 in mouse thymic epithelial cells

All three types of cell lines treated with RANKL exhibited a significant increase in the number of adherent thymocytes to the thymic epithelial cells (Figure 4). Simultaneous incubation of RANKL with OPG completely inhibited the effect of RANKL on the adhesion of thymocytes to these thymic epithelial cell lines (Figure 4). These results demonstrate that RANKL promotes thymocyte adhesion to the thymic epithelial cells. The effect of RANKL on the expression of ICAM-1 and VCAM-1 mRNA

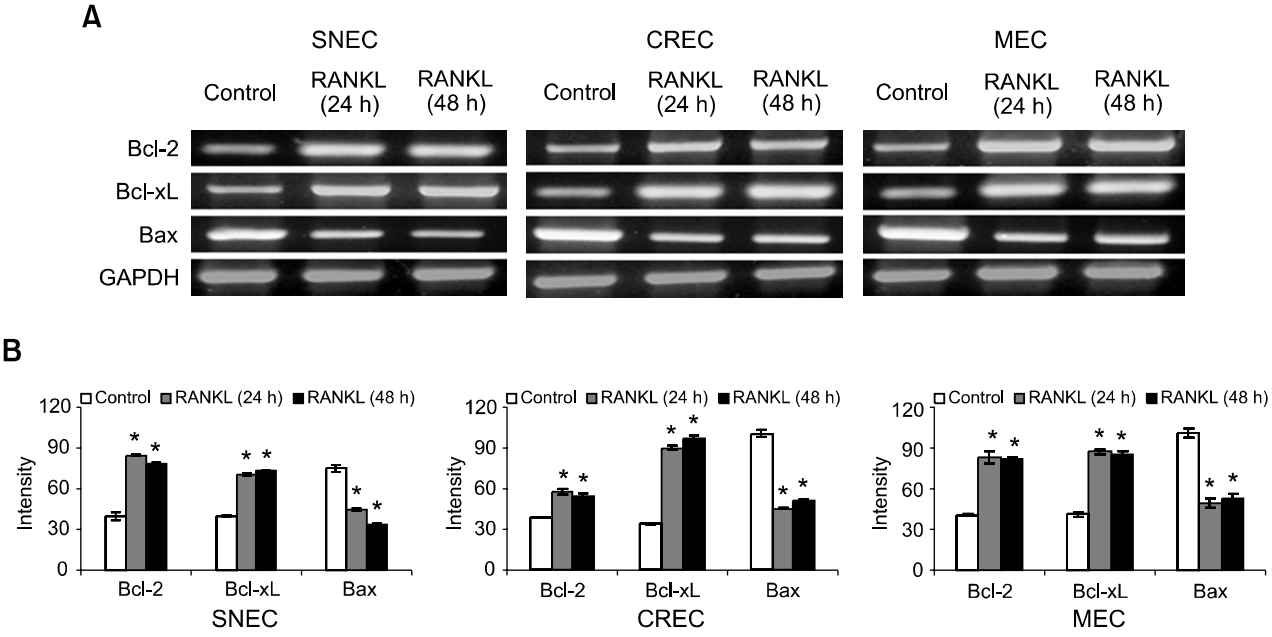

Figure 3. Analysis of Bcl-2, Bcl-xL and Bax expression in different types of mouse thymic epithelial cell lines. The three types of thymic epithelial cells (SNEC, CREC and MEC) were incubated for 1 or 2 day(s) after treatment with RANKL (0.5 μg/ml) and H_2O_2 (100 μM). Apoptosis was induced by H_2O_2 treatments. The control group was treated 100 μM H_2O_2. (A) RT-PCR analysis for gene expression of Bcl-2, Bcl-xL, Bax and GAPDH was performed. (B) The density of each band was measured by a scanning densitometry and then expressed as the mean ± SD. Data are expressed as ratios of Bcl-2, Bcl-xL and Bax mRNA normalized to GAPDH mRNA. *$P < 0.05$ compared with the corresponding control value as determined by *t*-test.

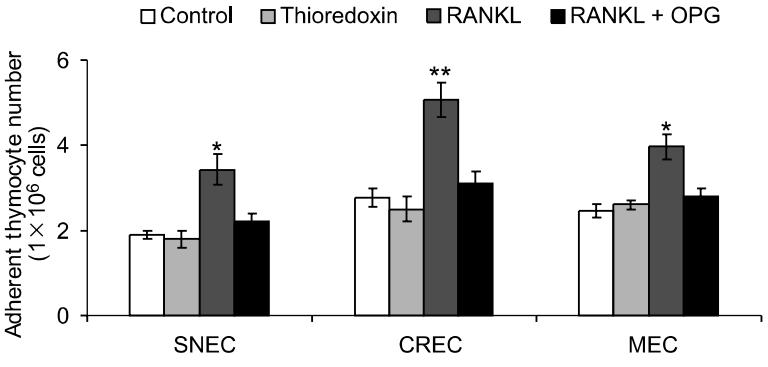

Figure 4. Quantitative adherence assay of thymocytes to different types of mouse thymic epithelial cell lines (SNEC, CREC and MEC). Thymocytes were seeded onto a layer of cultured thymic epithelial cells and then they were incubated for 4 h after treatment with thioredoxin (0.5 μg/ml), RANKL (0.5 μg/ml), and a mixture of RANKL (0.5 μg/ml) and OPG (2 μg/ml), respectively. Data are expressed as the mean ± SD. *$P < 0.05$ and **$P < 0.01$ compared with the corresponding control value as determined by *t*-test.

was also in turn examined in the three types of mouse thymic epithelial cell lines by RT-PCR. The expression of ICAM-1 mRNA was upregulated in the three types of thymic epithelial cells from 6 to 24 h after treatment with RANKL (Figure 5A). The expression of VCAM-1 mRNA was also increased in CREC and MEC from 6 to 24 h after treatment with RANKL but no difference was observed in SNEC under identical conditions (Figure 5A). This upregulatory effect of RANKL on the expression of ICAM-1 and VCAM-1 mRNA in these thymic epithelial cell lines was fully blocked by simultaneous incubation of RANKL with OPG (Figure 5B). This upregulatory effect of RANKL on the expression of ICAM-1 and VCAM-1 mRNA in these thymic epithelial cell lines was also confirmed at the protein level by immunofluorescent staining. The expression of ICAM-1 and VCAM-1 protein was increased in the thymic epithelial cell after treatment with RANKL, supporting the result obtained by RT-PCR analysis (Figure 6). These results suggest that RANKL enhances thymocyte adhesion to mouse thymic epithelial cell by upregulating the expression of ICAM-1 and VCAM-1.

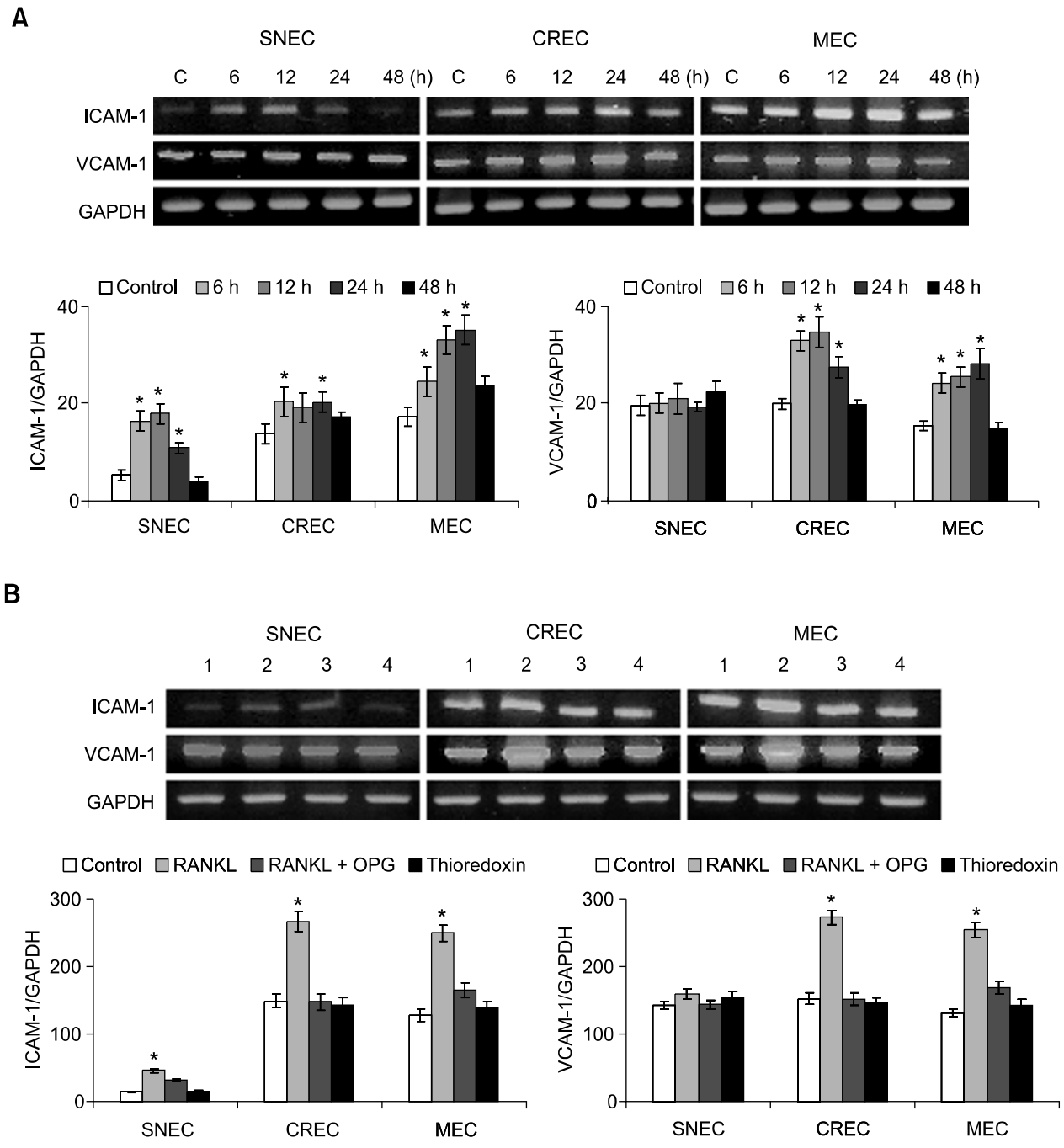

Figure 5. Analysis of ICAM-1 and VCAM-1 mRNA expression in different types of mouse thymic epithelial cell lines. (A) The three types of mouse epithelial cells (SNEC, CREC and MEC) were incubated for 6, 12, 24 and 48 h after treatment with RANKL (0.5 μg/ml). (B) These cells were incubated for 24 h after treatment with thioredoxin (0.5 μg/ml), RANKL (0.5 μg/ml), and a mixture of RANKL (0.5 μg/ml) and OPG (2 μg/ml), respectively. RT-PCR analysis for gene expression of ICAM-1, VCAM-1 and GAPDH was performed. Data are expressed as the mean ± SD. *$P < 0.05$ compared with the corresponding control value as determined by *t*-test. Lanes: 1, untreated control; 2, RANKL-treated group; 3, combined RANKL and OPG-treated group; 4, thioredoxin-treated group.

RANKL upregulates the expression of IL-7 and GM-CSF mRNA in mouse thymic epithelial cells

The effect of RANKL on the expression of IL-7 and GM-CSF mRNA in the three types of mouse epithelial cell lines was assessed by RT-PCR analysis. The expression of IL-7 and GM-CSF mRNA was progressively increased in all three cell types from 6 to 24 h after treatment of RANKL (Figure

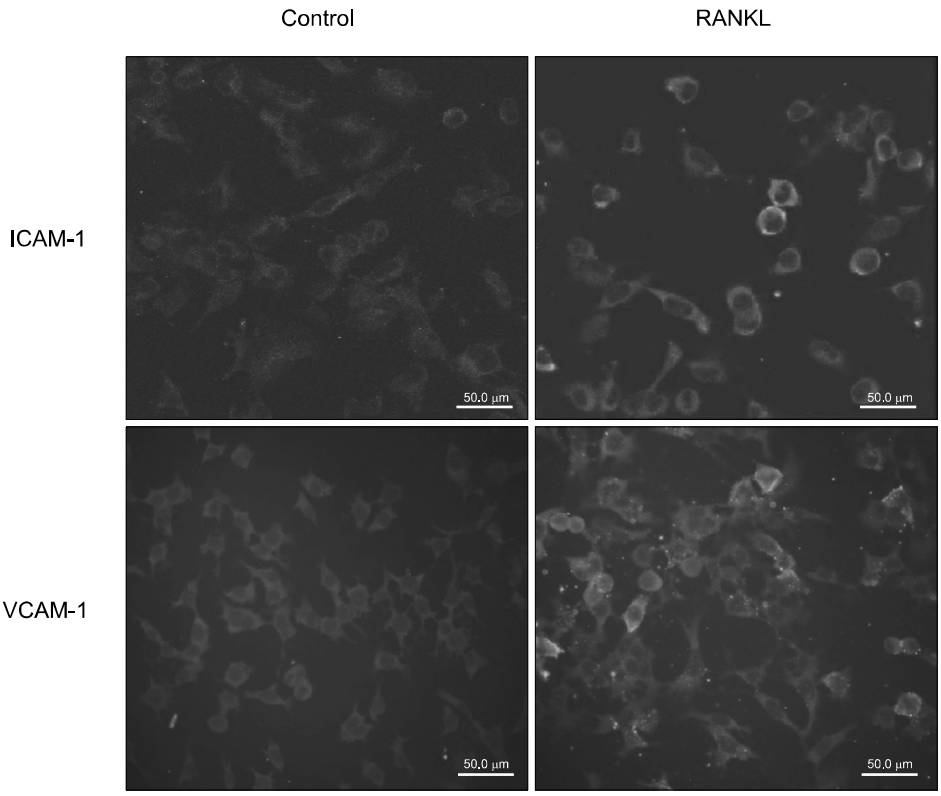

Figure 6. Analysis of ICAM-1 and VCAM-1 protein expression after RANKL treatment in different types of mouse thymic epithelial cell lines. The three types of mouse epithelial cells (SNEC, CREC and MEC) were incubated for 24 h after treatment with RANKL (0.5 μg/ml). Representative figures of immunofluorescent staining are shown for protein expression of ICAM-1 in CREC, and of VCAM-1 in MEC.

7A). This stimulatory effect of RANKL on the expression of IL-7 and GM-CSF mRNA in these thymic epithelial cells lines was completely prevented by simultaneous incubation of RANKL with OPG (Figure 7B).

RANKL activates thymic regeneration both *in vivo* and *ex vivo*

A significant increase in the size and weight of the thymus was found in the RANKL-treated group so that faster regeneration of the thymus following RANKL treatment was obvious macroscopically compared to the saline-treated control (Figure 8A and B). Subsequently, using the ATOC system to study T cell development *ex vivo*, we further attempted to determine whether this *in vivo* stimulative effect of RANKL on thymic regeneration is due to the direct action of RANKL to the thymus or whether it was mediated by the indirect or systemic effect of RANKL. By this ATOC experiment, it was revealed that the thymic cellularity was greatly increased in the group treated with RANKL (Figure 8C), indicating RANKL has the ability to exert direct trophic effects on the thymus to activate thymic regeneration.

Discussion

In line with our previous finding that RANKL is significantly upregulated in the thymic epithelial cells during thymus regeneration (Lee *et al.,* 2005), our data reveal that RANKL is able to enhance thymic regeneration in mice via multiple effects on the thymic epithelial cells. Furthermore, this study demonstrates that RANKL acts directly on the thymus to activate thymus regeneration regardless of its potential influences on thymic regeneration through an indirect or systemic effect. Thymic epithelial cells provide a key microenvironment for the proliferation, maturation, differentiation, and selection of thymocytes, (1) by interacting with the surface molecules of thymic epithelial cells and thymocytes, and (2) by secreting many biologically active factors including many types of cytokines, such as IL-7 and GM-CSF (Le *et al.,* 1988; Aspinall *et al.,* 2004). This study clearly shows that RANKL stimulates the functional activities of mouse thymic epithelial cells. It has been shown that RANKL-RANK signaling leads to NF-κB activation through cyclin D1 in mammary gland epithelial proliferation (Cao *et al.,* 2001). Recently, it was also reported that RANKL directly induces the proliferation of mouse mammary gland epithelial cells through the

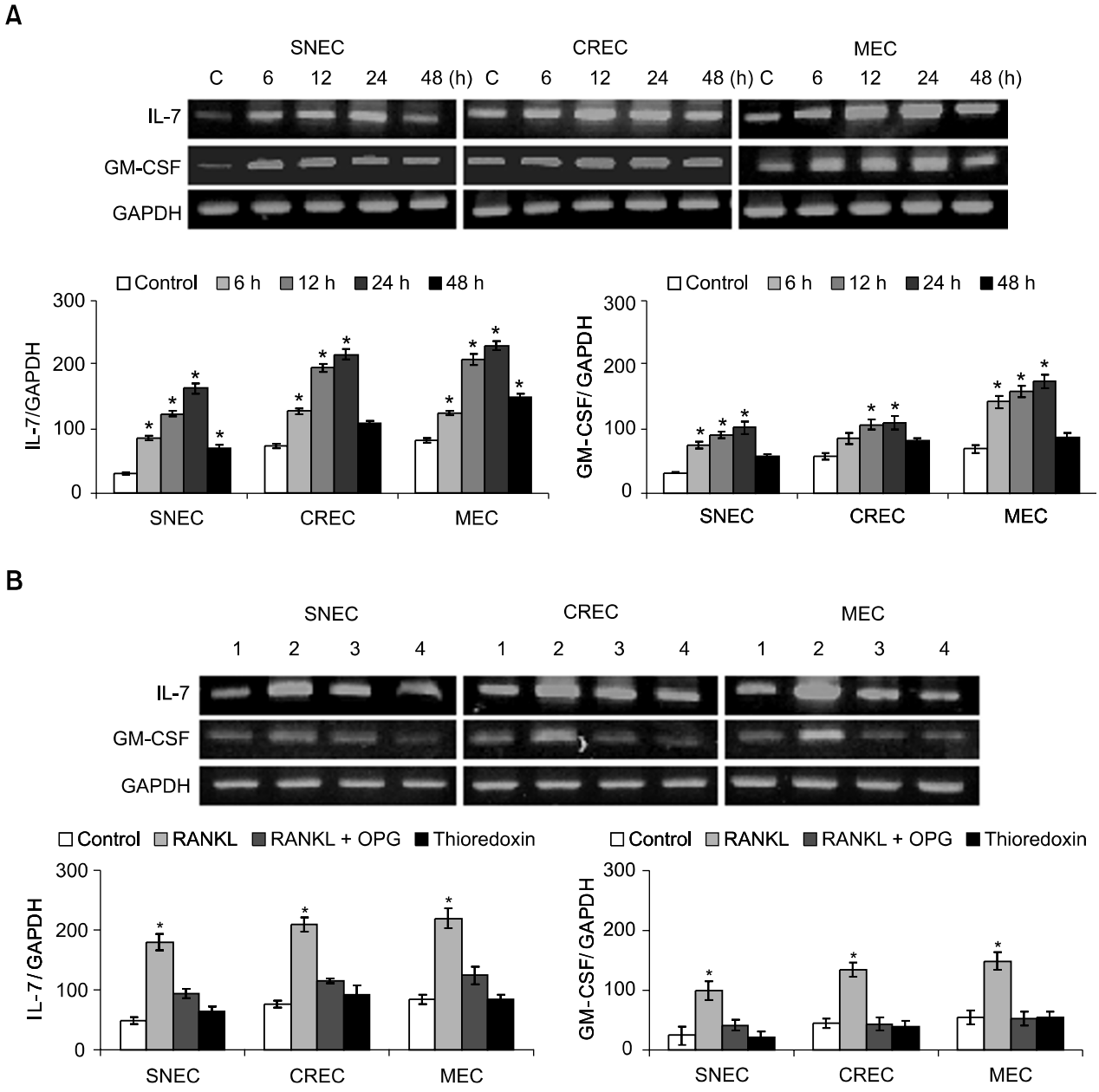

Figure 7. Analysis of IL-7 and GM-CSF mRNA expression in different types of mouse thymic epithelial cell lines. (A) The three types of mouse thymic epithelial cells (SNEC, CREC and MEC) were incubated for 6, 12, 24 and 48 h after treatment with RANKL (0.5 μg/ml). (B) These cells were incubated for 24 h after treatment with thioredoxin (0.5 μg/ml), RANKL (0.5 μg/ml), and a mixture of RANKL (0.5 μg/ml) and OPG (2 μg/ml), respectively. RT-PCR analysis for gene expression of IL-7, GM-CSF and GAPDH was performed. Data are expressed as the mean ± SD. *$P < 0.05$ compared with the corresponding control value as determined by *t*-test. Lanes: 1, untreated control; 2, RANKL-treated group; 3, combined RANKL and OPG-treated group; 4, thioredoxin-treated group.

nuclear translocation of Id2, a positive regulator of cell cycle progression (Kim *et al.,* 2006). In accordance with these data, it was demonstrated in the present study that RANKL enhances the proliferation of all three types of mouse thymic epithelial cells. Thus, these findings indicate that RANKL has a biologically significant capacity to stimulate the proliferation of multiple types of epithelial cells. In addition, RANKL upregulated the transcription of anti-apoptotic genes such as Bcl-2 and Bcl-xL, but at the same time downregulated that of the pro-apoptotic gene Bax in all three types of thymic epithelial cells, suggesting RANKL has survival-promoting effects on all three types of

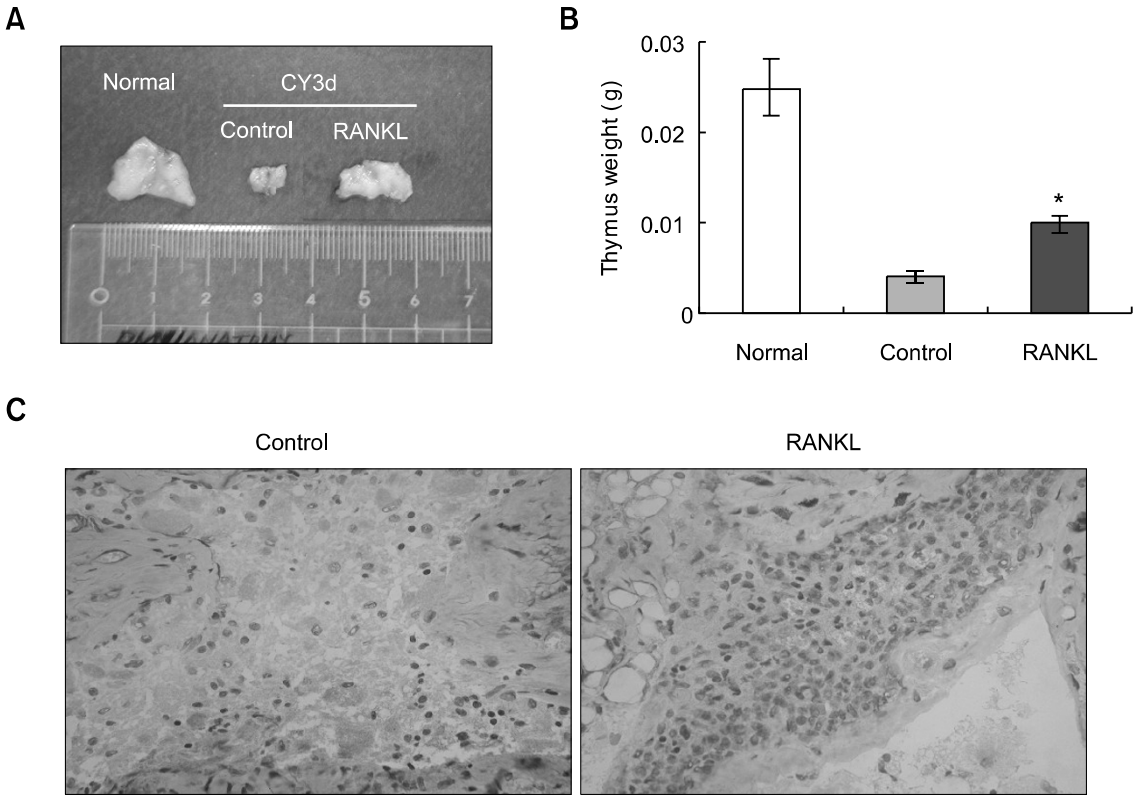

Figure 8. Effect of RANKL on thymic regeneration following acute mouse thymic involution induced by CY treatment in young adult male C57BL/6 mice. Either 10 μg of RANKL or normal saline was given intraperitoneally into the CY-treated mice daily for 3 days and then the size (A) and weight (B) of the thymus were measured from the untreated control mice (Normal) and from the saline-treated control mice (CY3d control) and the RANKL-treated mice (CY3d RANKL) at 1 day after their last injection. (C) Result of the ATOC experiment at the histological level. The untreated control and 0.5 μg/ml RANKL-treated thymus after 10-day ATOC using the thymus at day 3 after CY treatment. Data are expressed as the mean ± SD. *$P < 0.05$ compared with the corresponding saline-treated control value as determined by *t*-test. Hematoxylin-Eosin stain. × 400.

mouse thymic epithelial cells.

The enhanced thymocyte adhesion to thymic epithelial cells and the increased expression of ICAM-1 and VCAM-1 by thymic epithelial cells following RANKL treatment is interesting in view of the fact that the adhesion of immature T cells to the thymus is mainly mediated by the binding of the LFA-1 and VLA-4 integrins to ICAM-1 and VCAM-1 present on the thymic cortical epithelium, respectively, and these interactions constitute important events in T cell development (Fine and Kruisbeek, 1991; Salomon *et al.,* 1994, 1997). Thus, these facts indicate that RANKL plays a role in facilitating T cell differentiation through the up-regulation of ICAM-1 and VCAM-1 expression by the thymic epithelial cells and the enhancement of thymocyte adhesion to thymic epithelial cells, and thereby the increase of thymocyte-thymic epithelial cell interactions.

IL-7 is a cytokine produced predominantly by thymic epithelial cells, which plays a central role in the survival and proliferation of thymocytes (Aspinall *et al.,* 2004). It was found that IL-7 has the potential to stimulate T cell reconstitution and thymic regeneration (Alpdogan *et al.,* 2001; Mackall *et al.,* 2001). GM-CSF is locally produced in the thymus by different cellular elements such as thymic epithelial cells (Le *et al.,* 1988). It was previously reported that human GM-CSF affects the proliferation and differentiation of thymocytes (Yasuda *et al.,* 2002). Thus, the upregulated expression of IL-7 and GM-CSF by RANKL observed in the present study may account at least partly for the role of RANKL in the promotion of thymopoiesis as shown in this study.

Taken together, it is especially noteworthy that RANKL has a significant capability to facilitate thymic regeneration by stimulating mouse thymic epithelial cell activities including cell proliferation, thymocyte adhesion to thymic epithelial cells, and expression of death regulatory genes favoring cell survival, cell adhesion molecules such as ICAM-1 and VCAM-1, and thymopoietic factors including IL-7 and GM-CSF. In light of this, the present study provides a greater insight into the development of novel therapeutic strategies for effective thymus repopulation using

RANKL in the design of therapies for many clinical conditions in which immune reconstitution is required.

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