

# Differential Roles of Interleukin 15 mRNA Isoforms Generated by Alternative Splicing in Immune Responses In Vivo

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## Abstract

At least two types of interleukin (IL)-15 mRNA isoforms are generated by alternative splicing at the 5' upstream of exon 5 in mice. To elucidate the potential roles of IL-15 isoforms in immune responses in vivo, we constructed two groups of transgenic mice using originally described IL-15 cDNA with a normal exon 5 (normal IL-15 transgenic [Tg] mice) and IL-15 cDNA with an alternative exon 5 (alternative IL-15 Tg mice) under the control of an MHC class I promoter. Normal IL-15 Tg mice constitutively produced a significant level of IL-15 protein and had markedly increased numbers of memory type (CD44<sup>high</sup> Ly6C<sup>+</sup>) of CD8<sup>+</sup> T cells in the LN. These mice showed resistance to *Salmonella* infection accompanied by the enhanced interferon (IFN)- $\gamma$  production, but depletion of CD8<sup>+</sup> T cells exaggerated the bacterial growth, suggesting that the IL-15-dependent CD8<sup>+</sup> T cells with a memory phenotype may serve to protect against *Salmonella* infection in normal IL-15 Tg mice. On the other hand, a large amount of intracellular IL-15 protein was detected but hardly secreted extracellularly in alternative IL-15 Tg mice. Although most of the T cells developed normally in the alternative IL-15 Tg mice, they showed impaired IFN- $\gamma$  production upon TCR engagement. The alternative IL-15 transgenic mice were susceptible to *Salmonella* accompanied by impaired production of endogenous IL-15 and IFN- $\gamma$ . Thus, two groups of IL-15 Tg mice may provide information concerning the different roles of IL-15 isoforms in the immune system in vivo.

Key words: transgenic mice • memory T cells • CD8 T cells • Th1/Th2 • *Salmonella*

## Introduction

IL-15 uses  $\beta$ - and  $\gamma$ -chains of the IL-2R for signal transduction, and thus shares many properties of IL-2 in spite of having no sequence homology with IL-2 (1–3). IL-15 is produced by a wide variety of tissues, including placenta, skeletal muscle, kidney, and activated monocytes/macrophages, whereas IL-2 is exclusively produced by activated T cells (4, 5). It has been reported that IL-15 prevents apoptosis of lymphocytes in peripheral tissues induced by growth

factor deprivation via upregulation of antiapoptosis proteins, including Bcl-2 and Bcl-XL (6–8). Mice lacking interferon regulatory factor-1 (IRF-1<sup>-/-</sup> mice) have an impairment in IL-15 mRNA expression in bone marrow cells and exhibit a severe deficiency of NK cells and NK1.1<sup>+</sup> T cells (9, 10). Recently, Lodelce et al. (11) showed that IL-15R $\alpha$ <sup>-/-</sup> mice are deficient in NK cells, NK1.1<sup>+</sup> T cells, memory phenotype of CD8<sup>+</sup> T cells, and TCR- $\gamma/\delta$  intraepithelial lymphocytes. It thus appears that IL-15 has potential roles in the development and maintenance of significant fractions of lymphocytes.

IL-15 has also been shown to stimulate macrophages (12), mast cells (13), NK cells (14), T cells (4) and B cells (15) to proliferate, secrete cytokines, exhibit increased cytotoxicities and/or produce Ab, and it is thus involved in protection

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against infection with *Toxoplasma gondii* (16), *Cryptococcus neoformans* (17), *Candida albicans* (18), *Salmonella choleraesuis* (19), *Escherichia coli* (20), *Listeria monocytogenes* (21), *Mycobacterium tuberculosis*, *Mycobacterium leprae*, Bacillus Calmette-Guerin (22, 23), human immunodeficiency virus (24), human herpes virus-6 (25), -7 (26), or hepatitis C virus (27), and in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (28, 29) and inflammatory bowel disease (30, 31). Furthermore, several reports have suggested that IL-15 has anti-tumor effects (32, 33). Thus, IL-15 may play pleiotropic roles in immune responses in infection, tumor, allergy, and autoimmune diseases.

IL-15 translation and secretion are impeded by multiple control elements, including upstream AUGs of 5' UTR (5, 34), unusual signal peptides (34–37), and the COOH terminus of the mature protein (38). Hence, it has been difficult to demonstrate IL-15 protein in the supernatants of various cells/tissues that express messages for this cytokine. We have previously cloned several isoforms of IL-15 mRNA generated by alternative splicing in mice (34). One of these isoforms, which had an alternative exon 5 containing another 3' splicing site, exhibited a high translational efficiency as compared with other isoforms with the originally described exon 5, which is generated by internal splicing of the alternative exon 5. The translation product of the alternative isoform lacks hydrophobic domains of the signal sequence in the leader peptide, suggesting that the IL-15 protein derived from this isoform is located intracellularly, whereas the product encoded by the IL-15 mRNA with the normal exon 5 may be released extracellularly.

In the present study, to elucidate the potential roles of IL-15 encoded by mRNA isoforms in immune responses in vivo, we constructed two groups of transgenic (Tg)<sup>1</sup> mice using two isoforms of IL-15 cDNA with a normal exon 5 or alternative exon 5 under the control of an MHC class I promoter. We found that normal IL-15 Tg mice constitutively produced a significant level of IL-15 protein, whereas a large amount of intracellular IL-15 protein was detected but little was secreted extracellularly in alternative IL-15 Tg mice. Memory type (CD44<sup>high</sup> Ly6C<sup>+</sup>) of CD8<sup>+</sup> T cells were markedly increased in the lymph node of normal IL-15 Tg mice, whereas the numbers of NK1.1<sup>+</sup> T cells or NK cells were reduced in alternative IL-15 Tg mice. Normal IL-15 Tg mice showed resistance to *Salmonella* infection accompanied by enhanced IFN- $\gamma$  production, while alternative IL-15 transgenic mice were susceptible to the infection accompanied by impaired production of endogenous IL-15 and IFN- $\gamma$ . The implications of these findings for differential roles of IL-15 mRNA isoforms in vivo are discussed.

## Materials and Methods

**Transgene Construction and Generation of Transgenic Mice.** Full-length cDNA encoding the murine IL-15 gene with the originally described exon 5 (710 bp) (39) or alternative exon 5 (830

bp) (34) was cloned between the BamHI and Sall sites of a transgenic expression vector, pHSE-3', which contains the H-2K promoter and Ig enhancer, and  $\beta$ -globin splice site and poly A signal. Transgene DNAs were microinjected into the male pronucleus of fertilized single-cell embryos of C57BL/6 mice. Microinjected eggs were transferred to pseudopregnant C57BL/6 foster mothers. IL-15 Tg mice were identified by digesting genomic DNA with PstI, followed by Southern blot analysis using an IL-15-specific probe. In each experiment, age- and sex-matched littermates were used. C57BL/6 mice were obtained from Japan SLC. Mice were maintained under specific pathogen-free conditions and offered food and water ad libitum. All mice were used at 6–8 wk of age.

**Competitive Reverse Transcription PCR.** mRNA was prepared from the peritoneal macrophages of wild-type or IL-15 Tg mice using a QuickPrep Micro mRNA Purification Kit (Pharmacia LKB Biotechnology, Inc.). First-strand cDNA was synthesized from 2  $\mu$ g mRNA using reverse transcriptase (SuperScript II RT; Life Technologies, Inc.) and 20 pmol of random primer in 20  $\mu$ l reaction mixtures, according to the manufacturer's instructions. The synthesized first-strand cDNA (2  $\mu$ l) was amplified by means of the PCR using 20 pmol of each primer specific for murine  $\beta$ -actin or IL-15 with 2.5 U rTaq (Takara Shuzou) in a total volume of 50  $\mu$ l reaction buffer consisting of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin, and 0.2  $\mu$ M dNTP. The specific primers were as follows:  $\beta$ -actin sense, 5'-TTCTGCATCCTGTCAGCAAT-3'; antisense, 5'-TAAAA-CGCAGCTCAGTAACAGTCCG-3'; IL-15 exons 6 and 7 sense, 5'-GTGATGTTACCCAGTTGC-3'; and IL-15 exon 8 antisense, 5'-TCACATTCTTGCATCCAGA-3'. The cDNA was coamplified with a known amount of specific competitor "mimic" fragment. The cDNA was coamplified in a series of three reactions with a twofold dilution series of mimic concentrations. The products were separated on 1% agarose gels.

To estimate the mRNA expression derived from the transgene in macrophages of alternative IL-15 Tg mice, we amplified the cDNA from the peritoneal macrophages by RT-PCR with specific primers for exons 1 and 8, and the amplified products were hybridized with an internal specific probe for the isoform with an alternative exon 5. The specific primers were as follows: IL-15 exon 1 sense, 5'-GCTGTGTTTGGGAAGGCTGAGTT-3'; and IL-15 exon 8 antisense, 5'-ATGGAGCTGTGCTGCCTCT-3'. The PCR products were resolved by electrophoresis on a 1.8% agarose gel and transferred to a GeneScreen plus filter (NEN Research Products), and then hybridized with a <sup>32</sup>P-labeled oligo probe as follows: IL-15 exon 7, 5'-GCAATGAACTGCTTTCTCCTG-3' (39); and IL-15 additional sequence  $\phi$  probe, 5'-AAGCAACGGAACAATCAAGA-3' (37).

**Immunofluorescence Staining of Intracellular IL-15 Protein.** Peritoneal macrophages of IL-15 Tg or control mice were cultured on coverslips. The cells were fixed in cold acetone. Coverslips were then incubated with a solution of 5% newborn calf serum in HBSS (blocking solution) for 1 h at room temperature to reduce background levels. The cells were incubated with rat anti-murine IL-15 Ab diluted 1:10 in blocking solution for 2 h at room temperature, washed in excess PBS, and then incubated with a 1:50 dilution of FITC-conjugated goat anti-rat Ig (TAGO Inc.) in blocking solution for 1 h at room temperature. Anti-murine IL-15 sera were produced in F344 rats by immunization with an emulsion containing ~0.2 mg *Escherichia coli*-expressed murine IL-15 protein in the form of an insoluble inclusion body preparation in CFA (Difco Laboratories Inc.). A total of three booster injections was given, each at 2-wk intervals after primary injection. 2 wk after the last immunization, we collected blood from the heart.

<sup>1</sup>Abbreviations used in this paper: HKS, heat killed-*Salmonella*; MMC, mytomicin-C; Tg, transgenic.

Fluorescent images were viewed and recorded with an MRC-series confocal imaging system (Bio-Rad Laboratories).

**Antibodies and Reagents.** Phycoerythrin-conjugated anti-TCR  $\gamma\delta$  (UC7-13D5), anti-TCR  $\beta$  (H57-597), anti-CD4 (GK1.5), and anti-CD44 (IM7); FITC-conjugated anti-CD3 (145-2C11), anti-CD8 (53-6.7), anti-CD24 (M1/69), anti-CD44 (IM7), anti-CD45RB (16A), anti-CD62L (MEL-14), anti-Ly6C (AL-21), anti-IL-2R $\alpha$  (7D4), anti-IL-2R $\beta$  (TM- $\beta$ 1), anti-IL-2R $\gamma$  (TUGm3), and anti-IFN- $\gamma$  (XMG1.2); and biotin-conjugated anti-NK1.1 mAb (PK136) were purchased from PharMingen. Cy-Chrome-conjugated streptavidin was obtained from PharMingen. Anti-TCR  $\alpha\beta$  mAb (H57-597) was a gift from Dr. R. Kubo (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). Anti-CD3 mAb (145-2C11) was a gift from Dr. J.A. Bluestone (University of Chicago, Chicago, IL). Purified anti-murine IL-15 mAb (rat IgG1, G277-3588) and biotin-conjugated anti-murine IL-15 mAb (rat IgG1, G277-3960) were purchased from PharMingen.

**Flow Cytometric Analysis.** The cells were stained with PE-, FITC-, and biotin-conjugated mAbs. To block FcR-mediated binding of the mAb, 2.4G2 (anti-Fc $\gamma$ R mAb) was added. All incubation steps were performed at 4°C for 30 min. To detect biotin-conjugated mAb, cells were stained with Cy-Chrome-conjugated streptavidin after incubation with a primary mAb. The stained cells were analyzed by a FACSCalibur® flow cytometer (Becton Dickinson & Co.). Small lymphocytes were gated by forward and side scatter.

**Cell Culture.** Lymphocytes from the LN were cultured in 200  $\mu$ l of complete culture medium in a 96-well flat-bottom plate (Falcon Labware, Becton Dickinson & Co.) at a density of  $5 \times 10^5$  cells/well with anti-TCR $\alpha\beta$  mAb (100  $\mu$ g/ml) that had been immobilized on the plates by earlier incubation for 1 h. To estimate cytokine production, the supernatant was collected at indicated times.

**Cytokine Enzyme-linked Immunosorbent Assay.** The cytokine activity in the culture supernatants or serum from the mice was assayed using a DuoSet ELISA development system (Genzyme Diagnostics) for IL-4 or IFN- $\gamma$ . ELISA for mouse IL-15 was performed in triplicate using purified anti-mouse IL-15 mAb (capture mAb, G277-3588; PharMingen), biotin-conjugated anti-mouse IL-15 mAb (second mAb, G277-3960; PharMingen), and peroxidase-conjugated streptavidin (detection reagent; Genzyme Corp.).

**Analysis of Intracellular Cytokine Synthesis.** C57BL/6 mice or IL-15 transgenic mice were injected intravenously with 4  $\mu$ g of anti-CD3 mAb (145-2C11). After 90 min, the spleen cells were harvested, washed, and suspended at  $10^6$  cells/ml in complete culture medium, and then incubated for 3 h at 37°C in the presence of 10  $\mu$ g/ml Brefeldin A (Sigma Chemical Co.). These cells were harvested, washed, and incubated for 30 min at 4°C with PE-conjugated anti-CD44 mAb, biotin-conjugated CD8 mAb, and then Cy-Chrome-conjugated streptavidin. After surface staining, cells were subjected to intracellular cytokine staining using the Fast Immune Cytokine System according to the manufacturer's instructions (Becton Dickinson & Co.). The cells were washed and fixed in 100  $\mu$ l of FACS® lysing solution (Becton Dickinson & Co.) for 15 min at room temperature, and then washed again, and resuspended in 50  $\mu$ l of FACS® permeabilizing solution (Becton Dickinson & Co.) and incubated for 15 min at room temperature. After washing, the cells were stained with FITC-conjugated IFN- $\gamma$  mAb or FITC-conjugated isotype control rat IgG (PharMingen) for 30 min at room temperature and the fluorescence of the cells was analyzed by a flow cytometer.

**Experiments of Salmonella Infection.** *Salmonella* subspecies *chol-*

*eraesuis* serovar *Choleraesuis* strain 31N-1 (40) was maintained by several passages through C57BL/6 mice. The approximate LD<sub>50</sub> was  $2 \times 10^7$  CFU in C57BL/6 mice inoculated intraperitoneally. Heat-killed *Salmonella* (HKS) was prepared by incubating viable *S. choleraesuis* at 74°C for 120 min. Mice were inoculated intraperitoneally with  $2 \times 10^6$  viable *S. choleraesuis* in 0.2 ml PBS. On day 6 after inoculation, mice were anesthetized with ether and killed by cutting their cervical arteries. Peritoneal contents were lavaged with 5 ml of PBS and harvested after gentle massage. Samples were serially diluted with PBS. The organ was homogenized thoroughly, and the homogenates were serially diluted with PBS. Samples were spread on tripto-soya agar (Nissui Pharmaceutical) plates, and colonies were counted after incubation for 24 h at 37°C. Peritoneal exudate cells were prepared by centrifuging peritoneal exudates at 110 *g* for 10 min and suspended at a concentration of  $10^6$  cells/ml in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml of streptomycin, and 10 mM HEPES. Cells were plated in the wells of 100-mm tissue culture dishes (Nunc, Inc.) and allowed to adhere for 1 h at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Nonadherent cells were used for flow cytometric analysis. In some experiments, 400  $\mu$ g of anti-CD8 mAb (2.43, rat IgG2b), 400  $\mu$ g of anti-CD4 mAb (GK1.5, rat IgG2b) or isotype control rat IgG was administered to IL-15 Tg mice 2 d before an i.p. challenge with *S. choleraesuis* ( $2 \times 10^6$  CFU).

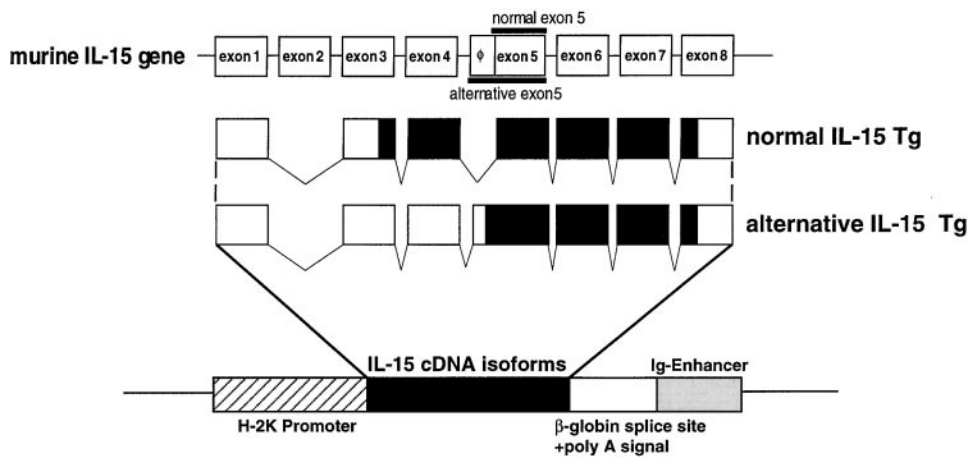
**T Helper 1 Response to Salmonella Antigen.** CD4<sup>+</sup> or CD8<sup>+</sup> T cells purified by MACS (Miltenyi Biotechnology) in LN from *Salmonella*-infected IL-15 Tg mice or control mice on day 7 were cultured with HKS in the presence of syngeneic naive mytomycin C (MMC)-treated splenocytes for 72 h, and supernatants were examined for the following cytokine. The purity of CD4<sup>+</sup> or CD8<sup>+</sup> T cells was confirmed to be more than 98% by FACS® analysis.

**Statistical Analysis.** The statistical significance of the data was determined by Student's *t* test. *P* < 0.05 was taken as significant.

## Results

**Generation of Two Groups of IL-15 Tg Mice.** Full-length cDNA encoding murine IL-15 gene with the originally described exon 5 or alternative exon 5 was inserted into a transgenic cassette that contained the H-2K promoter, Ig enhancer, and  $\beta$ -globin splice site and polyA signal (Fig. 1). The resulting IL-15 transgenic constructs were injected into C57BL/6 blastocysts. Three and two founders from 113 and 81 independent mice of each group were found to be incorporated with the IL-15 transgene with the originally described exon 5 (normal IL-15 Tg mice) or alternative exon 5 (alternative IL-15 Tg mice), respectively. Homozygous transgenic mice were generated by breeding heterozygous littermates to obtain maximal levels of IL-15 mRNA expression. All lines of each transgenic group were born healthy and survived normally up to 12 mo. As the analysis of offsprings of each of the three normal and two alternative IL-15 transgenic lines showed very similar results in each group, we demonstrate the representative results obtained with offsprings from normal IL-15 transgenic line 3.2 and alternative IL-15 transgenic line 1.2, which contain  $\sim 10$  and 2 copies of the transgene, respectively.

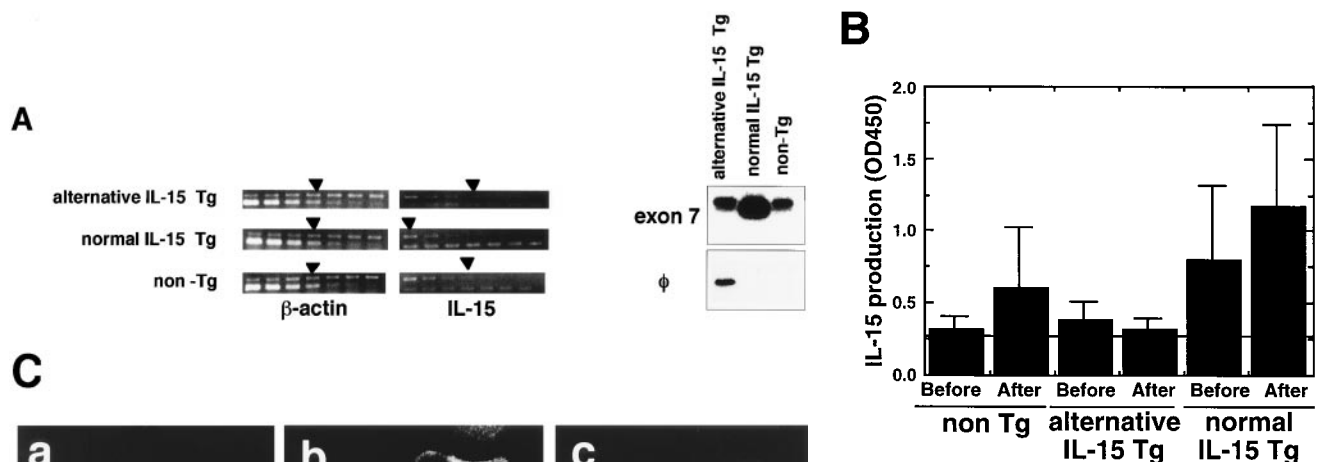
RT-PCR was used to estimate the mRNA expression derived from the transgene in macrophages of IL-15 Tg mice because the molecular sizes of these isoforms are too



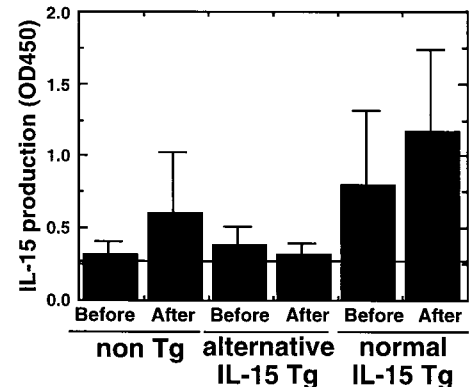
**Figure 1.** Schematic illustration of the organization of the mouse IL-15 gene and IL-15 transgenes. Exons 1–8 are shown as open boxes and introns as disconnected lines in the mouse IL-15 gene organization above the schematic representation of the transgene construct.  $\phi$  denotes an alternatively spliced additional sequence of exon 5. The H-2K promoter,  $\beta$ -globin splice site + poly A signal, and Ig enhancer are shown in the transgenic vector. Coding regions are indicated by black boxes and noncoding region by stippled boxes.

close to separate each isoform by Northern blot analysis. First, we examined the expression of the total IL-15 mRNA by competitive RT-PCR using specific primers for exons 6 and 8. As shown in Fig. 2 A, the expression level of IL-15

mRNA in the peritoneal macrophages of normal IL-15 Tg mice was eightfold higher than that of C57BL/6 control mice, while the expression level of IL-15 mRNA in alternative IL-15 Tg mice was very similar to that in control



**B**



**Figure 2.** Expression of the transgenes in the IL-15 Tg mice. (A) Quantitative RT-PCR analysis of IL-15 Tg mice (left). mRNA from macrophages of normal IL-15 Tg mice, alternative IL-15 Tg mice, and control C57BL/6 mice was transcribed into cDNA and amplified by PCR using common primers for exons 6–7, and 8. cDNA were adjusted to identical concentrations of  $\beta$ -actin cDNA by coamplification of constant amounts of cDNA and twofold dilutions of mimic. Identical amounts of cDNA were used for PCR with IL-15-specific primer in the presence of twofold dilution of mimic. Arrows indicate the titration point when equal band intensities were obtained for target cDNA and mimic. Southern blot analysis of RT-PCR products amplified with primers for exons 1 and 8 (right). The cDNA was amplified by PCR using primers for exons 1 and 8. The PCR products were hybridized with an internal probe specific for exon 7 or additional sequence  $\phi$ . (B) Production of IL-15 protein in the serum of IL-15 Tg mice. The serum was collected

from normal IL-15 Tg mice, alternative IL-15 Tg mice, and control C57BL/6 mice noninfected or i.p. infected with *Salmonella choleraesuis* ( $2 \times 10^6$  CFU) on day 6, and the IL-15 protein levels were determined by mouse IL-15 ELISA. The production level of IL-15 protein was expressed as net OD absorbance at 450 nm. The IL-15 level at the line in the figure was shown as the amount of the detection limit. Data were obtained from three independent experiments and expressed as the means of triplicate determinations  $\pm$  SD. (C) Intracellular IL-15 expression in IL-15 Tg mice. Macrophages from the peritoneum of normal IL-15 Tg mice (a), alternative IL-15 Tg mice (b), and control C57BL/6 mice (c) were stained with anti-mouse IL-15 antibody as described in Materials and Methods. The data are representative of three independent experiments, and typical confocal microscopic images are shown.

mice. We next amplified the same cDNAs from the peritoneal macrophages by RT-PCR with specific primers for exons 1 and 8 in the untranslated region of the IL-15 gene, and the amplified products were hybridized with an internal common probe for both isoforms, or a specific probe for the isoform with alternative exon 5. No alternative exon 5 was expressed in control mice and normal IL-15 transgenic mice, whereas the expression of mRNA containing alternative exon 5 was detected in alternative IL-15 Tg mice.

We next examined the IL-15 protein levels in serum from IL-15 Tg mice. Since recombinant murine IL-15 was not available, we expressed net OD for the IL-15 level in the serum. As shown in Fig. 2 B, a significant level of the IL-15 protein was constitutively detected in the serum of normal IL-15 Tg mice, whereas it was only at the background level in the serum from alternative IL-15 Tg or control C57BL/6 mice. We have previously reported that IL-15 production was induced in vivo by infection with *Salmonella choleraesuis* 31N-1 (19). Therefore, we further examined serum IL-15 levels on day 6 after *Salmonella choleraesuis* infection. IL-15 protein levels in the serum from normal IL-15 Tg mice and control C57BL/6 mice were upregulated after infection, whereas serum IL-15 protein was not detected in alternative IL-15 Tg mice after the infection. We have previously reported that the translational efficiency of the IL-15 mRNA isoform with the alternative exon 5 was significantly higher than that of the IL-15 mRNA isoform with the normal exon 5 (34). However, the signal peptide encoded by alternative exon 5 lacks hydrophobic domains, suggesting that the isoform of the IL-15 protein precursor is restricted intracellularly. Therefore, we examined the intracellular expression of the IL-15 protein in the macrophages of IL-15 transgenic mice as assessed by confocal laser microscopy with polyclonal anti-IL-15 Ab. As shown in Fig. 2 C, a large amount of intracellular IL-15 protein was detected in the macrophages from alternative IL-15 Tg mice, but not in those from normal IL-15 Tg or control C57BL/6 mice. Thus, consistent with our earlier finding in vitro, the IL-15 protein is effec-

tively synthesized by alternative IL-15 transgene, but may be restricted intracellularly. Our findings also suggest that endogenous IL-15 production by *Salmonella* infection may be suppressed in the alternative IL-15 Tg mice.

**Lymphocyte Development in the Thymus and Periphery of IL-15 Tg Mice.** To investigate T cell development in the IL-15 Tg mice, thymocytes and peripheral T cells from Tg or wild-type animals were analyzed by flow cytometry using antibodies specific for a variety of developmentally regulated T cell surface antigens. Thymi from newborn to 4-wk-old normal IL-15 Tg mice possessed an ~25% higher level of cells than did control mice, whereas the level in thymi from alternative IL-15 Tg mice was very similar to that from control C57BL/6 mice. The thymi from both IL-15 Tg mice contained normal proportions of double negative CD4<sup>-</sup>CD8<sup>-</sup>, double positive CD4<sup>+</sup>CD8<sup>+</sup>, and single positive CD4<sup>+</sup> or CD8<sup>+</sup> cells. Levels of CD3 and TCR- $\alpha/\beta$  expression were also indistinguishable among wild-type and both IL-15 Tg mice, as were the expression levels of TCR- $\gamma/\delta$  and heat-stable antigen (data not shown). Thus, overexpression of the normal or alternative IL-15 transgene did not appear to perturb thymocyte ontogeny. The populations of CD44<sup>+</sup>CD8<sup>+</sup> T cells in the spleen and the lymph node were threefold higher in normal IL-15 Tg mice (Table I and Fig. 3). The CD44<sup>+</sup>CD8<sup>+</sup> T cells showed a memory phenotype such as CD25<sup>-</sup>, CD69<sup>-</sup>, Ly-6C<sup>+</sup>, CD62L<sup>+</sup>, and CD45 RB<sup>+</sup> (Fig. 4, A and B). The expression of IL-2R $\alpha$  (CD25) was undetectable on CD44<sup>+</sup>CD8<sup>+</sup> T cells, but IL-2/IL-15R  $\beta$  and  $\gamma$  chains were highly expressed on memory-phenotype CD44<sup>+</sup>CD8<sup>+</sup> T cells. On the other hand, the proportion of memory-type CD8<sup>+</sup> T cells in alternative IL-15 Tg mice was very similar to that in control mice. However, it should be noted that NK1.1<sup>+</sup> cells in the spleen of alternative IL-15 Tg mice were significantly reduced in number compared with control mice (Fig. 3).

**Stimulation of LN Cells from IL-15 Tg Mice by IL-2/IL-15.** Our results suggest that the number of CD44<sup>+</sup>CD8<sup>+</sup> T cells constitutively expressing an IL-2/IL-15R  $\beta$  chain is in-

**Table I.** Proportion and Absolute Number of T Cell Subsets in the Lymph Node or the Spleen of Transgenic Mice Expressing Different IL-15 Isoforms

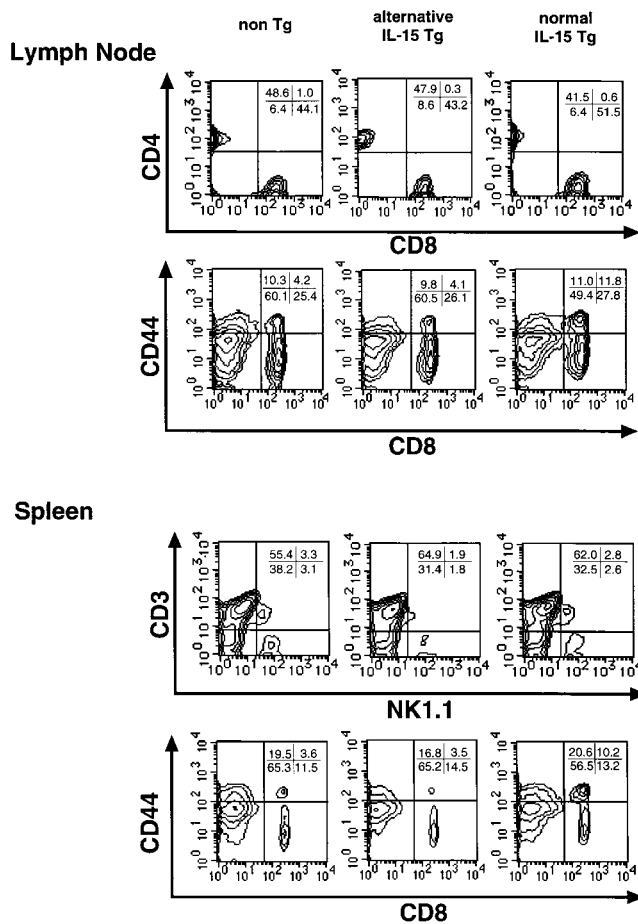
	Lymph node					Spleen		
	Total leukocyte number ( $\times 10^6$ )	In CD3 <sup>+</sup>		In Leukocytes		Total leukocyte number ( $\times 10^8$ )	In Leukocytes	
		CD4 <sup>+</sup> cells	CD8 <sup>+</sup> cells	CD44 <sup>+</sup> CD4 <sup>+</sup> Cells	CD44 <sup>+</sup> CD8 <sup>+</sup> Cells		NK1.1 <sup>+</sup> CD3 <sup>+</sup> cells	NK1.1 <sup>+</sup> CD3 <sup>-</sup> cells
		%	%	%	%		%	%
Non-Tg	7.3 $\pm$ 3.2*	58.2 $\pm$ 11.5	33.6 $\pm$ 5.5	4.0 $\pm$ 1.9	3.3 $\pm$ 3.7	1.2 $\pm$ 0.1	3.1 $\pm$ 0.4	3.1 $\pm$ 0.1
Alternative IL-15 Tg	5.3 $\pm$ 3.0	56.4 $\pm$ 8.5	32.9 $\pm$ 5.3	3.2 $\pm$ 1.0	3.2 $\pm$ 0.3	1.7 $\pm$ 0.2 <sup>‡</sup>	1.4 $\pm$ 0.7 <sup>‡</sup>	2.1 $\pm$ 0.4 <sup>§</sup>
Normal IL-15 Tg	7.7 $\pm$ 5.5	43.0 $\pm$ 8.6 <sup>‡</sup>	45.3 $\pm$ 5.1 <sup>‡</sup>	5.4 $\pm$ 2.4	8.8 $\pm$ 3.6 <sup>‡</sup>	2.0 $\pm$ 0.1 <sup>§</sup>	2.8 $\pm$ 0.1	4.2 $\pm$ 2.2

\*Values represent means and SEM of three to nine individual determinations.

<sup>‡</sup> $P < 0.05$  by Student's *t* test compared with the values of non-Tg mice.

<sup>‡</sup> $P < 0.01$  by Student's *t* test compared with the values of non-Tg mice.

<sup>§</sup> $P < 0.001$  by Student's *t* test compared with the values of non-Tg mice.



**Figure 3.** Flow cytometry analysis of the peripheral T cell subsets in IL-15 Tg mice. Lymphocytes were prepared from the LN or the spleen of normal IL-15 Tg mice, alternative IL-15 Tg mice, and control C57BL/6 mice as described in Materials and Methods. The cells from the LN were stained with FITC-CD3, PE-CD8 $\alpha$ , and Cy-Chrome-CD4, and then the cells were analyzed by a flow cytometer and the analysis gate was set on CD3<sup>+</sup> cells. The cells were also stained FITC-CD44 and PE-CD8 $\alpha$ . Cells from the spleen were stained with FITC-CD3 $\epsilon$  and PE-NK1.1 or FITC-CD44 and PE-CD8 $\alpha$ , and then they were analyzed by a flow cytometer. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical two-color profiles.

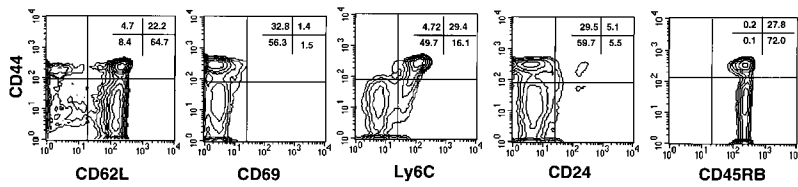
creased in normal IL-15 Tg mice. We next examined the effect of IL-2 or IL-15 on the expansion of CD44<sup>+</sup>CD8<sup>+</sup> T cells from normal IL-15 Tg mice. The LN cells from normal IL-15 Tg or control mice were cultured with human recombinant (r) IL-2 (100 ng/ml) or IL-15 (100 ng/ml) for 48 h, and then the blastoid cells were analyzed by FSC (forward scatter, cell size) and SSC (side scatter, granularity) on flow cytometer. As shown in Fig. 5 A, rIL-15 and rIL-2 significantly increased the proportion of large, blastoid cells after 48 h of culture of LN cells from normal IL-15 Tg mice. We stained the responding cells, as evidenced by their blastoid appearances to rIL-15 or rIL-2 with anti-CD44 and -CD8 mAbs. Flow cytometric analysis revealed that the blastoid cells after culture with rIL-15 represented CD44<sup>+</sup>CD8<sup>+</sup> T cells, indicating that CD44<sup>+</sup>CD8<sup>+</sup> T cells proliferated in response to rIL-15 as well as to rIL-2. The ability of the lymph node cells in normal IL-15 Tg mice to

respond to rIL-15 or rIL-2 was significantly higher than that in alternative IL-15 Tg mice. The CD44<sup>+</sup>CD8<sup>+</sup> T cells in Fig. 5 A were nonaddition,  $25.5 \pm 5.5$  vs.  $28.3 \pm 11.0\%$ ; IL-2,  $70.2 \pm 3.8$  vs.  $53.3 \pm 8.2\%$ ; IL-15,  $71.0 \pm 4.2$  vs.  $40.2 \pm 5.3\%$  (IL-15 transgenic mice versus control mice). On the other hand, the ability of the LN cells of alternative IL-15 Tg mice to respond to these cytokines was not different from that of control mice (data not shown).

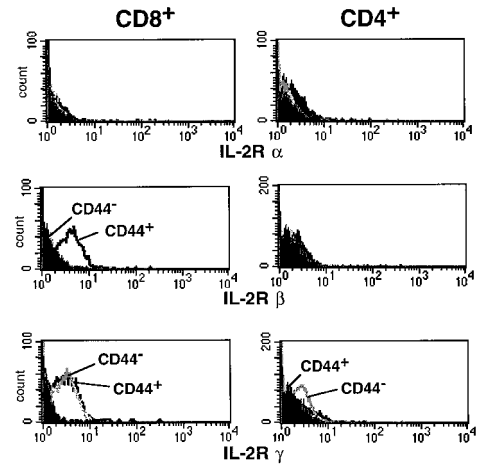
To assess whether cell proliferation by IL-2 or IL-15 induces cytokine production, we examined cytokine production in the culture supernatant of lymphocytes from IL-15 Tg mice or control mice. As shown in Fig. 5 B, LN cells from normal IL-15 Tg mice produced a larger amount of IFN- $\gamma$  in response to IL-2 or IL-15, while those from non-Tg or alternative IL-15 Tg mice produced only a marginal level of IFN- $\gamma$  in response to IL-2 or IL-15. Other cytokines, including IL-4, IL-10, TNF- $\alpha$ , and IL-13, were not detected in any culture supernatants. These results suggest that memory-phenotype CD44<sup>+</sup>CD8<sup>+</sup> T cells in the normal IL-15 Tg mice can expand and produce IFN- $\gamma$  in response to bystander stimulation with IL-2 or IL-15.

**Cytokine Production by LN Cells in IL-15 Transgenic Mice upon TCR Triggering.** Memory-type T cells are characterized by early cytokine production upon TCR engagement (41, 42). Therefore, we examined the cytokine production of LN cells from IL-15 Tg mice or control mice in response to immobilized anti-TCR $\alpha\beta$  mAb. IFN- $\gamma$  was rapidly produced by LN cells in normal IL-15 Tg mice within 24 h after stimulation with immobilized anti-TCR antibodies, while LN cells from alternative IL-15 Tg mice produced only a marginal level of IFN- $\gamma$ , if any, after stimulation (Fig. 6). To confirm that early IFN- $\gamma$  production depends on memory-type CD8<sup>+</sup> T cells in normal IL-15 Tg mice, we next examined the cytokine production of T cells in IL-15 Tg mice after in vivo administration of anti-TCR mAb. As shown in Fig. 7, IFN- $\gamma$ -producing cells were observed in populations of CD8<sup>+</sup>CD44<sup>+</sup> cells, but not in those of CD44<sup>-</sup> cells. In correlation with the number of memory-type CD8<sup>+</sup> T cells, the number of IFN- $\gamma$ -producing cells in normal IL-15 Tg mice was larger than that in control mice, while the number of IFN- $\gamma$ -producing cells in alternative IL-15 Tg mice was greatly reduced compared with that in control mice.

**Susceptibility to Salmonella Infection in IL-15 Tg Mice.** To investigate whether protective immunity against bacteria is affected in normal IL-15 or alternative IL-15 Tg mice, these mice were intraperitoneally injected with a sublethal dose of *Salmonella choleraesuis*. The growth kinetics of *S. choleraesuis* in the peritoneum were monitored for 6 d after an i.p. challenge with *S. choleraesuis*. As shown in Fig. 8 A, the number of bacteria in the peritoneal cavity of normal IL-15 Tg mice was significantly smaller than that in control mice on days 3 and 6 ( $P < 0.05$ ). On the other hand, alternative IL-15 Tg mice had increased numbers of *Salmonella* as compared with control mice. In correlation with the protection level as assessed by bacterial growth, the level of IFN- $\gamma$  in the serum of normal IL-15 Tg mice was significantly higher than that in control mice on day 6 after an

**A**

**Figure 4.** Cell surface markers on CD44<sup>+</sup>CD8<sup>+</sup> T cells in normal IL-15 Tg mice. (A) Expression of activation markers on the CD8<sup>+</sup> T cells from the LN of normal IL-15 Tg mice was examined by a flow cytometer. The cells were stained with FITC-CD44, Biotin-CD8 $\alpha$ , and PE-mAbs against various markers, and then they were analyzed by a flow cytometer, and the analysis gate was set on CD8<sup>+</sup> cells. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical two-color profiles. (B) Expression of IL-2 receptor subunits on CD8<sup>+</sup> or CD4<sup>+</sup> T cells from the LN of normal IL-15 Tg mice were examined. The cells were stained with PE-CD44, Cy-Chrome-CD4, or biotin-CD8 $\alpha$ , and FITC-mAbs against IL-2 receptor subunits, and they were then analyzed by a flow cytometer, and the analysis gate was set on CD44<sup>+</sup> or CD44<sup>-</sup> on CD4<sup>+</sup> or CD8<sup>+</sup> cells. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical single-color profiles.

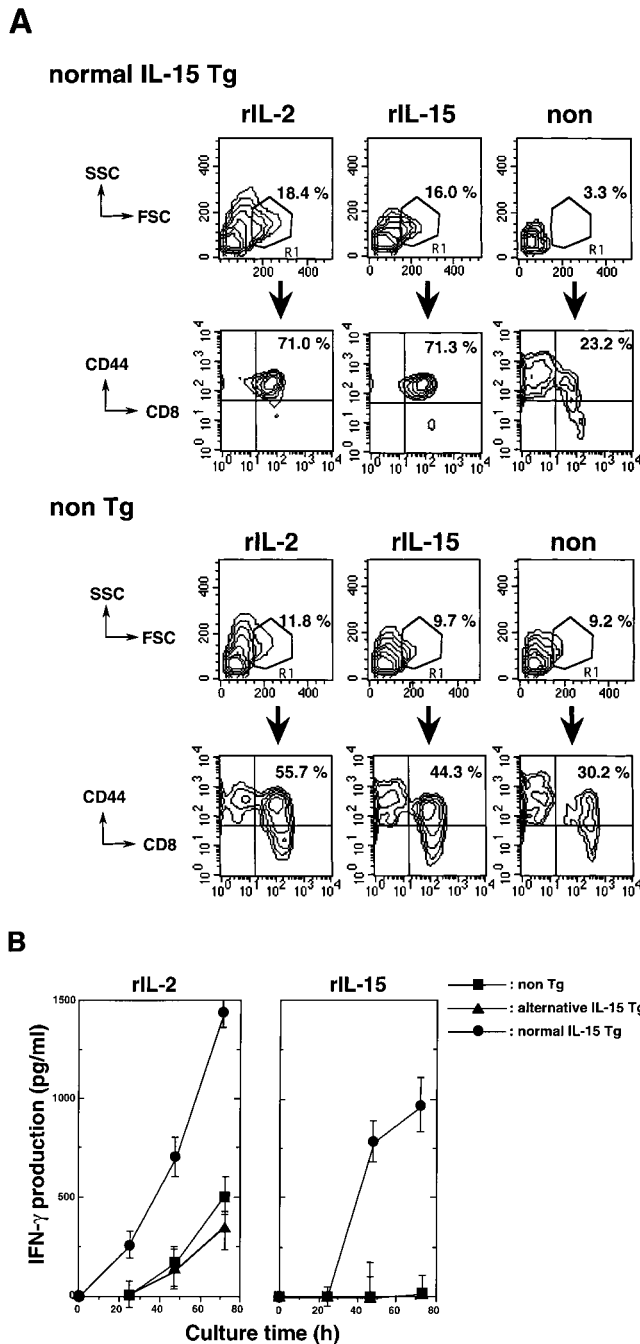
**B**

i.p. challenge with *S. choleraesuis* ( $P < 0.001$ ), while the IFN- $\gamma$  level in the serum of alternative IL-15 Tg mice was lower than that in control mice (Fig. 8 B). IL-4 was not detected in the serum at any stage during salmonellosis. We next compared the cellular response in the peritoneum between IL-15 Tg and control mice during *Salmonella* infection. The absolute number of peritoneal nonadherent cells in normal IL-15 Tg mice ( $1.2 \pm 0.2 \times 10^7$  cells/mouse) was larger than that in control mice ( $4.8 \pm 0.1 \times 10^6$  cells/mouse,  $P < 0.001$ ), whereas that in alternative IL-15 Tg mice was not different from those in control mice. A typical two-color profile is shown in Fig. 9. The CD8<sup>+</sup> T cells in the peritoneal cavity of normal IL-15 Tg mice were markedly increased on day 6 after the i.p. challenge with *S. choleraesuis* compared with that in alternative IL-15 Tg and control mice. The numbers of NK1.1<sup>+</sup>CD3<sup>-</sup> cells and NK1.1<sup>+</sup>CD3<sup>+</sup> cells were also significantly increased in the peritoneal cavity of normal IL-15 Tg mice on day 6 after infection with *Salmonella* compared with that in control mice, while the number of NK1.1<sup>+</sup>CD3<sup>-</sup> cells were significantly reduced in alternative IL-15 Tg mice after *Salmonella* infection.

Early IFN- $\gamma$  production plays an important role not only in host defense at the early stage, but also in determining differentiation of naive CD4<sup>+</sup> T cells to Th1 cells during infection with *Salmonella* species. Therefore, we next compared the generation of CD4<sup>+</sup> Th1 cells specific for *Salmonella* in normal and alternative IL-15 Tg mice. As shown in Fig. 10, CD4<sup>+</sup> T cells from normal IL-15 Tg mice on day 6 after the i.p. challenge with *S. choleraesuis* produced a larger amount of IFN- $\gamma$  in response to HKS than did those from control mice, while CD4<sup>+</sup> T cells in the LN of alternative IL-15 Tg mice produced a smaller amount of IFN- $\gamma$  than did those in control mice. CD8<sup>+</sup> T cells from normal IL-15 Tg mice or control mice produced only a small amount of IFN- $\gamma$  in response to HKS. On the other hand, no IFN- $\gamma$  production was evident in the CD8<sup>+</sup> T cells from alterna-

tive IL-15 Tg mice on day 6 after infection with *S. choleraesuis*. These results suggest that Th1 cells are efficiently generated in normal IL-15 Tg mice but poorly generated in alternative IL-15 Tg mice.

*Effect of CD8<sup>+</sup> or CD4<sup>+</sup> T Cell-depletion on Protection Against Salmonella Infection in the Normal IL-15 Transgenic Mice.* Our results raise the possibility that the increase in the number of memory-phenotype CD8<sup>+</sup> T cells in the normal IL-15 Tg mice augmented the protective immunity against *Salmonella* infection. To address the potential role of CD8<sup>+</sup> or CD4<sup>+</sup> T cells in normal IL-15 Tg mice, we examined the effect of in vivo administration of anti-CD8 or -CD4 mAb to deplete CD8<sup>+</sup> or CD4<sup>+</sup> T cells on the susceptibility to *S. choleraesuis* in the normal IL-15 Tg mice. The normal IL-15 Tg mice were injected intraperitoneally with anti-CD8 or -CD4 mAb 2 d before a *Salmonella* challenge. We confirmed by FACS<sup>®</sup> analysis that CD8<sup>+</sup> or CD4<sup>+</sup> T cells were almost completely depleted in the peritoneum and the LN 6 d after the i. p. challenge with *S. choleraesuis* (data not shown). As shown in Fig. 11 A, the number of bacteria was significantly increased in the peritoneal cavity of normal IL-15 Tg mice depleted of CD8<sup>+</sup> and CD4<sup>+</sup> T cells compared with that in mice treated with isotype control rat IgG ( $P < 0.001$ ). Thus, CD8<sup>+</sup> and CD4<sup>+</sup> T cells serve to protect against *Salmonella* infection in normal IL-15 Tg mice. We further examined the effect of CD4<sup>+</sup> cell depletion on the generation of CD4 Th1 cell response in normal IL-15 Tg mice after *Salmonella* infection. The CD4<sup>+</sup> T cells were harvested on day 6 after *Salmonella* infection and stimulated in vitro in the presence of HKS and APC. The CD4<sup>+</sup> T cells from anti-CD8 mAb-treated normal IL-15 Tg mice showed a significantly decreased production of IFN- $\gamma$  in response to heat-killed *Salmonella* compared with those from isotype control IgG-treated mice (Fig. 11 B). CD8<sup>+</sup> T cells from anti-CD4 mAb or control rat IgG-treated normal IL-15 Tg mice produced only a marginal level of IFN- $\gamma$  in response to HKS. These



**Figure 5.** Bystander activation of CD44<sup>+</sup>CD8<sup>+</sup> T cells in IL-15 Tg mice. (A) Flow cytometry analysis of LN cells from normal IL-15 Tg mice or control C57BL/6 mice after culture with rIL-2 or rIL-15 was performed. The LN cells were cultured with or without rIL-2 (100 ng/ml) or rIL-15 (100 ng/ml) for 48 h at 37°C. After the culture, the cells were stained with PE-CD8 $\alpha$  and FITC-CD44 mAb, and they were then analyzed by a flow cytometer. Blastoid cells were gated in FSC and SSC profiles as R1. CD44 expression on the responding cells, as evidenced by their blastoid appearance to rIL-15 or rIL-2, was presented as typical two-dimensional profiles. (B) Cytokine production of the LN cells in response to rIL-2 or IL-15 in IL-15 Tg mice was assessed by ELISA. The culture supernatant of A was collected and the IFN- $\gamma$  activity was determined. The data are representative of two separate experiments and are expressed as the mean of triplicates  $\pm$  SD.

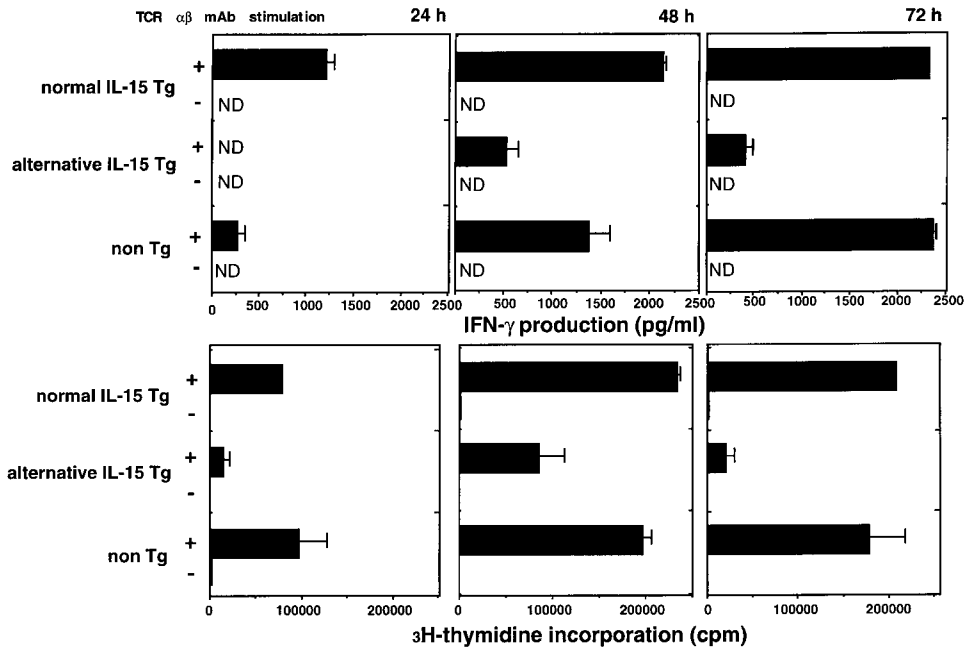
results suggest that the CD8<sup>+</sup> T cells may be at least partially responsible for the augmented Th1 response in normal IL-15 Tg mice infected with *Salmonella*.

## Discussion

In the present study, we generated two types of IL-15 Tg mice: normal IL-15 Tg mice expressing IL-15 cDNA with the originally described exon 5 and alternative IL-15 Tg mice expressing a novel IL-15 cDNA with an alternative exon 5. We found that the number of memory-phenotype CD8<sup>+</sup> T cells increased significantly in normal IL-15 Tg mice that constitutively produced a significant level of IL-15 in the serum. The CD8<sup>+</sup> cells were stimulated to produce IFN- $\gamma$  by exogenous IL-2/IL-15 in a bystander manner. The normal IL-15 Tg mice showed resistance to an i.p. infection with a virulent strain of *Salmonella choleraesuis* accompanied by increases in memory-type CD8<sup>+</sup> T cells and CD4 Th1 cells specific for *Salmonella*. These results suggest that overexpression of the normal IL-15 transgene may induce expansion of memory-type CD8<sup>+</sup> T cells, which serve to enhance the protective immunity against *Salmonella* infection through early IFN- $\gamma$  production and enhanced CD4 Th1 cell response. On the other hand, T cells from alternative IL-15 Tg mice exhibited impaired IFN- $\gamma$  production upon TCR engagement, and the alternative IL-15 Tg mice were susceptible to an i.p. challenge with *Salmonella choleraesuis* accompanied by impaired releases of IL-15 and IFN- $\gamma$  and poor CD4 Th1 response. The overexpression of the alternative IL-15 transgene may negatively regulate the endogenous IL-15 production, resulting in suppression of subsequent protective immunity against *Salmonella* infection.

It is now widely accepted that IL-15 is important for the maintenance of lymphocytes, especially memory-type CD8<sup>+</sup> T cells (11, 43). We showed that CD8<sup>+</sup> T cells expressing memory markers increased in normal IL-15 Tg mice. The memory-phenotype CD8<sup>+</sup> cells were found to express a high level of antiapoptotic protein Bcl-2, but not proapoptosis protein BAX (data not shown). This indicates that the memory phenotype CD8<sup>+</sup> T cells are maintained by overexpression of IL-15 through upregulation of an antiapoptotic protein such as Bcl-2 or Bcl-XL via the IL-2/IL-15R  $\beta$  chain. It is notable that normal IL-15 Tg mice were resistant to infection with a virulent strain of *S. choleraesuis*. We have previously reported that NK cells serve to protect against infection with avirulent *S. choleraesuis* (44). In the present study, besides NK cells, CD8<sup>+</sup> T cells were remarkably increased in normal IL-15 Tg mice compared with those in control mice after *Salmonella* infection and, concurrently, IFN- $\gamma$  production in the serum was augmented during the infection. Experiments with in vivo depletion of CD8<sup>+</sup> T cells revealed that CD8<sup>+</sup> T cells contribute to the resolution of *Salmonella* infection in normal IL-15 Tg mice. There are several possible explanations of how the CD8<sup>+</sup> T cells serve to protect against *Salmonella* infection in normal IL-15 Tg mice. First, the memory CD8<sup>+</sup> T cells may recognize the conserved antigens in *Salmonella*, which are common to

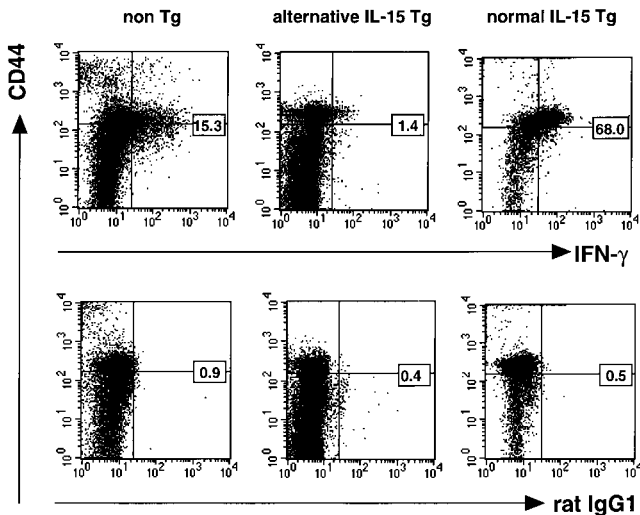




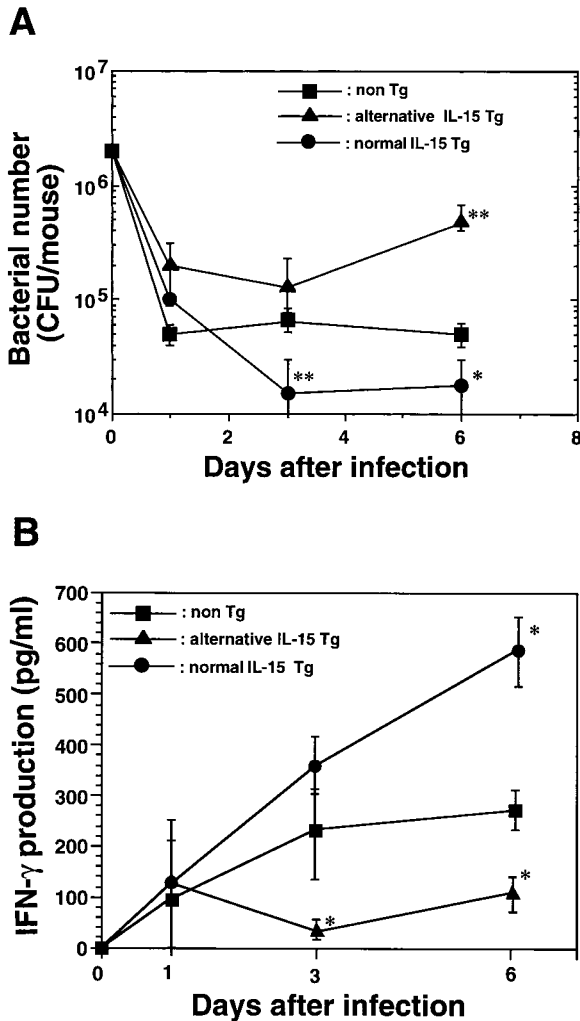
**Figure 6.** IFN- $\gamma$  production of LN cells in IL-15 Tg mice in response to TCR triggering. The LN cells from normal and alternative IL-15 Tg mice and control C57BL/6 were cultured in the presence (closed bars) or absence (open bars) of immobilized anti-TCR  $\alpha/\beta$  mAb. At 24, 48, or 72 h after incubation, culture supernatants were harvested, and IFN- $\gamma$  was measured by ELISA. The proliferation activity was assessed by incorporation of  $^3\text{H}$ -thymidine. The data are representative of three independent experiments using pooled cells from three transgenic mice or control mice and are shown as the mean of triplicate determinations  $\pm$  SD.

previously encountered Ag such as environmental Ag or self antigens. However,  $V\beta$  repertoires of the CD8 $^+$  T cells are diversified and not skewed (data not shown). Furthermore, CD8 $^+$  T cells in normal IL-15 Tg mice infected with *Salmonella* are not specialized to recognize heat-killed *Salmonella*. Therefore, it is unlikely that the memory T cells cross-react with a conserved Ag in *Salmonella*. There are several lines of evidence for the strong bystander stimula-

tion of CD8 $^+$  T cells in vivo elicited by various infectious agents (45–52). Lymphocytic choriomeningitis virus infection induces activation and expansion of CD8 $^+$  T cells. Limiting dilution analysis to quantitate virus-specific cytotoxic T lymphocytes has shown that only a small fraction (1–5% at most) of the activated CD8 $^+$  T cells are antigen specific at the peak of the primary response (45–47). Thus, memory-phenotype CD8 $^+$  T cells are subject to nonantigen-specific “bystander” stimulation through contact with various cytokines released during infection with pathogens. Zhang et al. (43) have reported that the strong bystander stimulation of CD8 $^+$  T cells in vivo elicited by various infectious agents is driven by IFN I-induced production of IL-15. We showed direct evidence for bystander stimulation of CD4 $^+$ CD8 $^+$  T cells from IL-15 transgenic mice by exogenous IL-2 or IL-15. Therefore, it is most likely that most of the CD8 $^+$  T cells expand in a bystander manner by endogenous IL-2 and/or IL-15 produced during *Salmonella* infection. *Salmonella* serovars are facultative parasites that are capable of surviving and persisting within mammalian host cells, and Th1-type response is essential for control of such intracellular pathogens (44, 53, 54). The normal IL-15 Tg mice appeared to generate CD4 Th1 cells more efficiently than did control mice after *Salmonella* infection. Early IFN- $\gamma$  production is thought to be important for induction of Th1 response mediated by  $\alpha\beta$  T cells (55, 56). Our present results revealed that CD4 $^+$  T cells play a potential role in protection against *Salmonella* infection in normal IL-15 Tg mice and that CD4 $^+$  T cells from anti-CD8 mAb-treated normal IL-15 Tg mice showed a significantly decreased production of IFN- $\gamma$  in response to HKS. Hence, early IFN- $\gamma$  produced by CD8 $^+$  T cells may serve to induce a Th1 response by  $\alpha\beta$  T cells against *S. choleraesuis* 31N-1. However, in vivo depletion of CD8 $^+$  T cells only partially inhibited CD4 $^+$  Th1 cell development in nor-

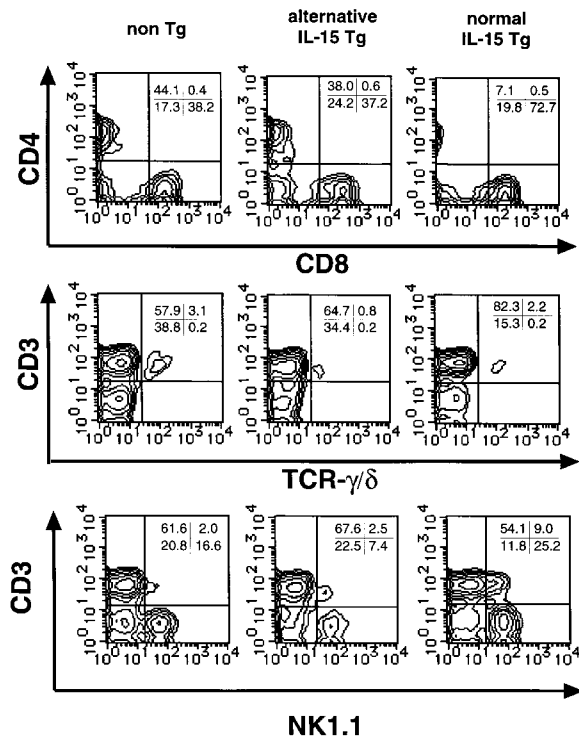


**Figure 7.** Expression of IFN- $\gamma$  in CD44 $^+$ CD8 $^+$  T cells after an i.v. injection of anti-CD3 mAb. Normal or alternative IL-15 Tg mice or control C57BL/6 mice were injected intravenously with 4  $\mu\text{g}$  of anti-CD3 mAb (145-2C11). After 90 min, spleen cells were harvested and cultured for 3 h at 37°C in the presence of 10  $\mu\text{g}/\text{ml}$  Brefeldin A. Intracellular cytokine-producing cells were examined by flow cytometry and analyzed by gating on CD8 $^+$  T cells. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical two-color profiles.



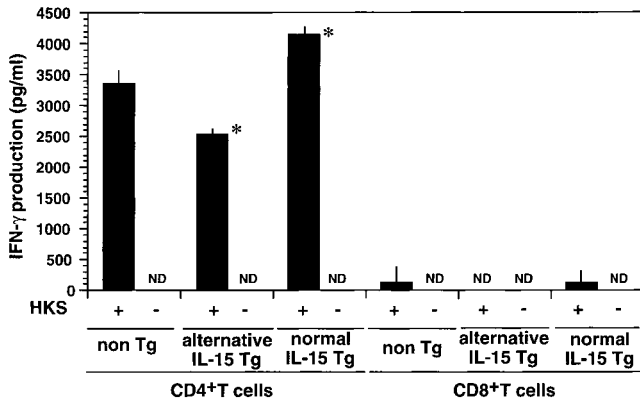
**Figure 8.** Susceptibility to *Salmonella* infection in IL-15 transgenic mice. (A) Bacterial growth of *S. choleraesuis* in the peritoneal cavity after an i.p. challenge with *S. choleraesuis*. Mice were injected intraperitoneally with  $2 \times 10^6$  CFU. The number of bacteria was determined by a colony formation assay in tripto-soya agar plates. Data were obtained from three separate experiments and are expressed as the mean  $\pm$  SD for nine mice at each point. Statistically significant differences between Tg mice and control mice are shown (\* $P < 0.05$ , \*\* $P < 0.001$ ). (B) IFN- $\gamma$  production in the serum of IL-15 transgenic mice after infection with *S. choleraesuis*. Serum was collected from normal or alternative IL-15 Tg mice or control mice after an intraperitoneal challenge with *S. choleraesuis*. The amount of IFN- $\gamma$  in the serum was determined by ELISA. Data were obtained from three separate experiments and are expressed as the mean  $\pm$  SD for nine mice at each point. Statistically significant differences between transgenic mice and control mice are shown (\* $P < 0.001$ ).

mal IL-15 Tg mice after *Salmonella* infection. Besides CD8<sup>+</sup> T cells, NK cells are significantly increased in normal IL-15 Tg mice after *Salmonella* infection. Therefore, not only CD8<sup>+</sup> cells, but also NK cells may be responsible for early IFN- $\gamma$  production, and in turn induction of CD4<sup>+</sup> Th1 development in these mice. It thus appears that IL-15 may serve to protect against microbial infection through not only activation of innate immunity, but also induction of adaptive immunity.



**Figure 9.** Flow cytometry analysis of the peritoneal exudate cells in IL-15 Tg mice infected with *S. choleraesuis* on day 6. Nonadherent peritoneal exudate cells of normal and alternative IL-15 Tg mice and control C57BL/6 mice were stained with FITC-CD3, PE-CD8 $\alpha$ , and Cy-chrom-CD4, and then the cells were analyzed by a flow cytometer and the analysis gate was set on CD3<sup>+</sup> cells. The cells were also stained FITC-CD3 $\epsilon$  and PE-TCR- $\gamma/\delta$  or -NK1.1. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical two-color profiles.

Another notable finding in the present study is that alternative IL-15 Tg mice are susceptible to a virulent *Salmonella* infection accompanied by impaired releases of IL-15 and IFN- $\gamma$  and poor responses of NK cells and CD4 Th1 cells. We also showed that early cytokine production by in vivo administration of anti-CD3 mAb was severely impaired in the Tg mice. The IL-15 protein is thought to be post-transcriptionally regulated by multiple control elements that impede translation, including upstream AUGs of 5'UTR, unusual signal peptides, and the COOH terminus of the mature protein (5, 34–38). We could not detect IL-15 in the serum of alternative IL-15 transgenic mice infected with *S. choleraesuis*, although these cells expressed an abundant level of intracellular IL-15. The alternative splicing pathway represents a mechanism whereby diversity is generated in a reversible fashion without a requirement for the expression of new genes (57–59). Changes in the alternative splicing of specific pre-mRNA molecules may be associated with a unique function of each isoform. Our results suggest that overexpression of the intracellular IL-15 isoform may suppress the endogenous IL-15 production. Abnormalities of IL-15 have been reported in inflammatory disorders of autoimmune diseases such as rheumatoid arthritis or inflam-



**Figure 10.** Generation of CD4 Th1 cells specific for *Salmonella* antigen in IL-15 Tg mice. CD4<sup>+</sup> T cells ( $2 \times 10^5$  cells) or CD8<sup>+</sup> T cells ( $2 \times 10^5$  cells) in LN of IL-15 Tg mice or control mice on day 6 after an i.p. challenge with *S. choleraesuis* ( $2 \times 10^6$  CFU) were purified by MACS and cultured with or without heat-killed *Salmonella* in the presence of MMC-treated splenocytes ( $10^6$  cells) for 3 d at 37°C. Thereafter, the supernatants were collected and cytokine activity was determined by ELISA. The data are representative of three independent experiments using pooled cells from five IL-15 Tg or control mice and shown as the mean of triplicate determinations  $\pm$  SD. Statistical analysis was performed by means of Student's *t* test. Significant differences compared with the values in control mice are shown: \**P* < 0.01.

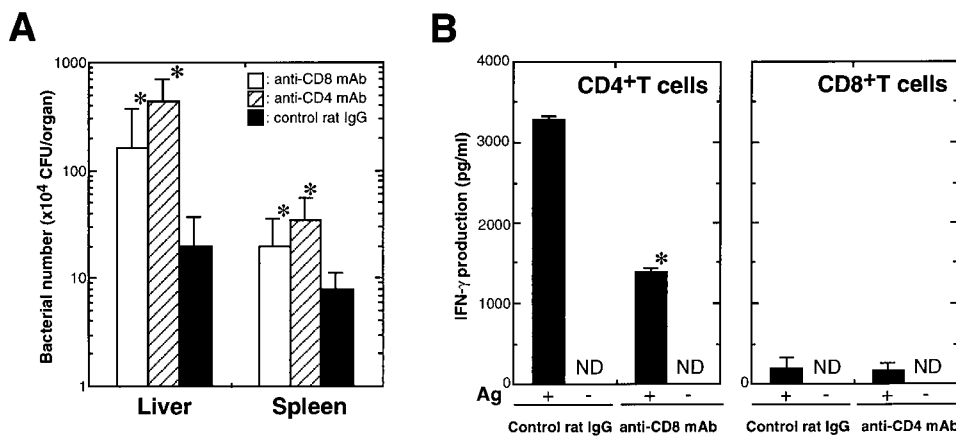
matory bowel disease (28–31). The broad constitutive expression of the IL-15 gene in several normal tissues suggests that mechanisms controlling protein synthesis of secretion must operate in these cells to prevent an inappropriate activation of the immune system. Thus, it is possible that the intracellular IL-15 isoform plays the role of negative feedback on the endogenous IL-15 production to prevent excessive immune responses. The mechanism whereby intracellular IL-15 isoform suppresses endogenous IL-15 production is

not known. Tagaya et al. (37) have reported that a part of the intracellular isoform of IL-15 is located in the nucleus, although the intracellular IL-15 isoform lacks a nuclear localization signal. Interestingly, IL-15R $\alpha$ , the specific binding subunit of the T/NK type I IL-15 receptor, possesses a potential nuclear localization signal in its signal peptide, indicating that the intracellular IL-15 isoform may be colocalized in the nucleus with IL-15R $\alpha$ . Taken together, we presume that intracellular IL-15/IL-15R $\alpha$  complex may affect transcription of endogenous IL-15 gene. But this is only speculation, further analysis of the impediment mechanism on the IL-15 gene expression by the intracellular isoform of IL-15 is required.

In conclusion, we found that the number of memory-phenotype CD8<sup>+</sup> T cells increased significantly in normal IL-15 Tg mice. They rapidly expanded and produced IFN- $\gamma$  in response to IL-2/15R $\beta$ -dependent cytokines. After an intraperitoneal challenge with *S. choleraesuis*, CD8<sup>+</sup> T cells markedly expanded in the peritoneum of normal IL-15 Tg mice at the early stage of infection accompanied by increased IFN- $\gamma$  production and protected against infection. On the other hand, we found that the expression of endogenous IL-15 mRNA with the original exon 5 was suppressed in the alternative IL-15 Tg mice. The alternative IL-15 Tg mice were highly susceptible to an i.p. challenge with *S. choleraesuis* accompanied by a lower level of IFN- $\gamma$  in the serum. Analysis of two groups of IL-15 Tg mice may provide information concerning the differential roles of IL-15 isoforms in the immune system in vivo.

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**Figure 11.** Effect of in vivo CD8 or CD4 depletion on Th1 response in normal IL-15 Tg mice. (A) Bacterial growth of *S. choleraesuis* in the spleen or the liver of CD8- or CD4-depleted normal IL-15 Tg mice on day 6 after an i.p. challenge with *S. choleraesuis*. Anti-CD8 mAb (400  $\mu$ g, rat IgG2b, 2.43), anti-CD4 mAb (400  $\mu$ g, rat IgG2b), or isotype control rat IgG was administered to normal IL-15 transgenic mice 2 d before an i.p. challenge with *S. choleraesuis* ( $2 \times 10^6$  CFU). The number of bacteria was determined by colony formation assay in tripto-soya agar plates. Data are obtained from three separate experiments and are expressed as the mean  $\pm$

SD for four mice at each point. Statistical analysis was performed by means of Student's *t* test. Significant differences compared with the values in control IgG-treated mice are shown: \**P* < 0.001. (B) CD4<sup>+</sup> T cells ( $2 \times 10^5$  cells) or CD8<sup>+</sup> T cells ( $2 \times 10^5$  cells) in LN of normal IL-15 Tg mice depleted CD8, CD4, or nondepleted on day 6 after an i.p. challenge with *S. choleraesuis* ( $2 \times 10^6$  CFU) were purified by MACS and cultured with or without heat-killed *Salmonella* in the presence of MMC-treated splenocytes ( $10^6$  cells) for 3 d at 37°C. Thereafter, the supernatants were collected, and cytokine activity was determined by ELISA. The data are representative of three independent experiments using pooled cells from five normal IL-15 Tg or control mice and shown as the mean of triplicate determinations  $\pm$  SD. Statistical analysis was performed by means of Student's *t* test. Significant differences compared with the values in control-IgG treated mice are shown: \**P* < 0.01.

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