Overexpression of IL-15 In Vivo Enhances Protection Against *Mycobacterium bovis* Bacillus Calmette-Guérin Infection Via Augmentation of NK and T Cytotoxic 1 Responses

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Overexpression of IL-15 In Vivo Enhances Protection Against Mycobacterium bovis Bacillus Calmette-Guérin Infection Via Augmentation of NK and T Cytotoxic 1 Responses

Masayuki Umemura,* Hitoshi Nishimura,* Kenji Hirose,† Tetsuya Matsuguchi,* and Yasunobu Yoshikai2*†

To investigate the immunomodulating effects of IL-15 in vivo on mycobacterial infection, we used IL-15-transgenic (Tg) mice, which were recently constructed with cDNA-encoding secretable isoform of IL-15 precursor protein under the control of a MHC class I promoter. The IL-15-Tg mice exhibited resistance against infection with Mycobacterium bovis bacillus Calmette-Guérin (BCG), as assessed by bacteria growth. IFN-γ level in serum was significantly higher in IL-15-Tg mice than in non-Tg mice after BCG infection. NK cells were remarkably increased, and Ag-specific T cytotoxic 1 response mediated by CD8+ T cells producing IFN-γ was significantly augmented in the IL-15-Tg mice following BCG infection. Neutralization of endogenous IFN-γ by in vivo administration of anti-IFN-γ mAb deteriorated the clearance of the bacteria. Depletion of of NK cells or CD8+ T cells by in vivo administration of anti-asialo-GM1 Ab or anti-CD8 mAb hampered the exclusion of bacteria. Thus, overexpression of IL-15 in vivo enhanced protection against BCG infection via augmentation of NK and T cytotoxic 1 responses. The Journal of Immunology, 2001, 167: 946–956.

Protection against infection with Mycobacterium tuberculosis depends on IFN-γ, as evidenced by severely diseminated tuberculosis in IFN-γ knockout mice (1, 2). Although IFN-γ is produced by mainly CD+ Th1 cells (3), Tc1 response mediated by CD8+ T cells producing IFN-γ was evident in mycobacterial infection (4–6). B2-microglobulin or TAP knockout mice, both of which lack functional CD8+ T cells, are susceptible to infection with M. tuberculosis (7, 8). Recent studies suggest that CD8 CTL releasing perforin and granulysin play a role in protection against M. tuberculosis infection via cytolysis mechanism (9, 10). However, the resistance to M. tuberculosis infection in both perforin- and fas-deficient mice was unaltered, suggesting that the cytotoxic function of CD8+ T cells may not be critical in protection to tuberculosis (11, 12). On the other hand, the critical function of CD8+ T cells is mediated by IFN-γ (6). Taken together, it thus appears that Tc1 response mediated by CD8+ T cells producing IFN-γ plays a requisite role in resistance to mycobacterial infection.

M. bovis bacillus Calmette-Guérin (BCG) is the vaccine approved for prevention of M. tuberculosis (13). Although M. tuberculosis DNA vaccines can induce substantial protective immune responses, the current vaccine preparations are not as protective as BCG in a mouse model of human pulmonary M. tuberculosis (13, 14). Protection against pulmonary tuberculosis requires a sustained cellular immune response, while it is not clear that BCG is effective for inducing long-term cellular immunity sufficient for protection against pulmonary disease and it confers incomplete protection against tuberculosis, at least in adults (15). Therefore, it is required to develop immune adjuvants to increase the effectiveness of BCG vaccination.

IL-15 uses β- and γ-chains of the IL-2R for signal transduction, and thus shares many properties of IL-2, despite having no sequence homology with IL-2 (16–18). IL-15 is reported to stimulate NK cells and TCRγδ intestinal intraepithelial lymphocytes (i-IEL) to produce IFN-γ and exhibit increased cytotoxicity (19–21). In addition to these cells, memory phenotype CD8+ T cells are reported to expand in response to exogenous IL-15 or various infectious agents that can elicit IL-15 via IFN regulatory factor-1/NF-κB activation (22). Recently, IL-15Ra−/− mice and IL-15−/− mice are reported to be deficient in NK cells, NKT cells, and TCRγδ i-IEL and memory phenotype CD8+ T cells (23, 24). It thus appears that IL-15 has potential roles in the development and maintenance of significant fractions of lymphocytes, including NK, NKT, TCRγδ i-IEL, and memory phenotype CD8+ T cells. There have been several lines of evidence for IL-15 production in infection with various microbes, including M. tuberculosis (25–35). We have demonstrated that IL-15 is involved in protection against infections with Escherichia coli (35) or avirulent Salmonella choleraesuis (36) via activation of γδ T cell or NK cells. Recently, we have constructed transgenic (Tg) mice expressing IL-15 cDNA encoding a secretable isoform and found that the IL-15-Tg mice contained a large number of memory phenotype CD8+ T cells expressing CD44highCD62L−Ly-6C− in the peripheral lymphoid tissues (37). IL-15-Tg mice may be useful to elucidate the roles of IL-15-dependent memory phenotype CD8+ T cells in host defense mechanism against infection with various pathogens including mycobacteria.
With the aim to study the immunomodulatory effects of IL-15 in vivo, we examined cell-mediated immunity against BCG in IL-15-Tg mice. We found that purified protein derivative (PPD)-specific CD8+ Tc1 responses producing IFN-γ were significantly augmented in IL-15-Tg mice coincident with a marked increase of NK cells. Neutralization of endogenous IFN-γ and depletion of NK or CD8+ T cells by in vivo administration of Abs deteriorated the clearance of the bacteria. Overexpression of IL-15 in vivo enhanced protection against BCG infection via augmentation of NK and Tc1 responses.

Materials and Methods

Animals

IL-15-Tg mice, which were constructed using originally described IL-15 cDNA under the control of an MHC class I promoter, have been described previously (37). In brief, full-length cDNA encoding the murine IL-15 gene with the originally described exon 5 (710 bp) was cloned between the BamHI and SalI sites of a Tg expression vector, pHSE-3'. which contains the H2-K promoter and Ig enhancer, and β-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 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 C57BL/6 mice, purchased from Charles River Japan (Hino, Japan), were used as a control. 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.

Microorganisms

Lyophilized M. bovis BCG were purchased from Kyowa Pharmaceuticals (Tokyo, Japan). BCG were dissolved in 7H9 medium (Difco, Detroit, MI), supplemented with albumin-dextrose-catalase enrichment (Difco). The viable bacterial numbers were determined by 7H10 (Difco) plate count using oleic acid-albumin-dextrose-catalase enrichment (Difco). Small aliquots of BCG suspended in 7H9 medium containing 10% glycerol were stored in −80°C until use. The concentration of bacteria was quantified by plate counting. Before use, the bacteria were washed three times with PBS, and resuspended in PBS. Mice were inoculated i.p. with 2–10 × 10^6 CFU of BCG in a volume of 100 μl PBS.

Abs and reagents

FITC-conjugated anti-C3d mAb (145-2C11), PE-conjugated anti-TCRβ mAb (H57-597), PE-conjugated anti-NK1.1 mAb (PK136), PE-conjugated anti-CD8 mAb (53-6.7), biotin-conjugated anti-TCRγδ mAb (GL3), Cy-Chrome-conjugated anti-CD4 mAb (GK1.5), FITC-conjugated anti-IFN-γ mAb (XM12), FITC-conjugated anti-IL-4 mAb (BVD4-1D11), FITC-conjugated rat IgG1 isotype control Ig, and FITC-conjugated rat IgG2b isotype control Ig were purchased from PharMingen (San Diego, CA). Staining with biotin-conjugated mAb was followed by treatment with streptavidin-Cy-Chrome (PharMingen). The 2G4 (anti-FcγRII/III-specific mAb, rat IgG1, producing hybridoma) was obtained from American Type Culture Collection (Manassas, VA). Anti-murine IL-15 sera were produced in Japan white rabbit (Japan SLC, Hamamatsu, Japan) by immunization with an emulsion containing approximately 0.5 mg E. coli-expressed murine IL-15 protein in the form of an insoluble inclusion body preparation in monophospholipid A, synthetic trehalose dicyclonolate, and cell wall skeleton exudate (Corixa, Hamilton, MT). A total of three booster injections was given each at 3-wk intervals after primary injection. One week after the last immunization, blood was collected from three booster injections was given each at 3-wk intervals after primary injection. A total of 3×10^7 CFU of BCG in a volume of 50 μl PBS and 100 μl Streptomycin, and 10 mM HEPES. Cells were plated and allowed to adhere for 2 h at 37°C in a humidified atmosphere of 95% air and 5% CO2 Nonadherent cells were used as mononuclear cells, and adherent cells were washed several times with HBSS. Adherent cells were collected by scraping with a rubber policeman, washed, and counted. More than 95% of the cells retained by this procedure were macrophages.

In vitro culture

Plastic nonadherent cells of PEC were subjected to an Ag stimulation assay for cytokine production. Nylon wool-passed plastic nonadherent cells of PEC from BCG-infected non-Tg or IL-15-Tg mice on days 0, 7, 14, and 28 were resuspended in RPMI 1640 and added to 96-well plates at a concentration of 2 × 10^5 cells/well. Cells were cultured without any stimulation, or with 5 μg/ml PPD (Japan BCG Association, Tokyo, Japan), or with 100 μg/ml anti-TCRβ mAb in the presence of mitomycin-treated splenocytes (2 × 10^5) from naive mice for 48 h at 37°C. Supernatants were collected and stored at −80°C until the cytokine assay. In some experiments, cells were pulsed with [3H]Tdr 6 h before harvesting; then [3H]Tdr incorporation was determined by liquid scintillation counting.

Cytokine ELISA

IFN-γ and IL-4 levels in the serum were determined by ELISA (Genzyme Diagnostics). ELISA for IL-15 in individual sera was performed in triplicate using purified anti-mouse IL-15 mAb (G277-3588; PharMingen), anti-mouse IL-15 rabbit antiserum, and anti-rabbit Ig, HRP-linked F(ab')2 (Amersham Pharmacia Biotech, Uppsala, Sweden), or biotin-conjugated anti-mouse IL-15 mAb (G277-3586; BD Pharmingen) and peroxidase-conjugated streptavidin. Murine rIL-15 was used as positive control.

Expression of the inducible isofrom of NO synthase (iNOS) and IL-15 genes

Adherent PEC of IL-15-Tg or non-Tg mice on day 0 or 14 after BCG infection were applied. Total RNA was extracted from adherent PEC at specific times, basically according to the method of Chomczynski and Sacchi (40). First strand cDNA was synthesized from 2 μg RNA using reverse transcriptase and 20 pmol random primer in 20 μl reaction buffer. Synthesized first strand cDNA were amplified by PCR using 20 pmol of each primer, and 2.5 μl AmpliTaq Kit (Takara Shuzo, Kyoto, Japan) in a total volume of 50 μl reaction buffer consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, and 0.2 mM dNTP. PCR cycles were run for 1 min at 94°C, 1 min at 54°C, and 30 s at 72°C. Before the first cycle, a denaturation step for 5 min at 94°C was included, and after 35 cycles, the extension was prolonged for 2 min at 72°C. The specific primers were as follows: iNOS sense, 5′-AGC TCC TCC CAG GAC CAC AC-3′, and antisense, 5′-ACG GTG AGT AGC TCA TTA TG-3′; IL-15 exon 7–8 sense, 5′-GGT ATG TTC ACC CCA GTT GC-3′, and antisense, 5′-TCA CAT TCT TGT CAT CCA GA-3′; and β-actin sense, 5′-TGG AAT CTC-3′.
GTG GCA TCC ATG AAA C-3′; and antisense, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′. The PCR product was subjected to electrophoresis on a 1% agarose gel and then was transferred to GeneScreen+ filter (Dupont NEN, Boston, MA). The internal oligonucleotide probes were labeled with 32P-PATP using Megalabel 5′-labeling kit (Takara Shuzo), according to the manufacturer’s instructions. The internal oligonucleotide probes were as follows: iNOS, 5′-AAG GCC GGT GGG CAA GCC CGG AGA CCC TGT GCC CTG CT CAT C-3′; IL-15 exon 7–8, 5′-GCA ATG AAC TGC TTT CTC CT-3′; and β-actin, 5′-TTC TGC ATC CTG TCA GCA AT-3′. Prehybridization was performed by incubating the membrane in 1 M NaCl, 1% SDS, and 10% dextran sulfate for 1 h. In hybridization, the filters were incubated in 1 M NaCl, 1% SDS, 10% dextran sulfate, and 100 mg/ml heat-denatured salmon sperm DNA with labeled probes for 18 h at 60°C, and then the filters were washed in 2× SSC, 1% SDS for 15 min at 60°C. The radioactivity of each band of PCR product was analyzed with the Fujix BAS2000 Bio-image analyzer (Fuji Photo Film, Tokyo, Japan).

Flow cytometric (FCM) analysis
Plastic nonadherent cells of PEC were preincubated with a culture supernatant of 2.4G2 to prevent nonspecific staining. After washing, cells were stained with various combinations of mAbs. Staining with biotin-conjugated mAb was followed by treatment with streptavidin-Cy-Chrome. For three-color analysis of T cell subsets, single-cell suspensions were stained with FITC-conjugated CD3ε, PE-conjugated TCRαβ, NK1.1 mAbs, or biotin-conjugated TCRγδ, and analyzed with a FACSCalibur flow cytometer (BD Biosciences). The live lymphocytes were carefully gated by forward and side scattering. The data were analyzed with CellQuest software (BD Biosciences).

Intracellular cytokine staining
Splenocytes (2 × 10⁶ cells/ml) were incubated without any stimulation, or with 25 ng/ml PMA and 1 μg/ml ionomycin, or with 5 μg/ml PPD (Japan BCG Association) and 100 pg/ml rIL-2 (Takeda Chemical, Tokyo, Japan) for 6 h at 37°C and 5% CO₂, with 10 μg/ml brefeldin A (Sigma) added for the last 2 h in 24-well flat-bottom plates (Falcon; BD Biosciences) in a volume of 1 ml RPMI containing 10% FCS. After 6 h of culture, the cells were harvested, washed once in HBSS containing 2.5% newborn horse serum and 0.1% NaN₃ (staining buffer), and then subjected to intracellular cytokine staining using the FastImmune Cytokine System (BD Biosciences), according to the manufacturer’s instructions. For intracellular cytokine staining, we used FITC anti-IFN-γ, IL-4 mAbs, or FITC-conjugated rat IgG1 or IgG2b as isotype control. Samples were acquired in a FACSCalibur flow cytometer and analyzed by CellQuest software.

### Results

**Bacterial growth in IL-15-Tg mice following BCG infection**

To elucidate the ability of the IL-15-Tg mice to limit the bacterial growth against BCG infection, we examined the kinetics of bacterial growth in the peritoneal cavity, the liver, the lung, and the spleen of IL-15-Tg mice following an i.p. infection with 4.8 × 10⁶ CFU of BCG. As shown in Fig. 1, the bacterial number in each organ was decreased with time both in non-Tg and IL-15-Tg mice. However, the bacterial numbers in the peritoneal cavity on day 28 and in the liver or the lung on days 14 and 28 after BCG infection were significantly lower in IL-15-Tg mice than in non-Tg mice (*, \( p < 0.05 \), **, \( p < 0.005 \)). Similar tendency in bacterial growth was observed in the spleen (data not shown). Thus, IL-15-Tg mice were resistant against BCG infection, as assessed by bacterial growth.

**Serum cytokines and iNOS mRNA expression in the peritoneal macrophages in IL-15-Tg mice infected with BCG**

To compare IL-15 synthesis between IL-15-Tg and non-Tg mice following BCG infection, we next examined IL-15 expression at mRNA and protein levels in IL-15-Tg and non-Tg mice infected with BCG (Fig. 2, A and C). Consistent with previous report (37), IL-15 mRNA expression in the adherent PEC and IL-15 production in the serum were detected in naive IL-15-Tg mice before infection. IL-15 expression was detected in non-Tg mice after BCG infection, and the levels were much higher in IL-15-Tg mice than those in non-Tg mice on days 14 and 28 after BCG infection (Fig. 2, A and C, \( p < 0.05 \)).

We next examined the serum levels of IFN-γ and IL-4 after BCG infection. As shown in Fig. 2B, the IFN-γ levels in serum of IL-15-Tg mice were significantly higher on days 14 and 28 than those in non-Tg mice. Serum IL-4 was not detected in either IL-15-Tg or non-Tg mice at any stage of BCG infection.

High output NO production induced by iNOS is a major antimicrobial mechanism, and iNOS mRNA induction is known to be significant in macrophages infected with mycobacteria (*, \( p < 0.05 \); **, \( p < 0.005 \)).
regulated by IFN-γ and TNF-α (41–43). We compared iNOS mRNA in macrophages from IL-15-Tg or non-Tg mice on day 14 after BCG infection. As shown in Fig. 2C, the macrophages in the peritoneal cavity of BCG-infected IL-15-Tg mice expressed a higher level of the iNOS mRNA as compared with those of BCG-infected non-Tg mice.

**Kinetics of peritoneal exudate lymphocytes in IL-15-Tg mice following infection with BCG**

FCM analysis for expression of CD3e, TCRαβ, TCRγδ, NK1.1, CD4, and CD8 was conducted on nonadherent PEC on days 7, 14, and 28 after infection. A typical result was shown in Fig. 3 and the data were summarized in Table I. The proportions of CD3− NK1.1+ cells in the peritoneal cavity of non-Tg mice and IL-15-Tg mice before infection were 3.02 ± 0.03% and 3.14 ± 0.09%, respectively. The proportions of CD3− NK1.1+ cells increased and reached to ~9.2% in non-Tg mice on day 7 after BCG infection and then rapidly decreased to 4.5% by day 28 after BCG infection. Similar to non-Tg mice, CD3− NK1.1+ cells were increased in IL-15-Tg mice on day 7 after BCG infection, whereas, in contrast to non-Tg mice, the proportions of CD3− NK1.1+ cells remained at a higher level on day 28 after BCG infection. Similarly, NK1.1+ T cells were increased more in IL-15-Tg mice than those in non-Tg mice on days 7 and 14 after BCG infection, and the number of NK1.1+ T cells remained at an increased level on day 28 in IL-15-Tg mice. The number of CD3− TCRγδ+ T cells in non-Tg mice and IL-15-Tg mice was significantly increased in the peritoneal cavity on days 7 and 14, and the increase was more prominent in IL-15-Tg mice on these stages. The TCRγδ+ T cells were decreased in both non-Tg and IL-15-Tg mice on day 28 after BCG infection. As reported previously (37), the number of CD8+ T cells was larger in the peritoneal cavity of naive IL-15-Tg mice without BCG infection compared with non-Tg mice. The number of CD8+ T cells remained at a high level during the course of BCG infection, although the proportion of CD4+ T cells gradually increased in the peritoneal cavity of IL-15-Tg mice by day 28 after BCG infection.

**Cytokine production by Ag-stimulated T cells in the PEC of IL-15-Tg mice after infection with BCG**

To investigate whether Ag-specific T cells were generated more efficiently in IL-15 mice during the course of BCG infection, T cells were isolated from PEC of the mice before and on day 14 post-i.p. infection with BCG, and were cultured with or without PPD in the presence of APC, or on anti-TCRαβ mAb-coated dishes, and the culture supernatants were examined for IFN-γ release by ELISA. The proliferative response and IFN-γ production of T cells from IL-15-Tg mice were much the same level in response to TCRαβ cross-linking for 48 h as those from non-Tg mice (Fig. 4). On the other hand, T cells from IL-15-Tg mice on day 14 after infection produced significantly higher level of IFN-γ in response to PPD than those from non-Tg mice. Those results suggest that BCG-specific Th1/Tc1 cells are more efficiently generated in IL-15-Tg mice following BCG infection (p < 0.05). We further examined IL-4 or IL-10 production by T cells from non-Tg mice or IL-15-Tg mice infected with BCG. T cells from neither non-Tg mice nor IL-15-Tg mice produced IL-4 or IL-10 in response to PPD, suggesting that PPD-specific Th2 or regulatory T cells (44) are not generated in IL-15-Tg mice (data not shown).

To determine which population of T cells produces IFN-γ in response to PPD, we utilized cytokine FACS analysis for expression of CD4, CD8, and intracellular IFN-γ. A typical result is shown in Fig. 5 and data were summarized in Table II. As shown [in Fig. 5](#), most of the IFN-γ-producing T cells in response to PPD and rIL-2 were of CD4+ phenotype in non-Tg mice infected with BCG 14 days previously, and the relative number of the CD4+ Tc1 cells was significantly higher in IL-15-Tg mice on day 14 after BCG infection (p < 0.05, Table II). Notably, large number of CD8+ T cells from the BCG-infected IL-15-Tg mice produced IFN-γ in response to PPD and rIL-2 (Fig. 5, Table II). Such Tc1 cells were not detected in non-Tg mice on day 14 after BCG infection. These results indicated that the generation of CD8+ Tc1

**FIGURE 2.** Cytokine production in serum and iNOS mRNA expression in adherent PEC in IL-15-Tg or non-Tg mice after BCG challenge. IL-15-Tg and non-Tg mice were challenged i.p. with 5 × 10⁶ CFU of BCG. A, ELISA for IL-15 in individual sera was performed using purified anti-mouse IL-15 mAb (G277-3588), anti-mouse IL-15 rabbit antiserum, and anti-rabbit Ig, HRP-linked F(ab′)₂. B, Concentrations of IFN-γ in individual sera were determined by ELISA. C, Total RNA extracted from adherent PEC pooled from three mice of each group by the acid guanidium thiocyanate-phenol-choroform method on day 0 or 14 after infection was reverse transcribed, and the cDNA was amplified using primers specific for iNOS, IL-15, and β-actin. Data were obtained from at least three separate experiments and were expressed as the mean ± SD of five mice of each group from a representative experiment. Statistical analysis was performed with Student’s t test. *, Significant difference from the value for non-Tg mice infected with BCG, p < 0.05.
cells was significantly increased in IL-15-Tg mice infected with BCG.

**Effects of neutralization of endogenous IFN-γ on bacterial growth in IL-15-Tg mice during BCG infection**

IFN-γ is known to play a critical role in protection against mycobacterial infection (1, 2). We examined the effects of neutralization of endogenous IFN-γ by in vivo administration of anti-IFN-γ mAb on mycobacterial growth in the peritoneal cavity, the liver, the lung, and the spleen of IL-15-Tg mice after BCG infection. IL-15-Tg and non-Tg mice were treated with anti-IFN-γ mAb or control rat IgG by s.c. implanted ALZET osmotic pump on day 0 after an i.p. infection with 5 × 10⁶ CFU of BCG. The numbers of bacteria recovered from the peritoneal cavity, the liver, or the lung of infected mice on day 14 were determined. As shown in Fig. 6, the bacterial numbers in the peritoneal cavity, the liver, or the lung of non-Tg mice and IL-15-Tg mice, both of which were treated with anti-IFN-γ mAb, were significantly higher on days 14 after BCG infection than those in isotype control Ab-treated non-Tg and IL-15-Tg mice, respectively. Similar tendency in bacterial growth was observed in the spleen (data not shown). Thus, these results indicated that IFN-γ was a key cytokine in control of mycobacterial infection both in non-Tg mice and IL-15-Tg mice.

**Effects of depletion of NK cells or CD8⁺ T cells on bacterial growth in IL-15-Tg mice after BCG infection**

Both NK cells and CD8⁺ cells capable of producing IFN-γ were increased in IL-15-Tg mice during BCG infection. To investigate the contribution of NK cells and/or CD8⁺ T cells to protection against BCG infection in IL-15-Tg mice, anti-asialo-GM1 Ab or anti-CD8 mAb were administered i.p. 2 days before, and 5 and 12 days after an i.p. inoculation with 5 × 10⁶ CFU of BCG. We
confirmed by FACS analysis that NK or CD8 \(^*\) T cells were almost depleted in the spleen and the liver of IL-15-Tg mice on day 14 after BCG infection (data not shown). Bacterial counts in the peritoneal cavity and the liver of the Ab-treated IL-15-Tg mice were determined on day 14 after BCG infection. As shown in Fig. 7A, the numbers in the peritoneal cavity and the liver of IL-15-Tg mice treated with anti-asialo-GM\(_1\) Ab were significantly larger on day 14 after infection than those in control Ab-treated IL-15-Tg mice (\(p<0.01\)). On the other hand, the number was only marginally increased in the liver of non-Tg mice treated with anti-asialo-GM\(_1\) Ab. When IL-15-Tg mice were treated with anti-CD8 mAb, the numbers of BCG were markedly greater in the peritoneal cavity and the liver on day 14 than those in isotype control Ab-treated IL-15-Tg mice (\(p<0.01\), Fig. 7B). Non-Tg mice depleted of CD8\(^*\) T cells showed a slight increase in number in the peritoneal cavity or the liver on day 14 after BCG infection. These results suggested that both NK cells and CD8\(^*\) T cells contributed to the enhanced protection against BCG infection in IL-15-Tg mice.

**FIGURE 4.** Proliferative response and IFN-\(\gamma\) production by Ag-stimulated T cells in the PEC of IL-15-Tg mice after infection with BCG. IL-15-Tg mice and non-Tg mice were challenged i.p. with \(5 \times 10^6\) CFU of BCG. The enriched peritoneal exudate T cells (\(2 \times 10^5\) cells) on day 0 or 14 after infection were cultured with PPD (5 \(\mu\)g/ml) in the presence of mitomycin C-treated spleen cells (\(1 \times 10^5\) cells) from naive mice or an immobilized anti-TCR\(\alpha\) mAb for 48 h at 37\(^\circ\)C. After the incubation period, proliferative responses were assayed by \[^{3}H\]thymidine incorporation during the final 6 h of incubation (A). The concentrations of IFN-\(\gamma\) in the culture supernatants of peritoneal exudate T cells from IL-15-Tg mice or non-Tg mice on day 0 or 14 after infection were determined by ELISA (B). Statistical analysis was performed with Student’s \(t\) test. *, Significantly different from the value for non-Tg mice infected with BCG (\(p<0.05\)).

### Table I. Lymphocyte subsets of the PEC from IL-15-Tg mice or non-Tg mice after BCG infection

<table>
<thead>
<tr>
<th>Days after (M.) (\text{bovis}) BCG Infection</th>
<th>No. of Cells ((\times 10^5)/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD3(^+)TCR(\alpha)(\beta)(^+)</td>
</tr>
<tr>
<td>Non-Tg</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6.78 (\pm) 2.21</td>
</tr>
<tr>
<td>7</td>
<td>18.43 (\pm) 3.56</td>
</tr>
<tr>
<td>14</td>
<td>31.64 (\pm) 5.42</td>
</tr>
<tr>
<td>28</td>
<td>17.87 (\pm) 3.04</td>
</tr>
<tr>
<td>IL-15-Tg</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8.28 (\pm) 2.43</td>
</tr>
<tr>
<td>7</td>
<td>28.22 (\pm) 4.27*</td>
</tr>
<tr>
<td>14</td>
<td>93.97 (\pm) 7.87*</td>
</tr>
<tr>
<td>28</td>
<td>81.11 (\pm) 9.81*</td>
</tr>
</tbody>
</table>

\(\alpha\) TCR, \(\gamma\) TCR, \(\beta\) TCR. Mice were infected i.p. with BCG (\(5 \times 10^6\) CFU). Nonadherent PEC were recovered on indicated day (days 0, 7, 14, and 28), and were stained with FITC-conjugated anti-CD3e mAb, biotin-conjugated anti-TCR\(\gamma\)\(\delta\) mAb, PE-conjugated anti-TCR\(\alpha\) mAb, or anti-NK1.1 mAb, and staining with biotin-conjugated mAb was followed by treatment with streptavidin-Cy-Chrome. Samples were analyzed by FACSCalibur flow cytometer. The absolute number of each subset was calculated by amplifying the absolute number of whole nonadherent PEC by the percentage of each subset. Data are represented as the mean \(\pm\) SD of five mice. *Significantly different from the value for non-Tg mice infected with BCG (\(p<0.001\)).
In this study, we investigated cell-mediated immunity against primary infection with avirulent strain of BCG (Tokyo strain) in IL-15-Tg mice and found that the IL-15-Tg mice showed resistance against primary BCG infection accompanied by marked increases in NK cells and Tc1 response mediated by PPD-specific CD8\(^+\) T cells producing IFN-\(\gamma\). IFN-\(\gamma\) level in serum was significantly higher in IL-15-Tg mice than in non-Tg mice after BCG infection, and neutralization of endogenous IFN-\(\gamma\) deteriorated the clearance of the bacteria in IL-15-Tg mice. The peritoneal macrophages expressed the higher amounts of iNOS mRNA, suggesting that macrophages in IL-15-Tg mice are more immunologically activated following BCG infection. Taken together, overexpression of IL-15 in vivo may enhance protection against BCG infection via increased IFN-\(\gamma\) production and macrophage activation.

**Discussion**

In this study, we investigated cell-mediated immunity against primary infection with avirulent strain of BCG (Tokyo strain) in IL-15-Tg mice and found that the IL-15-Tg mice showed resistance against primary BCG infection accompanied by marked increases in NK cells and Tc1 response mediated by PPD-specific CD8\(^+\) T cells producing IFN-\(\gamma\). IFN-\(\gamma\) level in serum was significantly higher in IL-15-Tg mice than in non-Tg mice after BCG infection, and neutralization of endogenous IFN-\(\gamma\) deteriorated the clearance of the bacteria in IL-15-Tg mice. The peritoneal macrophages expressed the higher amounts of iNOS mRNA, suggesting that macrophages in IL-15-Tg mice are more immunologically activated following BCG infection. Taken together, overexpression of IL-15 in vivo may enhance protection against BCG infection via increased IFN-\(\gamma\) production and macrophage activation.

**Table II.** Intracellular IFN-\(\gamma\)-positive cells in the spleen T cells from IL-15-Tg or non-Tg mice after BCG infection

<table>
<thead>
<tr>
<th>Stimulation With</th>
<th>Before Infection</th>
<th>After Infection</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>PPD + rIL-2</td>
</tr>
<tr>
<td>% of IFN-(\gamma)-producing T cells within CD4(^+) T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Tg</td>
<td>3.68 ± 2.32</td>
<td>5.82 ± 1.80</td>
</tr>
<tr>
<td>IL-15-Tg</td>
<td>2.02 ± 0.55</td>
<td>2.04 ± 0.92</td>
</tr>
<tr>
<td>% of IFN-(\gamma)-producing T cells within CD8(^+) T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Tg</td>
<td>0.96 ± 0.42</td>
<td>1.33 ± 0.91</td>
</tr>
<tr>
<td>IL-15-Tg</td>
<td>0.63 ± 0.47</td>
<td>0.57 ± 0.23</td>
</tr>
</tbody>
</table>

\(952\) M. bovis BCG INFECTION IN IL-15-Tg MICE

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non-Tg mice, but NK cells were markedly increased in IL-15-Tg mice on day 7 after BCG infection and remained high on day 28 after BCG infection. Depletion of NK cells hampered the exclusion of bacteria in IL-15-Tg mice after BCG infection. We have previously reported that NK cells increased at the earlier stage after avirulent strain of S. choleraesuis and neutralization of endogenous IL-15 by in vivo administration with anti-IL-15 mAb inhibited the appearance of NK cells and exacerbated the infection (25, 36, 37). Taken together, it appears that IL-15 may serve to induce proliferation and/or accumulation of NK cells during BCG infection, and the increase in number of NK cells is at least partly responsible for the enhanced resistance against BCG infection in IL-15-Tg mice.

IL-15 also plays important roles in proliferation, accumulation, and maintenance of NKT cells (24, 45). Our current results reveal that NKT cells were increased and remained at a higher level in IL-15-Tg mice compared with non-Tg mice following BCG infection (Fig. 3 and Table I). NKT cells are known to recognize glycolipids, including phosphatidylinositol containing compound from bacteria in the context of CD1d molecules (46, 47). CD1 gene knockout mice that are deficient in NKT cells are not susceptible to M. tuberculosis infection, suggesting that NKT cells made no contribution to immunity (8). However, it remains unknown whether NKT cells play protective role in infection with less virulent mycobacteria, such as BCG. Studies with TCR\(\gamma\delta\) gene knockout mice suggested that TCR\(\gamma\delta\) T cells play a role in granuloma formation to mycobacteria (48). We previously reported that TCR\(\gamma\delta\) T cells increased on day 7 after BCG infection well before Ag-specific TCR\(\alpha\beta\) T cells appeared (49). Consistent with this finding, TCR \(\gamma\delta\) T cells increased drastically on day 7 and thereafter rapidly decreased in IL-15-Tg mice following BCG infection. We have previously reported that TCR\(\gamma\delta\) T cells proliferated in response to IL-15 during the course of Salmonella (25) or E. coli (35). IL-15 may serve to induce accumulation and expansion of TCR\(\gamma\delta\) T cells in the inflamed sites, but may not be able to maintain TCR\(\gamma\delta\) T cells in contrast to the cases of NK cells and CD8\(^+\) T cells. TCR\(\gamma\delta\) T cells are reported to be easily subjected to activation-induced cell death by apoptosis (50, 51). Therefore, it is possible that activated TCR\(\gamma\delta\) T cells may be subjected to activation-induced apoptosis even in the presence of IL-15, whereas IL-15 may protect NK cells and CD8\(^+\) T cells from activation-induced cell death.

A notable finding in the present study is that IL-15-Tg mice developed a significant level of Ag-specific Tc1 response after BCG infection and the CD8\(^+\) T cells contribute to protection against BCG infection in IL-15-Tg mice. IL-15 is known to be important for proliferation and maturation of memory-type CD8\(^+\) T cells (22–24, 52). We previously reported that CD8\(^+\) T cells expressing memory markers, CD44\(^+\), Ly-6C\(^-\), and CD69\(^-\) of the phenotype, were increased in naive IL-15-Tg mice (37). Furthermore, we found that IL-15-Tg mice preferentially developed Ag-specific CD8\(^+\) Tc1 responses, producing IFN-\(\gamma\), and generated a significantly lower level of IgE, but a higher level of IgG2a specific for OVA after OVA sensitization (53). Thus, overexpression of IL-15 helps to induce Ag-specific CD8\(^+\) Tc1 cells and maintain the CD8\(^+\) T cells, exerting a strong antibacterial activity in IL-15-Tg mice. Recent studies suggest that CD8 CTL releasing perforin and granulysin play a role in protection against M. tuberculosis infection via cytolsis mechanism (9, 10). We do not know the relative contribution of cytotoxicity and IFN-\(\gamma\) production by CD8\(^+\) T cells to protection against BCG infection in IL-15-Tg mice. The resistance to M. tuberculosis infection in perforin-deficient mice was unaltered, suggesting that the cytotoxic function of CD8\(^+\) T cells may not be critical in protection to tuberculosis (11, 12). Studies with IFN-\(\gamma\)-knockout mice revealed that protection against infection with M. tuberculosis depends on IFN-\(\gamma\) (1, 2). Our results also showed that neutralization of endogenous IFN-\(\gamma\) hampered the resolution of BCG infection. Taken together, we speculate that IL-15-dependent Tc1 cells play a critical role in enhanced protection against BCG infection in IL-15-Tg mice via IFN-\(\gamma\) production. Maeurer et al. (54) have recently reported that administration of exogenous IL-15 3 wk after M. tuberculosis infection prolonged the survival rate of BALB/c mice, although apparent Tc1 response was not detected in these mice. IL-15-Tg mice, which constitutively produced IL-15 during the course of mycobacterial infection, exhibited an enhanced Tc1 response, whereas administration of IL-15 only at effector phase during infection might not affect the generation of Tc1 cells. Alternatively, we determined. Data were obtained from at least three separate experiments and were expressed as the mean \(\pm\) SD of three mice from a representative experiment. *, Significantly different from the value for mice treated with control Ab (\(p < 0.01\)).
M. tuberculosis is known to suppress in vitro response of T cells partly via NO induction (55), which may inhibit Tc1 response against M. tuberculosis-infected APC in vitro in their study. CD8+ T cells from BCG-infected IL-15-Tg mice produced IFN-γ in response to exogenous PPD in the presence of APC. Exogenous particulate Ags such as PPD are usually present in association with MHC class II, whereas recent reports have suggested that there is a degree of degeneracy in the processing pathways and that Ags in the extracellular milieu can also be processed and presented in association with MHC class I (56–59), which are usually required for activation of CD8+ T cells. Therefore, it is possible that PPD may be presented by MHC class I on APC in alternative processing pathway. However, TAP-deficient mice are reported to be susceptible to M. tuberculosis infection, indicating that TAP-dependent Ag presentation, a major pathway for activation of CD8+ T cells, is critical for protection against M. tuberculosis infection (8). PPD may contain Ag that can bind directly to MHC class I on APC, which activate Tc1 cells in IL-15-Tg mice infected with BCG.

The relative contribution of NK cells and CD8+ T cells to protection against BCG infection in IL-15-Tg mice remains to be elucidated. Either in vivo administration of anti-asialo-GM1 Ab or anti-CD8 mAb abrogated the antibacterial activity, suggesting that both NK cells and CD8+ T cells are required for protection against BCG infection in IL-15-Tg mice. Th1 cells, into which naive CD4+ T cells differentiate in the presence of IL-12 and IFN-γ, secrete IL-2, IFN-γ, and TNF-α for induction of cell-mediated immunity (60–62). Thus, early production of IFN-γ by NK cells may be responsible for development of Tc1 responses in IL-15-Tg mice. Alternatively, asialo-GM1 is expressed not only by NK cells, but also some subset of T cells such as native CD8+ T cells, antiviral CD8+ T cells, and alloreactive CD8+ T cells (63). In vivo treatment with anti-asialo-GM1 thereby may deplete asialo-GM1+ CD8+ Tc1 cells, which are responsible for antibacterial activity in IL-15-Tg mice. This speculation warrants further examination of the characteristics of Ag-specific CD8+ T cells in IL-15-Tg mice.

Infection of mice with the less virulent BCG consistently found that CD8+ T cells made no contribution to immunity in normal mice (64, 65). Our results also showed that depletion of CD8+ T cells in non-Tg mice was less effective on the bacterial clearance compared with IL-15-Tg mice. Although BCG has been used as a vaccine, it confers incomplete protection against tuberculosis, at least in adults (14). Therefore, it is important to establish effective vaccination to induce strong and long-lasting immunity against M. tuberculosis infection. It is likely that host cytokine cascade may have the most pronounced and long-lasting effects on the T cells’ responses. Recently, Freidag et al. (66) reported that IL-12 improved the efficacy of BCG vaccination in mice challenged with M. tuberculosis. Kremer et al. (67) showed that intradermal injection with DNA encoding IL-18 led to increased in vitro PPD-dependent IFN-γ production in mice infected with BCG. These results imply that these cytokines can be used as immune adjuvant given with BCG vaccination to increase efficacy of BCG vaccination. Since CD8+ Tc1 cells, besides CD4+ Th1 cells, are critical in protection against virulent M. tuberculosis infection (5, 8, 68), our current data with IL-15-Tg mice may also suggest that IL-15 can be used as immune adjuvant to increase the efficacy of BCG vaccination via enhancing Tc1 response. Additional experiments with aerosol challenge with M. tuberculosis might allow us to define conclusively whether IL-15 is useful for development of new immunoprotective approaches against mycobacterial infection.

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References


