

Pulmonary Tuberculosis in Various Gene Knockout Mice With Special Emphasis on Roles of Cytokines and Transcription Factors

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Abstract: The technique of gene targeting (knockout) has swept through biomedical research. Cytokine research has been revolutionized by knockouts and since then this technique has been widely utilized in various research fields including immunological, inflammation research and human disease model. This paper focuses on knockout mice in tuberculosis research among many infectious diseases. We have generated several knockout mice for inflammation research. After we infected various kinds of knockout mice suffering from *Mycobacterium tuberculosis* by aerosol infection, we investigated the roles of cytokines and transcription factors that regulate cytokines. We used knockout mice lacking IFN- γ , TNF- α , IL-18, IL-1 β , IL-4, IL-1 type 1 receptor, NF-IL6, TLR-2, TLR-6, interferon regulatory factor-1 (IRF-1), NK- B p50, signal transducer and activator of transcription (STAT)1, STAT4, NKT cells and MyD88 genes in our experimental tuberculosis research. *M. tuberculosis*-infected knockout mice displayed various histopathologies depending on the degree of importance of the molecules in defense against tuberculosis. IFN- γ , TNF- α , IRF-1, NF-IL6, NF- B p50, STAT1 and STAT4 knockout mice succumbed to *M. tuberculosis* infection over time. The results indicate that these molecules play major roles for defense against tuberculosis. These knockout mice are essential for investigating their roles in experimental tuberculosis.

Keywords: Knockout mice, tuberculosis, mycobacterial infection, cytokine, transcription factor.

INTRODUCTION

Cytokine research has been revolutionized by the technique of gene targeting. The technique provides an acid test of the function of a gene. It involves creating deletion in one designated gene in an embryonic stem (ES) cell line, and then producing mice with just one disrupted gene from that cell line. The mice are tested for physiological abnormalities. Hundreds of genes have now been knocked out and the results have changed many paradigms [1].

This technique is also utilized for tuberculosis research because tuberculosis development involves many cells, various cytokines, receptors and transcription factors. Although tuberculosis remains a major health problem worldwide, its molecular mechanism remains to be solved. We and other researchers have demonstrated that IFN- γ is critical to defense against mycobacterial infection. TNF- α , IL-1, IL-12 and IL-18, which belong to the group of proinflammatory cytokines and are produced by activated macrophages, are also important. Knockout mice are suitable for defining the roles of the molecules in antimycobacterial immunity. This paper focuses on the roles of cytokines and transcription factors that regulate cytokine functions in experimental tuberculosis.

GENERATION OF KNOCKOUT MICE AND AEROSOL INFECTION

1. Bacterial Strains

Virulent *M. tuberculosis* H37Rv (ATCC25618), Kurono strain (ATCC35812) and BCG Pasteur (ATCC27289) were

passed through mice and grown in 7H9 liquid medium once before storing in aliquots at -85°C and tittered. The cultured strains were filtered with a membrane filter of 5 μ m pore size before use so that they were dispersed evenly.

2. Generation of Various Knockout Mice

1) IFN- γ Knockout Mice

The *Bam* HI fragment (10.5 kb) of the murine IFN- γ genomic DNA clone was kindly provided by Dr. H. A. Young (NIH, USA). A *Kpn* I fragment was subcloned from the *Bam* HI fragment and placed into Bluescript II. Then *LacZ* gene and the neomycin-resistant gene (*neo*) were inserted into the newly created *Hind* III and the *Cla* I site of the IFN- γ gene in which exon 1 was disrupted. The targeting vector was linearized with *Not* I. After electroporation and selection of A3-1 embryonic stem (ES) cells from a 129/SvJ mouse with the targeting vector, 10 ES cells were injected into the blastocoelic cavity and the blastocysts were cultured for 1-2 hr in modified BWW medium. Embryos with visible blastocoele cavities were transferred to the uterus of pseudopregnant random-bred ICR recipients 2.5 days after coitus. The chimeric mice were crossed with wild-type mice, and the F2 generations were used to breed the homozygous IFN- γ -deficient mice that were used in the present experiments [2].

2) TNF- α Knockout Mice

In our knockout mice, a *Hind* III-*Hinc*II fragment containing exon III and part of exon IV was replaced by a 1.8 kb *pgk-neo* gene derived from pKJ1. Germline transmission of the TNF- α mutation was confirmed by Southern blot analysis of murine tail DNA. Homozygotes were crossed to each other to expand the colony, and their progeny was used in the experiments [3].

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3) *IL-18 Knockout Mice*

We disrupted the IL-18 gene by homologous recombination in E14.1 ES cells, and a targeting vector was constructed to replace a 3.0 kb fragment of genomic DNA containing exons 3, 4 and 5 of the IL-18 gene with the neomycin resistance gene [4]. Heterozygous mice were crossed to produce mice homozygous for the IL-18 gene mutation. IL-18-deficient C57BL/6 mice were born at the expected mendelian ratios and were phenotypically normal and fertile. Their serum IL-18 concentrations, assessed by ELISA, were below the detectable level.

4) *IL-1 / Double Knockout Mice*

IL-1 and IL-1 genomic clones were isolated from mouse 129 genomic phage libraries. For the IL-1 targeting vector, a 1.5 kb DNA fragment lying between the *Sau3AI* and *KpnI* sites in exon 5 and intron 5, including the NH₂ terminal coding region for mature IL-1, was deleted, and a *lacZ*-pA-PGK-*hpn*-pA cassette was inserted in its place. For the IL-1 targeting vector, a 2.45 kb DNA fragment situated between the *HincII* and *BstXI* sites in exons 3 and 5 including the NH₂ terminal coding region for mature IL-1, was deleted, and a *lacZ*-pA-PGK-*neo*-pA cassette was inserted in its place [5]. ES cells were electroporated with 20-25 µg of the linearized IL-1 and IL-1 targeting vectors per 10⁷ cells. Mice heterozygous for the resulting IL-1 and IL-1 mutations were intercrossed to yield mice homozygous for the mutation. All of the knockout mice were fertile, and the pups were born healthy. After birth, the IL-1 / knockout mice developed normally.

5) *IL-1 Type 1 Receptor Knockout Mice*

IL-1 R1-deficient mice with a C57BL/6 background were generated by Labow *et al.* [6]. They were purchased from Jackson Laboratories, Bar Harbor, Maine.

6) *IL-4 Knockout Mice*

The generation of gene-targeted IL-4 knockout mice of BALB/c origin and their immunocompetent counterparts, wild-type MALB/c mice has been described elsewhere [7].

7) *NF-IL6 Knockout Mice*

An 11-kb genomic fragment spanning from 8.5 kb 5' of the end of the exon was subcloned into pUC18 plasmid vector. The MC1-herpes simplex virus thymidine kinase was inserted into the unique *HindIII* site in the 5' end of the homologous region. E14-1 ES cells were electroporated in 800 µl of PBS with 32 µg of *SalI*-linearized targeting vector DNA in a Bio-Rad gene Pulser. G418- and gancyclovir-resistant colonies were picked up 10 to 12 days later. Homologous recombination was screened by PCR and subsequently confirmed by genomic southern blot hybridization [8].

8) *NF- B p50 Knockout Mice*

NF- B p50 knockout mice of C57BL/6 origin whose exon 6 of the *Nfkb1* gene had been disrupted by insertion of a vector containing the *neo* resistance gene were purchased from Jackson Laboratories [9].

9) *IRF-1 Knockout Mice*

C57BL/6 IRF-1 knockout mice were purchased from Jackson Laboratories [10].

10) *STAT1 and STAT4 Knockout Mice*

STAT1 knockout mice were purchased from Taconic Transgenic Models via Immuno-Biological Laboratories Co. (Gunma, Japan) [11]. BALB/c STAT4 knockout mice were purchased from Jackson laboratories [12].

11) *TLR2 and TLR6 Knockout Mice*

TLR2 and TLR6 knockout mice of C57BL/6 origin were generated as described previously [13]. The TLR2 and TLR6 knockout mice did not show any developmental abnormalities.

12) *MyD88 Knockout Mice*

C57BL/6 MyD88 knockout mice were supplied by Dr. Shizuo Akira, Osaka University [14]. These knockout mice showed no developmental abnormalities. All mice were housed in a biosafety level 3 facility and given mouse chow and water *ad libitum* after aerosol infection with virulent mycobacteria.

13) *NKT Cell Knockout Mice*

To generate NKT cell knockout mice, J 281 gene was disrupted by homologous recombination [15]. For demonstration of J 281-deficient mice, PCR using J 281-specific primer sets was performed. The 1.8 kb PCR product using primer sets (CJ-2 and IM-*neo* 2) indicated J 281 mutants and the 1.5 kb PCR product using primer sets (CJ-2 and CJ-4) indicated wild-type mice.

3. Experimental Aerosol Infection

H37Rv (ATCC25618) and A Kurono strain of *Mycobacterium tuberculosis* (ATCC 35812) was grown in Middlebrook 7H9 broth for 2 weeks, then filtered with a sterile acrodisc syringe filter with a pore size of 5.0 µm. Then, the aliquots of the filtrate bacterial solution were stored at -80°C until use. The mice were infected with the airborne route by placing them in the exposure chamber of the Glas-Col aerosol generator (Glas-Col, Inc., Terre Haute, Ind., USA). The nebulizer compartment was filled with 5 ml of a suspension containing 2x10⁶ cfu of H37Rv and Kurono tubercle bacilli so that approximately 200 bacteria might be deposited in the lungs of each animal. Inhalation infection experiments were carried out twice. The survival of groups of mice for 50 days after infection with Kurono strain was recorded, and survival curves were plotted.

4. CFU Assay

At 1, 3, 5, 7 and 12 weeks after aerosol infection, mice were anesthetized with pentobarbital sodium. The abdominal cavity was incised, and exsanguinations were performed by splenectomy. The left lobe of each lung and a part of spleen tissue separately were weighed and used to evaluate *in vivo* growth of mycobacteria. The lung and spleen tissues were homogenized with a set of mortar and pestle, and 1 ml of sterile physiological saline was added. Then, 100 µl of homogenates were picked up and plated in a 10-fold serial dilution on 1% Ogawa's egg media. Colonies on the media were counted after a 4-week incubation at 37°C.

5. Histopathology

For light microscopy, the rats were sacrificed 1, 3, 5, 7, and 12 weeks after infection. The tissue sections (5 μ m thick) that had been cut from paraffin blocks containing lung, liver, and spleen tissues were stained with hematoxylin and eosin or by the Ziehl-Neelsen method for acid-fast bacilli.

6. Cytokine Assays

Alveolar macrophages were prepared from the bronchoalveolar lavage (BAL) cells in order to determine which of the various cytokines they secreted (IL-12, TNF- α , IL-1 β and IFN- γ). After the mice had been anesthetized with 100 μ l pentobarbital, alveolar macrophages were obtained by bronchoalveolar lavage (BAL). Briefly, the murine trachea was cannulated and 1 ml physiological saline was poured in. The saline was recovered using a 1-ml disposable syringe. The cells thus obtained contained more than 99% macrophages as assessed by phagocytosis of BCG Tokyo. The cell suspensions (1×10^6 cells/well) were plated in 96-well culture plates and incubated for 24 hr at 37°C in 5% CO₂ in air, and the cells were stimulated with live *M. tuberculosis* Kuroono strain (multiplicity of infection; 10). The concentrations of IL-12, TNF- α , IL-1 β and IFN- γ in the culture supernatants of these cells were measured by sandwich enzyme-linked immunosorbent assay (ELISA) (Biosource International Inc., Calif., USA).

7. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Lung tissue samples were taken from infected mice 1, 3, 5, 7 and 12 weeks after infection, frozen in liquid nitrogen, and stored at -85°C until required for use, when RNA was extracted as described previously [21, 22]. Polymerase chain reaction was performed with equivalent amounts of cDNA of each sample, TAKARA EX *Taq* and gene-specific primer sets. Amplifications were performed with a DNA thermal cycler model 480 (Perkin-Elmer Cetus). Ten microliters of each PCR product was applied to electrophoresis in 4% agarose and NuSieve GTG (1:3) gel and visualized using ethidium bromide staining. The same amounts of β -actin RNA from the lung tissues were used as an internal control in the RT-PCR analysis.

RESULTS AND DISCUSSION

1. Roles of IFN- γ in Murine Tuberculosis

IFN- γ , a cytokine secreted by activated T cells, natural killer cells and natural killer T cells, has immunomodulatory effects on several cell types. IFN- γ is one of the major cytokines responsible for the activation of macrophages that mediate non-specific, cell-mediated host defense. To gain a better understanding of the pathological role of IFN- γ in specific mycobacterial granuloma formation, IFN- γ gene-deficient mice (BALB/c and C57BL/6) were produced. The IFN- γ gene in embryonic stem cells was disrupted by inserting the β -galactosidase gene (*lacZ*) and the neomycin-

resistant gene (*neo*) at the translation initiation site in exon 1 by homologous recombination (2). Six-week-old IFN- γ -deficient and wild-type mice were inoculated with 10^{3-7} tubercle bacilli of various strains of *M. tuberculosis* (Kuroono and H37Rv) and BCG Pasteur aerielly. The mice were examined 7 weeks later for pulmonary granuloma formation. The relatively avirulent BCG Pasteur and H37Ra strains induced granulomas in the lungs, spleen and liver of IFN- γ -deficient mice. Figure 1 shows a macroscopic view of the spleen. The IFN- γ knockout mice were infected with BCG Pasteur (B). Please note the prominent splenomegaly and granulomatous lesions of various sizes. No granulomatous lesions are seen in wild-type BALB/c mouse (A). The granulomas consisted of epithelioid macrophages and Langhans type multinucleated giant cells with central necrosis in long-term observation (9 months). The virulent Kuroono and H37Rv strains induced disseminated abscesses but not granulomas in various organs of IFN- γ -deficient mice and Mac-3-positive macrophages were not detected in the abscess lesions. These results suggest that IFN- γ may be primarily responsible for macrophage activation and that other factors may be involved in the granuloma formation mechanism [16].

2. Roles of TNF- α in Murine Tuberculosis

TNF- α is a cytokine with various activities that are induced by activated macrophages through signal transduction at two distinct receptors. It mediates inflammation and produces protective immunity against bacterial, parasitic, and viral infections, and it is thought to play a significant role in the pathogenesis of various diseases, including cancer. Of the several cytokines associated with the pathogenesis of tuberculosis, including IL-12 and IFN- γ , TNF- α is thought to be responsible for protection against the development of the disease. Kindler *et al.* showed that depletion of TNF- α using polyclonal antibodies blocked granuloma formation and impaired the ability to localize infection with BCG in mice [40]. Infusion of TNF- α has been shown increase resistance against *M. tuberculosis* and *M. avium* in mice. Clearly, there are conflicting data with respect to the role of TNF- α in granuloma formation. To study the role of TNF- α in mycobacterial infection, we generated TNF- α knockout mice, in which the third and fourth exons of the TNF- α gene were disrupted. The C57BL/6 KO mice were infected with virulent *M. tuberculosis* strain Kuroono or relatively avirulent bacillus BCG Pasteur (10^6 CFU), by inhalation exposure system (IES) as described previously. The major organs were removed at weekly intervals, and morphologic observation, assay of IL-1, IL-12, IFN- γ , and inducible nitric oxide synthase mRNA expression, and colony counts in the lungs and spleen were performed. Peritoneal and alveolar macrophages from BCG- and H37Rv strain-treated mice produced significant levels of nitric oxide after stimulation *in vitro*. Formation of abscesses was seen only in the Kuroono strain-treated groups, and these abscesses contained large numbers of mycobacteria. The administration of recombinant TNF- α significantly ameliorated the mycobacterial lesions. IFN- γ mRNA was expressed significantly in virulent H37Rv-treated groups with time, and the number of mycobacterial colonies per unit weight

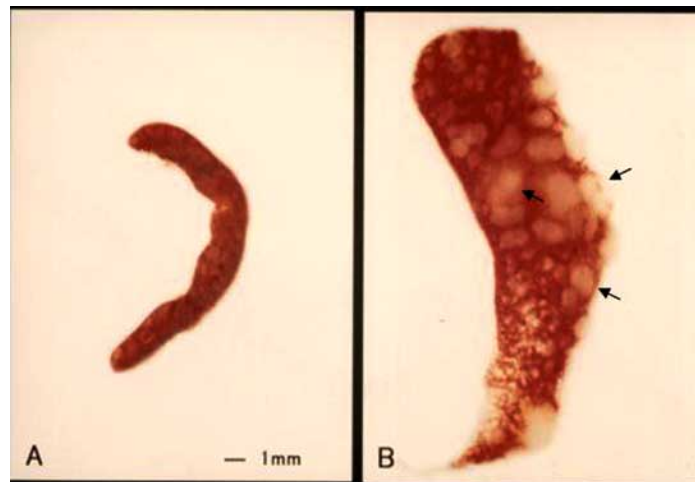


Fig. (1). Macroscopic views of the spleens from a wild-type BALB/c mouse infected with BCG Pasteur (A) and an IFN-gene-disrupted BALB/c mouse infected with BCG Pasteur. Arrows () indicate whitish nodules suggestive of granulomatous lesions.

increased remarkably with time. Nitric oxide production was not observed in H37Rv-treated groups but was seen in BCG-treated groups. Figure 2 shows effect of TNF- on survival of Mycobacterium-infected mice. C57BL/6 TNF- knockout mice with *M. tuberculosis* Kurono strain. These mice died of disseminated tuberculosis by the 60th day post-infection. We concluded that TNF- played an important role in protective immunity against virulent mycobacteria. Because avirulent mycobacteria did not induce granulomas in TNF-knockout mice, TNF- played an indirect role in granuloma formation [16].

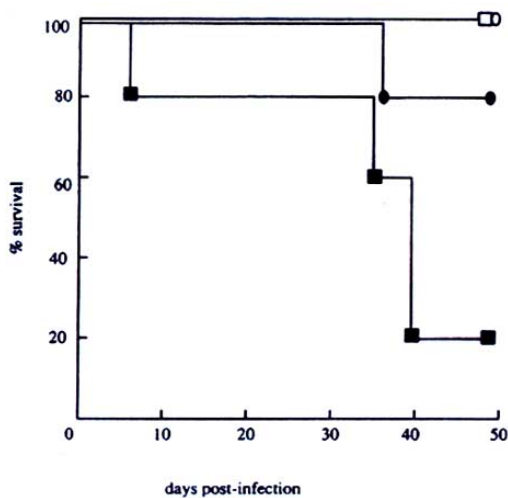


Fig. (2). Effect of TNF- gene disruption on survival of Mycobacterium-infected mice. The TNF- knockout mice were infected with BCG Pasteur (●), or Kurono strain (■). Wild-type mice (serving as controls) were infected with BCG Pasteur (○) or Kurono strain (□) and showed 100% survival.

3. The Roles of Other Cytokines

IL-18, IL-4, IL-1 type 1 receptor and IL-1 / as well as IFN- and TNF- play important roles in protective

immunity against mycobacterial infection. IL-18-, IL-4-, IL-1 R1 and IL-1 / knockout mice did not die when these KO mice were infected with virulent Kurono strain via an airborne route in our experiments [18-21]. It is thought that these cytokines are not essential for protection against mycobacterial infection or the functions by these cytokines are compensated by other cytokines. If we give a priority the cytokines in terms of roles in mycobacterial infection, we can describe a cytokine hierarchy in murine tuberculosis as shown in Figure 3. IFN- and TNF- are the most important cytokines in defense against tuberculosis.

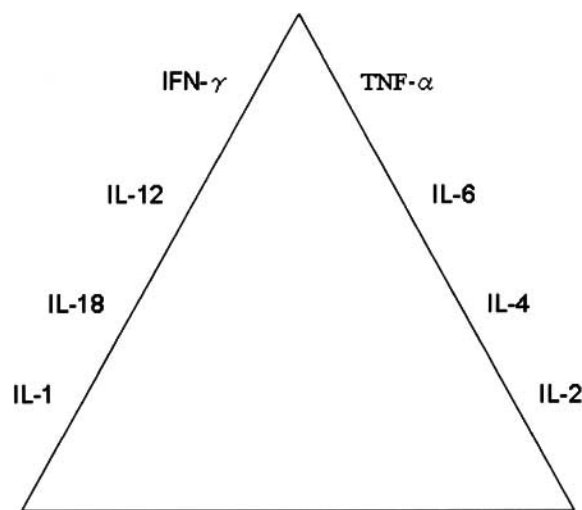


Fig. (3). Cytokine hierarchy in experimental tuberculosis. IFN- and TNF- are grand champions in defense against tuberculosis.

4. The Roles of Transcription Factors

NF-IL6 is one of several nuclear transcription factors. NF-IL6 and NF- B are expressed in macrophages and is induced by bacterial LPS. Airborne infection of these mice with *M. tuberculosis* induced disseminated tuberculosis

[22]. NF-IL6 is a critical transcription factor in mycobacterial control as well as in granulocyte-colony stimulating factor induction resulting in neutrophil activation.

The NF- B p50 knockout mice developed multifocal necrotic pulmonary lesions or lobar pneumonia. Pulmonary iNOS, IL-2, IFN- , and TNF- mRNA levels were significantly low [23].

IRFs are a family of 9 transcription factors possessing a novel helix-turn-helix DNA-binding motif [23]. Pulmonary iNOS mRNA expression level was significantly lower in these knockout mice. IRF-1 is indirectly responsible for iNOS mRNA expression and plays an important role in the pathogenesis of murine tuberculosis.

Signal transducer and activator of transcription (STAT) family consist of seven transcription factors. The STAT1 and STAT4 knockout mice succumbed to mycobacterial infection by the 35th and 80th day, respectively after infection [25, 26]. STAT1 regulates IL-12 expression and appear to be a critical transcription factor in controlling mycobacterial infection. Pulmonary iNOS, IFN- , - , -

mRNA levels were significantly lower in STAT4 knockout mice.

5. The Roles of TLR2 and MyD88

Several *in vitro* studies have suggested active participation of TLR2 in mycobacterial infection. Infected TLR2 knockout mice developed granulomatous pulmonary lesions with neutrophil infiltration, which were slightly larger in size than those in wild-type mice [27]. MyD88 acts as an adaptor molecule that plays an important role in TLR/IL-1 receptor/IL-18 receptor signaling. However, MyD88 deficiency did not influence the development of murine tuberculosis [28]. It seems that IFN- and TNF- play more important roles for defense against tuberculosis than TLR2 and MyD88 molecules.

6. The Role of NKT Cells

The NKT knockout mice developed granulomatous lesions in the lungs. The average CFU values increased 3 weeks post-infection, but decreased 9 and 11 weeks post-

Table 1. Characteristics of Various Knockout Mice Used in this Study (in order of Appearance)

Knockout mouse	Genetic deficiency	Mycobacterium	Outcome	Ref No.	Comment
IFN-	IFN-	<i>M. tuberculosis</i>	lethal	16	
		BCG Pasteur	larger granulomas		Langhans' giant cells
TNF-	TNF-	<i>M. tuberculosis</i> BCG Pasteur	lethal	17	
			larger granulomas		
IL-18	IL-18	<i>M. tuberculosis</i> BCG Pasteur	larger granulomas	18	
IL-1 /	IL-1 /	<i>M. tuberculosis</i>	larger granulomas	19	
IL-1	IL-1	<i>M. tuberculosis</i>	larger granulomas	20	
type 1 receptor	type 1 receptor				
IL-4	IL-4	<i>M. tuberculosis</i>	larger granulomas	21	
NF-IL6	NF-IL6	<i>M. tuberculosis</i>	lethal	22	
NF- B p50	NF- B p50	<i>M. tuberculosis</i>	lethal	23	
IRF-1	IRF-1	<i>M. tuberculosis</i>	lethal	24	
STAT1	STAT1	<i>M. tuberculosis</i>	lethal	25	
STAT4	STAT4	<i>M. tuberculosis</i>	lethal	26	
TLR2	TLR2	<i>M. tuberculosis</i>	larger granulomas	27	
TLR6	TLR6	<i>M. tuberculosis</i>	no difference	27	
MyD88	MyD88	<i>M. tuberculosis</i>	larger granulomas	28	
NKT	J 281	<i>M. tuberculosis</i>	no difference	29	

infection in the lungs of NKT knockout mice. Our data indicate that NKT cells play a detrimental role in late-phase mycobacterial infection, although Th1 cells are essential in early-phase mycobacterial infection [29].

Many knockout mice have been generated on a laboratory basis in the world and most of them are now available from Jackson laboratories (<http://www.jax.org>). We have described important findings obtained in a series of experimental murine tuberculosis research. What is clinical relevance to the murine tuberculosis? We have previously reported that serum IFN- γ levels are significantly low in advanced active TB patients [30]. It is also reported that people with IFN- γ receptor deficiency are susceptible to *M. tuberculosis* [31].

On the other hand, humanized anti-TNF- α neutralizing monoclonal antibody is applied to patients with rheumatoid arthritis and Crohn's disease whose serum TNF- α levels are low [32]. These patients develop tuberculosis readily. Thus, it is meaningful to study murine tuberculosis when we always bear clinical tuberculosis in mind. At the same time, it is critical to study the roles of transcription factors that regulate functions of cytokines, which I already mentioned.

We have described our work on tuberculosis of knockout mice. Let me briefly review other researchers' work here. There are two reports on tuberculosis of IFN- γ gene-disrupted mice already [33, 34]. These studies also stress the importance of IFN- γ in antimycobacterial immunity. Th2 cells and macrophages secrete IL-6. IL-6 knockout mice lacking Th2 cytokine, IL-6, succumb to lethal tuberculosis [34]. Alveolar macrophages are targets of mycobacterial infection and IL-12 secreted by activated macrophages plays a role in protective immunity against tuberculosis [35]. Nitric oxide (NO) is one of killing agents for *M. tuberculosis*. It is controlled by inducible NO synthase (iNOS). These iNOS-deficient mice proved highly susceptible to *M. tuberculosis* infection [37]. Natural resistance-associated macrophage protein (Nram) 1 is an important molecule related to resistance of infection with *M. bovis* BCG. Nram1-deficient mice were found to be as resistant as wild-type mice to infection with the virulent H37Rv strain of *M. tuberculosis* [38]. Nram1 is of limited importance in resistance to tuberculosis in mice. The mice that had had their β 2-microglobulin genes disrupted by homologous recombination died from a normally sublethal injection of *M. tuberculosis* [39]. Because β 2-microglobulin is an integral component of the class I MHC molecule, these mice are unable to sensitize CD8 T cells.

The knockout mice research has had great impact on cytokine and transcription factor research because they not only answer important questions, but they raise many more questions. It is our hope that answers to newly raised questions regarding the physiological roles of the cytokines and transcription factors will be prompted by other biological studies.

In summary, we briefly reviewed the roles of cytokines and transcription factors in experimental mycobacterial infection with special emphasis on roles of IFN- γ and TNF- α . Table 1 shows characteristics of various knockout mice used in our experimental tuberculosis study. IFN- γ and TNF- α are grand champions of all cytokines involved in mycobacterial infection. Therefore, it is very important to

investigate their roles and regulatory factors for IFN- γ and TNF- α in early-phase mycobacterial infection in more detail to diagnose and treat tuberculosis as soon as possible.

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REFERENCES

- [1] Durum SK, Muegge K Eds. Cytokine knockouts. Totowa, New Jersey, Humana Press. 1998.
- [2] Tagawa Y, Sekikawa K, Iwakura Y. Suppression of Con A-induced hepatitis in IFN- γ -/-, but not in TNF- α -/-, mice: role for IFN- γ in activating apoptosis in hepatocytes. *J Immunol* 1997; 159: 1418-1428.
- [3] Taniguchi T, Takata M, Ikeda A, Momotani E, Sekikawa K. Failure of germinal center formation and impairment of response to endotoxin in TNF- α -deficient mice. *Lab Invest* 1997; 77: 647-658.
- [4] Takeda K, Tsutsui H, Yoshimoto T, *et al.* Defective NK activity and Th1 response in IL-18-deficient mice. *Immunity* 1998; 8: 383-390.
- [5] Horai R, Asano M, Sudo K, *et al.* Production of mice deficient in IL-1 β , IL-1 α , IL-1 γ , and IL-1 receptor antagonist shows that IL-1 is crucial in turpentine-induced fever development and glucocorticoid secretion. *J Exp Med* 1998; 187: 1463-1475.
- [6] Labow M, Shuster D, Zetterstrom M, *et al.* Absence of IL-1 signaling and reduced inflammatory response in IL-1 type 1 receptor-deficient mice. *J Immunol* 1997; 159: 2452-2461.
- [7] Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G. Disruption of the murine IL-4 gene blocks Th2 cytokine response. *Nature* 1993; 363: 245-248.
- [8] Tanaka T, Akira S, Umemoto M, *et al.* Targeted disruption of the NF-IL6 gene discloses its essential role in bacterial killing and tumor cytotoxicity by macrophages. *Cell* 1995; 80: 353-361.
- [9] Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF- κ B leads to multifocal defects in immune response. *Cell* 1995; 80: 321-330.
- [10] Matsuyama T, Kimura T, Kitagawa M, *et al.* Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. *Cell* 1993; 75: 83-97.
- [11] Meraz MA, White JM, Sheehan KCF, *et al.* Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* 1996; 84: 431-442.
- [12] Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 1996; 382: 174-177.
- [13] Takeuchi O, Hoshino K, Kawai T, *et al.* Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity* 1999; 11: 443-451.
- [14] Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J Immunol* 2000; 165: 5392-5396.
- [15] Kawano T, Cui J, Koezuka Y, *et al.* CD1d-restricted and TCR-mediated activation of V α 14 NKT cells by glycosylceramides. *Science* 1997; 278:1626-1629.
- [16] Sugawara I, Yamada H, Kazumi Y, *et al.* Induction of granulomas in IFN- γ gene-disrupted mice by avirulent but not by

- virulent strains of *Mycobacterium tuberculosis*. J Med Microbiol 1998; 47: 871-877.
- [17] Kaneko H, Yamada H, Mizuno S, *et al.* Role of TNF- in Mycobacterium-induced granuloma formation in TNF- deficient mice. Lab Invest 1999; 79: 379-386.
- [18] Sugawara I, Yamada H, Kaneko H, Mizuno S, Takeda K, Akira S. Role of IL-18 in mycobacterial infection in IL-18-gene-disrupted mice. Infect Immun 1999; 67: 2585-2589.
- [19] Yamada H, Mizuno S, Horai R, Iwakura Y, Sugawara I. Protective role of IL-1 in mycobacterial infection in IL-1 / double-knockout mice. Lab Invest 2000; 80: 759-767.
- [20] Sugawara I, Yamada H, Hua S-C, Mizuno S. Role of IL-1 type 1 receptor in mycobacterial infection. Microbiol Immunol 2001; 45: 743-750.
- [21] Sugawara I, Yamada H, Mizuno S, Iwakura Y. IL-4 is required to defense against mycobacterial infection. Microbiol Immunol 2000; 44: 971-979.
- [22] Sugawara I, Mizuno S, Yamada H, Matsumoto M, Akira S. Disruption of nuclear factor-IL6, a transcription factor, results in severe mycobacterial infection. Am J Pathol 2001; 158: 361-366.
- [23] Yamada H, Mizuno S, Reza-Gholizadeh M, Sugawara I. Relative importance of NF- B p50 in mycobacterial infection. Infect Immun 2001; 69: 7100-7105.
- [24] Yamada H, Mizuno S, Sugawara I. Interferon regulatory factor 1 in mycobacterial infection. Microbiol Immunol 2002; 46: 751-760.
- [25] Sugawara I, Yamada H, Mizuno S. STAT1 knockout mice are highly susceptible to pulmonary mycobacterial infection. Tohoku J Exp Med 2004; 202: 41-50.
- [26] Sugawara I, Yamada H, Mizuno S. Relative importance of STAT4 in murine tuberculosis. J Med Microbiol 2003; 52: 29-34.
- [27] Sugawara I, Yamada H, Li C-Y, Mizuno S, Takeuchi O, Akira S. Mycobacterial infection in TLR2 and TLR6 knockout mice. Microbiol Immunol 2003; 47: 327-336.
- [28] Sugawara I, Yamada H, Mizuno S, Takeda K, Akira S. Mycobacterial infection in MyD88-deficient mice. Microbiol Immunol 2003; 47: 841-847.
- [29] Sugawara I, Yamada H, Mizuno S, Li C-Y, Nakayama T, Taniguchi M. Mycobacterial infection in natural killer T cell knockout mice. Tuberculosis 2002; 82: 97-104.
- [30] Jitsukawa T, Nakajima S, Sugawara I, *et al.* Characterization of murine monoclonal antibodies to human IFN- and their application for sandwich ELISA. Microbiol Immunol 1987; 31: 809-820.
- [31] Jouanguy E, Lamhamedi-Cherradi S, Altare F. Partial IFN-receptor 1 deficiency in a child with tuberculoid BCG infection and a sibling with clinical tuberculosis. J Clin Invest 1997; 11: 2658-2664.
- [32] Kean J, Gershon S, Wise RP. Tuberculosis associated with infliximab, a tumor necrosis factor- neutralizing agent. N Engl J Med 2001; 345: 1098-1103.
- [33] Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme I. Disseminated tuberculosis in IFN- gene-disrupted mice. J Exp Med 1993; 178: 2243-2247.
- [34] Flynn JL, Chan JJ, Tribold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for IFN- in resistance to Mycobacterium tuberculosis infection. J Exp Med 1993; 178: 2249-2254.
- [35] Ladel CH, Blum C, Deher A, Reifenberg K, Kopf M, Kaufmann SH. Lethal tuberculosis in IL-6-deficient mice. Infect Immun 1997; 65: 4843-4849.
- [36] Cooper AM, Magram J, Ferrante J, Orme IM. IL-12 is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. J Exp Med 1997; 186: 39-45.
- [37] MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc Natl Acad Sci USA 1997; 94: 5243-5248.
- [38] North RJ, LaCourse R, Ryan L, Gros P. Consequence of Nramp1 deletion to *Mycobacterium tuberculosis* infection in mice. Infect Immun 1999; 67: 5811-5814.
- [39] Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility complex class I-restricted T cells are required for resistance to *M. tuberculosis* infection. Proc Natl Acad Sci USA 1992; 89: 12013-12017.
- [40] Kindler V, Sapping A-P, Grau GE, Piguet P-F, Vassalli P. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell 1989; 56:731-740.