

# Short Communication

## Disruption of Nuclear Factor-Interleukin-6, a Transcription Factor, Results in Severe Mycobacterial Infection

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**Nuclear factor-interleukin-6 (NF-IL-6) is one of several nuclear transcription factors (NF-IL-6, NF- $\kappa$ B, PU.1, interferon-regulatory factor 1, Egr-1, and Stat-1). NF-IL-6 and NF- $\kappa$ B are expressed in macrophages and is induced by bacterial lipopolysaccharides. To evaluate whether NF-IL-6 is required for the inflammatory immune response to mycobacterial infection, in which epithelioid macrophages comprise the leading cell population, we generated NF-IL-6 knockout (KO) mutant mice. Airborne infection of these mice with *Mycobacterium tuberculosis* strains induced disseminated tuberculosis lacking granuloma formation, although interferon- $\gamma$ , tumor necrosis factor- $\alpha$ , and interleukin-12 mRNA expression levels were within the normal range compared with those of wild-type mice. Generation of O<sub>2</sub><sup>-</sup> and mycobacterial killing by neutrophils from these mice were impaired severely compared with wild-type mice. We conclude that NF-IL-6 is a critical transcription factor in mycobacterial control as well as in granulocyte-colony stimulating factor induction resulting in neutrophil activation. (Am J Pathol 2001, 158:361–366)**

Nuclear factor-interleukin-6 (NF-IL-6) is one of several nuclear transcription factors (NF-IL-6, NF- $\kappa$ B, PU.1, interferon-regulatory factor 1, Egr-1, and Stat-1). Especially, NF-IL-6 and NF- $\kappa$ B are expressed in macrophages and induced by bacterial lipopolysaccharides.<sup>1,2</sup> NF-IL-6 was originally identified as a nuclear factor that binds to the IL-1 response element of the human IL-6 gene.<sup>3</sup> It is reported that NF-IL-6 expression level is dramatically

increased during macrophage differentiation.<sup>4,5</sup> Thus, NF-IL-6 may be closely related to macrophage functions.

NF-IL-6-deficient mice displayed a high susceptibility to *Salmonella*, *Listeria* and *Brucella abortus* infections, indicating that NF-IL-6 plays a role in controlling intracellular parasite proliferation by an as yet unknown mechanism. Macrophages acquire enhanced bactericidal capacity when fully activated after their interaction with *Mycobacterium tuberculosis*. Tuberculosis is a chronic disease induced by *M. tuberculosis*, one of typical intracellular pathogens, and characterized by clusters of epithelioid macrophages with central caseous necrosis.<sup>6</sup> Thus, it is of great interest to examine the relationship between NF-IL-6 expression and tuberculosis, because the direct role of NF-IL-6 in mycobacterial infection has not been established fully. To evaluate whether NF-IL-6 is required for the inflammatory immune response to mycobacterial infection, in which epithelioid macrophages comprise the leading cell population, we generated NF-IL-6 knockout (KO) mutant mice<sup>7</sup> and demonstrated that NF-IL-6 is an essential transcription factor in defense against mycobacterial infection.

### Materials and Methods

#### Generation of NF-IL-6 KO Mice with C57BL/6 Background

An 11-kb genomic fragment spanning from 8.5 kb 5' of the NF-IL6 transcription initiation site to 2.3 kb 3' of the end of the exon was subcloned into pUC18 plasmid vector. The MC1-herpes simplex virus thymidine kinase was inserted into the unique *Hind*III site in the 5' end of the homologous region. E14–1 ES cells were electroporated in 800  $\mu$ l of phosphate-buffered saline (PBS) with

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32  $\mu\text{g}$  of *Sall*-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 polymerase chain reaction (PCR) and subsequently confirmed by genomic Southern blot hybridization.<sup>7</sup> These NF-IL-6 mice with C57BL/6 background were back-crossed over six generations. After birth, the NF-IL-6 KO mice developed normally and they have normal number of lymphocytes, leukocytes, and leukocyte subsets. They were kept under specific pathogen-free conditions in an environmentally controlled clean room at the laboratory animal facility at the Research Institute of Tuberculosis.

### *Bacterial Strains and Mouse Infections*

Virulent *M. tuberculosis* H37Rv (ATCC358121) and more virulent Kurono (ATCC25618) strains were passed through mice and grown in 7H9 liquid medium once before storing in aliquots at  $-85^{\circ}\text{C}$  and titered. The cultured strains were filtered with a membrane filter of 4  $\mu\text{m}$  pore size (Millipore, Bedford, MA) before use so that they were dispersed evenly. The NF-IL-6 KO and wild-type (WT) mice were infected by an airborne route by placing them in the exposure chamber of an airborne infection apparatus (Model 099CA4241; Glas-Col, Inc., Terre Haute, IN). The nebulizer compartment was filled with 5 ml of a suspension of  $10^6$  CFU of H37Rv or Kurono strain.<sup>8</sup> The concentration was calculated to result in uptake of  $\sim 200$  viable bacilli by the lungs just after inhalation exposure for 90 minutes under the experimental conditions for this study.<sup>19</sup> The survival of groups of mice for 50 days after infection with Kurono strain was recorded, and survival curves were plotted. The lungs and spleens from NF-IL-6 KO and WT mice infected with H37Rv were retrieved 1, 2, 3, 4, 5, 6, and 7 weeks after infection, homogenized, diluted with physiological saline, plated on Ogawa slant agar, and incubated at  $37^{\circ}\text{C}$  for 21 days. The resulting CFU were counted. Permission (no. 991) to experiments on animals was given by the Animal Experiment Committee of The Research Institute of Tuberculosis.

### *Histology*

Some mice were sacrificed just before death after infection. Tissue sections from paraffin blocks containing lung, liver, and spleen tissue were stained with hematoxylin and eosin or the Ziehl-Neelsen method for acid-fast bacilli.

### *Reverse Transcriptase (RT)-PCR*

Pulmonary tissue samples were taken from infected NF-IL-6 KO and WT mice just before death after infection (usually 35 days), frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until required for use, when RNA was extracted as described previously.<sup>8</sup> RT-PCR was carried out with gene-specific primer sets for interferon- $\gamma$  (IFN- $\gamma$ ), IL-12 p40, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and IL-1 $\beta$  (CLP

Inc., San Diego, CA). The granulocyte-colony stimulating factor (G-CSF) cDNA was amplified by primer sets specific for G-CSF.<sup>7</sup> The size of  $\beta$ -actin used as a positive control was 514 bp. The same amounts of  $\beta$ -actin RNA from the lung tissues used as an internal control were used in RT-PCR analysis.

### *Macrophage Nitric Oxide (NO) Assay*

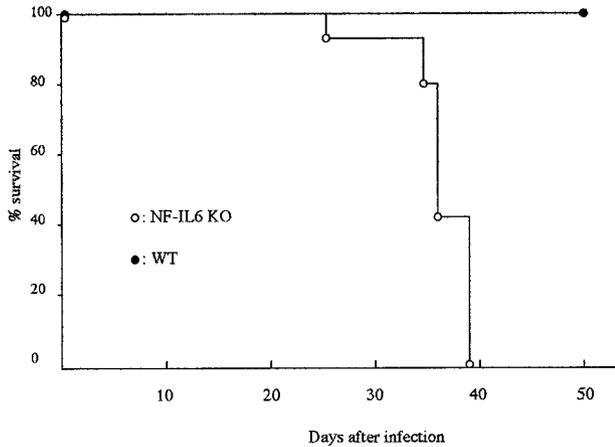
Alveolar macrophages were obtained by bronchoalveolar lavage. Briefly, the murine trachea was cannulated and 1 ml of physiological saline was poured in. The saline was recovered using a 1-ml disposable syringe. The cells thus obtained contained more than 99% macrophages as assessed by phagocytosis of BCG and anti-Mac-1 immunostaining. The alveolar macrophages were cultured with H37Rv overnight at the multiplicity of infection of 5:1. Nitrite concentrations were determined by the Griess assay as described previously.<sup>9</sup>

### *Enzyme-Linked Immunosorbent Assay (ELISA)*

The spleens were collected from the NF-IL-6 KO and WT mice and pooled, and a single-cell suspension was prepared. The cells were treated for 5 minutes with a 0.155 mol/L ammonium chloride/0.01 mol/L potassium bicarbonate solution for hemolysis of RBC, washed, and resuspended in RPMI 1640 medium with heat-inactivated 10% fetal calf serum. The cell suspensions were plated at  $5 \times 10^5$  cells/well in 96-well flat-bottomed culture plates and incubated for 3 days at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  in air. The cells were stimulated with either medium alone or live H37Rv ( $10^5$  CFU). The concentrations of IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , and IL-12 in cell culture supernatants from the cells incubated in the presence of H37Rv were measured by sandwich ELISA (Biosource International, Camarillo, CA). The values were expressed as the mean and SE (in picograms per milliliter) of triplicate determinations for pooled cells from five animals.

### *$\text{O}_2^-$ and Neutrophil CFU Assays*

The 10% peptone broth-elicited peritoneal exudate cells were plated and cultured on Petri dishes for 6 hours. Non-adherent cells were collected after gentle shaking. The neutrophils ( $10^5$ ; more than 98% pure as evaluated by morphology) from NF-IL-6 KO and WT mice were stimulated with H37Rv strain ( $5 \times 10^6$  CFU) *in vitro* overnight. Their  $\text{O}_2^-$  production was examined using a nitroblue tetrazolium (NBT) reagent.<sup>10</sup> Briefly, 0.1 ml of 0.1% HEPES-saline-glucose buffer containing NBT was added to 0.1 ml neutrophil cell suspension ( $1 \times 10^6/\text{ml}$  PBS) and the cell suspension was incubated at  $37^{\circ}\text{C}$  for 7 minutes. Then, 10  $\mu\text{l}$  of 0.2 mol/L EDTA solution was added. The cell suspension was placed on a hemocytometer. The 200 cells were counted, and cells possessing NBT formazan in cytoplasm (blue-stained neutrophils) were judged as positive. At the same time, the supernatants containing neutrophils subjected to lysis with 0.05% sodium dodecyl sulfate were cultured in Lo-

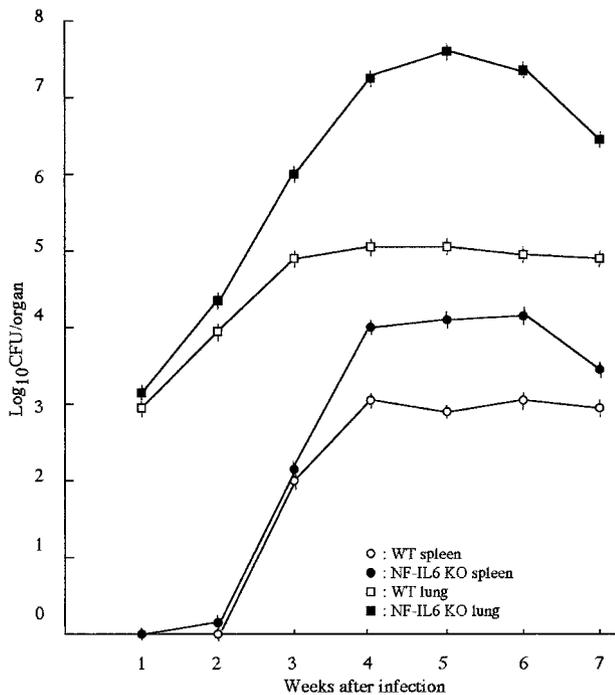


**Figure 1.** Survival of mice infected with *M. tuberculosis* Kurono strain. WT and NF-IL-6 KO mice were infected with  $10^6$  CFU of the Kurono strain by an airborne route. Percentages of surviving WT (solid circles) and NF-IL-6 KO mice (open squares) are shown.

wenstein-Jensen media for 4 weeks before counting mycobacterial colonies.

### Reconstitution of NF-IL-6 KO Mice with Exogenous G-CSF

The mice were injected subcutaneously with 2  $\mu$ g of recombinant murine G-CSF (PeproTech, Rocky Hill, NJ) in PBS or PBS alone three times at weekly intervals to see whether or not recombinant G-CSF affects development of mycobacterial infection. The lungs, spleens, and livers



**Figure 2.** CFU in lung and spleen tissues of NF-IL-6 KO and WT mice exposed to  $10^6$  CFU of H37Rv strain by the airborne route. At the indicated weeks after infection, four mice from each group were sacrificed and homogenates of lung and spleen tissues were plated. Error bars indicate standard errors of the means.

from NF-IL-6 KO mice treated subcutaneously with recombinant G-CSF were retrieved from the infected mice 7 weeks after infection, and their histopathology was evaluated.

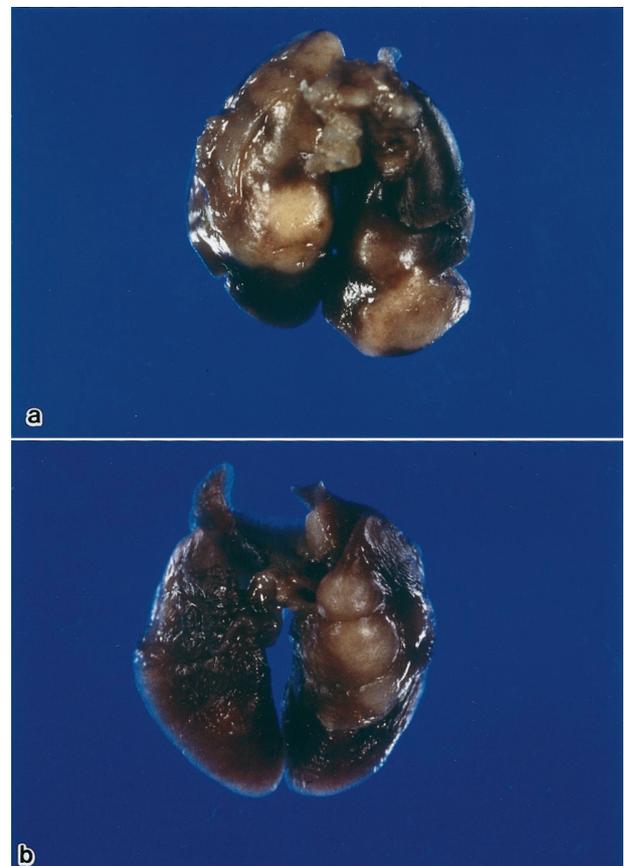
### Statistical Method

The values were compared by Student's *t*-test. For all statistical analyses, a *P* value <0.01 was considered significant.

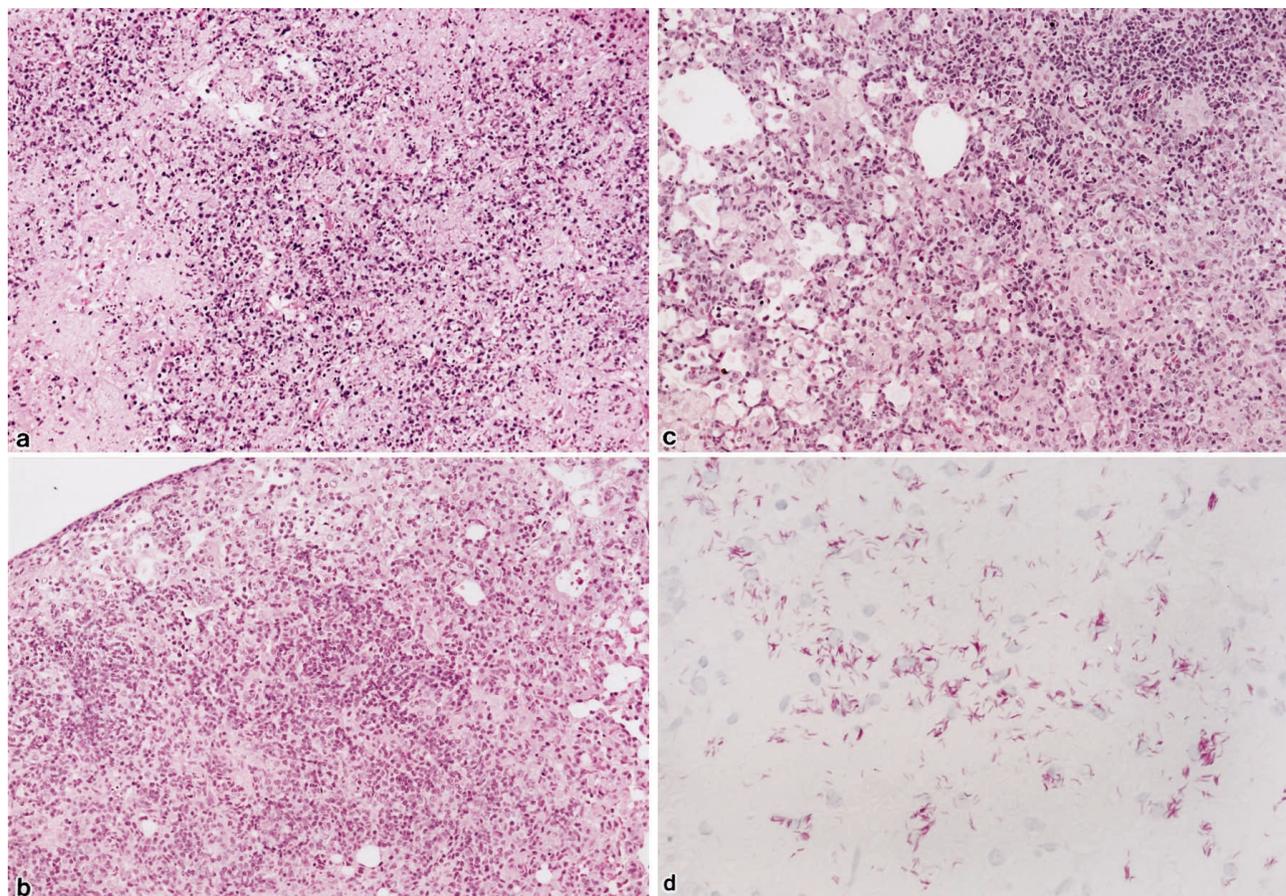
### Results

#### Course of *M. tuberculosis* Infection in NF-IL-6 Mice

We infected WT and NF-IL-6 KO mice with either H37Rv (ATCC358121) or Kurono (ATCC25618) *M. tuberculosis* strains ( $10^6$  each). All of the mice infected with the Kurono strain (each group of 10) died of mycobacteremia within 40 days, whereas WT mice survived until the date of sacrifice at 50 days (Figure 1). When NF-IL-6 KO mice were infected with H37Rv, the CFU number was much higher than that from WT mice at weeks 2 through 7 after infection (Figure 2). Figure 3 shows that there were ex-



**Figure 3.** Macroscopic appearance of lungs from NF-IL-6 KO (a) and WT (b) mice infected with the Kurono strain by an airborne route. Whitish necrotic nodules are noted in the lungs of NF-IL-6 mice, but nodular lesions are noted in the lungs of WT mice.

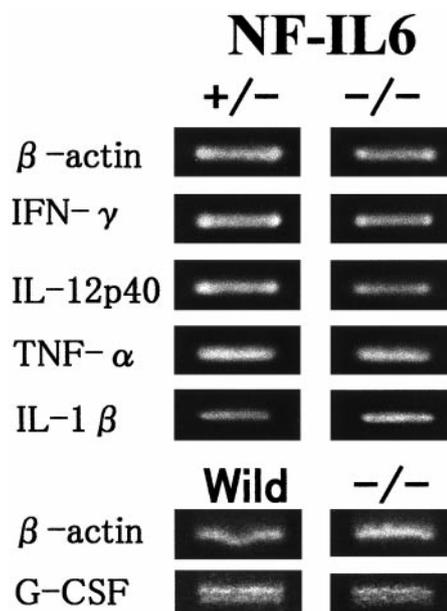


**Figure 4.** Histological analysis of lung sections after infection with *M. tuberculosis* (Kuro no strain). Lungs were removed from NF-IL-6 KO (a) and WT mice (b) 38 days and 50 days, respectively, after infection with Kuro no strain ( $10^6$  CFU) by an airborne route. Hematoxylin and eosin stain; original magnification,  $\times 200$ . c: NF-IL-6 KO mice infected with Kuro no strain were injected subcutaneously three times at day 0 and at weekly intervals with recombinant murine G-CSF (2  $\mu$ g/mouse). Thirty-eight days later, the mice were sacrificed and the issues were fixed with 10% buffered formalin. Hematoxylin and eosin stain; original magnification,  $\times 200$ . d: Stain for acid-fast bacilli in the lung of NF-IL-6 infected with Kuro no strain. Original magnification,  $\times 600$ .

tensive necroses in the lungs, spleen, and liver of NF-IL-6 KO mice infected with Kuro no strain. On the contrary, pulmonary granulomas were observed in WT mice. Histopathologically, multiple foci of abscesses consisting of neutrophils and macrophages were noted in the lungs, spleen, and liver, whereas WT mice developed granulomatous lesions lacking neutrophil infiltration (Figure 4). When infected with H37Rv by an airborne route, NF-IL-6 KO mice survived but developed severe lobar pneumonia with pulmonary edema. Necrotic lesions induced in NF-IL-6 KO mice were inhibited significantly by treatment with exogenous recombinant murine G-CSF, as shown in Figure 4c. No neutrophil infiltration was recognized in the lung treated with recombinant G-CSF. The NF-IL-6 KO mice treated with recombinant murine G-CSF lived longer than the WT mice.

#### RT-PCR and Cytokine Assay

As shown in Figure 5, RT-PCR showed that NF-IL-6 KO mice expressed IFN- $\gamma$ , TNF- $\alpha$ , and IL-12 mRNA as strongly as did those from WT mice. G-CSF mRNA expression (704 bp) was lower in the lungs of NF-IL-6 KO mice than in the lungs of WT mice. Fibroblasts other than alveolar macrophages expressed G-CSF mRNA. To de-



**Figure 5.** RT-PCR analysis of cytokine mRNA expression. Total RNA was isolated from lung tissues of NF-IL-6 KO and WT mice infected with *M. tuberculosis* and subjected to RT-PCR using cytokine-specific primers. G-CSF mRNA expression is lower in the lung of NF-IL-6 mice than that of WT mice. Lung fibroblasts from NF-IL-6 KO mice G-CSF-mRNA significantly.

termine whether the absence of NF-IL-6 might influence the induction and control of various cytokines, the levels of several cytokines in the splenocyte cultures were determined. Table 1 shows that H37Rv-driven release of IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , and IL-12 was similar for the two types of mice.

### NO Assay

We examined the production of NO in culture supernatants of alveolar macrophages stimulated with H37Rv overnight from WT and NF-IL-6 KO mice. NO production was detectable in NF-IL-6 KO mouse macrophages to the same extent as in WT mouse macrophages ( $63 \pm 5$  vs.  $60 \pm 3$   $\mu\text{mol/L}$ ).

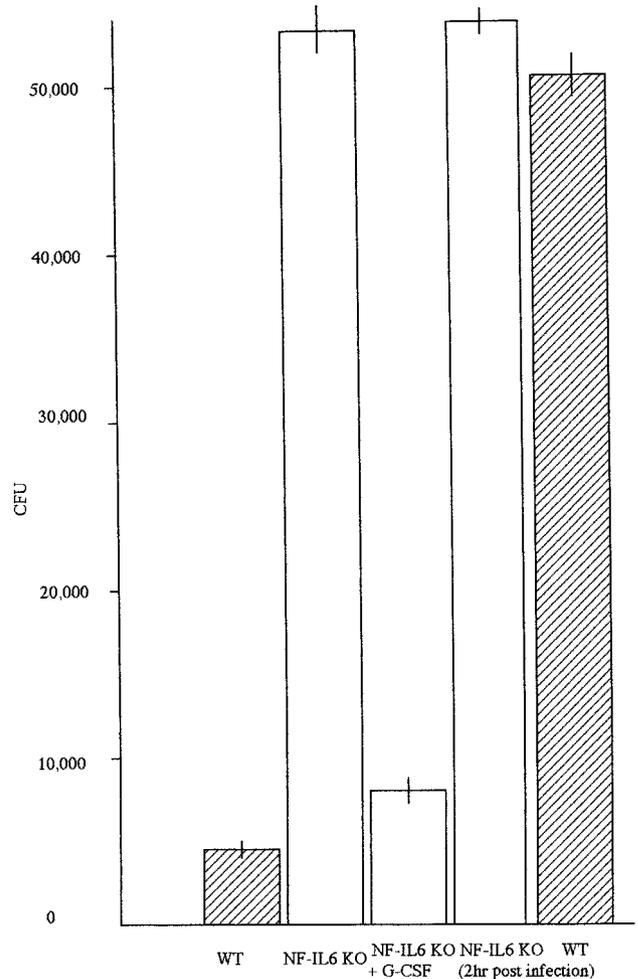
### Neutrophil Function Assay

Because induction of G-CSF production was impaired in NF-IL-6 KO mice, we next examined neutrophil functions by two methods. First, 10% peptone broth-elicited peritoneal neutrophils ( $10^5$ ) from either NF-IL-6 KO or WT mice were cultured *in vitro* overnight in the presence of *M. tuberculosis* H37Rv strain ( $5 \times 10^6$  CFU). The supernatants containing neutrophils subject to lysis with 0.05% sodium dodecyl sulfate were cultured in Lowenstein-Jensen media for 4 weeks before counting of mycobacterial colonies. The numbers of mycobacterial colonies in these supernatants from NF-IL-6 KO mice were significantly higher than those from WT mice ( $54,3215 \pm 2850$  vs.  $4356 \pm 248$  CFU;  $P < 0.01$ ) (Figure 6).

Next, 10% peptone broth-elicited peritoneal neutrophils ( $10^5$ ) from NF-IL-6 KO and WT mice were stimulated with *M. tuberculosis* H37Rv strain ( $5 \times 10^6$ ) *in vitro* overnight. Their  $O_2^-$  production was examined using a NBT reagent.<sup>10</sup> The ratio of  $O_2^-$  producing neutrophils in NF-IL-6 KO mice was 15%, whereas in WT mice it was 95% ( $P < 0.01$ ) (Table 2). The  $O_2^-$  producing ability of neutrophils was significantly lower in NF-IL-6 KO mice.

### Discussion

The purpose of our study was to determine the role of NF-IL-6 as a transcription factor in the pathogenesis of murine tuberculosis. The results obtained with our NF-IL-6 KO mouse model demonstrated that NF-IL-6 was indispensable for the control and survival of the *M. tuberculosis* infection. As these KO mice died of mycobacteremia within 40 days, it was speculated that levels of IFN- $\gamma$ , IL-12, and TNF- $\alpha$ , which are important cytokines in defense against mycobacterial infection, were lower



**Figure 6.** CFU assay of peritoneal neutrophils from NF-IL-6 mice. The peritoneal neutrophils ( $10^5$ ) were cultured in the presence of H37Rv ( $5 \times 10^6$ ) in 96-well flat-bottomed microculture plates 2 hours after infection with H37Rv or overnight. The neutrophils were also cultured with H37Rv in the presence of recombinant murine G-CSF ( $2 \times 10^{-3}$   $\mu\text{g}$ ) overnight. Thereafter, the suspension containing peritoneal neutrophils were cultured for 4 weeks in Lowenstein-Jensen media and the resulting colonies were counted.

than those of WT mice. However, the expression levels of these cytokines were within normal range as evaluated by RT-PCR and ELISA.

NO production was detectable significantly in NF-IL-6 KO mouse macrophages. There was no significant difference in NO production by alveolar macrophages between WT and NF-IL-6 mice ( $P < 0.01$ ). Taken together with RT-PCR and ELISA data, these results suggest that macrophages of NF-IL-6 KO mice function normally to exert normal mycobactericidal activity.

What is the mechanism by which NF-IL-6 KO mice succumb readily to mycobacterial infection? Killing ability

**Table 1.** Cytokine Release by Splenocytes from WT and NF-IL6 KO Mice

Stimulus	Mouse type	Cytokine level			
		IL-1 $\beta$	IFN- $\gamma$	TNF- $\alpha$	IL-12
H37Rv	WT	250 $\pm$ 14	120 $\pm$ 10	750 $\pm$ 35	110 $\pm$ 11
	NF-IL6 KO	245 $\pm$ 12	130 $\pm$ 10	800 $\pm$ 32	95 $\pm$ 9

**Table 2.** Percentage of  $O_2^-$  Producing Neutrophils

Neutrophils	$O_2^-$ producing neutrophils (%)
WT mice	95
NF-IL6 KO mice	15

The 200 peritoneal neutrophils were counted, and neutrophils containing NBT formazan were judged to be positive.

of mycobacteria and  $O_2^-$  producing ability by neutrophils was significantly lower in NF-IL-6 KO mice compared with those of WT mice. Since induction of G-CSF production by alveolar macrophages was impaired severely in NF-IL-6 KO mice,<sup>7</sup> we examined neutrophil functions in terms of mycobacterial killing ability by neutrophils,  $O_2^-$  generation, and reconstitution of NF-IL-6 KO mice with recombinant murine G-CSF. Consequently, neutrophils of NF-IL-6 KO mice did not function properly and granulomatous formation in NF-IL-6 KO mice was abrogated significantly with exogenous murine G-CSF. Since no neutrophils were recognized in the granulomatous lesions, recombinant murine G-CSF reversed neutrophil function significantly. It is also reported that G-CSF induces proliferation and differentiation of neutrophil precursors.<sup>11–13</sup> Electron microscopy revealed tubercle bacilli in phagosomes of macrophages from NF-IL-6 KO mice, but we saw no evidence of *M. tuberculosis* having escaped from phagosomes to the cytoplasm, although this has been demonstrated in infection with *L. monocytogenes* (data not shown).<sup>7</sup> Therefore, it is concluded that bactericidal activity by neutrophils may be impaired severely in these NF-IL-6 mice.

Alveolar macrophages are targets of mycobacterial infection and activated macrophages, as well as IFN- $\gamma$ , TNF- $\alpha$ , IL-12, IL-18, and IL-1, play major roles in protective immunity against tuberculosis.<sup>13–19</sup> Our results demonstrate that neutrophils play an important role in the early phase of defense against murine tuberculosis when aerosol infection was used. Since G-CSF is impaired in NF-IL-6 KO mice,<sup>4</sup> NF-IL-6 appears to be essential for inducing G-CSF production in neutrophil proliferation and differentiation.<sup>14–18</sup>

It has been very recently reported that addition of G-CSF, whose induction is impaired in NF-IL-6 KO mouse macrophages, restores both endocytosis and morphology of endosomes, together with bactericidal activity.<sup>20</sup> Taken together with our data, both bactericidal activity by neutrophils and modulation of endocytosis by macrophages may be impaired in these NF-IL-6 KO mice. We showed that neutrophils played an important role in preventing development of granulomatous lesions. Tuberculosis is of global importance. This NF-IL-6 KO mouse model may offer a strategy for developing novel anti-tuberculosis drugs that augment the NF-IL-6 expression that is closely associated with neutrophil functions.

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