STAT1 Knockout Mice are Highly Susceptible to Pulmonary Mycobacterial Infection

ISAMU SUGAWARA, HIROYUKI YAMADA and SATORU MIZUNO

Mycobacterial Reference Center, The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo 204-0022

SUGAWARA, I., YAMADA, H. and MIZUNO, S. STAT1 Knockout Mice are Highly Susceptible to Pulmonary Mycobacterial Infection. Tohoku J. Exp. Med., 2004, 202 (1), 41-50 —— This study was designed to determine the roles of STAT1 protein in defense against mycobacterial infection. Airborne infection of STAT1 knockout (KO) mice with a Mycobacterium tuberculosis Kurono strain induced multiple necrotic lesions in lungs, spleen and liver, while that in wild-type (WT) mice did not. The STAT1 KO mice succumbed to mycobacterial infection by the 35th day after infection. Compared with the levels in WT mice, inducible nitric oxide synthase (iNOS), tumor necrosis factor-α, interferon-γ and IL-12 mRNA levels were significantly lower in the lung of STAT1 KO mice. Interestingly, granulomatous lesion development in STAT1 KO mice was inhibited significantly by treatment with exogenous recombinant murine IL-12. Therefore, STAT1 regulates IL-12 expression and appears to be a critical transcription factor in controlling mycobacterial infection.

© 2004 Tohoku University Medical Press

Activation mechanisms of signal transducer and activator of transcription (STAT) was clarified in the biological responsive system stimulated with interferon (IFN)-γ. STAT family consists of seven transcription factors (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) (Darnell 1997). STAT proteins are cytoplasmic proteins that are activated to participate in gene control when cells encounter various extracellular polypeptides. Among them, STAT1 is activated by stimuli with IFN-α/β and IFN-γ and is essential for cell growth suppression in response to IFN-γ. The STAT1 signaling pathway appears to negatively regulate the cell cycle by inducing cyclin-dependent kinase inhibitors in response to cytokines (Chin et al. 1996). STAT1 knockout (KO) mice are highly susceptible to vesicular stomatitis virus (VSV) and Listeria monocytogenes infections (Meraz et al. 1996).

Tuberculosis is a chronic, airborne infectious disease induced by M. tuberculosis. The target cells of M. tuberculosis are alveolar macrophages and alveolar macrophages are activated by IFN-γ so that they can kill tubercle bacilli.
(Sugawara et al. 1998). Thus, it is important to study the roles of IFN-γ and transcription factors that regulate expression of IFN-γ. There are several reports suggesting the roles of STAT proteins in mycobacterial infections. In our previous report, we have shown that STAT4, but not STAT6, was a critical transcription factor in controlling mycobacterial infection (Sugawara et al. 2003). In the absence of STAT4, development of Th1 type helper T cells abrogated. Lymphocyte proliferation and expression induction of IFN-γ by IL-12 and natural killer cell activation by IL-12 are also inhibited. There are several in vitro reports suggesting the close relationship between STAT1 protein and mycobacterial infection. *M. tuberculosis* inhibits IFN-γ transcriptional responses without inhibiting activation of STAT1 (Ting et al. 1999). *M. tuberculosis* infection activates JAK2/STAT1-pathway (Rojas et al. 2002). Selective expression of type I IFN genes is induced in human dendritic cells infected with *M. tuberculosis* (Remoli et al. 2002). We have been interested in the roles of transcription factors that regulate cytokine expression in mycobacterial infection. Nuclear factor (NF)-IL-6 is critical in mycobacterial control as well as in the induction of granulocyte-colony-stimulating factor (G-CSF) in alveolar macrophages that results in neutrophil activation (Sugawara et al. 2001a). NF-κB p50 KO mice developed multifocal necrotic pulmonary lesions or lobar pneumonia (Yamada et al. 2001). STAT4 KO mice infected with *M. tuberculosis* developed large granulomas with massive neutrophil infiltration over time, while STAT6 KO mice did not (Sugawara et al. 2003). In the absence of STAT6, development of Th2 type helper T cells is abrogated and class switch of immunoglobulins to IgE is not recognized. Thus, STAT6 has nothing to do with the development of tuberculosis. These transcription factors regulate IFN-γ, TNF-α and IL-12 that play critical roles in defense against tuberculosis development. These findings prompted us to explore the roles of STAT1 protein in mycobacterial infection further. We report here that STAT1 plays a critical role in defense against mycobacterial infection.

**MATERIALS AND METHODS**

**Animals**

Six-week-old C57BL/6 wild-type (WT) mice were purchased from Japan SLC Co. Ltd. (Shizuoka), and STAT1 knockout (KO) mice were purchased from Taconic Transgenic Models via Immuno-Biological Laboratories Co. (Gunma) (Meraz et al. 1996). These KO 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.

**Experimental infections**

The experimental procedures were in accordance with the ARVO resolution on the use of animals in research. Permission to experiments on animals was granted by the Animal Experiment Committee of the Research Institute of Tuberculosis. A virulent Kurono strain of *Mycobacterium tuberculosis* (ATCC 358121) was grown in Middlebrook 7H9 broth for 2 weeks, then filtered with a sterile acrodisc syringe filter (Pall Corp., Ann Arbor, MI, USA) with a pore size of 5.0 µm. Then, the aliquots of the filtrate bacterial solution were stored at −80°C until use. Mice were infected via the airborne route by placing them into the exposure chamber of the Glas-Col aerosol generator (Model 099CA4212; Glas-Col, Inc., Terre Haute, Ind.). The nebulizer compartment was filled with 5ml of a suspension containing 10⁶ CFU of Kurono strain tubercle bacilli so that approximately 100 bacteria might be deposited in the lung of each animal (Sugawara et al. 1999; Yamada et al. 2001).

**cfu assay**

At 1, 3, and 5 weeks after aerosol infection, mice were anesthetized with pentobarbital sodium, and the abdominal cavity was incised, and exsanguination was performed by splenectomy. Lungs, spleens and livers were excised and weighed. The left lobe of each lung and a part of
spleen tissues were separately 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 then 1ml of sterile physiological saline was added. Then, 100µl of homogenate was picked up and plated in a 10-fold serial dilution on 1% Ogawa’s egg media. Colonies on the media were counted after a four-week incubation at 37°C (Yamada et al. 2001).

**RT-PCR**

Parts of right lobe of lung and spleen tissues that had been left after cutting off for CFU examination were used to perform RT-PCR analysis for mRNA expression for several cytokines and iNOS in these organs during TB infection. These tissues samples were snap-frozen in liquid nitrogen, and stored at −85°C until use. RNA extraction was performed as described previously (Sugawara et al. 2001b; Yamada et al. 2001). Briefly, frozen tissues were homogenized with a microcentrifuge tube and tip-closed 1ml pipet tip in liquid nitrogen. Then homogenates were treated with total RNA isolation reagent, TRIZol™ Reagent (GIBCO BRL) according to a manufacturer’s instructions. After RNA isolation, total RNA was reverse transcribed into cDNA with M-MLV reverse transcriptase (GIBCO BRL) following measurement of total RNA concentration, and agarose gel electrophoresis was performed.

Polymerase chain reaction was performed with gene-specific primer sets for β-actin, IFN-α, IFN-β, IFN-γ, TNF-α, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-18, TGF-β, and iNOS. DNA sequences of primer sets and corresponding PCR conditions are listed as described previously (Sugawara et al. 2003). The PCR primer sets for ICAM-1 mRNA are as follows: sense, 5’-TGCGTTTTGGAGCTAGCGGACCA-3’ antisense, 5’-CGAGGACCATAACAGCAGTCGCAG-3’. The expected product length is 326 bp. Amplification was carried out with a DNA thermal cycler 480 (Perkin-Elmer Cetus). PCR product (10 µl each) was applied for electrophoresis in 4% agarose and NuSieve GTG (1:3) gel and visualized using ethidium bromide staining. Relative ratios of various cytokines and iNOS mRNA to β-actin mRNA as an internal control were determined (Sugawara et al. 2003).

**Light microscopic examination**

For light microscopic examination, the right middle lobe of each lung was excised and fixed with 20% formalin buffered methanol solution, Mildform 20NM (containing 8% formaldehyde and 20% methanol, Wako Pure Chemical Co., Osaka), dehydrated with grading series of ethanol, treated with xylene, and embedded in paraffin. The 5 µm-thick sections were cut from each paraffin block and stained for either hematoxylin and eosin or Ziehl-Neelsen staining.

**Reconstitution of STAT1 KO mice with exogenous recombinant IL-12**

As the IL-12 mRNA level was reduced significantly in the STAT1 KO mice, reconstitution experiments of STAT1 KO mice with exogenous murine IL-12 were performed twice. The three mice were injected subcutaneously with 10 µg of recombinant murine IL-12 (210-12, Pepro Tech, London, UK) in PBS or PBS alone four times at weekly intervals. The biological activity of the recombinant murine IL-12 was evaluated by determining the stimulation of IFN-γ production by murine splenocytes co-stimulated with IL-12. The ED₅₀ for this effect was 0.1 ng/ml, corresponding to a specific activity 1×10⁷ units/mg. The lungs from STAT1 KO mice treated subcutaneously with recombinant IL-12 were retrieved from the infected mice 5 weeks after aerosol infection (Sugawara et al. 1999). The sizes of the 10 granulomas were measured with a micrometer (Nikon Optical Co., Tokyo) (Sugawara et al. 2001a).

**Statistical methods**

The values were compared by Student’s t-test. For all statistical analyses, differences at p<0.01 were considered significant.
RESULTS
Mycobacterial burden in the lungs and spleens of STAT1 KO mice

STAT1 KO mice died of disseminated tuberculosis by the 35th day after aerosol infection, whereas WT mice survived until the day they were sacrificed at 60 days (Fig. 1). After one week post-infection, when STAT1 KO mice were infected with Kurono strain, the cfu number was

Fig. 1. Survival of mice infected with *M. tuberculosis* Kurono strain. WT and STAT1 KO mice were infected with $10^6$ cfu of the Kurono strain by an airborne route. Percentages of surviving WT (open circle) and STAT1 KO mice (solid circle) are shown.

Fig. 2. Mycobacterial titers in lungs (solid circle) and spleens (solid square) of STAT1 KO mice exposed to $10^6$ cfu *M. tuberculosis* Kurono strain by the airborne route. Lungs (open circle) and spleens (open square) of WT mice were included in each experiment. At the indicated times after infection, four mice from each group were sacrificed and homogenates of lung and spleen tissues were plated on 7H10 agar. Error bars indicate standard deviation (S.D.).
Fig. 3. Histological analysis of lung sections with *M. tuberculosis* Kurono strain. Lungs were removed from STAT1 KO (A) and WT mice (C) 35 days after infection with Kurono strain (10⁶ cfu) by an airborne route. Necrosis (→) is recognized in the lung of the STAT1 KO mouse. Hematoxylin and eosin stain; original magnification, ×200. B: Stain for acid-fast bacilli in the lung of STAT1 KO infected with Kurono strain. Original magnification, ×600. D: Pulmonary tissue (5 weeks after infection) from STAT1 KO mice infected with *M. tuberculosis* Kurono strain and treated four times subcutaneously with recombinant IL-12. The granuloma became smaller. Original magnification, ×200.
much higher than that from WT mice at week 1 through 5 after aerosol infection (Fig. 2). At 3 and 5 weeks after infection, there were statistically significant differences in both lung and spleen counts between WT and STAT1 KO mice ($p<0.01$).

**Light microscopic observations of infected lungs**

The mice were sacrificed 5 weeks after being infected by an airborne route and formalin-fixed sections were stained with hematoxylin and eosin and by the Ziehl-Neelsen method. Macroscopically, there were extensive necroses in the lungs, spleen, and liver of STAT1 KO mice infected with *M. tuberculosis* Kurono strain. On the contrary, pulmonary discrete granulomatous lesions were recognized in WT mice and these granulomas lacked necrosis. Histopathologically, multiple foci of abscesses consisting of neutrophils and epithelioid macrophages were noted in the lungs, spleen, and liver, whereas WT mice developed granulomatous lesions lacking neutrophil infiltration (Fig. 3). Epithelioid macrophages and lymphocytes were not recognized near the necrotic lesions. The necrotic lesions displayed central necrosis, neutrophils and cell debris. Ziehl-Neelsen staining revealed diffuse proliferation of tubercle bacilli in the necrotic lesions (Fig. 3B).

![Fig. 4. RT-PCR analysis of cytokine, TGF-$
\beta$, iNOS and ICAM-1 mRNA expression. Total RNA was isolated from lung tissues of STAT1 KO and WT mice at 1, 3, and 5 weeks after aerosol infection and subjected to RT-PCR using the specific primer sets. IL-12 mRNA expression level is low significantly in the lung tissues of STAT1 KO mice.](image-url)
RT-PCR analysis

Fig. 4 shows the results of the RT-PCR data from infected lung tissues at 1, 3 and 5 weeks after aerosol infection (three mice each). In STAT1 KO mice, expression of IFN-γ, TNF-α, IL-10, IL-18 and iNOS mRNA was low at weeks 3 after infection compared with that of WT mice. Expression of IL-12 p40 mRNA was very low at weeks 3-5 after infection than that of WT mice and the difference in IL-12 mRNA expression between STAT1 KO and WT mice was statistically significant (p<0.01). Expression of IL-1, IL-2, IL-4, IL-6 and ICAM-1 mRNA was similar in both STAT1 KO and WT mice.

Reconstitution of STAT1 KO mice with exogenous recombinant murine IL-12

As the defect in the STAT1 KO mice was genetically defined, the possibility of recovering immune response function by administering exogenous recombinant IL-12 subcutaneously to the KO mice was investigated. As shown in Table 1, when recombinant IL-12 was given four times subcutaneously, the sizes of the granulomatous pulmonary lesions 5 weeks after aerosol infection with M. tuberculosis were reduced significantly (mean diameter, 450±20 μm), and there were virtually no mycobacteria in the pulmonary lesions (Fig. 3D). The sizes of the granulomatous lesions without recombinant IL-12 treatment 5 weeks after aerosol infection were 5, 500±105 μm. There was a statistically significant difference in the sizes of pulmonary granulomas between STAT1 KO and STAT1 KO mice treated with IL-12 WT (p<0.01). The sizes of WT and WT mice treated with recombinant IL-12 were 400±25 and 350±15 μm, respectively. There were no apparent side effects of recombinant murine IL-12 when it was given four times subcutaneously to both STAT1 KO and WT mice.

DISCUSSION

The purpose of this study was to determine the roles of STAT1 protein as a transcription factor in defense against mycobacterial infection. STAT1 KO mice developed multifocal necrotic lesions in the lung, liver and spleen tissues and died of disseminated tuberculosis by the 35th day of infection. We have shown that STAT1 is essential for the control and survival of the M. tuberculosis infection. We made aerosol infection experiments twice and the results were reproducible. We also noted marked reduction of IL-12 mRNA expression in STAT1 KO mice. It is reported that IL-12 is crucial to the development of protective immunity in M. tuberculosis-infected mice (Cooper et al. 1997). This marked reduction of IL-12 may be explained as follows. Interferon regulatory factor (IRF)-1 mRNA is significantly reduced in IFN-γ-treated macrophages from STAT1 KO mice (Meraz et al. 1996). Stat 1 is placed upstream from IRF-1 (Darnell 1997). It is known that IL-12 production by macrophages is impaired in IRF-1 KO mice (Lohoff et al. 1997;
Hermann et al. 1998). We have also reported that IRF-1 KO mice developed multifocal necrotic lesions in the lung, liver and spleen tissues and died of disseminated tuberculosis within 43 days of infection (Yamada et al. 2002).

There are seven STAT proteins. We have recently reported the role of STAT4 in mycobacterial infection (Sugawara et al. 2003). STAT4 KO mice infected with M. tuberculosis developed large granulomas with massive neutrophil infiltration over time, but did not die of disseminated tuberculosis. Pulmonary iNOS, and IFN-γ mRNA levels were low in STAT4 KO mice, but expression of IL-12 mRNA was not affected in STAT4 KO mice. No significant difference was recognized in severity of mycobacterial infection between WT and STAT6 KO mice. Therefore, STAT1 plays the most essential role for defense against mycobacterial infection among seven STAT proteins.

IFN-γ, TNF-α, and IL-18 mRNA expression was lower in STAT1 KO mice than WT mice. Their reduced expression contributes to the progression of murine tuberculosis. We and other researchers have already reported that IFN-γ, TNF-α and IL-18 play major roles for defense against murine tuberculosis (Cooper et al. 1993; Flynn et al. 1993; Flynn et al. 1995; Sugawara et al. 1998; Kaneko et al. 1999; Sugawara et al. 1999). It is reported that STAT4 and extracellular regulated kinase 1 activation by IL-12 was intact, whereas the activation of STAT1, -3, and -5 by IL-12 was lost in a patient with atypical mycobacterial infection (Gollob et al. 2000). This impairment of STAT activation was specific for IL-12 because STAT activation IL-2, IL-15, and IFN-γ was unaffected. In our mycobacterial experiments, STAT1 activation is impaired by IL-12 because IL-12 mRNA expression was severely lost. This indicates that the activation of STAT4 alone is not sufficient for IL-12-induced IFN-γ production. There is another interesting report stating that M. tuberculosis inhibits IFN-γ transcriptional responses without inhibiting activation of STAT1 (Ting et al. 1999). One mechanism for M. tuberculosis to evade the immune response is to inhibit the IFN-γ signaling pathway and the mechanism of inhibition is distinct from that reported for Leishmania donovani or cytomegalovirus (CMV) infection (Nandan and Reiher 1995; Miller et al. 1998). It, however, seems that mice with no STAT1 have no innate response to either vesicular stomatitis virus (VSV) or Listeria monocytogenes infection because the first line of defense against potential pathogens requires the IFN response (Durbin et al. 1996; Meraz et al. 1996).

When recombinant murine IL-12 was administered subcutaneously to the infected STAT1 KO mice, the sizes of the pulmonary granulomas were reduced significantly (p<0.01). Therefore, changing the therapeutic regimens administered to the experimental model mice in our study may enable mycobacterial infection to be prevented completely (Phillpotts et al. 2003). Treatment with exogenous IL-12 also reduced the bacterial load, indicating that IL-12 plays an important role in the immune response to M. tuberculosis (Cooper et al. 1997). However, other factors than IL-12 may be involved in tuberculosis of STAT1 KO mice because IL-12 addition did not cure pulmonary tuberculosis completely.

In summary, STAT1 KO mice were highly susceptible to M. tuberculosis infection. Our data demonstrate clearly that the STAT1-mediated cytokine regulation pathway is critical for the development of protective immunity against tuberculosis.

Acknowledgements

This study was supported in part by an International Collaborative Study Grant awarded to Dr. I. Sugawara from the Ministry of Health, Labor and Welfare, Japan.

References


