Relative importance of STAT4 in murine tuberculosis

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This study was designed to determine the roles of STAT proteins in defence against mycobacterial infection. Airborne infection of STAT4 knockout (KO) mice with a *Mycobacterium tuberculosis* strain induced large granulomas with massive neutrophil infiltration over time, while that in STAT6 KO mice did not. The STAT4 KO mice succumbed to mycobacterial infection by the 80th day after infection. Compared with the levels in wild-type (WT) and STAT6 KO mice, pulmonary inducible nitric oxide synthase, interferon- α , - β and - γ mRNA levels were significantly lower in STAT4 KO mice, but expression of interleukin-2, -6, -12 and -18 mRNAs was slightly higher up to the fifth week after aerial infection. Therefore, STAT4, but not STAT6, appears to be a critical transcription factor in mycobacterial regulation.

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INTRODUCTION

Interferon (IFN)-stimulated gene factor 3 is a heterotrimeric transcription factor that consists of p48, p91 and p113. p91 was named 'signal transducer and activator of transcription' (STAT) 1 and p113 has been named STAT3 (Darnell, 1997). Other STAT proteins have since been found, and the STAT family consists of seven transcription factors. Among them, STAT4 is activated in response to stimulation with interleukin (IL)-12. In STAT4 knockout (KO) mice, Th1-helper T-cell development is inhibited and lymphocytic proliferation in response to IL-12, induction of IFN- γ expression and activation of natural killer (NK) cells are abrogated (Kaplan *et al.*, 1998). It is thought that the STAT4 and STAT6 proliferation signals are regulated via inhibition of induction of p27^{Kip1} (Kaplan *et al.*, 1998). However, STAT6 is activated after stimulation with IL-4. In STAT6 KO mice, the disappearance of Th2 lymphocytes and proliferative abrogation of lymphocytes by IL-4 have been reported (Darnell, 1997).

Tuberculosis is a chronic, airborne infectious disease. It has been reported that IFN- γ , a Th1 cytokine, and tumour necrosis factor (TNF)- α , IL-12 and IL-18, which affect Th1 T-cell development, are implicated in the pathogenesis of mycobacterial infection (Flynn *et al.*, 1993, 1995; Cooper *et al.*, 1993, 1997; Sugawara *et al.*, 1998, 1999; Kindler *et al.*, 1989; Kaneko *et al.*, 1999). We are interested in the roles of transcription factors that regulate cytokine expression in mycobacterial infection. We found that the transcription factor nuclear factor (NF)-IL-6 is critical in mycobacterial control as well as in the induction of the granulocyte-colony-

stimulating factor in alveolar macrophages that results in neutrophil activation (Sugawara *et al.*, 2001). We have also reported that NF- κ B p50 KO mice develop multifocal necrotic pulmonary lesions or lobar pneumonia and that the interaction of NF- κ B with host cells plays an important role in the pathogenesis of tuberculosis (Yamada *et al.*, 2001). These findings, together with reports on roles of STAT4 and STAT6 proteins in lymphocyte development, prompted us to explore the roles of STAT proteins in mycobacterial infection. We report here that STAT4, but not STAT6, plays an important role in defence against mycobacterial infection.

METHODS

Animals. Six-week-old BALB/c wild-type (WT) mice were purchased from Japan SLC Co. Ltd and BALB/c STAT4 KO and STAT6 KO mice (Kaplan *et al.*, 1996a, b) were purchased from Jackson Laboratories (20 mice per group). The KO mice showed no developmental abnormalities. All mice were housed in a biosafety level 3 facility and were given mouse chow and water *ad libitum* after aerosol infection with virulent mycobacteria.

Experimental infections. The virulent Kuroko strain of *Mycobacterium tuberculosis* (ATCC 35812) was grown in Middlebrook 7H9 broth for 2 weeks and then filtered using a sterile acrodisc syringe filter (Pall Corp.) with a pore size of 5.0 μ m. Aliquots of the bacterial solution were stored at -80 °C until use. The mice were infected via the airborne route by placing them into the exposure chamber of a Glas-Col aerosol generator. The nebulizer compartment was filled with 5 ml of a suspension containing 10⁶ c.f.u. of Kuroko tubercle bacilli so that approximately 100 bacteria would be deposited in the lungs of each animal (Sugawara *et al.*, 1999; Yamada *et al.*, 2001). Several infected mice were followed up to 80 days for survival.

Titration of mycobacteria (c.f.u. assay). At 1, 3, 5 and 7 weeks after aerial infection, mice were anaesthetized with pentobarbital sodium, the abdominal cavity was opened and the mice were exsanguinated after splenectomy. Lungs, spleens and livers were excised and weighed. The

Abbreviations: IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; KO, knockout; TGF, transforming growth factor; TNF, tumour necrosis factor.

left lobe of each lung and a part of the spleen tissue were weighed separately and used to evaluate *in vivo* growth of mycobacteria. The lung and spleen tissues were homogenized with a mortar and pestle and 1 ml sterile saline was added. Next, 100 µl homogenate was plated in a 10-fold serial dilution on 1% (v/v) Ogawa's egg medium. Colonies on the medium were counted after a 4-week incubation at 37 °C (Yamada *et al.*, 2001).

RT-PCR. Parts of the right lobe of the lung and the spleen tissue that remained after the c.f.u. assay were used to perform RT-PCR analysis for mRNA expression of several cytokines and inducible nitric oxide synthase (iNOS) during infection. These tissue samples were snap-frozen in liquid nitrogen and stored at -85 °C until use. RNA extraction was performed as described previously (Sugawara *et al.*, 2001; Yamada *et al.*, 2001). Briefly, frozen tissues were homogenized using a microcentrifuge tube and a tip-closed 1 ml pipette in liquid nitrogen. The homogenates were treated with the total RNA isolation reagent TRIzol (Gibco-BRL) according to the manufacturer's instructions. After RNA isolation, the total RNA concentration was measured and the RNA was then reverse-transcribed into cDNA with M-MLV reverse transcriptase (Gibco-BRL) and agarose gel electrophoresis was performed.

The PCR was performed with gene-specific primer sets for β -actin, IFN- α , - β and - γ , TNF- α , IL-1 β , -2, -4, -6, -10, -12p40 and IL-18, transforming growth factor (TGF)- β and iNOS. The DNA sequences of the primer sets and corresponding PCR conditions are summarized in Table 1. Amplification was carried out with a DNA thermal cycler 480

(Perkin-Elmer Cetus). The PCR product (10 µl) was applied for electrophoresis on a 4% (w/v) agarose and NuSieve GTG (1:3) gel and visualized using ethidium bromide staining. Thereafter, we conducted a densitometric analysis of the electrophoretic RT-PCR results using the NIH Image software version 1.62. Relative densitometric ratios were determined with β -actin mRNA as an internal control (Sugawara *et al.*, 2002).

Light and electron microscopic examination. For light microscopic examination, the right middle lobe of each lung was excised and fixed with a 20% (v/v) formalin buffered methanol solution, Mildform 20 NM [containing 8% (v/v) formaldehyde and 20% (v/v) methanol, Wako Pure Chemical Co.], dehydrated with a graded series of ethanol, treated with xylene and embedded in paraffin. Sections 5-mm-thick were cut from each paraffin block and stained with either haematoxylin and eosin or Ziehl-Neelsen stains.

For electron microscopy, the right lower lobe of each lung was fixed with 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C overnight, washed three times with cold phosphate buffer, post-fixed with 1% (w/v) osmium tetroxide in phosphate buffer at 4 °C for 1 h, dehydrated with a graded series of ethanol containing 10% (v/v) methanol and finally embedded in Spurr's low-viscosity resin. Ultrathin sections were cut using a Reichert Ultracut ultramicrotome and stained with uranyl acetate and Sato's lead solution. Stained ultrathin sections were examined with a JEOL JEM-1230 electron microscope (Yamada *et al.*, 2001).

Table 1. PCR primer sets used in this study

Target	Sequence	Annealing temperature (°C)	Cycles (n)	Product length (bp)
β -actin	Sense: 5'-TGTGATGGTGGGAATGGGTGAC-3' Antisense: 5'-TTTGATGTCACGCACGATTTC-3'	65	23	514
IFN- α	Sense: 5'-ATGGCTAGGCTCTGTGCTTTCCT-3' Antisense: 5'-AGGGCTCTCCAGAYTTCTGCTCTG-3'	68	30	524
IFN- β	Sense: 5'-CATCAACTATAAGCAGCTCCA-3' Antisense: 5'-TTCAAGTGGAGAGCAGTTGAG-3'	65	33	354
IFN- γ	Sense: 5'-TACTGCCACGGCACAGTCATTGAA-3' Antisense: 5'-TAGGCGACTCCTTTTCCGCTTCCT-3'	65	30	405
TNF- α	Sense: 5'-ATGAGCACAGAAAGCATGATC-3' Antisense: 5'-TACAGGCTTGTCACTCGAATT-3'	65	30	276
IL-1 β	Sense: 5'-CAGGATGAGGACATGAGCACC-3' Antisense: 5'-CTCTGCAGACTCAAACCTCCAC-3'	65	30	447
IL-2	Sense: 5'-CTTCAAGCTCCACTTCAAGCT-3' Antisense: 5'-CCATCTCCTCAGAAAGTCCAC-3'	65	40	400
IL-4	Sense: 5'-ACGGAGATGGATGTGCCAAACGTC-3' Antisense: 5'-CGAGTAATCCATTGTCATGATGC-3'	65	40	279
IL-6	Sense: 5'-CATCCAGTTGCCTTCTTGGGA-3' Antisense: 5'-CATTGGGAAATTGGGGTAGGAAG-3'	65	40	463
IL-10	Sense: 5'-GTGAAGACTTTCTTTCAAACAAAG-3' Antisense: 5'-CTGCTCCACTGCCTTGCTCTTATT-3'	56	40	273
IL-12p40	Sense: 5'-ATCTCCTGGTTTGCCATCGTTTG-3' Antisense: 5'-TCCCTTGGTCCAGTGTGACCTTC-3'	65	30	527
IL-18	Sense: 5'-ACTGTACAACCGCAGTAATACGG-3' Antisense: 5'-TCCATCTTGTTGTGCTCTGG-3'	58	35	319
TGF- β	Sense: 5'-CGGGGCGACCTGGGCACCATCCATGAC-3' Antisense: 5'-CTGCTCCACCTTGGGCTTGGGACCCAC-3'	65	24	405
iNOS	Sense: 5'-TGGAATGGAGACTGTCCAG-3' Antisense: 5'-GGGATCTGAATGTGATGTTT-3'	65	30	306

Statistical methods. Values were compared using Student's *t* test. For all statistical analyses, a value of $P < 0.01$ was considered significant.

RESULTS

Mycobacterial burden in the lungs and spleens of STAT KO mice

STAT4 KO mice died of disseminated tuberculosis by the 80th day after aerosol infection, whereas WT and STAT6 KO mice survived until the day they were killed (data not shown). Up until 3 weeks after aerial infection, there were no significant differences in lung and spleen titres between STAT4 KO mice and BALB/c WT mice; both groups possessed similar titres, approximately 10^5 c.f.u. in the lungs. However, after 9 weeks post-infection, many STAT4 KO mice succumbed to the mycobacterial infection. Mycobacterial titres in the lung and spleen tissues exceeded 10^6 c.f.u. At this time-point, there were statistically significant differences in both lung and spleen counts between WT and STAT4 KO mice ($P < 0.01$) (Fig. 1a). There were no statistically significant differences in lung and spleen counts between STAT6 KO and WT mice ($P < 0.01$) (Fig. 1b).

Light and electron microscopic observations of infected lungs

Consistent with the changes in mycobacterial burden, the histopathological findings from STAT4 KO and WT mice showed similar changes at 5 weeks after infection, but at 9 weeks, STAT4 KO mice showed multifocal necrotic lesions in the lung, liver and spleen. Each necrotic lesion was characterized by central necrosis, with massive accumulation of tubercle bacilli. These severe histopathological changes were not observed in the WT or STAT6 KO mice.

Electron microscopy demonstrated that alveolar macrophages in STAT4 KO mice phagocytosed more tubercle bacilli and that the engulfed tubercle bacilli were located in phagosomes and appeared to escape the killing mechanisms of the host cells. No escape of *M. tuberculosis* from phagosomes to cytoplasm was observed. The tubercle bacilli in the phagosomes of epithelioid macrophages were relatively long and contained many large vacuole-like structures (Fig. 2).

RT-PCR analysis

Fig. 3 shows the results of densitometric analysis of the RT-PCR data from infected lung tissues. In STAT4 KO mice, expression of IFN- α , IFN- β , IFN- γ and iNOS mRNA was low at weeks 1–5 after infection compared with that of WT mice. Expression of TNF- α , IL-1 β , IL-4, IL-10 and IL-12 mRNA was similar in STAT4 KO mice and WT mice. However, IL-2, IL-6, IL-18 and TGF- β mRNA were expressed at slightly higher levels in STAT4 KO mice than in WT mice. No significant differences were found between STAT6 KO and WT mice in expression of cytokine, TGF- β or iNOS mRNAs (data not shown).

Fig. 1. Mycobacterial titres in lungs (□) and spleens (○) of STAT4 KO (a) and STAT6 KO (b) mice exposed to 10^6 c.f.u. *M. tuberculosis* Kurono strain by the airborne route. Lungs (■) and spleens (●) of WT mice were included in each experiment. At the indicated times after infection, four mice from each group were killed and homogenates of lung and spleen tissues were plated on 7H10 agar. Error bars indicate SEM.

DISCUSSION

The purpose of our study was to determine the roles of STAT proteins as transcription factors in the pathogenesis of murine tuberculosis. We have shown that STAT4, but not STAT6, is essential for the control and survival of the *M. tuberculosis* infection. At the same time, the levels of IFN- α , IFN- β , IFN- γ and iNOS mRNA expression were low until the fifth week after infection in STAT4 KO mice. We repeated the experiments twice and the results thus obtained were reproducible. It has been reported that STAT4 is critical in IFN- α and IFN- β signalling (Darnell, 1997; Frucht *et al.*, 2000). Because STAT4 is absent in STAT4 KO mice, this explains the low expression of IFN- α and IFN- β mRNA. IFN- α , in combination with IL-18, induces IFN- γ expression efficiently in NK cells (Matikainen *et al.*, 2001). Although IL-18 mRNA expression is high in STAT4 KO mice, IFN- α mRNA expression is low. This explains why IFN- γ mRNA expression is also low in STAT4 KO mice.

Alveolar macrophages are not activated in STAT4 KO mice because IFN- γ mRNA expression is low and IFN- γ is unable

Fig. 2. Electron micrographs of lung lesions from STAT4 KO (a) and WT (b) mice obtained 7 weeks after airborne infection with *M. tuberculosis* Kurono strain. Phagosomes in the STAT4 KO mice contain more tubercle bacilli than those from the WT mice. Bars, 2 μ m.

to activate alveolar macrophages. This explains the low expression of iNOS mRNA, the product of which is responsible for production of NO. NO is essential for killing tubercle bacilli and it is known to play an important role in mycobacterial killing mechanisms. It has been reported that, in STAT4 KO mice, induction of IFN- γ expression and activation of NK cells are abrogated, so our present findings are consistent with these observations (Kaplan *et al.*, 1996).

The levels of expression of IL-2, IL-6, IL-12 and IL-18 mRNA were slightly higher in STAT4 KO mice than in WT mice. Although it is known that development of Th1-type T-helper cells is inhibited strongly in STAT4 KO mice, this does not explain the relatively high expression of IL-2, -6, -12 and -18 mRNA (Kaplan *et al.*, 1996). The development of Th1 cells in response to *Listeria monocytogenes*, a common intracellular pathogen, is impaired in the absence of STAT4 (Kaplan *et al.*, 1996), but there have been no reports on cytokine mRNA expression. Further studies will be required to explain this relatively high mRNA expression. The level of IL-12 mRNA expression is rather high in STAT4 KO mice. Therefore, we conclude that T cells from *M. tuberculosis*-infected STAT4 KO mice fail to respond to IL-12 due to impaired IL-12 receptor expression. Susceptibility to *M. tuberculosis* infection is not due to the inability to produce IL-12, rather to a lack of IL-12 responsiveness (Rodriguez-Sosa *et al.*, 2001).

On the other hand, STAT6 KO mice did not develop large pulmonary granulomas, similar to WT mice. STAT6 is activated by IL-4 stimulation. Lymphocytic proliferation in

response to IL-4 and the class switch to IgE and Th2-type T-helper cell development are inhibited significantly in STAT6 KO mice (Kaplan *et al.*, 1996). However, Th1-type T-helper cell development is not affected in STAT6 KO mice and IFN mRNA was expressed in STAT6 KO mice (data not shown). It is safe to say that Th2 T-helper cells do not affect *M. tuberculosis*-induced granuloma formation substantially.

There have been two recent reports on the relationship between STAT4 activation and mycobacterial infection (*Mycobacterium leprae* and *Mycobacterium avium*) (Kim *et al.*, 2001; Gollob *et al.*, 2000). IL-12 induced STAT4 phosphorylation and DNA binding in *M. leprae*-activated T cells from tuberculoid patients (Kim *et al.*, 2001). It has also been reported that activation of STAT4 alone is not sufficient for IL-12-induced IFN- γ production and proliferation and that other STAT proteins play a role in responses to IL-12 (Gollob *et al.*, 2000). However, there have been no reports of a role for STAT4 protein in *M. tuberculosis* infection. STAT4 seems to be required for promoting Th1 development and also plays a role in the inhibition of Th2 differentiation in *M. tuberculosis* infection. In the absence of STAT4, T cells are unable to produce IFN- γ or to proliferate in response to IL-12.

In summary, STAT4 KO mice succumbed to *M. tuberculosis* in the late phase of murine tuberculosis. Our data demonstrate that the STAT4-mediated pathway is critical for the development of protective immunity against tuberculosis.

Fig. 3. Densitometric analysis of *in vivo* expression of mRNA for various cytokines, iNOS and TGF-β in STAT4 KO (○, dotted lines) and WT (●, solid lines) mice infected with *M. tuberculosis* Kurono strain. RT-PCR data were obtained and densitometric ratios relative to β-actin mRNA were then obtained using NIH Image.

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REFERENCES

- Cooper, A. M. D., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G. & Orme, I. M. (1993). Disseminated tuberculosis in interferon- γ gene-disrupted mice. *J Exp Med* **178**, 2243–2247.
- Cooper, A. M., Magram, J., Ferrante, J. & Orme, I. M. (1997). Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. *J Exp Med* **186**, 39–45.
- Darnell, J. E., Jr (1997). STATs and gene regulation. *Science* **277**, 1630–1635.
- Flynn, J. L., Chan, J., Triebold, K. J., Dalton, T. K., Stewart, T. A. & Bloom, B. R. (1993). An essential role for interferon- γ in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* **178**, 2249–2254.
- Flynn, J. L., Goldstein, M. M., Chan, J., Triebold, K. J., Pfeffer, K., Lowenstein, C. J., Schreiber, R., Mak, T. W. & Bloom, B. R. (1995). Tumor necrosis factor- α is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity* **2**, 561–572.
- Frucht, D. M., Aringer, M., Galon, J. & 8 other authors (2000). Stat4 is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages at sites of Th1-mediated inflammation. *J Immunol* **164**, 4659–4664.
- Gollob, J. A., Veenstra, K. G., Jyonouchi, H. & 8 other authors (2000). Impairment of STAT activation by IL-12 in a patient with atypical mycobacterial and staphylococcal infections. *J Immunol* **165**, 4120–4126.
- Kaneko, H., Yamada, H., Mizuno, S., Udagawa, T., Kazumi, Y., Sekikawa, K. & Sugawara, I. (1999). Role of tumor necrosis factor- α in *Mycobacterium*-induced granuloma formation in tumor necrosis factor- α -deficient mice. *Lab Invest* **79**, 379–386.
- Kaplan, M. H., Sun, Y. L., Hoey, T. & Grusby, M. J. (1996a). Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* **382**, 174–177.
- Kaplan, M. H., Schindler, U., Smiley, S. T. & Grusby, M. J. (1996b). Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* **4**, 313–319.
- Kaplan, M. H., Daniel, C., Schindler, U. & Grusby, M. J. (1998). Stat proteins control lymphocyte proliferation by regulating p27^{Kip1} expression. *Mol Cell Biol* **18**, 1996–2003.
- Kim, J., Uyemura, K., Van Dyke, M. K., Legaspi, A. J., Rea, T. H., Shuai, K. & Modlin, R. L. (2001). A role for IL-12 receptor expression and signal transduction in host defense in leprosy. *J Immunol* **167**, 779–786.
- Kindler, V., Sappino, A. P., Grau, G. E., Piguet, P. F. & Vassalli, P. (1989). The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell* **56**, 731–740.
- Matikainen, S., Paananen, A., Miettinen, M., Kurimoto, M., Timonen, T., Julkonen, I. & Saraneva, T. (2001). IFN- α and IL-18 synergistically enhance IFN- γ production in human NK cells: differential regulation of Stat4 activation and IFN- γ gene expression by IFN- α and IL-12. *Eur J Immunol* **31**, 2236–2245.
- Rodriguez-Sosa, M., Monteforte, G. M. & Satoskar, A. R. (2001). Susceptibility to *Leishmania mexicana* infection is due to the inability to produce IL-12 rather than lack of IL-12 responsiveness. *Immunol Cell Biol* **79**, 320–322.
- Sugawara, I., Yamada, H., Kazumi, Y. & 7 other authors (1998). Induction of granulomas in interferon- γ gene-disrupted mice by avirulent but not by virulent strains of *Mycobacterium tuberculosis*. *J Med Microbiol* **47**, 871–877.
- Sugawara, I., Yamada, H., Kaneko, H., Mizuno, S., Takeda, K. & Akira, S. (1999). Role of interleukin-18 (IL-18) in mycobacterial infection in IL-18-gene-disrupted mice. *Infect Immun* **67**, 2585–2589.
- Sugawara, I., Mizuno, S., Yamada, H., Matsumoto, M. & Akira, S. (2001). Disruption of nuclear factor-interleukin-6, a transcription factor, results in severe mycobacterial infection. *Am J Pathol* **158**, 361–366.
- Sugawara, I., Yamada, H., Mizuno, S., Li, C. Y., Nakayama, T. & Taniguchi, M. (2002). Mycobacterial infection in natural killer T cell knockout mice. *Tuberculosis* **82**, 97–104.
- Yamada, H., Mizuno, S., Reza-Gholizadeh, M. & Sugawara, I. (2001). Relative importance of NF- κ B p50 in mycobacterial infection. *Infect Immun* **69**, 7100–7105.