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Progression of Pulmonary Tuberculosis and Efficiency of Bacillus Calmette-Guérin Vaccination Are Genetically Controlled via a Common sst1-Mediated Mechanism of Innate Immunity

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Using a mouse model for genetic analysis of host resistance to virulent Mycobacterium tuberculosis, we have identified a genetic locus sst1 on mouse chromosome 1, which controls progression of pulmonary tuberculosis. In vitro, this locus had an effect on macrophage-mediated control of two intracellular bacterial pathogens, Mycobacterium tuberculosis and Listeria monocytogenes. In this report, we investigated a specific function of the sst1 locus in antituberculosis immunity in vivo, especially its role in control of pulmonary tuberculosis. We found that the sst1 locus affected neither activation of Th1 cytokine-producing T lymphocytes, nor their migration to the lungs, but rather controlled an inducible NO synthase-independent mechanism of innate immunity. Although the sst1 macrophages responded to stimulation with IFN-γ in vitro, their responsiveness to activation by T cells was impaired. Boosting T cell-mediated immunity by live attenuated vaccine Mycobacterium bovis bacillus Calmette-Guérin or the adoptive transfer of mycobacteria-activated CD4+ T lymphocytes had positive systemic effect, but failed to improve control of tuberculosis infection specifically in the lungs of the sst1 animals. Thus, in the mouse model of tuberculosis, a common genetic mechanism of innate immunity mediated control of tuberculosis progression in the lungs and the efficiency of antituberculosis vaccine. Our data suggest that in immunocompetent humans the development of pulmonary tuberculosis and the failure of the existing vaccine to protect against it, in some cases, may be explained by a similar defect in a conserved inducible NO synthase-independent mechanism of innate immunity, either inherited or acquired. The Journal of Immunology, 2007, 179: 6919–6932.

One-third of the human population is estimated to be infected with virulent Mycobacterium tuberculosis (MTB),4 while <10% of the infected immunocompetent individuals are at lifetime risk of developing clinical tuberculosis (1). Epidemiological (2) and genetic (3, 4) studies demonstrate that genetic variation within human populations significantly contributes to the host heterogeneity in terms of tuberculosis resistance. Analysis of tuberculosis infection using experimental animals also demonstrated a role of host genetic variation in determining outcomes of host-pathogen interactions (5–8).

The mouse model of tuberculosis infection is most widely used and was instrumental in elucidation of essential pathways of antituberculosis immunity (reviewed in Ref. 9). Using a mouse model of infection with virulent MTB, it has been demonstrated that targeted mutations in genes essential for Th1-mediated immune responses or macrophage responsiveness to IFN-γ and TNF-α result in systemic failure of host resistance to MTB (10–13). Subsequently, mutations that affect those pathways in humans have been shown to result in extreme susceptibility to mycobacterial infection, including disseminated disease caused by otherwise non-pathogenic mycobacteria (reviewed in Ref. 4).

It has been well-established that immunocompetent inbred strains of laboratory mice dramatically differ in their susceptibility to infection with virulent MTB. To date, several studies have been performed to dissect the genetic control of host resistance to tuberculosis using classical linkage analysis in crosses between resistant and susceptible inbred mouse strains. In all of those studies, genetic control of tuberculosis resistance was found to be multigenic. Apt and colleagues (14) mapped three quantitative trait loci in a cross between A/Sn and I/St mouse strains. Mitsos et al. (15, 16) using a cross between DBA/2 (susceptible) and C57BL/6 (resistant) inbred mouse strains mapped four tuberculosis-resistance quantitative trait loci. Importantly, these authors found that the same locus on mouse chromosome 7 controlled the survival of mice after i.v. challenge as well as multiplication of MTB in their lungs after infection by aerosol.
Using a cross of the C3HeB/FeJ (susceptible) with the tuberculosis-resistant C57BL/6j (B6) inbred mice, we mapped five host resistance loci (17–19). The major distinction of tuberculosis progression in the C3HeB/FeJ mice was the development of large necrotic lesions in their lungs starting as early as 3 wk after systemic i.v. infection and 6 wk after an aerosol challenge with MTB. The first susceptibility locus identified in our studies, ss1 (super-susceptibility to tuberculosis), was responsible for early death of the infected mice (18). Substitution the ss1<sup>R</sup> allele for the ss1<sup>S</sup> allele in C3H.B6-sst1 congenic mice increased their survival after i.v. infection with 10<sup>5</sup> CFU of MTB to ~10–12 wk, as compared with 3.5–4 wk of the parental C3HeB/FeJ mice. Most importantly, it prevented the formation of necrotic lung lesions after both systemic (i.v.) and low dose aerosol infection (20).

In humans, damage to lung tissue by virulent MTB is necessary for the pathogen transmission. Progression of the disease leads to formation of necrotic lung lesions. When the expanding necrotic lesions erupt into airways, their contents containing live mycobacteria are released and spread to other hosts via aerosol. Therefore, the ability to induce formation of large necrotic lesions in the lung is considered a key element of a highly successful virulence strategy of MTB. This, however, occurs only in a relatively small proportion of infected individuals, most of which are immunocompetent but susceptible to tuberculosis infection. Importantly, the existing antituberculosis vaccine, live attenuated Mycobacterium bovis bacillus Calmette-Guérin (BCG), is inefficient in preventing pulmonary tuberculosis in adults, while it protects against disseminated disease in children (21, 22). From this perspective, understanding mechanisms of particular vulnerability of lung tissue to tuberculosis infection in a specific context of a susceptible, but otherwise the immunocompetent host is critical for developing efficient preventive and therapeutic interventions (9). However, the mouse model was considered inadequate for those studies, because most of the standard inbred mouse strains do not develop typical necrosis within tuberculosis lung lesions (23). Therefore, identification of the ss1 locus that controls formation of necrotic lung lesions in mice and generation of the ss1<sup>S</sup> congenic inbred mouse strains provided a unique opportunity to address this critical aspect of tuberculosis pathogenesis using mouse model of infection with virulent MTB.

Previously, we have demonstrated that the ss1-dependent mechanism of immunity was expressed by the bone marrow-derived cells. In vitro, macrophages isolated from the ss1<sup>S</sup> mice were more efficient than the ss1<sup>R</sup> ones in controlling the intracellular growth of two unrelated intracellular pathogens, MTB and Listeria monocytogenes. Using positional cloning we have identified a candidate gene within the ss1 locus, Ipr1 (intracellular pathogen resistance 1) (20). Expression of the Ipr1 gene was strongly up-regulated in the ss1<sup>R</sup> macrophages upon infection with MTB or priming with type 1 and type 2 IFNs, while this gene was not expressed in the ss1<sup>S</sup> macrophages under similar conditions. In vitro, expression of the Ipr1 transgene in the ss1<sup>S</sup> macrophages improved their ability to control multiplication of the two intracellular pathogens. It also prevented necrotic death of the infected macrophages and facilitated a switch in the mechanism of their death to an apoptotic pathway.

In this report, we further address a specific role of the ss1 locus in host resistance to tuberculosis infection in vivo, especially in control of pulmonary disease. We demonstrate that the extent of lung inflammation caused by MTB infection as well as the efficiency of vaccination with M. bovis BCG against pulmonary tuberculosis are controlled by the ss1-mediated mechanism of innate immunity, although two major known components of host resistance to tuberculosis—activity of CD4<sup>+</sup>Th1 cells and the NO-dependent effector mechanism—are not compromised in the ss1<sup>R</sup> mice.

**Materials and Methods**

Four C3H substrains—C3HeB/FeJ, C3H/HeJ, C3H/HeOuJ, C3H/HeSnJ—as well as C3Smn.CB17-Prkd<sup>S/L1</sup> and B6.129P2-Nos2<sup>2m1m2</sup> (B6.Nos2<sup>2</sup> mice were purchased from The Jackson Laboratory. The congenic C3H.B6-sst1 was generated by introgression of a 25 cM ss1-containing segment from C57BL/6j (B6) into the C3HeB/FeJ strain as previously described (20). The B6.C3H-sst1 congenic mice were obtained using the similar strategy, but an ~12-cM interval of C3H-derived susceptible ss1<sup>S</sup> allele was transferred into B6 genetic background (19). To generate the ss1<sup>R</sup>-susceptible congenic mouse strain, in which the Nos2 gene encoding iNos was inactivated, the B6.C3H-sst1 strain was crossed with B6-Nos2<sup>2m1</sup> (the iNos knockout strain, which was purchased from The Jackson Laboratory). Their F<sub>1</sub> offspring were backcrossed on B6-Nos2<sup>2m2</sup> and the Nos2<sup>2</sup> homozygous and the ss1 heterozygous progeny of that backcross were intercrossed to produce a population of Nos2<sup>2m2</sup> mice segregating at the ss1 locus. These mice were tested for susceptibility to MTB infection and also were used to establish the B6.C3H-sst1, Nos2<sup>2</sup> congenic mouse strain homozygous for the ss1<sup>S</sup>-susceptible allele and Nos2<sup>2m2</sup> mutation on the B6 genetic background (B6.C3H-sst1<sup>ss1</sup>, Nos2<sup>2</sup>). The original B6-Nos2<sup>2m2</sup> mice, which carried the B6-derived ss1<sup>R</sup> allele, were used as a control.

The scid mutation was introduced from the C3Smn.CB17-Prkd<sup>S/L1</sup> (C3H.scid) mice into the C3H.B6-sst1 genetic background to obtain the C3H.B6-sst1, scid strain. From this strain, the Prkd<sup>S/L1</sup> mutation was further moved on the C3HeB/FeJ background (Ipr1-negative). Thus, the two strains that carry the ss1<sup>R</sup> and the ss1<sup>S</sup> alleles in the presence of scid mutation on the C3H genetic background were generated. Mice were bred and maintained under specific-pathogen-free conditions in animal facilities at the Harvard Medical School and given autoclaved chow and water ad libitum. All experiments were performed with the full knowledge and approval of the Standing Committee on Animals at Harvard Medical School (protocol no. 03000).

**Bacteria and infection of mice**

MTB (Erdmann strain; Trudeau Institute, Saranac Lake, NY) was grown to mid-log phase in Middlebrook 7H9 liquid medium, washed, resuspended in PBS containing 1% FCS and 10% glycerol, aliquoted, and frozen at ~80°C until use. For infection of mice, the frozen stock was melted, sonicated, and used on the same day. The bacteria were diluted by PBS containing 0.05% Tween 80. Each mouse was infected via tail vein injection with 1 × 10<sup>6</sup> CFU of MTB in 100 μl. For infection of macrophages in vitro, the bacteria from a frozen stock was grown in liquid medium 7H9 (BD Biosciences Microbiology Systems) supplemented with 10% oleic acid/albumin/dextrose/catalase (Difo), 0.2% glycerol, and 0.05% v/v Tween 80. The bacteria were grown with agitation for 4–6 days, washed twice, sonicated using a cup sonifier (Branson Sonifier) twice for 5 s and filtered through 5.0-μm filter (Micron Separation). Bacterial density was adjusted by OD at 600 nm. The precise numbers of bacteria were estimated by plating serial dilutions of the initial inoculums on 7H10 agar. The colonies were counted after incubation for 3 wk at 37°C.

For BCG vaccination, the mice were infected i.v. with 1 × 10<sup>6</sup> CFU of live attenuated strain of M. bovis BCG Pasteur and challenged with MTB i.v. 10 wk after immunization. Aerosol infection was performed using a Wisconsin aerosol chamber as described (20). For all experiments, three to four animals per group were tested at each time point. Mice were sacrificed using halothane anesthesia. The bacterial loads in infected organs were determined by plating 10-fold dilutions in PBS with 0.05% Tween 80 of organ homogenates on 7H10 Middlebrook agar enriched with 10% oleic acid/albumin/dextrose/catalase for 21–28 days at 37°C.

**Isolation and MTB infection of murine bone marrow-derived macrophages (BMDM) in vitro**

BMDM were isolated from femurs and tibias of mice (6–8 wk old). The cells were cultured in a complete culture medium mixed with 50% DMEM and 50% HAM F-12 (HyClone) containing 10% FCS (HyClone), and 1 ng/ml IL-3 (Sigma-Aldrich) and 20% L-929 fibroblast-conditioned medium were used as a source of macrophage colony stimulation factor. After a 2-day culture, the nonadherent cells were harvested by a cup sonifier twice for 5 s and filtered through 5.0-μm filter (Micron Separation). Bacterial density was adjusted by OD at 600 nm. The precise numbers of bacteria were estimated by plating serial dilutions of the initial inoculums on 7H10 agar enriched with 10% oleic acid/albumin/dextrose/catalase for 21–28 days at 37°C.

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were maintained in complete medium containing 20% of L-929-conditioned medium to form monolayers. BMDM monolayers were infected with MTB at a multiplicity of infection (MOI) 1 bacteria per 10 macrophages (MOI 1:10). The cells were washed by PBS with 1% FCS at 6 h postinfection and incubated in complete medium. At indicated time points, three coverslips with macrophage cells were transferred in 2 ml of sterile distilled water with 0.1% Triton X-100 to lyse the cells. A 10-fold serial dilution of the cell lysates was plated on 7H10 agar, and colonies were counted to determine the number of intracellular MTB after incubation at 37°C for 3 wk.

RNA preparation and quantitation of mRNA expression

RNA samples from homogenized organs or cells were prepared using TRIzol reagent (Invitrogen Life Technologies) followed by the DNase I treatment and purification using RNeasy column (Qiagen) according to the manufacturer’s recommendations. Total RNA was reverse transcribed using RETROscript kit (Ambion) and random decamers, diluted with sterile water to 100 μl and 4 μl of the final sample, were used for PCR amplification.

Gene expression was analyzed by either quantitative real-time PCR or semiquantitative PCR. For quantitative real-time PCR, each reaction was prepared using the following reagents and concentrations in 25 μl: 1.25 U of AmpliTaqGold DNA polymerase (Applied Biosystems), 1× GeneAmp PCR buffer II, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.1 μM primers, 1/30,000 diluted 10X SYBR Green nucleic acid stain (Molecular Probes), 100 nM 6-carboxy-X-rhodamine (ROX reference dye: Invitrogen Life Technologies), and 5 μl of cDNA sample. The reaction was run using an ABI7900 (Applied Biosystems) as per the following condition: 10 min at 95°C, 40 cycles (95°C, 20 s; 60°C, 30 s; 72°C, 40 s), 5 min at 72°C, and followed by one cycle (95°C, 15 s; 60°C, 15 s; 72°C, 15 s) for a dissociation curve analysis which was performed on all samples to detect nonspecific products. cDNA standards were generated by serially diluting purified ampiclon of each gene over a range of 10⁶–10² copies. 18S rRNA was used as an internal control and each sample was set up in triplicate. The semiquantitative RT-PCR was performed as described in Ref. 24. In brief, 15 μl of reaction mixture contained 0.75 U of AmpliTaqGold DNA polymerase, 1× GeneAmp PCR buffer II (both from Applied Biosystems), 2.5 mM MgCl2, 0.2 mM dNTPs, 0.2 μM primers, 0.2 μl of cDNA sample. PCR was performed according to the following program: 95°C for 10 min, 35 cycles of 20 s at 95°C, 30 s at 60°C, 40 s at 72°C, and a final step at 72°C for 10 min. A total of 1 μl of PCR product was added in 10 μl of DNA-loading buffer. Radioactively labeled PCR products were separated on acrylamide gel. The gels were dried and incorporated radioactivity was quantitated by PhosphorImager Storm 860 (Molecular Dynamics) using ImageQuant software. The primers for PCR were designed to have an annealing temperature of 55°C to 60°C as following sequences: inducible NO synthase (iNOS): forward, 5′-CTT CAA TGG TTG GTA CAT GGG CAC-3′; reverse, 5′-TCA ACA TCT CCT GGT GGA ACA CAG-3′. 18S rRNA: forward, 5′-AGT CCC TGC CTT TTG TAC ACA-3′; reverse, 5′-CGA TCC GAG GCC CTC ACT A-3′. LRG-47: forward, 5′-TAGT TTG CCC AGG ACA AGA ACA ACA-3′; reverse, 5′-TGG CTT CTG TGG TAG AAG AAG GGT-3′.

Histopathology

Organs of infected mice were fixed with 10% buffered formalin for >24 h and then embedded in paraffin. These tissues were sectioned and stained with H&E by standard procedure at the Harvard Rodent Histopathology manufacturers’ protocol. Mice were infected i.v. with MTB (four per group). At 2 and 4 wk postinfection, the lungs were flushed and inflated with 4% paraformaldehyde in PBS and fixed for 24 h and embedded. TUNEL assay was performed using the ApopTag Apoptosis Detection kit (Serologicals) according to the manufacturer’s protocol.

Isolation of lung cells

Isolation of cells from tuberculosis lung lesions was performed essentially as described (25, 26). Briefly, mice were anesthetized with sodium pentobarbital (100 μl with 64.8 mg/ml). Following flushing of the blood vessels with 20 ml of PBS containing 10 U heparin/ml, bronchoalveolar lavage was performed with 5 ml of PBS via cannulated trachea. The lungs were collected and massaged in a petri dish containing PBS and subsequently transferred into a petri dish containing digestion medium (L-15 medium, 10 mM HEPES, 5% FCS, kanamycin (0.05 μg/ml), collagenase IV (150 U/ml; Worthington) and DNase I (50 μg/ml; Sigma-Aldrich)). A total volume of 10 ml of digestion buffer was used to digest the lungs of each mouse during incubation at 37°C in a shaker for 90 min. The cells were disaggregated by repeated pipetting and filtered through a 100-μm cell strainer to remove clumps. The intestinal lung cell suspension was washed three times with PBS supplemented with 1% FCS. The total number of lung cells from each mouse was counted in a 1:10 suspension of 2% acetic acid. The viability of the cells as determined by trypan blue exclusion was >97% at each time point.

Immunization and isolation of lymph node cells

Mice were immunized with CFA (Difco Laboratories) in the footpad of the hind feet. Popliteal lymph nodes were removed after 10–14 days, rinsed in PBS, and placed in a petri dish containing DMEM supplemented with 1% FCS. The lymph nodes were teased apart to release the lymph node cells. Following filtration through a 100-μm cell strainer, the lymph node cell suspension was washed three times with DMEM supplemented with 1% FCS and resuspended in complete culture medium. For detection of intracellular cytokines by mycobacteria-reactive T cells, the lymph node cells were labeled with CFS (Molecular Probes) at 2 μM for 30 min in serum-free medium at room temperature. The cells were washed and stimulated with purified protein derivative (PPD; 10 μg/ml) for 9 h and subsequently with PMA (50 ng/ml) and ionomycin (1 μM) for 6 h. Monensin (3 μM) was added to the cultures during the last 4 h. The gate was set on proliferating (CFSElow) cells.

Adaptive transfer of T lymphocytes in scid mice

A total of 50 × 10⁵ splenocytes obtained from immunocompetent C3H.B6-sst (sst16) and C3HeB/FeJ (sst17) mice were depleted from plastic adherent cells and transferred i.v. into C3H.B6-sst16, scid mice, which were infected with 1 × 10⁶ CFU of MTB 7 days later. CD4⁺ T cells were isolated from inguinal lymph nodes of C3H.B6-sst16 mice 2 wk following s.c. vaccination into two points with 1.4 × 10⁷ CFU of M. bovis BCG Pasteur and separated via high-affinity negative selection on the Mouse T Cell CD4 Subset Column (R&D Systems) according to the manufacturer’s instructions. A total of 4.4 × 10⁶ freshly purified cells (99% CD3⁺ CD4⁺ CD8⁻ cells by flow cytometry) were injected i.v. 24 h following infection of recipients with 10⁶ CFU of MTB Erdmann by i.v. route.

Culture of cells from tuberculosis lung lesions

Unfractionated lung cells were cultured at 2 × 10⁶/ml in DMEM/F12 plus 10% heat-inactivated FCS, 10% L-929-conditioned media (10% heat-inactivated FCS, 10% L-929-conditioned media; Mycos Research Laboratories). Cultures were incubated at 37°C/5% CO₂ for 4 days. Culture supernatants were harvested and stored at −80°C. In other assays, macrophages were depleted from lung cell suspensions by adherence onto tissue-culture flasks. The resulting nonadherent cells were cultured at 1 × 10⁶/ml in media coated with anti-CD3 (2 μg/ml) or stimulated with PPD in the presence of gamma-irradiated (2000 rad) syngeneic splenocytes. Culture supernatants were harvested after 24 h in the anti-CD3-stimulated cultures and 3 days in the cultures containing APC and PPD. Culture supernatants were kept at −80°C. For detection of intracellular cytokines, lung T cells were enriched by adherence and stimulated with PPD (10 μg/ml) in the presence of gamma-irradiated syngeneic splenocytes for 96 h, and monensin (3 μM) was added to the cultures during the last 4 h. The cells were fixed and stained as described below.

Flow cytometry

mAb specific for mouse CD3 (145-2C11, hamster IgG1), CD4 (L3T4 clone H129.19, rat IgG2a), CD8 (Ly-2 clone 53-67, rat IgG2a), CD69 (H1.2F3, hamster IgG1), L-Ab (clone 11-5.2, mouse IgG2b), and CD25 (IL-2Rα chain clone 7D4, rat IgM) were purchased from BD Biosciences Pharmingen, CD28 (clone 30H4, mouse IgG2b, PerCP), or biotin. mAb specific for mouse collagenase IV (clone F4/80 (clone BM8, rat IgG2a) was purchased from Caltag Laboratories as direct conjugate to PE. Biotinylated Ab to Tim3 was gifted by Dr. V. Kuchroo (Brigham and Women’s Hospital, Boston, MA). Unseparated
lung and lymph node cells were washed in PBS containing 1% BSA and 0.01% NaN₃ and incubated for 10 min at 4°C in the same buffer containing FcR-blocking Ab (CD16/CD32; BD Biosciences). After an additional wash, cells were triple stained for 30 min at 4°C with directly or indirectly conjugated Abs according to the manufacturer’s instructions. Stained cells were washed three times in PBS containing 1% BSA and 0.01% NaN₃, fixed in PBS containing 2% paraformaldehyde, and analyzed by flow cytometry using FACSCalibur cytometer (BD Biosciences) and CellQuest software (BD Biosciences).

Intracellular cytokine staining
Stained for surface markers and fixed cells were permeabilized by washing two times in perm/wash buffer (BD Biosciences) and incubated for 15 min in BD Biosciences perm/wash buffer. Pellet cells were resuspended in BD Biosciences perm/wash buffer containing PE-conjugated IFN-γ Ab (clone MXG1.2, rat IgG1; BD Biosciences) and incubated on ice for 30 min. After incubation, cells were washed twice BD Biosciences perm/wash buffer and resuspended in staining buffer before flow cytometric analysis (see above).

Statistical analyses
CFU data were analyzed by two-way ANOVA (type III). Survival curves were analyzed using GraphPad Prism 3.02. The influence of time postinfection on recruitment of different T cell populations was done by Spearman rank correlations. Values of \( p < 0.05 \) were considered significant.

Results
The sst1 locus prevents formation of necrotic lung lesions and limits lung inflammation in vivo
To detect the earliest correlates of the sst1-mediated resistance in vivo, we studied the kinetics of disease progression in the sst1ˢ mice...
infection with 5 × 10^4 CFU of MTB Erdman. During the first 11 days postinfection, the initial rate of multiplication of MTB was similar in both sst1^S and sst1^R congenic mice. The first difference in the bacterial loads was detected at 15 days postinfection: it was five times higher in the spleens and only 1.5 times higher in the lungs of the sst1^S mice (Fig. 1A). Within the next 3 days, however, the bacteria continued to grow rapidly in the lungs of the sst1^S mice, while the number of viable MTB in their spleens started to decline. MTB continued rapid multiplication in the lungs of the sst1^S mice until the mice died at 25–28 days postinfection with ~10^9 CFU of MTB in their lungs. In contrast, the bacterial load in the lungs and the spleens of the sst1^R congenic mice remained stable during that period.

The first microscopic differences between the tuberculosis lung lesions of the sst1^R and sst1^S mice were also detected at 15 days postinfection: we observed formation of necrotic microfoci within the lung inflammatory lesions of the sst1^S, but not the sst1^R animals (Fig. 2A, upper panels). Upon blinded microscopic examination of H&E-stained tissue sections, the extent of necrosis was significantly higher in the lungs of the sst1^S mice 15 and 18 days postinfection (Fig. 1C). By day 21, large coalescing necrotic lesions were formed in the lungs of the sst1^R mice (Fig. 2A, lower panels); these contained numerous clusters of the bacteria, associated with necrotic areas (Fig. 1D, left panels). At the same time, necrosis was virtually absent in the lungs of the sst1^R mice, although some sst1^R macrophages contained many bacterial cells (Fig. 1D, right panels).

To assess cell death within the tuberculosis lung lesions of the sst1 congenic mouse strains, we performed TUNEL assay, which detects 3′-OH DNA ends, generated by caspase-activated DNase in the nuclei of cells undergoing apoptosis. TUNEL-positive staining was detected within tuberculosis lung lesions of both sst1^S and sst1^R congenic mice. However, the distribution of TUNEL-positive material within the cells was strikingly different. At 4 wk postinfection, TUNEL staining was localized to the nuclei in >70% of all TUNEL-positive cells in the lungs of the sst1^R mice (Fig. 2B, right panels). Meanwhile, TUNEL-positive apoptotic nuclei were detected only in 8% of all TUNEL-positive cells within the necrotic lung lesions of the sst1^S mice, and the rest of the TUNEL-positive cells displayed strong cytoplasmic staining (Fig. 2B, left panels). Thus, at 4 wk postinfection, the proportion of typical apoptotic cells with TUNEL-positive nuclei was significantly higher in the lungs of the sst1^S congenic mice (p < 0.01), although the total number of TUNEL-positive cells was much higher within the necrotic lung lesions of the sst1^R mice.

Expression of proinflammatory chemokines in the lungs increased as the infection progressed (Fig. 1B). The levels of proinflammatory chemokines MIP-1α, MIP-1β, MIP-2, and MCP-1 mRNA were higher in the lungs of the infected mice as compared with their spleens (data not shown). At day 11, the cytokine levels were similar on both genetic backgrounds, but at day 15, the levels of MIP-2 and MCP-1 mRNA were 3- to 5-fold higher in the lungs of the sst1^S mice (Fig. 1, E and F).

Between the second and the fourth week of infection, we observed increased influx of inflammatory cells in the lungs of the sst1^S mice. At 4 wk, total numbers of inflammatory interstitial cells, which were isolated from the lungs using collagenase digestion, were twice as much as compared with the sst1^R congenics. At this time, CD11b^+ myeloid cells represented 46% of all inflammatory interstitial cells in the lungs of the sst1^S mice, while this population comprised only 28% of interstitial cells in the lungs of the sst1^R mice, as determined by FACS analysis (p < 0.01).

To summarize, formation of necrotic microfoci and increased production of proinflammatory chemokines were the earliest signs associated with the sst1-susceptible phenotype in vivo that we were able to detect two weeks after systemic infection. After this point in time, the differences between the sst1^S and sst1^R congenic mice rapidly increased: expanding necrotic inflammation, increased influx of myeloid cells including granulocytes, spread MTB throughout the lung tissue, and rapid growth of the bacteria with a doubling time of 28 h (similar to the growth rate of the bacteria in culture broth) were characteristic for the lungs of the sst1^S mice, which rapidly succumbed to infection. The bacterial loads in the lungs of the sst1^S mice were stable during the same period, an inflammatory reaction in the lungs was much less severe, limited to interstitial spaces and contained no areas of necrosis.

**The sst1-mediated mechanism of innate resistance is iNOS independent**

Next, we wanted to determine whether the sst1-mediated control of pulmonary tuberculosis progression was due to the NO-dependent or independent effector mechanism. At day 18 after systemic i.v. infection with MTB, the expression of macrophage-specific iNOS
mRNA was strongly up-regulated in the spleens and in the lungs of the MTB-infected sst1s and sst1R congenic mice, and its expression level was similar on both genetic backgrounds (Fig. 3A). The expression of the iNOS protein was also detected on sections of tuberculosis lung lesions using staining with iNOS-specific Abs. The iNOS protein levels were similar in both the sst1R and the sst1s mice three weeks after infection (data not shown).

We also compared expression of iNOS mRNA in the lungs of the sst1R and sst1s congenic mice with several other substrains of C3H mice. The C3HeB/FeJ mouse strain is more susceptible to MTB infection than the C3H substrains (20). Other C3H substrains have been previously shown to express the Ipr1 gene, which is a candidate gene encoded within the sst1 locus (20). Other C3H substrains have been previously shown to express the Ipr1 gene, and to be more resistant to MTB infection than the Ipr1-negative sst1s C3HeB/FeJ mice. At 25 days postinfection, expression of iNOS mRNA (Fig. 3B, left panel) as well as the bacterial loads (Fig. 3B, right panel) were the highest in the lungs of the Ipr1-negative sst1s C3HeB/FeJ mice. Most likely, the higher iNOS mRNA content in the lungs of the MTB-infected sst1s mice was due to higher MTB loads in their lungs. These data demonstrate that the induction of the iNOS gene expression after MTB infection in vivo was not compromised in the sst1s mice.

To assess the enzymatic activity of iNOS in isolated macrophages, we tested NO production by the sst1 congenic BMDM in vitro. When these cells were stimulated with IFN-γ (50 U/ml) plus LPS (10–50 ng/ml) in vitro, production of NO by the sst1s and the sst1R cells was similar (data not shown), also demonstrating normal NO-production capacity of the sst1s macrophages. However, when the BMDM were primed with IFN-γ and infected with MTB, the sst1R macrophages produced approximately two times higher levels of NO as compared with the sst1s macrophages (Fig. 3C). This was observed using the sst1 congenic pair on the C3H genetic background, C3HeB/FeJ (sst1s) and C3H.B6-sst1 (sst1R) (Fig. 3C, right panel) as well as another sst1 congenic pair on the B6 genetic background, B6 (sst1m2) and B6.C3H-sst1 (sst1s) (Fig. 3C, left panel).

The above data suggested a possibility that more efficient control of MTB in sst1R mice might be due to higher levels of NO production by the MTB-infected macrophages. Therefore, we wanted to test the effect of the sst1 locus on progression of tuberculosis infection in iNOS knockout (Nos2tm1) mice in vivo. To combine the Nos2tm1 and sst1R alleles on B6 genetic background, we intercrossed the B6.C3H-sst1 (sst1s) and B6.Nos2tm1 mice as described in Materials and Methods. The resultant sst1R congenic Nos2 knockout mice were infected with a low dose of MTB via aerosol. The bacterial loads in the lungs of the B6-sst1s,
Nos2\(^{-/-}\) mice were 10-fold higher as compared with the B6-sst1\(^R\), Nos2\(^{-/-}\) mice at 3 and 6 wk after the aerosol infection (Fig. 4A). The 10-fold difference was also observed in the lungs of the iNOS-sufficient B6 (sst1\(^R\)) and B6.C3H-sst1 (sst1\(^S\)) congenic mice (Fig. 4B), although the bacterial loads in these mice were ~100-fold lower as compared with those in the corresponding strains of the iNOS-deficient mice. Although the iNOS-deficient mice failed to control MTB multiplication in their lungs, spleens, and livers, the effect of the sst1 locus was limited to the lung environment in both iNOS knockout and in wild-type mice (Fig. 4, A and B).

The survival time of the Nos2 knockout mice segregating at the sst1 locus (sst1\(^R\), sst1\(^S\) and sst1\(^R/S\) heterozygotes) also correlated with their sst1 allele; the sst1\(^R/S\) and sst1\(^R/S\) Nos2\(^{-/-}\) mice survived significantly longer than their sst1\(^S/S\) Nos2\(^{-/-}\) littermates after either the i.v. (Fig. 4C) or aerosol (Fig. 4D) infections. Of note, the survival of the Nos2\(^{-/-}\) knockout mice of either sst1 genotype was much shorter as compared with their wild-type counterparts. For example, median survival time of the B6.C3H-sst1 mice after a low-dose aerosol infection was 225 days (19), while the B6.C3H-sst1, Nos2\(^{-/-}\) mice survived for 55 days only (Fig. 4D). Thus, in agreement with previously published data, the iNOS deficiency conferred severe systemic defect in host resistance to MTB. Importantly, even in this environment the effect of the sst1 locus was significant, i.e., iNOS independent.

The BMDM isolated from the sst1\(^R\) Nos2\(^{-/-}\) mice were superior in their ability to control MTB multiplication in vitro, as compared with the sst1\(^S\) Nos2\(^{-/-}\) macrophages, both in the presence and in the absence of IFN-\(\gamma\) (Fig. 4E). Therefore, iNOS function was not required for the sst1-mediated control of MTB by macrophages. Taken together, these data demonstrate that the sst1 locus mediates the iNOS-independent mechanism on innate immunity to MTB.

The sst1 locus confers no apparent deficiency on Th1 function

To determine whether the sst1 locus affected T lymphocyte migration to the lungs, activation status or cytokine production after the infection, we characterized T cell populations present in the lungs of the sst1\(^R\) and the sst1\(^S\) congenic mice at various times after aerosol infection. Between the 3rd and the 12th week after a low-dose aerosol challenge with MTB, the bacterial loads increased in the lungs of the sst1\(^S\) mice (Fig. 5A). Meanwhile, the bacterial loads in the lung of the sst1\(^R\) mice and in the spleens (Fig. 5B) and livers (data not shown) in both sst1\(^R\) and sst1\(^S\) mice decreased.

During this period, the total number of interstitial cells progressively increased in the lungs of both strains, but was significantly higher in the sst1\(^R\) mice at 5 and 11 wk postinfection (Table 1; \(p < 0.01\) and \(p < 0.001\), respectively). The total numbers of the CD3\(^+\)
T cells, as well as the CD4$^+$ and CD8$^+$ T cell populations, in the lungs were not significantly different between the sst1$^S$ and sst1$^R$ strains before the infection. The number of T cells, which were recruited to the lungs at 5 wk, was higher in the sst1$^S$ mice with both CD4$^+$ and CD8$^+$ T cells being more abundant ($p < 0.01$ and $p < 0.02$, respectively). At 11 wk postinfection, total numbers of the CD3$^+$ T cells were not significantly different between the strains and the difference in the absolute numbers of CD4$^+$ and CD8$^+$ T cells were only marginally significant ($p < 0.05$, in both cases).

Following the aerosol infection with MTB, we observed a progressive increase in the proportion of CD4$^+$ and CD8$^+$ T cells that expressed activation markers CD69 and CD25 as compared with the uninfected mice ($p < 0.001$, in all cases, Table I). At 5 wk postinfection, a significantly higher proportion of CD69$^+$ activated CD4$^+$ and CD8$^+$ T cells were present in the lungs of the sst1$^S$-susceptible mice ($p < 0.02$ and $p < 0.01$, respectively). The proportions of CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ T cells were also significantly higher in the sst1$^S$ ($p < 0.01$ and $p < 0.001$, respectively), perhaps, reflecting higher antigenic load in their lungs. The proportion of CD4$^+$ T cells expressing Tim3, a novel surface marker of differentiated Th1 cells (27), in the lungs was 2-fold higher as compared with the sst1$^R$ congenic mice (Fig. 5C).

Next, we studied the expression of IFN-γ and IL-4 genes in the tuberculosis lung lesions in vivo, as well as production of these cytokines by the T cells, which were isolated from the lung tuberculosis lesions, after stimulation in vitro. Expression of IL-4...
mRNA in the lungs was very low on both genetic backgrounds as determined by RT-PCR and it did not increase after the infection. Meanwhile, the levels of IFN-γ mRNA in the lungs increased after the MTB infection, but were not different between the sst1R and sst1S mice (data not shown). The ability of the lung CD4+ T cells to produce IFN-γ, IL-4, and IL-10 was also evaluated by intracellular cytokine staining either after polyclonal (anti-CD3) activation or stimulation with mycobacterial Ag, PPD, in vitro (Fig. 5D). We were unable to detect IL-10- and IL-4-producing T cells on either genetic background by this technique. The proportion of IFN-γ-producing CD4+ T cells within the T cell population following polyclonal stimulation with anti-CD3 was 2.4-fold higher in the sst1S mice (Fig. 5D, left panels). Comparable proportions of IFN-γ-producing CD4+ T cells were observed in the sst1S and sst1R mice in response to PPD stimulation (Fig. 5D, right panels). Similar levels of IFN-γ were also detected in supernatants of these cells using IFN-γ-specific ELISA (data not shown).

To compare functional activity of lymphocytes isolated from the naive sst1R and sst1S congenic mouse strains in vivo, we performed their adoptive transfer into immunodeficient C3H.B6-sst1I, scid recipients (see Materials and Methods for details) and challenged the reconstituted mice with a low dose of MTB i.v. In three independent experiments, lymphocyte transfer significantly increased the survival of the scid mice after MTB infection. However, there was no difference in survival between the recipients of either the sst1R or the sst1S lymphocytes (data not shown).

The above studies suggested to us that the severe lung disease in the sst1R mice was not due to an intrinsic defect of their T lymphocytes or a defect in recruitment of T cells to the tuberculosis lung lesions.

Phenotypic expression of the sst1 locus during the course of MTB infection is modulated by the macrophage interactions with T cells

To examine whether the sst1-mediated mechanism of host resistance would improve control of MTB infection in the absence of adaptive immunity in vivo, we compared susceptibility to tuberculosis of the immunodeficient scid mice that were sst1R congenic, i.e., carried either the sst1-resistant (sst1R) or the sst1-susceptible (sst1S) allele on the C3H.scid genetic background (see Materials and Methods). These sst1R congenic scid mice infected with a low dose of MTB (3600 CFU) i.v. survived slightly longer (median survival time = 33 days) than their sst1S scid (median survival time = 20.5 days) counterparts (p < 0.005). However, the kinetics of the MTB growth in the organs of the two immunodeficient sst1 congenic strains was practically identical: the highest MTB burden in both strains of the scid mice was observed in the spleens and no difference of the bacterial loads in lungs was detected (see Fig. S4, □, and data not shown). This pattern is clearly distinct from the progression of tuberculosis observed after i.v. infection of the immunocompetent sst1R congenic C3H.B6-sst1I and C3H.B6F1 (sst1R) animals, in which the sst1 locus largely affected the MTB multiplication in the lungs (Figs. 1A and 5A) (20). These experiments demonstrate that in the absence of intact adaptive immunity the sst1-mediated mechanism of host resistance is not sufficient to confer significant protection against tuberculosis infection in vivo.

Next, we tested the effect of the sst1 locus on T cell-macrophage interactions in coculture experiments in vitro. To generate the MTB-specific T cells, the sst1S and the sst1R congenic mice were immunized with CFA, which contained heat-killed MTB, and lymphocytes were isolated from the regional lymph nodes 12–14 days later. Both the sst1R and the sst1S lymphocytes responded to stimulation with mycobacterial Ag PPD in vitro equally well by proliferation and produced similar levels of IFN-γ. No IL-10 or IL-4 production was detected on either genetic background, as determined by ELISA and intracellular cytokine staining (data not shown). These lymphocytes were depleted of plastic adherent cells and cocultured with either the sst1R or the sst1S BMDM, which had been infected with MTB in vitro.

The sst1R and the sst1S lymphocytes were equally capable of decreasing the bacterial load of the infected macrophages after 3 and 4 days of coinoculation (Fig. 5E). The sst1R macrophages controlled the MTB growth better than the sst1S ones even in the absence of T cells and they benefited the most from the coculture with MTB-specific T cells. The bacterial burden in the sst1R macrophages after the coculture with either the sst1R or sst1S lymphocytes was reduced equally, by ~50% (p < 0.001), while the MTB burden in the sst1S macrophages decreased by <20% under similar conditions. Thus, the efficiency of MTB control in the coculture experiments correlated with the sst1 genotype of the MTB-infected macrophages, but not with the sst1 genotype of the MTB-specific T lymphocytes.

The sst1R macrophages do respond to stimulation with IFN-γ

Because IFN-γ is a most potent macrophage-activating factor produced by Ag-specific T cells and known to be essential for antmycobacterial immunity (10, 11), we wanted to determine whether the sst1R macrophages were impaired in their ability to respond to stimulation with IFN-γ and tested several parameters of IFN-γ-dependent macrophage activation in vitro. First, we found that the expression of MHC class II (I-A^d) molecule on the surface of the sst1R and the sst1S macrophages was induced equally well with both low (1–5 U/ml) and standard (50–100

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**Table 1. T cells in the lungs of the sst1S and sst1R congenic mice after aerosol infection with MTB**

<table>
<thead>
<tr>
<th>Time (wk)</th>
<th>Mouse Strain</th>
<th>Total Cells (×10⁶)</th>
<th>CD3+ (×10⁶)</th>
<th>CD4+ (×10⁶)</th>
<th>CD8+ (×10⁶)</th>
<th>CD45° (%)</th>
<th>CD69° (%)</th>
<th>CD25° (%)</th>
<th>CD69° (%)</th>
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<tbody>
<tr>
<td>0</td>
<td>C3H</td>
<td>5</td>
<td>0.93</td>
<td>0.55</td>
<td>0.27</td>
<td>9.73</td>
<td>11.90</td>
<td>3.21</td>
<td>7.30</td>
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<tr>
<td></td>
<td>C3H.B6-sst1R</td>
<td>6</td>
<td>1.04</td>
<td>0.67</td>
<td>0.30</td>
<td>7.45</td>
<td>16.77</td>
<td>3.31</td>
<td>11.87</td>
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<tr>
<td></td>
<td></td>
<td>(NS)</td>
<td>(NS)</td>
<td>(NS)</td>
<td>(p &lt; 0.05)</td>
<td>(p &lt; 0.01)</td>
<td>(NS)</td>
<td>(p &lt; 0.01)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>C3H</td>
<td>12</td>
<td>4.88</td>
<td>2.25</td>
<td>1.98</td>
<td>10.60</td>
<td>44.54</td>
<td>20.61</td>
<td>64.9</td>
</tr>
<tr>
<td></td>
<td>C3H.B6-sst1R</td>
<td>7</td>
<td>2.39</td>
<td>1.22</td>
<td>0.75</td>
<td>5.12</td>
<td>32.51</td>
<td>5.47</td>
<td>45.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.02)</td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.01)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>C3H</td>
<td>30</td>
<td>4.33</td>
<td>1.38</td>
<td>1.70</td>
<td>21.23</td>
<td>48.29</td>
<td>26.46</td>
<td>84.68</td>
</tr>
<tr>
<td></td>
<td>C3H.B6-sst1R</td>
<td>16</td>
<td>4.24</td>
<td>2.11</td>
<td>1.35</td>
<td>16.15</td>
<td>47.96</td>
<td>30.04</td>
<td>61.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.001)</td>
<td>(NS)</td>
<td>(p &lt; 0.05)</td>
<td>(p 0.01)</td>
<td>(NS)</td>
<td></td>
<td>(p &lt; 0.01)</td>
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</table>

A Intestinal lung cells were isolated from mice infected via the respiratory route with 30–50 CFU of MTB.

B At indicated time postinfection, the cells were isolated from interstitial lung tissue by collagenase digestion and analyzed by FACS (10,000 events per sample). One representative experiment of two is presented.
U/ml) concentrations of IFN-γ (Fig. 6A). Second, the sst1S and the sst1R macrophages produced similar levels of an IFN-γ-inducible chemokine IFN-γ-inducible protein 10 (IP-10) after activation with rIFN-γ in vitro (data not shown). We also determined that, in vitro, the sst1S and the sst1R macrophages produced similar amounts of proinflammatory mediators MIP-1α, MCP-1, KC, and IL-1β both after infection with MTB or stimulation with LPS in vitro (data not shown). Priming of BMDMs with IFN-γ before infection with MTB suppressed the secretion of these cytokines by the sst1S and sst1R MTB-infected macrophages to the same extent (Table II). Therefore, basic components of IFN-γ signaling were intact in the sst1R macrophages.

The LRG-47 protein is a member of IFN-γ-regulated p47 GTPase family known to mediate IFN-γ-dependent, but iNOS-independent mechanism of innate immunity to tuberculosis (28). Therefore, we wanted to determine whether the sst1 polymorphism affected the IFN-γ-inducible expression of LRG-47. As shown in Fig. 6B, BMDMs isolated from the sst1R mice of either B6 or C3H genetic backgrounds up-regulated expression of LRG-47 mRNA after activation with IFN-γ to similar or higher levels as compared with their sst1R congenic counterparts. Furthermore, the sst1S and sst1R macrophages expressed similar levels of LRG-47 mRNA after infection with MTB in vitro (MOI = 1:1) (data now shown).

These data demonstrate that major pathways of IFN-γ signaling are intact in the sst1R macrophages; however, it does not exclude a possibility that more subtle differences in IFN-γ responsiveness between the sst1 disparate macrophages may exist.

The sst1 locus limits the protective efficiency of BCG vaccination and CD4+ T cells in the lung

We wanted to examine whether stimulation of T cell-mediated immunity ameliorates the sst1-susceptible phenotype in vivo. The sst1S and the sst1R congenic mice were vaccinated with 1 × 106 CFU of live attenuated vaccine strain of M. bovis BCG i.v. Ten weeks after the vaccination, when the BCG CFU in the lungs were below the level of detection (<100 CFU), the mice were challenged i.v. with MTB. As shown in Fig. 7A, the BCG-vaccinated sst1S mice succumbed to the MTB infection even before the nonvaccinated sst1R congenic mice, while the survival time of the sst1R mice increased substantially after the BCG vaccination.

Initially, we observed positive effect of the BCG vaccination on control of MTB growth in both the sst1R and the sst1S mice: at 4 wk after the MTB infection the bacterial burdens in spleens, lungs, and livers of the BCG-vaccinated mice were lower as compared with their nonvaccinated counterparts and were similar on both genetic backgrounds (Fig. 7, B and C). However, between the fourth and the eighth weeks of infection, the bacterial burden in the lungs of the BCG-vaccinated sst1S mice increased almost 100-fold, while it remained stable in the lungs of the sst1R mice during the same period (Fig. 7C, left panel). In the spleens, the difference in the MTB burden between the strains was only 5-fold at 8 wk postinfection (Fig. 7C, right panel). Rapid increase of MTB burden in the lungs of the sst1S mice between weeks 4 and 8 postinfection correlated with formation of extensive necrotic lung lesions, which contained both intra- and extracellular acid fast bacteria (Fig. 7D, left panels). The tuberculosis lung lesions in the sst1R congenic mice were smaller contained only the intracellular bacteria and no necrotic areas within the lesions were observed (Fig. 7D, right panels). This is similar to what have been observed previously in the lungs of nonvaccinated sst1S and sst1R mice 3–4 wk after the i.v. infection (Figs. 1D and 2A). These data demonstrate that the BCG vaccination had a positive systemic effect irrespective of the sst1 allele of the host. However, the vaccine did not prevent, but rather delayed, the breakdown of immunity in the lungs of the sst1R animals. Therefore, the overall benefit of the BCG vaccine was much greater in the sst1R mice.

Next, we purified mycobacteria-primed CD4+T lymphocytes from the BCG-vaccinated sst1R donors, C3H.B6-sst1, and adaptively transferred the identical T cell populations into the sst1R or sst1S congenic immunodeficient scid mice of the C3H genetic background—C3H, scid, and C3H-sst1R, scid. The scid mice were

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>C3H.B6-sst1</th>
<th>C3H</th>
<th>p Valuesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP-1α</td>
<td>99.7</td>
<td>99.4</td>
<td>0.0664</td>
</tr>
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<td>MCP-1</td>
<td>70.8</td>
<td>67.1</td>
<td>0.322</td>
</tr>
<tr>
<td>KC</td>
<td>98.3</td>
<td>97.9</td>
<td>0.2077</td>
</tr>
<tr>
<td>IL-1β</td>
<td>98.6</td>
<td>97.5</td>
<td>0.2667</td>
</tr>
</tbody>
</table>

b Numbers in columns represent the percent of inhibition of cytokine production calculated as follows: (concentration of a cytokine after MTB infection produced by nontreated macrophages − concentration of a cytokine after MTB infection produced by IFN-γ-treated macrophages)/concentration of a cytokine after MTB infection produced by nontreated macrophages × 100.

Values of p were obtained by comparing levels of individual cytokines in supernatants of the sst1S and sst1R macrophages treated with IFN-γ (10 U/ml) and infected with MTB (1:3) for 18 h. Experiment was repeated twice.
infected with $10^4$ CFU of MTB Erdman i.v. a day before the adoptive transfer. In these settings, possible effects of the $\text{sst1}$ locus on T cell priming and of residual BCG vaccine on the course of MTB infection were eliminated. First, we compared the disease progression in the $\text{sst1}$ congenic $\text{scid}$ mice with and without adoptively transferred CD4$^+$ T cells. The $\text{scid}$ mice without adoptively transferred T cells had the highest MTB loads and succumbed to the infection 4–5 wk postinfection. As shown in Fig. 8A, at 3 wk postinfection the effect of the adoptively transferred CD4$^+$ T cells was most pronounced in the spleens, where the bacterial loads were reduced several hundred fold. In the lungs the effect of T cells was significant, but less prominent. Overall, the organ distribution of MTB in mice, which received the mycobacteria-primed CD4$^+$ T cells was similar to this of the immunocompetent BCG-vaccinated mice (Fig. 7B). At 3 wk, both the $\text{sst1}^R$ and $\text{sst1}^S$ $\text{scid}$ mice, which received the T cells, had similar bacterial loads. Again, a dramatic, 1000-fold increase of MTB load was observed in the lungs of the $\text{sst1}^S$ $\text{scid}$ mice even in the presence of the BCG-primed CD4$^+$ T cells between 3 and 6 wk postinfection, while the bacterial growth was efficiently controlled in their spleens and livers (Fig. 8B). A severe necrotic inflammation was observed in lungs, but not other organs, of these mice at the 6-wk time point (Fig. 8C). This was prevented in $\text{scid}$ mice that carried the $\text{sst1}^R$ allele and received adoptively transferred CD4$^+$ T cells. The later experiments excluded a possibility that the development of necrotic lung lesions was mediated by cytotoxic CD8$^+$ T cells and

**FIGURE 7.** Protective effect of vaccination with *M. bovis* BCG is dependent on the sst1 genetic polymorphism. A. Survival of naive (open symbols) and BCG-vaccinated (filled symbols) $\text{sst1}^S$ and $\text{sst1}^R$ congenic mice after i.v. infection with $10^3$ CFU of MTB, $S = \text{sst1}^S$ (triangles), $R = \text{sst1}^R$ (squares). B. MTB burden in the organs of naive and BCG-vaccinated $\text{sst1}^S$ (S) or $\text{sst1}^R$ (R) mice 4 wk after infection with $10^5$ CFU of MTB i.v.

C. Kinetics of MTB growth in the lungs (left panel) and spleens (right panel) of the BCG-vaccinated $\text{sst1}^S$ (S) and $\text{sst1}^R$ (R) congenic mice after infection with $10^3$ CFU of MTB i.v.

D. Histopathology of the lungs of the BCG-vaccinated $\text{sst1}^S$ and $\text{sst1}^R$ congenic mice 8 wk after infection with $10^5$ CFU of MTB i.v.: upper panels, H&E (original magnification, ×40); lower panels, acid fast fluorescent staining of MTB (AFF, original magnification, ×400).
directly demonstrated the importance of the sst1-mediated innate immune mechanism in the genetic control of this process.

**Discussion**

An important finding in our studies is that, despite their extreme susceptibility to tuberculosis, the C3HeB/FeJ mice are not generally immunodeficient. The fulminant course of tuberculosis infection in these mice is not a result of systemic failure of host immunity, as observed in scid, T cell-, IFN-γ-, TNF-α- or iNOS-deficient animals. Instead, a major characteristic of tuberculosis progression in the sst1-susceptible mice in vivo is the development of necrotic lung lesions and rapid multiplication of MTB within those lesions, while progression of the tuberculosis infection in other organs is controlled much more efficiently.

To establish a specific role for the sst1-mediated mechanism in control of pulmonary tuberculosis, we compared progression of the infection in the sst1<sup>S</sup> and sst1<sup>R</sup> animals at short intervals. A dichotomy was first observed at 2 wk postinfection. The sst1-susceptible phenotype was distinguished by formation of necrotic microfoci. At that time, the difference in bacterial loads between the lungs of the sst1<sup>S</sup> congenic mice was <2-fold, and the MTB loads in the lungs of the sst1<sup>R</sup> C3HeB/FeJ mice were ~5–10 million per organ (Fig. 1A). At these MTB loads, no necrosis was evident in the lungs of other immunocompetent mouse strains, such as B6, BALB/c, SJL, C3H.B6-sst1 congenics as well as other substrains of C3H. Formation of necrotic lung lesions in these strains may be observed at a terminal stage of the disease, when the bacterial load in the lungs reach ~1–2 × 10<sup>8</sup> CFU or higher. This is similar to necrosis in the organs of the IFN-γ-deficient mice (10), which also occurs due to a very high bacterial load. In contrast, signs of the necrotic lung inflammation in the lungs of the sst1-susceptible C3HeB/FeJ mice were detected when the MTB burden was ~20-fold lower. Therefore, initial formation of the necrotic microfoci in the lungs of the sst1<sup>S</sup> mice could not be explained by extremely high MTB bacterial burden. Instead, formation of necrotic microfoci in the sst1-susceptible C3HeB/FeJ mice preceded uninhibited multiplication of the bacteria in the lungs and most likely generated an environment in which the bacteria thrived and disseminated throughout the lung tissue. A similar dichotomy of tuberculosis progression in the sst1 congenic mice was observed after a low-dose aerosol infection, albeit at a much slower pace (20). Thus, the sst1 locus appeared to control sensitivity of the inflammatory lung tissue to virulent MTB and its effect was not dependent on the route of infection.

Previously, Chackerian et al. (29) reported that there were more IFN-γ-producing CD4<sup>+</sup>-positive T cells in the tuberculosis lesions in the resistant parental B6 mice as compared with the susceptible C3HeB/FeJ mice. Deficit of functionally active T cells in the tuberculosis lesions of the extremely susceptible C3HeB/FeJ mice could provide a plausible explanation, as to why the adaptive immunity, although active systemically, failed to control tuberculosis progression in the lungs of the sst1<sup>S</sup> mice. However, using the H-2 congenic mouse strains Kamath et al. (30) have found that the greater number of the Th1-type T cells in the lungs was controlled by the MHC locus on chromosome 17 and associated with the H-2<sup>B</sup> haplotype, which, nevertheless, did not correlate with protection. In contrast, we have shown that progression of tuberculosis infection in the lungs was controlled by the sst1 locus.
(18), although the sst1 polymorphism did not affect the recruitment of T cells to the lungs of the MTB-infected mice, nor the balance of Th1/Th2 cytokine production. Our data suggested that formation of necrotic inflammatory lesions in the lungs of the sst1R animals could not be attributed to functional deficiency of T lymphocytes in that organ (Fig. 5, C and D, and Table I).

Using coculture experiments in vitro, we found that the effect of the sst1 locus could be explained by intrinsic properties of macrophages (Fig. 5E). At the same time, we have demonstrated that two basic mechanisms of tuberculosis resistance were intact in the sst1R macrophages, which responded to stimulation with IFN-γ (Fig. 6 and Table II) and produced NO after infection with MTB in vitro (Fig. 3C). In vivo, total macrophage unresponsiveness to IFN-γ would lead to systemic failure of antimycobacterial immunity and lack of control of even avirulent vaccine strain M. bovis BCG (31). This was not the case in the sst1-susceptible mice, in which the effect of the sst1 locus was more subtle, and was revealed specifically in the lung microenvironment, rather than systemically. We have previously established that progression of lung disease caused by virulent strain M. bovis Ravelen was also under the sst1 control (I. Kramnik, unpublished observations). However, the attenuated vaccine strain M. bovis BCG was efficiently controlled in our experiments by the sst1-independent mechanism: after systemic infection with BCG the bacterial burden in the organs of both the sst1S and sst1R congenic mouse strains declined in agreement with the well-established fact that both strains carried the resistant allele of the Nrampl gene (18, 20). Two months after i.v. injection of the sst1S and sst1R congenic mice with M. bovis BCG, only several hundred CFU of the vaccine strain could be detected in the spleens of both mouse strains, while the numbers of live M. bovis BCG in the lungs were below the level of detection (<50 CFU) and no inflammatory lung lesions were observed. Thus, the susceptible allele of the sst1 locus did not compromise host resistance to attenuated vaccine strain of M. bovis BCG. Taken together, our data demonstrate that principal components of macrophage responsiveness to activation with IFN-γ were intact in the sst1R mice.

Production of reactive NO by activated macrophages is an important component of macrophage-mediated mechanism of host resistance to tuberculosis (32–34). In the mouse model, lack of NO production led to pronounced systemic defect in control of MTB growth (33). However, both in humans and in mice existence of NO-independent mechanisms of innate immunity have been demonstrated (28, 35, 36) and postulated to play a prominent role in the human disease. We have shown that the antituberculosis effect of the sst1 locus on macrophage function in vitro, as well as on control of MTB multiplication in the lungs of immunocompetent mice and their survival, were detectable in the sst1 congenic Nos2−/− mouse strains. Therefore, the sst1-dependent mechanism was not mediated by control of the iNOS activity. However, the iNOS function was undoubtedly necessary for maximal antituberculosis resistance, because both the sst1S and sst1R congenic mouse strains were significantly more resistant to MTB infection as compared with their iNOS-deficient counterparts (Fig. 4, A and B).

To assess the effect of the sst1 locus on tuberculosis progression in the absence of adaptive immunity in vivo, we have generated immunodeficient mouse strains that carry the C3HeB/FeJ-derived sst1R or the B6-derived sst1K alleles on the C3H.scid genetic background and, therefore, due to mutation in the Prkdc gene are unable to generate mature T and B lymphocytes. The scid mice were very susceptible even to a low dose (600 CFU) of virulent MTB irrespective of their sst1 allele, although the sst1R mice survived slightly longer. The course of the disease in the scid mice differed from that in corresponding immunocompetent mice: the spleens, not the lungs, were the major site of the bacterial multiplication (Fig. 8A). In scid mice, reconstituted with mycobacteria-specific CD4+ T lymphocytes the MTB growth was controlled much more efficiently. The effect of the adoptive transfer of mycobacteria-specific T lymphocytes was especially pronounced in the spleens, where the bacterial loads were reduced almost 1000-fold. However in the lungs, the mycobacteria-specific CD4+ T cells were less efficient. In these settings, the sst1 genotype of the scid recipient mice, not of the mycobacteria-specific CD4+ T cells (that carried the sst1R allele), determined the outcome of the infection: similar to the sst1K immunocompetent mice C3HeB/FeJ, the sst1K scid mice reconstituted with sst1K CD4+ T cells developed necrotic lung lesions and were unable to control multiplication of MTB specifically in that organ. These experiments established that the sst1 locus directly affected innate immunity to tuberculosis, but functional CD4+ T cells were necessary for its phenotypic expression. From the genetic perspective, this is an example of epistatic gene interactions between the scid and sst1 loci. The scid mutation conferred a severe systemic defect on antituberculosis immunity due to the lack of adaptive immune response and almost completely obliterated beneficial effect of the sst1K allele.

We also studied whether augmentation of T cell-mediated immunity could ameliorate the sst1-susceptible phenotype. Initially, the BCG vaccination did produce a notable decrease in bacterial burdens in all organs of both the sst1S and sst1R congenic mice. However, boosting the T cell immunity by vaccination with BCG failed to protect the sst1S mice efficiently. Perhaps, in the vaccinated mice, the numbers of the bacteria in circulation was decreased and initial deposition of the pathogen in the lungs was reduced. That delayed, rather than prevented, the development of necrotic inflammation in the lungs of the sst1S mice, because the vaccine did not remedy the genetic defect of macrophages.

It has been proposed that necrotic centers in tuberculosis granulomas develop as a result of T cell-mediated immunity leading to excessive inflammation via cytokine production and activity of cytolytic T cells (37). In our adoptive transfer experiments we excluded a possibility that the necrosis in the lungs of the sst1S mice was due to cytotoxicity of CD8+ T lymphocytes, because we transferred only purified CD4+ T cells and still observed formation of necrotic lung lesions. Orme and coworkers (38) proposed an alternative hypothesis that the initial development of a necrotic core within the granuloma after the primary infection is triggered by innate immune responses before the emergence of the acquired immunity, and an effective vaccination appeared to prevent the formation of early necrosis due to very rapid lymphocytic responses in vaccinated animals. Our data indicate that the sst1-mediated effect is more consistent with the Orme hypothesis. However, in mice that carry the susceptible allele at the sst1 locus, the necrotic lung lesions develop even in the presence of primed mycobacteria-specific CD4+ T lymphocytes. Although, the precise mechanism of macrophage cell death in the lungs of the sst1S and sst1R mice remains to be established, we hypothesize that the defect in the yet unknown sst1-mediated molecular pathway makes macrophages intrinsically more sensitive to cytotoxic factors secreted by virulent MTB, for example ESAT-6 protein (39, 40). Therefore, irreversible damage to the sst1K macrophages upon infection occurs at much lower bacterial loads as compared with the sst1K animals. In the lungs, where proportion of inflammatory macrophages after MTB infection is especially high, this may result in inefficient clearance of the damaged cells, release of their cytoplasmic content leading to extensive inflammation and, eventually, tissue destruction. This promotes unrestricted multiplication and further spread of the virulent mycobacteria within the lung.
tissue including airspaces. In humans, similar course of events may generate conditions for the pathogen transmission via aerosol.

Our observations support the prediction that, if in susceptible hosts the failure to resolve the infection in the lungs was due to an intrinsic deficiency in macrophage responsiveness, the number of MTB-specific T cells produced might not matter (9). This notion confers a theoretical limit on how efficient even the ideal antituberculosis vaccine that predominantly activates CD4+ Th1-type T cells could be and raises a practical question of how defects in innate immunity could be identified and corrected most efficiently. Identification of genetic loci and genes, in which variation increases susceptibility of otherwise immunocompetent hosts to pulmonary tuberculosis, will help uncover specific pathways that are effectively exploited by this evolutionary successful pathogen and suggest new approaches to the development of preventive and therapeutic strategies that specifically target individuals with genetic or acquired defects in innate immunity predisposing to tuberculosis.

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Disclosures

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