The Host Resistance Locus *sst1* Controls Innate Immunity to *Listeria monocytogenes* Infection in Immunodeficient Mice[1]

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Epidemiological, clinical, and experimental approaches have convincingly demonstrated that host resistance to infection with intracellular pathogens is significantly influenced by genetic polymorphisms. Using a mouse model of infection with virulent *Mycobacterium tuberculosis* (MTB), we have previously identified the *sst1* locus as a genetic determinant of host resistance to tuberculosis. In this study we demonstrate that susceptibility to another intracellular pathogen, *Listeria monocytogenes*, is also influenced by the *sst1* locus. The contribution of *sst1* to anti-listerial immunity is much greater in immunodeficient *scid* mice, indicating that this locus controls innate immunity and becomes particularly important when adaptive immunity is significantly depressed. Similar to our previous observations using infection with MTB, the resistant allele of *sst1* prevents formation of necrotic infectious lesions in vivo. We have shown that macrophages obtained from *sst1*-resistant congenic mice possess superior ability to kill *L. monocytogenes* in vitro. The bactericidal effect of *sst1* is dependent on IFN-γ activation and reactive oxygen radical production by activated macrophages after infection, but is independent of NO production. It is possible that there is a single gene that controls common IFN-dependent macrophage function, which is important in the pathogenesis of infections caused by both MTB and *L. monocytogenes*. However, host resistance to the two pathogens may be controlled by two different polymorphic genes encoded within the *sst1* locus. The polymorphic gene(s) encoded within the *sst1* locus that controls macrophage interactions with the two intracellular pathogens remains to be elucidated. ***The Journal of Immunology*****, 2004, 173: 5112–5120.**

Host populations are heterogeneous in terms of their susceptibility to infectious agents. A variety of epidemiological, clinical, and experimental approaches have convincingly demonstrated a significant contribution of host heredity in resistance to infection (reviewed in Refs. 1–3). From those studies it has become increasingly clear that genetic control of immunity to infection is a complex genetic trait (4, 5). Therefore, the focus of this field has shifted from the search for major qualitative genetic regulators of immunity (6) toward identification of multiple genetic factors, in which polymorphisms have a smaller, quantitative effect on the outcome of host-parasite interactions (7).

The contribution of genetic polymorphisms to human variation in tuberculosis resistance has been amply demonstrated in twin, association, and linkage studies (8). As a result of this ongoing work, several genes (*NRAMP1* (*SLC11A1*), vitamin D receptor, and HLA-DQ) have been shown to contribute to variation in tuberculosis susceptibility in human populations. However, the effect of these genes is accountable for only a small proportion of the overall genetic variation, justifying the need for multiple approaches for identifying additional host resistance factors. As suggested by the work performed with NRAMP1, the identification of gene polymorphisms in mouse models of infection susceptibility can provide a ready resource of gene candidates to evaluate in human models of infection (9, 10).

Recently, experimental tuberculosis infections of C57BL/6J (resistant) and C3HeB/FeJ (susceptible) inbred strains of mice have been used to define a region that encodes a genetic determinant of tuberculosis susceptibility (11). This locus (*sst1*, supersusceptibility to tuberculosis) has been mapped to a 5-cM interval (49–54 cM) on mouse chromosome 1. A C3H.B6-*sst1* congenic mouse strain that carries the C57BL/6J-derived resistant allele at the *sst1* locus ($sst1^{B6}$) was generated by introgressing a 20-cM interval (43–64 cM) of mouse chromosome 1 from C57BL/6J to the C3HeB/FeJ background in a series of 10 backcrosses.[4] Using this congenic strain we have demonstrated that the presence of $sst1^{B6}$ on the susceptible C3HeB/FeJ genetic background results in better control of multiplication of virulent *Mycobacterium tuberculosis* (MTB),[5] prevention of formation of necrotic lesions in the lungs and prolonged survival of mice after both i.v. and aerosol challenges with MTB. The identity of the tuberculosis resistance gene encoded within the *sst1* locus and its precise function remain unknown. At this step, it is important to determine whether the *sst1* effect is specific for MTB and whether this locus controls innate or adaptive immunity to an intracellular pathogen(s).

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[5] Abbreviations used in this paper: MTB, *Mycobacterium tuberculosis*; AMG, aminoguanidine hemisulfate; BMDM, bone marrow-derived macrophage; HE, hydroethidine; HPRT, hypoxanthine phosphoribosyltransferase; LLO, listeriolysin O; MOI, multiplicity of infection; NAC, *N*-acetyl-L-cysteine; NGMMA, N^G-monomethyl-L-arginine; PEC, peritoneal exudate cell; ROI, reactive oxygen intermediate; SOD, superoxide dismutase.

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Infection of mice with *Listeria monocytogenes* is widely used for studies of innate and adaptive immunity (12–14). Intravenous challenge with this pathogen causes acute disseminated infection, and inbred mouse strains differ in their ability to control the acute primary infection (15, 16). Previously, deficiency of the C5 component of complement, located on mouse chromosome 2, was found to influence the susceptibility of some mouse strains (17, 18). More recently, genetic studies of differential sensitivity to infection with *L. monocytogenes* using BALB/cByJ and C57BL/6ByJ strains of mice identified two resistance loci on chromosomes 5 and 13 (19). In addition, in those studies a minor locus was mapped to a distal part of chromosome 1, in close proximity to the *sst1* locus.

In this study we assessed whether alleles of *sst1* could affect natural host resistance to *L. monocytogenes*. Natural resistance to systemic infection with *L. monocytogenes* was tested using a set of *sst1* congenic strains on a *scid* background. Mice that carry the *scid* mutation lack mature T and B cells because of an inactivating mutation in the DNA-activated protein kinase *Prkdc* gene (20, 21). We have found that the resistant allele of *sst1* ($sst1^{B6}$) confers an increase in resistance to *L. monocytogenes*, especially in the *scid* background, where the effects of the adaptive immune response are greatly, if not completely, removed. These findings establish that the *sst1* locus controls innate immunity, and its effect is independent of the function of T and B cells in the *L. monocytogenes* model in vivo. We have also demonstrated that the presence of the B6-derived resistant allele at the *sst1* locus ($sst1^{B6}$) correlates with the superior ability of the congenic bone marrow-derived macrophages to kill the bacteria in vitro. Activation of macrophages by IFN-γ before infection was necessary to reveal the effect of the *sst1* locus in vitro. The effect of the *sst1* locus correlated with higher production of reactive oxygen intermediates (ROI) by the $sst1^{B6}$ (resistant) macrophages, and the *sst1*-dependent killing of *L. monocytogenes* in our model was mediated by ROIs, but was not dependent on NO production.

Materials and Methods

Animals

C3HeB/FeJ, C3Smn.CB17-$Prkdc^{scid}$/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The C3H.B6-*sst1* and C3H.B6-*sst1,scid* mouse strains were generated in our laboratory. The C3H.B6-*sst1* mice were obtained by introgression of an ~20-cM interval of C57BL/6J-derived chromosome 1 (43–64 cM) encompassing the mouse tuberculosis susceptibility locus *sst1* (11) on the C3HeB/FeJ genetic background using 10 backcrosses.[4] To obtain the C3H.B6-*sst1,scid* mouse strain, C3HSmn.C-$Prkdc^{scid}$/J mice that carry the *scid* mutation in the *Prkdc* gene on the C3H genetic background were crossed to C3H.B6-*sst1* mice. The resulting F_1 hybrids were backcrossed on the C3HSmn.C-$Prkdc^{scid}$, and the backcross progeny were intercrossed to produce immunodeficient C3H.B6-*sst1,scid* mice homozygous for both the resistant allele at the *sst1* locus and a mutant allele of the *Prkdc* gene. The abbreviations used for the congenic inbred strains used in this study are as follows: C3HeB/FeJ, C3H; C3Smn.CB17-$Prkdc^{scid}$, C3H-*scid*; C3H.B6-*sst1*, C3H-$sst1^{B6}$; and C3H.B6-*sst1,scid*, C3H-$sst1^{B6}$,*scid*. Mice were housed under specific-pathogen-free conditions in barrier animal facilities at the Harvard Medical School and were provided 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.

Bacterial strains

In this study virulent *L. monocytogenes* strain 10403S was used for in vivo infection and in vitro experiments, and a listeriolysin O $(LLO)^-$ mutant strain of *L. monocytogenes* containing in-frame deletion of the *hly* gene (DP-L2161) was used for in vitro experiments (22). Bacteria were stored as frozen aliquots at ~10^8 CFU/ml.

Infection of mice and determination of the bacterial load

Before infection, a frozen aliquot of *L. monocytogenes* strain 10403S was thawed, diluted 10-fold in tryptic soy broth medium (Difco, Detroit, MI), and recovered at 37°C for 1 h and 15 min. The resulting bacterial culture was diluted 200-fold in sterile PBS to obtain 3–10 $\times$ 10^4 CFU/ml. Three hundred microliters of diluted culture containing 1–3 $\times$ 10^4 CFU of *L. monocytogenes* was injected via lateral tail vein. The dose of bacteria allowing reliable differentiation between resistant C57BL/6J and susceptible animals of the C3H background was determined in a dose-response experiment. Intravenous infection of animals by tail vein injection of 1–3 $\times$ 10^4 CFU of *L. monocytogenes* 10403S led to death of C3HeB/FeJ, C3H/HeJ, and C3H.SmnJ mice at ~94 h, whereas all animals of the C57BL/6ByJ background were able to recover. Our observation schedule defined an 8-h window for determination of the death time point. Livers and spleens of infected animals were harvested at defined time points. Before organ harvest, infected animals were euthanized by CO_2 asphyxiation. Spleens and 0.3-g fragments of liver were removed using a sterile technique and were placed in 2.5 ml of sterile 0.02% Nonidet P-40. After determination of the precise weight of the removed tissue, cell lysis was induced by homogenization using a Polytron homogenizer (Brinkmann Instruments, West Orange, NJ). Five microliters of tissue homogenates and serial 5-fold dilutions were plated in triplicate on TSB agar (Difco) plates containing 100 μg/ml streptomycin. After overnight incubation, the number of bacteria per milligram of tissue was determined by counting colonies at the appropriate dilution.

Histology

Livers and spleens were removed aseptically after death from infected animals and were fixed in 10% buffered formalin. The sections were prepared and stained with H&E according to established techniques at the Harvard Rodent Histopathology Core Facility.

FACS analysis

Splenocyte isolation. For FACS analysis, mice were infected with 1 $\times$ 10^5 *L. monocytogenes* i.v. Single cell suspensions were prepared from the spleens 48 h after the infection. The spleens were removed, and half of each spleen was fragmented in 5 ml of DMEM containing 1% FCS, followed by filtration through a 100-μm pore size mesh cell strainer (BD Biosciences, Mountain View, CA) to obtain a single-cell suspension. The suspension was treated with ACK lysis buffer (Quality Biological, Gaithersburg, MD) to remove erythrocytes and was washed three times in PBS supplemented with 1% FCS. The viability of the cells, as determined by trypan blue exclusion, was >98%.

Peritoneal exudate cell (PEC) isolation. Mice were infected with 10^6 CFU of *L. monocytogenes* i.p. as previously described (23). Cells were isolated from peritoneal cavity of mice 6 h after the infection by flushing with 10 ml of PBS plus 5 μg/ml gentamicin. Cells were stained with Gr-1, Ly-6C-specific Abs. For the analysis of superoxide anion production by myeloid cells in vivo, PECs isolated from the infected mice were stained with hydroethidine (HE; described below), either alone or in combination with Gr-1-specific (clone RB6-8C5) and Ly6C-specific (clone AL-21) mAbs, as described below.

Surface staining for FACS analysis. Cells were washed in PBS containing 0.05% BSA and 0.01% NaN_3 and were incubated for 30 min at 4°C in the same buffer containing FcR-blocking Ab (CD16/CD32; BD Pharmingen, San Diego, CA). After an additional wash, cells were triple-stained with directly or indirectly conjugated Abs according to the manufacturer's instructions. All Abs, except Tri-color-anti-F4/80 (Caltag Laboratories, Burlingame, CA), were purchased from BD Pharmingen: FITC-anti-CD4, FITC-anti-B220/CD45R, FITC-anti-CD8a, FITC-anti-Gr1, FITC-anti-CD11c, FITC-anti-F4/80, PE-anti-CD3, PE-anti-CD11b, PerCP-anti-CD8, biotin-anti-NK, biotin-anti-I-A, and PerCP-anti-streptavidin. Stained cells were washed three times with PBS containing 0.05% BSA and 0.01% NaN_3, fixed in PBS containing 2% paraformaldehyde, and analyzed using a FACSCalibur flow cytometer (BD Biosciences). The absolute numbers of cells in individual populations were calculated by multiplication of the absolute number of cells per organ by the percentage of corresponding cells in the total population, as determined by FACS.

Quantitation of cytokine mRNA expression in vivo by real time RT-PCR

RNA was isolated from the spleens of *L. monocytogenes*-infected and noninfected C3H-*scid* and C3H.$sst1^{B6}$,*scid* mice. Spleens were aseptically removed and snap-frozen in liquid nitrogen. Frozen organs were homogenized using a Polytron homogenizer (Brinkmann Instruments, West Orange, NY) in TRIzol (Invitrogen Life Technologies, Carlsbad, CA) according to the TRIzol protocol for isolation of RNA from tissues. Isolated RNA was dissolved in 50 μl of RNase-free water (Ambion, Austin, TX). DNase treatment of RNA was then performed using an RNeasy Mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA). RNA

integrity was checked by running denatured samples on a formaldehyde gel. After DNase I treatment and cleanup, 2 μg of RNA/sample was reverse transcribed to cDNA using an Ambion RETROscript kit. The cDNA was diluted 1/25 in nuclease-free H_2O to use in quantitative PCR.

Quantitative PCR was performed on each sample in duplicate for each gene using a 50-μl reaction mixture with SYBR Green as the reporter. Hypoxanthine phosphoribosyltransferase (HPRT) was used as an internal standard, and known dilutions of control RNA were also run to test the efficiency of each primer during the reaction. Each reaction mixture contained the following reagent concentrations: 1.25 U ABI AmpliTaq Gold (Applied Biosystems, Foster City, CA), ABI 1× PCR buffer (2.5 mM $MgCl_2$, 0.125 mM dNTPs, and 0.25 nM primers), 1/60,000 dilution of 10,000× SYBR Green nucleic acid stain (FMC Bioproducts, Rockland, ME), and 5 μl of cDNA sample. The reaction was run using DNA Engine Opticon 2 (MJ Research, Cambridge, MA). After denaturation at 95°C for 10 min, a three-step PCR cycle was used as follows: 95°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min for 35 cycles, followed by a 5-min extension at 72°C. Because nonspecific fluorescence reporter was used, DNA was run on an agarose gel to confirm the presence of a single band to ensure accurate results. The threshold cycle of each sample was obtained using MJ Research Opticon 2 software by subtracting the baseline fluorescence from each sample's curve and measuring the threshold cycle slightly above the threshold of the curve. Relative values of expression between samples were then calculated using the standard curve of diluted samples representing the efficiency of each primer pair, corrected by expression values of the internal control, HPRT.

Primers were designed to produce an ~100-bp product and to have an annealing temp of 55–60°C. The following primer sequences were used: IFN-γ: forward, ACAGCACTCGAATGTGTCAGGTAG; reverse, TTCAGCTGTATAGGGAAGCACCAG; TNF-α: forward, GCACCAC CATCAAGGACTCAAATG; reverse, ATTCTGAGACAGAGGCAACCT GAC; IL-10: forward, GCTTCTATTCTAAGGCTGGCCACA; reverse, TAGGAGCTCTGAACTCAGGGATGA; IL-12p40: forward, GTGGGA GCTGGAGAAAGACGTTTA; reverse, TCATCTTCTTCAGGCGTGT CACAG; and HPRT: forward, TACGAGGAGTCCTGTTGATGTTGC; reverse, GGGACGCAGCAACTGACATTTCTA.

Infection of murine bone marrow-derived macrophages (BMDM) in vitro

BMDM were isolated from femurs and tibias of male C3H and C3H.B6-*sst1* mice (6–10 wk old). The cells were grown in a complete culture medium (1/1 mix of DMEM and Ham's F-12 (HyClone, Logan, UT)) supplemented with 10% of FCS (HyClone), 1 ng/ml rIL-3 (Sigma-Aldrich, St. Louis, MO), and 20% L929 fibroblast-conditioned medium as a source of M-CSF in 75-cm^2 tissue culture flasks (Falcon; BD Biosciences, Franklin Lakes, NJ) for 2 days for enrichment of macrophage precursors and depletion of mature adherent cells. Cells were collected and seeded onto circular 12-mm diameter glass coverslips placed in a 60-mm diameter petri dish and differentiated into macrophages in medium containing 40% L929 fibroblast-conditioned medium for 4 days, and maintained in complete medium containing 20% L929 fibroblast-conditioned medium. Cells were stimulated with 50 U/ml murine rIFN-γ (R&D Systems, Minneapolis, MN) 18 h before infection. Macrophage monolayers were infected with *L. monocytogenes* for 30 min at a multiplicity of infection (MOI) of one bacterium per 10 macrophages (MOI, 1:10). After 30 min, the cells were washed with PBS containing 1% FCS (PBS-1% FCS) and incubated for an additional 30 min in complete medium without antibiotics to allow for internalization of the bacteria. After that, the cells were incubated in complete medium containing 10% FCS and 20% L929-conditioned medium and gentamicin (10 μg/ml). Three coverslips were removed from the culture at appropriate time intervals and separately placed in 15-ml conical polypropylene tubes containing 5 ml of sterile distilled H_2O with 0.1% Triton X-100. The tubes were vortexed for 15 s to lyse macrophages, and dilutions of lysates were plated on Luria-Bertoni agar to determine the number of intracellular bacteria. The colonies were counted after 24-h incubation at 37°C. Macrophage monolayers were stained using Diff-Quik (VWR, West Chester, PA) directly on coverslips.

Superoxide anion and NO production by BMDM

To measure nitrite production in vitro, 50 μl of culture supernatants were mixed with 50 μl of Griess reagent (*N*-1-naphthylethylenediamine hydrochloride (0.1%); Sigma-Aldrich) and prepared with distilled water, and 1% sulfanilamide (Sigma-Aldrich) was prepared with 5% H_3PO_4 as described previously (24). Absorbance was measured after 10 min at 550 nm in an ELISA microreader (Molecular Devices, Sunnyvale, CA). A standard curve of $NaNO_2$ was used to establish the NO_2^- concentration in the samples.

Superoxide anion production was measured by FACS using oxidation of HE (Molecular Probes, Eugene, OR). HE is a nonfluorescent lipophylic molecule that is oxidized by superoxide anion alone or by hydrogen peroxide in the presence of peroxidases to produce ethidium, which is a hydrophilic fluorescent compound (25). The stock solution of HE was prepared in *N,N*-dimethylformamide (ICN Biochemicals, Cleveland, OH). To determine superoxide anion production by BMDM, coverslips containing infected macrophages were transferred into 24-well plates containing medium with 2.5 μM HE (Molecular Probes) for 15 min at 37°C at the indicated time points after infection. After incubation, the cells were removed with cold PBS-EDTA (0.37 mg/ml), and the ethidium fluorescence was determined in a FACSCalibur (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences).

Inhibitors

To inhibit superoxide production, BMDM were treated with superoxide dismutase (SOD; 100 U/ml; Sigma-Aldrich) and *N*-acetyl-L-cysteine (NAC; 10 μM; Sigma-Aldrich) for the duration of infection. Aminoguanidine hemisulfate (AMG; 2 mM; Sigma-Aldrich) and N^G-monomethyl-L-arginine (NGMMA; 10 μM; Sigma-Aldrich) were used to inhibit NO production.

Statistical analysis

Bacterial burdens were compared using Student's *t* test (PRISM 4; GraphPad, San Diego, CA). Cytometric analyses were made using the Windows Multiple Document Interface (WinMDI, from http://facs.scripps.edu/) software (release 2.8). All in vitro experiments were performed in triplicate and independently repeated at least three times. Results are presented as the mean ± 95% confidence interval for the mean. Data were analyzed by ANOVA. Statistical significance was considered to be $p < 0.05$. For all analyses, Statgraphics Plus (release 2, 1996; Statgraphics, Rockville, MD) was used.

Results

The sst1 *locus controls innate immunity to* L. monocytogenes

The* sst1 *locus controls multiplication of* L. monocytogenes *and lesion morphology in vivo. According to the literature, the standard inbred strains of mice, C57BL/6 (B6) and C3H, can be classified as relatively resistant to i.v. infection with *L. monocytogenes* compared with the genetically susceptible BALB/c inbred strain (19). Thus, in our experiments the effect of the *sst1* locus was studied on a relatively resistant genetic background (C3H). The bacterial loads in the livers and spleens of an *sst1*-congenic pair of inbred strains C3H (*sst1*-susceptible) and C3H-*sst1*^{B6} (*sst1*-resistant) were determined at 48 and 72 h after i.v. infection with 1–3 × 10^4 CFU of *L. monocytogenes* in three independent experiments. We observed three to eight times lesser bacterial loads in the spleens and livers of the *sst1*-resistant congenic mice C3H-*sst1*^{B6} compared with the *sst1*-susceptible C3H mice (data not shown). However, the bacterial burden even in the *sst1*-susceptible C3H mice was much less than that in susceptible BALB/c inbred mice infected with a similar dose of the bacteria. Thus, the *sst1*-attributable difference in immunocompetent C3H mice was small, but statistically significant, and was reproducible in three independent experiments.

To test the role of the *sst1* locus in innate immunity, we have generated a pair of the *sst1* congenic inbred strains that also carry the *scid* mutation, C3H-*scid* and C3H-*sst1*^{B6},*scid* (see *Materials and Methods*). These strains were infected with 1 × 10^4 CFU of *L. monocytogenes* i.v. As shown in Fig. 1, the bacterial loads in the spleens and livers 72 h after i.v. infection were 500- to 1000-fold less in the organs of the *sst1*-resistant *scid* mice (C3H-*sst1*^{B6},*scid*). A similar difference was observed at 48 h in an independent experiment using the same strains and dose of infection. In this experiment, histologic sections of liver and spleen 48 h after i.v. challenge were compared in addition to CFU counts. Representative findings are shown in Fig. 2. Histologic sections of liver contained 6–17 microabscesses/representative cross-section in C3H-*scid* animals (Fig. 2*A*). At these foci, there were central zones of

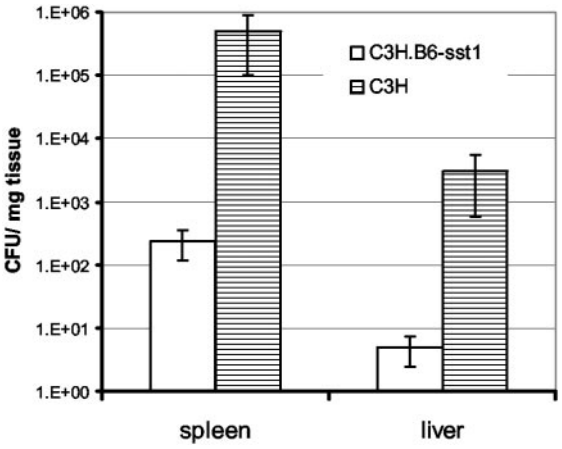

FIGURE 1. Bacterial loads in organs of the *sst1*-susceptible C3H-*scid* and the *sst1*-resistant C3H-*sst1*^{B6},*scid* immunodeficient mice 72 h after i.v. infection with 1×10^4 CFU of *L. monocytogenes* ($p < 0.05$).

necrosis containing inflammatory cells as well as tissue debris. Often there were intact neutrophils on the periphery or adjacent to the necrotic foci. In contrast, three of four C3H-*sst1*^{B6},*scid* mice had no microabscess formation, whereas one mouse had only a single microabscess (Fig. 2*B*). Representative splenic tissue from four C3H-*scid* mice contained multiple microabscesses similar in appearance to those seen in liver (Fig. 2*C*), whereas no microabscesses were noted in splenic tissue from four C3H-*sst1*^{B6},*scid* mice (Fig. 2*D*). Thus, formation of the necrotic lesions in spleens and livers was a hallmark of *L. monocytogenes* infection in *sst1*-susceptible *scid* mice (C3H-*scid*).

These results demonstrate that the *sst1* polymorphism plays a role in control of *L. monocytogenes* infection. In the presence of the *sst1*-resistant allele, the development of massive necrosis in C3H-*sst1*^{B6},*scid* animals was prevented. However, prevention of the development of necrotic lesions might result from a direct effect of the *sst1*-encoded polymorphic gene on mechanisms of cell death or might be due to an indirect effect of the *sst1* locus via more efficient control of *L. monocytogenes* by the *sst1*-resistant phagocytic cells. The *sst1* contribution to host resistance is much more pronounced in immunodeficient *scid* animals. From the genetic perspective, this phenomenon can be described as an interaction of the *scid* and the *sst1* genetic loci, in which phenotypic expression of the *sst1* locus is enhanced by the *scid* mutation.

The* sst1 *locus does not correct the T and B lymphocyte deficiency conferred by the* scid *mutation. Next, we determined whether the *sst1* polymorphism might affect the phenotypic expression of the *scid* mutation. Despite the mutation in *Prkdc*, low numbers of T and B lymphocytes might be present in C3Smn.CB17-*Prkdc*^{scid} (C3H-*scid*) mice, due to the "leakiness" of the *scid* mutation (26, 27). To address the possibility that the presence of the B6-derived resistant allele at the *sst1* locus alleviated the effect of the *scid* mutation and thus increased the resistance to the infection, we tested the *sst1*-congenic *scid* mice (*Materials and Methods*) for the presence of T and B cells before and after infection with *L. monocytogenes*. The numbers of T and B lymphocytes in the spleens and peripheral blood of noninfected *sst1* congenic *scid* mice were similar and greatly diminished compared with their immunocompetent counterparts. As shown in Table I, the proportion of CD3$^+$ T cells in the total population of splenocytes in noninfected mice was 0.5–1%, i.e., reduced 50- to 100-fold as compared with that in normal immunocompetent mice. The number of B220-positive cells, of which B cells are a dominant subset, was also drastically reduced.

After infection with *L. monocytogenes*, the proportion of CD3-positive cells slightly increased (Table I). However, no statistically significant differences in the numbers of total and activated (CD69-positive) T cells were observed between the *sst1*-resistant and the *sst1*-susceptible *scid* mice. We concluded that the increased resistance to *L. monocytogenes* of the *sst1*-resistant *scid* mice was not due to correction of the immunodeficiency conferred by the *Pkrdc* mutation.

Taken together, our results demonstrated that the *sst1* polymorphism plays a role in control of *L. monocytogenes* infection, and its contribution to host resistance is much more pronounced in immunodeficient *scid* animals. Therefore, the *sst1* locus mediates a mechanism(s) of innate immunity to this pathogen. A subsequent

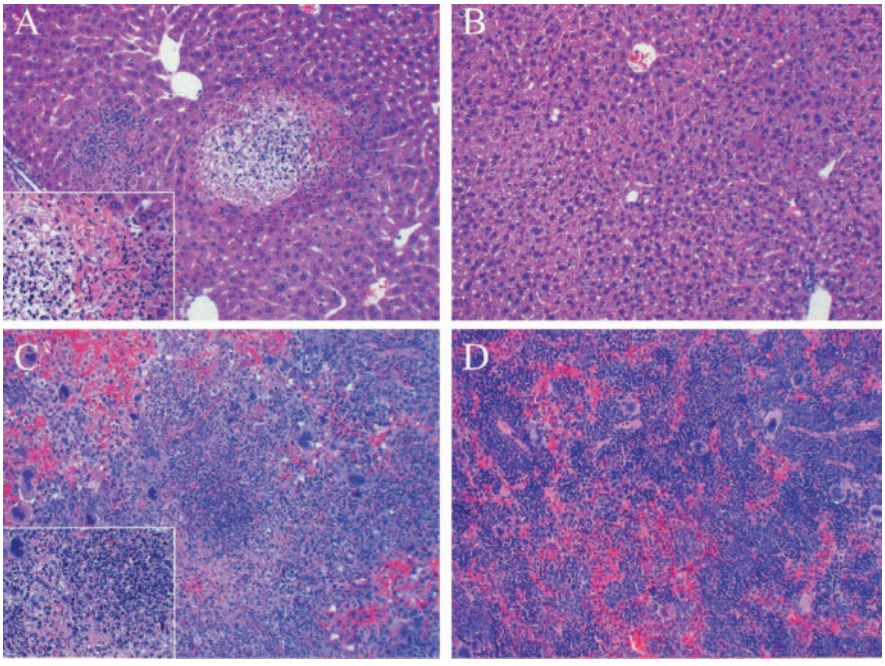

FIGURE 2. Resistant allele of the *sst1* prevents formation of abscesses in the livers and spleens of *L. monocytogenes*-infected *scid* mice. Histologic sections of livers (*A* and *B*) and spleens (*C* and *D*) of the C3H-*scid* (*A* and *C*) and C3H.B6-*sst1*,*scid* (*B* and *D*) mice 48 h after i.v. infection with 1×10^4 CFU of *L. monocytogenes*. H&E staining. Magnification, ×100; *insets* in *A* and *C*, ×400.

Table I. *Proportion of lymphoid populations in the spleens of the* sst1 *congenic scid mice before and after infection with* 1×10^5 *CFU of* L. monocytogenes[a]

	sst1 Allele		% $Dx5^+$ $CD3^-$					
			% Total NK $Dx5^+CD3^-$	% NK $Dx5^+CD69^+$	% NK^+ $Dx5^+CD69^-$	% of Activated NK Cells	% CD3	% B220
Noninfected	R	Average	11.6	2.6	9.0	22.5	0.49	4.75
		SD	3.69	1.61	2.12		0.47	3.03
Infected	R	Average	12.1	4.6	7.5		2.05	8.09
		SD	3.83	1.86	1.36	37.0	1.31	5.61
		p value, infected vs noninfected	NS	NS	NS	NS	NS	NS
	S	Average	16.6	11.1	5.5		1.70	10.62
		SD	3.56	2.69	0.92	67.1	1.06	5.21
		p value, R vs S	NS	<0.002	NS		NS	NS

[a] The splenocytes were isolated from three to four individual animals of each strain 48 h after i.v. infection. The total numbers of the splenocytes were similar on both genetic backgrounds. Values of *p* were calculated using *t* test.

series of experiments was performed to identify the cellular mechanism(s) of the *sst1* action.

Effects of the sst1 *locus on inflammatory cells in vivo*

Activation of NK cells is not affected by the* sst1 *allelic polymorphism. IFN-γ-producing NK cells and phagocytic myeloid cells are known to be the major cell types that contribute to control of *L. monocytogenes* in *scid* mutants (28–31). Therefore, we determined whether there were any differences between the *sst1*-resistant and -susceptible *scid* mice in the numbers or activation status of corresponding cell types in vivo. The proportion of $Dx5^+CD3^-$ NK cells in the spleens were similar in noninfected *sst1* congenic *scid* mice, and the total numbers of NK cells in the spleens did not significantly increase after infection (Table I). However, we have observed a significant increase in activated $Dx5^+CD69^+$ NK cells 48 h after infection (Table I). At that time, the proportion of the $CD69^+$ activated NK cells in the total pool of NK cells increased from ~40 to 77% and became significantly higher in the *sst1*-susceptible *scid* mice compared with the *sst*-resistant congenics ($p < 0.002$; Table I). In the *scid* mice, activated NK cells are major producers of IFN-γ, which is necessary for activation of listeriocidal activity of phagocytic cells (32). Determination of levels of IFN-γ mRNA expression in the infected spleens of the *sst1* congenic *scid* mice demonstrated that the *sst1*-susceptible splenocytes expressed significantly higher levels of mRNA encoding this cytokine. Although the absolute numbers of activated ($CD69^+$) NK cells was only 2-fold higher in the *sst1*-susceptible mice, the IFN-γ mRNA levels in the spleens of those mice were ~6-fold higher (Table II), which probably reflects higher bacterial loads in the susceptible animals at this time. These data suggest that in both genetic backgrounds, NK cells become activated and are capable of producing IFN-γ after *L. monocytogenes* infection in vivo. Therefore, the availability of IFN-γ for macrophage activation is not likely to be affected by the *sst1* polymorphism. We hypothesized that the *sst1* locus may, instead, affect the responsiveness of phagocytic cells to this cytokine or other inflammatory mediators.

Recruitment and activation of phagocytic cells in vivo. Levels of mRNA expression of several cytokines were used to monitor the functional status of inflammatory myeloid cells in spleens of the infected *sst1* congenic *scid* mice in vivo. The levels of TNF-α, IL-10, and IL-12 mRNAs were increased in the spleens as a result of infection (Table II). The expression of TNF-α and IL-12 mRNAs was several-fold higher in the spleens of the *sst1*-susceptible *scid* mice. The expression of IL-10 mRNA was also higher in the infected spleens, but the differences between the resistant and susceptible congenic mice were not statistically significant due to high individual variation in the expression of this cytokine (Table II). Therefore, the effect of the *sst1* locus could not be accounted for by decreased levels of protective (TNF-α and IL-12) or increased levels of suppressive (IL-10) cytokines in the susceptible animals. The higher expression of IFN-γ, TNF-α, IL-10, and IL-12 is, perhaps, a function of a higher bacterial burden in the susceptible animals. This approach does not allow measurement of the production of these cytokines at the individual cell level. However, it does demonstrate that there is no overt deficiency of these cytokines that might explain the dramatic differences in susceptibility to *L. monocytogenes* conferred by the *sst1* locus on the *scid* genetic background.

To test whether the *sst1* polymorphism affected recruitment of phagocytic cells to the site of infection and their activation in vivo, we have performed experiments in which the *sst1* congenic *scid* mice were infected i.p. with 10^6 CFU of *L. monocytogenes*. The inflammatory cells were isolated 6 h after the infection and analyzed by FACS for the myeloid lineage-specific surface markers and production of ROIs (Fig. 3). The composition of resident peritoneal cells in noninfected mice was identical on both genetic backgrounds, and the majority of the cells were represented by $Ly6C^-$,$Gr\text{-}1^{low}$ resident macrophages (Fig. 3*A*, *gate R2*), which also were $F4/80^+$ (not shown). Rapid recruitment of inflammatory cells to the peritoneal cavity at 6 h postinfection was detected on both genetic backgrounds (Fig. 3*B*). The inflammatory cells were represented mostly by $Gr\text{-}1^{high}Ly6C^-$ neutrophils (*gate R3*) and $Ly6C^+$,$Gr\text{-}1^{low}$ monocytes (*gate R1*). Influx of granulocytes and monocytes at 6 h was similar in the *sst1*-resistant and -susceptible mice. At later time points, more neutrophils were present in the

Table II. *Induction of cytokine mRNA expression in spleens of the* sst1 *congenic scid mice 48 h after i.v. infection with* 1×10^5 *CFU of* L. monocytogenes[a]

Cytokine	Fold of Induction		*p* Value (R vs S)
	C3H-$sst1^{B6}$, *scid*	C3H-*scid*	
IFN-γ	3.90	24.20	0.022
TNF-α	1.18	9.51	0.016
IL-12	1.57	6.21	0.018
IL-10	3.79	19.52	0.119

[a] The relative content of mRNA species in the spleen of the *sst1* congenic C3H-*scid* and C3H-$sst1^{B6}$, *scid* mice was determined using real time RT-PCR (*Materials and Methods*). Fold of induction was determined as compared to the spleens of noninfected mice. Three to four individual mice were analyzed per group.

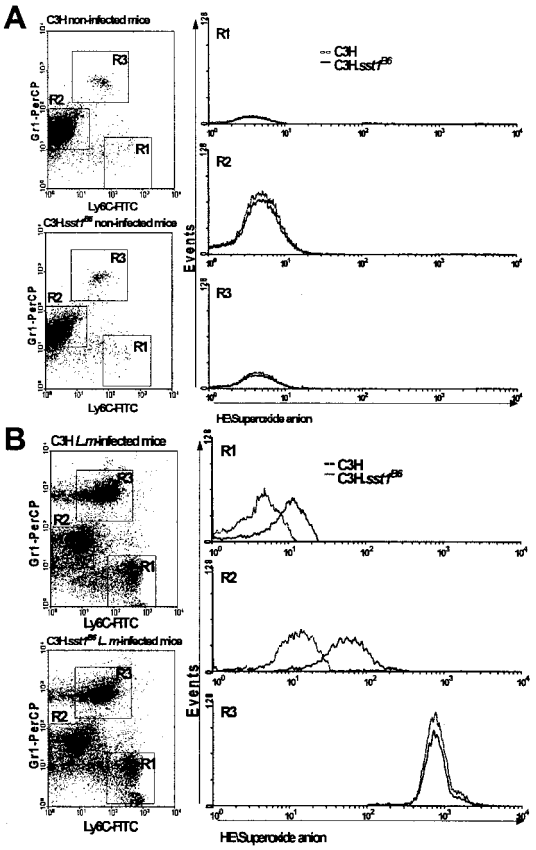

FIGURE 3. Recruitment of inflammatory cells and production of ROIs by the inflammatory cells after i.p. infection with *L. monocytogenes*. The *sst1*-congenic *scid* mice were infected with 1×10^6 *Listeria* i.p. Six hours later, PEC were collected by flashing with cold PBS containing gentamicin (5 μg/ml). Cells (1×10^6) were stained with HE and/or Ly6C- and Gr-1-specific Abs. The production of superoxide anion was analyzed in the indicated gates. We have determined in a separate experiment using four-color FACS analysis with lineage-specific markers that R1-gated ($Gr\text{-}1^{low}$,$Ly6C^{high}$) and R2-gated ($Gr1^{low}$,$Ly6C^{neg}$) populations corresponded to monocytes and macrophages, respectively, and neutrophils were in the R3 population ($Gr1^{high}$,$Ly6C^{neg}$).

sst1-susceptible mice (data not shown). We also studied recruitment of neutrophils to the spleens after systemic i.v. infection and found no defect in neutrophil recruitment to the spleens in *sst1*-susceptible animals (data not shown).

To assess early activation of different populations of inflammatory myeloid cells, we isolated PEC from the i.p. infected *scid* animals and measured the production of superoxide by HE staining in combination with lineage-specific markers Gr-1 and Ly-6C (Fig. 3*B*). We observed that the highest level of ROI production was by neutrophils (*gate R3*); however, it was identical on both genetic backgrounds. On the contrary, both the resident macrophages (*gate R2*) and the newly recruited monocytes (*gate R3*) produced higher levels of ROIs in the *sst1*-resistant mice. From these data we concluded that the *sst1*-resistant macrophages were activated more readily at early times postinfection, and the effect of the *sst1* locus on innate immunity was due to differential activation of macrophages for bactericidal or bacteriostatic activities.

The sst1 *locus controls activation of macrophages for killing of* L. monocytogenes *by BMDM in vitro*

To investigate the role of the *sst1* polymorphism in controlling intrinsic macrophage functions, we tested highly purified BMDM isolated from mice that differed at the *sst1* locus, C3H and C3H-*sst1*^{B6}. Those macrophages were obtained from immunocompetent mice, because there was no possibility of contamination with T or B lymphocytes in our cultures (see *Materials and Methods*). In one experiment, BMDM obtained from a pair of the *sst1* congenic *scid* mice were tested, and the results were identical with those obtained using macrophages obtained from immunocompetent mice (M. Rojas, unpublished observations).

***The* sst1 *locus controls IFN-γ-inducible ability of macrophages to kill* L. monocytogenes.** BMDM obtained from the *sst1* congenic C3H and C3H.B6-*sst1* mice were infected with *L. monocytogenes* in vitro (Fig. 4). The macrophages were either naive or pretreated with rIFN-γ for 18 h before the infection. The experiments were performed at a low MOI of approximately one bacterium per 10 macrophages in the presence of a bacteriostatic concentration of gentamicin (*Materials and Methods*) to inhibit extracellular multiplication of the bacteria. The rate of bacterial multiplication in nontreated BMDM was equal regardless of the macrophage *sst1* genotype (Fig. 4*A*, □ and ○). Pretreatment of macrophages with IFN-γ decreased the bacterial loads on both genetic backgrounds. At 2 and 4 h postinfection, the bacterial load of corresponding macrophage cultures was equal on both genetic backgrounds (Fig. 4*A*, ■ and ●). A statistically significant difference in bacterial loads between the IFN-γ-treated *sst1* congenic macrophages was first observed 6 h after infection, and the difference was highly significant 8–24 h after infection.

To determine whether the *sst1* affects bactericidal or bacteriostatic activity of IFN-γ-treated macrophages, we performed similar experiments using the LLO^- mutant of *L. monocytogenes*. This mutant has been shown to remain in the phagosome and is therefore unable to multiply intracellularly (33, 34). If the host cell does not kill the microorganism, the number of intracellular mutant bacteria remains constant, but it will decrease due to bactericidal effects displayed by the host cells. The experiments presented in Fig. 4*B* demonstrated that BMDM pretreated with IFN-γ (50 U/ml) acquired bactericidal activity. Initially, this activity appeared to be independent of the *sst1* allelic polymorphism, because ~90% of

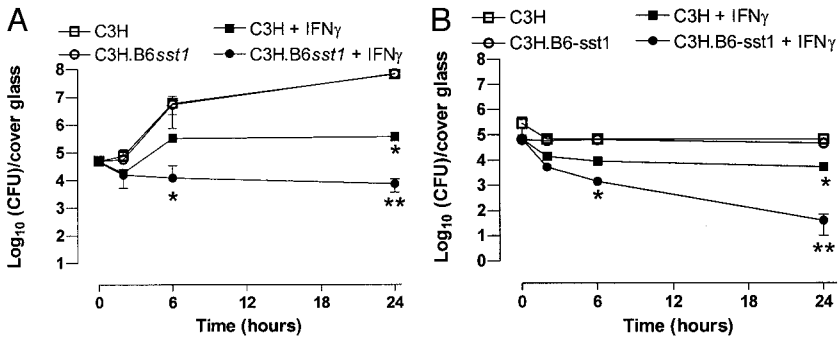

FIGURE 4. Intracellular growth of *L. monocytogenes* in BMDM obtained from the *sst1* congenic susceptible (C3H) and resistant (C3H-*sst1*^{B6}) mice. *A*, Macrophages were infected with wild-type *L. monocytogenes* 10403S at an MOI of one bacterium per 10 macrophages; *B*, macrophages were infected with an LLO^- mutant of *L. monocytogenes* at an MOI of 1:10. Intracellular bacteria (*y*-axis) were enumerated at the indicated time points (*x*-axis). A statistically significant difference is indicated: ∗, C3H vs C3H plus IFN-γ, $p < 0.05$; ∗∗, C3H plus IFN-γ vs C3H.B6-*sst1* plus IFN-γ (C3H-*sst1*^{B6}), $p < 0.01$.

LLO-*L. monocytogenes* were eliminated by the IFN-γ-activated macrophages of both genetic backgrounds. These data are in agreement with our observations using the wild-type bacteria described above. However, at 6 h postinfection, *sst1*-resistant macrophages demonstrated superior bactericidal activity, and the number of viable bacteria inside *sst1*-resistant macrophages continued to decline until the end of the experiment at 24 h postinfection. In contrast, the number of LLO^- mutant bacteria did not decrease significantly in the *sst1*-susceptible, IFN-γ-treated macrophages between 2 and 24 h postinfection (Fig. 4*B*).

Production of ROI, but not production of NO, is responsible for differential killing of L. monocytogenes *by the IFN-γ-activated,* sst1 *congenic macrophages in vitro*

To identify the effector mechanism that accounts for differential killing of *L. monocytogenes* by the *sst1* congenic macrophages in vitro, we used inhibitors of NO and scavengers of ROI. As shown in Fig. 5*A*, inhibitors of ROI (NAC and SOD; see *Materials and Methods*) significantly reduced killing of the bacteria by the IFN-γ-treated macrophages, whereas the inhibitors of NO production (AMG and NGMMA) had no effect. Moreover, differential killing of the bacteria by the IFN-γ-treated *sst1* congenic macrophages was preserved in the presence of NO inhibitors and was completely ablated by the inhibitors of ROIs. These findings are consistent with greater production of ROIs by the *sst1*-resistant peritoneal macrophages in vivo, as described above (Fig. 3*B*).

Next we wanted to determine whether differential production of ROIs by the *sst1* congenic macrophages infected with *L. monocytogenes* was macrophage cell autonomous. Therefore, we determined the kinetics of ROI production by BMDM after infection in vitro. As shown in Fig. 6, nontreated macrophages, which were unable to control the bacterial multiplication, produced the lowest amount of ROIs. The IFN-γ-pretreated *sst1*-resistant and -susceptible macrophages produced equal amounts of ROIs 2 h postinfection and killed approximately the same proportion of the initial bacterial inoculum. At 2 h, the proportion of macrophages producing ROI, as determined by FACS analysis, was ~16%, i.e., roughly equal to the proportion of the infected macrophages at a 1:10 MOI, which was used in this experiment. At 8 h postinfection, however, a significant difference between the IFN-γ-treated, *sst1* congenic macrophages was observed in both the amount of ROI per cell, as determined by the mean fluorescence intensity (*x*-axis; $p < 0.001$), as well as the number of ROI-producing cells (*y*-axis; $p < 0.001$). Of note, the proportion of ROI-producing cells among the IFN-treated macrophages was considerably higher than the number of *L. monocytogenes*-infected macrophages, as determined by differential staining of macrophage monolayers, indicating that the *sst1*-dependent activity may be mediated by soluble mediators.

The above experiments using purified macrophage populations indicate that the effect of the *sst1* polymorphism can be expressed by macrophages in a cell autonomous manner. The *Prkdc*^{scid} mutation is not necessary for phenotypic expression of *sst1*-mediated macrophage function.

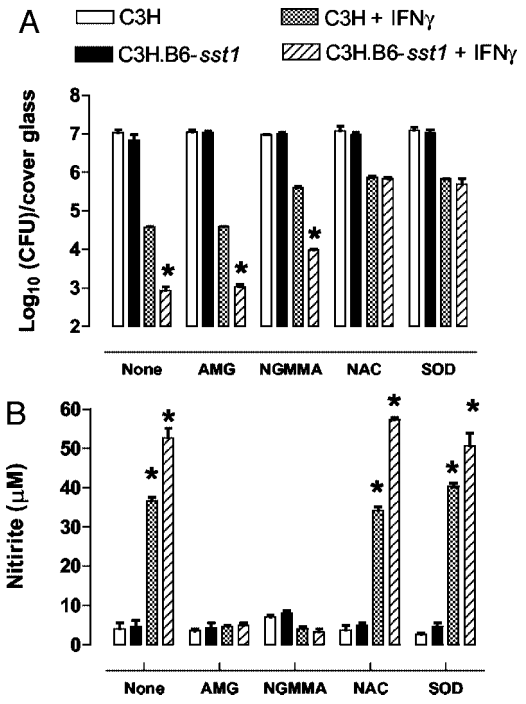

FIGURE 5. Intracellular growth of *L. monocytogenes* (*A*) and NO production (*B*) by BMDM obtained from the *sst1* congenic susceptible (C3H) and resistant (C3H-*sst1*^{B6}) mice in the presence of NO and ROI inhibitors. The inhibitors of NO (AMG and NGMMA) and ROI (NAC and SOD) were added at the indicated concentrations for the duration of infection (24 h): SOD, 100 IU/ml; NAC, 10 μM; AMG, 2 mM; and NGMMA, 10 μM (see also *Materials and Methods*).

Discussion

Genetic studies of host susceptibility to infections in experimental models as well as in human populations have demonstrated that host resistance to a number of intracellular pathogens is a complex genetic trait (reviewed in Ref. 7). The contribution of an individual polymorphic genetic locus to host defense may vary with the virulence strategy of a particular pathogen, the nature of a genetic polymorphism (quantitative vs qualitative effects), its interaction with other polymorphic host genes, and environmental factors.

Similarities in genetic control may reveal new pathogenic mechanisms shared by dissimilar microorganisms. Genetic polymorphisms controlling innate mechanisms of resistance to several intracellular pathogens have been identified previously (35, 36). Sometimes those polymorphisms control resistance to several taxonomically unrelated pathogens (37, 38). Perhaps this is a result of common pathogenic mechanisms used by different microorganisms. For example, the *Slc11a1* gene (formerly known as *Nramp1*) encodes a member of the proton-coupled divalent metal ion transporter family that is recruited to the bacterial phagosome (39). Several intracellular bacteria (*Mycobacterium bovis BCG*, *Mycobacterium lepraemurium*, and *Salmonella typhimurium*) and protozoa (*Leishmania donovani*) that are known to reside and multiply within the phagocytic vacuole are affected by the *Slc11a1* polymorphism. However, pathogens that are capable of destroying the phagosomal membrane, such as *L. monocytogenes*, and gain access to the cytoplasm of the host cell are unlikely to be dependent on this transporter; indeed, the *Slc11a1* polymorphisms do not affect intracellular multiplication of *L. monocytogenes*.

In this study we have determined that genetic polymorphism at the *sst1* locus, which has been identified by our group as a genetic determinant of host resistance to tuberculosis, significantly affects natural host resistance to *L. monocytogenes*. To elucidate the cellular mechanism of *sst1*-mediated resistance, we have generated a pair of the *sst1* congenic strains that lacked mature lymphocytes by introducing the *sst1*-resistant allele from C3H-*sst1*^{R} into the C3H-*scid* mouse strain, in which maturation of T and B lymphocytes is disrupted by a mutation in the *Prkdc* gene. The *scid* mutation confers a very high degree of susceptibility to virulent MTB and eliminates the effect of the *sst1* locus on host resistance to this pathogen. However, during primary infection with *L. monocytogenes*

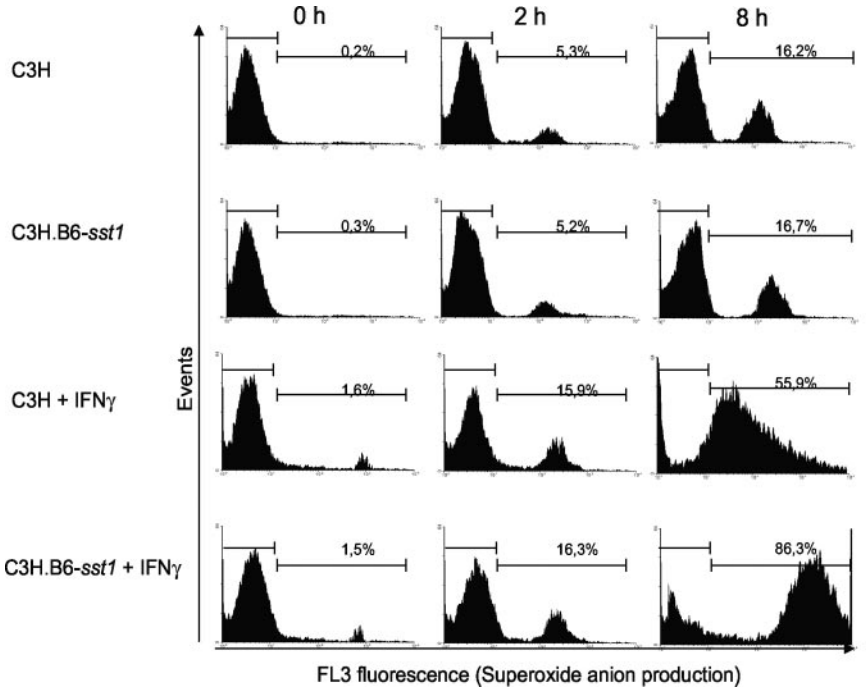

FIGURE 6. Production of superoxide anion by BMDM obtained from the *sst1* congenic mice C3H and C3H.B6-*sst1* (C3H-*sst1*^{B6}) was measured at 0, 2. and 8 h after infection in vitro. Untreated or IFN-γ-treated BMDM were infected with wild-type *L. monocytogenes* at an MOI of 1:10. At the indicated time points the cells were incubated for 15 min with HE before FACS analysis, as described in *Materials and Methods*. The *x*-axis shows ethidium fluorescence (FL3).

(40) within the initial 3-day period, the infection is primarily controlled by mechanisms of innate immunity, and no help of T and B lymphocytes is necessary for the initial containment of the pathogen (12, 32, 41). Adaptive immunity is implicated in clearance of the bacteria at later time points as well as in the recall response (42, 43). The ability to control *L. monocytogenes* infection within the first 2–3 days after primary infection serves as a classical test of macrophage function (13, 44). In our studies, systemic infection of *sst1* congenic *scid* mice with a sublethal dose of *L. monocytogenes* demonstrated a very significant effect of the *sst1* locus. In contrast to MTB infection, the effect of the *sst1* locus was not only preserved on the *scid* genetic background, but its effect was markedly enhanced compared with that in mice that carry the wild-type allele of the *Pkrdc* gene. These data suggest that the *sst1* locus controls a mechanism of innate immunity that is responsible for clearance of *L. monocytogenes* in *scid* mice.

Neutrophils, NK cells, and macrophages are the major effector cells of innate immunity to *L. monocytogenes* (45). It has been demonstrated that in *scid* mice protection is mediated by IFN-γ, which is produced by NK cells. The NK cell-generated IFN-γ activates macrophages, which clear the bacteria. Neutrophils represent the first line of defense against *L. monocytogenes* (45, 46). However, we found no evidence of neutrophil deficiency in *sst1*-susceptible *scid* animals. Instead, our data indicate that the *sst1* locus affects the macrophage cell-autonomous mechanism of resistance. Experiments using the LLO^{-} mutant indicate that *sst1* affects the ability of the macrophage to kill bacteria, and the effect of the *sst1* locus in vitro was dependent on macrophage activation by IFN-γ. Recently, we have found that the listeriocidal activity of the *sst1*-resistant macrophages was also induced more efficiently by type I IFNs, both α and β, which contribute to early macrophage activation (M. Rojas, unpublished observation).

It has been demonstrated that IFN-γ-activated BMDM and macrophage cell lines infected with *L. monocytogenes* become bactericidal (12) and produce TNF-α, IL-1β, and IL-6 (47). These cytokines can also enhance the resistance to the pathogen when administered before infection (48), and their blockade increased the growth of bacteria (49). The mechanisms by which these cytokines modulate the growth of *L. monocytogenes* involve the production of toxic compounds, such as ROI, NO, and peroxynitrite (50). The mechanism of the *sst1*-mediated control of host resistance to *L. monocytogenes* appeared to be IFN-γ-dependent, but NO-independent, because only treatment with scavengers of ROI (NAC and SOD) significantly increased bacterial multiplication in the resistant macrophages, whereas inhibition of NO production had no effect on *sst1* function. However, production of ROIs may not be the only mechanism responsible for the *sst1*-mediated control of *L. monocytogenes* in vivo. The differences in ROI production may reflect differential activation of the *sst1* congenic macrophages in general.

High bacterial loads in spleens and livers of *sst1*-susceptible *scid* mice correlated with the formation of necrotic lesions in these organs. Previously, it has been shown that the death of *L. monocytogenes*-infected murine BMDM follows the necrotic, not the apoptotic, pathway (51). More recently, signaling through type I IFNs was implicated in sensitizing macrophages to *L. monocytogenes*-induced cell death, which also requires production of LLO by the bacteria (52). Thus, both bacterial products and signals generated from within the host environment may participate in *L. monocytogenes*-induced cell death and may be counterbalanced by an unknown *sst1*-dependent mechanism in *sst*-resistant congenic mice. Formation of necrotic lesions in the lungs is also a hallmark of tuberculosis infection in *sst1*-susceptible mice. Thus, both intracellular pathogens, *L. monocytogenes* and MTB, share this pathogenic mechanism on the *sst1*-susceptible background. In both cases the presence of the *sst1*-resistant allele prevents formation of necrotic lesions. However, prevention of the development of necrotic lesions might result from a direct effect of the *sst1*-encoded polymorphic gene on mechanisms of cell death or might be due to an indirect effect of the *sst1* locus via more efficient control of the intracellular pathogens by the *sst1*-resistant phagocytic cells.

We have observed that the importance of the *sst1* locus in control of *L. monocytogenes* is greatly increased in the absence of adaptive immunity. It is not totally unexpected, because enhancement of several mechanisms of innate immunity to *L. monocytogenes* has been reported in *scid* mice. Until identification of the

candidate gene(s) encoded within the *sst1* locus, it may not be possible to determine whether this locus controls one of the known functions of myeloid cells or a novel mechanism of innate immunity. Understanding the *sst1*-mediated mechanism of innate resistance may suggest approaches to protect immunocompromised individuals, who are particularly susceptible to *L. monocytogenes* infection.

It is possible that there is a single gene encoded within the *sst1* locus that controls common IFN-dependent macrophage function, which is important in the pathogenesis of infections caused by both MTB and *L. monocytogenes*. However, the host resistance to the two pathogens may also be controlled by two different polymorphic genes encoded within the *sst1* locus. Ultimately, identification of the gene(s) that controls host resistance to intracellular pathogens within the *sst1* locus will provide excellent candidates for study of a potential role in human infectious diseases.

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