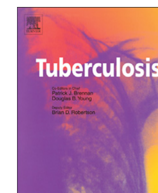
Tuberculosis 107 (2017) 175–180

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube



IMMUNOLOGICAL ASPECTS

CISH controls bacterial burden early after infection with *Mycobacterium tuberculosis* in mice

Berit Carow [a], Yu Gao [a], Graciela Terán [a, e], Xuexian O. Yang [b], Chen Dong [c], Akihiko Yoshimura [d], Martin E. Rottenberg [a, *]

[a] *Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, 17177 Stockholm, Sweden*
[b] *Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, NM 87131, USA*
[c] *Institute for Immunology and School of Medicine, Tsinghua University, Beijing 100084, China*
[d] *Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo 160-8582, Japan*
[e] *Immunoparasitology Unit, Universidad Mayor de San Andrés, La Paz, Bolivia*

ARTICLE INFO

Article history:
Received 26 July 2017
Received in revised form
8 September 2017
Accepted 17 September 2017

Keywords:
SOCS
Mycobacterium tuberculosis
Cytokine-inducible SRC homology 2 domain gene
T cell
Macrophage
Cytokines

ABSTRACT

CISH gene has been associated with increased susceptibility to human tuberculosis. We found that *cish*$^{-/-}$ mice had higher *M. tuberculosis* load in spleens and lungs up to 2.5 weeks after infection but not later compared to controls. *Cish* mRNA levels were increased in lungs at early and late time points after *M. tuberculosis* infection. In relation, the titers of *inos* and *tnf* mRNA in lungs were reduced early after infection of *cish*$^{-/-}$ mice.

The transfer of *cish*$^{-/-}$ and control T cells conferred *rag1*$^{-/-}$ mice similar protection to infection with *M. tuberculosis*.

Macrophages showed increased *cish* mRNA levels after *M. tuberculosis* infection *in vitro*. However, mycobacterial uptake and growth in *cish*$^{-/-}$ and control macrophages was similar.

Thus, we here show that CISH mediates control of *M. tuberculosis* in mice early after infection via regulation of innate immune mechanisms.

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1. Introduction

One third of the world's population is latently infected with *Mycobacterium tuberculosis*, the causative agent of TB, and around 2 million deaths are caused by TB yearly. The interaction between bacterial and host genes and the environment determine the outcome of infection and disease progression. While human and mouse genetic studies have identified various immune components crucial for the control of *M. tuberculosis* infection, the detailed understanding of bacterial-host interactions is deficient.

Cellular immune responses resulting in intracellular bacterial killing are crucial for controlling the infection with *M. tuberculosis*. However, a balanced strength is required since the very same cellular effector mechanisms that mediate mycobacterial killing might damage the host mammalian tissues. To achieve this balance mechanisms that can limit efficient bacterial killing for the cost of preventing infection-induced immune damage exist.

The “Suppressor of Cytokine Signaling” (SOCS) is a family of eight proteins (SOCS1-7 and CISH) that hamper the activation of different STAT transcription factors [1–3]. SOCS proteins bind to the JAKs and/or to the cytokine receptors targeting the receptor complex for ubiquitination and subsequent proteasome-mediated degradation [4] or inhibit the kinase activity of JAK [5,6]. Previously, others and we have shown that the expression of the SOCS family members SOCS1 and SOCS3 influence the outcome of *M. tuberculosis* infection in mice [7–10].

The “cytokine-inducible SRC homology 2 (SH2) domain protein” CISH is the founder member of the SOCS family. CISH has been described as an inhibitor of STAT5 and STAT6 activation and also as a regulator of T cell receptor (TCR) signaling in a STAT-independent manner [3,11–14].

Of importance, single nucleotide polymorphisms (SNPs) in *CISH* gene were associated with increased risk of TB [15–18]. Five *CISH* SNPs were associated with an increased susceptibility to bacteremia, TB and malaria in African and Asian cohorts [16]. Associations of these CISH polymorphisms with susceptibility to infection with Hepatitis B and leptospirosis have also been reported [16,19–21].

* Corresponding author. Nobels väg 16, 17177 Stockholm, Sweden.
E-mail address: Martin.Rottenberg@ki.se (M.E. Rottenberg).

https://doi.org/10.1016/j.tube.2017.09.007
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Three of the SNPs studied (−639, −292 and −163) are located in the promoter region, one near the translation start site and one in the third intron of the *CISH* locus. Two *CISH* genetic variants (−292 and + 1320) in the Chinese Han population also associated with increased risk of TB and SNP -292 correlated to susceptibility in Chinese children [15,17,18]. The SNP-292 also showed reduced *CISH* expression in IL-2-stimulated PBMCs [16]. Moreover, the combination of CISH, SOCS3, JAK2 and IL-2R expression in T cells has been suggested as a biomarker distinguishing between active and latent TB [22]. Whether CISH has a non-redundant protective role during infection with *M. tuberculosis* in vivo has not been studied.

Here, the role of CISH in the outcome of infection with *M. tuberculosis* was studied using genomically deficient mice.

2. Methods

2.1. Mice

Cish$^{-/-}$ [14] and *rag1*$^{-/-}$ mice, generated as described previously, were bred and kept under specific pathogen-free conditions. Mice were backcrossed to C57Bl/6 background that was used as wild type controls (WT). All animal experiments were approved by the Stockholm North Ethical Committee on Animal Experiments permit number N197/13.

2.2. In vivo infection with M. tuberculosis

Mice were infected with 250 CFU *M. tuberculosis* Harlingen using a nose-only aerosol exposure unit (In-tox Products). Mice were sacrificed at indicated time points after infection and bacteria in organ lysates were plated on Middlebrook 7H11 agar plates (Difco Laboratories) and quantified 3 weeks after 37 °C culture.

2.3. Adoptive transfer of T cells

Briefly, single CD90 $^{+}$ T cell suspensions from spleens were selected with magnetic beads (Miltenyi Biotech) following instructions of the manufacturer. 2×10^6 T cells were inoculated i.v. into *rag1*$^{-/-}$ mice. Three weeks after transfer, mice were infected with *M. tuberculosis* Harlingen.

2.4. Bone marrow-derived macrophages (BMM)

Bone marrow cells from tibia and femurs were suspended in DMEM containing glucose supplemented with 2 mM L-glutamine, 10% FCS, 10 mM Hepes, 100 μg/ml streptomycin, 100 U/ml penicillin (all Sigma) and 30% L929 cell-conditioned medium. Cells were filtered through a 70 μm cell strainer, plated, incubated for 6 days at 37 °C, 5% CO_2 and then washed, trypsinized, counted and cultured on round cover slides or in cell culture dishes for one day before infection.

2.5. In vitro infection of BMM

BMM were incubated with *M. tuberculosis* at the indicated multiplicity of infection (MOI). After 4 h, BMM were washed twice with PBS to remove extracellular bacteria and either fixed with 2% PFA or further incubated for three days. Fixed cells were stained with phalloidin to label F-actin (Life technologies, 1:100), DAPI (1:500) and auramine-rhodamine T to label mycobacteria (BD). Micrographs from infected macrophages (400×) were obtained and a total of at least 1000 BMM from 3 independent cultures and categorized as infected or uninfected. The intracellular *M. tuberculosis* were enumerated. BMM harboring 5 or more bacteria were considered as containing 5. In some cultures, mycobacterial CFU from BMM 6 days after infection were determined.

2.6. Real time PCR

Total RNA from lungs or infected BMM was extracted (TRI Reagent) and reversed transcribed. Transcripts were quantified using *hprt* (primer sense (S) 5′-CCCAGCGTCGTGATTAGC-3′, anti-sense (AS) 5′-GGAATAAACACTTTTTCCAAATCC-3′) as a control housekeeping gene to calculate the ΔC_t values for individual samples. Used primers for target genes: *il-6* (S) 5′-ACAAGTCGGAGGCTTAATTACACAT-3′, (AS) 5′-TTGCCATTGCACAACTCTTTTC-3′, *tnf* (S) 5′-GGCTGCCCCGACTACGT-3′, (AS) 5′-GACTTTCTCCTGGTATGAGATAGCAAA-3′, *inos* (S) 5′-CAGCTGGGCTGTACAAACCTT-3′, (AS) 5′-CATTGGAAGTGAAGCGTTTCG-3′, *il-1b* (S) 5′-TGGTGTGTGACGTTCCCATT-3′, (AS) 5′-CAGCACGAGGCTTTTTTGTTG-3′, *cish* (S) 5′-AGAGAATGAACCGAAGGTG-3’ (AS) 5′-CAGTACCACCCAGATTCC-3′.

The relative cytokine/*hprt* transcript amounts were calculated using the $2^{-(\Delta\Delta Ct)}$ method. These values were then used to calculate the fold increase of cytokine mRNA in uninfected compared to infected cells and tissues.

2.7. Neutrophil determination by flow cytometry

Lungs were mechanically minced into small pieces and digested with collagenase D/DNase I for one hour at 37 °C. Single-cell suspensions were prepared by filtering lung tissue through 70-μm nylon cell strainers and subjected to a 40/70% Percoll gradient centrifugation for 30 min at room temperature. Cells at the interphase were collected and washed. Lung cells were stained for CD11b, CD11c, Ly6C and Ly6G (eBioscience), fixed and analyzed by flow cytometry (CyAn™ ADP, Beckman Coulter) and FlowJo software (Tree Star).

3. Results

3.1. Role of CISH during experimental infection with M. tuberculosis in vivo

The bacterial levels in lungs and spleens from *cish*$^{-/-}$ and WT mice at different time points after aerosol infection with *M. tuberculosis* were first compared. *Cish*$^{-/-}$ mice showed increased bacterial burdens in lungs at 1.5 and 2.5 weeks (Fig. 1A). Spleens showed no bacteria at 1.5 weeks, but *cish*$^{-/-}$ mice contained higher splenic bacterial loads at 2.5 weeks after infection than WT controls (Fig. 1B). The *M. tuberculosis* titers in lungs and spleens of WT and *cish*$^{-/-}$ mice at 4 and 8 weeks after infection were similar.

We then studied whether differences in bacterial load associated with levels of *cish* transcripts during infection. *Cish* mRNA levels were increased in lungs from mice at early and late time points after aerosol *M. tuberculosis* infection (Fig. 1 C).

Since human *CISH* polymorphisms may affect the capacity of T cells to respond to *M. tuberculosis* [17], we studied if CISH expression in T cells is required for protection against *M. tuberculosis*. For this purpose, WT or *cish*-deficient T cells were transferred into *rag1*$^{-/-}$ mice three weeks before *M. tuberculosis* infection. Bacterial titers in lungs and spleens of mice transplanted with WT or *cish*$^{-/-}$ T cells one month after infection were similar (Fig. 1D and E). Mycobacterial levels in mice transferred with mutant or control T cells were lower in comparison to the non-transferred control mice (Fig. 1D and E). Thus, the regulation by CISH of T cell-mediated control of *M. tuberculosis* infection is redundant.

The levels of *tnf, inos, il6 and il1b* mRNA in *M. tuberculosis*-infected *cish*$^{-/-}$ and WT mice were compared. Lungs from *cish*$^{-/-}$ mice had lower levels of *tnf* and *inos* mRNA than controls 10 but not

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