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IMMUNOLOGICAL ASPECTS

Mycobacterium tuberculosis components stimulate production of the antimicrobial peptide hepcidin

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SUMMARY

We investigated the *in vitro* production of the antimicrobial peptide hepcidin by cells of the innate immune system that harbor *Mycobacterium tuberculosis*. Stimulation of mouse lung macrophages with *M. tuberculosis* or IFN-γ + *M. tuberculosis* induced hepcidin mRNA. In human alveolar A549 epithelial cells, lipoglycans of *M. tuberculosis*, in particular mannose-capped lipoarabinomannan and phosphatidyl-myo-inositol mannosides, were strong inducers of hepcidin mRNA. In mouse dendritic cells, hepcidin mRNA was increased by subcellular fractions and culture filtrate proteins of *M. tuberculosis* and by TLR2 and TLR4 agonists, but not by TLR9 agonists, IL-1α, IL-6 or TNF-α. Flow cytometry evaluation of human peripheral blood mononuclear cells demonstrated that $CD11c^+$ myeloid dendritic cells stimulated with killed *M. tuberculosis* or live *M. bovis* BCG produced hepcidin. The production of the antimicrobial peptide hepcidin by cells that interact with *M. tuberculosis* suggests a host defense mechanism against mycobacteria.

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1. Introduction

It is estimated that one third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis that continues to kill more than 1.7 million people every year.[1] The increased susceptibility of HIV-infected individuals to tuberculosis and the global emergence of multi-drug resistant strains of *M. tuberculosis*[1] are aggravating both the incidence and prevalence of the disease. The development of effective prevention and treatment strategies is urgently needed.

Following an aerosol infection with mycobacteria, alveolar macrophages are targeted by the bacilli for entry and subsequent replication. Dendritic cells also play a central role during a mycobacterial infection due to their ability to uptake, process and transport antigens to draining lymph nodes for presentation to naive lymphocytes, which fosters the development of an effective adaptive immune response.[2] Recently, it has been demonstrated that mycobacteria can also interact with and invade epithelial cells of the respiratory tract.[3,4] The epithelium lining the airways and alveoli is an essential host defense system to counteract the continuous microbial challenge to which the lung is exposed. Epithelial cells contribute to mucociliary clearance, produce inflammatory mediators such as cytokines and chemokines, and are also a major source of antimicrobial peptides, surfactant components and other host defense proteins (reviewed in Ref. 5).

Antimicrobial peptides have recently gained attention as potential therapeutic agents against tuberculosis,[6,7] as they participate in innate immunity and may act in concert with cytokines and other host defense mechanisms to clear *M. tuberculosis*. In particular, hepcidin is a liver-produced antimicrobial peptide with broad spectrum antimicrobial activities.[8–10] Hepcidin also acts as an iron-regulatory hormone by inhibiting iron absorption by the duodenum and macrophage recycling of iron from senescent erythrocytes.[11,12]

Abbreviations: APC, allophycocyanin; BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; CFP, culture filtrate protein; CW, cell wall; IFN, interferon; IL-, interleukin; irr M. tb, γ-irradiated *M. tuberculosis*; LM, lipomannan; ManLAM, mannose-capped lipoarabinomannan; MEM, cell membrane; PBMCs, peripheral blood mononuclear cells; PerCP, Peridinin Chlorophyll Protein; PIMs, phosphatidyl-myo-inositol mannosides; TDM, trehalose-6,6′-dimycolate; TLIP, total lipid; TLR, Toll-like receptor; TNF-α, tumor necrosis factor α; UPL, Universal ProbeLibrary; WCL, whole cell lysate.

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Nemeth et al. have shown that hepcidin regulates iron efflux by binding to ferroportin 1 on cell membranes, resulting in the internalization and degradation of ferroportin 1.[13] Hepcidin is induced in response to iron overload and inflammatory stimuli,[14–17] but is decreased during anemia or hypoxia.[15] Although hepcidin is predominantly expressed in the liver, it has also been detected in other organs, including the lung, pancreas and heart.[8,9,14] We previously demonstrated the induction of hepcidin in bone marrow derived mouse macrophages by the synergistic interaction of IFN-γ and *M. tuberculosis*.[10] Moreover, we showed that hepcidin localized to the mycobacteria-containing phagosomes and that hepcidin possessed antimicrobial activity against *M. tuberculosis in vitro*.[10]

Apart from macrophages, the ability of other cells of the innate and adaptive immune system to induce hepcidin in response to a mycobacterial infection is unknown. In the present study, we extend our previous findings by investigating the expression of hepcidin in different cells of the innate immune system that are crucial to host defense against mycobacteria. Our findings that hepcidin is expressed in lung macrophages, the human alveolar epithelial cell line A549, as well as in mouse and human dendritic cells upon stimulation with *M. tuberculosis* provides evidence that hepcidin is produced by multiple cells of the innate immune system. These findings may have an implication for the survival of *M. tuberculosis* in the host and the development of antimycobacterial peptide-based interventions for the treatment of tuberculosis.

2. Materials and methods

2.1. Mycobacteria

M. tuberculosis H37Rv suspensions were generated as described elsewhere.[18] *M. bovis* BCG Danish strain 1331 was obtained from the Food and Drug Administration (Bethesda, MD). Mycobacterial components (total lipids, phosphatidyl-*myo*-inositol mannosides, mannose-capped lipoarabinomannan, lipomannan, cell wall fraction, cell membrane fraction, cytosol fraction, culture filtrate proteins, whole cell lysate and γ-irradiated *M. tuberculosis*) were obtained from Colorado State University, TB Vaccine Testing and Research Materials (Contract # US0103). Trehalose-6,6′-dimycolate (TDM; also known as cord factor) was obtained from Sigma–Aldrich Milwaukee, WI. Purified proteins, lipoglycans and subcellular fractions were resuspended in PBS while lipids and glycolipids were resuspended in DMSO.

2.2. Mice

Pathogen-free female BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA) at 6–8 weeks of age. Mice studies were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee at the Centers for Disease Control and Prevention (CDC).

2.3. Cell culture

The human alveolar type II epithelial cell line A549 (American Type Culture Collection (ATCC), Manassas, VA) was cultured in Dulbecco's Modified Eagle Medium (DMEM) and maintained in the CDC Cell Culture Facility.

2.4. Mouse dendritic cells

Dendritic cells were isolated from bone marrow of BALB/c mice.[19] Bone marrow cells were depleted of T cells using antibodies to CD4, CD8 and complement. Cells were cultured with GM-CSF and IL-4 (20 ng/ml) for 7 days. A granulocyte-macrophage colony stimulating factor (GM-CSF) expressing J558L cell line (supplied by Dr. I. Mellman, Yale University School of Medicine) was the source of mouse GM-CSF. Flow cytometric analysis revealed that >90% of cells were $CD11c^+$ after 7 days of culture.

2.5. Human myeloid dendritic cells

Peripheral venous blood was obtained from healthy tuberculin skin test negative volunteers (IRB Protocol 1652, CDC). Human myeloid dendritic cells were differentiated from peripheral blood mononuclear cells (PBMCs) that were subjected to leukapharesis. Monocytes were enriched and resuspended in medium supplemented with 100 ng/ml GM-CSF and 20 ng/ml IL-4 for four days.[20]

2.6. Lung macrophages

Mouse lungs were digested with 1 mg/ml collagenase type IV and 25 U/ml DNase in complete RPMI for 1 h at 37 °C. After RBC lysis, macrophages were isolated by overnight adherence to plastic at 37 °C.

2.7. RNA isolation and quantitative RT-PCR (qRT-PCR)

RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). Residual DNA was removed during RNA purification by on-column DNase digestion. RNA was reversed transcribed using the Promega reverse transcription system (Promega, Madison WI). qRT-PCR was performed using Lightcycler Taqman Master and FastStart TaqMan Probe Master based on the Universal ProbeLibrary (UPL) assay (Roche Applied Science). Mouse G3PDH was detected using primers GACCCAAACGGGTCATCA (sense) and CATATTTCTCGTGGTTCACACC (antisense) and UPL#29 probe; Mouse hepcidin 1 was detected using GATGGCACTCAGCACTCG (sense) and GCTGCAGCTCTGTAGTCTGTCT (antisense) and UPL#27 probe; Human G3PDH was detected using AGCCACATCGCTCAGACAC (sense) and GCCCAATACGACCAAATCC (antisense) and UPL#60 probe; Human hepcidin (also called HAMP) was detected using CTGTTTTCCCACAACAGACG (sense) and TTCGCCTCTGGAACATGG (antisense) and UPL#78 probe. Data were collected using the Roche Lightcycler 480 at 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 58 °C for 20 s, 72 °C for 1 s and 40 °C for 30 s. Relative mRNA expression was calculated using G3PDH as the reference standard and the delta Ct method.[21]

2.8. Flow cytometry

PBMCs were stimulated with *M. tuberculosis* whole cell lysate (WCL), γ-irradiated *M. tuberculosis* or live *M. bovis* BCG for 4 h at 37 °C, treated with brefeldin A and monensin and further incubated for 4 h, permeabilized with BD™ phosflow Perm buffer II and stained first with monoclonal anti-human hepcidin antibody (Santa Cruz Biotechnology, Inc) and later with FITC labeled goat anti-mouse IgG. Cells were subsequently stained with a cocktail of pre-titrated anti-human antibodies comprising of HLA-DR-Peridinin Chlorophyll Protein (PerCP), CD11c- Allophycocyanin(APC), CD3-AlexaFluor 700 (A700), CD14- Pacific blue and CD20- APC- cyanine7 (APC-Cy7). PBMCs were fixed with 0.5% paraformaldehyle and acquired using the BD LSR II system. FlowJo software was used to analyze the results.

2.9. Statistical analysis

Results were analyzed by one-way ANOVA using SAS version 9.1 (SAS Institute, Cary, NC). Mean values of relative mRNA expression levels of hepcidin were compared between control and treated