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Requirement of SIRPα for protective immunity against *Leishmania major*

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ABSTRACT

Signal regulatory protein α (SIRPα) is a transmembrane protein that binds the protein tyrosine phosphatases SHP-1 and SHP-2 through its cytoplasmic region and is abundantly expressed on dendritic cells and macrophages. Wild-type (WT) C57BL/6 mice are known to be resistant to *Leishmania major* infection. We here found that C57BL/6 mice that express a mutant version of SIRPα lacking most of the cytoplasmic region manifested increased susceptibility to *L. major* infection, characterized by the marked infiltration of inflammatory cells in the infected lesions. The numbers of the parasites in footpads, draining lymph nodes and spleens were also markedly increased in the infected SIRPα mutant mice, compared with those for the infected WT mice. In addition, soluble leishmanial antigen-induced production of IFN-γ by splenocytes of the infected SIRPα mutant mice was markedly reduced. By contrast, the ability of macrophages of SIRPα mutant mice to produce nitric oxide in response to IFN-γ was almost equivalent to that of macrophages from WT mice. These results suggest that SIRPα is indispensable for protective immunity against *L. major* by the induction of Th1 response.

1. Introduction

The protozoan parasites *Leishmania* spp. are known to infect a variety of mammalian hosts and cause diseases ranging from localized cutaneous to systemic visceral forms [1,2]. Experimental cutaneous mouse leishmaniasis is widely used as a model for this parasitic disease. After the subcutaneous infection of mice with *Leishmania major*, *L. major* promastigotes are first taken up by skin resident macrophages and transform into amastigote life form of the parasite [3,4]. Infection does not lead to activation of macrophages, in which the obligate intracellular amastigotes reside before they are released into the tissue by lysed macrophages. In contrast, skin dendritic cells (DCs), such as Langerhans cells (LCs) or dermal DCs, are thought to phagocyte released amastigotes and process the parasites for presentation of *Leishmania* antigens with MHC molecules on the cell surface [3,4]. The DCs subsequently migrate to draining lymph nodes (LNs), where they make contact with naive $CD4^+$ T cells. During such migration, these DCs also mature and express costimulatory molecules such as CD80, CD86, and CD40 on their surface [5]. The mature DCs thus present *Leishmania* antigens to naive $CD4^+$ T cells together with costimulatory molecules that are essential for priming of the T cells by DCs and release IL-12, inducing development of T helper (Th) 1 cells. Ultimately, such development of Th1 cells producing interferon (IFN)-γ is indispensable for the elimination of *L. major* from the host organism [3,6]. Indeed, macrophages kill the intracellular parasites by means of nitric oxide (NO) production that is promoted by IFN-γ [3,7].

Signal regulatory protein α (SIRPα), also known as SHPS-1/BIT/p84, is a transmembrane protein whose extracellular region comprises three immunoglobulin (Ig)-like domains and whose cytoplasmic region contains immunoreceptor tyrosine-based inhibition motifs that mediate the binding of the protein tyrosine phosphatases SHP-1 and SHP-2 [8,9]. Tyrosine phosphorylation of SIRPα is triggered by various growth factors and cytokines as well as by integrin-mediated cell adhesion to extracellular matrix proteins. SIRPα thus functions as a docking protein to recruit and activate SHP-1 or SHP-2 at the cell membrane in response to extracellular stimuli, and these protein phosphatases are thought

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to be important for signaling downstream of SIRPα. SIRPα is especially abundant in DCs (including LCs) and macrophages, whereas it is barely detectable in T or B lymphocyte [9–13]. The extracellular region of SIRPα interacts with the ligand CD47, which is also a member of the Ig superfamily [8,9,14]. In contrast to the relatively restricted distribution of SIRPα, CD47 is expressed in most cell types including a variety of hematopoietic cells [14].

SIRPα on DCs is thought to be important for development of mouse models for autoimmune diseases [15–17]. Indeed, mice expressing a mutant form of SIRPα that lacks most of the cytoplasmic region of the protein are markedly resistant to experimental autoimmune diseases, such as experimental autoimmune encephalomyelitis, collagen-induced arthritis, and skin contact hypersensitivity [15–17]. IL-17-producing $CD4^+$ effector T cells, designated Th17 cells, are now thought to be the principal pro-inflammatory $CD4^+$ effector T cells that promote development of these mouse models for autoimmune diseases [18,19]. The antigen-induced production of IL-17 by T cells from immunized SIRPα mutant mice was also reduced in extent compared with those apparent for such cells from immunized wild-type (WT) mice [15,16]. Given that SIRPα is especially abundant in DCs, it is likely that SIRPα on DCs promotes the activation of T cells (presumably through interaction with CD47 on the T cells) in a manner functionally similar to that apparent for other costimulatory molecules on DCs [9,15,16]. However, it remains unknown whether SIRPα is important for protective immunity against infection of microorganisms by induction of Th cells.

It is well known that the C57BL/6 mice manifest self-healing skin lesions at the site of parasite inoculation because these mice are able to control the pathogens by strong induction of IFN-γ-producing Th1 cells [3,4]. Thus, we have here investigated the roles of SIRPα in the control of *L. major* infection in mice on C57BL/6 background.

2. Materials and methods

2.1. Mice

Mice that express a mutant version of SIRPα that lacks most of the cytoplasmic region were described previously [10,15,20] and were backcrossed onto the C57BL/6 background for ten generations. The BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). Mice were bred and maintained at the Institute of Experimental Animal Research of Gunma University under specific pathogen-free conditions and were handled in accordance with the animal care guidelines of Gunma University.

2.2. Parasites and infections

L. major (MHOM/SU/73/5-ASKH) promastigotes, that had been kindly provided by Dr. S. Hamano (Nagasaki University) and Dr. Y. Hashiguchi (Kochi University), were cultured in Schneider's Drosophila medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS) (Sigma–Aldrich, St. Louis, MO), 2 mM L-glutamine as previously described [21]. The virulence of the isolate was maintained by monthly passage in BALB/c mice. For infection with *L. major* parasites, stationary phase promastigotes were washed with phosphate-buffered saline (PBS) and counted. Mice were injected in left hind footpad with 5×10^6 parasites suspended in 50 μl of PBS. The development of footpad lesion was monitored weekly by measuring footpad thickness with a dial micrometer caliper (Mitutoyo; Kawasaki, Japan) and was expressed as the increase in thickness of the infected left hind footpad compared with the uninfected right hind footpad. Parasite loads in the infected footpads, draining popliteal LNs or spleens were determined by limiting dilution analysis [22]. Briefly, footpads, draining popliteal LNs or spleens from mice 12 weeks after *L. major* infection were minced and resuspended in RPMI1640 medium (Wako, Osaka, Japan) containing 20% FBS, after which serial five fold dilutions of the tissue preparations were made and the cells were plated in round-bottom 96-well plates in duplicate followed by culturing at 26 °C for 2 weeks. The wells containing motile parasites were identified with a microscope and scored for parasite number. For preparation of soluble *L. major* antigen (SLA), the parasites were subjected to five cycles of rapid freezing and thawing, after which the samples were centrifuge at 17,500*g* for 15 min. The resulting supernatant was filtered through with a 0.45 μm Millipore filter (Millipore, Billerica, MA) and was used as SLA.

2.3. Histological analysis

Eight weeks after infection with *L. major*, infected mouse footpads were subjected to histopathology. The infected footpads dissected from anesthetized animals were fixed, decalcified, and processed for paraffin embedding. Sections (4 μm) were stained with hematoxylin and eosin before microscopic observation.

2.4. Cytokine production by splenocytes stimulated with SLA

Eight weeks after *L. major* infection, splenocytes were prepared from infected mice and cultured in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine as previously described [21]. For measurement of cytokine production by splenocytes, the culture supernatants that had been obtained by incubation of the cells for 48 h with SLA were collected and assayed for IFN-γ and IL-4 by the use of ELISA kits (R&D systems, Minneapolis, MN).

2.5. Isolation of peritoneal macrophages (PEMs) and assays for production of NO

PEMs were prepared essentially as described [20]. For measurement of NO production by PEMs, the cells were incubated with 10 ng/ml IFN-γ (ENDOGEN, Woburn, MA) for 48 h. Culture supernatants were then collected and assayed for nitrite (NO_2^-) content with Griess Reagent System (Promega, Madison, WI), according to the manufacture's instructions.

2.6. Statistical analysis

Data were analyzed by Student's *t* test or by Mann–Whitney's *U*-test. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Increased susceptibility of SIRPα mutant C57BL/6 mice to L. major infection

To investigate whether SIRPα is important for the control of *L. major* infection, we examined the susceptibility of SIRPα mutant mice that express a mutant version of SIRPα lacking most of the cytoplasmic region [10,15,20] to *L. major* infection. The mutant protein expressed in the transgenic animals does not undergo tyrosine phosphorylation nor does it form a complex with SHP-1 or SHP-2 [20]. Given the importance of its cytoplasmic region for signaling by SIRPα, the function of this protein is thought to be eliminated in the mutant mice. Furthermore, the cellular abundance of the mutant protein is markedly reduced compared with that of the full-length protein in WT cells [10,15,20]. Either WT or SIRPα mutant C57BL/6 mice were infected with stationary phase