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Original Article

Mice Lacking Protein Tyrosine Kinase Fyn Develop a T Helper-Type 1 Response and Resist *Leishmania major* Infection

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Abstract

Fyn is a Src family protein tyrosine kinase associated with TCR/CD3 complex. Fyn appears to play a role in the activation of T cells based on its enzymatic activation and tyrosine phosphorylation following the ligation of TCR/CD3, and it also plays a critical role in the calcium flux and interleukin-2 (IL-2) production. The protective response against murine *Leishmania major* infection is associated with the T helper-type 1 (Th1) responses and the ability to modulate Th1 cytokines such as IL-2 and interferon-γ, respectively. The role of Fyn tyrosine kinase *in vivo* was directly examined by the response to infection with *L. major* in C57BL/6 *fyn*-deficient mice. Despite the absence of Fyn, the mice remained resistant to this infection with only mild lesion development, and, they demonstrated Th1 responses as assessed by the delayed-type hypersensitivity response and cytokine milieu. The findings in the *fyn*-deficient mice failed to support a relationship between the anticipated functions of Fyn *in vitro* and the immune response to *L. major* infection *in vivo*. As a result, in leishmanial disease, Fyn probably plays a minor role in the protective immune response and is, therefore, not a key factor in such a response.

Key words: Fyn, protein kinase, gene-deficient mice, Th1/Th2, *Leishmania major*, cytokine

Introduction

Leishmaniasis is one of the most widespread diseases in tropical and subtropical countries. The global burden of leishmaniasis emphasizes the need for the development of studies of its infectious mechanisms for controlling the disease in the context of preventive medicine. In this respect, the clarification of the function of $CD4^+$ T cells should be important for the consequence of protection against *Leishmania major* infection. The $CD4^+$ T cells can be divided into two subsets of effector cells, termed T helper-type 1 (Th1) and Th2 cells, based on their cytokine profiles. The distinctive cytokine profiles correlate with the functional differences of the two subsets, which has provided a concept for how the immune system works against diverse infectious diseases such as an *L. major*. The mice infected with *L. major* are models of chronic human disease caused by intracellular protozoa[1]. The *L. major* infection to different genetic backgrounds of mice results in one of two contrasting profiles of the disease. In BALB/c mice, the infection is not controlled by the immune response and the disease disseminates, eventually involving the visceral organs and, thus, leading to a fatal outcome. In contrast, infecting C57BL/6 mice causes a localized lesion that heals spontaneously. It is widely accepted that the protective immunity against an *L. major* infection is associated with the development of a Th1 response and the production of interleukin-2 (IL-2) and interferon-γ (IFN-γ)[2,3], whereas susceptibility is associated with the development of a Th2 response and the production of IL-4 and IL-10[4,5]. Due to these reliable outcomes, an infection with *L. major* is accepted as the prototypic model to study the regulation of the Th1/Th2 responses *in vivo*.

The importance of Fyn in T cell activation has been demonstrated by the finding that Fyn was activated by the engagement of the TCR/CD3[6] and the kinase was able to mediate the phosphorylation of intracellular proteins and calcium mobilization[7–9]. Studies with *fyn*-knockout mice confirmed that Fyn plays a critically important role in TCR-mediated T cell activation[10,11]. In addition, an over expression of Fyn in T cell hybridoma resulted in both an increase in tyrosine phosphorylation in cellular proteins and an enhancement of the IL-2 production after TCR stimulation[12]. Furthermore, Tamura et al. demonstrated that Fyn was activated in Th1 clones but not in Th2 clones shortly after stimulation with anti-CD3 antibody[13].

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Since C57BL/6 mice, which are resistant strains with *L. major*, develop a Th1 response, we hypothesized that a depletion of Fyn may, thus, negatively affect the establishment of protective immunity. C57BL/6 *fyn*-deficient mice were, therefore, infected with *L. major*, and, thereafter, the course of the disease was monitored. The outcome of the responses in the deficient mice was also discussed.

Materials and Methods

Animals and parasites

Female mice with a homozygous and heterozygous disruption of the gene encoding *fyn* on a C57BL/6 background were provided by Professor H. Nariuchi (Institute of Medical Science, University of Tokyo). Specific pathogen-free female wild-type C57BL/6 mice and BALB/c mice were purchased from SLC (Shizuoka, JAPAN) and used for the experiments at 6 weeks old. Infections were induced using stationary-phase promastigotes of *L. major* (MHOM/SU73/5KSKH) grown at 27°C in Schneider medium, pH 6.5 (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 20% heat-inactivated fetal calf serum (HyClone, Logan, UT, Lot No.: AGD6389). The mice were injected in the left hind footpad with 1×10^6 stationary-phase promastigotes, and the course of the disease was monitored by weekly measurements of the footpad thickness with a dial gauge caliper. Soluble leishmanial antigen (SLA) was prepared from promastigotes of *L. major* by four cycles of freezing and thawing in PBS followed by centrifugation at 20,000×*g* for 10 min. The antigen was finally passed through a 0.2 μm filter and stored at −80°C until use.

Delayed-type hypersensitivity

Delayed-type hypersensitivity (DTH) reactivity was assessed by injecting 50 μg of SLA in 50 μl of PBS into the contralateral footpad of the mice at 12 weeks post-infection. The increase in footpad thickness as a response was measured for up to 72 hours at 24 hour intervals with a dial gauge caliper.

In vitro stimulation of the lymph node cells

Popliteal lymph node cells were prepared from individual mice 14 weeks after an infection of *Leishmania*. Syngenic spleen cells γ-irradiated at 2,000 rads were used as the antigen presenting cells (APC). The lymph node cells (LNC, 2×10^6 cells) from individual mice and APC (6×10^6 cells) were dispensed in triplicate onto 24-well plates and then were cultured in 1 ml of RPMI 1640 medium with 10% FCS in the presence or absence of 50 μg/ml of SLA. Seventy-two hours after cultivation at 37°C in 5% CO_2, the supernatants were collected for an assay of cytokines. The levels of IFN-γ, IL-2 and IL-4 in culture supernatants were quantified by an enzyme-linked immunosorbent assay (ELISA) using a commercial ELISA kit (PerSeptive Diagnostics, Cambridge, MA) according to the manufacturer's instructions.

Statistic analysis

All values were expressed as the mean±SEM for each group. In the assessment of results, Dunnett's *t* test was performed for the comparison of means by the General Linear Models equipped in the Statistical Analysis System (SAS Institute Inc., Cary, NC). The p value<0.05 was regarded as statistically significant.

Results

C57BL/6 fyn-deficient mice resist infection with L. major

The physiological function of Fyn has been shown to correlate with both the T cell activation and IL-2 production that concomitantly up-regulate the Th1 response, thus indicating a correlation between the cell mediated immune response and the ability to kill intracellular *L. major*. To analyze the role of Fyn on the immune response to infection with *L. major*, $fyn^{-/-}$ and $fyn^{+/-}$ mice on the C57BL/6 background, wild-type control C57BL/6 mice and genetically susceptible BALB/c mice were infected with a lethal dose of *L. major*. The course of infection with respect to the footpad lesion was monitored for up to 14 weeks (Fig. 1). The $fyn^{-/-}$ mice controlled the infectious disease with only moderate lesion development for up to 6 weeks post-infection and the infection was subsequently resolved. There was no significant difference in the lesion development during the course of infection between the $fyn^{-/-}$ and $fyn^{+/-}$ mice. As expected, the Th2 response competent BALB/c mice infected with *L. major* developed progressive disease as assessed by footpad swelling. In contrast, C57BL/6 wild-type control mice were resistant to infection and only a mild lesion development was observed. As a result, in terms of the disease outcome in genetically resistant mice, the presence or absence of Fyn was not considered to be a consequential factor.

C57BL/6 fyn-deficient mice reveal a cell mediated response with L. major infection

It is generally accepted that a Th1 response is accompanied by the establishment of a cell mediated response, whereas a Th2 response is characterized by a poor response. To clarify the cell mediated response, we assessed the DTH response against SLA of $fyn^{-/-}$ mice, $fyn^{+/-}$ mice, C57BL/6 mice and BALB/c mice after being infected with *L. major* (Fig. 2). Groups of $fyn^{-/-}$ mice, $fyn^{+/-}$ mice, and wild-type C57BL/6 infected with *L. major* revealed enormous swelling of the footpad, thus indicating a strong cell mediated immunity. However, no significant difference was

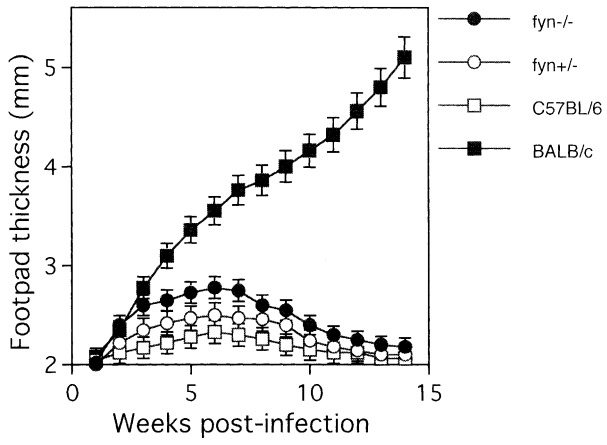

Fig. 1 The course of *L. major* infection in C57BL/6 *fyn*-deficient mice. Groups of mice (n=6) were infected with 1×10^6 of *L. major* in the left hind footpad and the course of the disease was monitored using a metric caliper to measure footpad swelling. The infected mice included genetically resistant C57BL/6 (open squares), with a homozygous (closed circles) and heterozygous (open circles) disruption of the *fyn* gene, and BALB/c mice (closed squares). The weekly measurements of the footpad thickness represent the mean score±SEM of six mice per group.

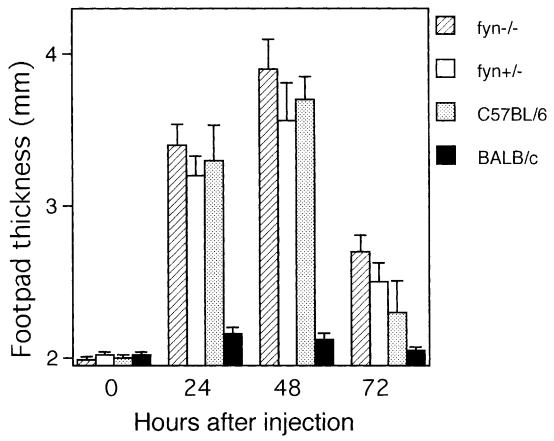

Fig. 2 C57BL/6 *fyn*-deficient mice previously infected with *L. major* demonstrate a strong DTH response to SLA. The groups of mice were injected with SLA into the contralateral footpad 12 weeks after being infected with *L. major* and the increase in footpad size was measured using a metric caliper. The injected mice included genetically resistant C57BL/6 (dotted bars), with a homozygous (shaded bars) and heterozygous (open bars) disruption of the *fyn* gene, and BALB/c mice (closed bars). Each bar represents the mean±SEM for six mice per group.

observed in DTH response between $fyn^{-/-}$ and $fyn^{+/-}$ mice. In contrast, BALB/c mice infected with *L. major* developed a minimal DTH response. As a result, the findings obtained in mice with a genetic deletion of *fyn* failed to support a simple causal relationship between Fyn and the cell-mediated response to *L. major* infection in C57BL/6 mice.

L. major infection induces a Th1 response in C57BL/6 fyn-deficient mice

It is generally accepted that a Th1 response is accompanied by the preferential production of IFN-γ, whereas a Th2 response is characterized by IL-4. To assess the Th1 and Th2 response, we measured the cytokine production in the supernatant after the SLA stimulation of LNC obtained from the draining lymph nodes in $fyn^{-/-}$, $fyn^{+/-}$, C57BL/6 wild-type and BALB/c mice 14 weeks after an *L. major* infection. Figure 3A shows that the C57BL/6 wild-type mice developed a clear Th1 response, with relatively high amounts of IFN-γ and low amounts of IL-4. BALB/c mice infected with *L. major* showed a large excess of IL-4 over IFN-γ production, thus indicating a strong Th2 response. In contrast, $fyn^{-/-}$ and $fyn^{+/-}$ mice did not show any development of a Th2 response and the IFN-γ levels were significantly higher than the IL-4 levels. Similarly, relatively high levels of IL-2 were also observed in $fyn^{-/-}$, $fyn^{+/-}$ and C57BL/6 wild-type mice compared with that of BALB/c mice (Fig. 3B). The cytokine profiles indicated that Fyn does not play a major role for the IFN-γ dependent Th1 response during an *L. major* infection in resistant C57BL/6 mice.

Discussion

To address the functional role of Fyn in the control of immune response, we used C57BL/6 *fyn*-deficient mice to investigate the response to *L. major* infection. We found that the *fyn*-deficient mice, which were infected with *L. major*, showed mild lesion development and the lesions all subsequently healed. The infected mice showed a strong DTH response and IFN-γ dominant

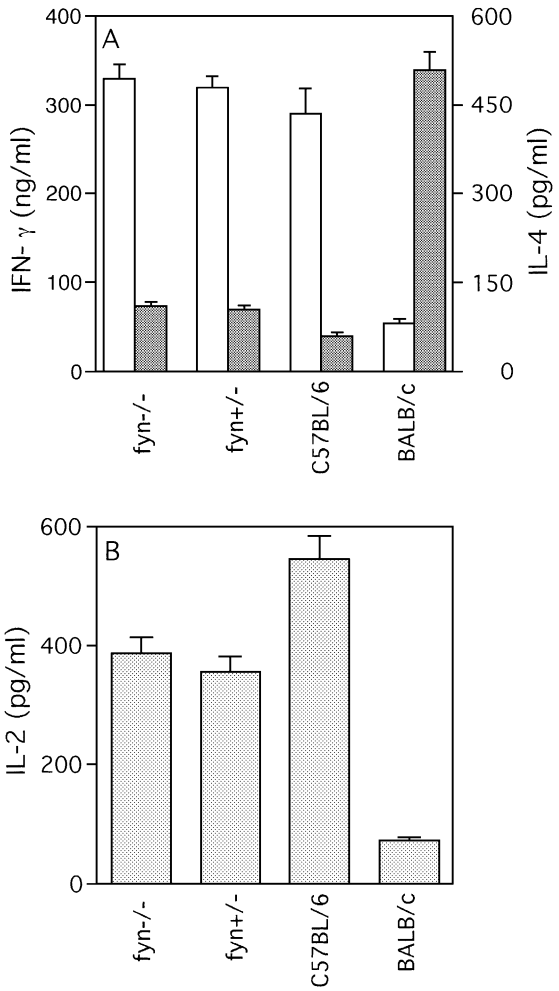

Fig. 3 C57BL/6 *fyn*-deficient mice induced an IFN-γ dominant profile against *L. major* infection. Single cell suspensions from the popliteal lymph nodes of individual mice in the designated groups ($fyn^{-/-}$, $fyn^{+/-}$, C57BL/6 and BALB/c mice) were prepared 14 weeks after infection and then were cultured for 72 hr in the presence of SLA and X-ray irradiated splenocytes from syngenic mice as APC. The supernatants were assayed for IFN-γ (A, open bars), IL-4 (A, closed bars) and IL-2 (B) by ELISA. Each bar represents the mean±SEM for six mice per group. The values are representative of two experiments.

profiles. As a result, the *fyn*-deficient mice were able to control leishmanial disease, and this phenotype was also correlated with the Th1 response. From the *in vitro* findings of the Th1 cell associated function of Fyn[12,13], a correlation was expected between the function of Fyn and the establishment of Th1 response. However, since we found that the $fyn^{-/-}$ mice exhibited a mild lesion development of up to 6 weeks post-infection, which was closely similar to that of the $fyn^{+/-}$ mice, the present findings might suggest that Fyn plays a minor role in the C57BL/6 mice with leishmanial disease.

Previous evidence has indicated Fyn to play a critical role in TCR-mediated signal transduction, and IL-2 production upon antigen stimulation[12,14]. In addition, Fyn regulates the stage in thymocyte development during which the T cell repertoire is established[11]. In *fyn*-deficient mice, thymocytes and mature T cells failed to induce a calcium flux in response to TCR-cross-linking. There is evidence that an increase in the calcium flux in T cells is closely associated with the activation of both Th1 and Th2 cells[13]. This increase in the calcium flux in Th1 cells is herbimycin A sensitive, while the increase in Th2 cells is

insensitive[13]. In the absence of Fyn, T cells may utilize other T cell growth factor(s) or calcium-independent pathway(s). Such findings may support the outcome of the response to *L. major* infection in *fyn*-deficient mice in this study.

It was suggested that a lack of Fyn may lead to a Th2 response with an *L. major* infection. We observed that Fyn mediated-signaling had little effect on the production of IFN-γ in response to *L. major* infection. The significant production of IFN-γ in response to the infection in *fyn*-deficient mice might be due to the activity of another Src family protein tyrosine kinase in T cells, such as Lck or might result from another T cell activation pathway. Alternatively, TCR associated protein kinase ZAP-70 may play a role in the response to the infection in *fyn*-deficient mice[15–17]. A plausible explanation for this unexpected finding is that alternative kinase(s) are able to replace the function of Fyn[18,19]. These possibilities may support the observation that both of the $fyn^{-/-}$ and $fyn^{+/-}$ mice produced high levels of IL-2 as shown in Fig. 3B.

The initial cytokine milieu is considered to be important for the expansion of Th1/Th2 cells, but the precise function of Fyn underlying Th1/Th2 differentiation in resistant and susceptible mice after *L. major* infection has yet to be elucidated. In this study, the absence of Fyn did not convert C57BL/6 mice into a susceptible phenotype resembling that seen in BALB/c mice. The Th1 response in *fyn*-deficient mice against *L. major* infection does not correlate with the findings demonstrating that Fyn facilitates the production of IL-2 upon TCR stimulation[12]. It has been shown in previous studies that the production of Th1 cytokines such as IFN-γ was optimized by IL-2 in *L. major* infected resistant strains of mice[20]. However, no significant difference was observed in the level of IFN-γ between *fyn*-deficient mice and wild-type C57BL/6 mice (Fig. 3A). As a result, the findings in mice with a genetic deletion of *fyn* failed to support a simple causal relation between Fyn and the immune response to an *L. major* infection in genetically resistant C57BL/6 mice. The findings in this study will hopefully provide insight into the host mechanisms that may contribute to the development of subclinical infections and active cutaneous disease in human *L. major* infection.

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