Role of T lymphocytes in liver abscess formation

by Bacteroides fragilis in mice

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Running title: Role of T cells in Bacteroides fragilis liver abscess
Abstract

The underlying mechanisms of liver abscess formation with regard to the interaction between bacterial virulence factors and the immune response have not been fully elucidated. The objective of this study was to determine the role of the host T cells in liver abscess formation caused by \textit{Bacteroides fragilis}. We developed a liver abscess mouse model with inoculation of \textit{B. fragilis} through hepatic portal vein and examined the role of T cells by studying T cell-deficient mice as well as conducting adoptive T cell transfer experiments. No microabscess was formed in the \(\alpha\beta\) TCR\(^+\) T cell-depleted mice in contrast to the control mice. In addition, the \(\alpha\beta\) TCR knockout (KO) mice showed a significantly lower number of microabscesses that were smaller in size compared to the wild-type mice. Adoptive transfer of T cells purified from the wild-type mice into the \(\alpha\beta\) TCR KO mice, resulted in liver abscess formation in those mice. These findings suggest that T cells play an essential role in liver abscess formation caused by \textit{B. fragilis} in mice.

Keywords: Portal vein; Kupffer cells; CD4-positive T-lymphocytes; Immune system; Polysaccharides
Introduction

The incidence of pyogenic liver abscess has remained relatively constant during this century (16). The major organisms causing pyogenic liver abscess are aerobic Gram-negative Enterobacteriaceae including *Escherichia coli*, *Klebsiella pneumoniae* and anaerobic bacteria, especially *Bacteroides fragilis* (9). *B. fragilis* has been recognized as one of the most important organisms causing pyogenic liver abscess, usually along with other enteric pathogens (3, 16). In general, infection is initiated after bacterial entry into liver through the hepatic portal vein from lesions at a site drained by the portal vein, or through the biliary tract from the intestinal lumen (11, 19). The pathological findings of abscess formation are characterized by infiltration of neutrophils and deposition of fibrin, resulting in a pus-filled cavity with capsule formation. The formation of an abscess is thought to be the result of the response of the host defense mechanisms that act to prevent dissemination of the bacterial infection.

Active research on the pathogenesis of intraperitoneal abscess formation has documented the interaction between the **virulence factors** of *B. fragilis* and host T lymphocytes (20-22). *B. fragilis* is the most important anaerobe that causes intraperitoneal infections (15), although it accounts for less than 0.5% of the microorganisms resident in the human colon (7). Capsular polysaccharides of *B. fragilis*, which have a distinct zwitterionic structure, are known to be essential **virulence factors** required for intraperitoneal abscess formation by this organism. T
cell immune responses, directly activated by these polysaccharides, play an important role in
the development of intraperitoneal abscess (20-22). That is, intraperitoneal abscess formation is
regulated by the immune response mediated by T cells that are activated by bacterial
polysaccharides.

Thus, the interaction between the polysaccharides of \textit{B. fragilis} and the host T cell responses
play an essential role in the development of intraperitoneal abscess formation (20). However,
there is no data on the development of liver abscess with regard to the role of T cells and their
interaction with \textit{B. fragilis}. The liver is a reticuloendothelial organ critical in the host defense
system against bacterial invasion. From an immunological point of view, the liver is a unique
organ in the peritoneal cavity. Kupffer cells are resident tissue macrophages found in the
sinusoids of the periportal area; they are the first macrophages encountered by the
microorganisms or microbial products transported from the intestines to liver (2). Their function
of rapid clearance of the bacteria is thought to be due to a complex interaction between the
Kupffer cells and immigrant neutrophils (8). In order to demonstrate the mechanism involved in
the development of pyogenic liver abscess in response to \textit{B. fragilis}, independent investigations
utilizing animal models of liver abscess formation are required.
The purpose of this study was to investigate the role of T cells in liver abscess formation caused by *B. fragilis*. We developed a mouse model of liver abscess by inoculating *B. fragilis* through the hepatic portal vein, and examined the role of T cells by immunological methods.
Materials and Methods

Animals.

C57BL/6 mice (4-6 weeks, male) were obtained from the Orient Bio, Inc. (Korea). The αβ TCR\(^{-/-}\) (B6.129P2-Tcrb) and wild-type mice were obtained from the Jackson Laboratory (Maine, USA). All animals were provided with food and water ad libitum. The animal experiments were performed in accordance with the guidelines established by the Hallym University College of Medicine Standing Committee on Animals.

Preparation of B. fragilis inoculums.

B. fragilis NCTC 9343, successfully used for the development of an intraperitoneal abscess model (22), was used for bacterial inoculums to induce liver abscess formation in the mice. Stock bacteria were inoculated onto Brucella blood agar plates and cultured at 37°C over 24 hours in an anaerobic jar (GasPak system, BBL, USA). After subculture in thioglycollate broth for 18 hours, diluted broth was used to inoculate fresh thioglycollate broth. The bacterial suspension was obtained at the exponential phase of growth. We prepared B. fragilis inoculums with a dose of \(2 \times 10^7\) CFU/mouse in a volume of 0.1 mL phosphate buffered saline (PBS).

Mouse model of pyogenic liver abscess formation.
The mice were anesthetized with a single intraperitoneal injection of 0.15 ml 1:5 (vol/vol) diluted pentobarbital sodium solution (10 mg/ml, Hanlim Pharm. Co. Ltd., Korea). After shaving and disinfection with povidone iodine solution, an anterior midline incision was made through the abdominal wall and peritoneum. The intestines were carefully pulled out, laid laterally over a sterile drape, and then the hepatic portal vein was identified. The bacterial inoculum prepared in a 0.1 ml volume was injected into the hepatic portal vein using a 30-gauge insulin syringe. For hemostasis, following removal of the needle, the vein was lightly compressed using sterile gauze. The abdominal wall was closed with two layers of interrupted sutures. Animals that had massive bleeding or died during this procedure and those who died within 12 hours after the procedure were excluded from the outcome analysis. The animals were sacrificed using CO2 euthanasia and examined for liver abscess formation 21 days later.

Examination for B. fragilis-induced liver abscess formation in mice.

In order to study liver abscess formation caused by *B. fragilis*, small liver sections were cultured under anaerobic conditions. Isolated bacterial colonies were Gram-stained and anaerobic identification was performed with the RapID™ ANA II kit (Remel Inc., Lenexa, KS, USA). The presence of a liver abscess was histopathologically confirmed. The liver was fixed in 4% paraformaldehyde and embedded in paraffin. Histological sections were stained with
hematoxylin-and-eosin (H&E). The serial sections included all of the hepatic lobes that were examined for histological evidence of liver abscess formation. To exclude simple neutrophilic infiltrations from our analysis, a microabscess was defined as a focal infiltration of neutrophils that was 50 μm or larger in diameter and that was accompanied by the destruction of a normal hepatic parenchyma. The number of microabscesses observed in 100 microscopic fields at a magnification of ×100 was determined for each mouse. The maximal diameter of the microabscesses was also determined. The median of the number of microabscesses and the maximal diameter were compared among the study groups. The size of the microabscess was measured using the ocular micrometer (Nikon Instruments).

**Induction of liver abscess formation in the αβ TCR+ T cell-depleted mice.**

T cells bearing αβ TCR in the C57BL/6 mice were depleted with the treatment of 300 μg TCRβ chain-specific mAb H57-597 (BD Pharmingen, USA) via the intraperitoneal route four days before surgery (6). The control mice were treated with the same amount of isotype-matched antibody. The bacterial inoculum was injected through the portal vein and the development of hepatic microabscesses was assessed as described above. Depletion of the αβ TCR+ T cells was confirmed by FACS analysis four days after treatment with antibody. Peripheral blood mononuclear cells were purified from the mice injected with
mAb H57-597, and those receiving control antibodies, and then tested for the proportion of αβ TCR+ T cells. The cells were stained with FITC (fluorescein isothiocyanate)- or PE (phycoerythrin)-labeled mAbs to CD3 and TCRβ, and isotype control antibodies (BD Pharmingen, USA). The stained cells were analyzed on a BD FACSCalibur (BD Biosciences, USA), CELLQuest™ (BD Biosciences, USA), and WinMDI 2.8 analysis software (http://facs.scripps.edu; Scripps Research Institute).

Induction of liver abscesses formation in αβ TCR−/− mice.

The αβ TCR−/− mice and wild-type mice were injected with B. fragilis inoculums through the hepatic portal vein and were assessed for the development of liver abscess formation as described above.

Adoptive T cell transfer.

Splenic mononuclear cells from the wild-type mice were separated by centrifugation with Ficoll-Hypaque gradient (Lymphoprep®, AXIS-SHIELD, Norway), and the T cells were purified on a nylon wool column (Polysciences, PA, USA). T cell-enriched populations (>90% T cells) were transferred to the αβ TCR−/− mice (2×10⁷ cells/mouse) by the intracardiac route 24 hours before inoculation of the B. fragilis. The control group received intracardiac injection of PBS instead of
purified T cells. As another control, the wild-type mice were inoculated with *B. fragilis* inoculums without T cell transfer.

**Statistical analyses.**

Evaluation of the differences in the number and maximal diameter of the microabscesses was performed by the Mann-Whitney U test (SPSS version 11.0, SPSS Inc., Chicago, USA). A *P* value of < 0.05 was considered to be statistically significant.
Results

Development of *B. fragilis*-induced liver abscess model in mice.

To demonstrate the liver abscess formation caused by *B. fragilis*, a small section of the liver from the mice was studied on day 21 after inoculation. The section was cultured anaerobically in thioglycollate broth in an anaerobic jar at 37°C over 48 hours. When diluted broth was subcultured into the Brucella blood agar, small, white, and even colonies were observed (Fig. 1A).

The Gram stain revealed pleomorphic Gram-negative organisms (Fig. 1B). The disk diffusion test showed resistance to vancomycin, kanamycin, and colistin on the Brucella blood agar plates.

Culture of the Bacteroides bile esculin agar showed black colonies and black pigmentation of the agar that was identified as *B. fragilis* by the RapID™ ANA II system (Remel Inc., Lenexa, KS, USA). Histopathological examination of the liver section revealed many microabscesses with variable diameter (50 ~ 250 µm) (Fig. 1C & 1D). Most infiltrating cells were composed of polymorphonuclear leukocytes.

Induction of liver abscess formation in T cell-depleted mice.

Flow cytometry analysis showed that H57-597, a mAb specific for the αβ TCR β chain, successfully depleted αβ TCR+ T cells in the mice (Fig. 2A). All eight mice that were given control antibody developed microabscesses with the diameter greater than 50 µm 21 days after inoculation.
inoculation with *B. fragilis* (Fig. 2B & 2D). The number of microabscesses with a diameter greater than 50 µm, in 100 microscopic fields at a magnification of ×100, was 10 (range 7 ~ 18) in mice given the control antibody (Fig. 2B). The median for the maximal diameter of the microabscesses was 150 µm (range 80 ~ 220) (Fig. 2C). By contrast, none of the mice depleted of αβ TCR+ T cells developed microabscesses (Fig. 2E).

**Induction of liver abscess formation in αβ TCR KO mice.**

To further demonstrate the role of αβ TCR+ T cells in the development of liver abscess formation caused by *B. fragilis*, αβ TCR−/− mice were studied. Among the 10 αβ TCR−/− mice injected with *B. fragilis* inoculums, one died two days later and three died three to four days later; these mice were excluded from the analysis. Only two out of six TCR KO mice developed microabscesses, in contrast to the all 12 wild-type mice that developed hepatic microabscesses with a diameter greater than 50 µm. The median number of microabscesses in the wild-type mice was 11 (range, 5~32) and the median for the maximal diameter was 150 µm (range, 90~250). By contrast, the number of microabscesses found in the αβ TCR−/− mice was one and two, respectively, and the maximal diameters were 70 µm and 90 µm (Fig. 3A & 3B).

αβ TCR+ T cells are critical for the liver abscess formation caused by *B. fragilis*. 
To further demonstrate the role of the TCR$^+$ T cells in the development of liver abscess formation caused by *B. fragilis*, we performed T cell transfer experiments in which the αβ TCR$^{-/-}$ mice, previously shown to be genetically impaired in their ability to develop liver abscesses, were used as recipients. A total of $2 \times 10^7$ T cells were transferred into each mouse, and flow cytometric analysis showed that 81% of the cells were CD3$^+$ T lymphocytes. Among eight αβ TCR$^{-/-}$ mice that received intracardiac injection of phosphate-buffered saline, three died three to four days after inoculation of *B. fragilis*. By contrast, two out of five αβ TCR$^{-/-}$ mice given purified T cells died seven and nine days after *B. fragilis* inoculation, respectively.

The number of microabscesses in the five surviving αβ TCR$^{-/-}$ mice, receiving saline instead of T cells as a control, was significantly less than the number in the wild-type mice ($P = 0.002$) (Fig. 4A). However, the number of microabscesses in the αβ TCR$^{-/-}$ mice, after adoptive transfer of the T lymphocytes, did not show a significant difference from the wild-type mice (Fig. 4A). The maximal diameter of the microabscesses did not differ between the αβ TCR$^{-/-}$ mice, after adoptive transfer of the T lymphocytes, and the wild-type mice; this was in contrast to the very small diameter found in the αβ TCR$^{-/-}$ mice that received saline transfer (Fig. 4B).
Discussion

The results of this study show that T lymphocytes play an essential role in the development of liver abscess caused by *B. fragilis*. To demonstrate the role of T lymphocytes, we developed a mouse model of *B. fragilis*-induced liver abscess formation and studied the mice with a T cell deficiency. Intraperitoneal injection of H57-597, a monoclonal antibody specific for the αβ TCR β chain, removed the αβ TCR⁺ T cells effectively, as shown by the results of the flow cytometric analysis. These αβ TCR⁺ T cell-deficient mice were impaired to develop microabscess formation in contrast to the mice that received the control antibodies. The role of T lymphocytes in the liver abscess formation was further tested using knockout mice. The αβ TCR KO mice developed fewer microabscesses and they were much smaller compared to the wild-type mice. The findings that adoptive transfer of T cells enabled these KO mice to fully recover the capability of abscess formation clearly showed that T lymphocytes are essential for the liver abscess formation by *B. fragilis*. In addition, the findings that some of αβ TCR⁺ T cell-deficient mice showed a few microabscesses suggest that γδ TCR⁺ T cells might also have some roles. There is also the possibility that NK-T cells, which express αβ TCR, might involve the development of liver abscess.

Previously reported animal models of pyogenic liver abscess formation most commonly used injection of anaerobic bacterial inoculums through the mesenteric vein in rabbits, through the
hepatic portal vein in rats, or intraperitoneal injections in mice (1, 10, 14, 18). We chose mice for
our study because monoclonal antibodies targeting surface antigens of T lymphocytes were
broadly available, and knockout mice could also be studied. We injected the B. fragilis inoculum
into the hepatic portal vein to mimic the common natural route of transmission in the
development of human liver abscess.

When the mice died during the inoculation through the hepatic portal vein procedure or died
immediately after surgery, they were excluded from the analysis because the mortality was
considered to be related to non-infectious causes such as bleeding, overdose of anesthesia, or
hypothermia. After excluding these animals, the wild-type mice generally survived until day 21
when they were sacrificed. This low mortality in mice infected with B. fragilis can be explained
by the low endotoxin activity of the lipopolysaccharides of B. fragilis, which seldom induce
disseminated intravascular coagulation and severe sepsis (12). In part, successful localization of
the bacterial infection to the liver, with effective killing and abscess formation, might have
contributed to protecting the mice from a high grade bacteremia and mortality. By contrast, a few
of the mice, with a T cell deficiency, died several days after inoculation of the B. fragilis. This
might have been due to their poor immune response to the bacterial invasion of the liver, as
suggested by the inability to develop liver abscesses in the mice that survived.

It is not clear how T cells specifically contribute to the liver abscess formation caused by B.
The alleged mechanism involved in the intraperitoneal abscess formation caused by *B. fragilis* might partially explain it. The activation of CD4\(^+\) T cells, by the zwitterionic polysaccharide antigens, is one of the critical factors involved in the complex host response to bacterial infection that results in abscess formation (5, 21). Subsequent release of cytokines, from activated T cells, stimulates the entry of neutrophils into the infection site. Recently, this process has been found to depend on the initial interaction of the zwitterionic polysaccharides with the Toll-like receptor (TLR) 2 (23). Based on the findings in murine models of intraperitoneal abscess formation, it is likely that zwitterionic capsular polysaccharides of *B. fragilis* activate T cells, and the cytokines secreted by these T cells play an important role in the recruitment of neutrophils into the liver that are the predominant inflammatory cells in abscess formation.

Further experiments using knockout mice defective in production of the capsular polysaccharides will be necessary to demonstrate the role of the capsular polysaccharides in T cell-mediated liver abscess formation by *B. fragilis*.

The innate immunity of the liver against bacterial invasion is unique and complex. The anatomical structure of the liver enables maximal contact of circulating blood with hepatocytes, which aids the host’s defense (2). Kupffer cells, located at the luminal surface of the endothelial cells, become activated in response to bacterial invasion, and produce proinflammatory cytokines and chemokines (4, 13, 17). However, the resident immune responses in the liver are not limited...
to Kupffer cells. Additional cells include natural killer cells, CD4$^+$ T cells, and CD8$^+$ T cells. Furthermore, monocytes and neutrophils that circulate through the rich blood supply in the liver, participate in the hepatic immune system (2). Therefore, the role of T cells in the liver abscess formation caused by *B. fragilis* is part of the complex interaction between the host’s immune system and bacterial virulence. The possible mechanisms involved in the liver abscess formation caused by *B. fragilis* include activation of Kupffer cells following the invasion of *B. fragilis* into the liver, followed by the release of cytokines and chemokines, activation of T cells and natural killer cells, followed by the release of the cytokines, rapid recruitment of circulating inflammatory cells, and encapsulation by fibrosis.

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References


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Figure legends

FIG. 1. Demonstration of liver abscess formation in mice injected with *B. fragilis* inoculums.

(A) Bacterial colonies isolated in Brucella blood agar plates under anaerobic conditions. Small, white, and even colonies are observed. (B) Gram stain revealed pleomorphic Gram-negative organisms. (C) Histopathological evidence of microabscess on day 7 (×100, inlet ×400, H&E stain). Infiltration of polymorphonuclear leukocytes is seen along with destruction of normal hepatic parenchyma. (D) Microabscess formation on day 21 (×100, inlet ×400). The results shown are representative of two independent experiments.
FIG. 2. Loss of liver abscess-forming capability in mice depleted of αβ TCR+ T cells. (A)

Depletion of αβ TCR+ CD3+ T cells in mice injected with the monoclonal antibody H57-597. (B)

Comparison of the number of hepatic microabscesses between mice injected with the H57-597 monoclonal antibody and control antibody (*P = 0.003, Mann-Whitney U test). (C) Comparison of the maximum diameter of the microabscesses between the two groups. The horizontal bar indicates median (**P = 0.003, Mann-Whitney U test). (D) Microabscesses induced by *B. fragilis* in control antibody-treated mice (×100, H&E stain). (E) No microabscesses were found in the H57-597-treated mice (×100). The results shown are representative of two independent experiments.
FIG. 3. Loss of liver abscess-forming capability in the αβ TCR knockout (KO) mice. (A)

Comparison of the number of hepatic microabscesses between the αβ TCR KO mice and the wild-type mice (\(^{'}P = 0.001\), Mann-Whitney U test). (B) Comparison of the maximum diameter of the microabscesses between the two groups. The horizontal bar indicates the median (\(^{''}P = 0.001\), Mann-Whitney U test). (C) Microabscesses induced by B. fragilis in the wild-type mice (×100, H&E stain). (D) Few microabscesses were found in the αβ TCR KO mice (×100). The results shown are representative of two independent experiments.
FIG. 4. Adoptive transfer of T cells enables liver abscess formation in αβ TCR KO mice. (A) Comparison of the number of hepatic microabscesses among αβ TCR KO mice repleted with T cells, the αβ TCR KO mice injected with saline, and the wild-type mice (°P = 0.002 compared to wild-type control; °°P = 0.017 compared to saline-transfer control; °°°P = 0.926 compared to wild-type control; Mann-Whitney U test). (B) Comparison of the maximum diameter of the microabscesses. The horizontal bar indicates the median (°P = 0.003 compared to wild-type control; °°P = 0.017 compared to saline-transfer control; °°°P = 0.708 compared to wild-type control; Mann-Whitney U test). (C) Microabscesses induced by B. fragilis in the wild-type mice (×100, H&E stain). (D) Few microabscesses were induced by B. fragilis in the αβ TCR KO mice injected with saline (×100). (E) Microabscesses induced by B. fragilis in the αβ TCR KO mice repleted with T cells by adoptive transfer (×100). The results shown are representative of two independent experiments.