Chapter

Role of Kupffer Cells in Systemic Anti-Microbial Defense

Hiroyuki Nakashima, Masahiro Nakashima, Manabu Kinoshita and Shuhji Seki

Abstract

The liver has long been recognized as important in digestion. However, the liver's abundance of innate immune cells strongly suggests that it has specific defense mechanisms. A characteristic anatomical feature of the liver is its large blood flow. The blood flowing out from the whole alimentary tract is transported to the liver via the portal vein and distributed to peripheral structures called sinusoids. Kupffer cells, a typical example of resident macrophages, are located in sinusoids and are in continuous contact with various portal blood components. They have vigorous phagocytic activity and eliminate bacteria coming from the gut before they enter systemic circulation. Based on this framework, Kupffer cells were considered a filter for portal blood pathogens. However, recent evidence reveals that they exert crucial functions in systemic host defense against bacterial infection. To defend against various sources of bacterial pathogens, Kupffer cells construct an efficient surveillance system for systemic circulation, cooperating aggressively with other immune cells. They collaborate with non-immune cells such as hepatocytes and platelets to potentiate defense function. In conclusion, Kupffer cells coordinate immune cell activity to efficiently defend against infections, making them crucial players in systemic antibacterial immunity.

Keywords: liver, Kupffer cells, innate immunity, macrophages, bacteria

1. Introduction

The liver is one of the largest organs in the mammalian body and plays an essential role in maintaining health [1, 2]. The hepatic vascular system has a unique and distinct anatomical structure. All veins from the digestive tract unite and form the portal vein. Interestingly, this sizable venous vessel branches into capillaries called sinusoids (indicated by arrows in **Figure 1**) for peripheral microcirculation in the liver. Venous blood from the digestive tract flows into the liver and is processed by hepatocytes before returning to systemic circulation (**Figure 2**). This unique vascular structure of the liver constitutes an ideal environment for innate immune cells to eliminate harmful materials in the blood. Portal blood is filled with beneficial nutrients and unwanted microorganisms ingested along with food. The gastrointestinal tract is also filled with numerous commensal bacteria that form the microbiota. Furthermore, 70% of intravenously injected bacteria accumulate in the liver and are removed therefrom [3]. Thus, bacterial materials in systemic circulation and the portal vein are brought

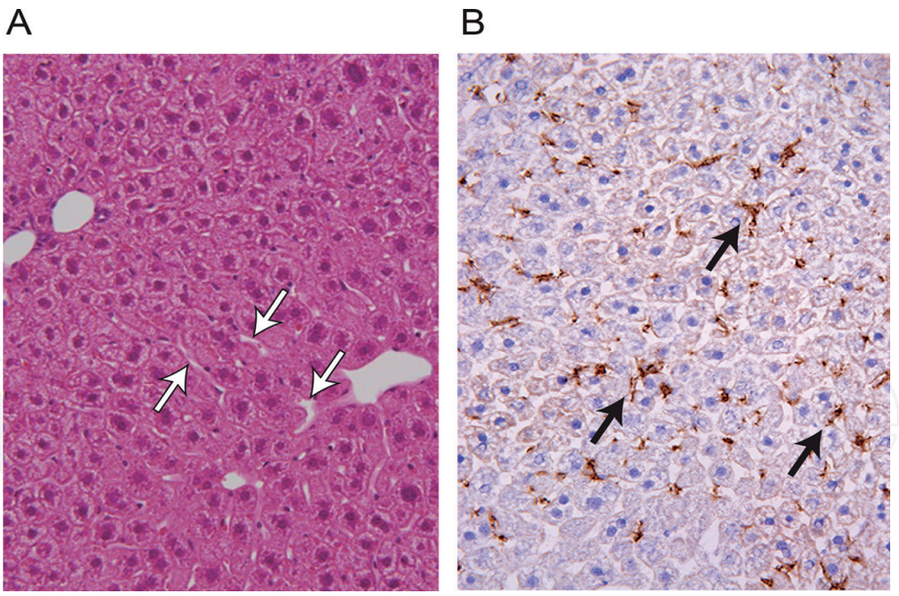

Figure 1.
Microstructure of the liver. (A) Hematoxylin and eosin (HE) staining of the liver (× 400). The portal venous blood and systemic arterial blood are mixed and flow through the sinusoidal space, which is a narrow space for microcirculation between numerous hepatocytes (white arrows). (B) Immunohistochemical staining of the mouse liver (× 400). The primary antibody against F4/80 antigen, which is a specific marker for the macrophage in mice, was reacted and followed by horseradish peroxidase staining (brown area). Counterstaining was performed by hematoxylin to distinguish hepatocytes (blue area). The sinusoidal space is lined with a large number of F4/80-positive Kupffer cells (black arrows). Overall, the blood stream passes through two types of filters, nutritional processing and immunological surveillance.

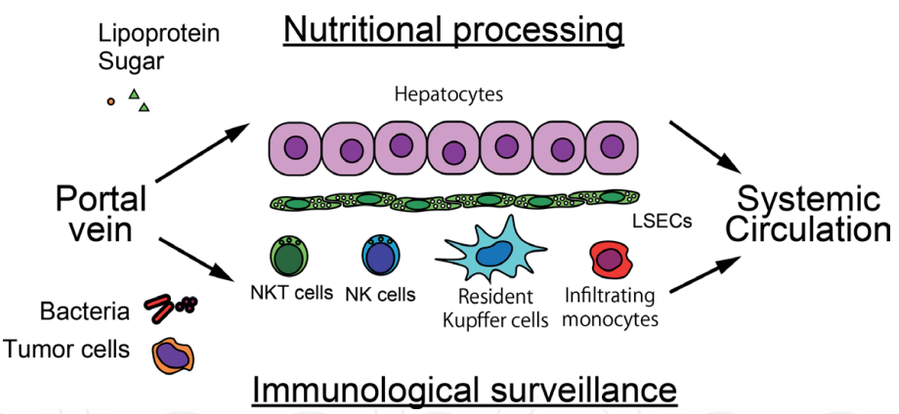

Figure 2.
The two kinds of filtering systems in the liver. One involves the nutritional processing of absorbed sugars and lipids, which is supported by hepatocytes. The other involves immunological surveillance of external pathogens, such as bacteria and tumor cells, through a unique innate immune cell network. These two cell types are separated by liver sinusoidal endothelial cells (LSECs).

to the liver and activate innate immune cells, which are essential for eliminating pathogenic organisms in the host. The narrow space of the sinusoids and slow blood flow form an ideal environment for eliminating pathogenic microorganisms entering the liver. Recently, many researchers have examined the liver as an innate immune organ based on anatomical and immunological viewpoints [4, 5].

2. The liver demonstrates the structure required for antibacterial responses

The liver contains unique innate immune cells, including natural killer (NK) cells, natural killer T (NKT) cells, and Kupffer cells [1]. These innate immune cells

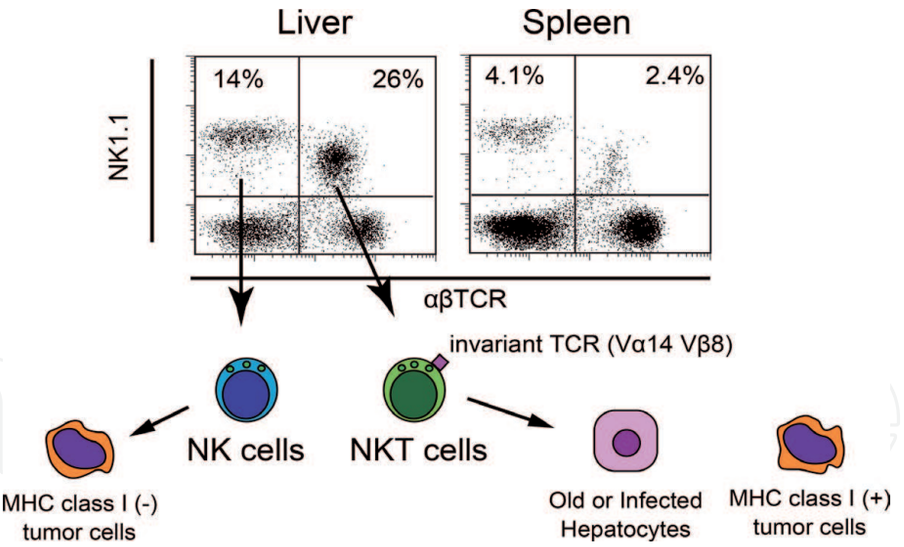

Figure 3.
The distinct composition of T cells in the liver. Liver and spleen lymphocytes were isolated from C57BL/6 mice and subjected to flow cytometry analysis. Isolated cells were developed into two-dimensional histograms with the αβ T-cell receptor (TCR) and NK1.1 antigen. In the liver, double-positive natural killer T (NKT) cells, and single-positive natural killer (NK) cells comprised a larger population than in the spleen. NK cells exert strong anti-tumor cytotoxicity against major histocompatibility complex (MHC) class I negative tumors. NKT cells can induce apoptosis in old or infected hepatocytes and MHC class I-positive tumor cells.

carry out essential bilateral immunological functions, such as antibacterial and anti-tumor immunity. Kupffer cells are the most well-known tissue-resident macrophages and are pivotal effectors of antibacterial immunity [6]. They are characterized by vigorous phagocytic activity [7]. Most Kupffer cells exist in the zone 2 region of the sinusoids, where the blood flow is the slowest [8] (**Figure 1B**). They express scavenger receptors and constantly engulf exogenous materials, such as bacteria. NKT cells comprise approximately 25% of the hepatic lymphocytes, which is a high percentage compared to other organs [1] (**Figure 3**). Typical NKT cells have an invariant T-cell receptor (TCR). In contrast to conventional T cells, their TCR shows much less variation; approximately 90% of them express Vα14-Jα18 in mice, which may recognize antigen "patterns" rather than specific antigen structures. The invariant TCR of NKT cells is reported to recognize a synthetic glycolipid, α-galactosylceramide, or some bacterial structures [9]. However, the natural ligands of NKT cells remain to be elucidated. Along with NK cells, the essential function of NKT cells is now considered to be anti-tumor response [10–12]. In contrast, macrophage populations are essential cellular factors for bacterial defense in the liver [13].

3. Two distinct macrophage subsets in the liver

Each organ has a specific macrophage subset. Generally, bone marrow-derived monocytes infiltrate tissues and differentiate into tissue-resident macrophages [14]. The constitution of macrophages in the liver is more complex. The liver tissue-resident macrophages or Kupffer cells are derived from yolk sac-originated progenitor cells and are self-renewed in the liver, independent of the bone marrow [15]. In contrast, bone marrow-derived infiltrating monocytes coexist in the sinusoidal space and play essential roles in inflammatory reactions (**Figure 4**) [16, 17] . They are positive for the lymphocyte antigen 6 complex (Ly6C), which is a typical marker for bone marrow-derived immune cells. Interestingly, these two macrophage subsets possess various differing features. Kupffer cells exhibit vigorous phagocytic activity and longer self-renewal time. They disappear in response to clodronate liposome

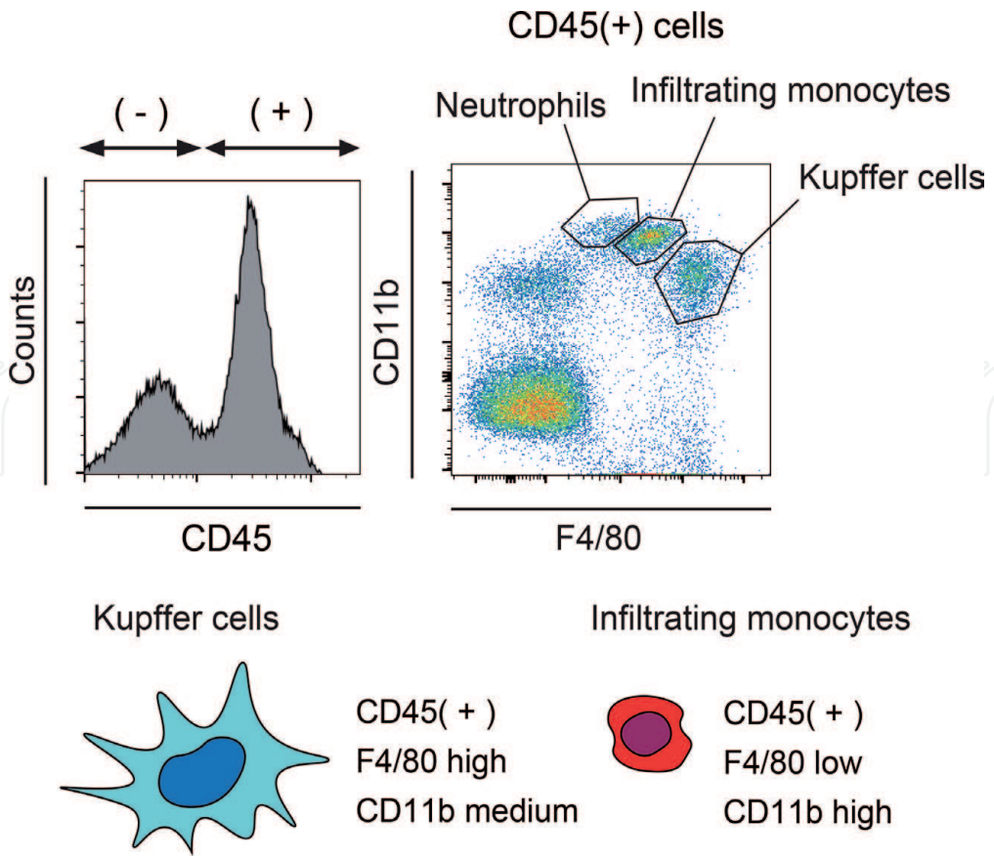

Figure 4.
The composition of macrophages and neutrophils in the liver. Non-parenchymal cells were isolated from the mouse liver and examined by flow cytometry to analyze macrophage composition. Immune cells were selected with the CD45 antigen, and a two-dimensional histogram was plotted against F4/80 and CD11b antigens. F4/80 high and CD11b medium cells were Kupffer cells; F4/80 low and CD11b high cells were infiltrated monocytes; neutrophils comprised the CD11b highest population; eosinophils, which are also F4/80 positive, were excluded using the Siglec-F antigen.

treatment [18, 19], which induces apoptosis of macrophages after phagocytosis. Their proliferation is independent of bone marrow, and their longer turnover cycle confers resistance to radiation exposure [20, 21]. In contrast, infiltrating monocytes potently secrete inflammatory cytokines and accelerate inflammation; they are less phagocytic and are rapidly supplied from the bone marrow [16, 22]. Furthermore, they are resistant to clodronate liposome treatment and are susceptible to radiation exposure [23]. These two lineages of macrophages cooperate to eliminate exogenous pathogens from the bloodstream.

4. Vigorous phagocytic activity of Kupffer cells

Kupffer cells are characterized by their vigorous phagocytic activity. They can engulf fluorescein isothiocyanate (FITC)-labeled *Escherichia coli* (FITC-*E. coli*) more efficiently than the infiltrating monocytes (**Figure 5**). The immediate initial response was also a remarkable feature. Kupffer cells phagocytose FITC-*E. coli* immediately after *in vivo* administration, which was much faster than that by infiltrating monocytes (**Figure 6**). This feature suggests they have a sophisticated ability to distinguish foreign pathogens, such as bacteria. From this viewpoint, it is natural to recognize them as key players in eliminating systemic bacterial loads, such as in severe sepsis. Notably, they can actively phagocytose both gram-negative and positive bacteria [23]. In 1959, Benacerraf et al. reported that the blood clearance rate of gram-positive *Staphylococcus aureus* (*S. aureus*) was much faster than that

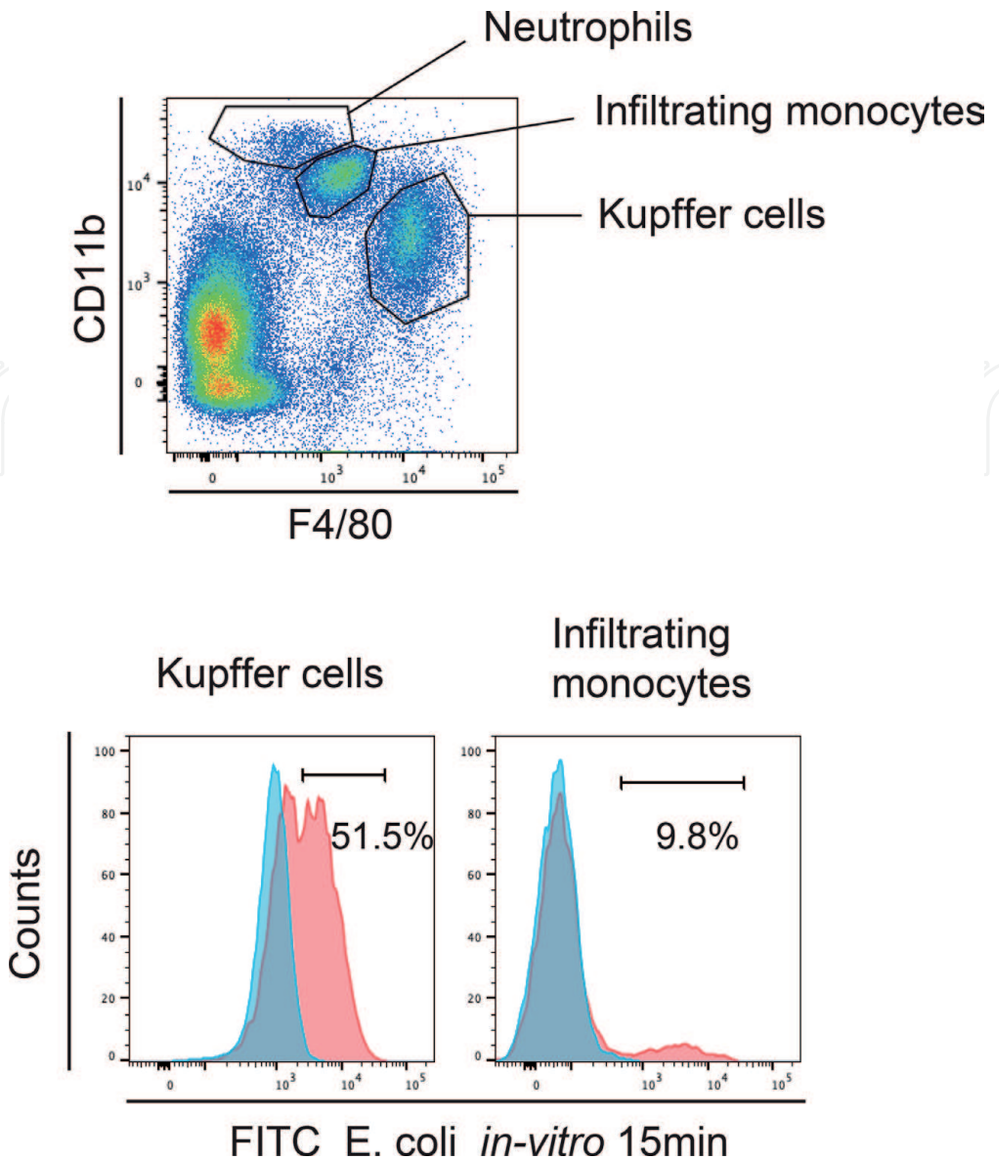

Figure 5.
Evaluation of phagocytosis by liver immune cells in vitro. Liver immune cells were isolated and incubated with FITC-labeled Escherichia coli (E. coli). After 15 minutes (min) of incubation, the cells were collected and analyzed using flow cytometry. Approximately half of the Kupffer cells engulfed the bacteria (red area), which is much more efficient than monocytes. The blue area represents the sample with no bacteria and is set as a negative control. Kupffer cells showed strong auto-fluorescence, and the blue area was shifted to the positive side.

of gram-negative *E. coli*, and almost all of them were trapped in the liver [3]. They also suggested that opsonization by immunoglobulin was not necessary because the clearance rate was very rapid. This report strongly suggests that Kupffer cells play a significant role in the clearance of gram-positive cocci in the blood stream. *S. aureus* usually invades the bloodstream from inflammatory lesions in the skin, oral cavity, and respiratory system. As Kupffer cells actively phagocytose this type of bacteria, it is evident that they play an essential role in protecting against pathogens derived from systemic circulation, not only from the portal vein. One of the characteristic genes of Kupffer cells is the complement receptor of the immunoglobulin superfamily (CRIg) [24]. CRIg directly binds to gram-positive bacteria through lipoteichoic acid, independent of complement [25]. This process is essential for effectively eliminating gram-positive bacteria from the bloodstream in the liver. Consistently, after elimination of Kupffer cells by treatment with clodronate liposomes, the survival rate after intravenous challenge with live *S. aureus* was significantly decreased [23] (**Figure 7A**). The Kupffer cell elimination blunts the liver's clearance ability and renders the mice more susceptible to the *S. aureus* (**Figure 7BC**).

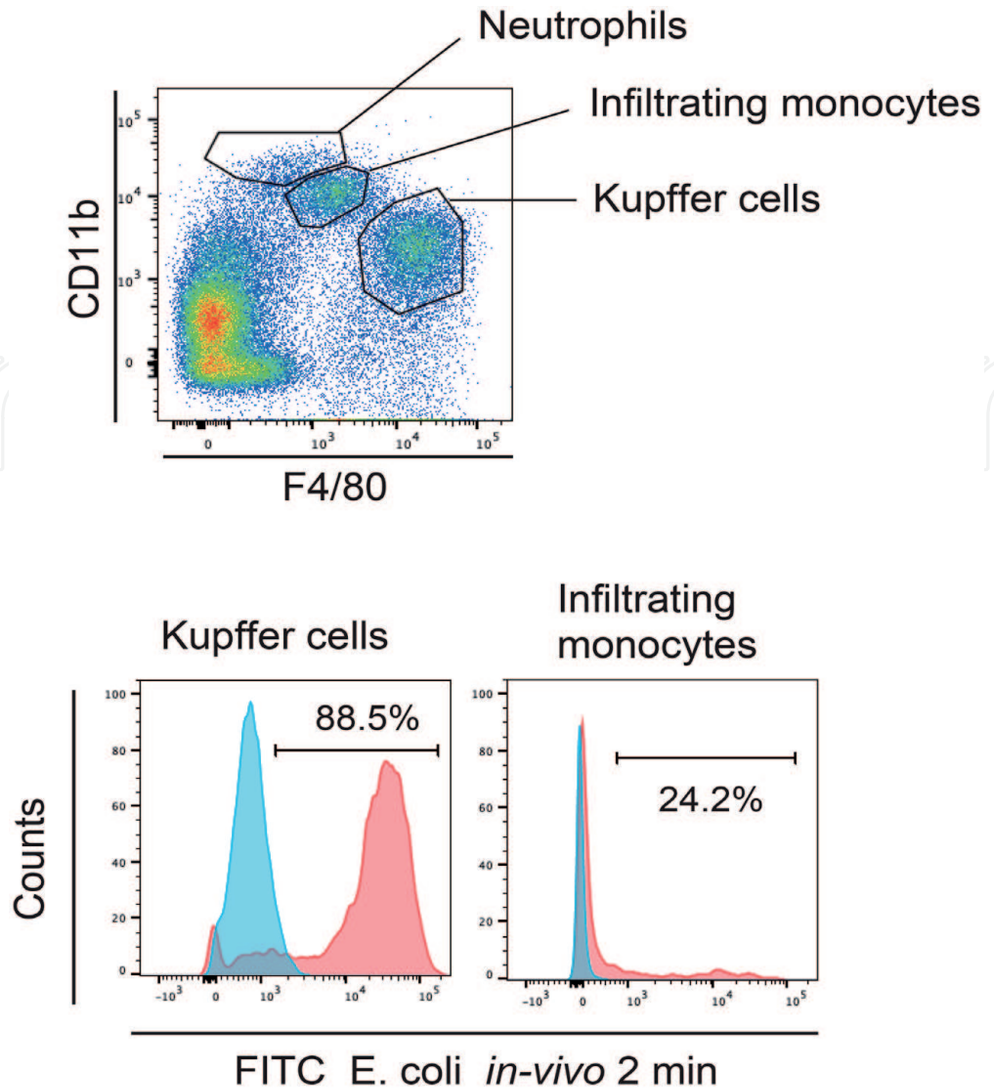

Figure 6.
Evaluation of phagocytosis by liver immune cells in vivo. Mice were intravenously injected with FITC-labeled E. coli via the tail vein. Liver immune cells were isolated 2 min after injection and analyzed by flow cytometry. The blue area is the sample from the mice injected with unlabeled control bacteria, set as a negative control. Approximately 90% of Kupffer cells engulf or attach the bacteria after only 2 min (red area), demonstrating their rapid and vigorous phagocytic activity.

5. Activation of Kupffer cells by infiltrated monocytes

A substantial number of monocytes exist in the liver, as well as in other organs. These can be isolated even after intense perfusion from the portal vein, and their numbers are markedly increased by systemic inflammation or experimental hepatitis [26]. These phenomena indicate that they are not aberrant bystander cells in the liver. They are recruited from the bone marrow, actively attach to the sinusoidal space, and play a specific role in the hepatic immune mechanism. Their definition and nomenclature are still controversial; some investigators call them infiltrating monocytes, whereas others refer to them as monocyte-derived macrophages. Both M1-like proinflammatory and M2-like immunomodulatory populations were present in this subset. These complexities have stimulated much discussion and controversy. Although their strict definition still requires future study, some of their primary functions are already known [21, 27]. Regarding immune reactions, $Ly6C^{+}$ monocytes produce proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-12

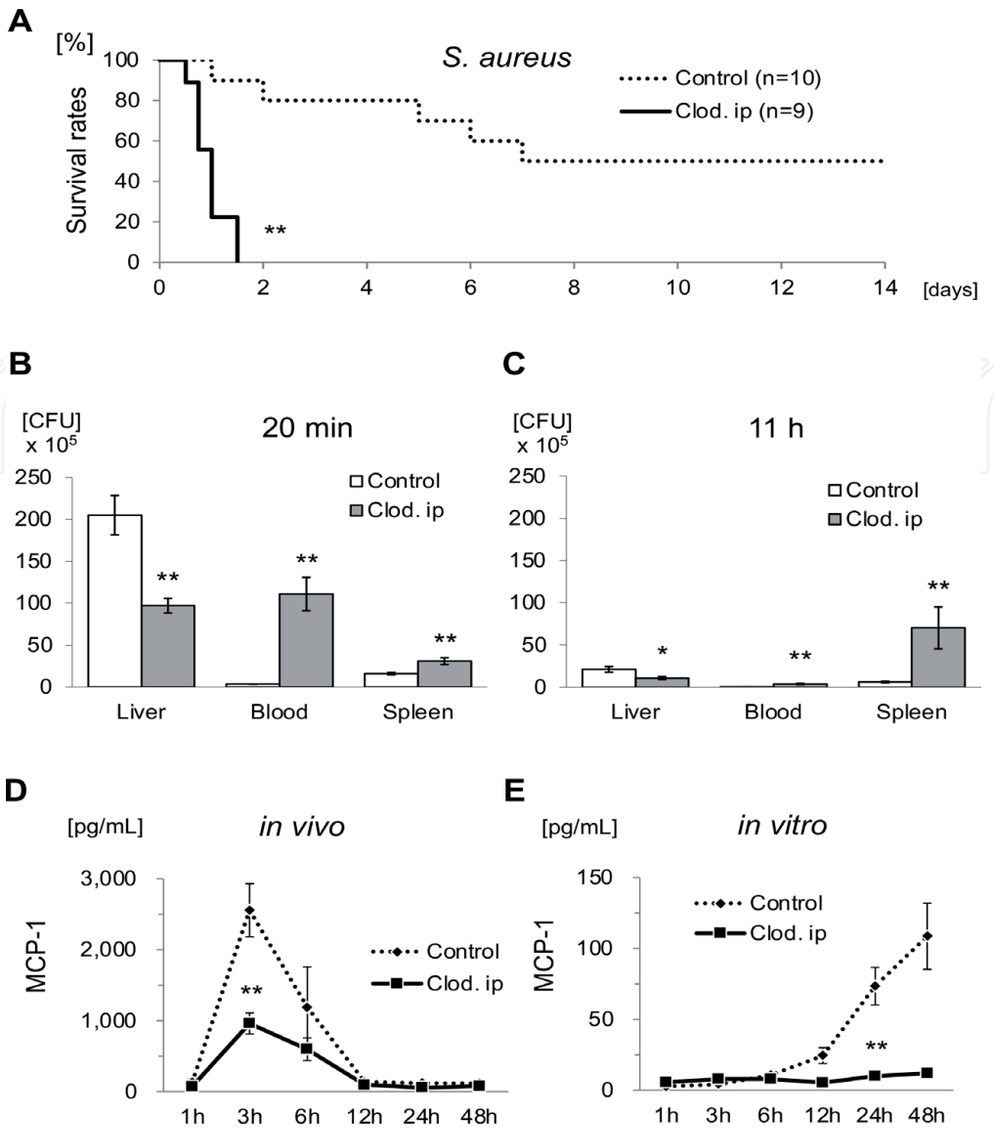

Figure 7.
*Clodronate pretreatment made mice susceptible to Staphylococcus aureus (S. aureus) infection. (A) In clodronate liposome-pretreated mice, the survival rate of mice infected with S. aureus was significantly decreased (solid line) compared to control mice (dotted line). (B) The number of bacteria trapped in the liver was decreased in clodronate treated mice (gray columns) compared to control mice (white columns). The un-trapped bacteria were remaining in the blood and the spleen. After 20 minutes of S. aureus injection, each organ was collected, homogenized and colony forming units (CFUs) were analyzed. (C) After 11 hours, the certain number of bacteria remaining in the spleen in clodronate-pretreated mice. (D) The MCP-1 level in sera after injection of S. aureus significantly decreased in clodronate-pretreated mice (solid line) compared to control mice (dotted line). (E) The MCP-1 production of liver immune cells by incubation with S. aureus was inhibited in clodronate-pretreated mice (solid line) compared to control (dotted line), which means Kupffer cells are the main source of this chemokine. *P < 0.01, **P < 0.05 versus control in unpaired student t test [23].*

(IL-12) [22]. In some experimental hepatitis models, FasL expressed by these cells acts as a final effector to injure hepatocytes that express Fas [26], inducing Fas–FasL-dependent apoptosis [28, 29]. In bacterial defense, Kupffer cells engulf bacteria and produce chemokines such as monocyte chemoattractant protein-1 (MCP-1) (**Figure 7DE**) and recruit these monocytes into the sinusoidal space. Such recruited monocytes produce inflammatory cytokines such as TNF and facilitate Kupffer cell's antibacterial activity [23]. If this pathway is blocked using a recombinant TNF antibody, reactive oxide production from Kupffer cells is inhibited, and their bactericidal activity is reduced [30, 31]. This cell population is thus essential for effective elimination of bacteria by Kupffer

cells, and the combination of these two macrophage populations is crucial for an effective immune response against bacteria.

6. Regulation of Kupffer cell functions by C-reactive protein (CRP)

CRP is an acute-phase protein produced by hepatocytes during inflammation. The serum level of this protein is recognized as a marker for evaluating inflammation severity. The sensitivity and specificity of serum CRP levels are high enough to detect even minor inflammation in the body. According to recent research, this acute-phase protein is a clinical marker as well as an important protein that drives macrophage activity into a preferable and reasonable state [32, 33]. Pretreatment with synthetic CRP improved survival after intravenous bacterial challenge (**Figure 8**). The mechanism underlying this reaction is the increased phagocytic activity of Kupffer cells and the suppression of excessive inflammatory cytokines from activated monocytes. Overall, treatment with synthetic CRP drives the immune cell system to a preferable state and improves survival in bacterial infections. In addition to the beneficial effect of synthetic CRP, the natural form of CRP reportedly has various means of modulating immune functions [34]. Although the primary functions of hepatocytes is commonly accepted to be involved in processing nutrition, it is suggested that hepatocytes have immunomodulatory functions, based on the fact that they are involved in the production of complement proteins and acute phase proteins such as CRP. This aspect of hepatocytes is consistent with the theory that the liver is a crucial organ in systemic antibacterial immunity.

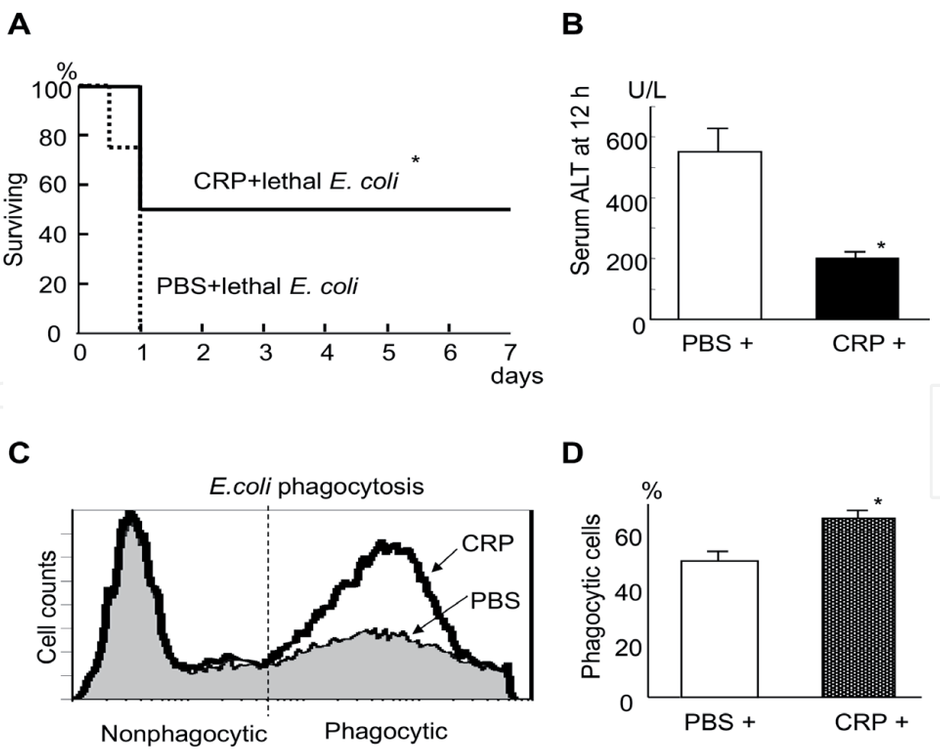

Figure 8.
*Synthetic CRP improved the survival rate of lethal E. coli infection in mice. (A) C57BL/6 mice were pretreated with synthetic CRP (C-reactive protein) or phosphate buffered saline (PBS) and were challenged intravenously with a lethal dose of E. coli. Survival rate was improved by synthetic CRP. (B) Liver dysfunction after 12 hours (h) of E. coli injection was ameliorated in CRP treated mice (black column). (C) CRP- or PBS-pretreated mice (1 hour before) were injected intravenously with FITC labeled E. coli. Liver immune cells were isolated after 20 minutes and analyzed with flow cytometry. Kupffer cells were gated, and phagocytosis of FITC-E. coli was demonstrated. (D) The proportion of phagocytosing Kupffer cells is increased in CRP treated mice. *P < 0.01 versus other groups in unpaired student t test [32].*

7. Relationship with neutrophils

The liver is highly responsive to invasion by external antigens from various origins [5]. Kupffer cell show the ability to engulf microorganisms. However, they have one serious disadvantage. Namely, their self-renewal speed is slower than that of other immune cells. For instance, after injection of clodronate liposomes, which can eliminate almost all Kupffer cells, at least two weeks are required to restore Kupffer cell numbers [6]. Upon exposure to an excessive number of bacteria, their phagocytic ability reaches its limit by repeated phagocytosis, and they easily undergo apoptosis and disappear from the sinusoidal space [35]. Their ability to attract other immune cells with chemokines seems to be a compensatory reaction to overcome this adverse effect. They recruit monocytes and neutrophils into the sinusoidal space to support the clearance of an excess number of bacteria. A previous report described that Kupffer cells attach bacteria on their cell surface and that the main effectors phagocytosing bacteria are neutrophils [36]. Consistent with this report, Kubes *et al.* reported that neutrophils clear the bacteria by cooperating with Kupffer cells in the presence of platelets [37]. Neutrophils phagocytose bacteria and form neutrophil extracellular traps (NETs) in the sinusoidal space to facilitate bacterial clearance.

8. Relationship with platelets

C-type lectin 2 (CLEC2) is a characteristic marker of Kupffer cells [38]. All Kupffer cells showed high expression of this antigen, which has been recognized as a marker for their identification in flow cytometric analyses. CLEC2 is a receptor for platelets, and it may be unclear why this antigen is highly expressed in Kupffer cells. The primary function of platelets is hemostasis, which is profoundly different to the immunological defense mechanism. However, platelets also express various immunological markers, such as toll-like receptors, and contribute to immunological functions [39, 40]. The specific role of platelets in liver immune reactions was previously reported in 1992 [41]. In this report, platelets in the blood were found to migrate rapidly to the liver after systemic bacterial antigen administration. The mechanism underlying this reaction was reported in 2013 [42]. Under normal conditions, platelets maintain continuous contact with Kupffer cells. However, in systemic gram-positive bacterial infection, Kupffer cells bind bacteria transported via the bloodstream, attach them to their cell surface, and form aggregates with platelets. These aggregated complexes facilitate NET development by neutrophils in the sinusoidal space. Along with the vigorous phagocytosis by Kupffer cells, this reaction also contributes significantly to the clearance of harmful bacteria from blood [43]. Interestingly, this reaction is augmented by complement component C3, which is produced by hepatocytes [42]. Thus, this reaction exemplifies a sophisticated collaboration network of Kupffer cells with platelets, neutrophils, and even hepatocytes in the systemic bacterial defense mechanism.

9. Conclusion: Kupffer cells are crucial immune cells for systemic antibacterial defense

The remarkable immunological abilities of Kupffer cells, such as phagocytosis, reactive oxygen species production, and antigen presentation, strongly suggest their enormous contribution to immunological responses. Based on the vascular

architecture of the liver, Kupffer cells have been recognized as playing pivotal roles in eliminating portal vein-derived pathogens from the intestinal tract. However, increasing evidence indicates that they are crucial effectors in systemic defense mechanisms against bacteria, cooperating with other immune cells such as monocytes, neutrophils, and even non-immune such as hepatocytes, and platelets. From this viewpoint, Kupffer cells are phagocytic scavengers and conductors orchestrating the effective elimination of blood-borne bacteria. Thus, Kupffer cells play a crucial role in systemic antibacterial defenses.

Author details

Hiroyuki Nakashima*, Masahiro Nakashima, Manabu Kinoshita and Shuhji Seki
Immunology and Microbiology, National Defense Medical College,
Tokorozawa, Saitama, Japan

*Address all correspondence to: hiro1618@ndmc.ac.jp

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