PD-1 EXPRESSION ON IMMUNE CELLS IN SEPSIS MOUSE MODEL AND THE SUSCEPTIBILITY TO SECONDARY FUNGAL INFECTION

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การแสดงออกของพีดี-1
บนเซลล์ภูมิคุ้มกันในแบบจำลองหนูเมาส์ภาวะพิษเหตุติดเชื้อและความไวต่อการติด เชื้อราทุติยภูมิ

น.ส.โจว เจิง เบ๋า หวู

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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โจว เจิง เบ่า หวู : การแสดงออกของพีดี-1 บนเซลล์ภูมิคุ้มกันในแบบจำลองหนูเมาส์ภาวะพิษเหตุติดเชื้อและความไวต่อการติดเชื้อราทุติยภูมิ. (PD-1 EXPRESSION ON IMMUNE CELLS IN SEPSIS MOUSE MODEL AND THE SUSCEPTIBILITY TO SECONDARY FUNGAL INFECTION) อ.ที่ปรึกษาหลัก : พัชรี ฤทธิ์ประจักษ์, อ.ที่ปรึกษาร่วม : อัษฎาศ์ ลีพหวนิชกุล,อาสา ธรรมหงส์

วัตถุประสงค์: การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อ i) ตรวจวัดการเปลี่ยนแปลงแบบจลน์ของฟีโนไทป์ของเซลล์ภูมิคุ้มกัน โดยเฉพาะการแสดงออก PD-1 ในม้ามของหนูที่มีภาวะพิษเหตุติดเชื้อ ii) ศึกษาความไวของการติดเชื้อราทุติยภูมิหลังจากเกิดภาวะพิษเหตุติดเชื้อ iii) ทดสอบประสิทธิผลของแอนติ-บอดีต่อพีดี-1 ในการรักษาการติดเชื้อราซ้ำซ้อนภายหลังภาวะพิษเหตุติดเชื้อ

วิธีการทดลอง : ในการศึกษานี้ใช้วิธีผูกเจาะลำไส้ใหญ่ส่วนซีกัม (ซีแอลพี) สำหรับแบบจำลองหนูเมาส์ภาวะพิษเหตุติดเชื้อและเก็บม้ามจากหนูเมาส์ดังกล่าวมาตรวจวัดด้วยโฟลวไซโตเมทรี จากนั้นทำการศึกษาการติดเชื้อราทุติยภูมิทางระบบโดยการฉีดเชื้อราแคนดิดา อัลบิแคนส์หรือเชื้อราแอสเปอร์จิลลัสในวันที่ 5 หลังจากทำซีแอลพี นอกจากนี้ทำการทดสอบประสิทธิภาพของแอนติบอดีต่อพีดี-1 ในการรักษาการติดเชื้อราแอสเปอร์จิลลัสแบบทุติภูมิ โดยให้ยาแอมโฟเทอริซินบีร่วมกับการให้แอนติบอดี หรือให้ยาเพียงอย่างเดียว

ผลการทดลอง: ผลการศึกษาพบว่าภายหลังที่หนูเกิดภาวะพิษเหตุติดเชื้อ จำนวนทีเซลล์และบีเซลล์ลดลง แต่ไม่มีผลต่อจำนวนแมโครฝาจ พบการแสดง ออกที่เพิ่มขึ้นของพีดี-1 (โมเลกุลบ่งชี้ภาวะเหนื่อยล้าของภูมิคุ้มกัน) บนทีเซลล์และบีเซลล์ ในวันที่ 5 และวันที่ 12 หลังจากทำซีแอลพี ในขณะเดียวกัน พบว่าการแสดงออกพีดี-1 บนแมโครฝาจเพิ่มขึ้นในวันที่ 1 หลังทำซีแอลพี และลดลงในวันที่ 12 หลังทำซีแอลพี นอกจากนี้จำนวนเซลล์ที่มีการแสดงออกของซีดี86 (โมเลกุลบ่งชี้ภาวการณ์กระตุ้นของแมโครฝาจ) ในกลุ่มของแมโครฝาจ มีการลดลงในวันที่ 5 หลังทำซีแอลพี และเพิ่มขึ้นในวันที่ 12 หลังทำซีแอลพี ซึ่งการเปลี่ยนแปลงของแมโครฝาจนี้บ่งชี้ถึงภาวะเหนื่อยล้าของภูมิคุ้มกันโดยกำเนิด ในระยะแรกของภาวะพิษ เหตุติดเชื้อ (1-5 วันหลังทำซีแอลพี) และมีการสร้างเซลล์แมโครฝาจขึ้นมาทดแทนในระยะหลัง (12 วันหลังทำซีแอลพี) จากผลการทดลองนี้ ดังนั้นจึงฉีดเชื้อราในวันที่ 5 หลังจากทำซีแอลพี ซึ่งในวันที่ 5 นี้ จากการตรวจสอบอัตราการมีชีวิตรอด และการบาดเจ็บของอวัยวะ พบว่าหนูมีความไว ต่อการติดเชื้อราแคนดิดา อัลบิแคนส์ และ แอสเปอร์จิลลัส ฟูมิกาตัส การรักษาการติดเชื้อราแอสเปอร์จิลลัสแบบทุติยภูมิ ด้วยยาต้านเชื้อรา แอมโฟเทอริซินบีไม่ให้ผลดี ในทางตรงกันข้ามการรักษาด้วยยาต้านเชื้อราเสริมด้วยแอนติบอดีต่อพีดี-1 ช่วยลดความรุนแรงของการติดเชื้อ การยับยั้ง การทำงานของพีดี-1 ลดการเกิดภาวะเหนื่อยล้าของภูมิคุ้มกันในม้าม ซึ่งตรวจวัดได้จากการแสดงออกที่เพิ่มขึ้นของซีดี86 ระดับที่เพิ่มขึ้นของอินเตอร์ เฟียรอน-แกมมาในซีรั่ม และการลดลงของอินเตอร์ลิวคิน-10 ในซีรั่ม นอกจากนี้เมื่อนำเซลล์ม้ามจากหนูที่ได้รับการรักษาด้วยแอนติบอดีต่อพีดี-1 มากระตุ้นซ้ำด้วยแอนติบอดีต่อซีดี3 พบว่ามีการผลิตอินเตอร์เฟียรอน-แกมมาเพิ่มขึ้น และมีการลดลดลงของอินเตอร์ลิวคิน-10

สรุปผลการทดลอง: การศึกษานี้ให้ความรู้พื้นฐานที่แสดงถึงภาวะเหนื่อยล้าและการกระตุ้นแมโครฝาจ เป็นตัววัดที่มีนัยสำคัญในการบ่งชี้ความไวต่อ การติดเชื้อราทุติยภูมิการรักษาเสริมด้วยแอนติบอดีต่อพีดี-1 ในหนูเมาส์ที่มีการติดเชื้อราทุติยภูมิน่าจะช่วยฟื้นแอนติเจนพรีเซนติงเซลล์และทีเซลล์จาก ภาวะความเหนื่อยล้าผ่านทางการเพิ่มขึ้นของการแสดงออกของซีดี 86 การเพิ่มขึ้นของการผลิตอินเตอร์เฟียรอน-แกมมา และการลดลงของการผลิตอินเตอร์ลิวคิน-10 ดังนี้การรักษาเสริมด้วยแอนติบอดีต่อพีดี-1 น่าจะมีประโยชน์ในการรักษาการติดเชื้อรารุนแรงด้วยวิธีภูมิคุ้มกันบำบัด

สาขาวิชา	ชีววิทยาช่องปาก	ลายมือชื่อนิสิต .....
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Chau Tran Bao Vu : PD-1 EXPRESSION ON IMMUNE CELLS IN SEPSIS MOUSE MODEL AND THE SUSCEPTIBILITY TO SECONDARY FUNGAL INFECTION. Advisor: Asst. Prof. PATCHAREE RITPRAJAK, Ph.D. Co-advisor: Asst. Prof. ASADA LEELAHAVANICHKUL, Ph.D., ARSA THAMMAHONG, Ph.D.

Objectives: This study aimed to i) determine the kinetic changes of immune cell phenotypes, including an exhaustion marker PD-1, in spleens of murine sepsis ii) investigate the susceptibility against secondary fungal infection after sepsis, and iii) investigate the efficacy of an anti-PD-1 treatment in post-sepsis fungal infection.

Methods: Cecal ligation and puncture (CLP) was used as a sepsis model and splenocytes post-CLP were assessed by flow cytometry. In addition, secondary post sepsis systemic fungal infections by *Candida albicans* or *Aspergillus fumigatus* administration at 5-day post-CLP were performed. Moreover, secondary aspergillosis was treated with Amphotericin B with or without anti-PD-1 to explore anti-PD-1 effectiveness.

Results: T cells and B cells, but not macrophages, in mouse spleens were decreased post-CLP. Increased expression of PD-1 (immune exhaustion marker) on T cell and B cell was demonstrated at 5 and 12 days post-CLP. In parallel, PD-1 expression on macrophage was increased at 1 day post-CLP and decreased at 12 days post-CLP. Meanwhile, the numbers of CD86+ cells (marker of macrophage activation) in macrophage population were decreased at 5 days post-CLP and increased at 12 days post-CLP. These implied early innate immune exhaustion (day 1-5 post-CLP) with the late immune reconstitution (12 days of CLP). Hence, fungi were introduce at 5 days post-CLP. Indeed, higher susceptibility to *C. albicans* and *A. fumigatus* at 5 days post-CLP was demonstrated by survival study and organ injuries, respectively, suggesting an impact of secondary fungal infection post-sepsis. Amphotericin B treatment alone was not effective to treat the CLP-mice with secondary aspergillosis. In contrast, the adjunctive treatment with anti-PD-1 attenuated the disease severity. PD-1 blockade attenuated immune exhaustion in spleens as determined by increased CD86 expression, augmented serum IFN-γ and dampened serum IL-10. In addition, anti-CD3 restimulated splenocytes from anti-PD-1 treated mice highly produced IFN-γ and reduced IL-10 production.

Conclusion: Our study provide fundamental knowledge about macrophage exhaustion and reactivation as a significant determinant for susceptibility to secondary fungal infection. The adjunctive anti-PD-1 treatment in mice with secondary fungal infection presumably reinvigorated exhausted antigen-presenting cells and T cells by upregulating CD86 expression and IFN-g production, diminished IL-10 production, and attenuated disease severity. The adjunctive anti-PD-1 therapy may be expedient for the advanced immunotherapy against lethal fungal infection.

Field of Study:	Oral Biology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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Chapter 1: Introduction

Sepsis is specified as the severe inflammatory response to fatal infection, which results in multi-organ injury and mortality in patients in the intensive care units (ICUs) [1]. Septic patients encountered the early days of hyper-inflammatory response, before undergoing the second period of immunosuppression; meanwhile these two phases could happen immediately after sepsis [1-3]. In response to sepsis, innate immune cells such as neutrophils, dendritic cells, macrophages recognize pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), thus become activated to provide the early defense against infection. Inflammatory mediators secreted by innate immune cells favor endothelial permeability, recruit more immune cells to defend against infection and drive inflammation. Overwhelming inflammation is the main cause of septic shock which results in early death of sepsis [1, 4, 5]. Indeed, “cytokine storm” leads to pathologic manifestations such as low cardiac output, thrombosis, acute renal failure, abnormal metabolism [1, 3, 4, 6]. If the patients survive the early days of sepsis, they would enter the prolonged immunosuppressive phase characterized by the functional failure and apoptosis of immune cell populations. This phenomenon is referred as “immunoparalysis”. Deaths during this phase are due to the inability of the immune system to eliminate primary infection and acquisition of secondary infection by opportunistic pathogens [1, 7, 8].

One mechanism of immune suppression post sepsis is the expression of immune checkpoint molecules, in which the programmed death-1 (PD-1) pathway is the most-characterized. PD-1 is the inhibitory receptor expressed on numerous immune cells as $CD4^+$ T cells, $CD8^+$ T cells, B cells, dendritic cells (DCs) and macrophages [1, 9, 10]. High expression of PD-1 on T cells imposes the phenomenon termed “T cell exhaustion” which is found in infection and cancer. “T cell exhaustion” was described as the decreased effector function of T cells, thus T cells proliferate less, secrete less cytokine and unable to kill infected cells [1, 9, 11].

The two ligands of PD-1 are PD-L1 (B7-H1) and PD-L2 (B7-DC) which are expressed on different cell types. PD-L1 is found on both hematopoietic cells and non-hematopoietic cells such as T lymphocytes, B lymphocytes, macrophages, dendritic cells (DCs), endothelial cells. Meanwhile, the PD-L2 expression is found on limited immune cells as macrophages, dendritic cells, bone marrow derived mast cells [9, 12, 13]. The PD-1-PDL pathway induce phosphatase SHP-2 to dephosphorylate the signaling pathway of T cell receptor (TCR), B cell receptor (BCR), thus inhibit the effector function of T cells and B cells [9, 12].

The relation of PD-1 expression and T cell exhaustion is well-established in chronic infection. For example, in the murine model of viral infection by lymphocytic choriomeningitis virus (LCMV), $CD8^+$ T cells expressed PD-1 and lost the effector function. The inhibition of PD-1-PD-L1 pathway restores the function of $CD8^+$ T cells

and boost anti-viral immune response [14]. In human infected with human immunodeficiency virus (HIV), PD-1 expression on $CD8^+$ T cells correlated with exhaustion such as the reduced proliferation, cytokine secretion and cytotoxicity [15, 16]. In terms of murine bacterial sepsis, PD-1 expression on macrophages induced cellular dysfunction and PD-$1^{-/-}$ mice were resistant to septic mortality [17]. Recent human studies revealed that there were high expression of PD-1 on circulating immune cells and postmortem splenocytes including $CD4^+$ T cells, macrophages, dendritic cells [7, 18].

Our first purpose was to investigate the kinetic change of immune populations as well as activation markers CD86, MHCII and immune checkpoint molecules PD-1 post murine bacterial sepsis. We aimed to evaluate the time point of immune paralysis and figure out the possible role of PD-1 expression on immune population as candidate bio-marker for the susceptibility to secondary fungal infection.

As mentioned earlier, septic patients in immunosuppressive phase are vulnerable to secondary infection by opportunistic organisms [1, 7, 8]. Several reports had revealed the prevelence of aspergillosis in patients in intensive care unit (ICU) [19-21]. Aspergillosis had been found in ICU patients with the underlying conditions as: chronic heart failure, neutropenia, cancer, organ transplant, immunosuppressive drug, sepsis [19].

Aspergillus spp. are the environmental opportunistic fungi. Humans inhale hundreds of *Aspergillus* spp. spores everyday due to its ubiquitous spread in the air. In immunocompetent humans, alveolar macrophages phagocytose the spores, thus *Aspergillus* spp. do not cause disease. Meanwhile, in immunocompromised patients and critically ill patients, *Aspergillus* spp. could germinate into hyphae and cause aspergillosis. *Aspergillus fumigatus* remained to be the main cause of aspergillosis [22-24]. Invasive aspergillosis was defined as the invasion of hyphae into tissue, while allergic aspergillosis was characterized by the colonization of hyphae on the mucosal surface [22-24].

As discussed above, the PD-1-PD-L pathway was responsible for immunoparalysis during chronic infection [1, 9-11]. In fungal infection as murine model of *Histoplasma capsulatum*, macrophages, DCs, T cells from infected mice highly expressed PD-L1. PD-$1^{-/-}$ mice had higher survival rate and less fungi in organs than wild type mice [25]. In another model of murine candidiasis, Chang et al had found that there were high expression of PD-1 on $CD4^+$ T cells, $CD8^+$ T cells. Treatment with anti-PD-1 rescued the function of lymphocytes and ameliorate the survival rate of fungal infected mice [8]. However, several recent reports showed the break-through of anti-PD-1 treatment in tuberculosis and aspergillosis in cancer patients [26]. Hence, controversial outcome of anti-PD-1 therapy on infectious diseases still require further studies. In this research, we investigated the efficacy of

an anti-PD-1 treatment in post-sepsis aspergillosis by observing the mortality, fungal burdens in conjunction with the alteration of the immune status.

Research Questions

1. How does PD-1 expression on innate and adaptive immune cells relate to survival of sepsis and susceptibility to fungal infection?
2. Does treatment with anti-PD-1 rescue immune exhaustion in secondary fungal infection?

Objectives and Hypothesis

1. **Question 1:** How does PD-1 expression on innate and adaptive immune cells relate to survival of sepsis and susceptibility to fungal infection?

Objective 1

To investigate the kinetic expression of immune checkpoint molecule PD-1 on innate and adaptive immune cells in CLP mouse model.

Hypothesis

The expression of immune checkpoint PD-1 on innate and adaptive immune cells in septic mice is different from that in sham mice, which relate to survival of sepsis, susceptibility to secondary fungal infection.

Experimental design

1.1 To determine the relation of PD-1 expression and survival of sepsis, we studied the kinetic expression of PD-1 at day 1, day 5, day 12 post sepsis.

We aimed to study day 1, day 5, day 12 post sepsis due to the reason that these days represent the phases of innate and adaptive response. During day 1 post sepsis, the innate immune cells are activated and hold the main role in defense against pathogens. At day 5, the adaptive immune system begin to be activated and contribute to eliminate bacteria. Day 12 is the effector phase of adaptive immunity. The effector T cells and B cells function at their best to exclude infection. After that,

the lymphocytes die by apoptosis, the immune response decline to maintain homeostasis. The survived antigen specific lymphocytes hold the role of memory [5].

With regards to the survival of sepsis, the murine dead rate was high at the first 4 days after CLP. Mice survive up to day 5 post CLP had higher chance to survive. Mice survive at day 12 are considered recovered [27].

Mice underwent cecal ligation and puncture (CLP) as characterized in previous researches [3, 8, 27-29]. At day 1, day 5, day 12 post sepsis, mice were sacrificed to collect blood and spleens.

a. To determine the expression of PD-1 on innate immune cells post sepsis, splenocytes were stained with fluorochrome-tagged anti-F4/80, anti-CD86, anti-MHCII, anti-PD-1 antibodies. Data were processed and analyzed by flow cytometry. The $F4/80^+$ cells which are macrophages were gated and the expression of CD86, MHCII, PD-1 were determined on $F4/80^+$ gate. CD86, MHCII are the activation markers of macrophages. Shame-operated mice were used as control.

b. To determination the expression of PD-1 on adaptive immune cells post sepsis, splenocytes were stained with fluorochrome-tagged anti-CD3, anti-B220, anti-CD4, anti-CD8, anti-PD-1 antibodies. Data were processed and analyzed by flow cytometry. The $CD3^+$ cells, $B220^+$ cells, $CD4^+$ cells, $CD8^+$ cells which are T cells, B cells, $CD4^+$ T cells, $CD8^+$ T cells respectively were gated and determined the expression of PD-1. Shame-operated mice will be used as control.

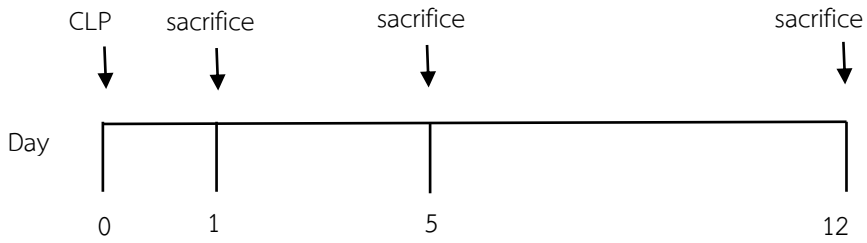


Figure 1.1: Time point of spleen and blood collection after CLP operation

1.2 To investigate and confirm the hyper-inflammatory response at early days of sepsis, we collected blood of mice at day 1, day 5, day 12 post CLP- induced sepsis. Serum was collected from whole blood by centrifugation. Serum cytokine such as TNF-α, IL-6, IFN-γ, IL-10 was determined by ELISA. Shame-operated mice was used as control.

1.3 To determine the relation of PD-1 expression on immune cells post sepsis and susceptibility to secondary fungal infection, we investigated the survival rate of secondary fungal. We supposed that PD-1 could be used as a bio-marker to determine the susceptibility to secondary fungal infection.

Mice underwent cecal ligation and puncture (CLP) as characterized in previous researches [3, 8, 27-29]. At day 5 or day 12 post sepsis, mice was injected intravenously via tail vein with *Candida albicans* SC5314 [30-34]. Mice was observed

within 20 days after fungal infection to obtain the survival rate. Shame-operated mice was injected with *Candida albicans* SC5314 and will be used as control.

2. **Question 2:** Do treatment with PD-1 antagonist rescue immune exhaustion in secondary fungal infection?

Objective 2

To determine immune reactivation after anti-PD-1 treatment in secondary fungal infection.

Hypothesis

Anti-PD-1 treatment in secondary fungal infection induces immune reactivation.

Experimental design

Mice underwent cecal ligation and puncture (CLP) as characterized in previous researches [3, 8, 27-29]. In the earlier experiments, we showed that mice in the immunosuppressive phase at day 5 after CLP-induced sepsis were susceptible to secondary infection (Figure 4.5). At day 12 post CLP- induced sepsis, macrophages started to recover to provide the protective response (Figure 4.3, 4.5). Therefore, we chose day 5 post CLP to study secondary fungal infection by the opportunistic fungi *Aspergillus fumigatus.*

At day 5 post sepsis, mice were injected intravenously via tail vein with *Aspergillus fumigatus* clinical isolate strain. We chose *Aspergillus fumigatus* clinical isolate strain due to the reason that this strain was isolated from lung tissue in patient succumbing to invasive aspergillosis, thus this would be a virulent strain. Mice were given two dose of amphotericin B at dose of 1 mg/kg in 100 μl PBS subcutaneously at 6h and 24h post *Aspergillus* infection to mimic the standard anti-fungal treatment protocol. The treatment group of mice was injected intraperitoneally with anti-PD1 (1 mg/ml for 200 μl) at 6 hour and 24h after secondary fungal infection. At day 7 post CLP-induced sepsis, mice were sacrificed to collect blood, spleen, organs. We aimed to study the effectiveness of treatment with PD-1 antagonist in secondary aspergillosis. Shame-operated mice, CLP-operated mice, CLP-operated mice infected with *Aspergillus fumigatus* were used as control.

a. Experiment to determine the dose of *Aspergillus fumigatus*

To select the proper fungal dose for the infection, the varied number of *A. fumigatus* spores ($1x10^3$, $1x10^4$, and $1x10^6$ spores) were administered into intact mice, a dose-related mortality rate and fungal burden in blood was determined.

b. Experiment to determine the fungal burden in organs and blood after anti-PD-1 treatment

We investigated whether anti-PD-1 theray had the effect in reduction of fungal burden in organ and blood. After centrifugation to collect serum, 100 μl of blood

was plated on Sabouraud Dextrose Agar (SDA) plates at 37^0 C for 72 hours. The brain, lung, kidney were minced in PBS and plated on SDA plates at 37^0 C for 48 hours. Total colony was counted to determine colony forming unit (CFU).

c. Experiment to determine histology after anti-PD-1 treatment

To determine whether anti-PD-1 therapy ameliorate organ damage, kidney, brain, lung were collected and fixed in 10% formalin, sectioned and stained with Gomori Methenamine-Silver Nitrate (GMS) Stain [35, 36].

d. Experiment to determine change of serum creatinine after anti-PD-1 treatment

The raise of serum creatinine is marker of kidney injury [3]. Serum creatinine was quantitated by The QuantiChrom Creatinine Assay kit (BioAssay Systems, CA, USA).

e. Experiment to determine the change of serum cytokine after anti-PD-1 treatment

We would determine the change of serum cytokine to evaluate the effect of anti-PD-1 therapy. Serum cytokines such as IL-6, IL-17, IFN-**γ,** IL-10 was determined by ELISA.

f. Experiment to determine innate immune reactivation after anti-PD-1 treatment

We would investigate the effect anti-PD-1 therapy on innate immune cells. CD86, MHCII are the activation marker expressed on antigen presenting cells. Splenocytes was stained with fluorochrome-tagged anti-CD86, anti-MHCII, anti-PD1 antibodies. The expression of CD86, MHCII, PD-1 on splenocytes was analyzed by flow cytometry.

g. Experiment to determine T cell function after anti-PD-1 treatment

Anti-CD3 is a reagent that provide signal to T cell receptor (TCR), thus activate the effector T cells. The innate T lymphocytes are not activated because of the lack of costimulatory signal to CD28. Splenocytes were stimulated by plate-coated anti-CD3 for 24 hours. The supernatant was collected to measure the level of cytokine IFN-γ, IL-17, IL-10 by ELISA.

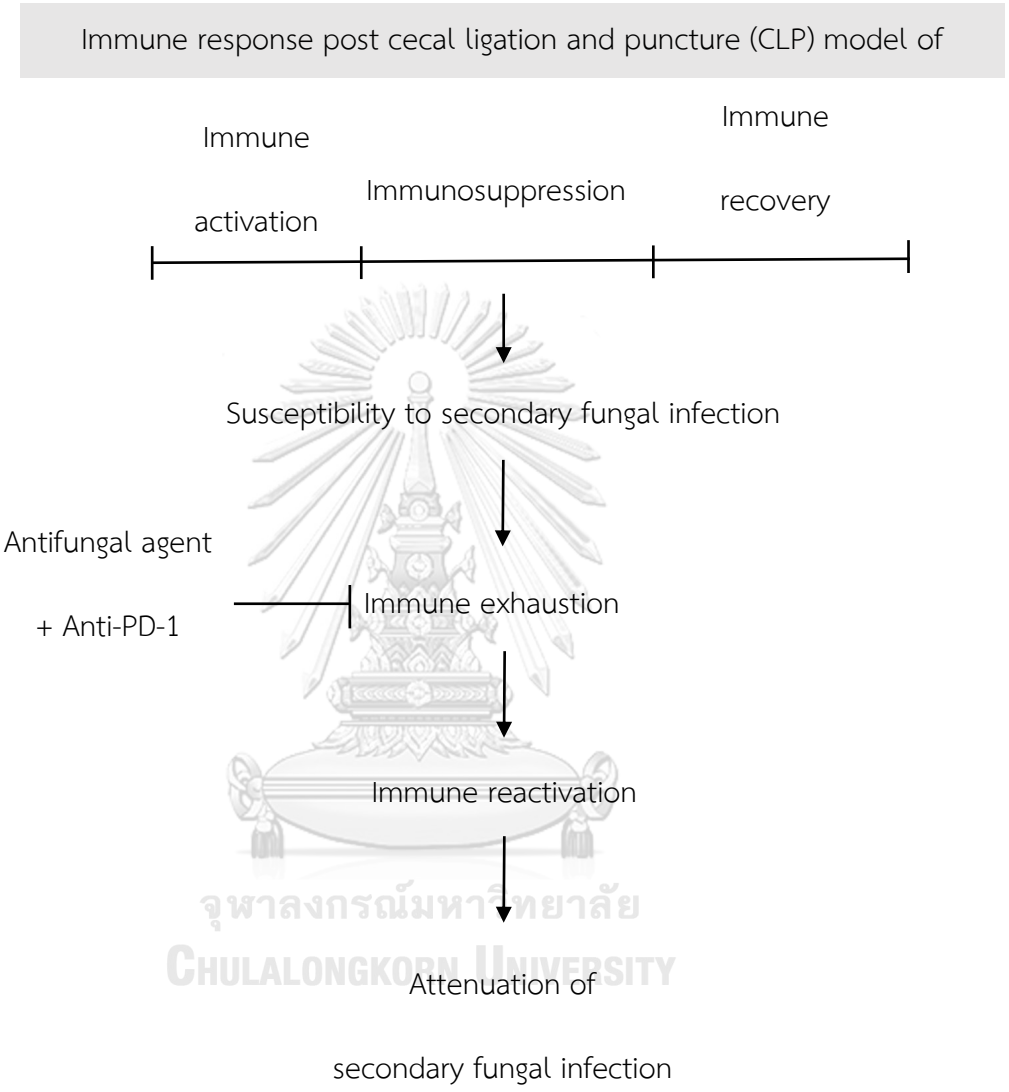
Benefits of the study

Our findings are supposed to provide profound insight about immune exhaustion, immune recovery post sepsis and immunotherapies against secondary nosocomial fungal infection. We demonstrated that the kinetic changes of innate immune phenotypes may be relevant to susceptibility to secondary fungal infection. In addition, the beneficial effect of anti-PD-1 treatment could be applied as a new candidate of immunotherapy against lethal fungal infection

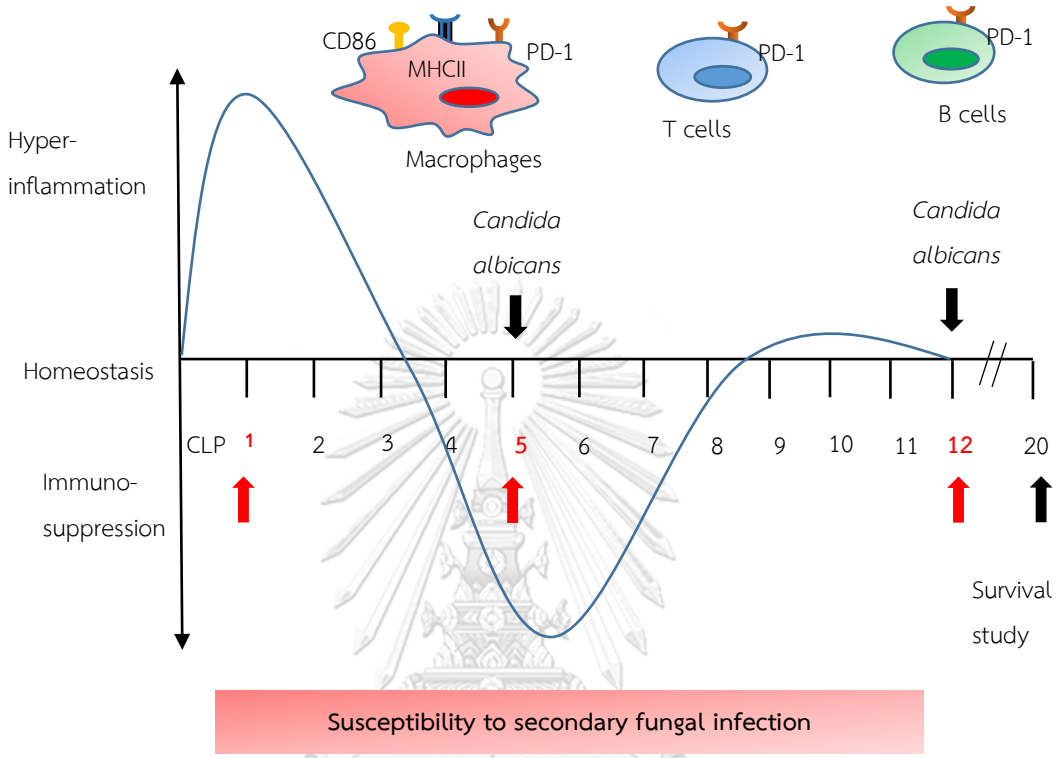
Research design

Laboratory experimental research

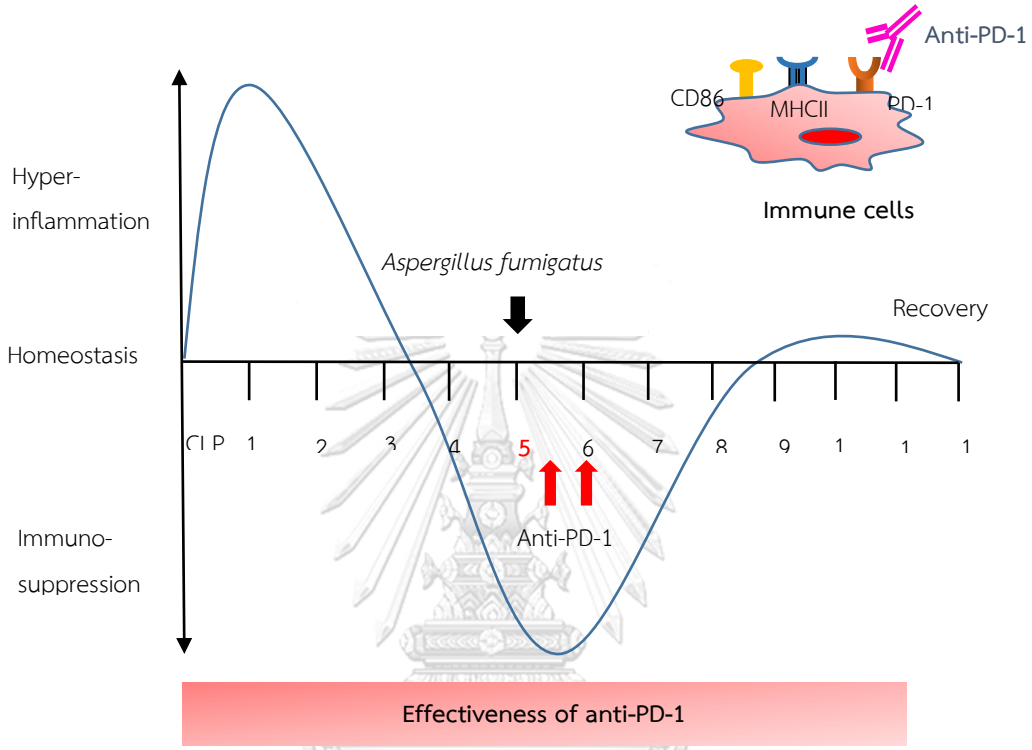
Conceptual framework



Experimental design 1



Experimental design 2



Chapter 2: Literature Review

2.1 Septic Pathology and Immune response against sepsis

Sepsis is referred as systemic inflammatory response to severe infection, which causes multiple organ failure [1]. The host response against sepsis could be described by the early phase of systemic inflammatory responses (SIRS) and late phase of immunosuppression, although these two phases could be overlapped and occur simultaneously [1-3]. During the onset of sepsis, innate phagocytes have pattern recognition receptors (PRRs) to recognize bacterial substances, thus the cytosolic receptor domains are phosphorylated and several signaling pathways are processed. This transcription factor NF-kB are is thus activated and promotes the expression of several inflammatory cytokines (including TNF, IL-6, IL-1, IL-12, IL-8), chemokines (including CC-chemokine ligand 2 (CCL2), CXC-chemokine ligand 8 (CXCL8), CXCL10), adhesion molecules (including E-selectin), and costimulatory ligands (including CD80, CD86) [5]. These inflammatory molecules boost the vascular permeability, favoring the accumulation of innate immune cells, inducing acute inflammation and stimulating adaptive immune response [5].

Acute inflammation is the mechanism of innate response to microbes but also the main cause of septic multisystem organ failure (MSOF) and clinical manifestation. Inflammatory cytokines not only have local effect but also have systemic protective effects and systemic pathologic effects [5].

2.1.1 Systemic protective effect of inflammatory cytokines

Some inflammatory cytokines as TNF-α, IL-6, IL-1β, which could be called endogenous pyrogenes in this circumstance, may exert the effect on hypothalamus, inducing production of prostaglandin by hypothalamic cells [37]. Prostaglandin is the main mediator which boost an increase in body temperature. The host temperature from equal to higher 38.3^0C is defined as fever [38]. A number of studies had reported the beneficial evidences of fever to the host. Ozveri et al demonstrated that hyperthermia at 42^0C for 15 minutes boost immune response by rescuing the deletion of $CD4^+$ T cells, B cells, resulting in higher survival rate in model of rat peritonitis [39]. With regards to clinical evidence, several researchers had found that hyperthermia resulted in higher survival rate of patients, in comparison with hypothermia [40, 41].

On the other hand, some interleukins as IL-1β, TNF-α, IL-6 boost the synthesis of some acute-phase reactants by hepatocytes such as C-reactive protein (CRP) and serum amyloid P (SAP) [42]. These plasma proteins are also called pentraxins base on their structure. CRP have the ability to recognize and bind to phosphocholine on microbial pathogens as well as phospholipids on damaged cells. Another pentraxins is SAP which can bind to phosphotidylethanolamine expressed on bacteria [43]. After binding to target cells, CRP and SAP induce the activation of complement system to eliminate these cells [42, 43].

2.1.2 Systemic pathologic effects of inflammatory cytokines

The large amount of inflammatory cytokines is the leading cause of septic pathology. In 1996, Kumar clarified that both TNF-α and IL-1β inhibited the contractile activity of myocardial cells, which result in septic hypotension [44]. In addition, Vincent et al had conducted a pilot study showing that treatment with anti-TNF-α antibody rescued the ventricular function in ten patients [45].

In addition, vascular endothelium is also a target of inflammatory cytokines. Healthy endothelium is an anticoagulant surface holding the function to maintain blood flow and inhibit thrombosis. Endothelium integrity is sustained by intracellular cytoskeleton and intercellular adhesion molecules. Thus endothelium help to evade displaying of collagen fibers and tissue factors to circulating von Willebrand factor, thus impeding the initiation of clotting. In septic circumstances, inflammatory cytokines rise the expression of adhesion molecules to induce neutrophil trafficking. The increased permeability of blood vessels and migration of white blood cells lead to the uncovering of tissue factor and collagen fibers to circulating von Willebrand factor and factor VII. Thus the clotting cascade and thrombosis is initiated [4, 46].

Additionally, the prolonged high amount of TNF-α in muscle impaired the muscular oxidative metabolism but enhanced glycolysis and lipolysis [47]. Moreover, TNF-α diminished tyrosine phosphorylation and expression of insulin receptor, resulting in septic insulin resistance [48]. The elevated muscular glycolysis and insulin

resistance are the leading cause of hyperglycemia in sepsis. Hyperglycemia had been considered as a marker of poor prognosis due to its detrimental effects on many organs [49]. In 2006, Van Den Berghe reported that insulin therapy provide the beneficial outcome to patients in intensive care units (ICUs) [50].

2.2 The animal model of sepsis

As described earlier, sepsis is a complex systemic infection which induces pro-inflammatory response to exerting many adverse effects on numerous vital organs [1, 4]. Thus the animal models are required for a reproducible system to study the septic pathophysiology, host immune response and drug therapy [3, 51]. Scientists had developed three categories of septic animal models: application of exogenous toxin (injection of lipopolysaccharide (LPS)), application of exogenous viable pathogens (injection of bacteria), modification of the endogenous protective barrier (induction of intestinal permeability as cecal ligation and puncture (CLP) or colon ascendens stent peritonitis (CASP)) [3, 51]. The gold standard of animal models is the one that reproduces and mimics the best human pathophysiology and host immune response, thus being the most appropriate to study therapeutic interventions [3, 51].

2.2.1 Application of lipopolysaccharide (LPS)

The method of administration of LPS is simple to perform and could induce the rapid increase of inflammatory cytokine in mice. However, these systemic mediators peaked only at 4 post after LPS inoculation and began to decline at 8 hours, thus

could not reproduce the sustained elevation of cytokines in human sepsis [52]. Moreover, the treatment of monoclonal antibodies against TNF-α enhanced survival rate in mice subjected to LPS injection but could only showed a modest reduction in human mortality [53, 54]. Additionally, mice injected with LPS suffered from immediate hypotension with low cardiac output but did not undergo the hemodynamic changes as human sepsis [3, 51].

2.2.2 Application of viable bacteria

This method allows researchers to study the specific pathophysiological response to a single pathogen. Several routes of bacterial infusion are via intraperitoneal, intravenous or pulmonary inoculation [51]. Each entrance provides different characteristics of host response and manifestation. Peritoneal and lung infection often induce inflammation and pathology to the local infected sites [51, 55, 56]. On the other hand, intravenous bacterial infection generate the systemic infection which boost high induction of pro-inflammatory cytokines, leading to injury of multiorgans [51, 55]. For example, Mizrachi-Nebenzahl demonstrated that mice infected intranasally with *Streptococcus pneumoniae* showed lung bacterial load, bacteremia, lung pathology and expression of inflammatory cytokines in lung [57]. Thus this model could be one choice to study pneumonia induced sepsis.

2.2.3 Modification of the endogenous protective barrier

Modification of the endogenous protective barrier refers to procedures generating leakage of the intestinal tract to release the fecal content into peritoneum and induce septic peritonitis. These procedures compose of the colon ascendens stent peritonitis (CASP) as well as cecal ligation and puncture (CLP).

2.2.3.1 Colon ascendens stent peritonitis (CASP)

This method is performed by surgically implanting of a stent to the colon ascendens of animals to induce the leakage of fecal materials. The septic severity could be adjusted by the diameter of stent [58]. Additionally, the stent could be remove to eradicate the infectious locus, thus mimicking some septic stages of patients after medical intervention [58]. This technique could induce bacteremia and systemic inflammatory response with higher amount than CLP [59] . One drawback of CASP is it requires challenging technique and budget [51].

2.2.3.2 Cecal ligation and puncture (CLP)

CLP was considered the gold standard of sepsis model due to its simplicity and capacity to mimic human sepsis [3, 51]. This procedure is manipulated by ligation of cecum and puncture with needle to release some fecal content and cause sepsis [51]. The severity of sepsis could be modified by the length of ligated cecum, size of needle and number of puncture [3, 51, 60]. Fluid and antibiotics are frequently supplemented to keep animal survival longer and mimic the clinical

intervention [3, 60]. CLP could induce bacteremia with numerous species of bacteria as well as the systemic inflammatory response with multiorgan injury, which mimic the human early phase of human sepsis [3, 51, 61, 62]. Moreover, CLP also cause phagocyte apoptosis which represent the immunosuppression phase of clinical sepsis [1, 3, 51]. In addition, CLP model could mimic the hemodynamic change and metabolic phase of human sepsis [51]. These advantages made CLP the gold standard to study the pathophysiological change, immune response and therapeutic intervention in sepsis [3, 51].

2.3 Immune Response to Fungal Infection

During homeostasis, healthy mycobiota reside on all human barrier surfaces. These fungal residents are diverse and outcompete pathogens for nutrients and niche, thus maintaining the metabolic mutualism with the host [63, 64]. Under dysbiosis triggered by environmental or genetic factors, the balanced mycobiota would be perturbed, which favors the overgrowth of opportunistic pathogens [63, 64]. Several opportunistic fungi such as *Candida spp.* or *Aspergillus spp.* could breach the epithelial barrier and cause infection when the host have medical intervention, neutropenia, immunodeficient state or HIV-mediated immunodeficiency [65]. In terms of fungal infection, innate immune cells use PRRs such as C-type lectin receptors (CLRs) or Toll-like receptors (TLRs) to recognize the fungal PAMPs. After that, the intracellular signaling pathways would be triggered to induce phagocytosis, secreting

of cytokines and chemokines, promoting inflammation and stimulating adaptive immune response [65-67].

2.3.1 Immune Response to *Candida spp.* infection

Candida spp. are the common commensal fungi which reside on human barrier surfaces as skin and mucosa. Under several immuno-compromised conditions as sepsis, HIV, cancer treatment or medical transplantation, the host become more susceptible to opportunistic candidiasis. When the fungi breach the barrier to cause infection, the polysaccharides on the cell walls would be recognized by different PRRs expressed on innate immune cells [68]. CLRs are crucial to interact with fungal polysaccharides. Dectin-1 recognizes β-glucans, whereas mannan would be recognized by dectin-2, mannose receptor (MR), galactin 3. Unopsonized *Candida* is caught by complement receptor 3 (CR3) and opsonized *Candida* is caught by Fc receptors for IgG (FcγRs). Engagement of dectin-1, dectin-2 and FcγRs induce activation of enzyme spleen tyrosine kinase (SYK) and SYK-dependent signaling pathway [65, 67, 68]. Dectin-1 had been shown to interact with TLR2, thus amplifying the anti-fungal response. In sum, these receptor-engagements and signaling pathways result in secretion of cytokines, chemokines, induce phagocytosis of fungi and activate adaptive immunity [65, 67, 68].

Effector T cell response is important for defense against *Candida spp*. T cell related cytokines provide the protective roles against candidiasis [67, 68]. IFN-γ

treatment had been showed to ameliorate morbidity in murine systemic infection to *Candida albicans* [69]. In 2014, Delsing et al. reported a case series demonstrating that IFN-γ treatment restore immune functions in patients succumbed invasive candidiasis [70]. In line with T_H1 cells, T_H17 cells hold the important role to fight against *Candida spp*. IL-17 receptor-deficient (IL-17RA$^{-/-}$) mice showed high susceptibility to systemic candidiasis, as seen by increased mortality and fungal burden in kidneys [71, 72]. In addition, Conti showed that T_H17 deficient (IL-23p19$^{-/-}$) mice, IL-17RA$^{-/-}$ mice suffered more severe oral infection with *Candida albicans* , thus illustrating the critical role of T_H17 in protection against mucosal candidiasis [73]. In terms of human with inborn defect in IL-17 pathway, they encountered severe mucosal candidiasis but not invasive candidiasis. This suggested the beneficial role of T_H17 in human mucosal anti-fungal immune response [74, 75].

2.3.2 Immune response to *Aspergillus spp.* infection

Aspergillus spp. are the common fungi which reside in soil and decaying biomass [23, 76]. *Aspergillus spp.* are spread ubiquitously in the air at the concentration 0.2 – 15 spores/ m^3 air [77], thus we inhales many spores every day. Meanwhile, immunocompetent human do not encounter aspergillosis due to the ability of innate immune system in host defense [22, 78-80]. Immunocompromised patients are more susceptible to aspergillosis which range into three categories: hypersensitivity disease, allergic aspergillosis, invasive aspergillosis [22].

Upon inhalation of *Aspergillus* spores, the mucous layer and epithelial cilia of respiratory tract function to remove the spores [22]. Meanwhile, some small spores which could arrive the air space lumen are caught by innate immune cells [22]. In immunocompetent host, *Aspergillus* spores are phagocytosed by alveolar macrophages without initiation of inflammatory response. In immunocompromised patients, the spores could geminate to form hyphae which induce recruitment of leukocytes, activation of adaptive immune cells to defend against the fungi [22, 24, 81]. Innate immune cells such as macrophages, neutrophils, dendritic cells (DCs) use PRRs to recognize PAMPs on the cell wall of *Aspergillus spp.* Dectin-1 recognize β-glucan, whereas dectin-2 recognize galactomannan. Meanwhile ligands of other PRRs which recognize *Aspergillus spp.* remain unknown [23, 24, 76, 79]. Upon recognition of *Aspergillus spp.* by PRRs, the signaling cascade is induced, innate immune cells secrete pro-inflammatory cytokine, which leads to activation of adaptive immune system [23, 24, 76, 79]. Dendritic cells which recognize *Aspergillus* spores migrate to lymphoid organ, present antigen via MHCII molecule and activate T cell. T_H1 provide the protective role against Aspergillus, as mice induced T_H1 priming showed resistance to aspergillosis [79, 82].

2.4 The function and regulation of programmed cell death 1 (PD-1) in sepsis

The programmed cell death-1 (PD-1) is immune checkpoint molecule, which refers as inhibitory receptors expressed on surface of immune cells such as T lymphocytes, B lymphocytes, macrophages, dendritic cells (DCs). The PD-1 pathway

holds the balance of immune response and tolerance in infection [9]. In the onset of sepsis, upon recognition of pathogens, activated APCs present antigen to naïve T cells via MHC-II molecule within lymphoid organs. At the same times, APCs expressed several costimulatory ligands to bind to the costimulatory receptor CD28 expressed on T cells. In sepsis, the prolonged duration and persistent antigen stimulation lead to the phenomenon described as “T cell exhaustion”. The exhausted T cells proliferate less, secrete less cytokine, have less cytotoxicity and highly express PD-1 [1, 9, 11].

The ligands of PD-1 are PD-L1 (B7-H1) and PD-L2 (B7-DC) which are expressed on different cell types. PD-L1 is expressed on hematopoietic cells [9, 13, 83-85] and non-hematopoietic cells [9, 12, 13]. Meanwhile, PD-L2 is expressed on limited hematopoietic cells as macrophages, dendritic cells, bone marrow-derived mast cells [9, 12, 13, 85]. Upon engagement of PD-1 and its ligands, the inhibitory cascade is initiated to dephosphorylate T cell receptor (TCR), suppress kinase Akt and glucose metabolism [86]. Several studies from septic patients had shown that there was high expression of PD-1, PD-L1 and PD-L2 in the circulating immune cells [18] and in splenocytes collected rapidly from mortal patients in the ICU [7]. The inhibition of PD-1-PD-L pathway had ameliorated murine mortality and mobility [10, 17, 87].

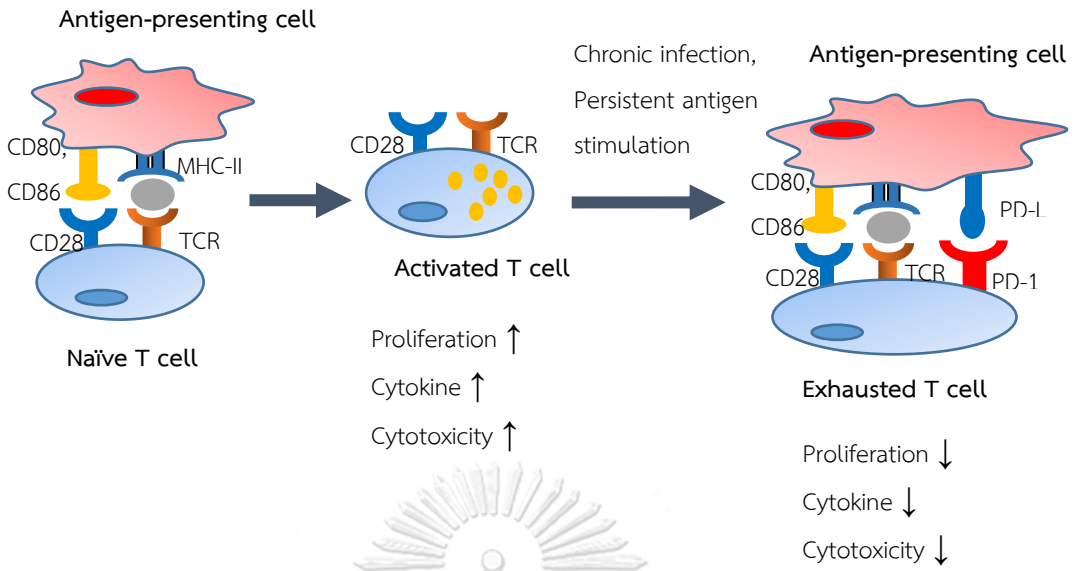


Figure 2.1: PD-1-PD-L pathway

Chapter 3: Materials and Methods

3.1. Mice

The protocol based upon National Institutes of Health (NIH), USA was revised and approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (protocol number SST 031/2561). Eight to ten-week old C57/BL6 female mice were purchased from the National Laboratory Animal Center of Mahidol University, Bangkok, Thailand and Nomura Siam International, Bangkok, Thailand. Animals were maintained in pathogen-free cages, under standard light cycle 12h-light/ 12h-dark, at 25 ± 2^0C, free allowance to food and water at Animal Center, Faculty of Medicine, Chulalongkorn University.

3.2. Antibodies for Flow Cytometry

The fluorescently tagged antibodies included: αF4/80- FITC (BM8), αCD86- PE (GL1), αMHCII- PC5.5 (M5/114.15.2), αPD1- APC/Cy7 (29F.1A12), αCD3- PerCP/Cy5.5 (145-2C11), αCD4- PE (RM4-5), αCD8- FITC (53-6.7), αB220- APC (RA3-6B2) from Biolegend (San Diego, CA, USA).

3.3. Cecal ligation and puncture (CLP) model

Cecal ligation and puncture (CLP) was operated to induce septic peritonitis in female C57BL/6 mice as described in previous researches [3, 8, 27-29]. In briefs, mice were anesthetized under isoflurance. The skin was cut to expose the abdominal

musculature which was cut to get access into peritoneum. After that, cecum was identified, exteriorized, ligated by 2.0 plastic suture, punctured by 21 gauze needle. The punctured cecum was compressed lightly between fingers to release a small amount of feces before being replaced into peritoneum, in order to induce septic peritonitis. The musculature and skin was closed at two layers respectively by 2.0 plastic suture. Mice received antibiotics treatment subcutaneously with Primaxin IV (Merck, Whitehouse Station, NJ, USA) at dose of 2.5 mg/day everyday post CLP operation, prior to secondary fungal infection.

3.4. Secondary candidiasis model

A standard strain *Candida albicans* SC5314 [30-34] was stored in 15% glycerol in -80^{0}C. Prior to murine infection, *Candida albicans* was plated on Yeast Extract Peptone Dextrose (YPD) plates (HIMEDIA, USA). The plates were cultured at 30^0 C for 48 hours to allow the growth of *Candida albicans*. The fungal cells then were harvested in PBS, washed, counted and resuspended in PBS with cellular concentration 10^6 cells/ml. At day 5 and day 12 post septic peritonitis, mice was anesthetized under isoflurance (Piramal Critical Care; PA, USA) and be inoculated intravenously with 10^5 cells of *Candida albicans* suspension.

3.5. Secondary aspergillosis model

Aspergillus fumigatus was isolated from a patient (Mycology Unit, King Chulalongkorn Memorial Hospital). Fungi was identified by morphology with Lactophenol cotton blue preparation and stored in 10% glycerol in -80°C. The Ethical Institutional Review Board, faculty of Medicine, Chulalongkorn University approved the sample accession process according to the declaration of Helsinki. The same strain of *Aspergillus fumigatus* was used in all experiments.

For the murine injection, *Aspergillus fumigatus* was sub-cultured on Sabouraud dextrose agar (SDA; BD Difco™, Sparks, MD, USA) at 37°C for 72 h and the spores were harvested in PBS/0.05% Tween 80, washed, counted and re-suspended in PBS at cellular concentration of 10^7 spores/ml. At day 5 post CLP, anesthetized mice was inoculated intravenously with 10^6 spores of *Aspergillus fumigatus* suspension. Amphotericin B (Amphotret, Maharashta, India) at the dose of 1 mg/kg in 100 μL PBS and anti-PD-1 monoclonal antibody (mAb) (clone RMP1-4) (200 ug in 200 μL PBS) were administered subcutaneously and intraperitoneally, respectively. The mouse blood and internal organs were collected at the end of experiment.

3.6. Determination of PD-1 expression on splenic immune cells

At the appropriate time point, mice will be sacrificed under overdose of isoflurane (Piramal Critical Care; PA, USA) to collect spleens. The collected spleens was minced with the plunger end of 3 ml-syringe through 0.7 μm cell strainers (Gibthai, Huay Kwang, Bangkok, Thailand) into the sterile plate containing 5 ml RPMI

1640 medium supplemented with 10% fetal bovine serum (FBS), 0.2 mM Glutamax, penicillin (100 U/mL) and streptomycin (100 μg/mL), and 50 μM 2-Mercaptoethanol (GIBCO, ThermoFisher Scientific, NY, USA). The cell suspensions were transferred to 15-ml tubes, then centrifuged at 300g in 5 minutes at 4^0C. Red blood cells was lyzed using 1X lysis buffer (GIBCO, ThermoFisher Scientific, NY, USA) for 1 minutes before 9 ml complete media was added for stopping the lytic reaction. After that, the splenocyte suspensions were filtrated through cell strainers. The cell suspensions were washed one more time by centrifugation at 300g in 5 minutes at 4^0C. The supernatant was removed and the cell pellets was resuspended in 10 ml completed media, filtrated through cell strainers. After these steps of preparation, we obtained the splenocyte suspensions without contamination of red blood cell and debris.

Splenocytes were then counted and resuspended at concentration $3x10^6$ cells/ml in Eppendorf tubes, incubated with 100 μl anti-CD16/32, 10 minutes, in dark, at 4^0C to block the Fc receptors on cells, in order to avoid unspecific binding of fluorochrome-conjugated antibodies. After this step, cells will be washed with FACS buffer (PBS contained 0.1 % sodium azide, 1% FBS) and stained with 100 μl fluorochrome-conjugated antibodies to F4/80 (BM8), CD86 (GL1), MHCII (M5/114.15.2), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), B220 (RA3-6B2), PD1 (29F.1A12), incubated 20 minutes in dark at 4^0C. Cells were washed with FACS buffer, resuspended in fixation buffer (PBS contained 4% paraformaldehyde), kept at 4^0C overnight. The next day, splenocyte suspensions were centrifuged at 300g in 5 minutes at 4^0C to remove

fixation buffer, resuspened in 200 μl FACS buffer and processed to flow cytometric operation on CytoFLEX (Beckman Coulter, CA, USA). Data was analyzed by Kaluza Software (Beckman Coulter, CA, USA). $F4/80^+$ macrophages, $CD8^+$ T cells, $CD4^+$ T cells, B cells were gated to determine the expression of PD-1.

3.7. Determination of serum creatinine

Mice were anesthetized under isoflurane, cardiac puncture technique was used to collect murine blood as previously described [88, 89]. Briefly, the skin was cut, the musculature was opened, 3ml syringe with 21-gauze needle was used to withdraw blood from heart. Blood was transferred to Eppendorf tube. To collect serum, blood was centrifuged at 10,000 RPM within 10 minutes, room temperature. The supernatant which was serum was transferred to another Eppendorf tube. For the purity of serum collection, serum was centrifuged a second time at 10,000 RPM, 10 minutes, room temperature. The supernatant was collected and transferred to a new Eppendorf tube, thus collected serum was pure and not contaminated by red blood cells. The serum was stored at -80^0C until assay.

The serum creatinine was quantitated by using QuantiChrom Creatinine Assay (BioAssay Systems, CA, USA). Briefly, 30 μl of diluted standard and samples were added into 96-well plate. To prepare working reagent, 100 μl Reagent A was mixed with 100 μl Reagent B. After that, 200 μl of working reagent was added to wells.

Optical density (OD) was measured immediately (OD_0) and at 5 minutes (OD_5) at absorbance 510 nm. Concentration of creatinine was determined as

$$\frac{OD_{\text{sample 5}} - OD_{\text{sample 0}}}{OD_{\text{STD 5}} - OD_{\text{STD 0}}} \times [\text{STD}]\ 2\ \text{mg/dL}$$

3.8. Determination of serum cytokine

Murine blood and serum were collected and stored at -80^{0}C as described above for determination of serum creatinine. The concentration of cytokine TNF-α , IL-6, IFN-γ, IL-10, IL-17 from serum was quantified by using ELISA set from Biolegend (San Diego, Ca, USA). Briefly, one day before the assay, capture antibody was diluted to 1X in coating buffer and added to 96-well plates. After that, the plates were incubated at 4^{0}C overnight to allow the binding of capture antibody to the bottom of wells. On day of assay, the plates were washed 4 times by 200 μl washing buffer (PBS with 0.05% Tween-20) to remove the unbound antibody. Assay diluent was added to wells to block unspecific binding and plates were incubated for one hour at room temperature. Next, plate were washed 4 times with 200 μl washing buffer. Standard serial dilution and diluted samples were added to plates. Next, plates were incubated two hours at room temperature to allow the binding to cytokines in samples to capture antibodies. Next, plates were washed before adding the detection antibody and incubating at room temperature for one hour, to allow the

binding of detection antibody to cytokines. The plates were washed and the enzyme Avidin-HRP was added. Plates were incubated 30 minutes at room temperature to allow the binding of enzymes. After that, plates were washed and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added and plates were incubated at room temperature about 15 minutes until the positive wells acquired the desired blue color. Last step, stop solution of 2N sulfuric acid was quickly added to allow blue color turning into yellow. The absorbance was read at 450 nm by using Microplate Spectrophotometer (BioTek Instruments, VT, USA).

3.9. Organ histology

At 2 days after aspergillus infection, the mice were sacrifice by isoflurane (Piramal Critical Care; PA, USA) and brain, kidney, lung were collected, keep in complete RPMI at 4^0C. After that, the organs were washed with PBS and fixed in 10% formalin and stained as described previously [90]. In brief, the tissues were sectioned to 4 μm thickness, fixed in paraffin. After that, sections were deparaffined in xylene, rehydrated by ethanol. Then sections were stained with Gomori Methenamine-Silver Nitrate Stain (GMS).

3.10. Determination of fungal burden in organs

As described previously for organ histology, brain, kidney, lung were collected at time of sacrifice, kept in complete RPMI at 4^0C. Organs were minced in 1ml PBS by

the plunger end of 3 ml-syringe and plated in SDA plates, incubated for 48 hours at 37^0C. Total colony of *Aspergillus fumigatus* was counted to determine fungal burden in organs.

3.11. Determination of fungal burden in blood

Blood was withdrawn by cardiac puncture technique at time of sacrificing mice as described above. Serum was collected by centrifugation to determine the amount of creatinine and cytokine as described above. 100 μl of blood pellet was plated in SDA plates, incubated at 37^0C for 48 hours. Fungal burden was determined by the total count of colony of *Aspergillus fumigatus*.

3.12. The *in vitro* re-stimulation assay

The *in vitro* anti-CD3 re-stimulation of splenocyte was performed as described in previous researches [8, 28]. Briefly, splenocytes were collected from the individual spleen, and cells were re-suspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 0.2 mM Glutamax, penicillin (100 U/mL) and streptomycin (100 μg/mL), and 50 μM 2-Mercaptoethanol (GIBCO, ThermoFisher Scientific, NY, USA) as described above. Purified anti-CD3 monoclonal antibody at the concentration of 1 mg/mL (Biolegend) were immobilized in 24-well plates for 18 h, and then the wells were wash twice with RPMI medium. The splenocytes at $3x10^6$ cells in 1 ml medium were seeded in anti-CD3 antibody pre-coated plates, and cultured for 24 hours at

humidified atmosphere with 5% CO2 at 37 °C. After culture, the supernatant was collected and the cytokines were analyzed by ELISA (Biolegend). The level of cytokine IFN-γ, IL-17, IL-10 in supernatant was quantified by using ELISA set from Biolegend (San Diego, Ca, USA) as described above.

3.13. Data analysis

Statistical analysis was operated using SPSS 23.0 (SPSS, Chicago, IL) and Prism 7.0 (GraphPad, San Diego, CA, USA). Data were showed as mean ± standard error (mean ± SEM). Differences between two groups and multiple groups were analyzed by two-tailed Student's *t*-test and One-way ANOVA with Bonferroni's post hoc analysis, respectively. Survival studies were analyzed by log-rank test. The differences at $P < 0.05$ were accepted as statistical significance.

3.14. Ethical consideration

Female C57BL/6 mice from eight to ten week-old, were purchased from the National Laboratory Animal Center (NLAC) at Mahidol University, Bangkok, Thailand. Mice were house at Animal Center, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Animal protocol was revised and approved by the Institute Animal Care and Use Committee (IACUC) of Faculty of Medicine, Chulalongkorn University, protocol number SST 031/2561.

Details of animal usage

Experimental groups	**Number of mice**
1) Titration of the number of yeast cells of *Candida albicans* SC5314 Number of yeast: 10^4 cells, 10^5 cells, 10^6 cells	5 mice/group x 3 groups = 15 mice
2) Titration of the number of *Aspergillus fumigatus* clinical isolate strain Number of spores: 10^3 cells, 10^4 cells, 10^6 cells, 10^7 cells	5 mice/group x 4 groups = 20 mice
3) Investigation of PD-1 expression on immune cells and serum cytokine at day 1 post sepsis Experimental groups - Sham - CLP- induced sepsis	5 mice/group x 2 groups = 10 mice
4) Investigation of PD-1 expression on immune cells and serum cytokine at day 5 post sepsis Experimental groups - Sham	5 mice/group x 2 groups = 10 mice

- CLP- induced sepsis	
5) Investigation of PD-1 expression on immune cells and serum cytokine at day 12 post sepsis Experimental groups - Sham - CLP- induced sepsis	5 mice/group x 2 groups = 10 mice
6) Investigation the survival rate of secondary candidiasis post sepsis. Experimental groups - Sham - Post CLP-induced sepsis at day 5 - Post CLP-induced sepsis at day 12	10 mice/group x 3 groups = 30 mice
7) Investigation the effect of treatment with anti-PD1 in secondary aspergillosis. Experimental groups - Sham - CLP- induced sepsis - CLP- induced sepsis and secondary aspergillosis - CLP- induced sepsis and secondary aspergillosis treated	5 mice/group x 4 groups = 20 mice

with anti-PD1 antibody	
8) Investigation the effect of treatment with anti-PD1 in survival rate Experimental groups - CLP- induced sepsis and secondary aspergillosis - CLP- induced sepsis and secondary aspergillosis treated with anti-PD1 antibody	10 mice/group x 2 groups = 20 mice
9) Total mice used	About 150 mice, mice survive 70% after CLP operation

Chapter 4: Result

Innate and adaptive immune response phase in CLP mouse model

To explore the phase of immune response following sepsis, several parameters were determined at day 1, day 5, day 12 post-CLP. At day 1 post-CLP, the serum innate pro-inflammatory cytokines, TNF-α and IL-6, were markedly upregulated, which defined the hyper-inflammatory state (Figure 4.1A-B). The increased serum IL-10 production of was also observed at day 1 post-CLP (Figure 4.1C). The levels of serum IL-6, TNF-α, IL-10 declined to the baseline level (equal to those of the sham control) from day 5 to 12 post-CLP (Figure 4.1A-C). Conversely, IFN-γ, an adaptive cytokine [91], was notably increased at day 5 and partially decreased at day 12 (Figure 4.1D). These data implied that the early phase of sepsis (1 day post-CLP) was an innate immune response with hyper-inflammation and the later phase (5-12 days post-CLP) was an adaptive immune response.

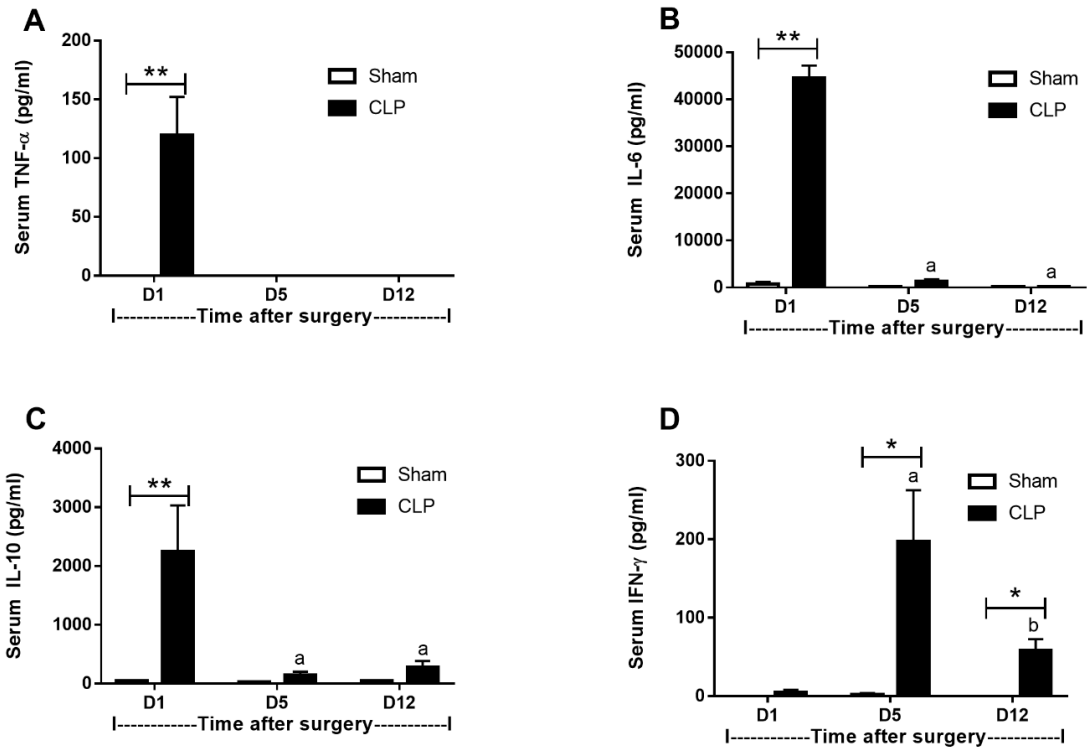


Figure 4.1. The kinetic change of serum cytokines (A-D) at day 1 (D1), day 5 (D5) and day 12 (D12) after cecal ligation and puncture (CLP) surgery or sham was demonstrated (n = 4/group/time point). * $p < 0.05$; ** $p < 0.01$; [a] $p < 0.05$ vs. D1; [b] $p < 0.05$ vs. D5.

Alteration of T cell, B cell and macrophage number during sepsis

Next, we observed whether the numbers of immune cells during sepsis correspond with the immune phases that were identified in Figure 4.1. CD4 T cells, CD8 T cells, and B cells were identified as $CD3^+CD4^+$, and $CD3^+CD8^+$, and $B220^+$ cells, respectively (Figure 4.2A). In CLP mice, total splenocyte numbers were reduced at day 1, restored at day 5, and significantly increased at day 12 post-CLP (Figure 4.2B). At day 1 post-CLP, CD4 T cell, CD8 T cell, and B cell numbers in CLP splenocytes were decreased in accordance with the total splenocyte numbers (Figure 4.2C-H). The loss of CD4 and CD8 T cells, and B cells in CLP splenocytes were further observed at day 5 and day 12 post-CLP (Figure 4.2C-H), however, these alteration did not support the increased total splenocyte numbers (Figure 4.2A).

Since an innate immune cell, macrophage, plays multiple roles in sepsis with their extensive effects on inflammatory and homeostasis process [92, 93], we therefore determined the macrophage numbers and activation. Macrophages were identified as $F4/80^+$ cells, and their activation state were determined based on the expression of CD86 and MHC class II (Figure 4.3A). Although, at day 1 post-CLP, the percentage of macrophage ($F4/80^+$ cells) in spleens of CLP mice was comparable to that of the sham control (Figure 4.3B), the macrophage numbers were significantly decreased (Figure 4.3C). Opposing to the reduction in the adaptive cell numbers, the macrophage numbers in CLP splenocytes was slightly increased at day 5 post-CLP, and the substantial numbers of macrophages were detected at day 12 post-CLP

(Figure 4.3C) in consistence with the large splenocyte numbers (Figure 4.2B). The percentage of $F4/80^{+}$MHC class II^{+} cell was decreased at day 5 and day 12 post-CLP (Figure 4.3D). Of note, the proportion of the $CD86^{+}F4/80^{+}$ cells was reduced at day 5 but it was greatly increased at day 12 post-CLP (Figure 4.3E).

Altogether, the numbers of splenic T cells and B cells were decreased throughout all the phases of sepsis. Meanwhile the splenic macrophage numbers were decreased in the early time of sepsis (1 day post-CLP) but their numbers were increased in the late phase (day 12 post-CLP). In addition, the splenic macrophages may lose their function as they lessened their activation markers at day 5 post-CLP, however, the cell numbers and the activation were restored at the late time of sepsis.

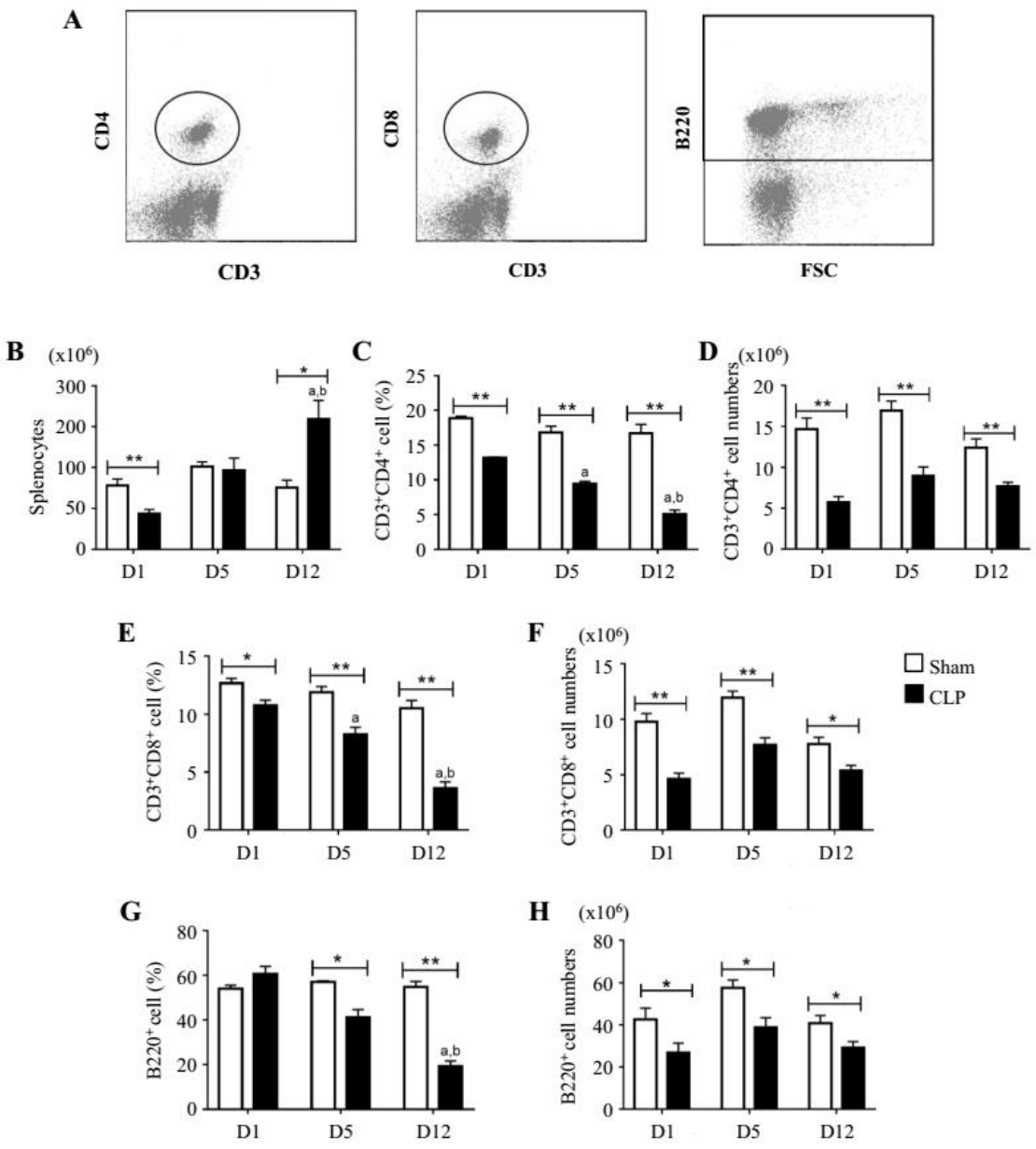


Figure 4.2. Alteration of T cell and B cell numbers during sepsis.

(A) Dot plot analysis of CD4 T cells ($CD3^+CD4^+$ cells), CD8 T cells ($CD3^+CD8^+$ cells) and B cells ($B220^+$ cells). (B) Total splenocyte numbers, (C) percentage of splenic CD4 T cells, (D) numbers of splenic CD4 T cells, (E) percentage of splenic CD8 T cells, (F)

numbers of splenic CD8 T cell, (G) percentage of splenic B cells, and (H) numbers of splenic B cell of CLP and sham control mice were assessed at day 1 (D1), day 5 (D5) and day 12 (D12) post-CLP. n = 5/group/time point. * $p < 0.05$; ** $p < 0.01$; [a] $p < 0.05$ vs. D1; [b] $p < 0.05$ vs. D5.

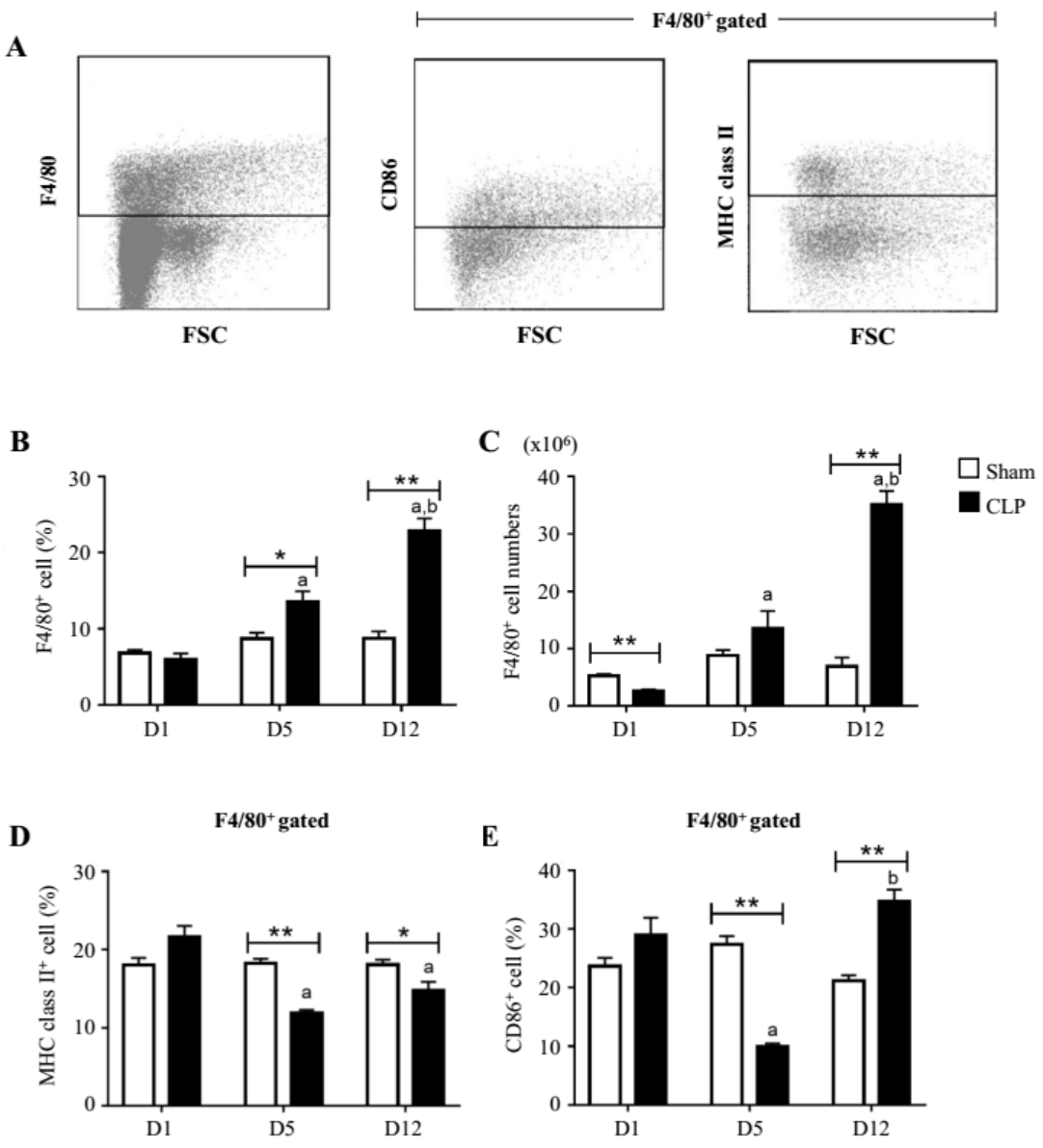


Figure 4.3. Changes in macrophage numbers and activation during sepsis.

(A) Dot plot analysis of macrophages. The bulk splenic macrophages were identified by F4/80 marker. The electronic gate was placed on F4/80^{+} population, and the macrophage activation markers, CD86 and MHC class II were analyzed. (B)

Percentage of splenic macrophage, (C) numbers of splenic macrophage, (D) percentage of MHC class II^+ macrophages ($F4/80^+$MHC class II^+ cells) and (E) percentage of $CD86^+$ macrophages ($F4/80^+CD86^+$cells) of CLP and sham control mice were determined at day 1 (D1), day 5 (D5) and day 12 (D12) post-CLP. *n* = 5/group/time point. Data represent two independent experiments. * $p < 0.05$; ** $p < 0.01$; [a] $p < 0.05$ vs. D1; [b] $p < 0.05$ vs. D5.

PD-1 expressing immune phenotype during sepsis

PD-1 expression is a characteristic marker of immune cell exhaustion in several fatal diseases including sepsis [8]. To verify the immune exhaustion state in CLP mouse model, we investigated PD-1 expressing immune phenotype of splenic CD4 T cells, CD8 T cells, B cells and macrophages in CLP mouse model. In flow cytometric analyses, each immune cell population was gated, and the PD-1^+ fraction was subsequently determined (Figure 4.4A). The proportions of PD-1^+ cells in splenic CD4 T cells ($CD3^+CD4^+$ cells) and B cells ($B220^+$ cells) from CLP mice were notably increased at day 5 post-CLP, and these increased levels were sustained until day 12 post-CLP, when compared to those of the sham control (Figure 4.4B, left and right panel). In the splenic CD8 T cell population ($CD3^+CD8^+$ cells), the high frequency of PD-1^+ cells was observed only at day 12 post-CLP (Figure 4.4B, middle panel).

Unlikely, the proportion of PD-1^+ cells in splenic macrophages ($F4/80^+$ cells) of sepsis mice was markedly increased at day 1 post-CLP, and were decreased to the baseline level at day 5 and day 12 post-CLP (Figure 4.4C, left panel). Similarly, the increased PD-1^+ cells in MHC class II^+F480^+ macrophages were found in CLP splenocytes at day 1 post-CLP, and these numbers were decreased at day 5 and day 12 post-CLP (Figure 4.4C, middle panel). The high frequency of PD-1^+ cells were also found in splenic $CD86^+F480^+$ macrophages of sepsis mice from day 1 to day 5 post-CLP (Figure 4.4C, right panel).

Collectively, our data indicated that the macrophages, and the adaptive immune cells exhibited the exhausted phenotype in the early phase (day 1 post-CLP), and in the late phase (day 5-12 post-CLP) of sepsis, respectively.

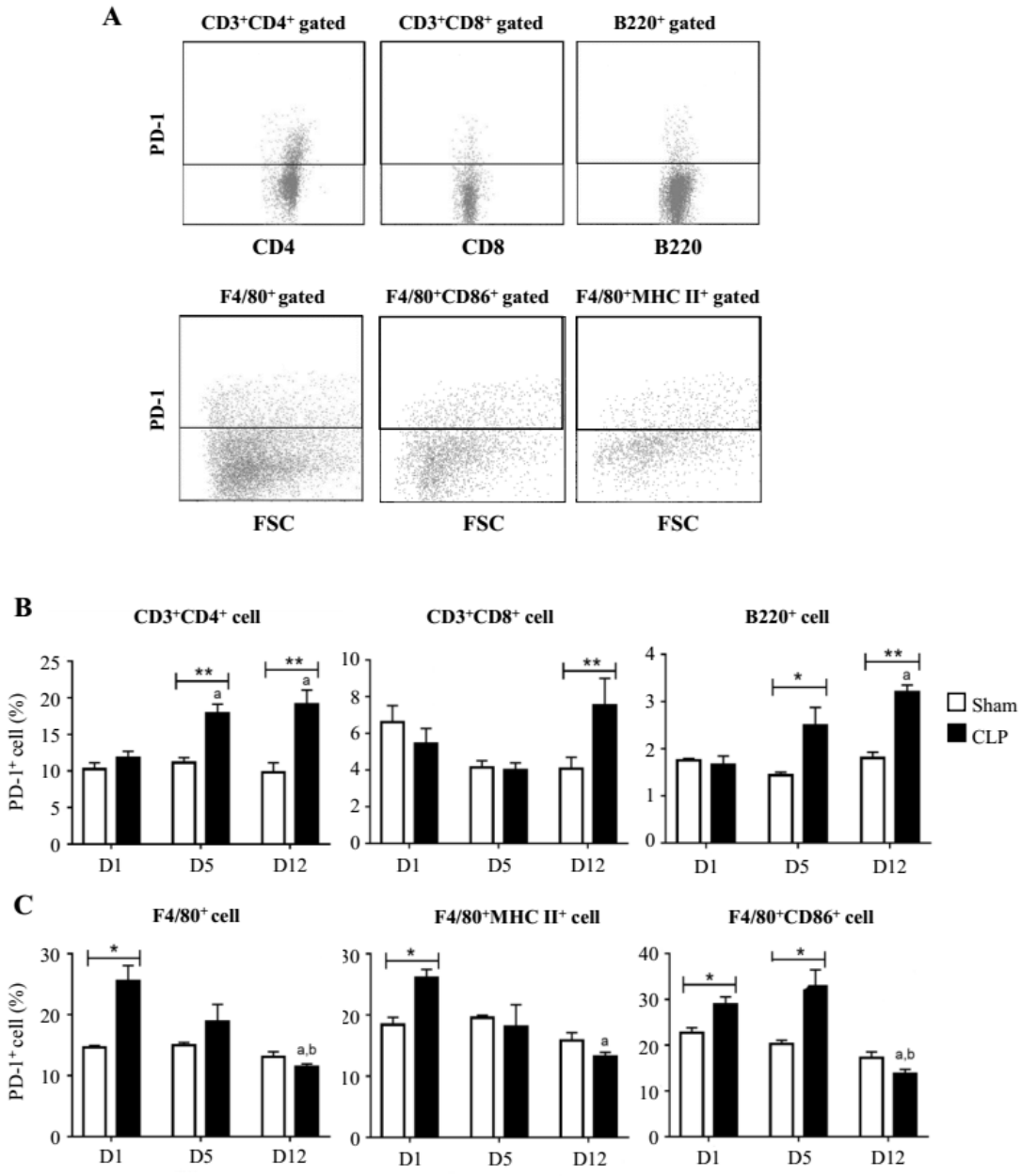


Figure 4.4. Kinetic expression of PD-1 molecules on immune cells during sepsis.

(**A**) Dot plot analysis of PD-1$^+$ cells. Upper panel, splenic CD4 T cells (CD3$^+$CD4$^+$ cells), CD8 T cells (CD3$^+$CD8$^+$ cells) and B cells (B220$^+$ cells) were identified by the electronic gates based on their lineage markers, and PD-1$^+$ fractions were subsequently gated in each immune cell subset. Lower panel, splenic macrophages

(F4/80$^+$ cells), CD86$^+$ macrophages (F4/80$^+$CD86$^+$cells), and MHC class II$^+$ macrophages (F4/80$^+$MHC class II$^+$ cells) were identified by the electronic gates, and PD-1$^+$ fractions were subsequently gated. Percentage of PD-1$^+$ cells in (B, left panel) CD3$^+$CD4$^+$ cells, (B, middle panel) CD3$^+$CD8$^+$ cells, (B, right panel) B220$^+$ cells, (C, left panel) F4/80$^+$ cells, (C, middle panel) F4/80$^+$MHC class II$^+$ cells, (C, right panel) F4/80$^+$CD86$^+$cells from spleens of CLP or sham control mice were examined at day 1 (D1), day 5 (D5) and day 12 (D12) post-CLP. *n* = 5/group/time point. Data represent two independent experiments. * $p < 0.05$; ** $p < 0.01$; [a] $p < 0.05$ vs. D1; [b] $p < 0.05$ vs. D5.

The influence of immune phenotype during sepsis on secondary fungal infection

To study the clinical significance of immune phenotype during sepsis, CLP and sham control mice were further systemically infected with *C. albicans* at day 5 and day 12 post-CLP. These time points were selected in regard to the reduction in macrophage activation (Figure 4.3), and the increase of adaptive immune cell exhaustion (Figure 4.4). In CLP group, the fungal infection at day 5 post-CLP (CLP D5-*Ca*) resulted in 100% mortality rate (Figure 4.5A) while the fungal infection at day 12 post-CLP (CLP D12-*Ca*) produced 40% mortality rate (Figure 4.5B). Our data suggested that the sepsis mice were highly susceptible to secondary *Candida* infection at day 5 post-CLP. The sepsis mice developed the resistance against secondary *Candida* infection after 12 days post-CLP.

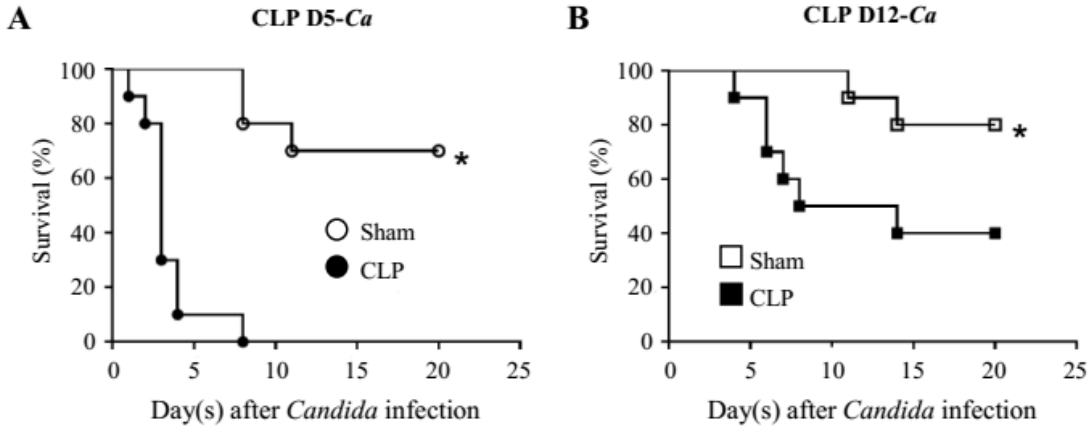


Figure 4.5. Survival rate of sepsis mice with secondary *Candida* infection.

Survival analyses of CLP or sham control mice infected with *C. albicans* at (A) day 5 post-CLP (CLP D5-*Ca*), and (B) day 12 post-CLP (CLP D12-*Ca*). $n = 10$. * $p < 0.05$

Generation of aspergillosis mouse model

The inhibition of PD-1-PD-L pathway is beneficial in murine infection models from bacteria [10, 17, 87] and fungi including post-sepsis secondary candidiasis [8, 25]. However, some case reports showed break-through tuberculosis and aspergillosis in anti-PD-1 treated cancer patients [26]. Hence, we studied the efficacy of anti-PD-1 treatment in post-sepsis aspergillosis.

To select the proper fungal dose for the infection, the varied number of *A. fumigatus* spores ($1x10^3$, $1x10^4$, and $1x10^6$ spores) were administered into intact mice, and a dose-related mortality rate was determined (Figure 4.6B). The dose of $1x10^6$ spores showed 75% mortality rate within 30 days after fungal infection (Figure 4.6B). Furthermore, at 1-day post-infection with the dose of $1x10^6$ spores, the highest fungal burden in the blood (fungemia) were observed, and it decreased at 7-day post-infection. Afterward, the high fungal number re-appeared at 21-day post-infection (Figure 4.6C). Regarding to the mortality and the relatively high blood fungal burdens, the spore number at $1x10^6$ was thus selected, and used in all secondary fungal infection experiments.

The secondary fungal infection occurs when the septic patients display the immune paralysis [1, 18]. As shown previously, 5-day post CLP mice deviated into the immunosuppressive state and were more susceptible to secondary candidiasis than 12-day post CLP mice. Therefore, we would choose day 5 post CLP to study anti-PD-1 treatment in secondary aspergillosis. A secondary aspergillosis was induced by the

intravenous inoculation of *Aspergillus fumigatus* spores at day 5 after CLP (Figure 4.6A). In the clinical therapy of septic patients with secondary fungal infections, the patients are usually received an anti-fungal medication. Following the clinical practice, all mice were medicated with an anti-fungal drug, amphothericin B, at 6 h and 24 h after the fungal infection. To examine whether blockade of PD-1 can attenuate a secondary aspergillosis, an anti-PD1 blocking antibody was administrated together with amphothericin B at 6 h and 24 h after the fungal infection (Figure 4.6A).

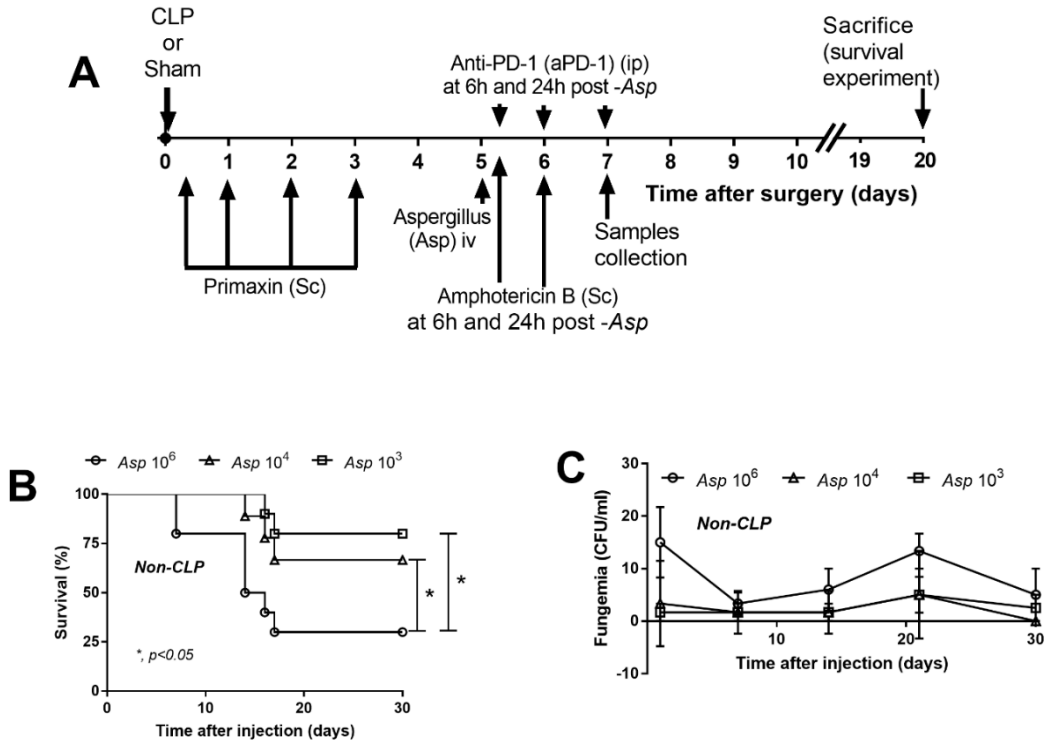


Figure 4.6 (A) Time line of the model of aspergillosis in sepsis-induced immune exhaustion by CLP was illustrated. (B) Survival rate of the mice intravenously infected with the various doses of *A. fumigatus* spores (n = 10) (C) The fungal burden in the blood at day 1, 7, 14, 21 and 30 was presented in colony forming unit per millilitre (CFU/ml) (n = 4-5 per time-point).). Log-rank (Mantel-Cox) test was performed for survival analysis.

Blockade of PD-1 attenuated secondary aspergillosis

Despite the anti-fungal treatment, the survival rate of the CLP mice with secondary aspergillosis (CLP-*Asp*) was 10% at 20 days follow-up (Figure 4.7A). Intriguingly, the blockade of PD-1 in the CLP mice with secondary aspergillosis (CLP-*Asp*-aPD1) significantly improved the survival rate of secondary aspergillosis from 10% to 40% at 20 days follow-up (Figure 4.7A). At day 2 after *Aspergillus* infection, the fungal burdens in kidney, lung, brain and blood were observed. Concomitant with the mouse mortality, the fungal numbers in all organs and blood from CLP-*Asp* group highly increased, and these fungal burdens were markedly decreased in CLP-*Asp*-aPD1 group (Figure 4.7B). The histology also clearly demonstrated the deposition of the mold in brain, lung and kidney of CLP-*Asp* mice (Figure 4.7C, middle panel), but not in CLP-*Asp*-aPD1 group (Figure 4.7C, left panel).

Along with the mold deposition in the kidney (Figure 4.7B and 4.7C), CLP-*Asp* mice showed the increased serum creatinine, which indicated the impaired renal function, when compared to the control CLP-PBS group (Figure 4.8A). The treatment with anti-PD-1 mAbs leaded to the reduction of serum creatinine (Figure 4.8A), which also associated to the decreased fugal number in the kidney (Figure 4.7B and 4.7C).

The serum innate cytokine, IL-6, and the adaptive cytokines IFN-γ, and IL-17, which played a critical role in the protective immunity against *Aspergillus* infection [70, 94, 95], were also determined. The serum IL-6 (Figure 4.8B), IL-17 (Figure 4.8C), and IFN-γ (Figure 4.8D) in CLP-*Asp* group did not differ from those of CLP-PBS control.

However, the treatment with anti-PD-1 mAbs in CLP-*Asp*-aPD1 group enhanced the level of IL-6 and IFN-γ (Figure 4.8B and 4.8D). The serum level of the anti-inflammatory cytokine, IL-10, were also assessed. CLP-*Asp* mice showed the augmented IL-10 level in the serum, and the anti-PD-1 treatment suppressed the IL-10 production (Figure 4.8E).

Altogether, the high mortality rate of CLP-*Asp* mice was related to the fungal burdens (Figure 4.7A and 4.7B), which consequently leaded to the kidney failure (Figure 4.8A), and the dampened immune responses that possibly resulted from the high anti-inflammatory cytokine, IL-10, production (Figure 4.8E), and the inability to produce the innate and adaptive cytokines for the protective immunity (Figure 4.8B-4.8D). Conversely, the blockade of PD-1 could boost IL-6 and IFN-γ production, as well as inhibited IL-10 production in secondary aspergillosis (Figure 4.8B, 4.8D, and 4.8E). Therefore, the treatment with anti-PD-1 mAbs may shift the immunosuppressive state to the immune activation, which leaded to the attenuation of secondary aspergillosis.

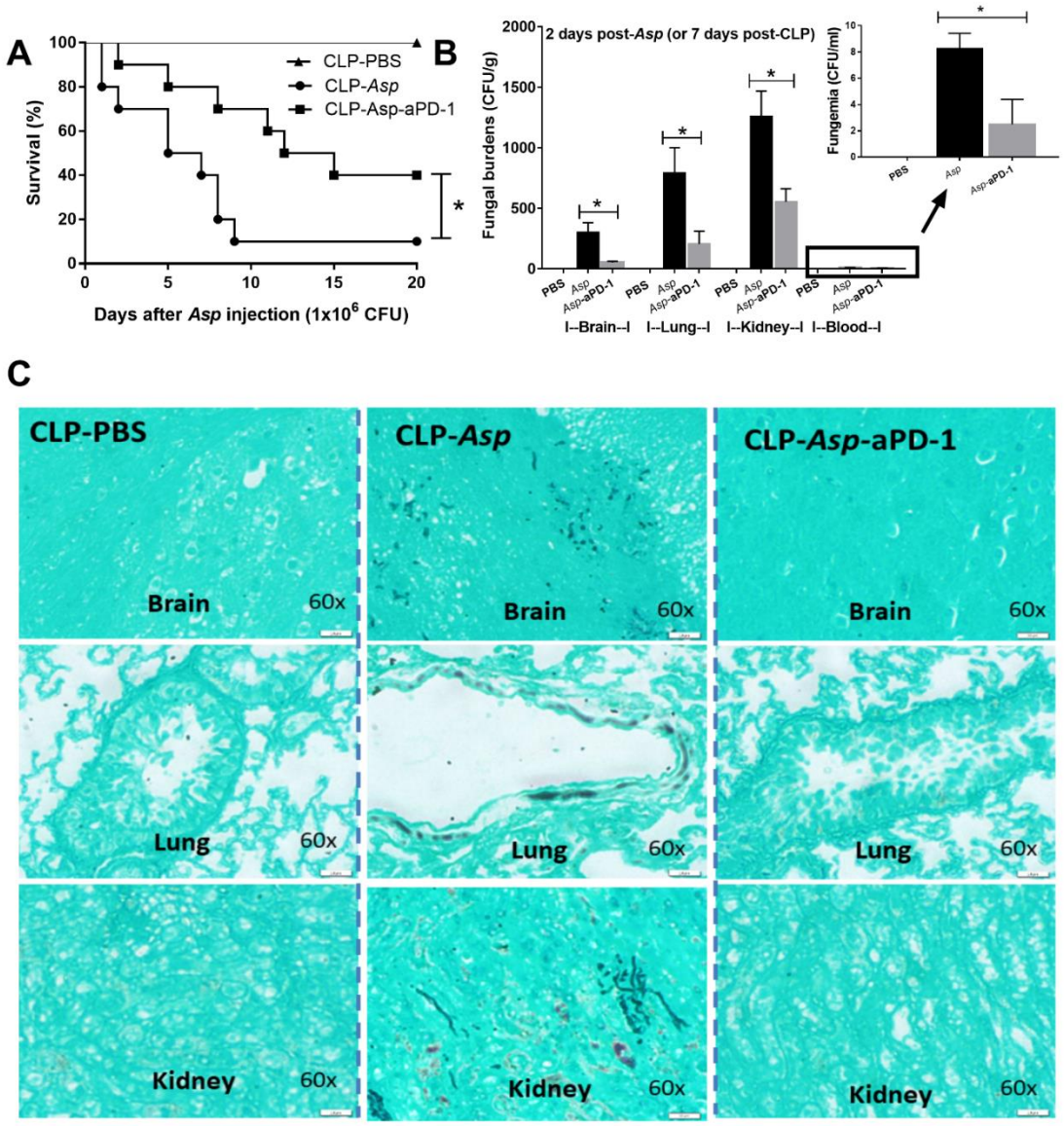


Figure 4.7. Eradication of the fungi upon anti-PD-1 treatment. (A) Survival analysis (n = 10). (B) Fungal burdens in the internal organs and in blood at 2-day post-aspergillus infection (or 7-day post CLP) (n = 4). (C) Representative histology of brain, lung, kidney and blood at 2-day post-aspergillus infection, 60x magnification. CLP-PBS, CLP with PBS injection as the control without infection; CLP-*Asp*, CLP with *A.*

fumigatus infection; CLP-*Asp*-aPD-1, CLP with *A. fumigatus* infection and treated with anti- PD-1 mAbs. * $p<0.05$

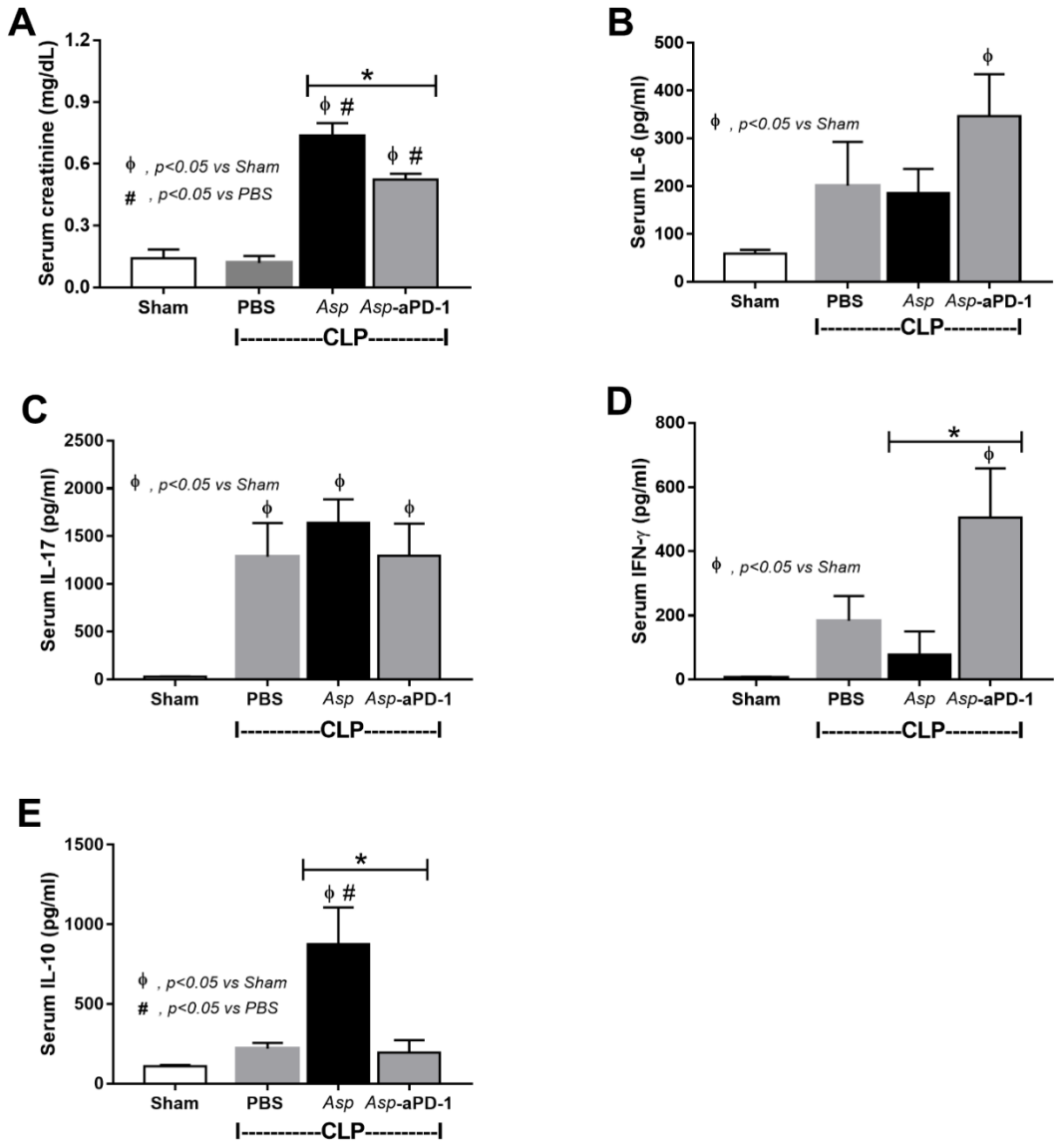


Figure 4.8. Alteration of serum cytokine profile following anti-PD-1 treatment. The serum (A) creatinine, (B) IL-6, (C) IL-17, (D) IFN-γ, and (E) IL-10 were determined. The sera were collected at 2-day post-aspergillus infection (or 7-day post CLP) (n = 4-5). Sham, the control procedure of CLP; CLP-PBS, CLP with PBS injection as the control without infection; CLP-Asp, CLP with A. fumigatus infection; CLP-Asp-PD-1, CLP with A.

fumigatus infection and treated with anti- PD-1 mAbs. ϕ p< 0.05 vs. sham; # p< 0.05 vs. CLP-PBS; * p<0.05

Anti-PD-1 attenuated secondary aspergillosis through the immune-stimulation of splenocytes

Accordingly, to investigate whether the blockade of PD-1 abrogates the immunosuppressive state in secondary aspergillosis, the innate and adaptive immune phenotypes were examined. The innate immune phenotype was assessed based on the expression of CD86 and MHC class II, and the immune cell exhaustion was determined based on expression of PD-1. At day 2 after *Aspergillus* infection. CLP-*Asp*-aPD1 splenocytes displayed the greatly increased percentage of $CD86^+$ cells, when compared to CLP-*Asp* splenocytes, and this increased number was similar to the sham control (Figure 4.9A and 4.9D). The percentages of MHC class II^+ cells and $PD\text{-}1^+$ cells among CLP-PBS, CLP-*Asp* and CLP-*Asp*-aPD1 group were comparable (Figure 4.9B, 4.9C, 4.9D). The data implied that the anti-PD-1 treatment probably retrieved the innate immune function via the induction of CD86 expression.

T cell responses were determined by an *in vitro* anti-CD3 re-stimulation assay. The splenocytes were re-stimulated with immobilized anti-CD3 mAbs (a T cell activator), and the secretion of IL-10, IL-17 and IFN-γ, which are the key cytokines of regulatory T cells, T helper (Th) 17, and Th1, respectively, was assessed. The IL-17 production from the re-stimulated CLP-PBS T cells was markedly decreased, and secondary aspergillus infection (CLP-*Asp*) did not alter the IL-17 level. Although the administration of anti-PD-1 in secondary aspergillosis (CLP-*Asp*-aPD1) did not significantly affect the IL-17 production from the activated T cells, there was a

tendency of enhanced IL-17 (Figure 4.10A). The activated T cells from CLP-PBS showed the reduced capability to produce IFN-γ, comparing to the sham control. Of note, IFN-γ production of the re-stimulated CLP-*Asp* T cells was strikingly impaired. The blockade of PD-1 could boost the IFN-γ production of the activated T cells (Figure 4.10B), which was coincide with the enhanced serum IFN-γ (Figure 4.8D). Consistent to the result in the serum (Figure 4.8E), IL-10 was highly produced from the anti-CD3 stimulated CLP-PBS splenocytes, and there was no difference of IL-10 level between anti-CD3 re-stimulated CLP-PBS and CLP-*Asp* splenocytes. The IL-10 production from anti-CD3 re-stimulated CLP-*Asp*-aPD1 splenocytes was not apparently reduced, however, this IL-10 production was significantly lower than the re-stimulated CLP-PBS splenocytes (Figure 4.10C).

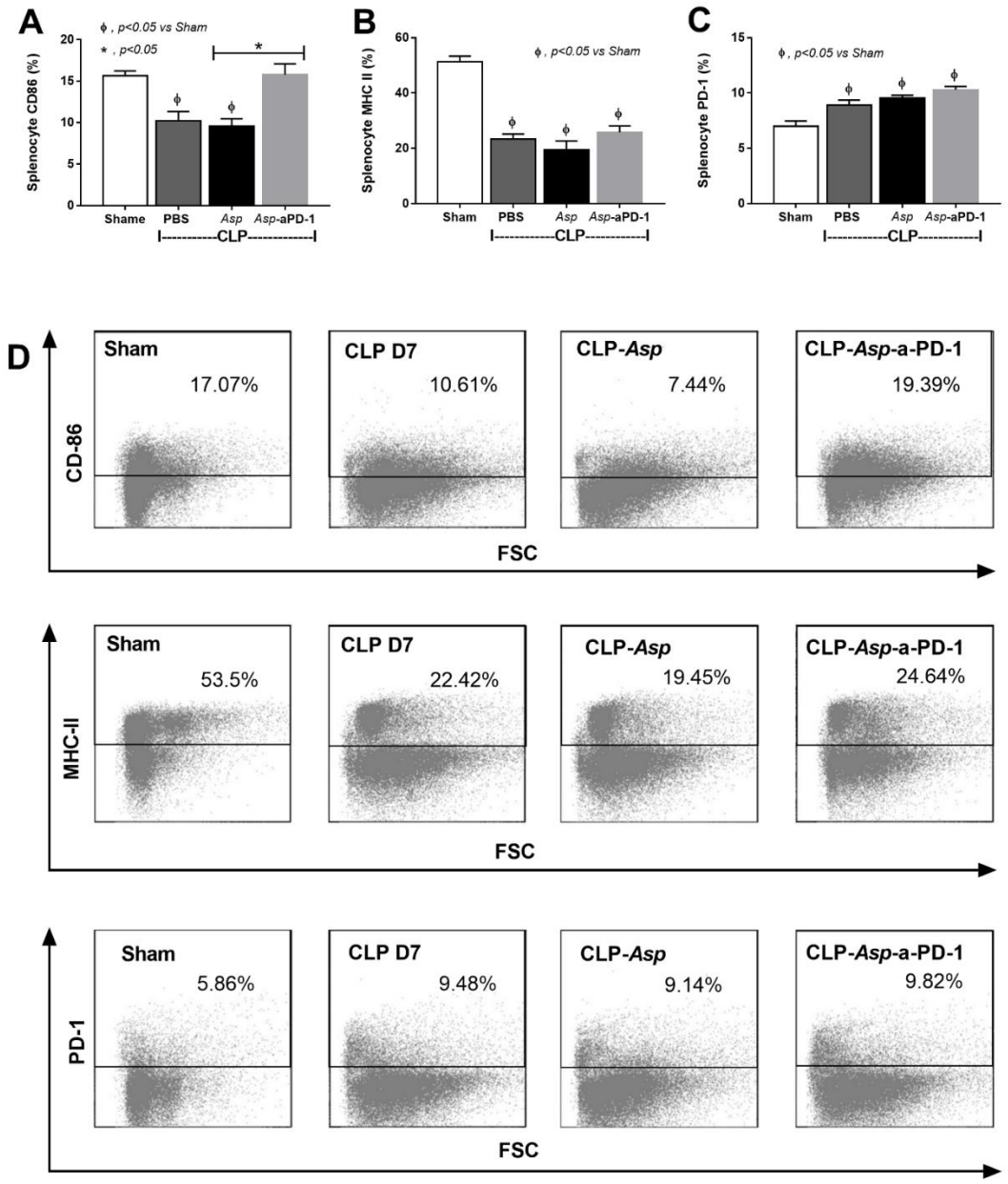


Figure 4.9. Amelioration of immune exhaustion upon anti-PD-1 treatment. The expression of CD86, MHC class II and PD-1 on splenocytes were assessed by flow cytrometric analysis. Live cells were defined based on side scatter (SSC) and forward scatter (FSC), and the percentage of (A) CD86+, (B) MHC class II+, and (C) PD-1+ cells

were determined (n = 4-5). (D) The dot plot analysis of CD86, MHC class II and PD-1. The number in the dot plot indicated the percentage of the positive cells. Sham, the control procedure of CLP; CLP-PBS, CLP with PBS injection as the control without infection; CLP-Asp, CLP with A. fumigatus infection; CLP-Asp-PD-1, CLP with A. fumigatus infection and treated with anti- PD-1 mAbs. ϕ $p<0.05$ vs. sham; *$p< 0.05$.

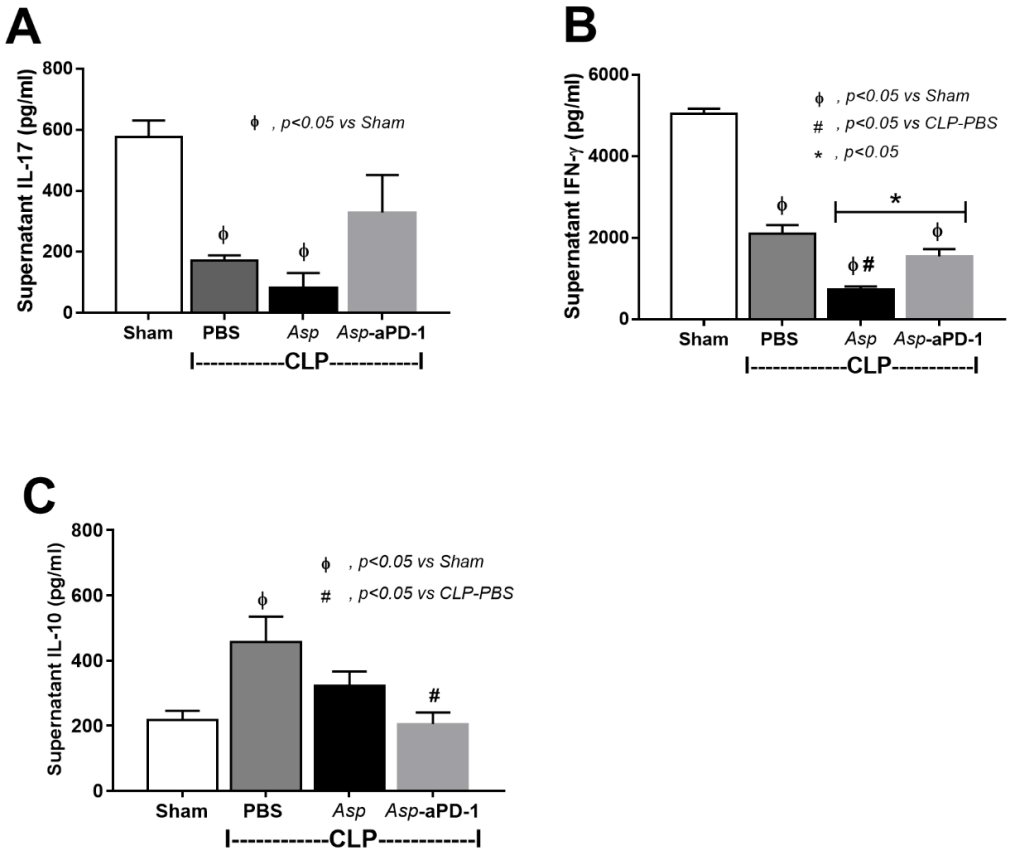


Figure 4.10. Reinvigoration of T cell function by anti-PD-1 treatment. Splenocytes were *in vitro* re-stimulated with immobilized anti-CD3 mAbs, and the levels of (A) IL-17, (B) IFN-γ, and (C) IL-10 were assessed from the culture supernatant (n = 4-5). Sham, the control procedure of CLP; CLP-PBS, CLP with PBS injection as the control without infection; CLP-*Asp*, CLP with *A. fumigatus* infection; CLP-*Asp*-aPD-1, CLP with *A. fumigatus* infection and treated with anti- PD-1 mAbs. $^{\phi}$ $p< 0.05$ vs. sham; $^{\#}$ $p<$ 0.05 vs. CLP-PBS; * $p<0.05$

Chapter 5: Discussion

Secondary fungal infection by opportunistic fungi frequently found in patients succumbed bacterial sepsis because of the concomitant immunosuppression [8]. The characteristics of our CLP-induced sepsis model exhibited hyperinflammation (cytokine storm) as early as 1 day after CLP, and immune exhaustion thereafter as demonstrated by the increased susceptibility against secondary *Candida* infection at day 5 post-CLP. The CLP mice eventually restored their immunity to overwhelm the fungal infection after 12 days post-CLP.

The hyperinflammation was developed in the early phase (day 1 post-CLP) of CLP mouse model, and this response is one underlying mechanism that perpetuate organ injuries (Figure 4.1) [96]. “Cytokine storm” of sepsis is the primary cause of septic shock and early death of sepsis by exerting the systemic pathologic effect such as low cardiac output, thrombosis, hyper-catabolism, acute renal failure [1, 4]. In 1996, Kumar found that IL-1β and TNF-α impeded myocardial cell contraction in vitro [44]. In the similar manner, Vincent et al reported the improvement of ventricular function following anti-TNF-α treatment in ten septic patients [45]. It is interesting to notice that TNF-α, IL-6, IL-10 were spontaneously returned into the control level (sham) in CLP survivors after 5 and 12 days post-CLP (Figure 4.1) supporting the cytokine suppression after sepsis [7, 97].

In the same early phase post sepsis, the reduction in total splenocytes, T cells, B cells, and macrophages were also observed (Figure 4.2 and Figure 4.3), and

this loss of immune cells is probably by virtue of the inflammation-induced apoptosis [96, 98]. Although an anti-inflammatory cytokine, IL-10, was also increased at day 1 post-CLP, it is merely a negative feedback of the inflammatory regulation to protect a self-damage [99].

The adaptive immune cells were persistently decreased throughout all the phases of sepsis (Figure 4.2), and the cell loss in the later phase of sepsis (day 5 to day 12 post-CLP) may be, in part, due to the immune exhaustion. Our result was consistent with previous research which showed the loss of adaptive immune cells in murine sepsis model at day 1 until day 7 [28]. Likewise, Hotchkiss reported the progressive loss of $CD4^+$ T cells and B cells in spleen of septic patients who died in ICUs, compared to critically ill non-septic patients [100]. PD-1 molecule plays a pivotal role in immune suppression owing to its diverse immunoregulatory properties, and the increased and sustained PD-1 expression is a hallmark of an exhausted immune phenotype in lethal infectious diseases including sepsis [8, 101]. One well-known function of PD-1 is it can mediate exhausted immune cells to undergo apoptosis [87]. Our study demonstrated the loss of adaptive immune cells during the late phase of sepsis (day 5 to 12 post-CLP) (Figure 4.2), and this cell loss is probably associated with the upregulation of PD-1 molecules (Figure 4.4). Our result is consistent with study from Guignant who found the increased PD-1 expression on circulating $CD4^+$ T cells and monocytes from septic patients [18]. Likewise, on splenocytes collected rapidly from septic patients who died in ICU, PD-1 was found

inducible expressed on $CD4^+$ T cells [7]. Notwithstanding the sustained exhaustion and reduction of T cells and B cells from 5 to 12 days after CLP, the sepsis mice displayed the high susceptibility to secondary *Candida* infection only at day 5, but not day 12, post-CLP.

The number of activated macrophages ($F4/80^+CD86^+$ and $F4/80^+$MHC class II^+ cells) at day 5 post-CLP was notably decreased, and about half number of $F4/80^+CD86^+$ population expressed PD-1 (Figure 4.3 and Figure 4.4). Our results were consistent to the observation in human and mouse sepsis, which the decreased number of $CD86^+$ cells and HLA-DR^+ (or MHC class II^+) cells were found in the blood circulation [7, 102]. MHCII is molecule belonged to class of major histocompatibility complex (MHC), found on dendritic cells, macrophages, some endothelial cells, B cells and hold the function of presenting antigen to T cells [103]. Extracellular proteins are endocytosed, processed in vesicles, loaded to MHCII molecules, and expressed on the membrane of cells [103]. CD86 or B7-2 is a protein belonged to the B7-CD28 family, expressed on macrophages, dendritic cells, and worked as costimulatory ligand [104, 105]. Upon the binding of CD86 on antigen presenting cells and CD28 on lymphocytes, T cells receive the signal to proliferate, activate and differentiate to effector lymphocytes [104, 105]. The reduction of CD86 and the decrease of MHCII on antigen presenting cells were used as hallmark of sepsis-induced immune exhaustion [1, 7, 8, 106, 107]. Study from Boomer revealed that there was a significant reduction in CD86, HLA-DR expression on antigen presenting

cells and macrophages in spleen collected from severe septic patients who died in ICUs [7]. Chang found the decrease in MHCII expression on splenic dendritic cells and macrophages from mice succumbed CLP and secondary candidiasis [8]. Likewise, Lukaszewicz reported the decrease in HLA-DR expression on monocytes from peripheral blood of septic patients in ICUs and weak recovery of HLA-DR was associated with secondary infection [107]. In addition, the substantial PD-1 expression was detected on macrophages from sepsis mice, and it was associated with the macrophage dysfunction [17]. The deficit of activated macrophages and their exhausted phenotype at day 5 post-CLP therefore may play role in the increased susceptibility to secondary *Candida* infection (Figure 4.5). On the contrary, the macrophages in sepsis mice at day 12-post CLP were reconstituted, and the increased macrophage numbers were related to the increased total splenocytes (Figure 4.2B and Figure 4.3C). At this time point, the large numbers of $CD86^{+}F4/80^{+}$ activated macrophages were also observed in accordance with the decreased PD-1 expression on macrophages (Figure 4.3E and Figure 4.4C), and these reinvigorated macrophages may account for the resistance to secondary *Candida* infection. In this regard, the activation and exhaustion of macrophages perhaps contribute to the host defense against secondary fungal infection during sepsis. Model of murine secondary infection after CLP-induced sepsis had been investigated by several groups to clarify disease pathology, immune response and treatment therapy [8, 28, 108]. Recently, Muenzer demonstrated that mice succumbed secondary infection with

Pseudomonas aeruginosa at day 7 post-CLP had better survival rate than mice challenged at day 4 post-CLP [28]. They explained that the improvement in survival rate was associated with reconstitution of natural killer cells, neutrophils, dendritic cells and increase IFN-γ secretion by stimulated splenocytes at day 7 post-CLP [28]. More translational studies are interesting.

T helper 1 (Th1) cell responses are important for defense against *Candida* bloodstream infection, and IFN-γ is a key cytokine mediating the anti-*Candida* immunity [91]. We, hence, investigated the systemic IFN-γ production in CLP mice. The level of serum IFN-γ from day 5 to 12 post-CLP was inconsistent with the reduced T cell numbers (Figure 4.1 and Figure 4.2). IFN-γ has been recently used as an adjunctive therapy against fungal infection [70, 109], however, the treatment with IFN-γ alone is not effective [110].

In the last decade, anti-PD-1 therapy has shown the high efficacy to promote immune responses against fungal infection and bacterial sepsis. Blockade of PD-1 pathway has improved the survival in primary and secondary *Candida* infection via the enhancement of IFN- γ production, and the restoration of antigen-presenting cell function [8]. In addition, the combination immunotherapy with an anti-PD-1 antibody (Nivolumab) and recombinant IFN-γ was highly effective to improve survival outcome of patient succumbed extensive abdominal mucormycosis and was unresponsive to regular therapy [109]. Very recent clinical trial has also demonstrated the high

efficacy and non-immune adverse effect of anti-PD-1 therapy in patients succumbed sepsis-associated immunosuppression [111]. The aforementioned evidences reveal that PD-1 is a target for an immunotherapy of sepsis and fungal infection.

Nosocomial fungal infection is one of the causes of death in ICU despite the standard anti-fungal therapies [8, 66]. In this regards, the adjuvant immunotherapy may be a promising strategy. Here, the efficacy of anti-PD-1 treatment against secondary aspergillosis was demonstrated in a mouse model of *Aspergillus fumigatus* intravenous administration at 5 days after (CLP). The adjunctive anti-PD-1 treatment reduced fungal burdens and improved mortality (Figure 4.7), at least in part, via the increased CD86 expression (Figure 4.9A), enhanced IFN-γ production (Figure 4.8D, 4.10B), and the dampened IL-10 level (Figure 4.8E).

The vulnerability toward secondary opportunistic infection is a major characteristic of the immunosuppressive phase of sepsis [1]. In past decades, the prevalence of secondary aspergillosis in patients with non-neutropenic condition in intensive care unit has been increasing [112]. Although the natural route of aspergillus infection is mostly through the respiratory system, the intravenous infection also resembles to invasive aspergillosis [113]. Amphotericin B is an anti-fungal drug used as a primary therapy for invasive aspergillosis [114], but this drug was not effective in CLP mice with secondary aspergillosis (Figure 4.7). The anti-PD-1 therapy has become a successful treatment in sepsis and secondary fungal infection [8, 115], however, the recent report demonstrated the progression of aspergillosis

following the anti-PD-1 administration in the cancer patient [116]. In addition, anti-PD-1 therapy also exhibited a paradoxical effect on tuberculosis [26]. Thus, the effect of anti-PD-1 against aspergillosis is still ambiguous.

As discussed previously, we demonstrated the immunosuppressive state at day 5 post CLP by the decreased $CD86^+$ macrophages (Figure 4.3E) and high susceptibility to secondary candidiasis (Figure 4.5). We found the spontaneous reconstitution of the innate immune response at 12-day post-CLP characterized by higher $CD86^+$ macrophages (Figure 4.3E) and higher resistance to secondary infection (Figure 4.5). Thus we select day 5 post CLP to study the model of secondary aspergillosis and investigated the outcome of anti-PD-1 treatment.

Mice succumbed aspergillosis at day 5 post CLP exhibited substantially high serum creatinine (Figure 4.8A). Creatine is generally synthesized in the liver, transported through blood to muscle and brain, where it is catalyzed to converse to phosphocreatinine and function as storage of ATP [117]. Creatinine, the waste molecule of muscular metabolism, is filtered through kidney without tubular reabsorption [117]. The raise of serum creatinine impose kidney injury which is associated with sepsis [3]. Septic acute kidney injury (AKI) are considered consequence of renal hypoperfusion causing ischemia-reperfusion injury [118]. Using an intravital video microscopy (IVVM) of the kidney in CLP model, Wu reported that sepsis-induced capillary dysfunction and reactive nitrogen species (RNS) generation were responsible for tubular injury [119]. RNS inhibitor should be a promising therapy

for septic AKI [119]. Uchino conducted a multinational prospective research including 29,269 critically ill patients at ICUs in 23 countries, reported that 6% patients succumbed AKI during their ICUs stay, septic shock being the main contributing factor to AKI (50%) [120]. Another retrospective study reported that 65% septic shock patients succumbed early AKI; and AKI was the independent risk factor for mortality [121]. The beneficial efficacy of anti-PD-1 treatment by reversing the increase of serum creatinine in secondary aspergillosis model (Figure 4.8A) propose the promising outcome of sepsis.

Secondary infection with *Aspergillus fumigatus* in CLP mice with the immune exhaustion status did not further reduced the proinflammatory cytokines IL-6, IL-17 and IFN-γ in the serum, however, the level of the immunosuppressive cytokine, IL-10 were substantially increased (Figure 4.8). The elevated concentration of IL-10 was associated with poor septic outcome in sepsis [122-124]. In 1999, Steinhauser found that *Pseudomonas aeruginosa* challenge at 24 following CLP operation in mice resulted in enhanced lung IL-10 production and impaired bacterial clearance; mortality and morbility was ameliorated after administration of IL-10 antibody [122]. In 2002, Kalechman showed that administration of ammonium trichloro(dioxoethyleneo,o)tellurate (AS101), an IL-10 inhibitor, at 12 hour post-CLP significantly improved survival of septic mice and restored the function of immune cells [125]. In the same manner, Muenzer reported that administration of AS101 following CLP significant enhanced survival of secondary infection by *Pseudomonas*

aeruginosa, restored level of IFN-γ secreted by splenocytes, increased serum pro-inflammatory cytokines, decreased bacterial burden in bronchoalveolar lavage (BAL) and blood [28]. A clinical study in 65 septic patients by Gogos showed that a high serum IL-10 to TNF-α ratio was associated with septic death and IL-10 was parallel with septic score [124]. In the same manner, Wu conducted clinical study in 76 septic patients and found that serum IL-10 level in non-survivors was more elevated than survivors [123]. Animal study by Clemons has demonstrated the deleterious function of IL-10 during systemic aspergillosis infection; IL-$10^{-/-}$ mice survived longer and carried lower fungal burdens in kidney and brain than wide type mice [35]. Therefore, in our present study, the high mortality rate and the high fungal burdens in secondary aspergillosis (Figure 4.7) feasibly caused by the augmented IL-10 level (Figure 4.8).

PD-1 negatively regulates the function of T cells and antigen-presenting cells such as dendritic cells, macrophages and B cells [1]. Our data revealed that PD-1 blockade in CLP with secondary aspergillosis recovered the immune activation and function (Figure 4.9-4.10). It has been reported that the increased $CD86^{+}$ cells in the blood of septic patients was associated to the patient recovery state [102]. Boomer collected rapid postmortem spleens in 40 severe septic patients who died in ICUs and control spleens from patients who were confirmed brain-dead or underwent splenectomy because of trauma, showed that CD86 expression on antigen presenting cells of septic patients was significant lower than control patients [7]. In the similar

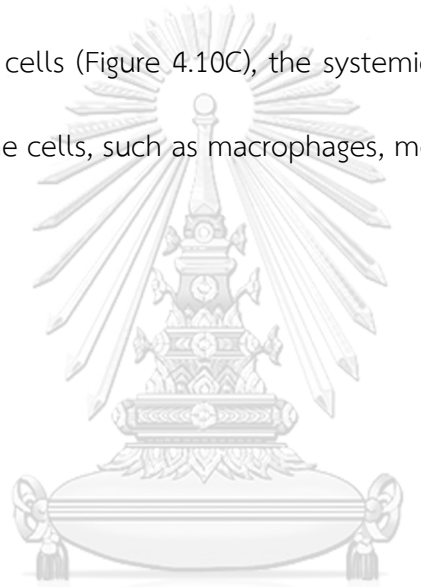
manner, Newton showed the decreased expression of CD86 on peritoneal macrophage of septic mice [126]. The anti-PD-1 treatment in CLP with secondary aspergillosis attenuated the diseases, which may be due to the increased number of $CD86^+$ cells (Figure 4.9A). CD86 is mostly expressed on antigen-presenting cells, and it functions as a T cell activator [127]. Therefore, the increased number of $CD86^+$ cells in the anti-PD-1 treated secondary aspergillosis possibly advocated the T cell functions (Figure 4.10).

The blockade of PD-1 in CLP with secondary aspergillosis also enhanced IFN-γ production, actuated T_H1 function, while it dampened the IL-10 production (Figure 4.8 and 4.10). T_H1 is the major source of IFN-γ production and provide the dominant protective immune response against fungal sepsis [66]. T_H1 and its signature cytokine IFN-γ provide the optimal activation of macrophages and monocytes [66]. The decreased in IFN-γ production by splenocytes post sepsis is the hallmark of immunosuppression [7, 28]. Armstrong-James studied aspergillosis model in mice immunosuppressed with cyclophosphamide/hydrocortisone acetate, claimed that death was due to the decrease IFN-γ response, increased TNF-α level in lung [128]. Shao overexpressed IFN-γ in the lung of mice by intranasally administration of adenovirus vector containing the murine IFN-γ cDNA (AdmIFN-γ), challenged with *Aspergillus fumigatus*; mice showed an increased level of IFN-γ in lung, decrease fungal burden and improve survival rate [129]. Muenzer showed that IFN-γ blockade

increased mortality upon murine secondary infection by *Pseudomonas aeruginosa* [28]. In the related manner, Chang revealed that anti-PD-1 treatment increased survival and splenocytes IFN-γ production in secondary candidiasis [8]. Recently, Jarvis reported that rhIFN-γ treatment accelerated the clearance of cryptococcal infection from the cerebrospinal fluid [130]. As IFN-γ plays a primary role in the protective immunity against aspergillus infection [128, 129], the elevated T_H1 function by the anti-PD-1 treatment (Figure 4.10) may facilitate the fungal clearance (Figure 4.7).

Our result showed that septic mice had lower IL-17 production by splenocytes and anti-PD-1 treatment did not help to increase IL-17 (Figure 4.10A). In line with T_H1, T_H17 pathway also provides protection against fungal sepsis by secreting IL-17 [66]. Huang showed that IL-17 receptor-deficient (IL-17RA$^{-/-}$) mice are highly susceptible to systemic candidiasis with increased mortality and fungal burden in kidneys [71]. In addition, T_H17 deficient (IL-23p19$^{-/-}$) mice and IL-17RA$^{-/-}$ mice succumbed severe oropharyngeal candidiasis, while T_H1 deficient (IL-12p35$^{-/-}$) mice was resistant to disease. Nevertheless, adaptive immune response against *Aspergillus fumigatus* is mediated by T_H1 cells rather than T_H17 cells [66, 79]. Chai showed that peripheral blood mononuclear cells secreted limited amount of IL-17 but high amount of IFN-γ upon exposure to live *Aspergillus fumigatus* [131].

The PD-1 pathway contributed to the infection-induced IL-10 production, and the absence of PD-1 down-modulated the IL-10 production [132]. As discussed above, IL-10 play deleterious role during infection and is associated with poor outcome of sepsis [35, 122-125]. Therefore, the treatment with anti-PD-1 mAbs in CLP with secondary aspergillosis exhibited the low level of serum IL-10 (Figure 4.8E). Although the blockade of PD-1 did not clearly showed the decrease in IL-10 production from the T cells (Figure 4.10C), the systemic IL-10 may be partly derived from the innate immune cells, such as macrophages, monocytes, dendritic cells [99].



Conclusion

This study characterized the kinetic alteration of immune cell population and immune phenotypes in CLP-induced sepsis, and their association with secondary fungal infection. Our findings demonstrated that an alteration of innate immune phenotypes during sepsis may reflect the innate immune activation state and function, which are possibly relevant to the susceptibility to secondary fungal infection. From a clinical point of view, our results prompt the necessity of further studies in patient with sepsis-associated immunosuppression to ascertain the implication of innate activation and exhaustion markers in accordance with secondary fungal infection. This fundamental knowledge will assist to design the therapeutic strategies to restore the immune function of patients with sepsis for their fungal clearance.

Secondly, we highlighted the important role of immune response in collaborate with an antifungal agent, and strengthens the beneficial effect of PD-1 blockade on post-sepsis aspergillosis. The adjunctive anti-PD-1 therapy presumably reinvigorated exhausted antigen-presenting cells and T cells by upregulating CD86 expression and IFN-γ production, and diminished IL-10 production, which consequently leaded to the attenuation of secondary aspergillus infection (Figure 5.1). The adjunctive anti-PD-1 therapy may be a new candidate of the advanced immunotherapy against lethal fungal infection.

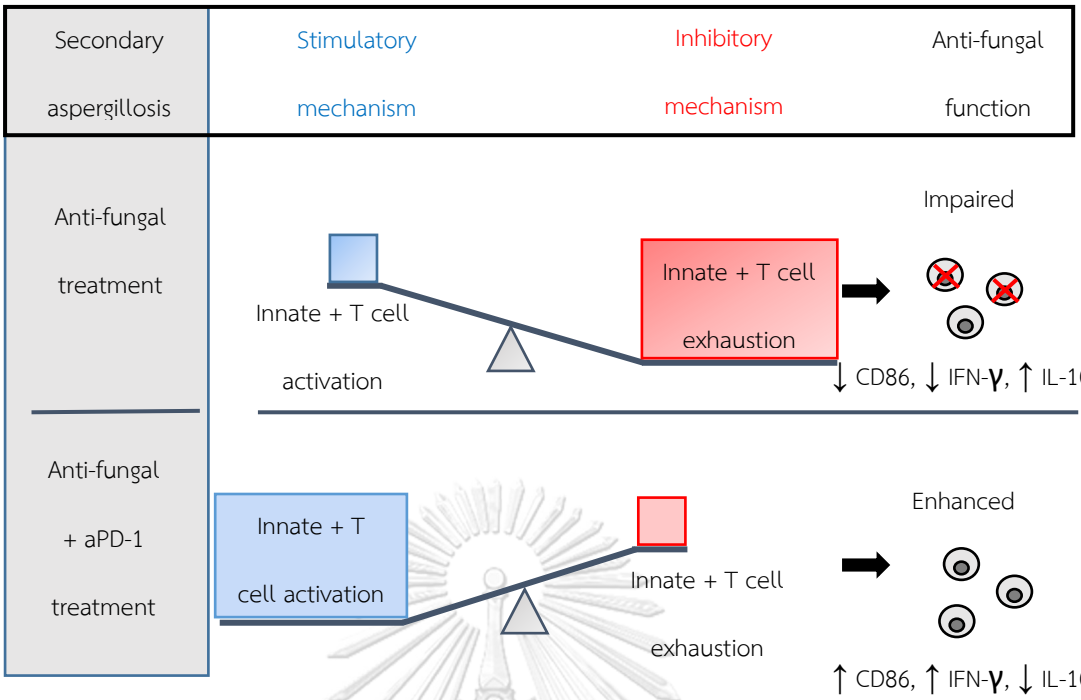


Figure 5.1. Suggestion mechanism of anti-fungal agent and anti-PD-1 treatment. The anti-fungal treatment alone did not reverse immunoparalysis. The combination of anti-fungal agent and anti-PD-1 treatment presumably reinvigorated exhausted antigen-presenting cells and T cells by upregulating CD86 expression and IFN-γ production, and diminished IL-10 production, which consequently leaded to the attenuation of secondary aspergillus infection.

REFERENCES

1. Hotchkiss, R.S., G. Monneret, and D. Payen, *Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy.* Nat Rev Immunol, 2013. **13**(12): p. 862-74.
2. Munford, R.S. and J. Pugin, *Normal responses to injury prevent systemic inflammation and can be immunosuppressive.* Am J Respir Crit Care Med, 2001. **163**(2): p. 316-21.
3. Doi, K., et al., *Animal models of sepsis and sepsis-induced kidney injury.* J Clin Invest, 2009. **119**(10): p. 2868-78.
4. Hotchkiss, R.S., et al., *Sepsis and septic shock.* Nat Rev Dis Primers, 2016. **2**: p. 16045.
5. Abbas, A.K., *Cellular and Molecular Immunology.* 2015. **Fifth Edition**.
6. Schrier, R.W. and W. Wang, *Acute renal failure and sepsis.* N Engl J Med, 2004. **351**(2): p. 159-69.
7. Boomer, J.S., et al., *Immunosuppression in patients who die of sepsis and multiple organ failure.* JAMA, 2011. **306**(23): p. 2594-605.
8. Chang, K.C., et al., *Blockade of the negative co-stimulatory molecules PD-1 and CTLA-4 improves survival in primary and secondary fungal sepsis.* Crit Care, 2013. **17**(3): p. R85.
9. Sharpe, A.H., et al., *The function of programmed cell death 1 and its ligands in*

regulating autoimmunity and infection. Nat Immunol, 2007. **8**(3): p. 239-45.

10. Brahmamdam, P., et al., *Delayed administration of anti-PD-1 antibody reverses immune dysfunction and improves survival during sepsis.* J Leukoc Biol, 2010. **88**(2): p. 233-40.

11. Wherry, E.J., *T cell exhaustion.* Nat Immunol, 2011. **12**(6): p. 492-9.

12. Keir, M.E., et al., *PD-1 and its ligands in tolerance and immunity.* Annu Rev Immunol, 2008. **26**: p. 677-704.

13. Yamazaki, T., et al., *Expression of programmed death 1 ligands by murine T cells and APC.* J Immunol, 2002. **169**(10): p. 5538-45.

14. Barber, D.L., et al., *Restoring function in exhausted CD8 T cells during chronic viral infection.* Nature, 2006. **439**(7077): p. 682-7.

15. Day, C.L., et al., *PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression.* Nature, 2006. **443**(7109): p. 350-4.

16. Trautmann, L., et al., *Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction.* Nat Med, 2006. **12**(10): p. 1198-202.

17. Huang, X., et al., *PD-1 expression by macrophages plays a pathologic role in altering microbial clearance and the innate inflammatory response to sepsis.* Proc Natl Acad Sci U S A, 2009. **106**(15): p. 6303-8.

18. Guignant, C., et al., *Programmed death-1 levels correlate with increased mortality, nosocomial infection and immune dysfunctions in septic shock*

patients. Crit Care, 2011. **15**(2): p. R99.

19. Taccone, F.S., et al., *Epidemiology of invasive aspergillosis in critically ill patients: clinical presentation, underlying conditions, and outcomes.* Crit Care, 2015. **19**: p. 7.

20. Vandewoude, K., et al., *Invasive aspergillosis in critically ill patients: analysis of risk factors for acquisition and mortality.* Acta Clin Belg, 2004. **59**(5): p. 251-7.

21. Khasawneh, F., et al., *Isolation of Aspergillus in critically ill patients: a potential marker of poor outcome.* J Crit Care, 2006. **21**(4): p. 322-7.

22. Park, S.J. and B. Mehrad, *Innate immunity to Aspergillus species.* Clin Microbiol Rev, 2009. **22**(4): p. 535-51.

23. Balloy, V. and M. Chignard, *The innate immune response to Aspergillus fumigatus.* Microbes Infect, 2009. **11**(12): p. 919-27.

24. Obar, J.J., T.M. Hohl, and R.A. Cramer, *New advances in invasive aspergillosis immunobiology leading the way towards personalized therapeutic approaches.* Cytokine, 2016. **84**: p. 63-73.

25. Lazar-Molnar, E., et al., *The PD-1/PD-L costimulatory pathway critically affects host resistance to the pathogenic fungus Histoplasma capsulatum.* Proc Natl Acad Sci U S A, 2008. **105**(7): p. 2658-63.

26. Reungwetwattana, T. and A.A. Adjei, *Anti-PD-1 Antibody Treatment and the Development of Acute Pulmonary Tuberculosis.* J Thorac Oncol, 2016. **11**(12): p. 2048-2050.

27. Cuenca, A.G., et al., *Cecal ligation and puncture.* Curr Protoc Immunol, 2010. **Chapter 19**: p. Unit 19 13.

28. Muenzer, J.T., et al., *Characterization and modulation of the immunosuppressive phase of sepsis.* Infect Immun, 2010. **78**(4): p. 1582-92.

29. Unsinger, J., et al., *Interleukin-7 ameliorates immune dysfunction and improves survival in a 2-hit model of fungal sepsis.* J Infect Dis, 2012. **206**(4): p. 606-16.

30. Koh, A.Y., et al., *Mucosal damage and neutropenia are required for Candida albicans dissemination.* PLoS Pathog, 2008. **4**(2): p. e35.

31. Pitarch, A., et al., *Analysis of the serologic response to systemic Candida albicans infection in a murine model.* Proteomics, 2001. **1**(4): p. 550-9.

32. MacCallum, D.M. and F.C. Odds, *Temporal events in the intravenous challenge model for experimental Candida albicans infections in female mice.* Mycoses, 2005. **48**(3): p. 151-61.

33. Bader, T., et al., *Role of calcineurin in stress resistance, morphogenesis, and virulence of a Candida albicans wild-type strain.* Infect Immun, 2006. **74**(7): p. 4366-9.

34. Westwater, C., et al., *Candida glabrata and Candida albicans; dissimilar tissue tropism and infectivity in a gnotobiotic model of mucosal candidiasis.* FEMS Immunol Med Microbiol, 2007. **51**(1): p. 134-9.

35. Clemons, K.V., et al., *Role of IL-10 in invasive aspergillosis: increased resistance of IL-10 gene knockout mice to lethal systemic aspergillosis.* Clin Exp Immunol,

2000. **122**(2): p. 186-91.

36. Caffrey, A.K., et al., *IL-1alpha signaling is critical for leukocyte recruitment after pulmonary Aspergillus fumigatus challenge.* PLoS Pathog, 2015. **11**(1): p. e1004625.

37. Netea, M.G., B.J. Kullberg, and J.W. Van der Meer, *Circulating cytokines as mediators of fever.* Clin Infect Dis, 2000. **31 Suppl 5**: p. S178-84.

38. Launey, Y., et al., *Clinical review: fever in septic ICU patients--friend or foe?* Crit Care, 2011. **15**(3): p. 222.

39. Ozveri, E.S., et al., *The effect of hyperthermic preconditioning on the immune system in rat peritonitis.* Intensive Care Med, 1999. **25**(10): p. 1155-9.

40. Peres Bota, D., et al., *Body temperature alterations in the critically ill.* Intensive Care Med, 2004. **30**(5): p. 811-6.

41. Ahkee, S., L. Srinath, and J. Ramirez, *Community-acquired pneumonia in the elderly: association of mortality with lack of fever and leukocytosis.* South Med J, 1997. **90**(3): p. 296-8.

42. Gabay, C. and I. Kushner, *Acute-phase proteins and other systemic responses to inflammation.* N Engl J Med, 1999. **340**(6): p. 448-54.

43. Bristow, C.L. and R.J. Boackle, *Evidence for the binding of human serum amyloid P component to Clq and Fab gamma.* Mol Immunol, 1986. **23**(10): p. 1045-52.

44. Kumar, A., et al., *Tumor necrosis factor alpha and interleukin 1beta are*

responsible for in vitro myocardial cell depression induced by human septic shock serum. J Exp Med, 1996. **183**(3): p. 949-58.

45. Vincent, J.L., et al., *Administration of anti-TNF antibody improves left ventricular function in septic shock patients. Results of a pilot study.* Chest, 1992. **101**(3): p. 810-5.

46. Opal, S.M. and T. van der Poll, *Endothelial barrier dysfunction in septic shock.* J Intern Med, 2015. **277**(3): p. 277-93.

47. Remels, A.H., et al., *TNF-alpha-induced NF-kappaB activation stimulates skeletal muscle glycolytic metabolism through activation of HIF-1alpha.* Endocrinology, 2015. **156**(5): p. 1770-81.

48. Marik, P.E. and M. Raghavan, *Stress-hyperglycemia, insulin and immunomodulation in sepsis.* Intensive Care Med, 2004. **30**(5): p. 748-56.

49. Hirasawa, H., S. Oda, and M. Nakamura, *Blood glucose control in patients with severe sepsis and septic shock.* World J Gastroenterol, 2009. **15**(33): p. 4132-6.

50. Van den Berghe, G., et al., *Intensive insulin therapy in mixed medical/surgical intensive care units: benefit versus harm.* Diabetes, 2006. **55**(11): p. 3151-9.

51. Buras, J.A., B. Holzmann, and M. Sitkovsky, *Animal models of sepsis: setting the stage.* Nat Rev Drug Discov, 2005. **4**(10): p. 854-65.

52. Remick, D.G., et al., *Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture.* Shock, 2000. **13**(2): p. 110-6.

53. Riedemann, N.C., R.F. Guo, and P.A. Ward, *The enigma of sepsis.* J Clin Invest, 2003. **112**(4): p. 460-7.

54. Marshall, J.C., *Such stuff as dreams are made on: mediator-directed therapy in sepsis.* Nat Rev Drug Discov, 2003. **2**(5): p. 391-405.

55. Zanetti, G., et al., *Cytokine production after intravenous or peritoneal gram-negative bacterial challenge in mice. Comparative protective efficacy of antibodies to tumor necrosis factor-alpha and to lipopolysaccharide.* J Immunol, 1992. **148**(6): p. 1890-7.

56. Rubins, J.B. and C. Pomeroy, *Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia.* Infect Immun, 1997. **65**(7): p. 2975-7.

57. Mizrachi-Nebenzahl, Y., et al., *Differential activation of the immune system by virulent Streptococcus pneumoniae strains determines recovery or death of the host.* Clin Exp Immunol, 2003. **134**(1): p. 23-31.

58. Zantl, N., et al., *Essential role of gamma interferon in survival of colon ascendens stent peritonitis, a novel murine model of abdominal sepsis.* Infect Immun, 1998. **66**(5): p. 2300-9.

59. Maier, S., et al., *Cecal ligation and puncture versus colon ascendens stent peritonitis: two distinct animal models for polymicrobial sepsis.* Shock, 2004. **21**(6): p. 505-11.

60. Freise, H., U.B. Bruckner, and H.U. Spiegel, *Animal models of sepsis.* J Invest Surg, 2001. **14**(4): p. 195-212.

61. Holly, M.K., et al., *Biomarker and drug-target discovery using proteomics in a new rat model of sepsis-induced acute renal failure.* Kidney Int, 2006. **70**(3): p. 496-506.

62. Yasuda, H., et al., *Chloroquine and inhibition of Toll-like receptor 9 protect from sepsis-induced acute kidney injury.* Am J Physiol Renal Physiol, 2008. **294**(5): p. F1050-8.

63. Underhill, D.M. and I.D. Iliev, *The mycobiota: interactions between commensal fungi and the host immune system.* Nat Rev Immunol, 2014. **14**(6): p. 405-16.

64. Iliev, I.D. and I. Leonardi, *Fungal dysbiosis: immunity and interactions at mucosal barriers.* Nat Rev Immunol, 2017. **17**(10): p. 635-646.

65. Brown, G.D., *Innate antifungal immunity: the key role of phagocytes.* Annu Rev Immunol, 2011. **29**: p. 1-21.

66. Romani, L., *Immunity to fungal infections.* Nat Rev Immunol, 2011. **11**(4): p. 275-88.

67. Wuthrich, M., G.S. Deepe, Jr., and B. Klein, *Adaptive immunity to fungi.* Annu Rev Immunol, 2012. **30**: p. 115-48.

68. Netea, M.G., et al., *Immune defence against Candida fungal infections.* Nat Rev Immunol, 2015. **15**(10): p. 630-42.

69. Kullberg, B.J., et al., *Recombinant interferon-gamma enhances resistance to acute disseminated Candida albicans infection in mice.* J Infect Dis, 1993. **168**(2): p. 436-43.

70. Delsing, C.E., et al., *Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series.* BMC Infect Dis, 2014. **14**: p. 166.

71. Huang, W., et al., *Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice.* J Infect Dis, 2004. **190**(3): p. 624-31.

72. van de Veerdonk, F.L., et al., *Differential effects of IL-17 pathway in disseminated candidiasis and zymosan-induced multiple organ failure.* Shock, 2010. **34**(4): p. 407-11.

73. Conti, H.R., et al., *Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis.* J Exp Med, 2009. **206**(2): p. 299-311.

74. Liu, L., et al., *Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis.* J Exp Med, 2011. **208**(8): p. 1635-48.

75. Puel, A., et al., *Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity.* Science, 2011. **332**(6025): p. 65-8.

76. van de Veerdonk, F.L., et al., *Aspergillus fumigatus morphology and dynamic host interactions.* Nat Rev Microbiol, 2017. **15**(11): p. 661-674.

77. VandenBergh, M.F., P.E. Verweij, and A. Voss, *Epidemiology of nosocomial fungal infections: invasive aspergillosis and the environment.* Diagn Microbiol Infect Dis, 1999. **34**(3): p. 221-7.

78. Rivera, A., T. Hohl, and E.G. Pamer, *Immune responses to Aspergillus fumigatus*

infections. Biol Blood Marrow Transplant, 2006. **12**(1 Suppl 1): p. 47-9.

79. Margalit, A. and K. Kavanagh, *The innate immune response to Aspergillus fumigatus at the alveolar surface.* Fems Microbiology Reviews, 2015. **39**(5): p. 670-687.

80. Cramer, R.A., A. Rivera, and T.M. Hohl, *Immune responses against Aspergillus fumigatus: what have we learned?* Current Opinion in Infectious Diseases, 2011. **24**(4): p. 315-322.

81. Hohl, T.M., *Immune responses to invasive aspergillosis: new understanding and therapeutic opportunities.* Curr Opin Infect Dis, 2017. **30**(4): p. 364-371.

82. Bozza, S., et al., *A dendritic cell vaccine against invasive aspergillosis in allogeneic hematopoietic transplantation.* Blood, 2003. **102**(10): p. 3807-14.

83. Augello, A., et al., *Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway.* Eur J Immunol, 2005. **35**(5): p. 1482-90.

84. Brown, J.A., et al., *Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production.* J Immunol, 2003. **170**(3): p. 1257-66.

85. Nakae, S., et al., *Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF.* J Immunol, 2006. **176**(4): p. 2238-48.

86. Parry, R.V., et al., *CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct*

mechanisms. Mol Cell Biol, 2005. **25**(21): p. 9543-53.

87. Zhang, Y., et al., *PD-L1 blockade improves survival in experimental sepsis by inhibiting lymphocyte apoptosis and reversing monocyte dysfunction.* Crit Care, 2010. **14**(6): p. R220.

88. Taratummarat, S., et al., *Gold nanoparticles attenuates bacterial sepsis in cecal ligation and puncture mouse model through the induction of M2 macrophage polarization.* BMC Microbiol, 2018. **18**(1): p. 85.

89. Panpetch, W., et al., *Oral administration of live- or heat-killed Candida albicans worsened cecal ligation and puncture sepsis in a murine model possibly due to an increased serum (1-->3)-beta-D-glucan.* PLoS One, 2017. **12**(7): p. e0181439.

90. Mouzon, B., et al., *Repetitive mild traumatic brain injury in a mouse model produces learning and memory deficits accompanied by histological changes.* J Neurotrauma, 2012. **29**(18): p. 2761-73.

91. Davidson, L., M.G. Netea, and B.J. Kullberg, *Patient Susceptibility to Candidiasis-A Potential for Adjunctive Immunotherapy.* J Fungi (Basel), 2018. **4**(1).

92. Kumar, V., *Targeting macrophage immunometabolism: Dawn in the darkness of sepsis.* Int Immunopharmacol, 2018. **58**: p. 173-185.

93. Qiu, P., Y. Liu, and J. Zhang, *Review: the Role and Mechanisms of Macrophage Autophagy in Sepsis.* Inflammation, 2019. **42**(1): p. 6-19.

94. Neveu, W.A., et al., *IL-6 is required for airway mucus production induced by inhaled fungal allergens.* J Immunol, 2009. **183**(3): p. 1732-8.

95. Delsing, C.E., et al., *Th17 cytokine deficiency in patients with Aspergillus skull base osteomyelitis.* BMC Infect Dis, 2015. **15**: p. 140.

96. Bosmann, M. and P.A. Ward, *The inflammatory response in sepsis.* Trends Immunol, 2013. **34**(3): p. 129-36.

97. Rigato, O. and R. Salomao, *Impaired production of interferon-gamma and tumor necrosis factor-alpha but not of interleukin 10 in whole blood of patients with sepsis.* Shock, 2003. **19**(2): p. 113-116.

98. Leelahavanichkul, A., et al., *Methyl-2-acetamidoacrylate, an ethyl pyruvate analog, decreases sepsis-induced acute kidney injury in mice.* Am J Physiol Renal Physiol, 2008. **295**(6): p. F1825-35.

99. Couper, K.N., D.G. Blount, and E.M. Riley, *IL-10: the master regulator of immunity to infection.* J Immunol, 2008. **180**(9): p. 5771-7.

100. Hotchkiss, R.S., et al., *Sepsis-induced apoptosis causes progressive profound depletion of B and CD4+ T lymphocytes in humans.* J Immunol, 2001. **166**(11): p. 6952-63.

101. Sharpe, A.H. and K.E. Pauken, *The diverse functions of the PD1 inhibitory pathway.* Nat Rev Immunol, 2018. **18**(3): p. 153-167.

102. Nolan, A., et al., *Differential role for CD80 and CD86 in the regulation of the innate immune response in murine polymicrobial sepsis.* PLoS One, 2009. **4**(8): p. e6600.

103. Holling, T.M., E. Schooten, and P.J. van Den Elsen, *Function and regulation of*

MHC class II molecules in T-lymphocytes: Of mice and men. Human Immunology, 2004. **65**(4): p. 282-290.

104. Collins, M., V. Ling, and B.M. Carreno, *The B7 family of immune-regulatory ligands.* Genome Biology, 2005. **6**(6).

105. Carreno, B.M. and M. Collins, *The B7 family of ligands and its receptors: New pathways for costimulation and inhibition of immune responses.* Annual Review of Immunology, 2002. **20**: p. 29-53.

106. Monneret, G., et al., *Monitoring immune dysfunctions in the septic patient: a new skin for the old ceremony.* Mol Med, 2008. **14**(1-2): p. 64-78.

107. Lukaszewicz, A.C., et al., *Monocytic HLA-DR expression in intensive care patients: interest for prognosis and secondary infection prediction.* Crit Care Med, 2009. **37**(10): p. 2746-52.

108. Delano, M.J., et al., *Sepsis Induces Early Alterations in Innate Immunity That Impact Mortality to Secondary Infection.* Journal of Immunology, 2011. **186**(1): p. 195-202.

109. Grimaldi, D., et al., *Nivolumab plus interferon-gamma in the treatment of intractable mucormycosis.* Lancet Infect Dis, 2017. **17**(1): p. 18.

110. El-Khoury, M., et al., *The in vitro effects of interferon-gamma, alone or in combination with amphotericin B, tested against the pathogenic fungi Candida albicans and Aspergillus fumigatus.* BMC Res Notes, 2017. **10**(1): p. 364.

111. Hotchkiss, R.S., et al., *Immune Checkpoint Inhibition in Sepsis: A Phase 1b*

Randomized, Placebo-Controlled, Single Ascending Dose Study of Antiprogrammed Cell Death-Ligand 1 Antibody (BMS-936559). Crit Care Med, 2019. **47**(5): p. 632-642.

112. Bassetti, M., M. Peghin, and A. Vena, *Challenges and Solution of Invasive Aspergillosis in Non-neutropenic Patients: A Review.* Infect Dis Ther, 2018. **7**(1): p. 17-27.

113. Desoubeaux, G. and C. Cray, *Rodent Models of Invasive Aspergillosis due to Aspergillus fumigatus: Still a Long Path toward Standardization.* Front Microbiol, 2017. **8**: p. 841.

114. Panackal, A.A., J.E. Bennett, and P.R. Williamson, *Treatment options in Invasive Aspergillosis.* Curr Treat Options Infect Dis, 2014. **6**(3): p. 309-325.

115. Chang, K., et al., *Targeting the programmed cell death 1: programmed cell death ligand 1 pathway reverses T cell exhaustion in patients with sepsis.* Crit Care, 2014. **18**(1): p. R3.

116. Uchida, N., et al., *Acute progression of aspergillosis in a patient with lung cancer receiving nivolumab.* Respirol Case Rep, 2018. **6**(2): p. e00289.

117. Leelahavanichkul, A., et al., *Comparison of serum creatinine and serum cystatin C as biomarkers to detect sepsis-induced acute kidney injury and to predict mortality in CD-1 mice.* American Journal of Physiology-Renal Physiology, 2014. **307**(8): p. F939-F948.

118. Ricci, Z., et al., *The implications and management of septic acute kidney injury.*

Nat Rev Nephrol, 2011. **7**(4): p. 218-25.

119. Wu, L.P., N. Gokden, and P.R. Mayeux, *Evidence for the role of reactive nitrogen species in polymicrobial sepsis-induced renal peritubular capillary dysfunction and tubular injury.* Journal of the American Society of Nephrology, 2007. **18**(6): p. 1807-1815.

120. Uchino, S., et al., *Acute renal failure in critically ill patients: a multinational, multicenter study.* JAMA, 2005. **294**(7): p. 813-8.

121. Bagshaw, S.M., et al., *Acute kidney injury in septic shock: clinical outcomes and impact of duration of hypotension prior to initiation of antimicrobial therapy.* Intensive Care Med, 2009. **35**(5): p. 871-81.

122. Steinhauser, M.E., et al., *IL-10 is a major mediator of sepsis-induced impairment in lung antibacterial host defense.* Journal of Immunology, 1999. **162**(1): p. 392-399.

123. Wu, H.P., et al., *Serial cytokine levels in patients with severe sepsis.* Inflamm Res, 2009. **58**(7): p. 385-93.

124. Gogos, C.A., et al., *Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options.* J Infect Dis, 2000. **181**(1): p. 176-80.

125. Kalechman, Y., et al., *Anti-IL-10 therapeutic strategy using the immunomodulator AS101 in protecting mice from sepsis-induced death: Dependence on timing of immunomodulating intervention.* Journal of

Immunology, 2002. **169**(1): p. 384-392.

126. Newton, S., et al., *Sepsis-induced changes in macrophage co-stimulatory molecule expression: CD86 as a regulator of anti-inflammatory IL-10 response.* Surg Infect (Larchmt), 2004. **5**(4): p. 375-83.

127. Greenwald, R.J., G.J. Freeman, and A.H. Sharpe, *The B7 family revisited.* Annu Rev Immunol, 2005. **23**: p. 515-48.

128. Armstrong-James, D.P., et al., *Impaired interferon-gamma responses, increased interleukin-17 expression, and a tumor necrosis factor-alpha transcriptional program in invasive aspergillosis.* J Infect Dis, 2009. **200**(8): p. 1341-51.

129. Shao, C., et al., *Transient overexpression of gamma interferon promotes Aspergillus clearance in invasive pulmonary aspergillosis.* Clin Exp Immunol, 2005. **142**(2): p. 233-41.

130. Jarvis, J.N., et al., *Adjunctive interferon-gamma immunotherapy for the treatment of HIV-associated cryptococcal meningitis: a randomized controlled trial.* AIDS, 2012. **26**(9): p. 1105-13.

131. Chai, L.Y., et al., *Anti-Aspergillus human host defence relies on type 1 T helper (Th1), rather than type 17 T helper (Th17), cellular immunity.* Immunology, 2010. **130**(1): p. 46-54.

132. McBerry, C., et al., *PD-1 modulates steady-state and infection-induced IL-10 production in vivo.* Eur J Immunol, 2014. **44**(2): p. 469-79.

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