การศึกษาอนุภาคทองคำระดับนาโนเมตรเพื่อลดความรุนแรงของ sepsis จากการกระตุ้นโดยวิธี CLP ในสัตว์ทดลอง และกระตุ้นในเซลล์แมคโครฟาจ

นางสาวสุจิตรา ธาราธรรมรัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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GOLD NANOPARTICLES IN CLP MODEL AND MECHANISM IN BONE MARROWS DERIVED MACROPHAGES CELL FOR SEPSIS SEVERITY ATTENUATION

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สุจิตรา ธาราธรรมรัตน์ : การศึกษาอนุภาคทองคำระดับนาโนเมตรเพื่อลดความรุนแรงของ sepsis จากการกระตุ้นโดยวิธี CLP ในสัตว์ทดลอง และกระตุ้นในเซลล์แมคโครฟาจ (GOLD NANOPARTICLES IN CLP MODEL AND MECHANISM IN BONE MARROWS DERIVED MACROPHAGES CELL FOR SEPSIS SEVERITY ATTENUATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. นพ. ดร. อัษฎาศ์ ลีฬหวนิชกุล, 63 หน้า.

ภาวะติดเชื้อในกระแสเลือดในปัจจุบันนี้เป็นปัญหาหนึ่งทางสาธารณสุขซึ่งมีผลทำให้อัตราการตายของผู้ป่วยเพิ่มสูงขึ้นทั่วโลก การติดเชื้อในกระแสเลือดนั้นคือภาวะการอักเสบทั่วทั้งร่างกายซึ่งตอบสนองต่อการติดเชื้อจุลชีพโดยเฉพาะแบคทีเรีย โดยความรุนแรงของโรคจะขึ้นกับลักษณะทางภูมิคุ้มกันของแต่ละบุคคลและความรุนแรงของเชื้อโรค การรักษาในปัจจุบันเน้นการรักษาตามอาการ โดยใช้ยาปฏิชีวนะสำหรับฆ่าเชื้อจุลชีพร่วมกับการดูแลประคับประคองอาการผู้ป่วยไม่ให้รุนแรงมากขึ้น อีกทั้งมีการใช้ยาบางชนิดเพื่อเสริมการรักษาซึ่งยังคงมีข้อจำกัดอยู่ในปัจจุบัน อนุภาคทองคำระดับนาโนเมตรมีการใช้มาตั้งแต่อดีตเพื่อการลดการอักเสบ และในปัจจุบันยังคงใช้เพื่อการรักษาโรครูมาติกอีกด้วย ผู้วิจัยจึงตั้งสมมติฐานว่าอนุภาคทองคำระดับนาโนเมตรจะสามารถลดการอักเสบจากภาวะติดเชื้อในกระแสเลือดได้เช่นกัน จึงได้ทดสอบความสามารถของอนุภาคทองคำระดับนาโนเมตรในการลดการอักเสบ ผ่านทางเซลล์แมคโครเฟจซึ่งได้จากไขกระดูกพร้อมทั้งบ่มร่วมกับอนุภาคทองคำระดับนาโนเมตร และ ทดสอบในหนูทดลองโดยใช้โมเดลผูกและเจาะลำไส้ในหนูในทดลอง (Cecal ligation and puncture; CLP) เพื่อกระตุ้นให้เกิดภาวะติดเชื้อในกระแสเลือด จากการศึกษาพบว่าอนุภาคทองคำระดับนาโนเมตรไม่เป็นพิษต่อเซลล์ มีผลลดปริมาณการหลั่งของไซโตไคน์ในซีรัมกลุ่มที่กระตุ้นให้เกิดการอักเสบ (TNF-α, IL-1β และIL-6) เช่นเดียวกันในเซลล์และหนูทดลอง แต่อย่างไรก็ตามปริมาณของไซโตไคน์ที่ลดการอักเสบ (IL-10) พบว่ามีปริมาณมากขึ้นในเซลล์ แต่ลดลงในหนูทดลอง อีกทั้งอนุภาคทองคำระดับนาโนเมตรยังมีผลต่อการกำจัดเชื้อแบคทีเรีย รวมถึงมีผลให้เกิดการเปลี่ยนแปลงเซลล์แมคโครเฟจให้เป็นเซลล์ที่ต่อต้านการอักเสบ (M2) จึงเป็นที่น่าสนใจว่าว่าอนุภาคทองคำระดับนาโนเมตรมีผลต่อการเปลี่ยนแปลงระบบภูมิคุ้มกันให้ช่วยลดความรุนแรงของภาวะติดเชื้อในกระแสเลือดได้ ซึ่งในการศึกษาต่อไปนั้น เป็นที่น่าสนใจที่จะศึกษาถึงกลไกการทำงานของอนุภาคทองคำระดับนาโนเมตรต่อภาวะติดเชื้อในกระแสเลือด และการปรับเปลี่ยนอนุภาคให้มีความจำเพาะและมีประสิทธิภาพมากขึ้นเพื่อการลดความรุนแรงของโรคในผู้ป่วยต่อไป

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ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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SUJITTRA TARATUMMARAT: GOLD NANOPARTICLES IN CLP MODEL AND MECHANISM IN BONE MARROWS DERIVED MACROPHAGES CELL FOR SEPSIS SEVERITY ATTENUATION. ADVISOR: ASST. PROF. ASADA LEELAHAVANICHKUL, M.D., Ph.D., 63 pp.

Sepsis is still a major worldwide- public health problem as a leading cause of the high mortality rate condition. Sepsis is the inflammatory condition in host due to bacterial infections. Sepsis severity depends on the individual's immune responses and the organism virulence factor. The current strategies for sepsis treatment are based, mainly on, symptomatic treatment with antibiotics. However, the adjuvant therapy in sepsis is interesting. Gold nanoparticles are anti-inflammatory drug from the ancient time which is still currently used for the treatment of rheumatoid arthritis. Thus, we hypothesized that gold nanoparticles could be effective in reducing an inflammation in sepsis and testes in bone marrow (BM)-derived macrophages (*in vitro*) and cecal ligation and puncture (CLP) mouse model (*in vivo*). After treat with gold nanoparticles, the pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) was reduced in both *in vitro* and *in vivo*. But, the anti-inflammation cytokine (IL-10) was increased *in vitro* but decreased *in vivo*. However, CLP mice showed the higher survival rate with gold nanoparticles treatment. the bactericidal property and the M2 macrophages induction property of gold nanoparticles are also demonstrated. In conclusion, gold nanoparticles attenuated sepsis through the immune modulation and bactericidal activity. Further studies on the mechanisms and the translation to patients are interesting.

Field of Study: Medical Microbiology Student's Signature .....

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LIST OF ABBREVIATIONS

ALT	Alanine transaminase
ANOVA	Analysis of variance
AuNPs	Gold nanoparticles
BM	Bone marrow
BUN	Blood urea nitrogen
CLP	Cecal ligation and puncture
DMEM	Dulbecco's Modified Eagle Medium
ELISA	Enzyme linked immunosorbent assays
FBS	Fetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-6	Interleukin 6
iNOS	inducible nitric oxide synthase
NSS	Normal saline solution
PBS	Phosphate buffer solution
qPCR	Quantitative polymerase chain reaction

Scr	Serum creatinine
SE	Standard error
TNF-α	Tumor necrosis factor-alpha

CHAPTER I
INTRODUCTION

Sepsis is life-threatening organ dysfunction due to a dysregulated host response against infection independent of the type of organisms, which is a major worldwide-public health problem (1). However, in intensive care units, bacterial infection is a major cause of death of the patients, especially gram-negative bacterial infection. Although the immune response in sepsis is complex, innate immune cells are important for the production of pro-inflammatory and anti-inflammatory cytokines. One of the important pathogenesis for determining sepsis severity is the imbalance of pro-inflammatory cytokines and anti-inflammatory cytokines, such as IL-6, TNF-α, IL-1β, IL-10 and IL-4. Moreover, malfunction of immune response against sepsis, including hyper-inflammatory response and immune suppression phase is danger for patients (2). These imbalance of the immune responses associated with organism burdens. Therefore, the rapid eradication of organisms is a most important strategy to control sepsis.

Despite several progresses on life-supporting system in critically-ill patients, treatment of sepsis still focuses on antibiotics, symptomatic treatments, supportive therapies and adjunctive therapies (3). Unfortunately, several adjunctive treatment of sepsis such as activated protein C, antithrombin III, intravenous immunoglobulin, statin and corticosteroids, etc. was mentioned, but do not show the significant benefit to sepsis in clinical practice (4). Extracorporeal therapies such as hemofiltration, hemoperfusion,

plasmapheresis incorporated with continuous renal replacement therapy and blood purification are of interest and are currently conducted as the ongoing clinical trials (4). However, these treatments are very expensive for Thai population. In all, sepsis mortality rate is still high and sepsis-treatments strategies are currently limited, the adjunctive therapies of sepsis are still interesting.

One of the interesting strategy is gold-based substance which have been used for the anti-inflammation since the ancient time (5). Gold nanoparticles (AuNPs) are inert metallic forms, which less than 100 nm in diameter, various shapes and sizes. Nano materials have an ability to conjugate with several interesting peptides or proteins or antimicrobial substances. Also, gold nanoparticle (AuNPs) also associated with several potentials of medical applications such as magnetic resonance imaging (MRI), drug and gene delivery, cancer treatment and gene therapy or bio-detection. The toxicity of AuNPs is also mentioned and is related to dose, surface charge, sizes, shapes, molecular gold location, and administration route. It has been shown that tail vein injection results the least toxicity in comparison between oral and intraperitoneal injection of mouse models (6). Interestingly, spherical AuNPs at the diameter of 20-30 nm is suitable properties that non-toxic and suitable for targeting macrophages (7). More importantly, AuNPs also down-regulates TNF-α and IL-6 mRNA expression in adipose cell (7).

Because RAW 264.7, macrophage-like cell line derived from mice injected with Abelson murine leukemia virus (8), shows lower phagocytic activity, slower phagosome

acidification and weaker proteolytic activity than bone marrow derived macrophages (BM-derived macrophages) (8), we used BM-derived macrophages for testing AuNPs in our experiments.

Regarding sepsis mouse model, several models were available with different limitations; lipopolysaccharide injection, cecal ligation and puncture (CLP) and bacterial infusion. LPS model is technically easy but there is no bacteremia different from patient sepsis. Cecal ligation and puncture (CLP) induced polymicrobial bacteremia with cytokine profiles that is similar to human. On the other hand, bacterial infusion coud induces sepsis by a single pathogen, but the overwhelm bacteria injection is not mimic sepsis pathogenesis in patients (9). In this study, we used CLP to induce sepsis due to the similarity of CLP with human sepsis.

Because AuNPs at a diameters of 21.3±0.7 nm showed a potent immune suppression in macrophage from the adepose cell of mice, we tested the effectiveness AuNPs at 21 nm diameters in BM-derived macrophages and in mice with CLP-induce sepsis.

Hypothesis

Gold nanoparticles (AuNPs) attenuate macrophage induced pro-inflammatory cytokines *in vitro* and reduce sepsis severity *in vivo*.

Objectives

1. To determine the effects of gold nanoparticles (AuNPs) in macrophage functions
2. To determine the effects of gold nanoparticles (AuNPs) for sepsis treatment

CHAPTER II
LITERATURE REVIEWS

Sepsis

The Third International Consensus Definitions for Sepsis and Septic Shock [Sepsis-3] has defined the new definition of sepsis “Sepsis is life-threatening organ dysfunction due to a dysregulated host response to infection” (1). Sepsis is an important cause of death which lead to high mortality rate in hospital and intensive care unit (ICU) (10). In addition, the increase mortality rate can be found in septic shock which is a subset of severe sepsis (1). Infection in sepsis that can begin in any parts of the body such as respiratory tract, central nervous system, cardiovascular, urinary tract, and soft tissue (11, 12).

Bacterial infection is the most common cause of sepsis, which including gram-negative and gram-positive bacteria. The most commonly isolated gram-negative bacteria are *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* spp and *Acinetobacter* spp and for the gram-positive bacteria are *Staphylococcus aureus* and *Streptococcus pneumoniae* (12, 13). The virulence factors of gram-negative and gram-positive bacteria are different. Gram-positive bacteria consist of peptidoglycan (PGN) and lipoteichoic acid (LTA) while gran negative is lipopolysaccharide (LPS) and peptidoglycan (PGN) (14, 15)(Figure1).

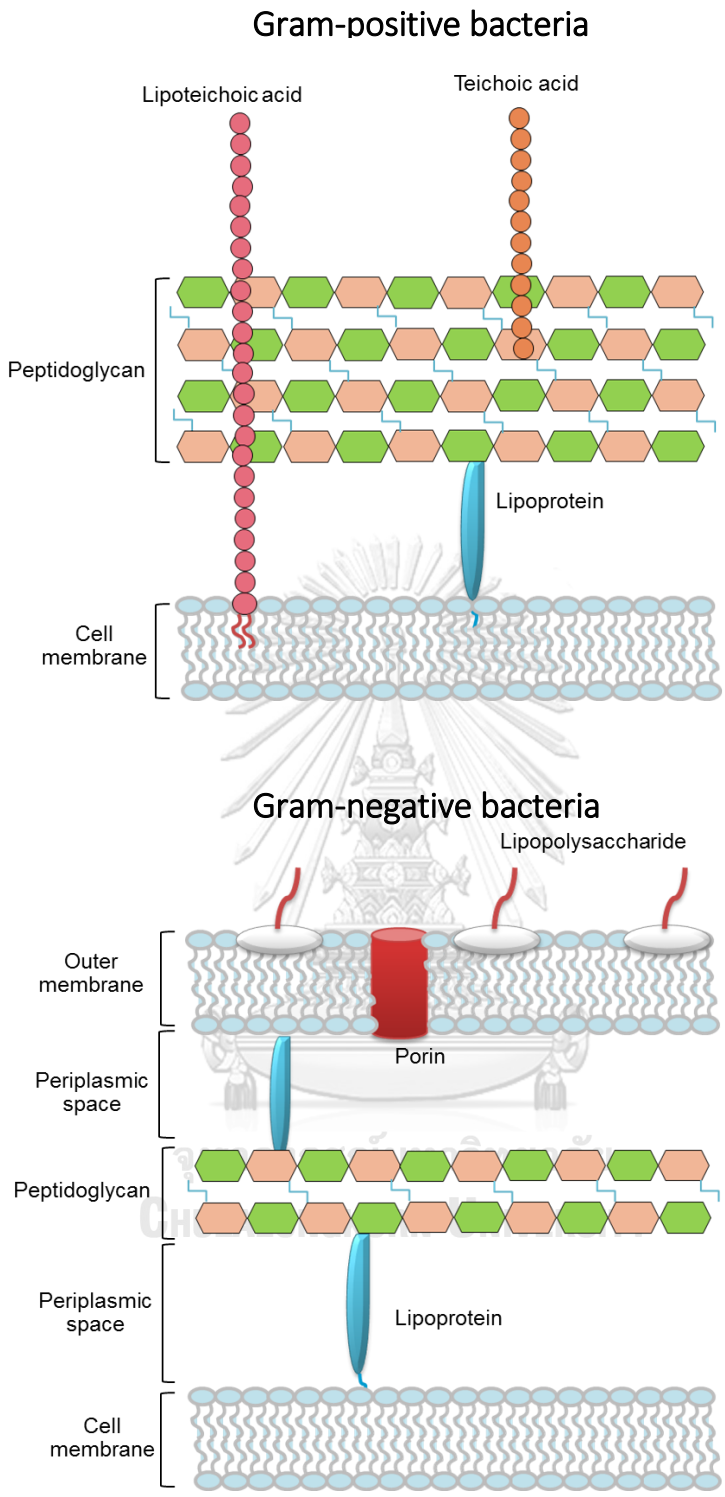


Figure 1 *The structure of gram-positive bacteria and gram-negative (15)*

Lipopolysaccharide (LPS) is a component of the outer membrane in gram-negative bacteria that is a major bacterial virulence factor (16). The lipopolysaccharide (LPS) molecule classically consist of

(i) Lipid A: This is a part of outter membrane that located at the inner region linked to the lipoprotein, nessesary to the inflammatory responsibility by the recognition with Toll-like receptor 4 and myeloid differentiation factor 2 (TLR4-MD2) which found on macrophages, monocytes and dendritic cells (16).

(ii) Core regions: This part is separated into two regions (inner core and outer core), that a set of sugar consist of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and L-glycero-D-manno-heptose.

(iii) O-antigen: This is a polysaccharide structure part consist of repeating oligosaccharide which more variation in different bacteria uses for bacterial differentiation or host immune evasion. O-antigen is important to exposure to the external environment directly.

Sepsis immune response

The response of immunity is working together between innate and adaptive immune system, but the innate immune system is the first one that comes to eradicate pathogens via a pattern-recognition receptors (PRRs). Pathogen-associated molecular patterns (PAMPs) are interactive with PRRs of host immune cells lead to the secretion of cytokines and chemokines for defense against pathogens (13). The central role of PRRs are Toll–like receptors (TLR) that express in mammalian immune cells and divided into 9-11 types (17)(Table1).

The Toll-like receptors 4 (TLR4) activation is one of the important role in sepsis physiology which interacts with LPS. LPS from the outer membrane of gram-negative bacteria are recognized with TLR4 on the cell wall and CD14 and MD-2 molecules. Then, TLR4 stimulation is reacting to the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88) and activate nuclear factor-κβ (NF-κβ). The pro-inflammatory genes in the nucleus were traslated into pro-inflammatory cytokines such as TNF-α, IL-1, IL-6. But, the overstimulation or the loss of immune homeostasis may lead to induce excessive inflammation and finally to sepsis (18).

Table 1 Pathogen-associated molecular patterns (PAMPs) and specific recognition by Toll-like receptors (TLRs)

PAMPs	Target microorganism	TLRs	Locations
Triacyl lipopeptides	Most bacteria	TLR1,TLR2	Cell membrane
Peptidoglycan	Most bacteria	TLR2	Cell membrane
Double-stranded RNA	Viruses	TLR3	Endosome
Lipopolysaccharide (LPS)	LPS-induced inflammation models	TLR4	Cell membrane
Flagellin	Flagellated bacteria	TLR5	Cell membrane
Diacyl lipopeptides	Mycobacteria	TLR6, TLR2	Cell membrane
Single-stranded RNA	Viruses	TLR7	Endosome
Single-stranded RNA	Viruses	TLR8	Endosome
CpG DNA	Bacterial DNA	TLR9	Endosome

The secretion of many pro-inflammatory cytokines from the innate immune system, including neutrophils and macrophages, lead to host inflammatory response within 1-3 days after the activation. However, the severity of inflammatory response depends on several factors, such as pathogen virulency, pathogen burdens, and host genetic. In the early state of sepsis, a cytokine storm causes symptoms of hyper-inflammatory response, including fever, shock, multiorgan failure, and hyper-catabolism (cause of death in within 3 days of sepsis). In contrast, if both of innate and adaptive immune system is loss of function (immune exhaustion), host could not clear pathogens and the risk of secondary

infections increased. The late sepsis death can occur over days 6 of sepsis-induction (immune exhaustion) (2, 13)(Figure2).

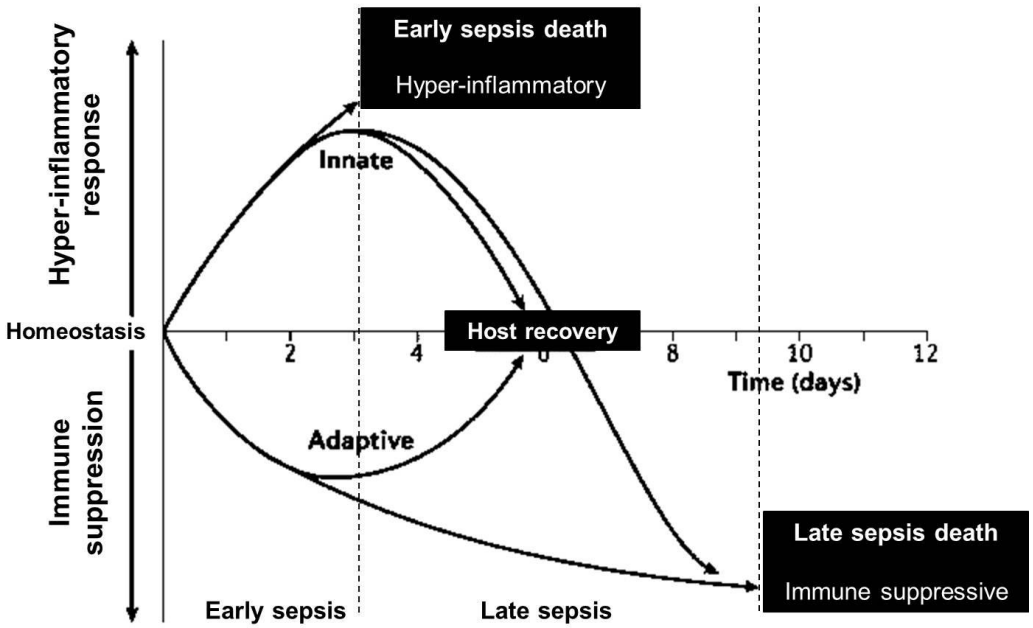


Figure 2 The sepsis immune response in host by dividing into early sepsis death and late sepsis death, associated with hyper-inflammatory response and immune suppression, respectively (13).

Cytokines in sepsis

Cytokines are a large group of small proteins, peptides or glycoproteins and low molecular weights (<40 kDa), which are secreted from many specific immune cells. Cytokines associate with proliferation, differentiation, cellular activation, signaling and migration of cells (19). Cytokine expression in sepsis mostly initiates by the innate immune cells, such as macrophage, neutrophil, dendritic cells. Moreover, the adaptive immune cells, including T cells are also produce and release cytokines (20). The important cytokines that involves in sepsis are pro-inflammatory cytokines, including TNFα, IL-6, IL-1β, IL-12 and IFN-γ, and anti-inflammatory cytokines, including IL-4, IL-10, IL-13, and TGF-β. A necessary role of pro-inflammatory cytokines is up regulates immune cells after pathogen recognition, while anti-inflammatory cytokines play a role to decrease immune response and limit host-immune response after pathogens elimination (21). Pro-inflammatory cytokines and anti-inflammatory cytokines in sepsis are mostly associated and secreted by several immune cell (Table2).

Table 2 Pro-inflammatory cytokines and anti-inflammatory cytokines, which is mostly associated with sepsis and macrophages (19-21)

Cytokines	Main source	Main functions
Pro-inflammatory		
TNFα	Macrophages, NK cells, T cells, fibroblasts	Activation of inflammation, coagulation, apoptosis and differentiation
IL-1β	Macrophages, dendritic cells, fibroblasts, endothelial cells, keratinocytes	Activation of inflammation, coagulation and differentiation of T_H17
IL-6	Macrophages, endothelial cells, T cells	Activation of inflammation, B, T lymphocytes and modulation of acute phase response
IL-12	Macrophages, dendritic cells	Activation of NK cells to produce IFN-γ and differentiation of T_H1
Anti-inflammatory		
IL-10	Macrophages, T cells	Inhibition of innate immune response to decrease pro-inflammatory cytokines and Inhibition of expression of IL-12, IFN-γ
IL-4	T cells (T_H2), mast ells	Differentiation of T_H2 but suppression of macrophage activity (no release during sepsis)
TGF-β	Macrophages, T cells	Induction of immune suppression of pro-inflammatory cytokines, induce tissue repair and inhibition of differentiating B, T cells
IL-13	T cells (T_H2)	Inhibition of expression of pro-inflammatory cytokines such as TNFα, IL-1

Macrophages

The major role of macrophage is phagocytosis of microorganisms and dead cells (22). Phagocytosis is a process of the engulfment of the particle. After the phagocytes, particles are surrounded in phagosome and the lysosome will come for released digested enzymes. Phagolysosomes is the combination of phagosome and lysosome. Macrophage is an important innate immune cell that is distributed throughout the body. Monocyte are circulating in blood and called macrophage in tissues, with diferrent names such as microglia in brain, kupffer cells in liver, alveolar macrophage in lung and langerhans cells in skin (23). Macrophage is the important cells in sepsis study as the major innate immune cells responsible to react with pathogens and releases both of pro-inflammatory cytokines and anti-inflammatory cytokines.

Sepsis therapy

There are several progression on a life-supporting system in critically-ill patients. But treatment of sepsis still focus on antibiotics and symptomatic treatments (3). After the diagnosis, the elimination of microorganism was focused on using antibiotics. Several supportive therapies are used to relieve the severity of the patient such as vasopressor therapy (inotropic drugs) and fluid replacement therapy (3).

Recently, several adjunctive therapies are performed including, corticosteroids, Intravenous immunoglobulin (IVIG), antithrombin III, statin and recombinant activated

protein C (rAPC), granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), IFN-γ and IL-7 (4). However, none of these are beneficial after the clinical control trial. In addition, the extracorporeal therapies (blood purification) such as hemofiltration for remove inflammatory cytokines, hemoperfusion for a decrease of toxin from pathogens, plasmapheresis for exchange the plasma and extracorporeal membrane oxygenation, are mentioned as the beneficial strategies but with very high cost (4, 10). Moreover, the specific nutritional supplementation is also used to increase the survival rate of the patient (4).

Nevertheless, in human, the treatment with recombinant activated protein C (rAPC) and several anti-cytokines are not useful. Hence, the adjunctive treatments strategies against sepsis are still limited (4).

Animal models of sepsis

Animal models of sepsis are important for the medical research of sepsis and septic shock to use as the *in vivo* study (24). Sepsis models are divided into 3 types, multi-organ involvement, and multi-organ involvement that superimposed to chronic diseases and local infection.

1. Infection with multi-organ involvement

The standard sepsis animal models in this group are (i) exogenous toxin injection (ii) infusion or instillation of exogenous bacteria which are not surgical models and (iii) alteration of the animal's endogenous protective barrier (surgical models).

1) exogenous toxin injection such as LPS-induced inflammation models; LPS is one of the endotoxins that is a component of gram-negative bacteria. LPS injection or infusion is a widely used procedure to induced inflammatory response, but not the infection. The toxin injection is simple and quite simple, but the drawback was found such as more severe than the sepsis in human and the increased inflammatory mediators are early and transient (9).

2) infusion or instillation of exogenous bacteria such as bacterial infusion; bacterial injected through the intravenous route to induced sepsis by a single bacteria. Because of the sepsis in human can be found by a single pathogens, this model is an attempt to mimic human condition. This model can simulate several human factors (not the intrarenal microcirculation) but requires for the large animals for study (9).

3) alteration of the animal's endogenous protective barrier such as cecal ligation and puncture (CLP) and colon ascendens stent peritonitis (CASP); CLP model is the most widely used for inducing sepsis

by polymicrobial pathogens. CLP model has several advantages in the immune response including mimic the hyperdynamic phase (pro-inflammatory cytokines and reactive oxygen species) in human sepsis (25). CLP model is performed by the ligation at the cecal tip, puncture twice with a needle (25). CASP model is a procedure for inducing polymicrobial sepsis by the insertion of a stent to the intestinal. This model represents the leakage of intestinal content into the peritoneal cavity. CLP and CASP model are similar but there are the variability of models by ages and strains of mice (9).

2. Infection with multi-organ involvement that superimposed to the chronic diseases

Infection is the most important complication of several chronic diseases (such as chronic kidney disease: CKD).Hence, this group of the models is induction of the infection in the model of chronic diseases. Indeed, the sepsis-CKD and sepsis-diabetes millitus models show the higher severity than the sepsis in former healthy. These models will help understanding the pathophysiology of infection in these chronic diseases (9).

3. Local infection.

Local infection induced sepsis models are performed by the injection of bacterial into an organ (muscle, brain, lung). The example of these models are pneumonia models, myositis model, gastritis model and cellulitis model which is beneficial for the experiments in these topics (26).

Nanotechnology

The study of nanotechnology focusing on the small size particles (27). Molecules of nano-sized material can define with lengths that range from 1-100 nm in the dimentions at 0-3 (28). Nanomaterials have several abilities depends on the variety of shapes, sizes and conjugated molecules. Nanomaterial can be defined into 3 groups;

(i) Nano rods and nano wires shapes

(ii) Tubes, fibers, platelets shapes

(iii) Particles, quantum dots, hollow spheres shapes

Applications of nanotechnology and gold nanoparticles

Nanotechnology has several applications in industry, engineering, computer technology, biology and medicine (29). The multi-directional applications of nanoparticles are useful in several fields (29)(Figure 3).

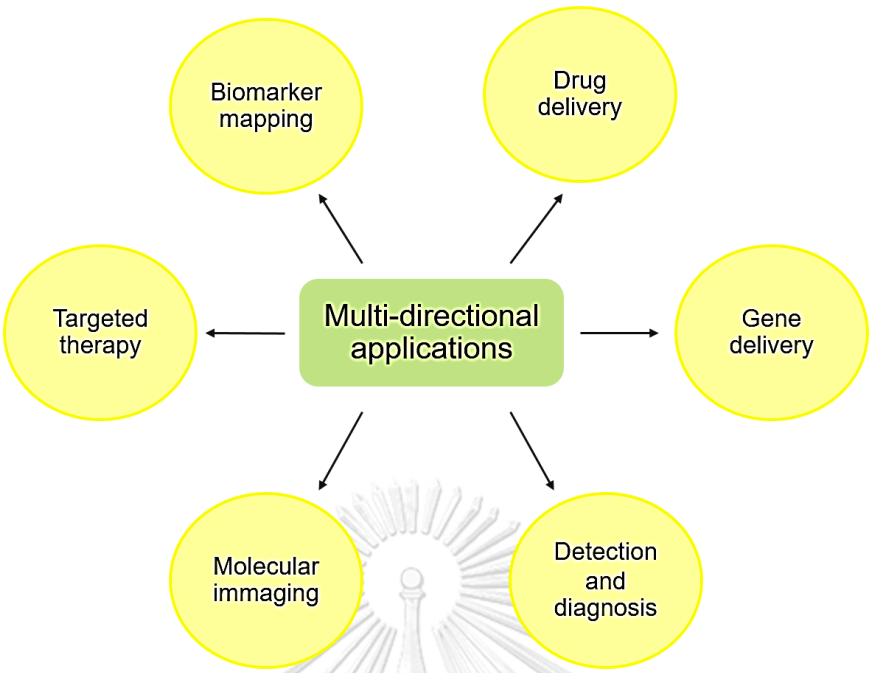


Figure 3 The multi-directional applications for the biomedicine (29)

The shapes and sizes of the particles such as the virus-like particle, nanoemulsions including of micelle, liposome and oil-in-water emulsion and simple nanoparticles including of dendrimer, spherical fullerene and cylindrical fullerene are important for the specific propertied (30).

Surface molecule conjugation and the administration route are also important for the properties of the particles (31). In the previous study, nanoparticles have many effects on the immune system both in immunostimulation and immunosuppression (32)(Table 3).

Table 3 A various nanoparticles effected to the immunomodulation (32)

Nanoparticles	size	Exposure routes/doses	Outcomes	Cytokines/ chemokines	Animal
PEG coated GNP	13 nm	Intravenously 0, 0.17, 0.85 or 4.26 mg/kg	Acute inflammation	MCP-1/CCL-2↑, MIP-1/CCL-3↑, MIP-1β↑, RANTES/CCL-5↑, IL-1β↑, IL-6↑, IL-10↑, IL-12β↑, TNF-α↑	BALB/c
Citrate-stabilized GNPs	40 nm	Oropharyngeal aspiration 0.8 mg/kg	Hypersensitivity	MMP-9↑, MIP-2↑, TNF-α↓, IL-6↓	TDI-sensitised mice (BALB/c)
GNP	21 nm	Intraperitoneally injection 7.85 g/g	Antiflammatory	TNF-αmRNA↓, IL-6 mRNA↓	Male C57BL/6
Citrate-stabilized GNPs	5 nm	100 nmol Au/kg	Antiflammatory	IL-1β↓	IL-1 β model mice (male C57BL/6)
Ag conjugated to core nanobeads	40–50 nm	Intradermally	Immunogenic	IFN-γ↑, antibody↑	$H\text{-}2K^{b}$ C57BL/6
Polystyrene NP	50 nm	ntratracheal administration of 200 μg/mouse	Anti-inflammation immunosuppression	IL-4↓, IL-5↓, IL-13↓	Allergen challenge mice
MPLA : NLP	6–25 nm	intraperitoneal injection ion of 1, 5, 10, 20 μg/mouse	Enhanced immunostimulatory	IL-6↑, TNF-α↑, MIP-1α↑	Female BALB/c mice

Gold nanoparticles (AuNPs) is one of the most widely used in the nanomedical applications such as for magnetic resonance imaging (MRI), drug and gene delivery, bio-detection, biosensors, gene therapy and cancer treatment (33). Because of the properties which can be modified to another forms or shapes, solubility function, chemical properties

as electrical conductivity and optical properties. The stability of AuNPs are high and the modification in different sizes and shapes are also easy to control. In 2010, Zhang et al. found that the toxicity of AuNPs is related to dose, surface charge, molecular gold location and administration route. It has been shown that tail vein injection results in the least toxicity in comparison with oral and intraperitoneal injection (6). The elimination of nanoparticles are still not clear, but currently defined into 3 organs including (i) kidneys, (ii) liver and (iii) reticuloendothelial system (34).

(i) nanoparticles at size <6 nm eliminate through kidneys into urine within hours to days by the renal clearance function.

(ii) nanoparticles at size >6 nm eliminate through liver as bile into feces in hours to weeks by hepatobiliary clearance function.

(iii) large and non-degradable AuNPs eliminate through the reticuloendothelial system within months to years.

Because of the non-toxic property of AuNPs, its applications in the medicine are increasing. In 2012, Pereira et al. demonstrated the treatment effect of ANPs against endotoxin-induced uveitis in rats through the down-regulation of TLR4 and NF-KB from macrophages (35).

CHAPTER III
MATERIALS AND METHODS

Gold nanoparticles (AuNPs) preparation

The gold nanoparticles were synthesized followed the standard citrate-reduction method as previously described (7, 36). Chloroauric acid solution ($HAuCl_4.3H_20$) at 1mM were added in Milli-Q water and heated with vigorously stirring. After solution boiled, 38.8 mM of sodium citrate was quickly added into the solution. That temperature altered the color of the solution from yellow into deep blue and, shortly afterwards, to deep red. Then the deep red color solution was continuously heat for another 15 minutes at room temperature. The final solution containing spherical gold nanoparticles (AuNPs) with an average size at 21.3±0.7 nM was stored at 4°C prevented from light until used. The determination of AuNPs size was confirmed by Ultra-Violet-Visible spectroscopy (Beckman Coulter DU 800 Spectrophotometer, Brea, CA, USA).

Bone marrow (BM) derived macrophage preparation

Macrophages were derived from bone marrow (BM) following the previously published methods (37, 38). BM-derived macrophages were obtained from femurs of mice and centrifuged at 6,000 rpm in 4°C for 10 minutes, then red blood cells were lysed by Red blood cells lysis buffer (Ammonium chloride lysis buffer consisting of KHCO, NH_4Cl and EDTA) in 4°C for 10 minutes and centrifuged at 1,000 rpm. The the pellets were counted and cells at $5x10^6$ cells/plate were incubated for 7 days with DMEM complete [High glucose Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% sodium pyruvate] plus 5% house serum and 20% media of the L929 cell line [containing a macrophage-colony stimulating factor (M-CSF)]. Then, cells were incubated in a humidified 5% CO_2 at 37°C. Cells were harvested with cold phosphate buffer solution (PBS) and the macrophage phenotype was analyzed with anti-F4/80 and anti-CD11b antibody staining (BioLegend, San Diego, CA, USA) by flow cytometry.

Cell viability determination (MTS assay)

Cell viability was measured by MTS assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Kit, Promega Corporation, WI, USA) according to the manufacturer's instruction. BM-derived macrophages at $1x10^5$ cells/well were seeded in 96-well culture plate with different concentrations and cultured for 24 hours. Cells were treated with various concentrations, including; 12.5, 25 and 50 ppm and combination of 100 ng/ml LPS with AuNPs 25 and 50 ppm, respectively. After an incubation time 20µl of Cell Titer 96 Aqueous One Solution was added into each well and incubated continuously in humidified 5% CO_2 incubator at 37°C for 2 hours. Cell viability was measured by micro-plate reader with wavelength at 490 nm.

Production of macrophage cytokine determination

BM-derived macrophages at $1x10^5$ cells/well were seeded in 96-well tissue culture plate with DMEM complete. Then 100 ng/ml of LPS in different concentrations of AuNPs (12.5, 25 and 50 ppm) in humidified 5% CO_2 at 37°C were incubated for 3, 6 and 24 hours (adjusted into the similar volume). At each time point, the culture supernatant was collected and stored at -80°C until analysis.

Cell culture supernatant was measured for cytokines (TNF-α, IL-6, IL-1β, IL-4 and IL-10) by sandwich ELISAs assay (eBioscience, San Diego, CA, USA) followed the manufacturer's instructions.

Antibiotic activity of gold nanoparticles determination

The procedure for determining antibiotic activity followed a previous publication (39). *Escherichia coli* ATCC 25922 (ATCC, Manassas, VA, USA) was sub-cultured in Tryptic soy broth (TSB) (Thermo Scientific, Waltham, MA, USA) at 37°C for 12 hours. *E. coli* approximately at $2.4x10^6$ CFU, as determined by a spectrophotometer (ELx808 absorbance reader; BioTek, Shoreline, WA) at optical density 600 nm at 0.003 (OD 600 nm at 0.003), were diluted in TSB and added with AuNPs in different concentrations; 12.5, 25 and 50 ppm, respectively. Then the solutions were incubated at 37°C for 1- 4 hours. After that, the supernatant in serial dilution was plated in Tryptic soy agar (TSA) (Thermo Scientific, Waltham, MA, USA), incubated at 37°C overnight before bacterial colony enumeration. *E. coli* in only TSB or in 100 µg/ml of gentamicin were used as the positive and negative control group, respectively.

Analysis of macrophage polarization by quantitative polymerase chain reaction (qPCR)

The procedure was adapted from a previously published method (40, 41). BM-derived macrophages at $5x10^5$ cells/well were seeded in 12-well tissue culture plate with DMEM complete. Cells were activated into M1 macrophage and M2 macrophage by LPS (100 ng/ml) and IL-4 (10 ng/ml), respectively, with AuNPs in the different concentrations (12.5, 25 and 50 ppm) or PBS control. All groups were incubated in humidified 5% CO_2 incubator at 37°C for 3 hours, then the culture supernatant was removed and 400 µl of

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added per well to extract RNA from the macrophages by followed to the manufacturer's protocol. The purified RNA was synthesized into complementary DNA, amplified by quantitative polymerase chain reaction (qPCR) by used of *iNOS* and *Arginase1* for characterize of M1 and M2 macrophages, respectively. The nucleotide sequences were as followed:

iNOS	forward	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3',
	reverse	5'-GGCTGTCAGAGCCTCGTGGCTTT G-3';
Arginase1	forward	5'-CAGAAGAATGGAAGAGTCAG-3',
	reverse	5'-CAGATATGCA GGGAGTCACC-3'.

The expression of each gene was normalized to the expression of β-actin by the calculation of $2^{-\Delta\Delta^{CT}}$

Animal models

The animal procedures followed the US National Institutes of Health (NIH) animal care and use protocols (#85-23, revised 1985) were used. Only male, 2-3-month-old, ICR mice (National Laboratory Animal Center, Nakornpathom, Thailand) were used to avoid the gender difference in sepsis severity (42). The animal protocols were approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The experiment were separated mice into 6 groups;

1. Sham
2. CLP with normal saline (NSS)
3. CLP with AuNPs 3.925 mg/g
4. CLP with AuNPs 7.85 mg/g
5. CLP with AuNPs 15.7 mg/g
6. CLP with AuNPs 31.4 mg/g

Cecal ligation and puncture (CLP) procedures followed a previous publication [19]. Briefly, 10 mm from the cecal tip was ligated with silk 2-0, punctured twice with a 21-gauge needle through an abdominal incision under isoflurane anesthesia. Tramadol (an analgesic drug) at 10 mg/kg in 0.2 ml of normal saline solution (NSS), for analgesia and imipenem/cilastatin (an antibiotic) at 14 mg/kg in 0.2 ml of NSS was administered intraperitoneally and subcutaneously, respectively, at post-operation and at 6h later.

AuNP diluted in 0.2 ml of NSS were administered through tail vein at post-operation. AuNP at 3.925, 7.85, 15.7 and 31.4 ug/ gram body weight were tested with survival experiments in CLP mice and the effective dose (7.85 ug/ g) were used in other experiments. Blood was collected through cardiac puncture under isoflurane anesthesia at 18 hours-post CLP. Whole blood was used for bacterial burden analysis and the rest of the blood was centrifuged at 13,000 g for 5 minutes to separate serum and kept on -80°C until analysis. In the survival study, tramadol and antibiotic were administered once daily for 3 days and until 5 days of the observation, respectively.

Mouse blood sample analysis

For quantitative bacterial analysis of blood and peritoneal cavity, 25 µl of blood and 1 µl of peritoneal fluid from the right para-mesenteric recess, respectively, were spread directly onto blood agar plates (Oxoid, Hampshire, UK), incubated at 37°C under aerobic conditions, and bacterial colonies were enumerated at 24 hours.

For serum creatinine (Scr) and alanine transaminase (ALT) was measured with QuantiChrom Creatinine Assay (DICT-500; Bioassay, Hayward, CA, USA) and EnzyChrom ALT assay (EALT-100, BioAssay), respectively.

For serum cytokines (TNF-α, IL-6, IL-1β, IL-4 and IL-10) were measured by sandwich ELISAs assay (eBioscience, San Diego, CA, USA). All assays were performed according to the manufacturer's protocol.

Statistical analysis

Data was analyzed as mean ±standard error (SE) and the differences between groups were examined for statistical significance by one-way analysis of variance (ANOVA) followed by Bonferroni analysis for the experiments with multiple time-point data collection. The survival analysis was determined by log-rank test. The repeated measures analysis of variance (ANOVA) with Bonferroni post hoc analysis was used for the analysis

of the time-course experiments. All statistical analyses were performed with SPSS 11.5 software (SPSS, IL, USA). A P value < 0.05 was considered to be statistically significant.

CHAPTER IV
RESULTS

Characterization of gold nanoparticles (AuNPs)

The measured size of AuNPs was shown by the Ultra-Violet-Visible spectroscopy, AuNPs size 21.3±0.7 nm was shown a single peak at wavelength 518 nm (Figure 4).

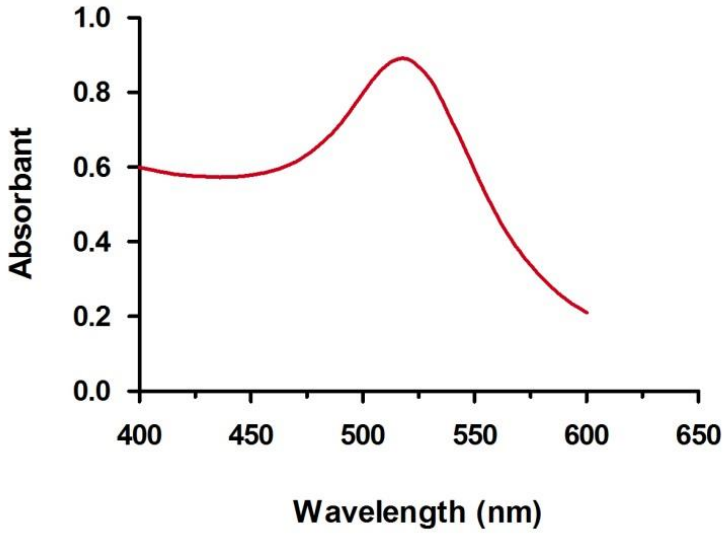
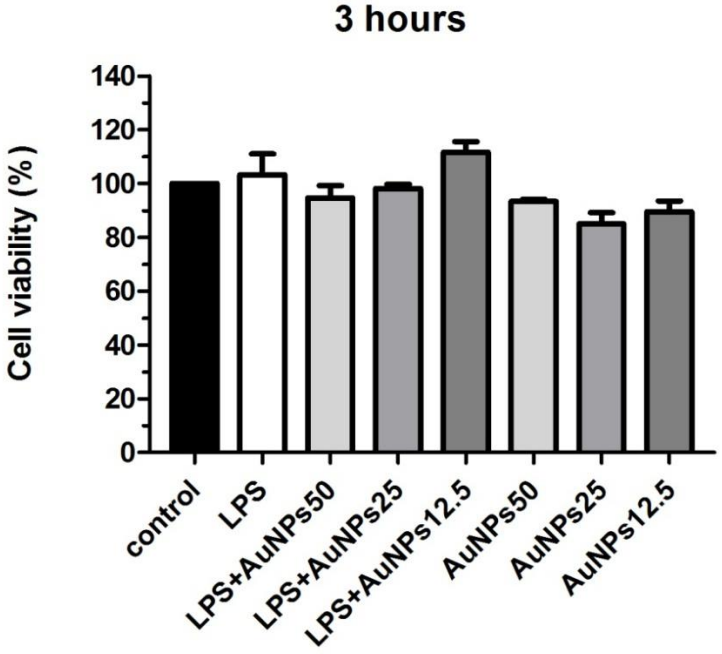


Figure 4 Characterization of gold nanoparticles (AuNPs) at size 21.3±0.7 nm

Cytoxicity measurement

The cell viability assay was used to measure the toxicity of AuNPs after incubation with BM-derived macrophage cells. To confirm that the cells were living, MTS assay was used in every condition of the experiments. The first group, cells were treated with various concentrations of AuNPs (12.5, 25 and 50 ppm). The second group, cells were treated with LPS and AuNPs at each concentration. All groups include of PBS control were incubated at 3, 6 and 24 hours. Cell viability was shown by the reaction between MTS tetrazolium compound and NAD (P) H-dependent dehydrogenase enzymes in metabolically active cells. The result showed that the percentage of viable cells did not significantly different between time points in all groups (Figure 5)



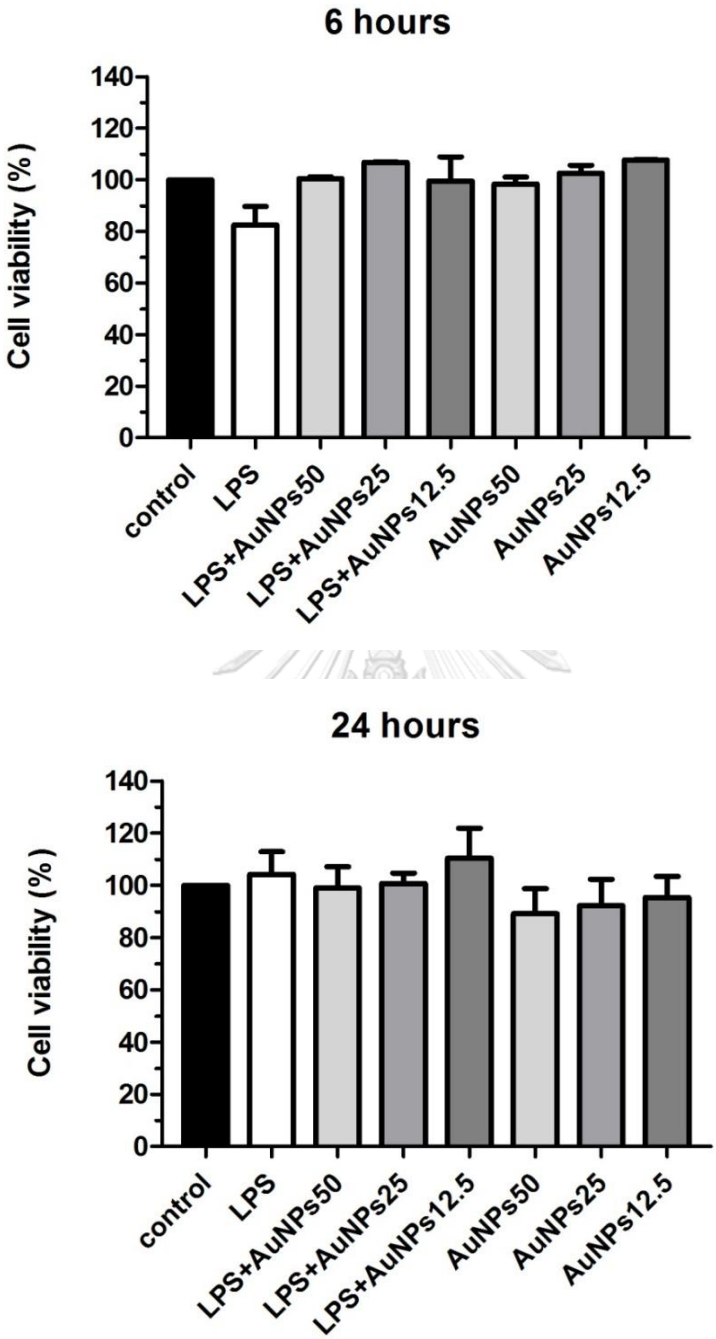
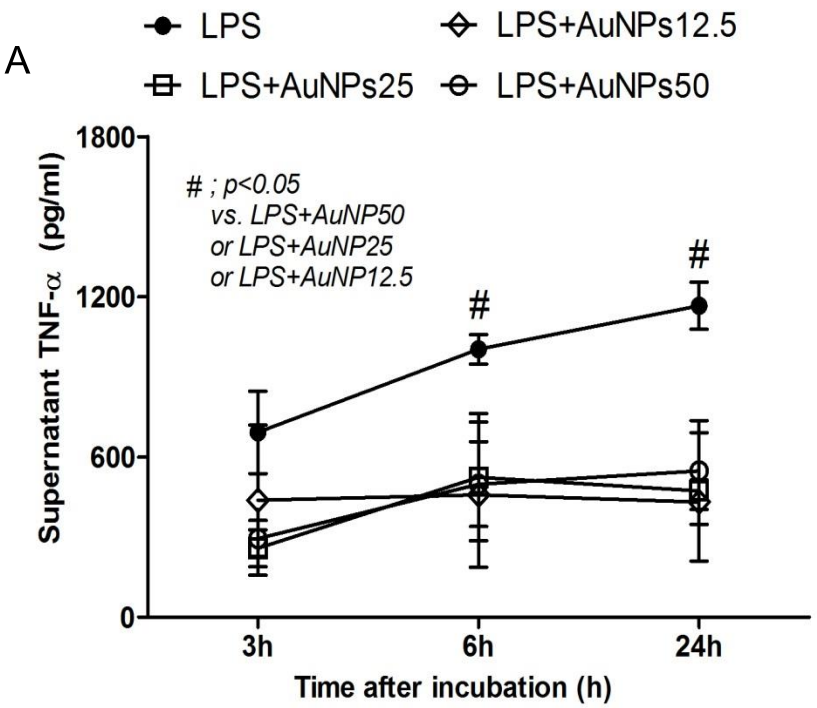
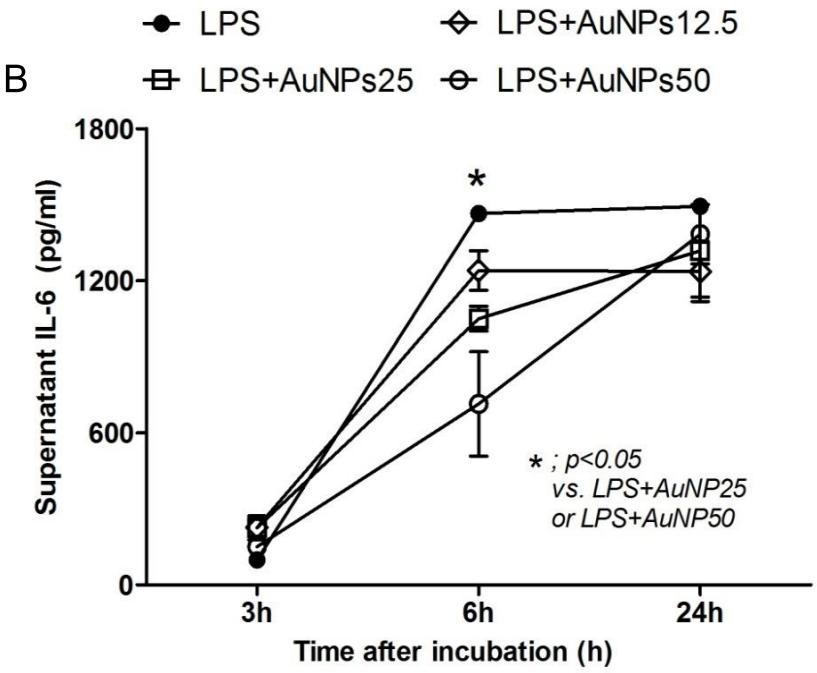


Figure 5 A viable cells after treating and incubated with LPS and/or various concentrations of AuNPs at 3, 6 and 24 hours. The percentage of cell viability didn't show the significantly different between groups.

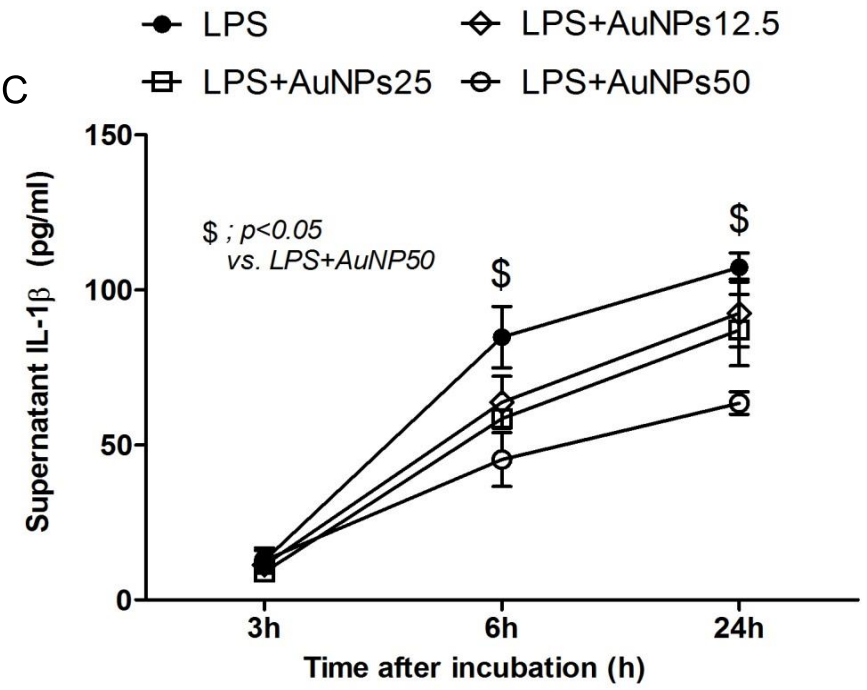
Production of macrophage cytokine

Cytokine production from BM-derived macrophages after incubated with LPS and AuNPs shown that pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 were decreased but IL-10, an anti-inflammatory cytokine, was increased. The result was shown the reduction of TNF-α and IL-1β at 6 and 24 hours of the incubation time, but IL-6 at 6 hours not 24 hours and IL-10 cytokine production was induced at 6 and 24 hours of the incubation time. However, AuNPs did not have any effects on IL-4 in every time point (Figure 6 A-E). Dose-related effect on macrophage cytokine production was not founded on TNF-α, IL-1β and IL-10 at 6 and 24 h of the incubation time, but was founded on on IL-6 at 6 h of the incubation time. Hence, AuNPs reduced several pro-inflammatory cytokines but demonstrated the different effects towards anti-inflammatory cytokines (increased IL-10 and no effect on IL-4).

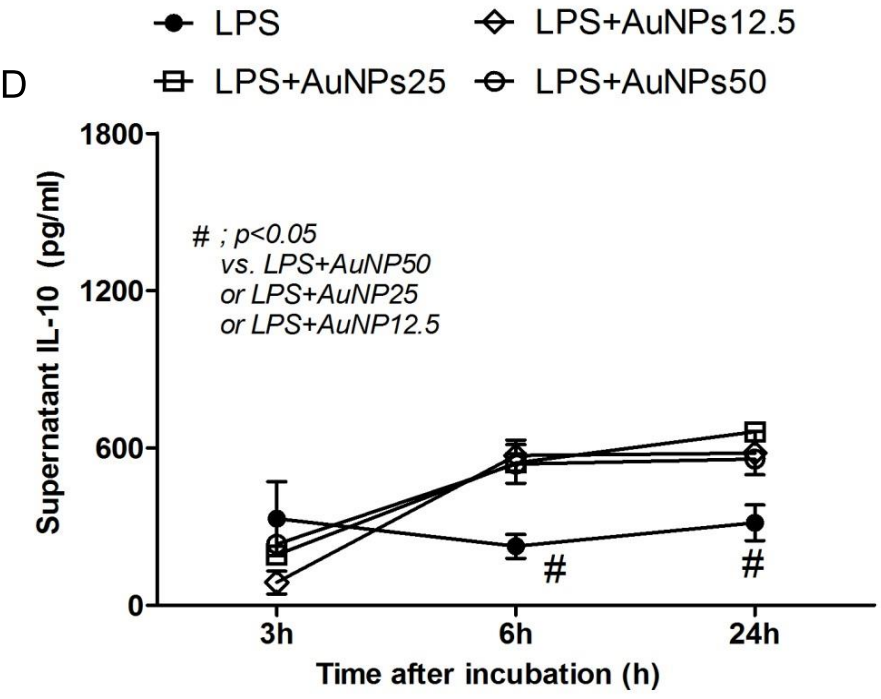

A
LPS
LPS+AuNPs12.5
LPS+AuNPs25
LPS+AuNPs50
Supernatant TNF-α (pg/ml)
1800
1200
600
0
; p<0.05
vs. LPS+AuNP50
or LPS+AuNP25
or LPS+AuNP12.5
#
#
3h
6h
24h
Time after incubation (h)


B
LPS
LPS+AuNPs12.5
LPS+AuNPs25
LPS+AuNPs50
Supernatant IL-6 (pg/ml)
1800
1200
600
0
*
* ; p<0.05
vs. LPS+AuNP25
or LPS+AuNP50
3h
6h
24h
Time after incubation (h)

C


LPS
LPS+AuNPs12.5
LPS+AuNPs25
LPS+AuNPs50
Supernatant IL-1β (pg/ml)
150
100
50
0
$; p<0.05
vs. LPS+AuNP50
$
$
3h
6h
24h
Time after incubation (h)

D


LPS
LPS+AuNPs12.5
LPS+AuNPs25
LPS+AuNPs50
Supernatant IL-10 (pg/ml)
1800
1200
600
0
; p<0.05
vs. LPS+AuNP50
or LPS+AuNP25
or LPS+AuNP12.5
#
#
3h
6h
24h
Time after incubation (h)

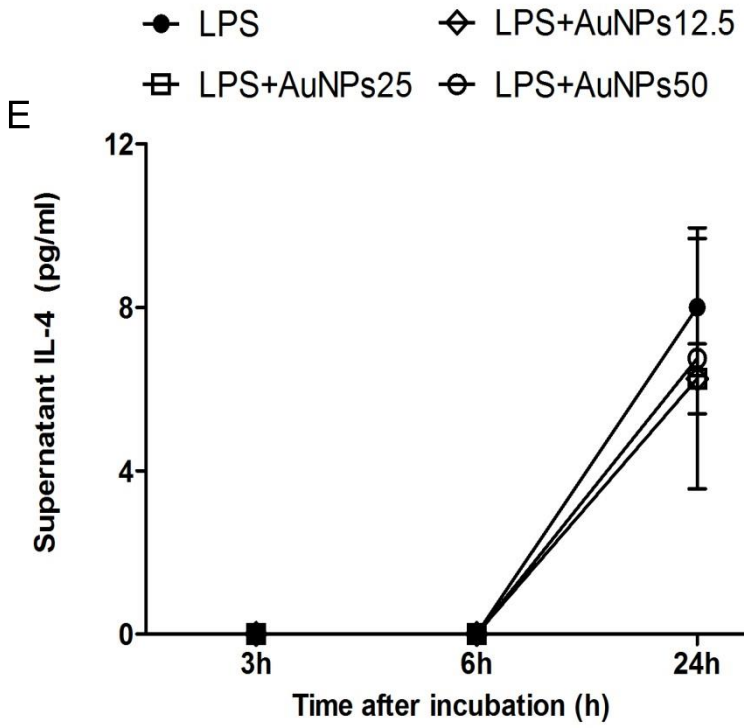
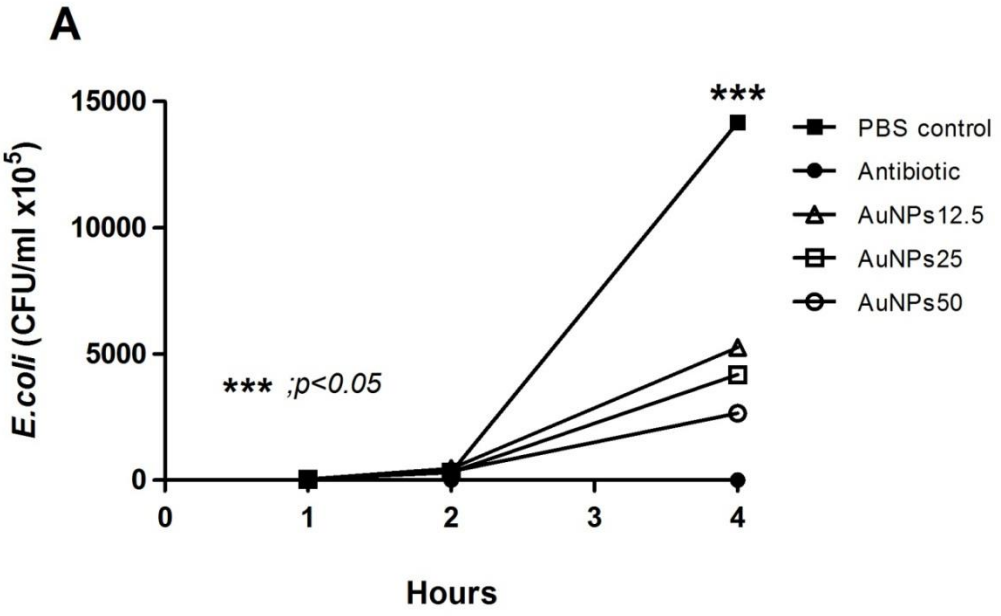


Figure 6 Macrophage cytokine production of TNF-α (A), IL-6 (B), IL-1β (C), IL-10 (D) and IL-4 (E) after incubation with endotoxin (LPS) and gold nanoparticles (AuNPs) in 20 different concentrations; 12.5, 25 and 50 ppm, respectively.

Antimicrobial activity of gold nanoparticles

The activity of AuNPs to antimicrobial effect was performed by incubating with AuNPs at various concentrations and controlled by PBS and gentamicin antibiotic. The result was shown to significantly reduce the amount of *E. coli* colonies after 4 hours of incubation time (Figure 7A). At 4 hours, the comparasion between PBS control and other groups with AuNPs was shown the reducing of *E. coli* after treat with AuNPs at 50, 25 and 12.5 ppm by dose-dependent from high to low concentration, but the antibiotic effect of AuNPs all concentration is weaker than gentamicin antibiotic (Figure 7B).



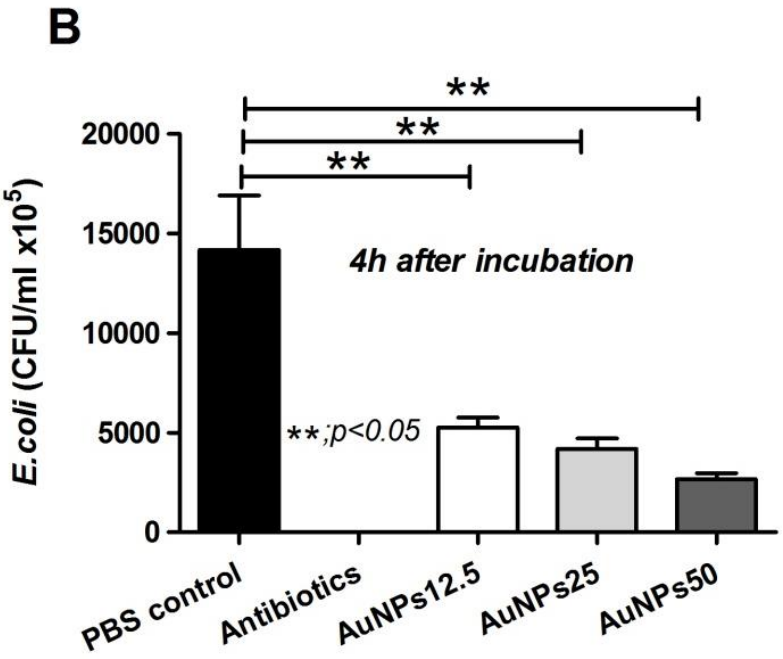
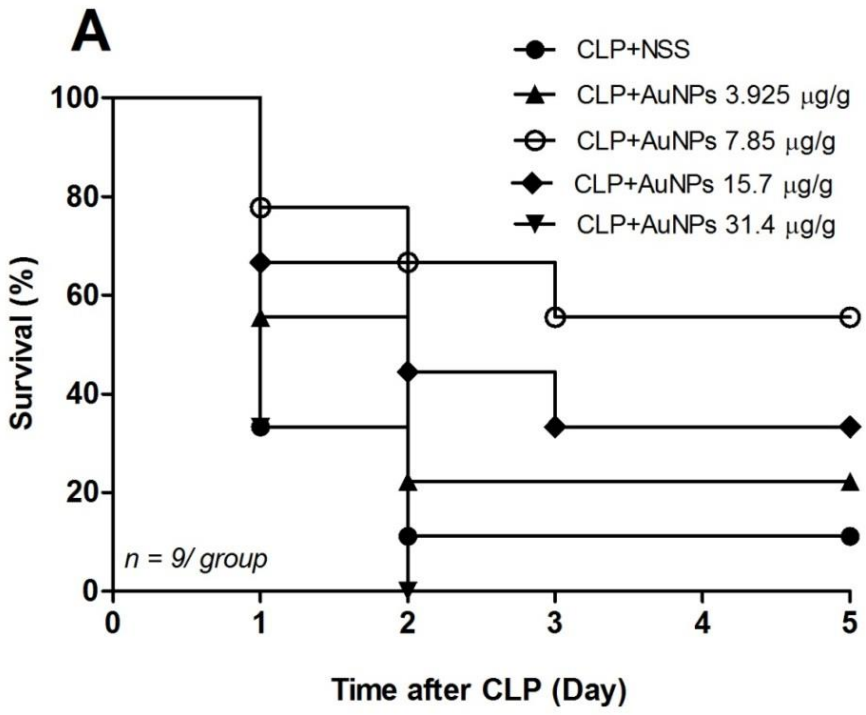


Figure 7 Antimicrobial activity of gold nanoparticles (AuNPs) was demonstrated with the incubation with PBS control, gentamicin antibiotic (antibiotic) and AuNP in different concentrations; 12.5, 25 and 50 ppm (A,B), respectively.

Effect of CLP and AuNPs for survival

The effect of AuNPs at various concentrations, 3.925, 7.85, 15.7 and 31.4 ug/ gram body weight, that were demonstrated in mice with CLP induce sepsis was shown the highest survival rate in mice use AuNPs at 7.85 ug/ gram body weight in comparison with a normal saline control group (Figure 8A). In other groups, after CLP and AuNPs injected at 3.925 and 15.7 ug/ gram body weight were shown the lower survival rate than the dose at 7.85 ug/ gram body weight group. None of mice in 31.4 ug/ gram body weight group was survive after 2 days (Figure 8A). Survival of AuNPs at 7.85 ug/ gram body weight shown a significant higher survival rate than the normal saline control group (Figure 8B).



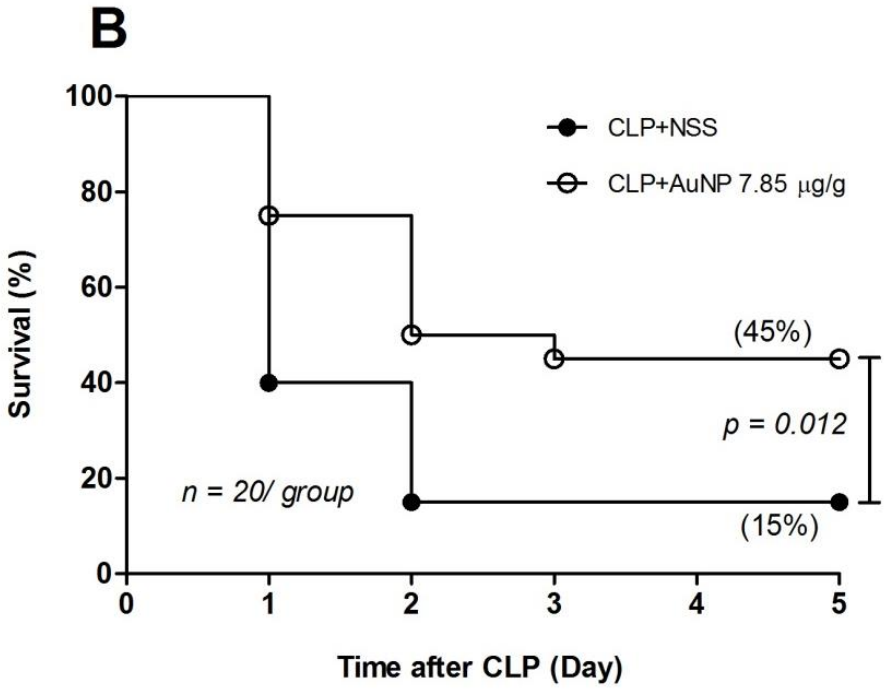
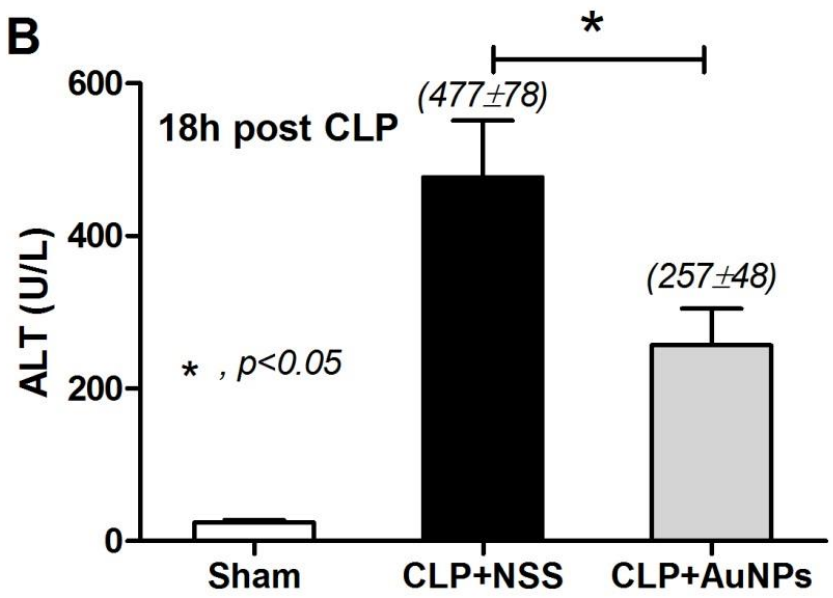


Figure 8 The survival of mice was demonstrated by CLP induce sepsis and AuNPs injected at various concentrations compared with normal saline control group (A). The highest survival group compared with control group (B).

Determination of organ injury

Serum creatinine (Scr) and alanine transaminase (ALT) were measured after CLP with AuNPs injection and sham to demonstrate kidney dysfunction and liver injury, respectively. AuNPs attenuated kidney injury (lower Scr) and liver injury (ALT) (Figure 9A, B).



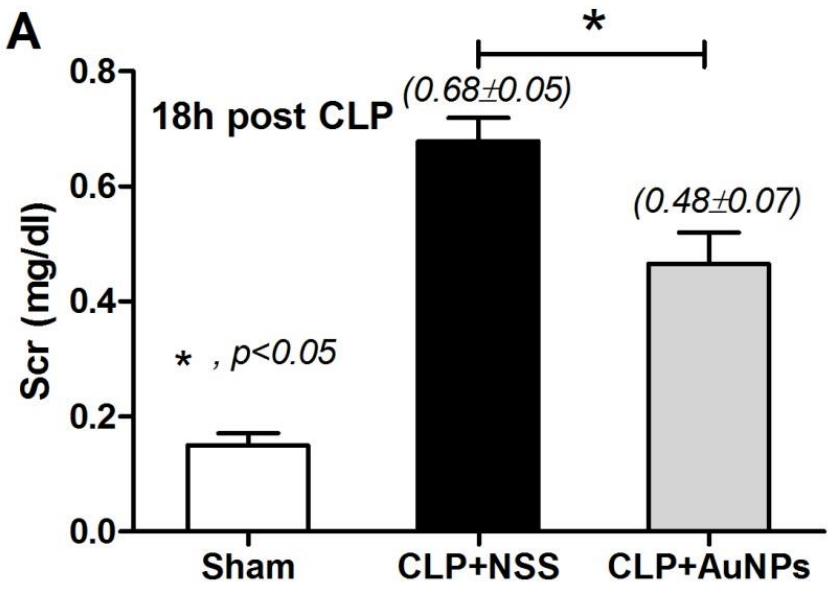
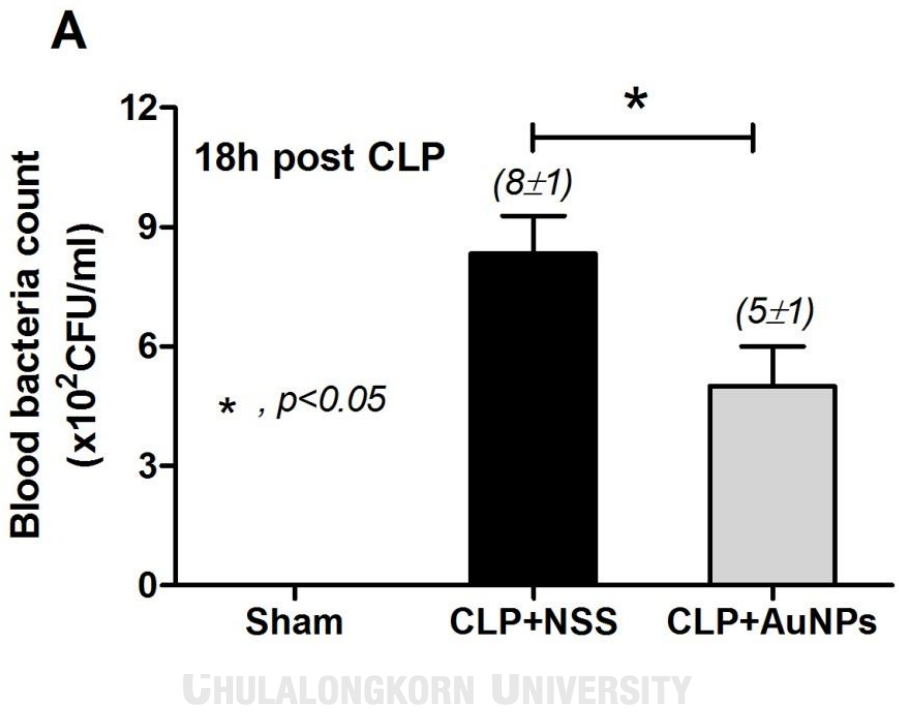


Figure 9 The levels of serum creatinine (A) and alanine transaminase (B) were demonstrated after CLP induce sepsis with an AuNPs injected group, a normal saline control group and the sham group.

Enumeration of bacteria from mice

Enumeration of bacteria from mice that induced by CLP was demonstrated by the bacterial burdens in blood and peritoneal fluid of mice. AuNPs attenuated blood bacterial burdens but not the burdens in peritoneal fluid (Figure 10A, B).



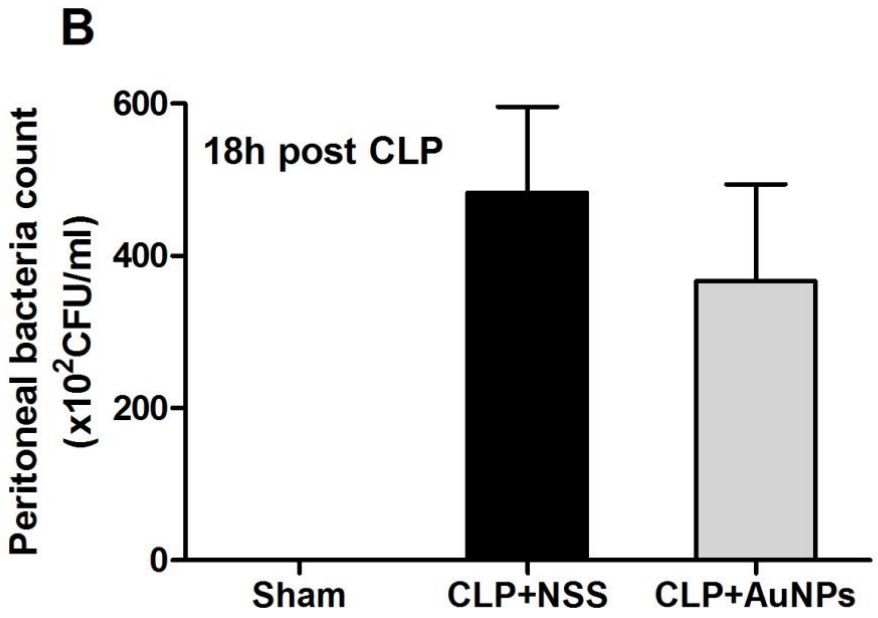
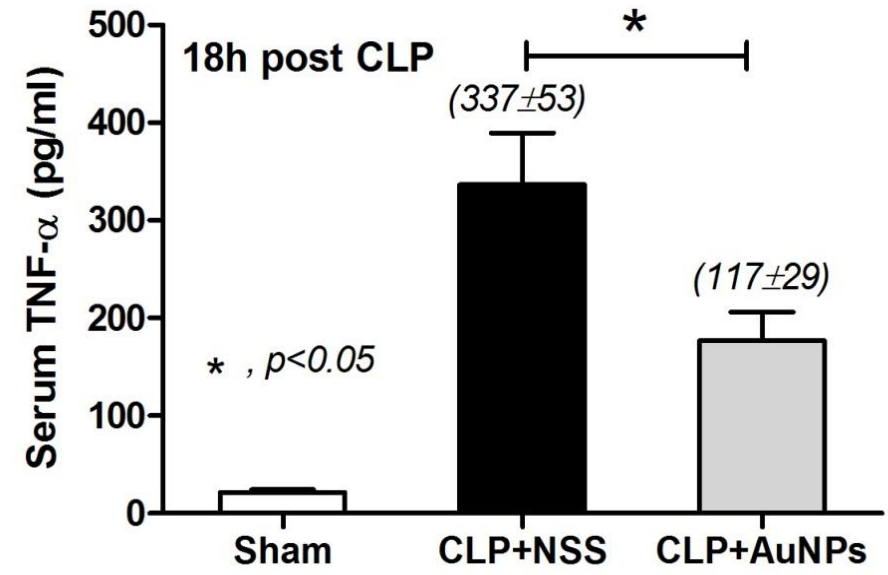


Figure 10 The bacterial burdens in blood and peritoneal lavage of mice were used for demonstrating the amount of bacteria after CLP induce sepsis with an AuNPs injected group, a normal saline control group and the sham group (A,B).

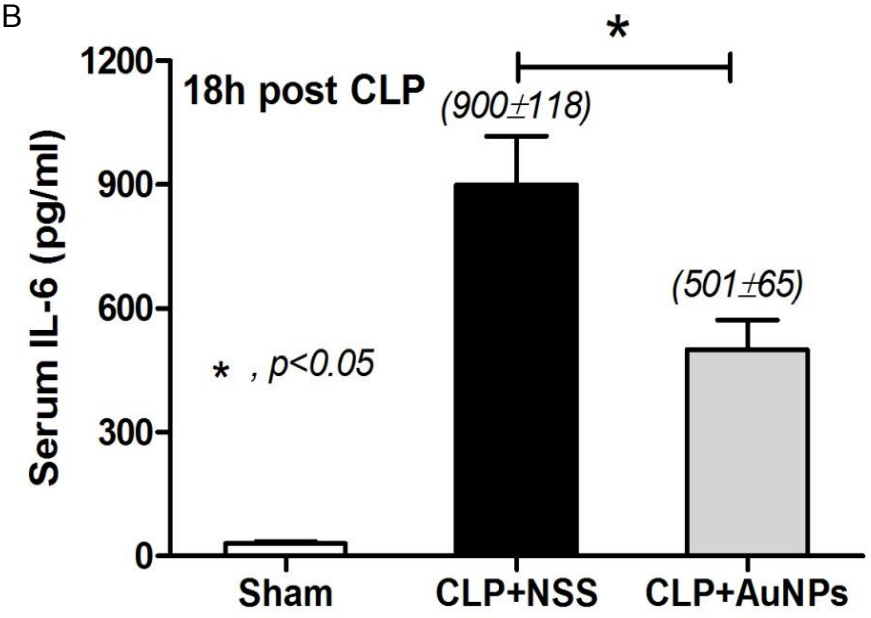
Production of cytokine from mice

In comparison between an AuNPs injected group and a normal saline injected group, the results were shown significant decreasing of TNF-α, IL-1β, IL-6 and IL-10 cytokines after injecting with AuNPs (Figure 11 A-D). But the production of IL-4 cytokine was not significantly different after injecting with AuNPs (Figure 11E).

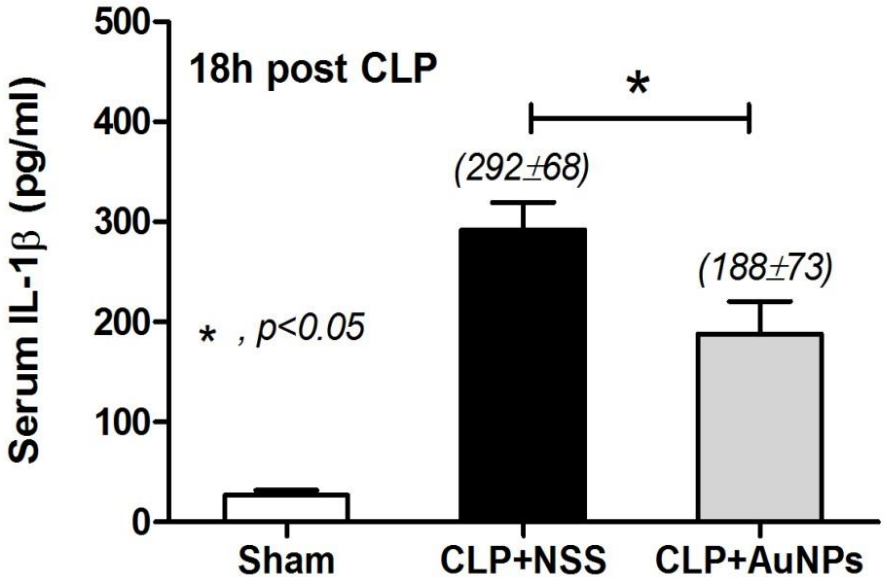
A



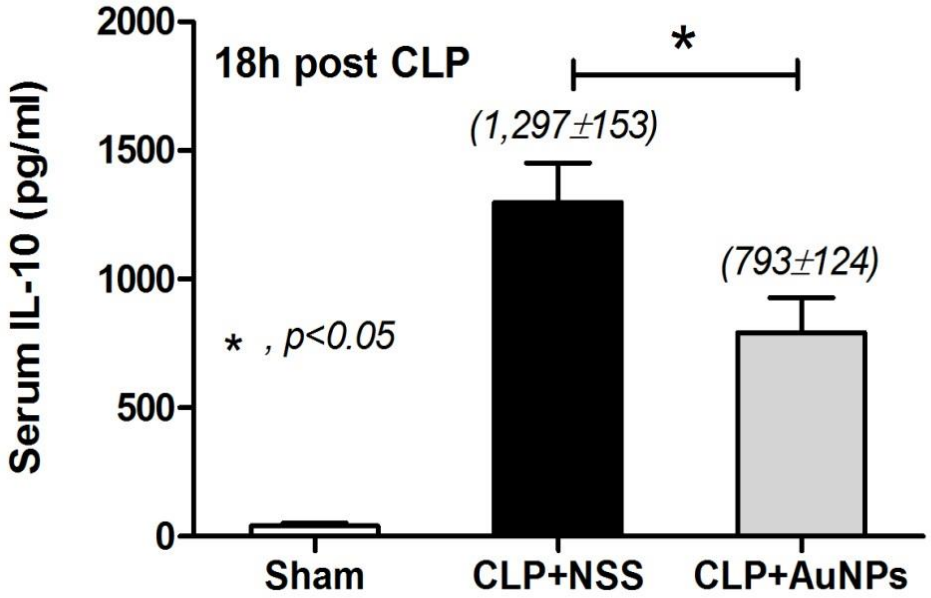
B


18h post CLP
Serum IL-6 (pg/ml)
1200
900
600
300
0
*
(900±118)
(501±65)
* , p<0.05
Sham
CLP+NSS
CLP+AuNPs

C


18h post CLP
Serum IL-1β (pg/ml)
500
400
300
200
100
0
*
(292±68)
(188±73)
* , p<0.05
Sham
CLP+NSS
CLP+AuNPs

D



E

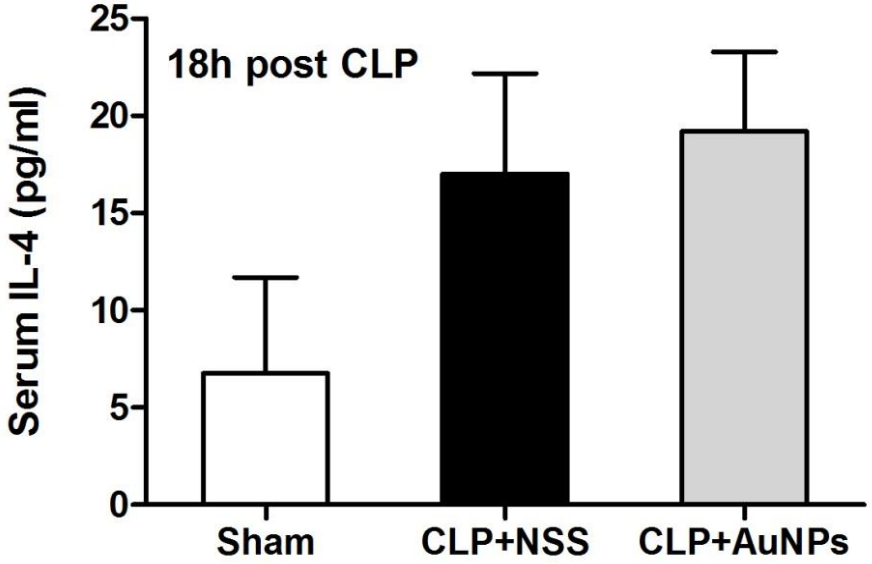


Figure 11 The production of cytokines from mice that induced sepsis by CLP and injected with AuNPs or normal saline, was shown the lower of TNF-α, IL-1β, IL-6 and IL-10 cytokines from AuNPs injected mice (A-D) but IL-4 was not different (E).

Macrophage polarization demonstrates by qPCR

This experiment, we used a quantitative polymerase chain reaction (qPCR) for testing mRNA expression of macrophage polarization. Bacause AuNPs induce high IL-10 and IL-10 production is the characteristic of anti-inflammatory macrophage (M2), we hypothesized that AuNPs could induced M2 macrophage. Indeed, AuNPs reduced inducible nitric oxide synthase (*iNOS*), a marker of pro-inflammatory macrophage (M1), as *iNOS* increased after LPS stimulation. AuNPs-treated macrophage showed the lower *iNOS* than LPS induction alone. Actually, *iNOS* in AuNPs-trated macrophage was not different from IL-4, a M2 polarization inducer (Figure 12A). In contrast, AuNPs increased *Arginase1* (a marker of M2 activation) and *Arginase1* from AuNPs-treated macrophage was not different from IL-4 induction (Figure 12B). Accordingly, the data were demonstrated the effect of AuNPs to the anti-inflammatory functions of macrophages.

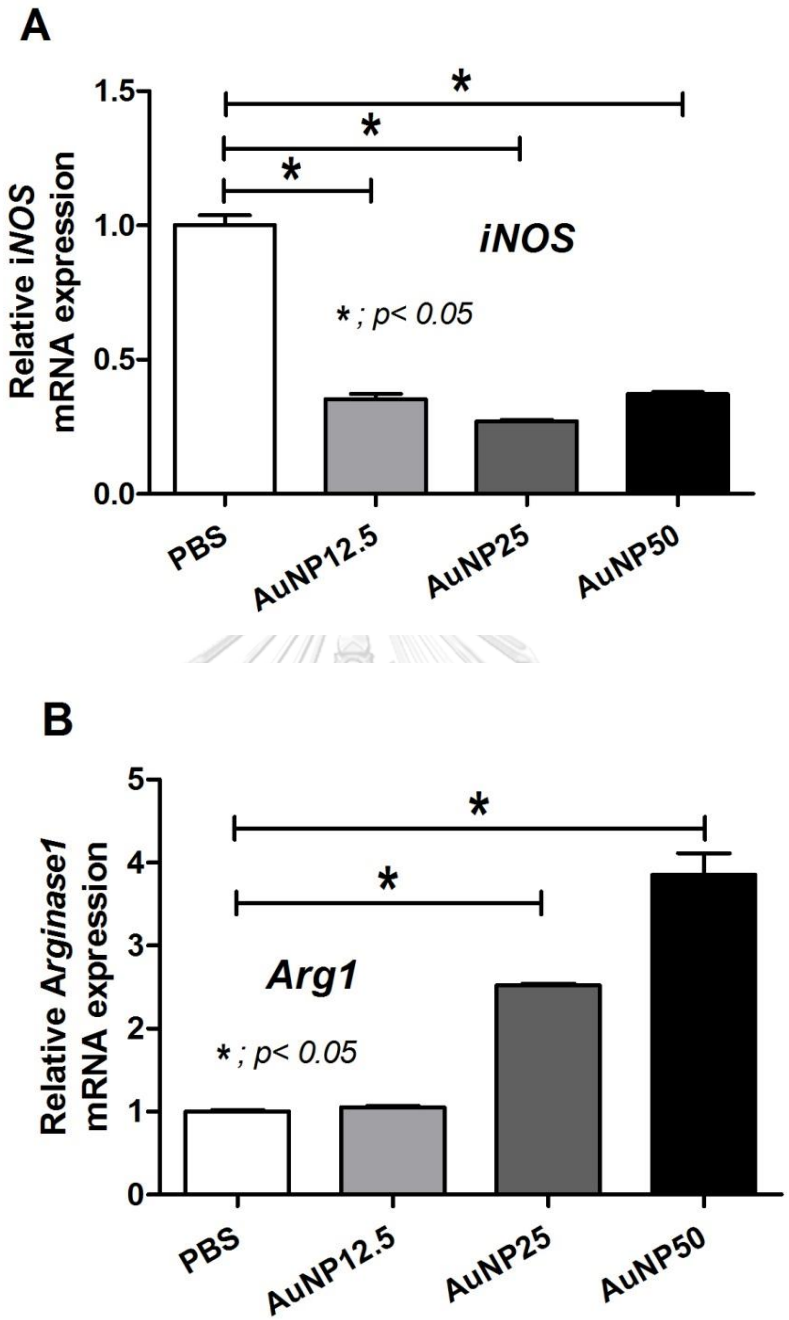


Figure 12 The relative expression of iNOS (A) and Arginase1 (B) after incubation of macrophages with AuNPs at various concentrations (12.5, 25 and 50 ppm), which represent the M1 and M2 macrophage polarization, respectively.

CHAPTER V
DISCUSSION

The effect of gold nanoparticles (AuNPs) toward BM-derived macrophages after LPS induction and in sepsis mouse model were demonstrated. AuNPs showed anti-bacterial property and decreased pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) but increased an anti-inflammatory cytokine (IL-10). AuNPs also altered macrophages toward the anti-inflammatory direction of M2 polarization. Moreover, the study on animal model showed the effect of AuNPs treatment as an antibiotic adjuvant therapy that attenuated organs dysfunction, reduced blood bacterial burdens, improved cytokine levels and also reduced mortality rate. This study revealed several new findings including i) the application to use AuNPs to improve CLP sepsis model and ii) AuNPs-induced M2 macrophage polarization. On the other hand, our finding supported the previous study of AuNPs induced anti-inflammation through the downregulation of TLR4 and NF-κβ expression levels (35) and the AuNPs antimicrobial activity (43).

Spherical colloidal AuNPs size at diameters 20-30 nm has been showed no-toxicity and also induced the anti-inflammatory effect in several studies on either cells or animals (7). AuNPs in the smaller size showed some toxicities toward several cells (44). Moreover, route of the administration is associated with the toxicity of AuNPs (the lowest toxicity is the intravenous injection in comparison with the oral and intra-peritoneal administration) (6). Although the surface conjugation of AuNPs with multiple modifications

is possible, the un-conjugated AuNPs is the easiest form of AuNPs for the cellular uptake (45). Several animal studies of AuNPs showed an anti-inflammatory effect in several models such as the endotoxin-induced uveitis in rats and traumatic muscle injury in rats (35, 46). Hence, the spherical un-conjugated AuNPs at 21 nm diameter is an interesting candidate for adjuvant therapy in sepsis.

The effect of AuNPs in BM-derived macrophages functions and anti-bacterial property

The innate immune response is the initial part of host immunity that rapid response against pathogens and macrophages are important cells of the innate immunity. The previous study showed the anti-inflammatory effect of AuNPs toward the responses of macrophage from abdominal fat pad (7). In this study, we investigated BM-derived macrophages induced by LPS (the endotoxin from gram-negative bacteria). AuNPs reduced LPS-induced pro-inflammatory cytokines of macrophage including TNF-α, IL-6 and IL-1β. From the previous study mentioned that the anti-inflammatory effect of AuNPs is due to TLR4 blocking (35). However, the detail mechanisms of AuNPs on TLR4 are still not clear. Possibly, AuNPs might attach to surface TLR4 directly (considering the opposite electrical charge of AuNPs) or bind with the down-regulation molecules after entering the cell. Indeed, the cell uptake processes of AuNPs are also not clear whether mainly with pinocytosis (without any specific receptors) or receptor-mediated phagocytosis. These are the further interesting topic on AuNPs studies.

It is interesting to note that AuNPs increase macrophage IL-10 (the anti-inflammatory cytokine), a cytokine of M2 polarization. Because one of the mechanisms for controlling the host immune response is the macrophage polarization which is dividing the macrophage into 2 types including M1 for the pro-inflammatory process and M2 for the anti-inflammatory activity (47), the alteration of macrophage into the proper polarization should be helpful for sepsis attenuation. Interestingly, IL-10 cytokine production is one of the properties of M2 macrophages (47). Indeed, we were the first group that demonstrated M2 macrophage polarization property of AuNPs. However, this effect was weak in comparison with IL-4, a major cytokine of M2 polarization.

The possible mechanism of AuNPs-induced M2 polarization is not clear, but in another study of nanoparticles, suggesting that size and arginine binding property of AuNPs may be the important factors. According to the previous study, copper nanoparticles is another metal nanoparticles with anti-inflammatory effect through the modification of arginase (an important enzyme of M2 polarization) (48). Probably, the proper size of AuNPs is allowing the maximal cell uptake and enhancing the optimal arginine binding on arginase (an important enzyme of M2 polarization). And AuNPs-induced arginase modifications activate M2 polarization.

In addition, our study showed that AuNPs have mild antimicrobial effect in comparison with gentamicin antibiotic support a previous study (38). The mechanism to antibacterial was mainly mentioned in silver nanoparticle (AgNPs) that interact with the

cell membrane and may interfere with the cell transportation (43). Silver ion was inserted into the bacterial cytoplasm and change the local pH that leads to bacterial cell lysis (43). However, another study suggests that the antibacterial effect of AuNPs was due to the AuNPs-aggregation in bacterial cytoplasm (39). Interestingly, this study propose AuNPs as a useful candidate for bactericidal drug with low toxicity (39).

AuNPs for sepsis treatment

The imbalance of inflammatory response between pro-inflammation and anti-inflammation causes sepsis severity (1). Therefore, the optimal management of sepsis could be done by the proper adaptation on the immune system (49). CLP mice with immediately injection of AuNPs through tail vein after antibiotic administration showed the highest survival only with AuNPs at the dose 7.85 μg/g, but not lower or higher doses. These data implied the U-curve phenomenon on AuNPs therapeutic effect which is a limitation for the further translation research in patients.

CLP mice with AuNPs showed the lower level of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) and serum IL-10 in comparison with the control group. Serum IL-10 is higher in AuNPs treatment but AuNPs induces higher IL-10 from macrophage. This might because of the diversity of the different responses against AuNPs of several cells *in vivo*. While AuNPs induced IL-10 could be easily demonstrated in macrophage, *in vitro*, the responses *in vivo* is more complex with several cell types. Serum IL-10 in mice with AuNPs

activation is the combination of AuNPs effect from macrophage, T cell, non-immune cells, etc. (50), hence, it is possible that AuNPs could not induce IL-10 from other cell types. In addition, the decreased serum IL-10, along with other pro-inflammatory cytokines suggesting a balance of the pro- versus anti- inflammatory immune responses, *in vivo* (2). Moreover, AuNPs did not increase IL-4 both in *in vitro* and *in vivo*, although IL-4 is an important cytokine of Th2 (51) implying a lower effect of AuNPs against Th2 compared with macrophage in sepsis (52).

However, the severity of sepsis can be attenuated by improving renal dysfunction, liver injury and decreased pro-inflammatory cytokine levels. And the supporting data of the antibacterial effect of AuNPs was demonstrated *in vivo* by the reduction of blood bacterial burdens. Hence, the adjuvant therapy with AuNPs, at the proper dose, may be interesting for the further study.

In conclusion, we demonstrated an adjuvant effect of spherical AuNPs in 21 nm diameter on BM-derived macrophages with LPS induction and CLP induced polymicrobial sepsis mouse model. This therapeutic effect was, at least in part, responsible from the induction of macrophage toward anti-inflammatory responses. The ability of AuNPs surface conjugation is interesting to improve the application to get more benefit. The further studies are certainly needed.

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APPENDIX

Reagent preparation

1. Tryptic soy broth (TSB)

Pancreatic digest of casein	15	g
Enzymatic digest of soybean	5	g
Sodium chloride	5	g
Distilled water	1000	ml

The solution was mixed and sterilized by autoclaving at 121°C for 15 minutes.

2. Tryptic soy agar (TSA)

Pancreatic digest of casein	15	g
Enzymatic digest of soybean	5	g
Sodium chloride	5	g
Agar	15	g
Distilled water	1000	ml

The solution was mixed and sterilized by autoclaving at 121°C for 15 minutes.

3. Fetal bovine Serum (FBS)

Heat-inactivated at 56 °C for 30 min, store at -20 °C

4. Phosphate buffer saline (PBS)

Stock solution (10X PBS)

NaCl	80	g
KCl	2	g

Na2HPO4	11.5	g
KH2PO4	2	g
Distilled water	1000	ml

Working solution (1X)

10X PBS	100	ml
Distilled water	900	ml

5. Dulbecco's Modified Eagle Media complete (DMEM complete media)

DMEM media	100	ml
(HyCloneTM, Thermo Scientific, Waltham, MA, USA)		
Fetal bovine serum (FBS)	10	ml
HEPES	1	ml
Sodium pyruvate	1	ml
Penicillin/Streptomycin	1	ml

6. L929 culture supernatant

Cells preparation

Thaw cell L929 by using DMEM serum free media

Plate cell on TC culture flask

Pass cell by using Trypsin-EDTA

Culture cell in 8ml of DMEM complete media in 5% CO_2, 37°C

Collection of supernatant

Collected supernatant when cell growth at 70-80%

Filtered by 0.2μm

Freeze cell by 10%DMSO in DMEM completed media, keep in -80°C overnight

Before transferring to liquid nitrogen for long storage

7. Bone marrows-derived macrophages Media (BMM media)

DMEM complete media	80	ml
L929 culture supernatant	20	ml
Horse serum	5	ml

8. Ammonium chloride lysis (Red Blood Cell lysis buffer)

KHCO	3 0.5	g
NH_4Cl	4.15	g
EDTA	0.018	g

Made up to 500ml with distilled water and autoclave before use.

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- The role of macrophages in the susceptibility of Fc gamma receptor IIb deficient mice to Cryptococcus neoformans

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