國 立 交 通 大 學

生物科技系暨研究所

碩士論文

# 探討兩種人類麻醉用藥戊巴比妥和異丙酚對於發炎反應之影響

# Investigate the Effects of two Anesthetics, Pentobarbital and Propofol on Inflammatory Response

研 究 生: 李其翰

指導教授: 廖光文 博士

中華民國九十七年七月

探討兩種人類麻醉用藥戊巴比妥和異丙酚對於發炎反應之影響

**Investigate the Effects of two Anesthetics, Pentobarbital and Propofol on Inflammatory Response**


研 究 生：李其翰 Student : Chi-Han Li

指導教授：廖光文 Advisor : Kuang-Wen Liao, Ph.D.

國 立 交 通 大 學

生物科技系暨研究所


碩 士 論 文

A Thesis

Submitted to Institute of Biological Science and Technology

College of Biological Science and Technology

National Chiao Tung University

in partial Fulfillment of the Requirements

for the Degree of

Master

in

Biological Science and Technology

June 2008

Hsinchu, Taiwan, Republic of China

中華民國九十七年七月

# 國 立 交 通 大 學

## 博碩士紙本論文著作權授權書

（提供授權人裝訂於全文電子檔授權書之次頁用）

本授權書所授權之學位論文，為本人於國立交通大學生物科技系所 ____________組， 96 學年度第 二 學期取得碩士學位之論文。

論文題目：探討兩種人類麻醉用藥戊巴比妥和異丙酚對於發炎反應之影響
指導教授：廖光文

■ 同意

本人茲將本著作，以非專屬、無償授權國立交通大學，基於推動讀者間「資源共享、互惠合作」之理念，與回饋社會與學術研究之目的，國立交通大學圖書館得以紙本收錄、重製與利用；於著作權法合理使用範圍內，讀者得進行閱覽或列印。

本論文為本人向經濟部智慧局申請專利(未申請者本條款請不予理會)的附件之一，申請文號為：____________________，請將論文延至____年____月____日再公開。

**授 權 人：李其翰**

**親筆簽名：** 李其翰

**中華民國** 97 **年** 7 **月** 23 **日**

# 國立交通大學
## 生物科技學系碩士班

## 論文口試委員會審定書

本校 生物科技學系 碩士班 李其翰 君

所提論文（中文） **探討兩種人類麻醉用藥戊巴比妥和異丙酚對於發炎反應之影響**

（英文） Investigate the Effects of two Anesthetics, Pentobarbital and Propofol on Inflammatory Response.

合於碩士資格水準、業經本委員會評審認可。

口試委員： ________ 教授　[illegible] 教授

廖光文 教授　鄧啓祿 教授

[illegible] 教授　吳彰哲 教授

指導教授： 廖光文 教授　________ 教授

系主任： 林志生 教授

中華民國 97 年 6 月 23 日

## 第一部分

# 戊巴比妥在清醒鼠內毒素模型中能夠降低的甲型腫瘤壞死因子分泌並降低組織因發炎反應所造成之傷害

研究生：李其翰　　　　　　　　　　指導教授：廖光文　博士

國立交通大學生物科技學系暨研究所碩士班

## 中文摘要

菌血症是重症病患最主要的致死原因之一，而戊巴妥是常利用在實驗動物麻醉所使用的麻醉藥物。利用清醒鼠實驗技術以及脂多醣（LPS）建立清醒鼠內毒素模型，並發現戊巴妥能夠降低該動物模型中發炎細胞激素甲型腫瘤壞死因子之分泌。實驗結果顯示，戊巴妥能夠降低在脂多醣刺激下甲型腫瘤壞死因子(TNF-α）的蛋白質以及mRNA的表現量；其作用機制是透過抑制轉錄因子 NF-κB, AP-1以及胞內蛋白 p38 MAPK的活性進而導致降低甲型腫瘤壞死因子的表現。除此之外，由於甲型腫瘤壞死因子會造成細胞組織器官的壞死，因此我們進行活體內毒性測試，我們發現在戊巴妥治療組中，老鼠血清中胺酸轉胺脢（alanine aminotransferase, ALT）、天冬氨酸轉氨酶（aspartate aminotransferase, AST）、乳酸脫氫脢（lactic dehydrogenase, LDH）、肌酸肌脢(creatine kinase, CK)、血清尿素氮(serum urea nitrogen)、澱粉酵素（amylase）的含量都較內毒素組明顯降低，顯示戊巴比妥能夠降低內毒素所造成之多器官傷害。由體外實驗發現戊巴比妥能夠在施予磺酸去鐵胺（deferoxamine mesylate, DFO）所造成的缺氧環境下降低細胞凋亡的發生率。以上結果顯示戊巴比妥不但能夠降低脂多醣所造成的發炎反應更能夠保護細胞免於直接或間接由甲型腫瘤壞死因子所引起的細胞凋亡。以上結果除了讓我們更了解戊巴比妥抗發炎的作用機制外，也提醒我們有許多在戊巴妥麻醉模式下所進行的藥物測試所觀察到的抗發炎反應結果有可能是由戊巴妥所協同作用引起的。

## 第二部分

# 異丙酚會刺激人類內皮細胞增加乙型轉型生長因子的表現進而抑制單核細胞之內吞活性

研究生:李其翰　　　　　　　　　　指導教授:廖光文　博士

國立交通大學生物科技研究所

## 中文摘要

以異丙酚進行麻醉或鎮靜之醫療行為時，往往伴隨著免疫細胞如巨噬細胞或自然殺手細胞之活性遭到抑制，然而已報導的相關機制並無提及是否與調控型細胞激素乙型轉型生長因子有關。為了更進一步探討臨床使用之異丙酚是否會對病人體內之乙型轉型生長因子造成變動，我們將外傷病患分為有接受或沒有接受異丙酚治療，並檢測這些病患血清中乙型轉型生長因子的濃度。我們首次發現相較於沒有接受異丙酚的病患，在有接受異丙酚治療的病患血清中，無論是總量還是已活化的乙型轉型生長因子都明顯較高。此外我們利用細胞實驗探討異丙酚究竟是促進哪一類的細胞分泌乙型轉型生長因子。在分別針對人類血液周邊單核細胞、單核球細胞株、T 淋巴球細胞株以及人類臍帶靜脈內皮細胞投與臨床用量之異丙酚並分析其乙型轉型生長因子分泌後顯示，異丙酚會增加人類血液周邊單核細胞、單核球細胞株、T 淋巴球細胞株之培養液中活化態乙型轉型生長因子之含量。不同於免疫類細胞，異丙酚反而不影響人類臍帶靜脈內皮細胞培養液中活化態乙型轉型生長因子之表現，但卻促使該細胞分泌更多的非活態之乙型轉型生長因子。並且當我們將以異丙酚前處理過的內皮細胞培養液與單核球細胞共同培養後，相較於控制組，單核細胞的吞噬活性被抑制將近百分之二十。最後，利用乙型轉型生長因子訊息傳遞途徑的阻斷劑 SB431542，我們證實了乙型轉型生長因子主導了有接受異丙酚治療的病人血清中能抑制單核細胞吞噬活性的相關機制。我們的發現有助於避免在臨床上使用異丙酚所造成的如免疫抑制等負作用。

# Part I

## The Reduction of Tumor Necrosis Factor-α Release and Tissue Damage by Pentobarbital in the Experimental Endotoxemia Model

Student: Chi-Han Li　　　　　Advisor: Dr. Kuang-Wen Liao

Institute of Biological Science and Technology

National Chiao Tung University

## ABSTRACT

Sepsis is the leading cause of death for intensive care patients. Lipopolysaccharide (LPS) administration to animals under anesthesia is a strategy for the study of uncontrolled release of proinflammatory cytokines. Anesthetics have been indicated that they can specially affect immune responses, such as the inflammatory response. Pentobarbital is an anesthetic used mainly in animal studies. Thus, the effect of pentobarbital on tumor necrosis factor-α (TNF-α) release was determined. The results revealed that pentobarbital suppressed the expression of TNF-α mRNA and its proteins, which may result from the decrease in the activities of nuclear factor-κB and activator protein 1 and the reduction of the expression of p38 mitogen-activated protein kinase by pentobarbital. After the inhibitory activity of the pentobarbital for TNF-α release was proven in vivo, the cytotoxic effects of LPS were examined in vivo with or without pentobarbital treatments. In vivo results indicated that plasma levels of alanine aminotransferase, aspartate aminotransferase, lactic dehydrogenase, creatine kinase, serum urea nitrogen, and amylase decreased dramatically in the anesthetic group with pentobarbital administration. Finally, the effect of pentobarbital on TNF-α–related cell death was monitored in vitro, and the results indicated that pentobarbital could directly enhance the viabilities of cells under the treatment of TNF-α and protected cells from apoptosis induced by deferoxamine mesylate-induced hypoxia. These results suggest that pentobarbital significantly influences the LPS-induced inflammatory responses and protects cells from death directly and indirectly induced by TNF-α. The information provides a perspective to re-evaluate the results of the experiments in which animals were anesthetized with pentobarbital. The anti-inflammatory effects of the drugs may have been caused by the synergistic effect of pentobarbital.

**Part II**

# The Treatment of Propofol Induced the TGF-β1 Expression in Human Endothelial Cells to Suppress Endocytosis Activities in Monocytes

Student: Chi-Han Li Advisor: Dr. Kuang-Wen Liao

Institute of Biological Science and Technology

National Chiao Tung University

## ABSTRACT

Propofol anesthesia or sedations downregulate the functions of many immuno-competent cells such as macrophage and neutrophils *in vivo*. However, the effects of propofol on regulatory cytokine TGF-β1 secretion in vivo are unknown. To investigate the clinical use whether affect the concentration of TGF-β1 in human, the human sera were obtained from the trauma patients treated with or without propofol and the levels of the total and active TGF-β1 expression was measured. Besides, the effects of propofol on TGF-β1 expression in human peripheral blood mononuclear cells (PBMC), vein endothelial cells (HUVECs), lymphocytes (Jurkat) and monocytes (THP-1) were also tested. Moreover, these sera were also tested in regards to their effects on monocytes endocytosis activity with or without TGF-β pathway inhibitor SB431542. Our results revealed that propofol would raise the total amount and active form of TGF-β1 in propofol-received patient sera (n=14) compared to non-propofol-received patient sera (n=10) within 24 h after surgical operations. Furthermore, propofol induced latent form TGF-β1 secretions in HUVEC cells (human umbilical vein endothelial cells) and enhanced TGF-β1 activation in THP-1 and JURKAT cells within clinical dosages (6.5μg/ml) *in vitro*. Besides, the sera from propofol-received patients would suppress the macrophage activity *ex vivo* (65.93% ± 5.36%, SE) and could be abrogated by TGF-β1 pathway inhibitor SB431542 (113% ± 9.48%, SE). These results implied that clinical treatments of propofol could trigger endothelial cells to express latent TGF-β1 which would be converted into the active form through monocytes or lymphocytes activities. This finding was conducive to predict the potential effects of propofol when used in medical operations during anesthesia.

# Acknowledgement

終於寫完了~此篇論文的完成雖然說不上曠日廢時，但確實是耗費我不少心思與時間，除了自己的投入之外，在這期間我受到很多人的幫助與鼓勵，我由衷的感恩在心！其中我最要感謝的是我的指導老師同時也是我的啟蒙恩師-廖光文博士。在將近一年半的大學專題生與兩年的碩士求學過程中，老師總是不厭其煩的提點我們實驗設計的要訣與點燃我追求科學真理的熱誠，總是維持許多良好的制度令我們的高度不斷提升，總是一再的循循善誘使我的心性更加堅強來面對實驗的挑戰…，我期許自己能以日後不斷的進步來回報對老師說不盡的感謝 !!

在這實驗室待了將近四年，可以算是這裡的“開朝元老”，大家一起粉刷的牆壁，不知道是誰烤壞的 pippet，每一張 bench，太多的回憶埋藏在各個角落，帶著不捨而離開是很艱難的決定，但面對不同的挑戰是讓自己更加成長的方式! 所有共事過的同學、學長姐和學弟妹們都是我的好夥伴、好朋友，我要特別感謝的是靜宜學姐，學姐是我實驗操作的入門導師，在這段期間我常常請教她、叨擾她，學姊總是不曾拒絕過我也不曾被我問倒，令我受益良多。還有彥谷學長 于鈴學姐、已經遠去的師兄等學長姐、很搞笑的詹姆士&小護士、資訊總管昱承、型男阿伯以及何佩、小利、小溫、筑婷等學弟妹 還有辛苦作值日生的大學部學弟妹們，難忘所有一同歡笑、一同愁眉苦臉 (被老師電的時候…)的時光!! 真的很幸運能和大家一起做研究與學習!! 真的很感謝你們! 希望能和大家一同在科學的道路上繼續不斷邁進!!

最後要感謝的是我的家人，從小父母親所提供的教育環境令我有充分的能力與心志面對求學過程中的挑戰，當然還要感謝有他們在經濟上的支持讓我可以衣食無缺，健健康康(白白胖胖??)的度過這段時間! 再來要感謝的也算是我的家人--我的咏馨(對不起!讓我偷偷放一下閃光~~)，她是我穩定的心靈寄託，全年無休的承受了我所有實驗成功的喜悅與失敗的落寞，讓我能夠在求學過程專心一致，不斷的尋求進步!!!!!!

其翰 2008.7

# Contents

**Abstract in Chinese……………………………………………….…....i-ii**

**Abstract……………………………………………………………...iii-iv**

**Acknowledment…………………………………………………………v**

**Contents……………………………………………………………...vi-xi**

**Lists of Tables…………………………………………………………xii**

**Lists of Figures…………………………………………………xiii-xiv**

**Abbreviations…………………………………………………………..xv**

**Chapter 1 Background**

**1.1 General view of anti-inflammation effects of intravenous anesthetics………..1**

**1.2 Modulation effects on proinflammatory cytokine release……………………..2**

**1.3 Modulation effects on other nitric oxide release………………………………..4**

**1.4 Modulation effects on antigen uptake and degradation……………………….5**

**1.5 Modulation effects on migration, adhesion and invasion……………………...6**

**1.6 Modulation effects on subpopulations of immune cells………………………..7**

**1.7 The protection effect on tissue damage and clinical implication……………...8**

**Chapter 2 Introduction (1)**

**2.1Pentobarbital…………………………………………………………….………12**

**2.2 Sepsis and Inflammation………………………………………………………13**

**Chapter 3 Material and Methods (1)**

**3.1 Material**

**3.1.1 Plasmid…………………………………………………………………7**

**3.1.2 Cell lines……………………………………………………………...7**

**3.1.3 Mice…………………………………………………………………..7**

**3.1.4 Pentobarbital…………………………………………………………...7**

**3.2 Methods**

**3.2.1 Spleen preparation and culture…………………………………………18**

**3.2.2 Cytokine measurement…………………………………………………18**

**3.2.3 TNF-α mRNA expression assay……………………………………...19**

**3.2.4 Transcription factor activity assay……………………………………...19**

**3.2.5 p38 MAPK expression assay……………………………………………20**

**3.2.6 TNF-α cytotoxicity assay……………………………………………..21**

**3.2.7 Cell apoptosis assay…………………………………………………...21**

**3.2.8 Preparation of animals………………………………………………..22**

**3.2.9 LPS shock……………………………………………………………...22**

**3.2.10 Experimental design…………………………………………………..23**

**3.2.11 Blood sample analyses………………………………………………..23**

**3.2.12 Blood biochemical analyses…………………………………………23**

**3.2.13 Statistical analysis……………………………………………………24**

**Chapter 4 Results (1)**

**4.1 Pentobarbital lowers the TNF-α concentration in serum at the presence of LPS in vitro and in vivo……………………………………………………25**

**4.2 Pentobarbital suppresses the activities of NF-κB and AP-1 at the presence of LPS……………………………………………………………………...26**

**4.3 Pentobarbital reduces tissue damages…………………………………………27**

**4.4 Pentobarbital protects cells from apoptosis……………………………...28**

**Chapter 5 Discussion (1)………………………………………...........29**

**Chapter 6 Figures (1)………………………………………………….32**

**Chapter 7 Introduction (2)**

**7.1 Propofol……………………………………………………………….....42**

7.1.1 The chemical properties of propofol……………………………….....42

7.1.2 The pharmaceutical properties of propofol……………………………42

7.1.3 The mechanism of propofol anesthetic action…………………………43

7.1.4 The effects of propofol on immune response…………………………..45

7.1.4.a The effects of propofol on leukocytes…………………………...45

7.1.4.b The effects of propofol on neutrophils…………………………..46

7.1.4.c The effects of propofol on macrophages………………………...47

7.1.5 The effects of propofol on inflammation induced by LPS……………48

7.1.6 The effects of propofol on endothelial cell……………………………...49

7.2 TGF-β...........................................................................................................50

7.2.1 The family TGF-β..................................................................................50

7.2.2 The biochemistry characteristic of TGF-β............................................50

7.2.3 Activation of latent TGF-β.....................................................................51

7.2.4 Cellular and tissue resource of TGF-β expression................................51

7.2.5 The eliciting stimuli of TGF-β...............................................................52

7.2.6 Signal transduction pathway of TGF-β.................................................53

7.2.7 The biological function of TGF-β..........................................................54

7.2.7.a TGF-β in angiogenesis...................................................................54

7.2.7.b TGF-β in atherosclerosis...............................................................56

7.2.7.c TGF-β in cancer progression……………………………………56

7.2.7.d TGF-β in wound healing…………………………………………58

7.2.8 TGF-β in immune modulation…………………………………………59

7.2.8.a Regulation of NK cell functions by TGF-β1……………………59

7.2.8.b Regulation monocytes/macrophage functions by TGF-β1…….60

7.2.8.c Regulation of dentritic cells by TGF-β1………………………...61

7.2.8.d Regulation of $CD4^+$ and $CD8^+$ T lymphocytes cell functions by

TGF-β1……………………………………………………………62

7.2.8.e Regulation of regulatory T lymphocytes functions by TGF-β1..64

7.2.8.f Regulation of B lymphocytes functions by TGF-β1…………….65

7.3 Strategy & Specific Aims…………………………………………………….67

Chapter 8 Material and Methods (2)…………………………………68

8.1 Material……………………………………………………………………….68

8.1.1 Reagents……………………………………………………………..…68

8.1.2 Animals………………………………………………………………...68

8.1.3 Plasmids……………………………………………………………….68

8.1.4 Bacterial strains………………………………………………………68

8.1.5 Patient serum and human white blood cells concentrate acquirement……………………………………………………………69

8.1.6 Cell lines………………………………………………………………69

8.2 Methods………………………………………………………………………71

8.2.1 Spleen preparation and culture………………………………………71

8.2.2 Midi-preparation of plasmid DNA……………………………………71

8.2.3 Transcription factor activity assay…………………………………...72

8.2.4 Mouse cytokine measurement………………………………………..73

8.2.5 Human peripheral blood mononuclear cells isolation………………...74

8.2.6 Human TGF-β1 measurement……......................................................74

8.2.7 Endocytosis activity measurement…………………………………...75

8.2.8 Statistical analysis……………………………………………………..77

Chapter 9 Results (2)……………………………………………79-91

9.1 The study of propofol action on mouse cells…………………………………..79

9.1.1 Propofol reduced TNF-α and IL-1β release in mouse splenocyes at the presence of LPS……………………………………………………………79

9.1.2 Propofol reduced IL-10 release in mouse-derived splenocyes at the presence of LPS……………………………………………………80
9.1.3 However, propofol enhanced TNF-α release in mice macrophage cell line P338/D1 at the presence of LPS…………………………………...81
9.1.4 Propofol induced TGF-β release in mouse-derived splenocytes at the presence of LPS…………………………….................................81
9.1.5 Propofol did not affect the TGF-β expression level in mouse macrophage cell P338/D1 and T lymphocyte cell EL-4………………..82
9.1.6 Propofol suppressed the activities of NF-κB and AP-1 at the presence of LPS……………………………………………………………………..82
9.1.7 Propofol promotes the activities of NF-κB and AP-1 without LPS stimulation………………………………………………………………...84
9.2 The study of propofol action on human TGF-β secretion and immune modulation…………………………………………………………………..85
9.2.1 Intravenous injection of propofol during medical procedure increased both total amount and active form of TGF-β in patient serum…………………………………………………………………...85
9.2.2 Clinical dosages of propofol have no significant effect on total amounts of TGF-β1 but slightly raised the amount of active TGF-β1 in the condition mediums of human peripheral blood mononuclear cells…………………………………………………………………….86
9.2.3 Clinical dosages of propofol slightly reduced total amounts of TGF-β1 but raised the amount of active TGF-β1 in the condition mediums of human Jurkat cells……………………………..…………………….86
9.2.4 Clinical dosages of propofol reduced total amounts of TGF-β1 but raised the amount of active TGF-β1 in THP-1 cells in a dose dependent

manner. …..……………………………………………87

9.2.5 Propofol inhibited the endocytosis activities of THP-1. ……………..87

9.2.6 Clinical dosages of propofol raised total amounts of TGF-β1 in HUVECs. ……………………………………………………..89

9.2.7 The condition mediums of HUVECs with propofol inhibited the endocytosis activities of THP-1…………………………………………89

9.2.8 The sera of patients received propofol could inhibit the endocytosis activities of THP-1 and abrogated by specific inhibitor of TGF-β pathway SB431542……………………………………………..90

9.2.9 Propofol might also induce IL-8 secretions THP-1…………………..91

Chapter 10 Discussion (2)………………………………………………..92

Chapter 11 Conclusion..…………………………………………………...100

Chapter 12 Tables…………………………………………………………102

Chapter 13 Figures (2)……………………………………………………108

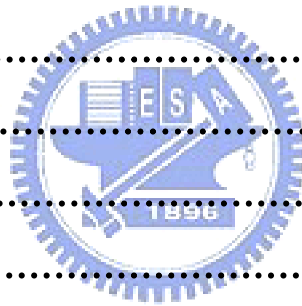
Reference…………………………………………………………………...133

Appendices…………………………………………………………………148

I. The map of pNFκB-hrGFP………………………………………………..148

II. The map of pAP1-hrGFP…………………………….…………………..148

III. Publishing..………………………………………………………………149



# Lists of Tables

**Table 1:** There have been different observations about immune-modulated effects of propofol in human beings.

**Table 2:** There are many inhibitors of TGF-β pathway in current study.

**Table 3:** The information of patients used in this study.

**Table 4:** There are many anesthetic agents affecting immune responses in current study.

# Lists of Figures

## Part I


**Fig.1.** The reduction of LPS-induced TNF-α from immune cells by pentobarbital.

**Fig.2.** The effect of pentobarbital on the expression of TNF-α mRNA.

**Fig.3.** The effect of pentobarbital on the production of TNF-α *in vivo*.

**Fig.4.** The effect of pentobarbital on the activities of NF-κB or AP-1 after LPS treatment.

**Fig.5.** p38 MAPK expression.

**Fig.6.** Improvement of blood biochemical factors reflecting multiple organ functions under pentobarbital anesthesia.

**Fig.7.** The protection of HEK293cells against TNF-α cytotoxicity by pentobarbital.

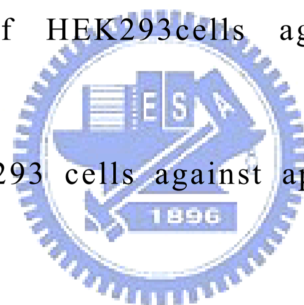


**Fig.8.** The protection of 293 cells against apoptosis by pentobarbital under hypoxia.


## Part II


**Fig.1.** The chemical structure of propofol.

**Fig.2.** Processing of TGF-β and formation of latent complex.

**Fig.3.** The signaling transduction pathway of TGF-β action.

**Fig.4.** The effect of propofol on IL-1β expression in mouse splenocytes.

**Fig.5.** The effect of propofol on TNF-α expression in mouse splenocytes.

**Fig.6.** The effect of propofol on IL-10 expression in mouse splenocytes.

**Fig.7.** Propofol induced TGF-β release of mouse splenocytes.

**Fig.8.** Propofol enhanced TNF-α release of mouse macrophages.

**Fig.9.** Propofol did not induce TGF-β expression in P338/D1 and EL-4

**Fig.10.** Propofol suppressed the activities of NF-κB and AP-1 at the presence of LPS.

**Fig.11.** Propofol raised the activities of NF-κB and AP-1.

**Fig.12.** Intravenous injection of propofol during medical procedure increased both total amount and active form of TGF-β1 in patient sera.

**Fig.13.** Clinical dosages of propofol have no significant effect on total amounts of TGF-β1 but slightly raised the amount of active TGF-β1 in the condition mediums of human peripheral blood mononuclear cells.

**Fig.14.** Clinical dosages of propofol slightly reduced total amounts of TGF-β1 but raised the amount of active TGF-β1 in the condition mediums of human Jurkat cells.

**Fig 15.** Clinical dosages of propofol reduced total amounts of TGF-β1 but raised the amount of active TGF-β1 in THP-1 cells in a dose dependent manner.

**Fig.16.** THP-1 uptake dextran-FITC ability was increased with dextran-FTIC concentrations.

**Fig.17.** Propofol inhibited endocytosis activity THP-1.

**Fig.18.** Clinical dosages of propofol raised total amounts of TGF-β1 in HUVECs.

**Fig.19.** The condition medium of HEVEC co-cultured with propofol inhibited endocytosis of THP-1.

**Fig.20.** The sera of patients who received propofol could inhibit the endocytosis activities of THP-1 and abrogated by TGF-β1 pathway inhibitor SB431542.

**Fig.21.** Continuous propofol treatments had potential to induced IL-8 secretion in THP-1.

**Fig.22.** Hypothesis of intravenous propofol on TGF-β expressions and activation *in vivo*.

# Abbreviations

**ALKs:** Activin receptor-like kinases

**AP-1:** Activating protein-1

**DFO:** Deferoxamine

**GABA:** Gamma-aminobutyric acid

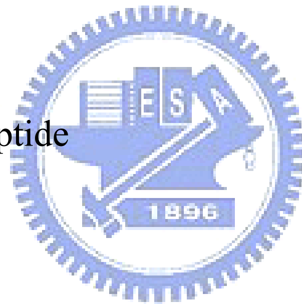
**HUVEC:** Human umbilical vein endothelial cells

**HEK 293:** Human embryonic kidney cell 293

**IL:** Interleukin

**IFN-γ:** Interferon-gamma

**LAP:** Latent associated peptide



**LPS:** Lipopolysaccharide

**LTBP:** Latent TGF-beta binding protein

**MODS:** Multiple organ dysfunction syndromes

**MOF:** Multiple organ failure

**NF-kB:** Nuclear factor-kappa B

**PBMC:** Peripheral blood mononuclear cells

**TGF-β:** Transforming growth factor-beta

**TNF-α:** Tumor necrosis factor-alpha

# Chapter 1 Background

## - General aspect: Immune modulation effects of intravenous anesthetics

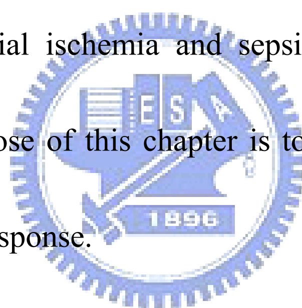
Intravenous anesthetics are large parts of drugs often used in anesthesia, and are administered directly into the patient's bloodstream. This allows them to reach a therapeutic level quickly and affect the brain quickly. Intravenous anesthetics nowadays include etomidate, midazolam, propofol, thiopentone and ketamine etc. There are some similar and some different side effects among these intravenous anesthetics. However, immune modulation effects, in particular anti-inflammation effects are general among these intravenous anesthetics.

## 1.1 General view of anti-inflammation effects of intravenous anesthetics

It is common in hospital that patients suffered different degrees of inflammation elicited by tissue damage or pathogen infections. Severe trauma, hemorrhage, and burns may initiate a cascade of inflammation and lead to septic complications, including multiple organ dysfunction syndromes (MODS) or multiple organ failure (MOF) [1, 2]. In addition to tissue damage-induced inflammation, endotoxin, more accurately referred to as lipopolysaccharide (LPS), is recognized as the most potent pathogenic mediator to induce local/systemic inflammations. Generally, LPS induce macrophages activation to priming the inflammation. LPS induce the activated macrophages to secreted proinflammatory cytokines and other mediators via MyD88-dependent Toll like

receptor 4 signaling pathway [3, 4]. The LPS-activated macrophage-derived inflammatory cytokines and other inflammatory mediators would result in generalized inflammation, procoagulant activity, tissue injury, and septic shock [5-7].

During the medical operations such as surgery or prospective care, intravenous anesthetics are the widespread used agents to induce anesthesia or sedation. By modulating the stress response or affecting the function of immune cells, anesthetics also cause immune suppression in surgical patients [8]. Besides, it had been reported that intravenous anesthetics had synergic effects to relieve inflammation syndrome, such as lung injury, myocardial ischemia and sepsis in clinical investigations or animal studies [9-13]. The purpose of this chapter is to summarize the effects of intravenous anesthetics on immune response.




**1.2 Modulation effects on proinflammatory cytokine release**

In the early stage of inflammation, many cells around the inflammation tissues would secrete many molecules served as chemokines or cytokines [14]. Until now, the studies about several intravenous anesthetics on proinflammatory cytokine release are quite discrepant. For example, midazolam suppressed the production of proinflammatory cytokine interleukin-1beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and chemokine interleukin-8 (IL-8) production in critically ill surgical patients during intravenous infusion [15]. Isoflurane succeed to

induce IL-1β, IL-6 and IL-8 secretion in patient alveolar macrophages [16]. There are arguments about the effects of propofol on proinflammatory cytokine release. These studies differ from the design of experiment and the subpopulations of immune cells and thus might offer confliction results. When study *in vitro*, propofol anesthesia increased the expression of IL-1β, IL-6, and IL-8 in patient derived alveolar macrophages [16]. However, Helmy showed that intravenous injection of propofol during surgery has no effect on IL-1β, IL-6, and TNF-α release in adult patients [17]. Nevertheless, the same group also found different observations when prolonged intravenous infusion (48h) in critically ill surgical patients. Propofol stimulated the production of the IL-1β, IL-6 TNF-α but caused suppression of IL-8 production [15]. The possible mechanism might be due to the difference in action dosages and timing between prolonged sedation and anesthesia.

When endotoxin like LPS stimulate the cytokine release, the synergic effects of these intravenous anesthetics were also been studied in cultured human whole blood. Thiopentone at pharmacologic concentrations inhibited LPS-stimulated TNF-α release. Ketamine significantly decreased LPS-stimulated TNF- α and IL-1β release. In addition, midazolam decrease the LPS-induced IL-8 secretions but even enhance LPS-induced IL-8 mRNA expression. There are still intangible when studying the effects of propofol on LPS-stimulated human whole blood. Propofol augmented LPS-stimulated

TNF-α release even in the presence of low concentrations. But propofol appears to inhibit IL-6, IL-8 and IL-10 production by LPS-stimulated PBMCs in vitro [18-21].

**1.3 Modulation effects on other nitric oxide release**

Besides cytokine release, immune cells would secrete other inflammatory mediators. For example, nitric oxide (NO) which generally generated by macrophages and neutrophils in response to stimulation, is a diffusible gas that plays an essential role in the course of inflammation. NO is toxic to bacteria and other human pathogens. In response, however, many bacterial pathogens have evolved mechanisms for NO resistance [4]. Besides, NO is also a major relaxing factor responsible for the vasodilation and systemic hypotension observed in septic shock. By short term administration of an inhibitor for nitric oxide synthesis, blood pressure in hypotensive patients with septic shock returns toward normal levels [22]. Ketamine reduce nitric oxide biosynthesis in animal studies and decrease NO productions in human umbilical vein endothelial cells by down-regulating endothelial nitric oxide synthase expression and intracellular calcium levels [23-26]. Thiopental also has been found that inhibit nitric oxide production in rat aorta [27]. Propofol could not only decrease the production of NO but also reduce the nitric oxide-induced apoptosis in testicular ischemia-reperfusion injury by downregulating the expression of inducible nitric oxide synthase [28-30]. Pentobarbital, midazolam and etomidate has also been reported that

caused a decrease in nitric oxide synthase activity resulting in decrease of NO production *in vitro* and *in vivo* [31, 32].

**1.4 Modulation effects on antigen uptake and degradation**

When neutrophils, macrophages or polymorphonuclear leukocytes (PMNL) arrive at the site of inflammation, they phagocyte and degrade substances such as pathogen, antigen or tissue debris. The degrade process would be accomplished by the action of oxygen-dependent or -independent manner [14]. The process of oxygen-dependent killing were also called "respiratory burst". In the course of this process, superoxide anion ($O_2^-$) is produced by NADPH-dependent oxidase. Several microbicidal oxidants like hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^.$) could be produced from $O_2^-$. Several agents contributed to oxygen-independent killing, for instance lysozyme, lactoferrin and defensins. Thiopentone, midazolam, etomidate, ketamine and propofol all significantly impair phagocytosis, chemotaxis and killing of *Staphylococcus aureus* and *Escherichia coli* by PMNL in vitro [33-35]. Furthermore, propofol and isoflurane reduce the phagocytotic and microbicidal activity of alveolar macrophages has been described during anesthesia [36, 37]. Furthermore, these intravenous anesthetics also significantly decrease the $O_2^-$, $H_2O_2$ and $OH^.$ production of neutrophils in a dose dependent manner. The possible mechanism also been purposed that the decreasing effects on calcium concentrations may responsible for the inhibition of neutrophils

functions [38-43].

### 1.5 Modulation effects on migration, adhesion and invasion

Under inflammation, a large number of immune cells are released into the circulation. Upon reaching the endothelium affected by inflammation, the leukocytes roll along the vascular lining, a process generally involving activation of selectins, integrins and ligands on the surface of leukocytes and the endothelium. To arrive at the extravascular tissue, leukocytes undergo morphologic change with pseudopodia and migrate through the intracellar junction of endothelial cells [14]. It has been reported that thiopental inhibits migration of human leukocytes through human endothelial cell monolayers in vitro [44, 45]. It has also been revealed that propofol reduce significantly the migration of leukocytes through endothelial monolayer by exerting inhibitory effects both on leukocytes and endothelial cells. Further *in vitro* studies showed that propofol has a greater inhibitory effect on endothelial cells [46]. Comparatively, Ketamine also reduce significantly the migration of leukocytes through endothelial cell monolayers. But Ketamine inhibits the function of leukocytes more than the function of endothelial cells [47]. The possible mechanism might be that ketamine has ability to attenuate the stimulated adhesion molecule like CD18 and CD62L expression of human neutrophils *in vitro* [48].

### 1.6 Modulation effects on subpopulations of immune cells

The balance among the immune-reactive cells is important in the reaction of the body to infection, injury, and surgical trauma. Drugs commonly used in anesthesia and intensive care may disturb the compositions of peripheral immune cells such as natural killer (NK) cells, B cells, and T lymphocyte subpopulations. It has been shown that anesthesia with thiopentone significant decrease the percentage of circulating NK cells but increase the percentage of B cells and $CD8^+$ T cells in the peripheral blood. The same set of experiments showed the percentage of $CD4^+$ T cells did not affect by thiopentone [49]. Similar to thiopentone, propofol significant increase the percentage of T cells and B cells and significant decrease the percentage of NK cells. However, different form thiopentone, changes in T lymphocyte subpopulations result from a significant increase in the percentage of CD4 T cells, which were accompanied only by a slight increase in CD8 T cells [50]. The possible mechanism still confused and conflicted. By *in vitro* and *in vivo* study, propofol downregulate the IL-2 and soluble IL-2 receptor production [15, 51], which is required for the proliferation of $CD4^+$ and $CD8^+$ cell, but induce the IFN-γ production [50, 52] , which is required for the proliferation of TH1 cells and $CD8^+$ cell. Although there have been no further research about the mechanisms, but the fluctuation of immune cells would be dangerous in patients with infection or extended surgical trauma.

### 1.7 The protection effect on tissue damage and clinical implication

Immune suppressive effects of intravenous anesthetics may expose the postoperative or intensive care patients to the danger of opportunity infection and sepsis. Due to bi-effects of inflammation cytokines such as TNF-α or other inflammation mediators like NO and reactive oxygen radical, tissue damages were commonly observed during inflammation and septic shock. It is rational that intravenous anesthetics with immune inhibitory effects could protect tissue damages caused by overpouring inflammatory mediators. Ketamine attenuates liver and gastric injury during endotoxemia [23, 25, 53]. Propofol could attenuate tissue damage in many inflammation associated situations, such as renal ischemia-reperfusion injury and ischemia-simulated cardiomyocyte dysfunction [54-56]. Propofol inhibited NF-κB activation in LPS-stimulated endothelial cells and was found to protect endothelial cells against LPS-induced barrier dysfunction. Pentobarbital could protect tissue functions from LPS-induced cytotoxic effects on hepatic, the heart or skeletal muscle, the renal, and the pancreatic. This protective effect might partly result from that pentobarbital could decrease LPS-induced TNF-α release and partly from that pentobarbital could decrease renal cell apoptosis induced by hypoxia or recombinant TNF-α [13]. Furthermore, immune suppression may facilitate the tumor progression in cancer patients who required anesthesia or long-term care. Thus using these immune

suppressive intravenous anesthetics should be more concerned.

Within know more clear about their immune modulated effects and mechanisms, we could take advantages using these intravenous anesthetics. For example, major burn injuries are widely recognized to engage most aspects of the immune system and to trigger a pronounced and often exaggerated activation of the inflammatory cascade. Propofol and ketamine anesthesia, as compared to the awaken state, has the potential of offering a certain degree of protection against burn injury in rats [57-60]. Up to the present, there are more studies about ketamine on burn injury than propofol. It has been reported that ketamine may improve the quality of long term sedation in severe burn injury patients in several case reports [61-63]. Besides, combination of propofol and ketamine also provided more effective sedation and analgesia than combination of propofol and fentanyl in pediatric patients undergoing burn dressing changes [64]. The present studies suggested that perfusing with intravenous anesthetics for sedation, particularly ketamine and propofol, could give good outcomes for burned patients after intensive care.

Therefore, further studies are needed to investigate the advantages and disadvantages of the current and newly developed intravenous anesthetics using in surgery and intensive care. Although inflammatory associated diseases subjected to treatment by intravenous anesthetics in clinical practices are certainty limited, our

current understanding and future insights into the mechanisms responsible for the wide range of inhibitory effects by these intravenous agents on the inflammatory cascade, may provide a safe and effective way for long term care patients suffering from inflammation-related effects such as lung injury, septic shock or myocardial ischemia. Comparatively, it should be controlled the use of these immune- modulated agents in immune-suppressed patients requiring anesthesia and intensive care, such as AIDS or cancer patients.

# Part I

## The Reduction of TNF-α Release and Tissue Damages by Pentobarbital in the Experimental Endotoxemia Model

# Chapter 2 Introductions (1)

Pentobarbital and propofol are the most used anesthetic agents in animal study and hospital. Both of them have been reported that suppress many immune responses. However, the mechanisms in regard to these reports are not enough and clear.

## 2.1 Pentobarbital

Pentobarbital is a short-acting barbiturate that is available as a free acid or a sodium salt, the former of which is only slightly soluble in water and ethanol. Pentobarbital is used to treat insomnia and to induce sleep before surgery. Pentobarbital exerts its antiepileptic action by enhancing gamma-aminobutyric acid receptor-coupled responses, thereby directly depressing neuronal excitability. For induction of barbiturate coma, pentobarbital is frequently chosen over other barbiturates for a number of reasons. Pentobarbital penetrates the brain faster and has a shorter half-life than phenobarbital; these attributes result in quicker antiepileptic activity and a shorter duration of coma when pentobarbital is stopped. The elimination half-life of pentobarbital is 15-60 hours. Despite a relatively shorter half-life, however, persistent pentobarbital drug levels can often be found in serum for days after discontinuation of therapy. Pentobarbital may also have a protective effect in cerebral hypoxia and may lower intracranial pressure [65]. The side effects of pentobarbital included difficulty breathing, hypotension and arrhythmia etc. Off-label uses of pentobarbital include reduction of intracranial pressure

in Reye's syndrome, traumatic brain injury and induction of coma in cerebral ischemia patients. High-dose pentobarbital has been the most commonly prescribed agent for the management of refractory status epilepticus (RSE) in children. Although this is a effective therapy for RSE, using high-dose pentobarbital has several disadvantages. In the present review, hypotension is a frequent complication and was reported in every pediatric patient who was treated with pentobarbital. Other side effects observed in pediatric patients treated with pentobarbital included pneumonia, delayed recovery, pulmonary edema, and ileus. Patients who are hemodynamically unstable or who have respiratory conditions may be particularly poor candidates for pentobarbital [66].

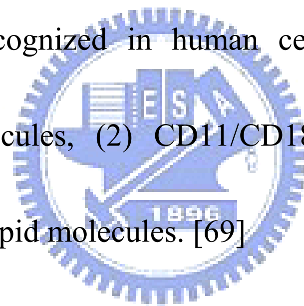
In recent years, due to the development of new anesthetic agents like propofol, the clinical uses of pentobarbital had been shrunken. But pentobarbital still often used as an intravenous agents to induce anesthesia and sedation in animals.

**2.2 Sepsis and Inflammation**

Sepsis is the leading cause of death in intensive care patients and it can cause persistent and uncontrolled release of proinflammatory cytokines [67, 68]. Despite advances in supportive care and the availability of potent antimicrobials, the mortality still exceeds 20 percents. This severe immune response would induce multiple organ failure.

Endotoxin, more accurately referred to as lipopolysaccharide (LPS), is recognized

as the most potent microbial mediator implicated in the pathogenesis of sepsis and septic shock. Despite its discovery over one century ago, the fundamental role of endotoxin in most patients with septic shock remains enigmatic and its value as a target for therapeutic intervention continues to be a contentious clinical issue [4]. LPS is viewed by the host as an alarm molecule indicating microbial invasion by gram-negative bacterial pathogens. LPS is a di-phosphorylated, polar macromolecule that contains hydrophobic elements within its lipid A core structure, and hydrophilic elements expressed on its repeating polysaccharide surface components. Three receptors for LPS have been recognized in human cells: (1) soluble or membrane-bound CD14-MD2-TLR4 molecules, (2) CD11/CD18 molecules (b2 integrins), and (3) scavenger receptors for lipid molecules. [69]



In human plasma and other body fluids, LPS trafficking is facilitated by a plasma protein known as LPS-binding protein (LBP). LBP performs a shuttle service picking up polymeric LPS aggregates and transferring LPS monomers to CD14 [70]. CD14 is a glycosyl phosphatidylinositol-linked protein found primarily on the cell surfaces of myeloid cells. Soluble and membrane-bound CD14 greatly potentiate the host response to small quantities of LPS and other microbial mediators. After docking to membrane-bound CD14, LPS is delivered to an essential extracellular adaptor protein known as MD2. This LPS–MD2 complex is then presented to the extracellular

leucine-rich domain of TLR4 where multimers of this complex aggregate on lipid rafts on the cell surface [71]. This series of events then triggers a signal to the intracellular space, subsequently activating LPS-responsive genes. CD14 also binds to bacterial peptidoglycan and lipopeptides and delivers these microbial ligands to TLR2 for intracellular signaling. Once TLR4 binds to its LPS ligand two possible pathways of cellular activation can occur through either the myeloid differentiation factor 88 (MyD88) or the TLR domain adaptor inducing interferon-beta (TRIF) pathway. A series of signaling events occur with sequential activation of specific tyrosine and threonine/ serine kinases [72, 73]. This signaling cascade ultimately leads to phosphorylation, ubiquitination, and degradation of inhibitory kappB (IκB) along with other transcriptional activators. IκB degradation releases nuclear factor kappaB (NF-κB) to translocate into the nucleus. Clotting elements, complement, other acute phase proteins, cytokines, chemokines, and nitric oxide synthase genes have NFκB-binding sites at their promoter regions [74, 75]. The outpouring of inflammatory cytokines and other inflammatory mediators after LPS exposure contributes to generalized inflammation, procoagulant activity, tissue injury, and septic shock. For example, studied revealed that LPS administration would induced TNF-α, IL-1β, IL-6 and inducible nitric oxide synthase etc. In particular, TNF rapidly increases in the circulation after exposure to LPS and is regarded as one of the earliest cytokines released [76-78].

LPS administration to animals under anesthesia is a strategy for inducing an inflammatory response. However, the anesthesia model has its drawbacks. First, the exposure of laboratory animals to the anesthetic agents might change their immune function [16, 37], including the production of cytokines [79-84] and the reduction in the activity of natural killer cells [9, 84, 85]. Second, hemodynamic changes after anesthesia enhance coagulation [84, 86, 87]. Because most animal studies were performed under anesthesia, their conditions are different from the clinical cases in which patients are in a conscious state. Therefore, the results obtained from anesthetized animals need to be re-examined. It has been demonstrated that such anesthetics may influence the immune response [16, 21, 37, 39, 88], but pentobarbital has not been discussed yet, which is an anesthetic used mainly in animal studies. In this present report, we compared the results of endotoxemia between pentobarbital-anesthetized and conscious animals. Surprisingly, the results suggest that pentobarbital not only reduce systemic TNF-α release but also decrease the degree of tissue damage under LPS administration. These results indicate that the medical effects of certain drugs which were performed on pentobarbital-anesthetized animals might have resulted from the synergistic effect of pentobarbital. This study provides a view to probe into the medical effects of anesthetics beside their anesthetic effects.

# Chapter 3 Materials and Methods (1)

## *3.1 Materials:*

### *3.1.1 Plasmid:*

pNF-kB/hrGFP and pAP-1/hrGFP plasmids containing the NF-κB and AP-1 transcription binding site, respectively, followed by a hrGFP reporter gene, were purchased from STRATAGENE, USA.. The higher the activity of transcription factors in a cell is, the higher the expression of hrGFP is.

### *3.1.2 Cell lines:*

HEK293 cell line and P338D1 cell line were obtained from BCRC (Food Industry and Development Institute, Taiwan) and cultured with DMEM supplemented with 10% FBS.

### *3.1.3 Mice:*

Animals were purchased from the National Laboratory Animal Center (Taipei, Taiwan, R.O.C.). C57BL/6JNarl mice were six-week female mice. Wistar Kyoto (WKY) rats were 16-week male rats.

### *3.1.4 Pentobarbital:*

It was purchased from MTC Incorporation (Cambridge, Ontario, Canada), whose trade name is “Somnotol.”

## *3.2 Methods:*

### *3.2.1 Spleen preparation and culture:*

C57BL/6JNarl mice were sacrificed by $CO_2$ asphyxiation. The spleen was taken, minced with DMEM, and filtered with a mesh. Soup was centrifuged at 1,200 rpm at 12 ℃ for 5 min. The supernatant was removed and 5 mL ACK buffer (150 mM NH4Cl, 10mM KHCO3, 0.1 mM Na2-EDTA) was added. After 5 min incubation, the mixture was centrifuged (at 1200rpm at 12℃ for 5 min) and washed twice with PBS to remove ACK buffer. 5 mL of RPMI 1640 was added to resuspend the cells. 2 x $10^6$ cells were then incubated in four conditions: 1) with RPMI 1640 (containing 10% FBS and 1% PS) (the control group), 2) with RPMI 1640 and 14 μg/mL of lipopolysaccharide (LPS; SIGMA, USA) (the LPS group), 3) with RPMI 1640 and pentobarbital (the pentobarbital group), 4) with RPMI 1640, LPS, and pentobarbital (the LPS + pentobarbital group). Each soup was collected after 48 hr and stored at -80℃.

### *3.2.2 Cytokine measurement:*

100 μl of capture antibody (0.8 μg/mL) was added into each well of an ELISA plate (COSTAR, USA) and the plate was incubated overnight. Wash buffer (0.05% Tween 20 in PBS, pH7.2~7.4) was applied three times. 300 μl of Block buffer was added and the plate was incubated for 1 hr at room temperature. Wash buffer was applied three times. 100 μl of samples were added into each well and incubated at room

temperature for 2 hr. The plate was then washed with wash buffer for three times. 100 μl of detection antibody (150 ng/mL) was added into each well. Samples were incubated at room temperature for 2 hr, and then washed three times with wash buffer. 100 μl of Tetramethylbenzidine substrate (CLINICAL, USA) was added into each well and the plate was incubated at room temperature for 20 min. To stop the reaction, 50 μl of stop solution (1N HCl) was added and the quantification was determined by the ELISA reader (SUNRISE, Switzerland) at the absorbance wavelength of 450 nm.

***3.2.3 TNF-α mRNA expression assay:***

P338D1 cells ($10^7$) were cultured and treated with LPS, and co-incubated without or with pentobarbital (final concentration = 12.5 μg/ml) for 6 hr. The treated cells were harvested and the total RNAs were extracted by phenol/chloroform method as described [89]. The cDNA were reverse-transcribed from total RNA by SuperScript™ Ⅲ First-Strand Synthesis SuperMix kit (Invitrogen, USA) and the TNF-α cDNA was amplified by PCR with the primer pairs (mouse TNF-alpha 5': ATgAgCACAgAAAgCATgATCCgCgA; mouse TNF-alpha 3': TCACAgAgCAATgACTCCAAAgTAgAC). The products of RT-PCR were analyzed by agarose electrophoresis and the results were photographed.

***3.2.4 Transcription factor activity assay:***

According to the manufacturer's instruction, pNF-kB/hrGFP and pAP-1/hrGFP

were transfected by Lipofectamine 2000 (Invitrogen, USA) into Balb/3T3 cells seeded in the 6-well plate, respectively. Twenty-four hr later, cells were passaged by versene (0.2g EDTA-4Na/L in PBS) and seeded into a 24-well plate. The transfectants were treated with LPS (14 μg/ml) and co-incubated without or with pentobarbital (12.5 μg/ml) for 16 hr, respectively. The transfectants were harvested and analyzed by flow cytometer. Specific FL-1 fluorescent intensities, representing the activities of the transcriptional factors, were calculated. In each plate, control plasmid pCMV/hrGFP was transfected into the target cells to measure the transfection efficiency, which was about 60 %.

***3.2.5 p38 MAPK expression assay:***

As previously described, p338D1 cell lines were treated with LPS and co-incubated with or without pentobarbital. The cells were harvested and mixed with the sample buffer (62.5 mM T ris-HCL pH 6.8, 2% SDS, 5% β-mercaptoethnol, 10% glycerol, 0.01% bromophenol blue). After the samples boiled, SDS-PAGE was performed and the products were transferred to a PVDF membrane. The samples were probed with rabbit anti mouse p38 polyclonal antibody (Santa cruz, Europe) or mouse anti-β-actin monoclonal antibody ( Biovision, USA). After washes, the membranes were re-probed with goat anti-rabbit IgG, HRP conjugated (MP Biomedicals, USA) or rabbit anti-mouse immunoglobulins/HRP polyclonal antibody (Dakocytomation, Denmark). Finally, the blots were washed, developed and visualized by ECL detection

according to the manufacturer's instructions. (PIERCE, USA).

***3.2.6 TNF-α cytotoxicity assay:***

Target cells were seeded into the 96-well plate. 12 hr later, pentobarbital at different concentrations was applied or not applied, with or without 2500 pg/mL TNF-α (the control group, the TNF-α group, the pentobarbital group, the TNF-α + pentobarbital group). The supernatant was removed after 16 hr. 100 μl of fresh medium was added with 20 μl MTS (Celltiter 96 Aqueous one solution cell proliferation assay, Promega, USA ). Cells were cultured in a $CO_2$ incubator at 37℃ for 4 hr. The absorbance was detected at 492 nm wavelength by an ELISA reader (SUNRISE, Switzerland). Relative cell survival (%) = Sample absorbance / Control absorbance * 100%. The control group was cultured in normal growth medium and its relative cell survival =100%.

***3.2.7 Cell apoptosis assay:***

The human renal epithelial cell HEK-293 ($2 \times 10^6$) in 3 ml growth medium were treated with 10 mM deferoxamine mysylate (DFO; SIGMA, USA) for 16 hr. The cells were harvested and suspended into 100 μl staining solutions (20 μl Annexin V-FITC labeling reagent and 20 μl propidium iodide in 1ml binding buffer). The mixture was incubated for 15 min and analyzed by flow cytometer. FL-1 represents Annexin V-FITC staining (apoptosis) and FL-3 represents PI staining (dead cells). The relative

apoptosis index = the fluorescent intensity of samples/the fluorescent average of the negative control x 100%.

### *3.2.8 Preparation of animals:*

Sixteen-wk-old male Wistar-Kyoto rats (WKY) were purchased from the National Animal Center and housed in the university animal rooms under a 12-hr light/dark cycle. Food and water were provided *ad libitum*. Animals were anesthetized with ether inhalation for about 10 min. During the period of anesthesia, a femoral artery was cannulated and connected to a pressure transducer to record the arterial pressure (AP) and the heart rate [87] on a polygraph recorder (PowerLab, AD Instruments Co., Mountain View, CA, USA). A femoral vein was catheterized for the intravenous (i.v.) administration of drugs. The operation procedure was completed within 15 min, and the section wound was smaller than 0.5 $cm^2$. After the operation, the animal was placed on a metabolic cage [88]. The rat awoke soon after the operation. During the experiment, the body temperature was measured rectally by a digital thermometer (HR 1300 thermometer, Yokogawa, Japanese) for every minute.

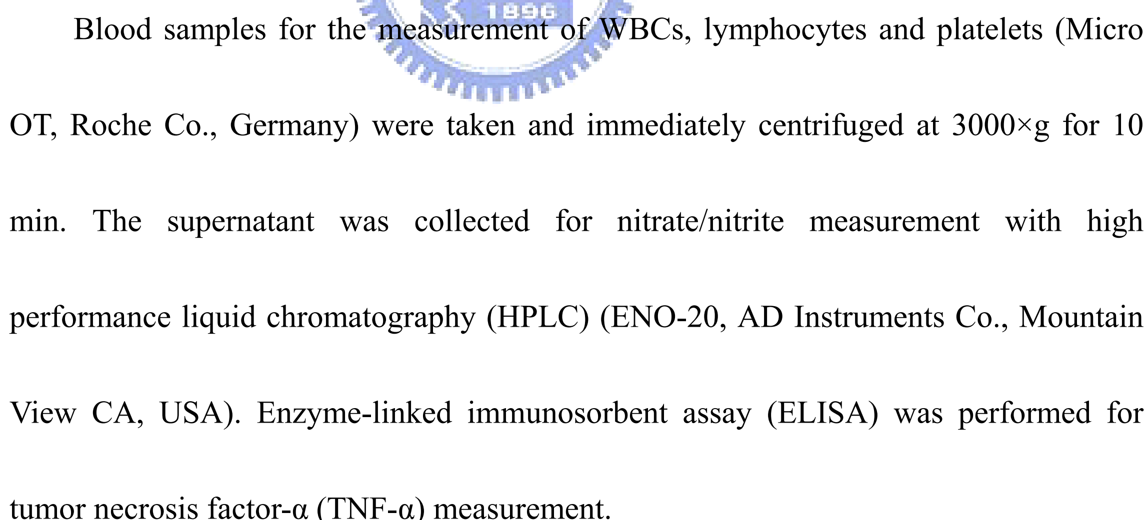
### *3.2.9 LPS shock*

LPS shock was induced by slow *i.v.* infusion of 10 mg/kg of LPS (Sigma Chemical Co., St. Louis, MO, USA) in 20 min. The infusion started 24 hr after the operation. The drug was dissolved in sterile physiological saline solution immediately before use. All

invasive procedures were operated under the aseptic condition. After the LPS administration, animals were observed for 48 hrs [90].

### *3.2.10 Experimental design*

Animals were divided into the NS, LPS and Pento groups (N=8). The NS group received a 1 ml injection of normal saline. The LPS group received 10 mg/kg of LPS (diluted in 1 ml) infusion. The Pento group received continuous infusion of pentobarbital at 10 mg/kg/hr after LPS. The blood samples were collected before normal saline and LPS and at 0.5 hr, 1 hr, 3hr, 6hr, 9 hr, 12 hr, 18 hr, 24 hr, 36 hr and 48 hr after the administration of saline or the drug.



### *3.2.11 Blood sample analyses*

Blood samples for the measurement of WBCs, lymphocytes and platelets (Micro OT, Roche Co., Germany) were taken and immediately centrifuged at 3000×g for 10 min. The supernatant was collected for nitrate/nitrite measurement with high performance liquid chromatography (HPLC) (ENO-20, AD Instruments Co., Mountain View CA, USA). Enzyme-linked immunosorbent assay (ELISA) was performed for tumor necrosis factor-α (TNF-α) measurement.

### *3.2.12 Blood biochemical analyses*

The plasma samples were diluted by 1:100 with distilled water before measurements. Plasma alanine aminotransferase (ALT), aspartate aminotransferase

(AST), lactic dehydrogenase (LDH), creatine phosphokinase (CPK), blood urea nitrogen [27] and amylase were measured with an autoanalyzer (Vitros 750, Johnson-Johnson Co., NY, USA) for evaluating various organ functions. ALT and AST are for the liver, LDH and CPK for the heart as well as other possible organs (such as muscle), and BUN for the renal, and amylase for the pancreatic function.

***3.2.13 Statistical analysis***

Data of *in vivo* experiments are expressed as mean ± SE. Multiple ANOVA and Scheffe test were used to compare the difference between and among groups (n = 8). All *in vitro* data were compared by student-test. The data of the transcriptional factor activity assay were obtained from 3 independent experiments and duplicated in each group (n=6). The data of the *in vitro* cytokine assay were obtained from 3 independent experiments and duplicated in each group (n=6). The data of TNF-α cytotoxic assay were obtained from 4 independent experiments (n=4). The data of the apoptosis assay were obtained from 3 independent experiments and duplicated in each group (n=6). P<0.05 was considered to be significant in all statistical analysis.

# Chapter 4 Results (1)

***4.1 Pentobarbital lowers the TNF-α concentration in serum at the presence of LPS in vitro and in vivo.***

Pentobarbital was assumed to be able to modify the inflammatory effects of LPS in endotoxemia. The results showed that pentobarbital significantly lowered the TNF-α release from P338D1 cells (mouse macrophage cells) under LPS stimulation (Fig. 1A). Moreover, pentobarbital also decreased the TNF-α expressions of splenocytes at the presence of LPS (Fig. 1B). In addition, the expression of TNF-α mRNA was reduced after pentobarbital treatment at the presence of LPS (Fig. 2). These results indicate that pentobarbital has the anti-inflammatory ability *in vitro*.

To study the effect of pentobarbital *in vivo*, an animal model of conscious rats with LPS treatment was established and utilized. After LPS treatment with or without pentobarbital administration, blood samples in each group were collected to measure the levels of inflammatory substances. LPS infusion caused a dramatic increase in TNF-α in sera of conscious rats *in vivo*. However, pentobarbital treatment reduced the seral concentration of TNF-α at the presence of LPS (Fig. 3A). The difference was observed within 12hrs after LPS treatment but no difference was detectable after 12h. The other indicator of LPS-induced inflammatory response, NO, increased in the conscious rat model after LPS treatment. In contrast, pentobarbital did not affect the expression of

NO in sera (Fig. 3B). *In vitro* and *in vivo* results indicate that pentobarbital has the ability to reduce the TNF-α release from immune cells. Because the effects could be mediated through the reduction of the body temperature, it was measured after different treatments. The body temperature of the animals in the LPS group (n=6) and the LPS+pento group (n=6) decreased (Fig. 3C). The decrease in the body temperature should be due to the LPS administration. The data of the two groups were no significantly different. Within 9 hours after the LPS administration, a conspicuous inhibitory effect of pentobarbital on TNF-α release was observed. However, the body temperature was not significantly different at this stage between the two groups. Therefore, a decrease in TNF-α release by pentobarbital should not be due to the change in the body temperature.

***4.2 Pentobarbital suppresses the activities of NF-κB and AP-1 at the presence of LPS.***

Previous literatures have reported that LPS activates NF-κB and AP1 pathways to enhance the TNF-α expression and release. Therefore, Balb/3T3 cells were transfected with plasmids containing the EGFP reporter gene under mini-promoter control (the mini-promoters were composed of several copies of NF-κB or AP-1 transcriptional factor binding sites) to determine the effects of pentobarbital on these signaling pathways. In our experiments, LPS increased the activities of NF-κB in cells (Fig. 4A) and slightly enhanced the activities of AP1 (Fig. 4B). However, pentobarbital reduced

the activities of NF-κB and AP-1 at the presence of LPS (Fig. 4). The result also showed that pentobarbital alone decreased the activities of NF-κB and AP-1 in cells at the concentration of 12.5 μg/ml. In addition, since LPS-induced TNF-α release involves p38 MAPK signaling pathway, we tested whether pentobarbital could interfere with it. Our result indicated that pentobarbital could reduce the amount of p38 MAPK at the presence of LPS (Fig.5).

***4.3 Pentobarbital reduces tissue damages***

TNF-α is a potent cytotoxic cytokine that results in tissue damages. As indicated in the previous results, pentobarbital decreases the TNF-α expression *in vitro* and *in vivo*. Whether pentobarbital could protect tissues from LPS-induced tissue damages was analyzed in the animal model of conscious rats *in vivo*. The results showed that LPS injection caused dramatic increases in ALT, AST, LDH, CPK, BUN and amylase in sera (Fig. 6) when compared to the normal control group, indicating that LPS injection deteriorated the hepatic (Fig. 6a, b), the heart or skeletal muscle (Fig. 6c, d), the renal (Fig. 6e) and the pancreatic functions (Fig.6f), as reflected by the changes of blood biochemical substances. However, pentobarbital infusion suppressed the increases in all bio-indicators at the presence of LPS (Fig. 6), indicating that pentobarbital could protect tissue damages from LPS-induced cytotoxic effects.

*4.4 Pentobarbital protects cells from apoptosis*

Besides the reduction of TNF-α release, the protective effects of pentobarbital on the survival of TNF-α target cells also needed to be determined. The HEK 293 human kidney cells are susceptive to TNF-α. Our results showed that the survival rates of HEK 293 cells were less than 40% under 2.5 ng/ml of TNF-α treatment for 16 hours (Fig. 7). Different concentrations of pentobarbital all increased the viabilities of cells at the presence of TNF-α , whereas pentobarbital alone did not significantly affect the cell survival rates of HEK 293 cells (Fig. 7).

TNF-α can increase the expression of adhesion molecules on the surface of immune cells and endothelial cells to cause the stagnant blood capillary effect and result in tissue hypoxia. When such hypoxia occurs, cells will undergo apoptosis. DFO has been shown to be able to induce apoptosis by the same mechanism as hypoxia and therefore, it was used to cause cell apoptosis in our *in vitro* experiment. We found that DFO alone increased the proportions of apoptosis of HEK 293 cells (Fig. 8). Nevertheless, pentobarbital protected the cells from apoptosis in DFO-induced hypoxia (Fig. 8).

# Chapter 5 Discussion (1)

In this study, the results suggest that pentobarbital infusion attenuates the multiple organ dysfunctions induced by LPS (Fig. 6). First, pentobarbital reduces the expression of TNF-α at the presence of LPS (Fig. 1 and 2). Gao and coworkers have demonstrated that LPS injection produces a large increase in the plasma TNF-α [91, 92]. In general, TNF-α is considered to be a principal mediator of endotoxemia and organ failure [93]. TNF-α has been implicated as an important mediator of the lethal effect of endotoxin, which can cause hepatic failure, etc. Several literatures have shown that inhibitors for reducing the activity or the expression of TNF-α significantly decrease the endotoxin-induced damages [93-95]. In addition, numerous studies have shown that the function of blood capillary is impaired and adhesion molecules aggregate to vessel walls under the septic progress [67, 82, 90, 96]. TNF-α strongly induces the expression of ICAM, VCAM, P-selectin on endothelial cells[97-99], which cause blood cells to adhere to endothelial cells [100]. The amount of TNF-α in serum can be associated with the degree of tissue damage because of the stagnant blood capillary. In fact, several anesthetic agents, including pentobarbital, have been demonstrated that they markedly suppress the TNF-α-induced neutrophil-venule adhesion [101]. For these reasons, the protective effect of pentobarbital might be due to the suppression of the systemic release of TNF-α.

In the second protective mechanism, pentobarbital directly protects tissue cells from the cytotoxic effect of TNF-α, which is a well-known cytotoxic cytokine for certain tissue cells (Fig. 6, 7). LPS raised the levels of several biophysical indicators (ALT, AST, LDH, CPK, BUN and amylase) in sera (Figure 6), which reflected the LPS-inducing damages of organ tissues. Pentobarbital increased the viability of the cells at the presentation of TNF-α, demonstrating its role in cell protection (Fig. 7). LPS stimulation can cause the stagnant blood capillary effect, resulting in hypoxia in tissues. When such hypoxia occurs, cells will undergo apoptosis. DFO can induce the same apoptosis as hypoxia and has been used to cause cell apoptosis *in vitro* [102, 103]. Pentobarbital can decrease the percentage of cells undergoing apoptosis at the presence of DFO treatment (Figure 8). Similar phenomenon has been reported that barbiturates have a protective effect against cerebral ischemia, and it has suggested that pentobarbital inhibits apoptosis to prevent ischemic neuronal death [10]. Therefore, the information indicates that pentobarbital should have the ability to protect tissue cells from the LPS-directly or -indirectly induced cytotoxicity of TNF-α.

In mammals, Toll-like receptor 4 (TLR-4) on macrophages send signals at the presence of LPS by associating with CD14 to activate its NF-κB pathway [3]. Besides NF-κB, previous literatures have also reported that LPS-induced endotoxemia can cause an increase in the p38 MAPK expression which is important for the LPS-induced

TNF-α release [104, 105] and AP-1 activities in cells [106]. In this study, the results reveal that pentobarbital suppresses the expression of p38 MAPK (Fig. 5) and the activities of NF-κB and AP-1 (Fig. 4) at the presence of LPS. Changes in the intracellular signaling pathway should be responsible for the decrease in TNF-α mRNA (Fig. 2) and the reduction of TNF-α protein expression caused by pentobarbital (Fig. 1A and B).

During endotoxemia, proinflammatory cytokines act both locally and systemically to aggravate the organ damage. Many investigations have shown that intravenous anesthetics have anti-inflammatory effects on endotoxemia both *in vitro* and *in vivo* [9, 11]. According to the results in this study, pentobarbital not only has an anti-inflammatory activity but also directly protects cells from apoptosis. Accordingly, pentobarbital may be beneficial in preventing organ dysfunction in endotoxemia or septicemia. But it should be used with caution regarding their potential immunomodulatory properties in critically ill patients.

# Chapter6 Figures (1)

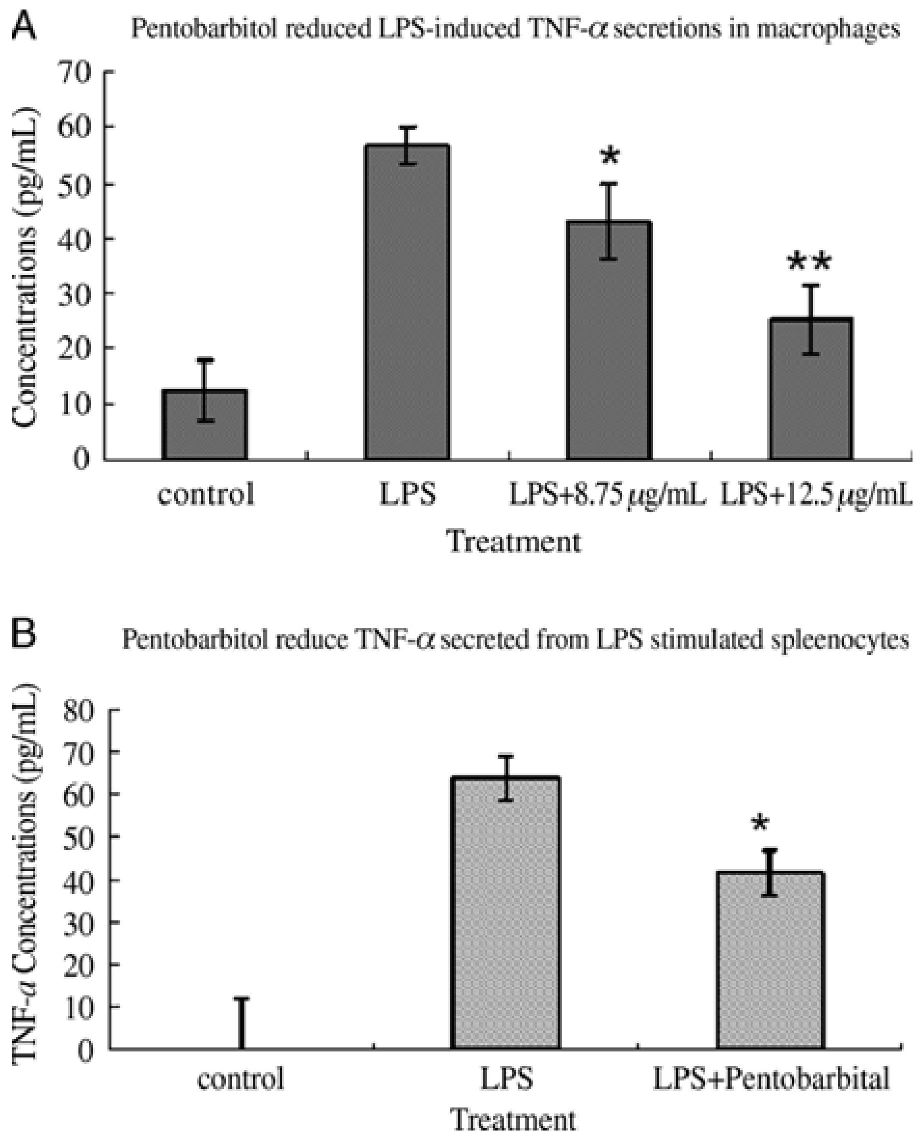



**Figure 1 The reduction of LPS-induced TNF-α from immune cells by pentobarbital.**

(A). P338D1 cells were treated with LPS (the LPS group). In the same condition, P338D1 cells were co-incubated with pentobarbital (the LPS+8.75μg/ml and the LPS+12.5μg/ml groups). The growth medium of untreated p338D1 cells served as the control group. *: $p < 0.05$ indicates a significant difference between the LPS+8.75μg/ml group and the LPS group，**: $p < 0.01$ indicates a significant difference between the

LPS+12.5μg/ml group the LPS group. (B). Splenocytes were treated with LPS (the LPS group). In the same condition, splenocytes were co-incubated with pentobarbital (the LPS+12.5μg/ml groups). The growth medium of untreated splenocytes served as the control group; *: $p < 0.05$ indicates a significant difference between the LPS+Pento group and the LPS group.

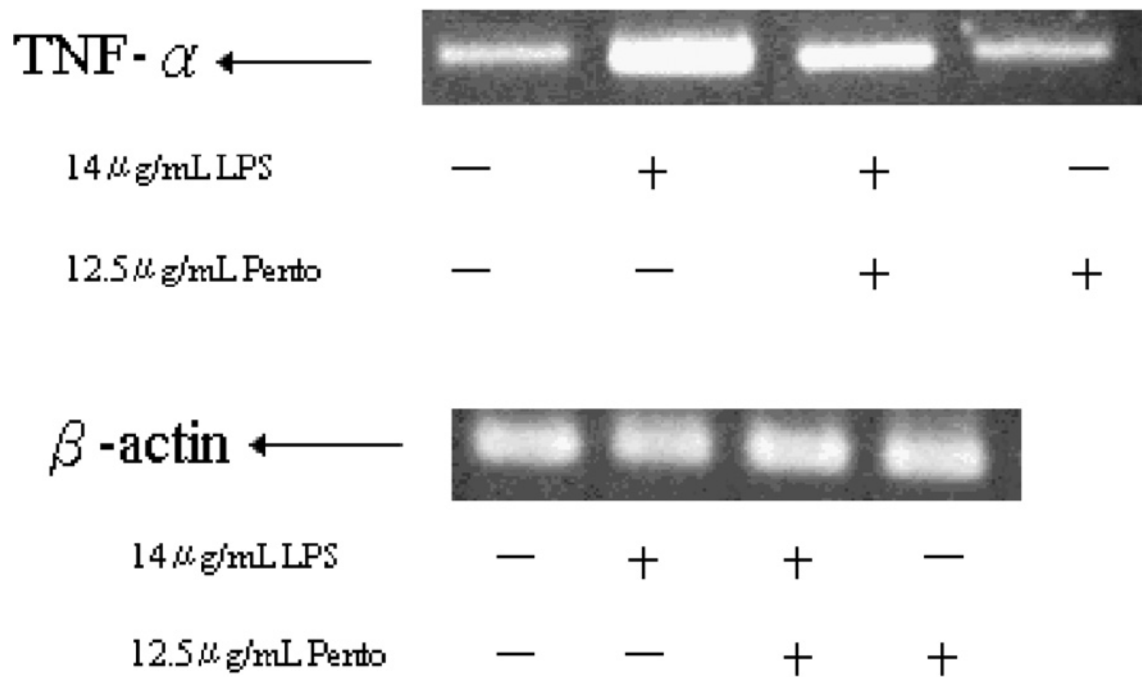



**Figure 2 The effect of pentobarbital on the expression of TNF-α mRNA.** P338D1 cells were treated with or without LPS and were co-incubated with or without pentobarbital. The mRNA levels of TNF-α were shown by RT-PCR. β-actin mRNA levels served as an internal control to normalize the sample loading.

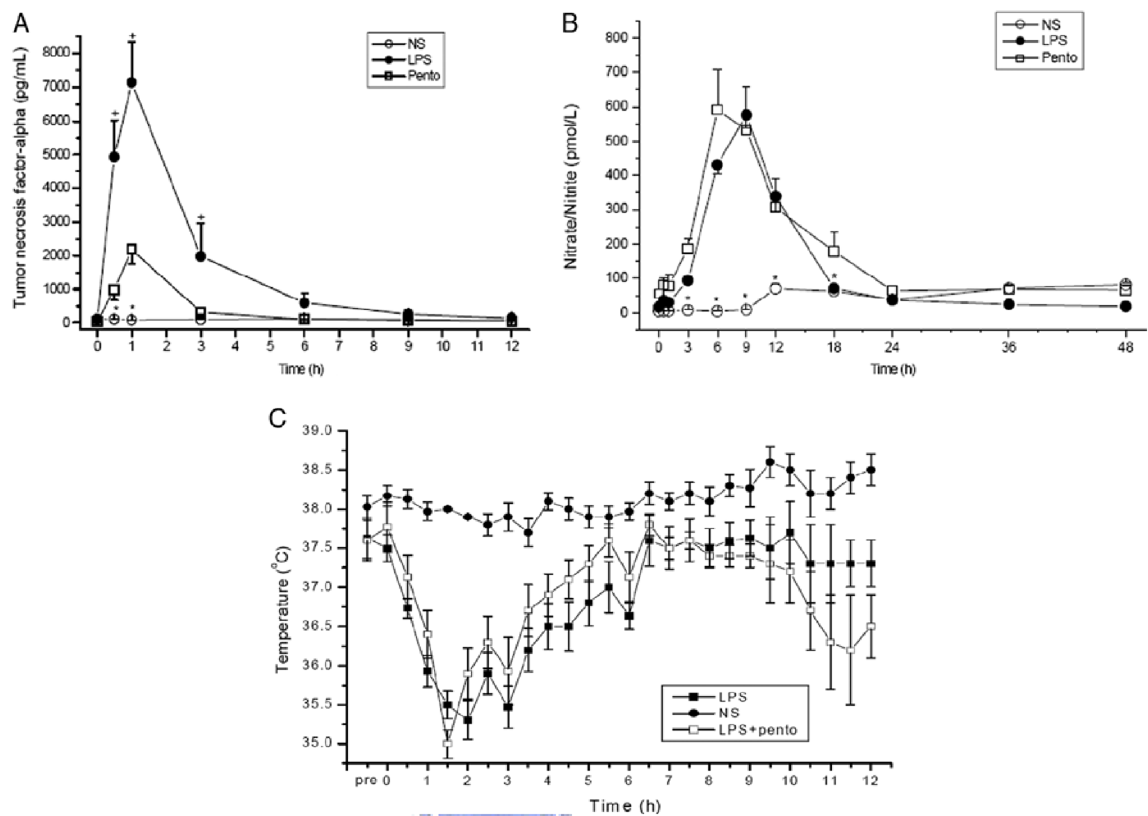



**Figure 3 The effect of pentobarbital on the production of TNF-α *in vivo*.** After LPS infusion, the levels of TNF-α (A) and nitric oxide (B) in sera of the mice were measured. The untreated mice served as the negative control (the NS group). (*: $P<0.05$ indicates a significant difference between the Pento group and the NS group. $^{+}$: $P<0.05$ indicates a significant difference between the Pento group and the LPS group.) (C)The body temperature of the rats was measured rectally by a digital thermometer. The untreated mice served as the negative control (the NS group).

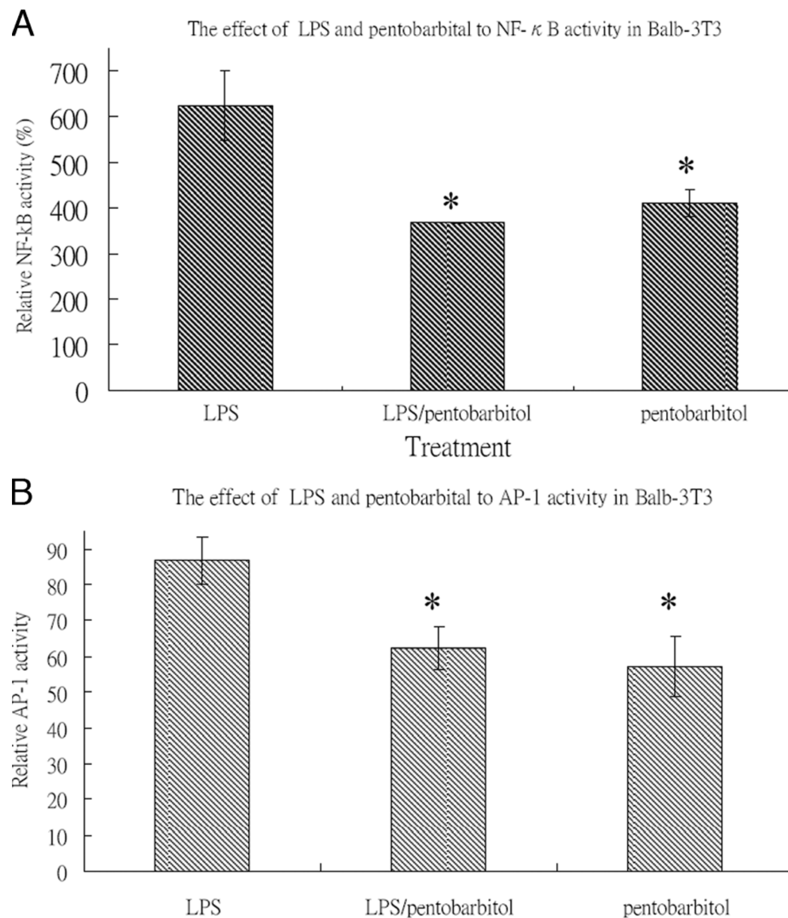



**Figure 4 The effect of pentobarbital on the activities of NF-κB or AP-1 after LPS treatment. (A)** pNF-κB/hrGFPor (B) pAP-1/hrGFP plasmids were transfected into Balb-3T3 cells. The untreated group was the control group. Transfectants were treated with LPS and co-incubated with (the LPS/pentobarbital group) or without (the LPS group) 12.5 µg/ml pentobarbital. Transfectants were also incubated with growth medium containing 12.5 µg/ml pentobarbital without LPS (the pentobarbital group). (*: $p < 0.05$ indicates a significant difference between the LPS+Pento or the Pento groups between the LPS group.)

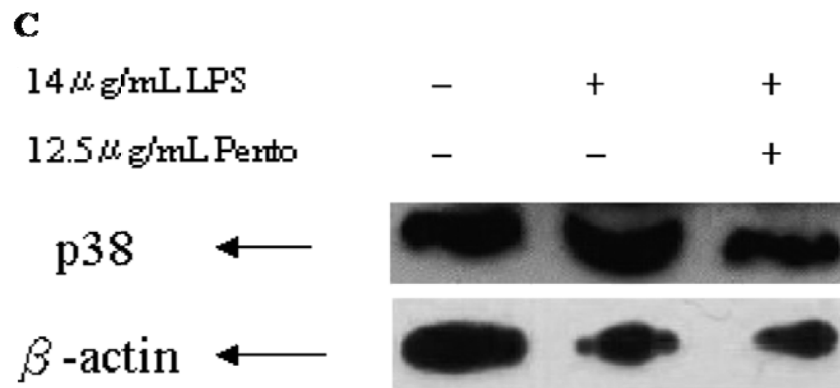



**Figure 5 p38 MAPK expression.** P338D1 cells with or without LPS treatment and co-incubated with or without pentobarbital. After treatment, the cells were probed to determine the expressions of p38 MAPK protein and β-actin protein.

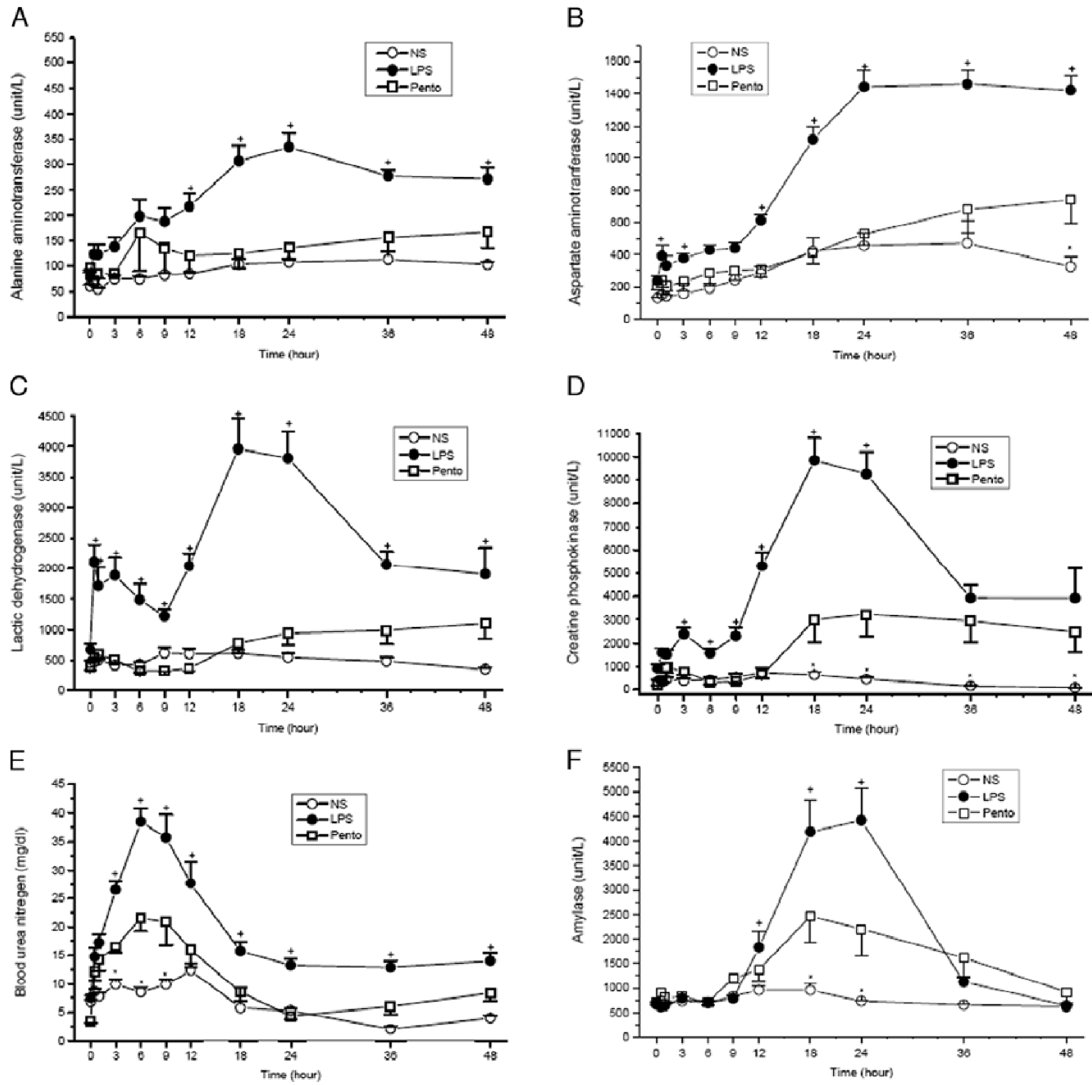



**Figure 6 Improvement of blood biochemical factors reflecting multiple organ functions under pentobarbital anesthesia.** Plasma levels of ALT **(a)**, AST **(b)**, LDH **(c)**, CPK **(d)**, BUN **(e)** and amylase **(f)** levels of rats in the Pento group, the LPS group and negative control group were measured. (*: $P<0.05$ indicates a significant difference between the concentration of the Pento group and that of the NS group. $^{+}$: $P<0.05$ indicates a significant difference between the concentration of the Pento group and that of the LPS group.)

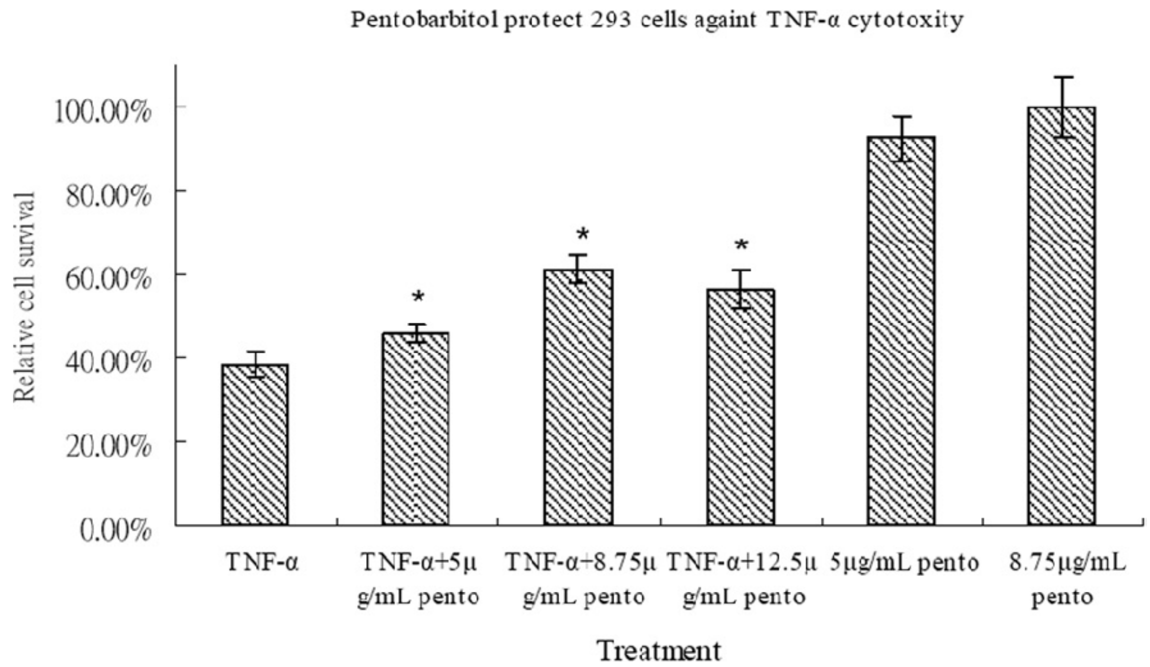



**Figure 7 The protection of HEK293cells against TNF-α cytotoxicity by pentobarbital.** HEK293 cells in the normal growth medium served as the control group. HEK293 cells were treated with or without TNF-α and co-incubated without or with different dosages of pentobarbital. (*$p < 0.05$ indicates the relative survival significantly differed from that of the TNF-α-treated group.)

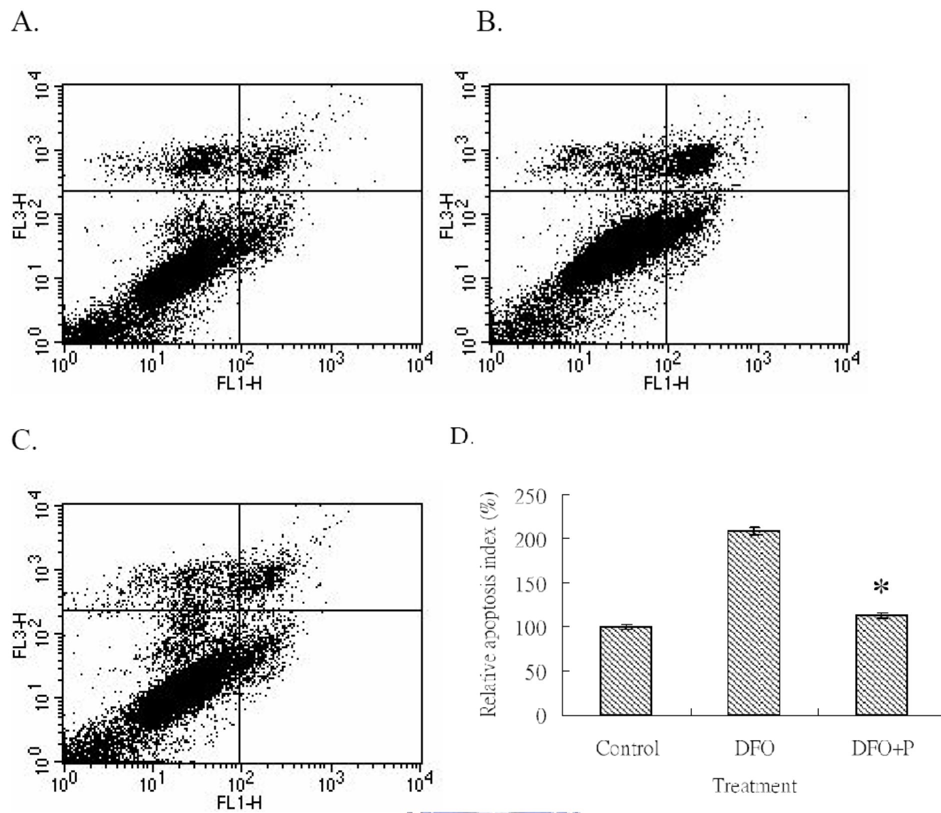
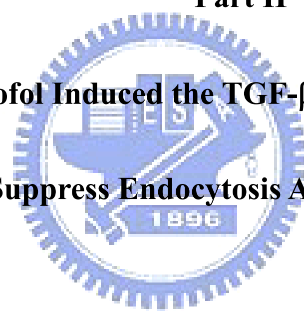



**Figure 8 The protection of 293 cells against apoptosis by pentobarbital under hypoxia.** The normal medium cultured HEK293cells served as the control group (A). The HEK293cells were treated with 10 mM DFO and co-incubated without (B). or with pentobarbital (C). The treated cells were probed with Annexin V-FITC (FL-1) and PI (FL-3), and analyzed by flow cytometer. The relative apoptosis index was calculated and shown in (D). (*: $p < 0.05$ indicates a significant difference between the DFO+P group and the DFO-treated group.)

# Part II



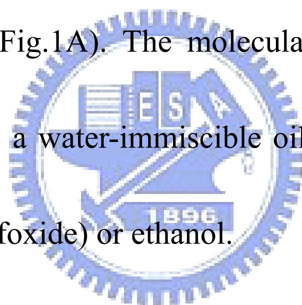
## The Treatment of Propofol Induced the TGF-β1 Expression in Human Endothelial Cells to Suppress Endocytosis Activities of Monocytes

# Chapter 7 Introductions (2)

## 7.1 Propofol

### 7.1.1 The chemical properties of propofol

Propofol is the most recently intravenous an aesthetics in clinical use. Propofol is approved for the induction and maintenance of anesthesia in more than 50 countries. The commonly used brand name is *Diprivan.* Another commonly used name is disoprofol. The molecular formula of propofol is $C_{12}H_{18}O$; molecular weight is 178.271 g/mol; and systematic (IUPAC) name is 2, 6-diisopropylphenol or 2, 6-Biss (1-methylethyl) phenol (Fig.1A). The molecular models of propofol were shown in Fig.1B [107], propofol is a water-immiscible oil but soluble in many organic solvents like DMSO (dimethyl sulfoxide) or ethanol.




### 7.1.2 The pharmaceutical properties of propofol

Due to propofol is not soluble in water and cannot be injected only by itself. Initial clinical trials were in 1977, in a form solving in cremophor EL. But cremophor formulation produced a marked increase in plasma histamine concentration; it was withdrawn from the market. It was subsequently reformulated as an emulsion formulation, and suggested that the emulsion formulation may produce less discomfort on i.v. injection based on animal test [108]. It was re-launched in 1986 by AstraZeneca with the brand name Diprivan. The current preparation is 1% propofol, 1.2% purified

egg phospholipid, 2.25% of glycerol, 10% soybean oil (emulsifier). In the United States, the products contain disodium edetate (0.005%) as a microbial growth retardant to inhibit the growth of microorganism in the event of accidental contamination.

Propofol is a short-acting intravenous anesthetic agent used for the induction of general anesthesia in adult patients and pediatric patients older than 3 years of age; maintenance of general anesthesia in adult patients and pediatric patients older than 2 months of age; and sedation in medical procedure. Propofol is rapidly and extensively distributed in the body. It crosses the blood-brain barrier quickly, and its short duration of action is due to rapid redistribution from the CNS to other tissues, high metabolic clearance. The elimination half-life of propofol has been estimated to be between 2–24 hours. The therapeutic concentrations of propofol in plasma is 1.5 to 6.5 μg/mL for anesthesia [109, 110]; 0.14 to 1.92 μg/mL for sedation [111] and 0.170-0.437 μg/mL for postoperative nausea and vomiting [112].

The side/adverse effects of propofol used as anesthetic agents clinically have been reported according to Apnea, bradycardia, bronchospasm, erythema, hypotension and so on [113, 114].

### 7.1.3 The mechanism of propofol anesthetic action

Its mechanism of anesthetic action has not been well-defined. The primary target of propofol act which been identified is the $GABA_A$ receptor [115]. Recent research has

also suggested propofol activate cannabinoid receptors in the endocannabinoid system may contribute significantly to propofol's anesthetic action [107]. There are three classes of GABA receptors: $GABA_A$, $GABA_B$, and $GABA_C$. $GABA_A$ and $GABA_C$ receptors are ligand-gated ion channels, whereas $GABA_B$ receptors are G protein-coupled receptors.

The $GABA_A$ receptors is formed a pentamer composed of $\alpha1\beta2\gamma2$ subunits. $GABA_A$ receptor channel is the most abundant, fast inhibitory, ligand-gated ion channel and is found throughout the central nervous system in the mammalian brain. The $GABA_A$ receptors mediate inhibitory neurotransmission in the central nervous system. Under physiological conditions, the receptors are activated by GABA, but several other compounds such as neurosteroids and barbiturates can also gate the channel. The $GABA_A$ receptors are a major target for drugs used for the induction and maintenance of general anesthesia and for the treatment of anxiety and epilepsy [116, 117].

Most volatile and intravenous anesthetics including propofol enhance the activity of gamma-aminobutyric acid type A ($GABA_A$) receptors and directly activate this ligand-gated chloride ion channel and, hence, increases the chloride conductance in the absence of its endogenous ligand, GABA. The current findings suggest that first transmembrane domain glycine 219 residue of the $\beta2$ subunit is critical for the rate at which desensitization occurs and that both GABA and intravenous anesthetics

implement an analogous pathway for generating desensitization [118]. Additionally, anesthetics are known to prolong GABA-induced $Cl^-$ channel opening, and depending on the type of anesthetic, this potentiation of GABA-gated currents appears to alter deactivation and/or desensitization. For example, halothane, a volatile anesthetic, has been shown to slow the dissociation of GABA from its receptor to slow deactivation. However, Propofol can slow both the rate of deactivation and the rate of recovery from desensitization by stabilizing the ligand-binding structure of $GABA_A$ receptors [119, 120].

**7.1.4 The effects of propofol on immune response**

Previous studies have demonstrated that propofol might have immune-modulating effects on various types of human immune cells like leukocytes, neutrophils and macrophage.

**7.1.4.a The effects of propofol on leukocytes**

There are researches which had showed that propofol is able to reduce significantly the migration of leukocytes through endothelial cell monolayers with a dose-dependent effect. These interactions between leukocytes and endothelial cells play a critical role during inflammatory processes to defense microorganisms. Leukocytes attack invading microorganisms after migrating from the intravascular space into tissue. To arrive at the extra-vascular tissue, leukocytes migrate through a monolayer of endothelial cells.

During this migration, leukocytes undergo morphologic changes from rounded, relatively smooth cells to elongated, ruffled cells with pseudopodia. The results of the investigation indicate that the influence of propofol both on leukocytes and endothelial cell monolayers [46]. The results would imply that propofol could inhibit leukocytes-dependent immune defense by acting directly on leukocytes.

**7.1.4.b The effects of propofol on neutrophils**

Neutrophils are the most numerous one of leukocytes. Neutrophils play a crucial role in the early line of anti-bacterial host defense mechanism as a component of nonspecific cell-mediated immunity. The neutrophi1 response to microbial invasion consists of chemotaxis, adherence, phagocytosis, and intracellular killing. Propofol significantly inhibited reactive oxygen species [22], chemotaxis, and phagocytosis of neutrophils in a dose-dependent manner. A clinically relevant concentration of propofol suppressed these neutrophils functions, this impairment observed in vitro, implying that leads to clinical immunological suppression during medical procedures [39]. Increase in intracellular calcium concentrations in neutrophils stimulated by N-formyl-L-methionyl-L-leucyl-L-phenylalanine was dose-dependent attenuated by propofol. This decreasing effect on intracellular calcium in neutrophils may be one of the mechanisms responsible for the propofol-induced suppression of chemotaxis and phagocytosis. Furthermore, the increase in intracellular $Ca^{2+}$ of neutrophils is thought to

be one important pathway by which extracellular stimuli are transmitted to activate enzymes responsible for the production of $O_2^-$ (nicotineamide adenine dinucleotide phosphate oxidase) in the cell membrane. However, the ROS produced by neutrophils that accumulate in various organs is thought to play a critical role in the pathogenesis of endotoxin-induced multiple organ failure. The ability of propofol to decrease neutrophi1 functions could be beneficial in particular situation.

### 7.1.4.c The effects of propofol on macrophages

Moreover, a clinically relevant concentration of propofol can suppress mouse macrophage-like Raw 264.7 cells functions of chemotaxis, phagocytosis, oxidant production, and IFN-γ mRNA synthesis in concentrationand time-dependent manners, possibly through inhibiting their mitochondrial membrane potential and adenosine triphosphate synthesis but did not affect cell viability [121]. Another evidence was showed that the number of phagocytic cells (ingesting at least one particle) of patient-derived alveolar macrophage would significantly decreased in 4h after induction of anesthesia [37]. Despite of many researches showed that propofol may impair macrophage functions; propofol anesthesia has also been reported to induce proinflammatory cytokines, including tumor necrosis factor-α, interferon-γ (IFN-γ), interleukin-1β, and interleukin-8 in orthopedic surgery patients [16]. However, multiple factors could be involved in modulating macrophage functions in the surgical

procedures, so that the *ex vivo* studies did not clarify whether propofol alone could modulate macrophage activities. Therefore, an *in vitro* study will be needed to verify the role of propofol in modulating macrophage functions to rule out the contribution of other factors. Nevertheless, there are few articles emphasizing on the human monocytes-like cells modulated by propofol within clinical dosages. This is one of our interests and makes our efforts to in this study.

**7.1.5 The effects of propofol on inflammation induced by LPS.**

In addition to its action on immune cells by itself, propofol also have been shown that it has anti-inflammatory and antioxidative effects on LPS-activated Raw 264.7 cell [122] and endotoxemia rats [123]. In response to LPS stimulation, propofol has also been shown to inhibit INF-γ mRNA synthesis in macrophages. During inflammation, macrophages destroy invaded microorganisms through a series of reactions, including chemotaxis, phagocytosis, oxidant synthesis, and cytokine release. Dysfunction of these activities will affect host macrophage-mediated immunity. Nitric oxide is an active oxidant that contributes to the physiology and pathophysiology of macrophages. It has been shown that propofol reduces nitric oxide biosynthesis in LPS-activated macrophages by down-regulating the expression of inducible nitric oxide synthase [124]. The present study also shows that propofol, at a therapeutic concentration, has anti-inflammatory and antioxidative effects on the biosyntheses of TNF-α, IL-1β, IL-6,

and NO in LPS-activated Raw 264.7. Furthermore, propofol enhances LPS-stimulated TNF-α and IL-1β but reduces the LPS-stimulated IL-8 expression [19, 21]. However, propofol also reduces the expression density of LPS-stimulated CD14 whereas unaffecting HLA-DR [18].

According above, two arguments can be made in regards to the propofol effect on the immune activity. There is no acceptable mechanism yet in regards to the effect of propofol in the immune regulation and inflammation induced by LPS (Table 1).

**7.1.6 The effects of propofol on endothelial cell**

Being an intravenous agent, the effects of propofol on endothelial cell also should be concerned. Propofol could reduce endotoxin-induced endothelial damages by extracellular and intracellular mechanisms. By extracellular mechanism, propofol could offers significant protection against endotoxin-induced pulmonary microvessel endothelial cell injury by scavenging the active oxygen species released in the extracellular space. By intracellular mechanism, propofol directly reduced the LPS-enhanced iNOS mRNA and prevent LPS-induced cell barrier dysfunction by reducing transcription factor NF- κB protein levels. Propofol also directly protect the endothelial cells reduces apoptosis by suppressing caspase-3 activity and recover the function in hydrogen peroxide-stimulated human umbilical vein endothelial cells [92, 125-128].

## 7.2 TGF-β

### 7.2.1 The family TGF-β

TGF-β purified from platelets, placenta, and kidney was subsequently named TGF-β1, to distinguish it from two other highly homologous isoforms, TGF-β2 and TGF-β3, which are interchangeable in a variety of biological assay, but which are encode by distinct genes. TGF-β1 is the predominant isoform in most cells and tissues.

### 7.2.2 The biochemistry characteristic of TGF-β

Each of three mammalian isoforms of TGF-β are encodes as 390-442 amino acid precursor proteins that contain s signal sequence and are processed proteolytically by furin [129], a member of the mammalian convertase family of endoproteases. In mammalian cells, the presence of pre-domain is required for the proper folding and secretion of TGF-β. TGF-β is secreted in noncovalent association with its own pro-domain (latency- associated peptide, LAP) in a "latent" form unable to bind receptors until activated. LAP contain N-linked glycosylation sites which be mutated would prevent the cellular secretion of TGF-β. The most critical posttranslational modification of TGF-β is the proteolytic processing at an RXXR site by the endoprotease furin resulting in release of this fragment with the remainder of the biologically active 112 amino acid C-terminal domain and the subsequent noncovalent association of the precursor called the latency-associated peptide, LAP (Fig.2). Secreted

TGF-β is either in the form of “small” (100 kDa) latent complex consisting only of LAP and mature TGF-β or a tertiary “large” (220 kDa) latent complex complex in which a secretory glycoprotein, the latent TGF-β binding protein (LTBP) is covalently bound to LAP through a disulfide bond from the third eight-cysteine repeat of the latter to the N-terminal-most cysteine in LAP [130]. LTBPs are important both in section of TGF-β and in targeting it to extracellar matrix.

### 7.2.3 Activation of latent TGF-β

Activation of latent TGF-β has been studied intensively *in vivo,* where extremes of pH, heat, protease or chaotropic agents have all been shown to release active TGF-β from the latent complex. These mechanisms include (1) a protease-independent mechanism involving the binding of thrombospondin, a component of platelet α granules and of extralcelluar matrix [131]; (2) proteolytic activation via transreglutaminase and plasmin.plasminogen activator [132]; (3) binding of RGD sequence of the LAP of the latent complex to the $\alpha_v\beta_6$ [133]; (4) uptake and activation of Fc receptors on macrophage of complexes of TGF-β bound to immunoglobulin [134]; (5) invasion of *Candidiasis* stimulates hepatocytes and monocytes to secrete active TGF-β, although the mechanism is unclear [135].

### 7.2.4 Cellular and tissue resource of TGF-β expression

Most cultured cells express at least one of the TGF-β isoforms. In many

mammalian cell types, all three isoforms are expressed significantly, while in other cell types one isoforms predominates. Immunoactive TGF-βs 1, 2, and 3 are present in four-cell embryos through to the blastocyst stage and continue to be expressed in all tissues throughout development. For example, TGF-β1 mRNA is expressed in the overlying epithelial cells. In contrast, TGF-β2 mRNA is often expressed in epithelium of morphogenetically active tissues and epithelial cells that are undergoing differentiation such as those in alveoli and palate, as well as superbasal keratinocytes.

TGF-β express widespread in the adult; most tissues express all three isoforms with some differences in the expression level. The most abundant source of TGF-β1 in humans are platelets, bone, and spleen; TGF-β2 predominates in fluids such as the aqueous and vitreous of the eye, breast milk, and amniotic fluid.

### 7.2.5 The eliciting stimuli of TGF-β

The main regulators of TGF-β expression in vivo are changes in levels of steroid hormone (either by addition of exogenous steroids or by manipulations to alter endogenous levels), as well as cellular injury, stress, or viral/parasitic infections. Hormone ablation or replacement strongly affects TGF-β expression. Retinoids have strong modulating effects on the expression of TGF-β isoform *in vivo* [136]. For example, β-Carotene treatment of patients with cervical intra-epithelial neoplasia leads to an increase in immunoreactive TGF-β1 in these cells [137].

### 7.2.6 Signal transduction pathway of TGF-β

The signals of cellular responses to TGF-β are through specific TGF-β transmembrane receptor type I and type II Ser/Thr kinase receptor. The signal pathway initiated by TGF-β binding to the TGF-β type II receptor. After ligand binding, TGF-β type II receptor recruits and phosphorylates the TGF-β type I receptor, also termed activin receptor-like kinase (ALK family) [138]. This results in a conformation change and activation of the type I receptor which can propagate the signal inside the cell to the nucleus by the phosphorylation of specific effectors [139]. TGF-β binds and transducts the signal through ALK-5 in most cells but through both ALK-5 and ALK-1 in endothelial cells. The specific effectors that play an important role in TGF-β signal transduction form the membrane receptor to the nucleus are called Smads. Smads (R-Smads) are recruited and phosphorylated after activation of ALK. The activation of ALK5 induced the phosphorylation Smad2 and Smad3, and activation of ALK1 induced the phosphorylation Smad1 and Smad5. Subsequently Smad4 forms heteromeric complexes with R-Smads and these complexes then translocate to the nucleus and regulated the target gene expression by collaboration with transcription factors (TFs) and cofactors (coactivators or corepressors) (Fig3). The end of signaling pathway is also important, and there are at least two identified mechanism for the termination of Smad signaling. One is that dephosphorylation of

R-Smads by unidentified phophatases. The other is ubiquitination and protease-dependent degradation of the activation R-Smads.

There are many TGF-β signaling pathway specific inhibitors at present (Table 3). We choose SB 431542 hydrate to inhibit the TGF-β signaling pathway in this study. SB 431542 inhibits the acitivity of (TGF-β1) activin receptor-like kinases (ALKs). It is a selective and potent inhibitor of the phylogenetically related subset of ALK-4, ALK-5, and ALK-7. Phosphorylation of Smad2 by ectopically expressed constitutively active ALK-4, ALK-5, and ALK-7 in transfected NIH 3T3 cells is completely abolished by SB 431542 at 10 μm. In addition, the compound inhibits TGF-β–induced Smad3 phosphorylation and nuclear localization.

### 7.2.7 The biological function of TGF-β

TGF-β is a typically multi-functional protein. It may have one or several effects like growth, differentiation, migration or apoptosis on a given cell or tissue. Thus TGF-β involved in many biological functions likes angiogenesis, atherosclerosis, wound healing and immune modulation. In addition, some diseases caused by aberrant TGF-β expression also stated here.

#### 7.2.7.a TGF-β in angiogenesis

Angiogenesis is a process of formation of new blood vessels from preexisting vasculature occurs in normal situations such as embryonic development, wound healing,

and during the female reproductive cycle. However, activated blood vessel growth is also found in many diseases, such as tumor progression and arthritis. TGF-β has been described to either activated or repress the process of angiogenesis. Angiogenesis can be divided into an activation phase and a resolution phase. Under normal conditions, the vessel is stabilized by mural cells. During activation phase, endothelial cells begin to proliferate and migrate to form a new tube. During resolution phase, smooth muscle cells will be recruited to cover the new tube and to inhibit the proliferation and migration of the endothelial cells. The use of a neutralizing antibody against TGF- β1 has been shown to strongly inhibit angiogenesis. Gene-targeting studies in mice have shown that the loss of TGF-β signaling component leads in general to abnormal differentiation and maturation of the primitive vascular plexus, resulting in fragile vessels with decreased integrity of vessel well [139]. It has been shown that TGF-β exerts bi-functional effects on endothelial cells *in vitro*. Low doses of TGF-β stimulate proliferation of endothelial cells but high doses of TGF- β inhibit proliferation of endothelial cells. These opposite results *in vitro* can be in agreement with that TGF- β both activate or repress the process of angiogenesis *in vivo*. Under most culture conditions, TGF-β inhibits the proliferation and migration of endothelial cells and significantly reduced the migration capacity of endothelial cells in Transwell assays. On the other hand, TGF- β1 also acts in a paracrine manner by stimulating the chemotaxis

of monocytes and the release of proangiogenic cytokines that can subsequently induce the activation of endothelial cells.

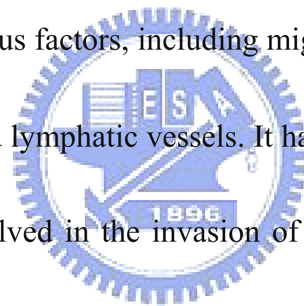
**7.2.7.b TGF-β in atherosclerosis**

Activation, proliferation and migration of arterial SMC are major events in the pathogenesis of atherosclerosis. In healthy vessels, the role of non-proliferative SMC is to maintain the structure of the vessel wall. However, they migrate into the arterial intima during the early stages of plaque formation, where they proliferate, and secrete proinflammatory cytokines under stress conditions during the later stages [140]. There are some evidences that TGF-β inhibits the process of atherosclerosis. An anti-atherogenic action of TGF-β signaling has been demonstrated by inhibition of its signaling in mouse models of the disease by use of neutralizing antibodies or expression of a dominant negative form of the receptor. Inhibition of TGF-β signaling accelerates atherosclerosis and induces an unstable plaque phenotype. In addition, specific inhibition of TGF-β signaling in T cells accelerates atherosclerosis and dramatically alters atherosclerotic lesion size and composition. These studies suggest that TGF-β slow down the pathogenesis of atherosclerosis.

**7.2.7.c TGF-β in cancer progression**

TGF-β also has bi-effects in cancer progression during different stages. During the early phase of epithelial carcinogenesis, TGF-β inhibits primary tumor development and

growth by inducing cell cycle arrest from the first growth G1 phase to the DNA synthesis S phase and by inducing apoptosis of epithelial cells by activation of caspase3 [141]. In late stages of tumor progression when tumor cells become resistant to growth inhibition by TGF-β due to inactivation of the TGF-β signaling pathway or downregulate TGF-β receptors at the cell surface, the role of TGF-β becomes one of tumor promotion. In this stage of tumor progression, TGF-β exert effects to stimulate invasion, angiogenesis, and metastasis. All human tumors overproduce TGF-β whose autocrine and paracrine actions promote tumor cell invasiveness and metastasis. Tumor metastasis relies on various factors, including migration and invasion first of the stroma, and then of the blood and lymphatic vessels. It has been shown the exogenous TGF-β in extracellular matrix involved in the invasion of malignant tumors. In a variety of cell types, TGF-β regulates deposition and breakdown of extracellular matrices, including basement membranes. Besides, TGF-β can upregulate the expression of cell-surface integrins which are cell adhesion receptors highly correlated with metastasis potential [142]. TGF-β also upregulate the matrix metalloproteinases which are a family of zinc-binding endopeptidase. Based on their ability to degrade most of the component of extracellular matrix, MMPs are believed to highly contribute to the invasion of tumor cells by clear the surrounding barrier [143-145]. In addition, TGF-β cooperates with activated Ras on the conversion of noninvasive to metastatic tumors and on the



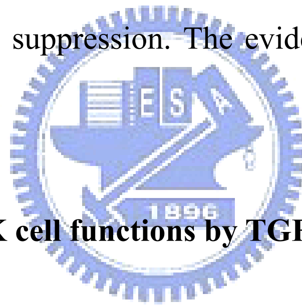
promotion of epithelial-to-mesenchymal transition, a differentiation switch that is required for transitory invasiveness of carcinoma cells [146, 147]. According to above, TGF-β plays the important role as a pro-oncogenic factor in the tumor metastasis. And targeting to signaling pathway would be effective way to retard tumorigenesis.

**7.2.7.d TGF-β in wound healing**

It is clear from studies on TGF-β deficient mice and analysis of the cytokine profile of chronic wounds, that TGF-β is necessary for wound healing. Furthermore, addition of TGF-β to wounds can accelerate the process of healing. The effect of exogenous platelet purified TGF-β in the healing of incisional wounds in the backs of male rats and reported that healing appeared to be accelerated by approximately 3 days [148]. Previous reports had shown that in doxorubicin (adriamycin) treated animals, application of TGF-β also reversed the wound healing deficit. The results therefore suggested that exogenous TGF-β could accelerate healing not only in normal but also in impaired wounds. In addition, TGF-β1, 2 significantly reduced scarring from a rat incisional wound. Scarring can cause adverse cosmesis, loss of function, e.g. strictures and adhesions following burns and abdominal surgery. It has adverse consequences in nearly every organ and tissue. Thus, these studies suggested TGF-β may have benefit on recovery of trauma or surgery patients

### 7.2.8 TGF-β in immune modulation

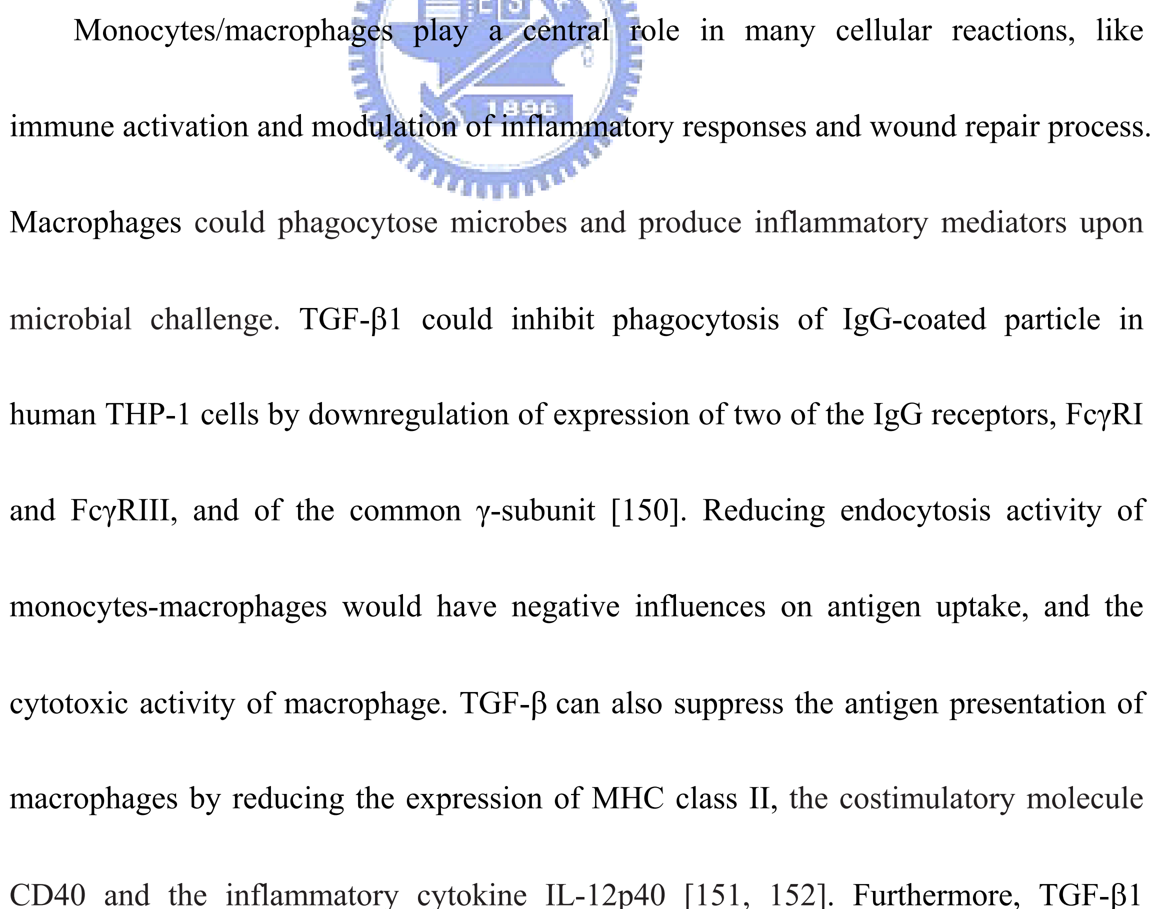
There are a lot of researches about TGF-β on immune function of cultured cells or tissues. Thus we make a special effort to focus the immune-modulation effects of TGF-β here. Lymphocytes and monocytes express high affinity TGF-β receptors, and TGF-β inhibits the proliferations of thymocytes, T cells, B cells, and natural killer cells. Additionally, it inhibits certain functions of lymphocytes including inhibition of immunoglobulin production by human B lymphocytes. Evidence is accumulating that TGF-β regulates immune function *in vivo* and that overproduction of TGF-β may be associated with immuno- suppression. The evidence of immunomodulation by TGF-β was stated below.



#### 7.2.8.a Regulation of NK cell functions by TGF-β1

NK cells are key contributors to the immune response against pathogens by modulating both innate and adaptive immune responses. They are directly cytotoxic for virus-infected cells and enhance the phagocytic activity of phagocytes. Exogenous bioactive TGF-β1 inhibited not only NK cell proliferation but also NK cell DNA synthesis and production of IFN-γ, TNF-α, and ganulocyte-macrophage CSF (GM-CSF) [149]. The cytotoxic activity of NK cells was also weakly inhibited by TGF-β1. The effect of TGF-β1 on proliferation and cytokine production of unstimulated NK cells were overcome by stimulatory cytokines such as IL-2 and IL-12. This suggests the

degree of NK cell activation is determined by the balance of inhibited (TGF-β1) and stimulatory (IL-2 or IL-12) cytokine in the micro-environment. TGF-β1 may also play a significant role in NK cell-mediated tumors rejection. A human TGF-β producing breast cancer cell line MDA-231 was tumorigenic and suppressed mouse spleen NK cell activity when injected into T cell-deficient nude mice. However, NK cell activity was restored and tumor formation was blocked after treatment with anti-TGFβ neutralized antibody. The similar phenomenon was also observed in murine model of head and neck cancer.



**7.2.8.b Regulation monocytes/macrophage functions by TGF-β1**

Monocytes/macrophages play a central role in many cellular reactions, like immune activation and modulation of inflammatory responses and wound repair process. Macrophages could phagocytose microbes and produce inflammatory mediators upon microbial challenge. TGF-β1 could inhibit phagocytosis of IgG-coated particle in human THP-1 cells by downregulation of expression of two of the IgG receptors, FcγRI and FcγRIII, and of the common γ-subunit [150]. Reducing endocytosis activity of monocytes-macrophages would have negative influences on antigen uptake, and the cytotoxic activity of macrophage. TGF-β can also suppress the antigen presentation of macrophages by reducing the expression of MHC class II, the costimulatory molecule CD40 and the inflammatory cytokine IL-12p40 [151, 152]. Furthermore, TGF-β1

suppress IFN-γ-induced NO production by multiple mechanisms. TGF-β1 inhibits of iNOS mRNA transcription and enhancement of iNOS protein degradation through suppressing the IFN-γ-induced signal transducer and activator of transcription 1 (STAT1) phosphorylation and activation [153]. It was also shown that TGF-β1 inhibits LPS-induced septic shock in the mouse. TGF- β1 arrested LPS-induced hypotension and decreased mortality. Blockade of TGF-β1 signaling pathway resulted in overreaction to endotoxin stimulation [154, 155]. TGF-β could inhibit LPS-stimulated mRNA and protein levels of iNOS, TNF-α and IL-1β *in vitro* and *in vivo* [156]. Toll-like receptors (TLRs) are types of receptors involved in the recognition of microbes. LPS is recognized by TLR4 with the assistance of CD14. TGF-β inhibits activation protein1 (AP-1)-mediated CD14 expression in LPS-stimulated macrophage. MyD88 is a key adapter molecule downstream of TLRs. TGF-β would promote MyD88 degradation in LPS-stimulated macrophage. Therefore, TGF-β attenuate TLR4 signaling and suppress LPS induced activation of macrophages [154, 157] .

**7.2.8.c Regulation of dentritic cells by TGF-β1**

Immature dentritic cells (DCs) located in peripheral nonlymphoid organs to filter the foreign antigens and pathogens. These immature DCs are highly specialized in antigen uptake and processing in nonlymphoid tissues. DCs from these nonlymphoid peripheral organs are capable of migrating to T-cell rich areas of secondary lymphoid

organs such as draining lymph nodes or spleen. These migrated DCs up-regulate major histocompatibility complex (MHC) class I and II molecules as well as T-cell costimulatory molecules such as CD80 and CD86. Thus, they lose potent antigen uptake and processing capacity, and in turn acquire potent T-cell stimulatory capacity. Several studies have shown that TGF-β1 inhibits in vitro activation and maturation of DCs. TGF- β1 inhibits upregulation of critical T-cell costimulatory molecules on the surface of DCs and reduces the antigen-presenting capacity of DCs. Thus, in addition to direct inhibitory effects of TGF-β1 on effector T lymphocytes, inhibitory effects on APCs like DC and macrophage may critically contribute to immunosuppressive effects of TGF- β1 [158]. In contrast to these negative regulatory effects of TGF- β1 on function and maturation of DCs, TGF-β1 positive stimulate development and maturation of certain subpopulations of immature DCs. Recent studies established that TGF- β1stimulation is absolutely required for the development of epithelial Langerhans cells (LCs) from hematopoietic progenitor cells in vitro and in vivo. As mentioned above, these results suggest TGF-β1 has stage-specific effects on DCS.

**7.2.8.d Regulation of $CD4^+$ and $CD8^+$ T lymphocytes cell functions by TGF-β1**

TGF-β1 is an important immunomodulatory cytokine that regulates the differentiation, proliferation, and apoptosis of $CD4^+$ T cells. In the present study, TGF-β1 knockout mice exhibit a striking expansion of $CD4^+$ T cells in the liver by 11

days of age, accompanied by $CD4^+$ T cell-dependent necroinflammatory liver disease. Further results indicate that $CD4^+$ T cells in TGF-$\beta1^{-/-}$ mice are activated by and respond to self-Ags present in the periphery, and define a key role for TGF-β1 in the peripheral regulation of Ag-specific $CD4^+$ T cell responses [159].

$CD8^+$ T cells often differentiate into highly cytotoxic cells. A cytotoxic T cell (also known as $T_C$, CTL or killer T cell) are capable of inducing the death of infected somatic or tumor cells; they kill cells that are infected with viruses (or other pathogens), or are otherwise damaged or dysfunctional. TGF-β1 potently suppressed CTL differentiation of human cord blood naïve $CD8^+$ T cells as determined by reduced induction of characteristic phenotypes of effector cells and cytotoxic activity. TGF-β1–null mice showed severe autoimmune inflammation in most organs, characteristics involved that infiltrations of large amount of inflammatory cells, elevated levels of nuclear autoantibodies and activated peripheral lymphocytes in tissue lesions. Inactivation of TGF-β type II receptor (TβRII) in $CD4^+$ and $CD8^+$ T cells in vivo leads to autoimmune inflammatory disease and spontaneous T-cell activation, showed the importance of TGF-β for these cells to control autoimmunity and T-cell homeostasis [160]. Another aspect also reflects that TGF-β1 associated with CTL activity closely. TGF- β1 on CTL cytotoxic activity is consistent with enhanced tumor eradication in mice with T cells lacking TGF- β signaling. Tumors secrete large amounts of TGF-β1, which limits

tumor-infiltrating $CD8^+$ T cells to fail to differentiate into effector or effector memory cells, leading to premature cell death.

#### 7.2.8.e Regulation of regulatory T lymphocytes functions by TGF-β1

There are lots of study suggesting that TGF-β1 closely associated with the maturation and bio-functions of regulatory T lymphocytes. It is widely accepted that $CD4^+CD25^+$ regulatory T lymphocytes are critical to maintain immune homeostasis and immunological tolerance. Naturally occurring $CD4^+CD25^+$ regulatory T cells (Treg cells) mediates immune suppression to limit immunopathogenesis associated with chronic inflammation, persistent infection and autoimmune disease. TGF-β1 not only mediate cell-contact dependent suppression between regulatory T cells and $CD4^+CD25^-$ or $CD8^+$ T cells, but also involved in conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ Treg cells [161]. In the present study, $CD4^+CD25^+$ Treg cells suppress the $CD4^+CD25^-$ T cells proliferation and B cell immunoglobulin production but anti-TGF-β would abolish the cell-contact suppression. Further studies suggest that high level of active form of membrane–bound TGF-βl expressed on $CD4^+CD25^+$ Treg cells would responsible to the suppression activity [162]. In addition to direct suppressive effect on $CD4^+$ cells, Treg cells also modulate the immune response through APC. Either soluble or surface bound TGF-β expression by Treg cells could deliver a suppressive signal to DCs, and these DCs would become tolerogenic with phenotypical and functional alterations and may

not prime the naïve T cells. In addition to suppression effect of Treg cells, TGF-β also involved in conversion Treg cells. This effect not only occurs in suppression of autoimmune disease but also occur in tumor evasion of the immune system. Tumor-derived TGF-β are responsible for converting $CD4^{+}CD25^{-}$ into $CD4^{+}CD25^{+}$ Treg cells and suppress the immune response in the tumor microenvironments. These conversions would facilitate tumor progression. Neutalization of TGF-β abrogated this conversion both in vitro and in vivo and reduces the tumor burden in mice [163].

**7.2.8.f Regulation of B lymphocytes functions by TGF-β1**

B lymphocytes play a large role in the humoral immune response. The antigen receptor on B lymphocytes is a cell-associated immunoglobulin. On activation by antigen, B cells differentiate into cells producing antibody of the same antigen specificity as this receptor. Exogenous TGF-β inhibits cytokine-induced proliferations and stimulates apoptosis of human B cells. TGF-β may function as an autocrine growth inhibitor that limits B lymphocyte proliferation [164, 165]. In addition, TGF-β is also an important regulator of B cell activation and differentiation. TGF-β stimulates activated murine and human B lymphocytes to isotype switch to IgA. Mice with a blockade of TGF-β signaling in B cells are almost devoid of serum IgA [166]. In addition to enhance IgA production, *in vivo* studies reveal a general inhibitory function for TGF-β on antibody production with the exception of IgA [167, 168]. Secretory IgA is

1896

important for the prevention of microbial infection in mucosa. The discovery that TGF-β stimulates isotype switching to IgA suggests that alterations in TGF-β expression could play a role in the pathogenesis of conditions associated with alterations in IgA.

### 7.3 Strategy & Specific Aims

First, we investigate the effects of propofol on mouse cells. From the study of mouse cells, we speculated that propofol has potential to induced TGF-β1. No studies have examined the correlations between propofol and TGF-β1 expression. Since propofol was one of most used anesthetic agents in hospital, we focus our study about the effects of propofol on human beings within clinical dosages.

To investigate the effects of propofol in clinical concentrations on the immunosuppression by determining the potent immunosuppressive TGF-β1 expression levels in patient sera, the consequential increase of TGF-β1 in sera induced lead the study to seek which responsive cell expresses TGF-β1 after propofol treatment. Human peripheral blood mononuclear cells (PBMC), vein endothelial cells (HUVECs), lymphocytes (Jurkat) and monocytes (THP-1) were candidates and tested in the study. In addition, whether the propofol-induced TGF-β1 is effective to decrease the activity of monocytes is also verified.

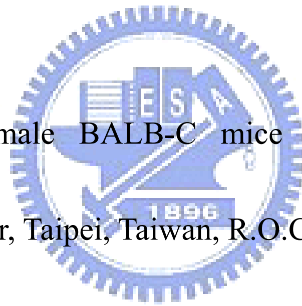
# Chapter 8 Material and Methods (2)

## *8.1 Materials*

### *8.1.1 Reagents:*

The propofol emulsion was purchased from Fresenius Kabi, Germany whose trade name was Fresofol (the license number of health department, Taiwan: 022868) and the content include 1% propofol, soyben oil, purified egg phosphatide, glycerol, oleic acid, sodium hydroxide and water for injections. LPS, Dextran-fluorescein isothiocyanate and SB431542 was purchase from Sigma, Louis, MO.

### *8.1.2 Animal:*




Six-eight week female BALB-C mice were purchased from the National Laboratory Animal Center, Taipei, Taiwan, R.O.C.

### *8.1.3 Plasmid:*

pNF-kB/hrGFP and pAP-1/hrGFP plasmids containing the NF-κB and AP-1 transcription binding site, respectively, followed by a hrGFP reporter gene, were purchased from Stratagene, Garden Grove, CA.

### *8.1.4 Bacterial strains*

*Escherichia coli* DH5 strain were purchased from Invitrogen, Carlsbad, CA and used for plamids amplications.

### *8.1.5 Patient serum and human white blood cells concentrate acquirement:*

The study was approved by the Human Research Ethics Committee of the Tzu Chi Medicine Center Hospital, Hualien, Taiwan. Sera from 24 trauma patients and heparinized white blood cells concentrates were taken from 3 healthy volunteers after receiving institutional approval and written, informed consent from the participants. All medical procedures followed the standard guidelines. The sera of patients from different ages, sex, department, diagnosis and operations (Table 3) were collected within 48 days after surgical operation and divided into two groups.

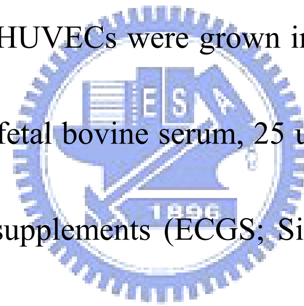
*Propofol-received group*: which received a daily intravenous injection of propofol for a minimal of 2 days (n=14).

*No-propofol-received group*: which did not receive any intravenous injection of propofol (n=10).

### *8.1.6 Cell lines:*

- P338/D1 (Mouse macrophage-like cell line; ATCC number: CCL-46.)
- EL-4 (Mouse T lymphocyte cell line, ATCC number: TIB-39)
- HEK293 (Human embryonic kidney cells; ATCC number: CRL-1573)
- THP-1 (Huamn monocytic cells; ATCC number: TIB-202.)
- JURKAT (Human T lymphocyte; ATCC number: TIB-152)
- HUVEC: Primary cultures of human umbilical vein endothelial cells;

All cell lines except were obtained from BCRC (Food Industry and Development Institute, Hsinchu, Taiwan). HEVEC were kindly provided by Dr. Ko-Jiunn Liu (Nation Health Research Institute, Taipei, Taiwan). P338/D1 and JURKAT cultured with RPMI1640 supplemented with 10% fetal bovine serum (Biological industries, Beit Haemek, Israel) and 1% penicillin-streptomycin amphotericin B (Biological industries); HEK293 cultured with Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen Co., Carlsbad, CA) supplemented with 10% FBS and 1% antibiotics PSA THP-1 was cultured with RPMI1640 with 10mM HEPES, Sodium pyruvate and 2-Mecaptomethnol, 10% FBS and 1% PSA. HUVECs were grown in Medium 199 (Gibco, Invitrogen Co.) supplemented with 20% fetal bovine serum, 25 units/ml heparin (Sigma,) and 30 μg/ml endothelial cell growth supplements (ECGS; Sigma), according to the instructions of the American Type Culture Collection (Manassas, VA). Passages between 3 and 7 were used for the subsequent experiments.



## 8.2 Methods

### 8.2.1 Spleen preparation and culture

BALB-C mice were sacrificed by $CO_2$ asphyxiation. The spleen was taken, minced with DMEM, and filtered with a mesh. Soup was centrifuged at 1,200 rpm at 12℃ for 5 min. The supernatant was removed and 5 mL ACK buffer (150 mM NH4Cl, 10mM KHCO3, 0.1 mM Na2-EDTA) was added. After 5 min incubation, the mixture was centrifuged (at 1200rpm at 12℃ for 5 min) and washed twice with PBS to remove ACK buffer. 5 mL of RPMI 1640 was added to resuspend the cells. 2 x $10^6$ cells were then incubated with treatment of LPS and/or propofol. The supernatant of each group was collected after 48 hr and stored at -80℃.

### 8.2.2 Midi-preparation of plasmid DNA

One ml of pre-cultured *E. coli* transformed with specific plasmids was added into 100 ml LB (1% tryptone, 0.5% yeast extract, 1% NaCl in sterile $ddH_2O$) broth with antibiotics and incubated at 37℃ with shaking (~225 rpm) for 12-16 hr. The broth was centrifuged at 8,000 rpm at 4℃ for 15 min. After discarding supernatant, 4 ml Buffer S1 (50 mM Tris-HCl, 10 mM EDTA, 100 μg/ml RNase A, pH8.0) was added and the solution was vortexed to dispense the pellet. Then 4 ml of Buffer S2 (200 mM NaOH, 1% SDS) was added. The lysate was mixed gently by inverting the tube 6~8 times and incubated at room temperature for 2~3 min. The solution was mixed with the 4 ml

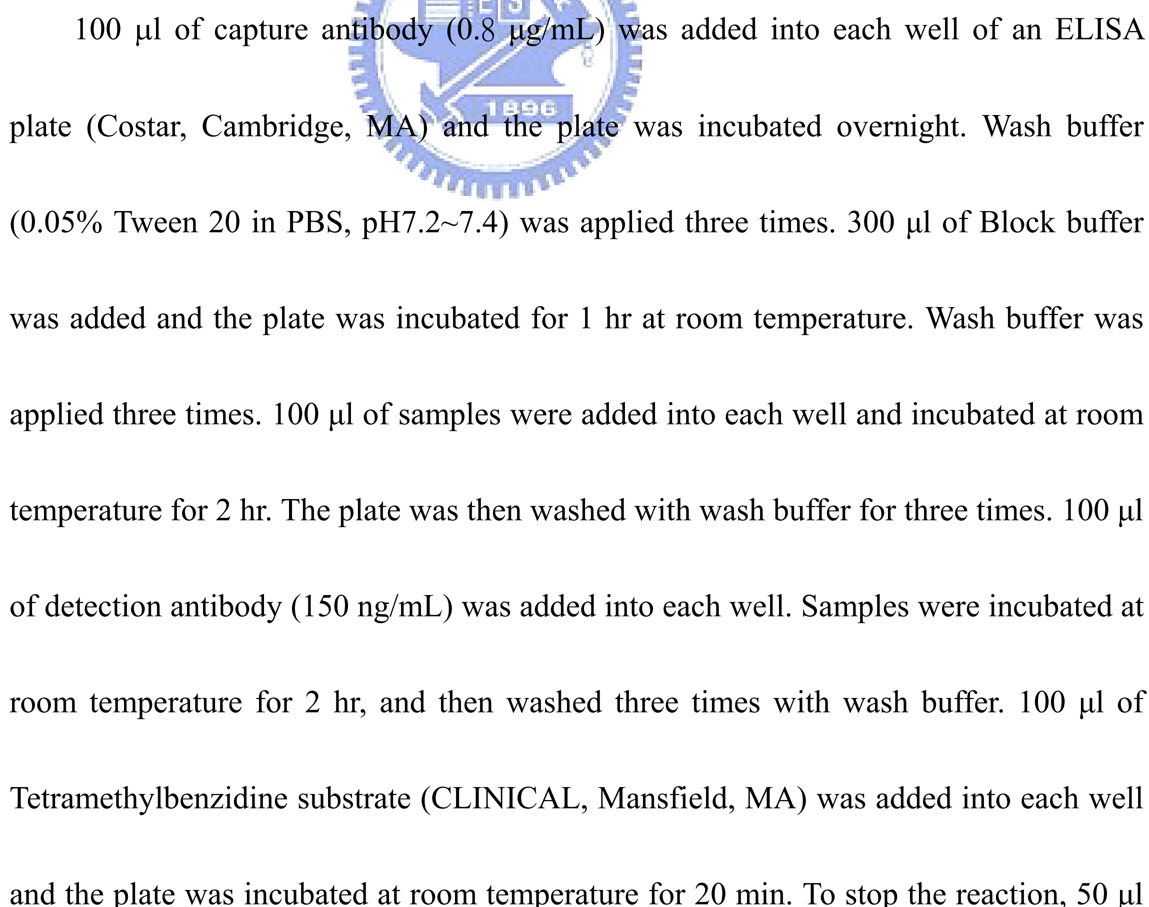
pre-cooled Buffer S3 (100 mM Tris, 15% ethanol, 900 mM KCl, 0.15% Triton X100, adjusted to pH 6.3) and inverted gently 6~8 times until a homogeneous suspension containing an off-white flocculate was formed. The suspension was incubated on ice for 5 min. A NucleoBond AX 100 column (MACHEREY-NAGEL, Düren, Germany) was equilibrated with 2.5 ml Buffer N2 (100 mM Tris, 15% ethanol, 900 mM KCl, 0.15% Triton X100, adjusted to pH 6.3 with $H_3PO_4$). The solution of bacterial lysate was centrifuged at 12,000 rpm at 4℃. The lysate was removed and the supernatant was loaded into the NuceloBond column. After the fluid flowing away by gravity flow, ten ml of Buffer N3 (100 mM Tris, 15% ethanol, 1M KCl, adjusted to pH6.3 with $H_3PO_4$) was added to wash the column and repeat this step once again. Plasmid DNA was eluted with 5 ml of Buffer N5 (100 mM Tris, 15% ethanol, 1M KCl, adjusted to pH 8.5 with $H_3PO_4$). Then 3.5 ml isopropanol was added to precipitate the eluted plasmid DNA. The mixture was incubated on ice for 10 min and centrifuged at 13,000 rpm for 30 min at 4℃. The supernatant was discarded. One ml 70% ethanol was added and stored at -20℃ or the solution was centrifuged at 13,000 rpm for 10 min, discarded ethanol carefully and the pellet was dissolved in 20 μl DWW for further application.

***8.2.3 Transcription factor activity assay***

According to the manufacturer's instruction, pNF-κB/hrGFP and pAP-1/hrGFP were transfected by Lipofectamine 2000 (Invitrogen) into Balb/3T3 cells seeded in the

6-well plate, respectively. Twenty-four hrs later, cells were passaged by versene (0.2g EDTA-4Na/L in PBS; PBS: 137 mM NaCl, 10 mM $Na_2HPO_4$, 2.7 mM KCl, 1.8 mM $KH_2PO_4$, Ph 7.4) and re-seeded into a 24-well plate. The transfectants were treated with LPS (14 μg/ml) and co-incubated without or with propofol for 16 hr, respectively. The transfectants were harvested and analyzed by flow cytometer. Specific FL-1 fluorescent intensities, representing the activities of the transcriptional factors, were calculated. In each plate, control plasmid pCMV/hrGFP was transfected into the target cells to measure the transfection efficiency, which was about 60 %.

***8.2.4 Mouse cytokine measurement***



100 μl of capture antibody (0.8 μg/mL) was added into each well of an ELISA plate (Costar, Cambridge, MA) and the plate was incubated overnight. Wash buffer (0.05% Tween 20 in PBS, pH7.2~7.4) was applied three times. 300 μl of Block buffer was added and the plate was incubated for 1 hr at room temperature. Wash buffer was applied three times. 100 μl of samples were added into each well and incubated at room temperature for 2 hr. The plate was then washed with wash buffer for three times. 100 μl of detection antibody (150 ng/mL) was added into each well. Samples were incubated at room temperature for 2 hr, and then washed three times with wash buffer. 100 μl of Tetramethylbenzidine substrate (CLINICAL, Mansfield, MA) was added into each well and the plate was incubated at room temperature for 20 min. To stop the reaction, 50 μl

of stop solution (1N HCl) was added and the quantification was determined by the ELISA reader at the absorbance wavelength of 450 nm.

***8.2.5 Human peripheral blood mononuclear cells isolation***

Human peripheral blood mononuclear cells were separated from white blood cells concentrate of healthy donors by Ficoll-Paque PLUS (GE Healthcare. Chicago, IL.) The operation processes are stated briefly below. The fresh human white blood cells concentrate (pre-treated with anticoagulant) were well mixed with the same volume of PBS. Then the diluted blood sample were carefully layered on Ficoll-Paque PLUS and centrifuged at 2000 rpm for 20 mins at 18℃. The upper layer was drawn off carefully. The white layer (mainly lymphocytes) and yellow Ficoll-Paque PLUS layer (contained monocytes and neutrophils) were carefully transferred to a clean centrifuge tube without removing the lower layer. A minimal 3 volumes of PBS was added to gently suspend the cells and centrifuged at 1500 rpm for 10 min at 18℃. The supernatant was removed and the cells were washed again with PBS. Finally, the cells were suspended in RPMI1640 (with 10 % FBS and 1 % PSA.) for the study.

***8.2.6 Human TGF-beta measurement***

Human peripheral blood mononuclear cells, Jurkat and THP-1 were seeded with the condition that $2x10^6$ cells in 1 ml growth medium respectively. Each culture co-incubated with the propofol for 24 h, yielding a final concentration of 0.45, 2 and 6.5

μg/ml, which was about the maximum therapeutic dosages of propofol for postoperative nausea and vomiting [112], for sedation [111], and for anesthesia [109, 110]. HUVECs were seeded in a gelatin-coated plate, conditioned with 600μl, $3x10^5$ cells, and co-incubated with 6.5 μg/ml propofol. After 24 h incubation, the supernatants were collected as day1 group or the same amount of propofol as stated previously was added again and the supernatants were collected after incubating for another 24 h, categorized as the day2 group. The human TGF-β1 existed in the cell culture supernatant and human sera were measured by TGF-β1 Emax ImmunoAssay System (Promega, Madison, WI) and operated as indicated by the manufacturer. The treated process of samples is stated briefly below. The human sera were diluted in DPBS and sample buffer. The cell culture supernatants were not diluted. The samples were acidified to approximately pH 2.6 and then to approximately pH 7.6 to measured the total amount TGF-β1. The amounts of active TGF-β1 were measured without acidification.

***8.2.7 Endocytosis activity measurement:***

THP-1 cells ($3x10^5$, 300μl) were seeded in RPMI1640 without FBS, and 300 μl condition medium of HEVEC from the day2 group was added for 16 h. Co-culturing the condition medium of HUVEC without propofol pretreated served as the control group and co-culturing the condition medium of HUVEC with propofol (6.5μg/ml) pretreated served as the propofol group. The cells were harvested and centrifuged (1500 rpm,4℃,

5 min). The supernatant was discarded and the 180 μl coolly fresh mediums were added to suspend the pellets. The cells were incubated at 0 ℃ for 5 min and 20 μl stock dextran-fluorescein isothiocyanate solution (dextran-FITC, 10mg/ml) was added and incubated at 0 or 37℃ for 2 h. The cells were washed three times by PBS and analyzed with flow cytometry (BD Biosciences, San Diego, CA). The FL-1 total fluorescent intensities at 0 ℃ represented the nonspecific dextran-FITC uptake of THP-1 cells. Thus, specific dextran-FITC uptake was represented as FL-1 total fluorescent intensities at 37 ℃ discount FL-1 total fluorescent intensities at 0 ℃. The relative endocytosis activity × 100% = specific dextran-FITC uptake of sample / specific dextran-FITC uptake of control.

The patient sera of each group (propofol and no-propofol received) were mixed respectively. 3 x $10^5$ THP-1 cells were seeded and cultured in different conditions for 16 h stated below: (a) Control group: THP-1 cells were cultured in 600 μl serum-free RPMI 1640. (b) SB431542 group: THP-1 cells were cultured in 600 μl serum-free RPMI 1640 with 15 μM SB431542. (c) Propofol group: THP-1 cells were cultured in 300 μl serum-free RPMI 1640 mixed with 300 μl human sera from the propofol-received patients. (d) Propofol with SB431542 group: THP-1 cells were cultured in 300 μl serum-free RPMI 1640 mixed with 300 μl human sera from the propofol-received patients and 15 μM SB431542. (e) No-propofol group: THP-1 cells

were cultured in 300 μl serum-free RPMI 1640 mixed with 300 μl human sera from the no-propofol-received patients. (f) No-propofol with SB431542 group: THP-1 cells were cultured in 300 μl serum-free RPMI 1640 mixed with 300 μl human sera from the no-propofol-received patients and 15 μM SB431542. Then, the dextran-FITC uptake activities were assayed as described above.

***8.2.8 Statistical analysis***

All in figures are expressed as mean ± SE. To measure the human TGF-β1 expression in patient sera, the sample size consisted of fourteen propofol-received patients and ten no-propofol-received patients; each sample was measured in two independent ELISA and duplicated. Kruskal Wallis test were used to compare the difference between groups. All other data were compared by student-test. The data of TGF-β1 expression in the condition medium human peripheral blood mononuclear cells were obtained from 2 independent experiments and duplicated in each group (n=4). The data of TGF-β1 expression in the condition medium Jurkat, THP-1 and HUVEC were obtained from 3 independent experiments and duplicated in each group (n=6). The data of the endocytosis activity assay of THP-1 co-cultured with condition medium of HUVECs were obtained from 2 independent experiments and duplicated in each group (n=4). The data of the endocytosis activity assay of THP-1 co-cultured with patient sera were obtained from 3 independent experiments and duplicated in each group (n=6).

Other results were performed at least 2 independent experiments. All statistical significant was set at $p<0.05$.

# Chapter 9 Results (2)

## 9.1 The study of propofol action on mouse cells

### *9.1.1 Propofol reduced TNF-α and IL-1β release in mouse splenocyes at the presence of LPS.*

As mentioned above, propofol was believed to have immuno-modulation effect. Here we used mouse-derived splenocytes to evaluate the effect of propofol on mouse immune cells. Six-week BALB-C mice were sacrificed by $CO_2$ asphyxiation and harvested the spleen cells with germfree manipulation as stated in methods. 2 x $10^6$ cells in 1ml were seeding with LPS and/or propofol and incubated 48hrs. The supernatants of each group were collected and the cytokine expressions were measured by ELISA. The mouse TNF-α and IL-1β expression were measured because TNF-α and IL-1β were thought as dominant proinflammatory cytokine during early stage of LPS-induced immune response. The results showed that LPS could strongly stimulate IL-1β (Fig.4) and TNF-α (Fig.5) expression; however, 30μg/mL propofol suppressed the expression of IL-1β and TNF-α significantly within the same concentration of LPS. At the same experiment condition, 14μg/mL and 30μg/mL propofol alone would inhibit the IL-1β (Fig.4) expression of mouse splenocyes compared to control group. Another interesting thing we observed was that propofol itself would stimulate TNF-α expression (Fig.5) compared to control group at both 14μg/mL and 30μg/mL

group.

***9.1.2 Propofol reduced IL-10 release in mouse-derived splenocyes at the presence of LPS.***

From the present study and our results, propofol would decrease the proinflammatory cytokine secretions of splenocyes when co-cultured with LPS. Thus, the sequential issue we concerned was that propofol down-regulate the proinflammatory cytokine by direct or indirect way. The direct way could be performed that propofol affect the intracellular physiology of the immune cells, like suppress the transcription factor activities associated to proinflammatory cytokine expression. The indirect way could be performed by inhibitory cytokine like IL-10 or TGF-β which induced by propofol. To investigate the mechanism of propofol action on immune-modulation, the IL-10 and TGF-β expression in supernatant of the mouse-derived splenocytes were measured. The cultured condition was the same as mentioned above (results 9.1.1). The results showed that LPS also induced IL-10 expression at this cultured condition (2 x $10^6$ cell in 1ml medium, 37℃, 48 hrs), but propofol decreased IL-10 expression in mouse splenocytes at the presence of LPS. In addition, propofol alone induced IL-10 expression at the concentration of 30μg/ml (Fig.6). The result implicated that the anti-inflammatory effect of propofol on mouse-derived splenocytes may not act through the effect of IL-10.

***9.1.3 However, propofol enhanced TNF-α release in mice macrophage cell line P338/D1 at the presence of LPS.***

In this study, we also investigated the effect of propofol on mouse macrophage P338/D1. Although some articles showed that propofol would suppress the inflammatory effect on LPS-activated mouse macrophage RAW 264.7 cells [122], our data showed that propofol would amplify the TNF-α expression induced by LPS in mouse macrophage P338/D1 (Fig.8). Furthermore, propofol itself induced TNF-α expression of P338/D1 compared to control group. It seemed that propofol would induce the TNF-α secretion and augment LPS-induced TNF-α secretion in P338/D1.

***9.1.4 Propofol induced TGF-β release in mouse-derived splenocytes at the presence of LPS.***

TGF-β is another well-known inhibitory cytokine responsible to immune suppression, thus we also investigated the expression level of TGF-β of mouse splenocytes under the effects of propofol. The cultured condition was the same as mentioned above, and total amounts of TGF-β were measured after acid treatment followed the manufacturer indicated. The results showed that LPS alone could not induce TGF-β compared to control group, however, propofol could increase total amounts of TGF-β whether at the presence of LPS or not (Fig.7). The result implied that TGF-β might be responsible for the anti-inflammatory effect of propofol in mouse

splenocytes.

***9.1.5 Propofol did not affect the TGF-β expression level in mouse macrophage cell P338/D1 and T lymphocyte cell EL-4.***

Due to mouse splenocytes were mixture of many type of immune cells, identifying which kinds of cells responded to propofol to secreted TGF-β was the aim which we did our effort to. Mouse cell P338/D1 and EL-4 were used to investigate the effect of propofol on the TGF-β expressions in mouse macrophages and T lymphocytes. $2x10^6$ cells were seeding in 1ml growth medium with or without propofol and incubated 48hrs. The results of ELISA showed the total amount of TGF-β did not statistically significantly affected by propofol significantly both in P338/D1 (Fig.9A) and EL-4 (Fig.9B) at 14 or 30 μg/ml. We did not exclude the possibility that higher concentrations of propofol would affect the TGF-β expression in P338/D1 and EL-4, but the effects of extremely high dose of propofol would not be discuss in this study.

***9.1.6 Propofol suppressed the activities of NF-κB and AP-1 at the presence of LPS.***

In addition to the cytokine secretions affected by propofol, another part we concerned was that transcription level of immune cells affected by propofol. To investigate the intracellular transcription factor regulation by propofol at the presence of LPS, what we needed to do was chosen some representative transcription factor during LPS-induced response. The present study has shown that transcription factor NF-κB and

AP-1 were activated at the presence of LPS and the activities of NF-κB and AP-1 were highly related to the LPS-induced inflammatory response like TNF-α production and endocytosis activity of macrophages. Here we examine the propofol effects on NF-κB and AP-1 at the presence of LPS by transcription factor activity assay. The reporter gene was transfected into Balb-3T3. We chose Balb-3T3 to perform this study because Balb-3T3 has toll-like receptor 4 expressed on its surface and has good transfection efficiency with Lipofectamine 2000. In addition, transcription factor NF-κB and AP-1 are critical intracellular regulators of macrophages function in the innate immunity. According to the manufacturer's instruction, pNF-kB/hrGFP and pAP-1/hrGFP were transfected into Balb/3T3 cells seeded in the 6-well plate, respectively. Twenty-four hrs later, cells were passaged by versene (0.2g EDTA-4Na/L in PBS) and re-seeded into a 24-well plate. The aim of this operation is prevention the wrong results or large deviations due to difference of transfection efficiency between each wells. The transfectants were treated with LPS (14μg/ml) and co-incubated without or with propofol for 16 hr, respectively. The transfectants were harvested and analyzed by flow cytometer. Specific FL-1 fluorescent intensities, representing the activities of the transcriptional factors, were calculated and compared to each control group. The control group was transfected respectively with pNF-kB/hrGFP or pAP-1/hrGFP without other treatments except growth medium In our experiments, LPS increased the activities of

NF-κB in cells (Fig.10A) and slightly enhanced the activities of AP1 (Fig.10B). However, 30μg/ml propofol reduced the activities of NF-κB and AP-1 at the presence of LPS (Fig.11). Previous literatures have reported that LPS activates NF-κB and AP1 pathways to enhance the TNF-α and IL-1β expression and release. Thus our result would illustrate that the intracellular regulation occurred when propofol reduce inflammatory response like TNF-αand IL-1β expression at the stimulation of LPS.

***9.1.7 Propofol promotes the activities of NF-κB and AP-1 without LPS stimulation.***

We also examine the propofol's effects on NF-κB and AP-1 activities without LPS stimulation. According to the inhibitory effect of propofol on macrophages, NF-κB and AP-1 activities would be downregulated with reasonable speculation. However, distinctive results showed that propofol would promote NF-κB activity at the concentration of 30μg/ml (Fig. 10A) and promoted AP-1 activity at the concentration of 14μg/ml (Fig.10B). The reasonable explanation of this result could be due to the complicated effect of propofol. The complicated effect of propofol may result from different concentration, timing, or cell types of action. Compare the results of 3.1.4 and 3.1.7 (Fig.8 and Fig.11), it seemed propofol could reduce the inflammatory response induced by LPS in mouse cells; however propofol itself might activated inflammatory response, especially in macrophage-like cells by activation of NF-κB and AP-1.

## 9.2 The study of propofol action on human TGF-β secretion and immune modulation

### *9.2.1 Intravenous injection of propofol during medical procedure increased both total amount and active form of TGF-β in patient serum.*

In order to study the effect of propofol used in hospitals, we were in corporation with Tzu Chi Medicine Center Hospital, Hualien, Taiwan to obtain the sample. All medical procedures were followed the standard operations. The sera of patients form different diseases and medical processes are collected within 2 days after surgery and divides into with propofol group which did receive intravenous injection of propofol (n=14) and without propofol group which did not receive intravenous injection of propofol (n=10). The sera were diluted and assayed by ELISA according to the manufacturer's instruction. The results of ELISA were retreat to the origin concentrations of each sample. The results show that successive two days propofol injections significantly increased the total amounts of TGF-β in patient sera in propofol group compared to without propofol group (Fig.12A). Interestingly, the active form TGF-β1 also significantly increased in propofol group for two days propofol injections (Fig.12B). According to these results, propofol used in hospitals could increase both total and active TGF-β1 in patients and implied that there were some matters needing

attention.

***9.2.2 Clinical dosages of propofol have no significant effect on total amounts of TGF-β1 but slightly raised the amount of active TGF-β1 in the condition mediums of human peripheral blood mononuclear cells.***

The next issue we interested was what type of cells responded to intravenous injections of propofol to increased production of TGF-β1. As mentioned above, almost all types of human cells could secrete TGF-β1, and our first candidate was human peripheral blood mononuclear cells (PBMCs) due to directly contact to propofol. After separations of human peripheral blood mononuclear cells from white blood cells concentrate of healthy donors, $2x10^6$ cells were cocultured with propofol and incubated at 37℃. In order to mimic the situation of propofol used in hospitals, we collected the supernatants 24 hrs after treatments (Day 1) or added propofol again and collected supernatants another 24 hrs after treatments (Day 2). The cells cultured without propofol were considered as control group. The results showed that propofol did not affect the total amount of TGF-β1 at all (Fig. 13A); whereas a fine change was observed (Fig. 13B) in which 6.5 μg/ml propofol treatment could slightly increased the active TGF-β1 expression when compared to control.

***9.2.3 Clinical dosages of propofol slightly reduced total amounts of TGF-β1 but raised the amount of active TGF-β1 in the condition mediums of human Jurkat cells.***

Because human PBMCs were mixtures of many kinds of immune cells, the next step we wanted study what types of immune cells responded to propofol to increase the active TGF-β1 production, as showed in human PBMC. Because T lymphocytes were the largest populations in human PBMCs, we chose the human T lymphocytic cell line JURKAT for study. The same cultured conditions were taken, $2x10^6$ JURKAT cells were co-cultured with propofol and incubated at 37℃ for 24hrs. The results showed the total amounts of TGF-beta were slightly reduced only when treated with 6.5μg/ml, the maximum concentrations used in clinical (Fig 14A). We also found that active TGF-β1 in the condition medium increased in a very slight manner when treated with 6.5μg/ml (20.85±3.24 pg/ml compared to 13.13±4.2 pg/ml of control group) (Fig 14B).

***9.2.4 Clinical dosages of propofol reduced total amounts of TGF-β1 but raised the amount of active TGF-β1 in THP-1 cells in a dose dependent manner.***

Since there were not significantly differences of TGF-β1 secretions between Jurkat cells with or without propofol, our next target was monocytic cells. $2x10^6$ human monocytic cells THP-1 cells were co-cultured with propofol and incubated at 37℃ for 24hrs. Interestingly, the results showed that total TGF-β in the THP-1 condition medium decreased by propofol in a dose dependent manner Fig15A). The active form of TGF-β1 decreased propofol groups (Fig15B). These implied that propofol did not affect the TGF-β1 secretions but affect the activation of TGF-β in the cultured mediums of human

monocytic cells THP-1.

### *9.2.5 Propofol inhibited the endocytosis activities of THP-1.*

Since active form TGF-β increased propofol co-cultured mediums in THP-1, we supposed it may have suppressive effects on THP-1. Here we used dextran-FITC uptake to measure the endocytosis activity of THP-1. In order to control the THP-1 endocytosis activity with normal growth conditions, we took a preliminary experiment to speculate the correlation between concentrations of dextran-FITC and uptake of dextran-FITC in THP-1. $5x10^5$ THP-1 cells were cultured in growth mediums for 24 hrs and harvested the cells by centrifugation. Remove the supernatant and add different amounts of cooled fresh medium to suspend the cells and 10mg/ml dextran-FITC (adjust the final volume equal to 200 μl). Incubate at 37℃ for 2 hrs and assay FL-1 fluorescent intensities by flow cytometer. The data showed that FL-1 fluorescent intensity of gated THP-1 cells increased in a dose-dependent manner with dextran-FITC concentrations (Fig.16.) Because it showed plateau at 15, 20 and 30 μl dextran-FITC, we chose the 1mg/ml as our experiment conditions following (20μl of 10 mg/ml dextran-FITC solution added into 180 μl cell suspensions).

With the same conditions, $5x10^5$ THP-1 cells were co-cultured with different dosages of propofol for 48 hrs (retreat in 24hrs.) The cells were harvested and incubated with 1mg/ml dextran-FITC at 37 ℃ or 0 ℃ for two hours uptake. The results showed

that the relative endocytosis activities of THP-1 decreased in a dose-dependent manner by propofol (Fig.17). The results that propofol could act on THP-1 and inhibited its activities were fitted with the present studies that propofol could directly inhibit monocytes / macrophages activities.

### *9.2.6 Clinical dosages of propofol raised total amounts of TGF-β1 in HUVECs.*

In addition to human peripheral blood mononuclear cells, we also considered whether blood vessel endothelial cells would be induced to secrete TGF-β1 by propofol. 5 x $10^5$ HUVECs were seeding and treated with 6.5 μg/ml propofol. The supernatant was collected after 24 hrs and 48 hrs as stated above. The results total TGF-β1 increased day 2 propofol compared to control groups. However, the levels of active TGF-β1 did not vary both in day 1 and day 2 propofol groups (Fig18). Thus, the above results suggested that propofol would induced the TGF-β secretion in human vein endothelial cells but did not affect the activation of latent TGF-β by human vein endothelial cells.

### *9.2.7 The condition mediums of HUVECs with propofol inhibited the endocytosis activities of THP-1.*

To verify whether the TGF-β1 in the condition media of HUVECs after propofol treatment have the bioactivities on immunity, the endocytosis of THP-1 cells were determined by dextran-FITC uptake. Next, the same supernatants of HUVECs condition medium were co-cultured with 3 x $10^5$ THP-1 for 16 hrs and measured the endocytosis

activity of THP-1. The data showed that the condition medium of HEVECs co-cultured with propofol inhibited endocytosis of THP-1 about 18% (Fig.19). This implied that propofol could induce HEVECs secrete TGF-β1 into mediums and might lead to inhibitory effects on the endocytosis activities of THP-1.

### *9.2.8 The sera of patients received propofol could inhibit the endocytosis activities of THP-1 and abrogated by specific inhibitor of TGF-β pathway SB431542.*

At last, we tried to connect the TGF-β elicited by propofol to the inhibitory effect on the endocytosis activities of THP-1. The patient sera of each group (with and without propofol) were mixed respectively. 3 x 105 THP-1 were seeded and treated in RPMI 1640 containing 50% human sera for 16hrs. THP-1 cultured in sera-free RPMI 1640 served as control group. The results from cytometr flow cytometer showed that the main peak of propofol group was shifted left compared to control group (Fig.20A). In other words, co-cultured with propofol-treated patient sera significantly reduced dextran-FITC uptake in propofol group. However, addition of SB431542 (the inhibitor of TGF-β receptor-associated kinase ALK-5) dramatically increased the endocytosis activity which be suppressed in propofol group (Fig.20C), and SB431542 did not affect the dextran-uptake in THP-1 (Fig.20B). Although the pattern changed by SB431542 compared to no propofol group, there was no significantly different between this two groups (Fig.20D). The quantifications of these results were shown in Fig.20E (n=7) and

implied that the sera from propofol-received patients inhibit endocytosis activity of monocytes ex vivo due to the contributions of TGF-β1. Interestingly, the sera of no propofol would also inhibit the dextran-FITC uptake of THP-1 ex vivo, however, did not abrogated by SB431542.

***9.2.9 Propofol might also induce IL-8 secretions THP-1.***

In addition to induced TGF-beta secretions in THP-1, we also founded that the IL-8 would also elevated when co-incubated with 20 μg/ml propofol for treatment twice with a 24hr interval (Fig.21). It implied that propofol might have potential to induced IL-8 secretion in human macrophages.

# Chapter10 Discussion (2)

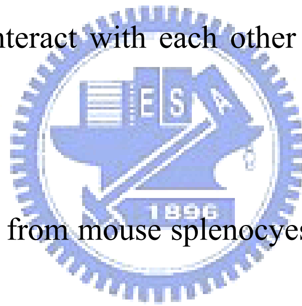
Up to present, there were many studies suggested that propofol could inhibit immune response in vitro or in vivo. Some of these studies indicated that propofol showed inhibitory effects on LPS stimulated response but propofol itself would not have effect on non-stimulated macrophage. On the contrary, some studies suggested that propofol could enhance inflammation response. And there were also some studies suggested that propofol could exert inhibitory without LPS stimulation. In addition to the effects on immune modulations, another controversial issue was the dosage of propofol for laboratory research, some studies investigated the effects of propofol within clinical dosages, and however, some used even ten folds of maximal clinical dosage to study. By the way, many studies used model or mouse cell line to study the effects of propofol on immune modulations; it might transmit confused information as propofol exerted on human beings.

In this study, we stood on a compromising position to investigate the effects of propofol on immune modulations. We used the mouse cells to study the synergistic effects of propofol and LPS on proinflammatory cytokine production, dosages of propofol were about two and four folds to clinical maximal use (about 6.5 μg/ml). On the other hand, we considered that propofol used generally in hospital but there were many situations without intervention of LPS. Thus, we investigate the clinical effects of

propofol and the effects by propofol itself within the clinical dosages on human cells. Furthermore, we took an *ex vivo* assay to clarify the immune modulation effects of propofol in hospital use.

The first part in this study began with the synergistic effects of propofol and LPS in mouse immune cells. Our data showed propofol indeed exerted opposite force against LPS stimulation. The mouse-derived spleen cells were induced to secrete TNF-α and IL-1β, two generally acknowledged proinflammatory cytokines induced by LPS (Fig4 and Fig5). There were many studies about propofol reduced the response stimulated by LPS. Chu SH et.al suggested that propofol exerts protective effects on the acute lung injury induced by endotoxin in rats. Chen RM et.al found that propofol had anti-inflammatory and anti-oxidative effects on LPS-activated macrophages. ELISA revealed that LPS increased macrophage inducible nitric oxide synthase [5], TNF-α, IL-1β, and IL-6 in both protein and mRNA levels, whereas propofol significantly reduced the levels of iNOS, TNF-α, IL-1β, and IL-6 at the presence of LPS both in protein and mRNA levels. In addition, Song HK et.al suggested that propofol allowed MNCs to retain their cytotoxicity in septic conditions by protecting immune cells from apoptosis. However, present reports did not expound the mechanisms of propofol actions. Besides cytokine profiles, in this study we also investigated the transcriptional regulations which involved in immune modulation of propofol. Here we suggested

propofol might inhibit the LPS-induced inflammation by two mechanisms. First, propofol could induce the inhibitory cytokine TGF-β but not IL-10 whether in the presence of LPS or not (Fig6 and Fig7) Second, propofol could suppress the activities of NF-κB and AP-1 (Fig.10) which both were crucial transcription factors involved in LPS-induced inflammation priming. Interestingly, propofol alone in our experimental conditions did not reduce but promote the activities of NF-κB and AP-1. Thus, we preliminary exclude the possibility that propofol inhibited the activities of NF-κB and AP-1 through induced TGF-β. Although we did not provide strong evidences whether these two mechanisms interact with each other or not, it might another landmark we could work for.



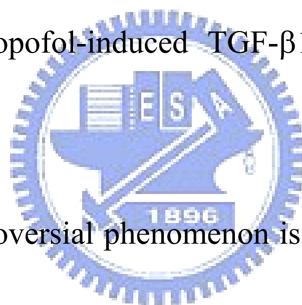

Through the analysis from mouse splenocyes and cell lines, we found that propofol had potentials to induced TGF-β secretions (Fig.7 and Fig.9). Thus another part of this study was to make a study of mechanisms and the biological effects of propofol-induced TGF-β in medication. In this study, we first reported that clinical dosages of propofol not only induced latent TGF-β1 expressions but also conversed to an active form in human sera (Fig. 12). This effect was accomplished by a two-step mechanism. On one hand, propofol induced endothelial cells to secrete more latent TGF-β1 (Fig. 17); on the other, propofol induced T lymphocytes (Fig. 14) and monocytes (Fig. 15) to activate the surrounding latent TGF-β1. By TGF-β pathway inhibitor SB431542, the results

indicated that the propofol-induced TGF-β1 in patient sera mediated the suppression activities against endocytosis in monocytes (Fig. 20).

Monocytes mediate many responses of the innate immunity. Reducing endocytosis activity of monocytes-macrophages might have negative influences on antigen uptake, presentation and immune activation. In addition to endocytosis activity, it is believable that propofol-elicited TGF-β1 could inhibit phagocytosis, the production of the reactive nitrogen intermediates, TNF-α expression, the common γ-subunit expression and TLR signaling pathways in monocytes [150, 169, 170]. Thus, the inhibitory activities of TGF-β1 on monocytes may partake in a key role to suppress inflammatory responses after clinical administration of the anesthetic propofol.

Two arguments can be made in regards to the propofol effect on the immune activity. First, propofol could attenuate CD14 expression on the surface of LPS-stimulated monocytes to reduce inflammatory response [18]. In addition, propofol also protect endothelial cells against LPS-induced barrier dysfunction by inhibiting NF-κB activation [127]. However, propofol also was shown to reinforce the effects on the releases of LPS-induced proinflammatory cytokines including TNF-α and IL-1β [19] which induce/enhance inflammatory responses through NF-κB activation [171]. Based on our findings, the bioactive TGF-β1 induced by propofol, it might help to explain the controversial effects of propofol found in previous literature. Studies have

shown that propofol can increase LPS-stimulated proinflammatory cytokine expression by activating NF-κB pathway; it may be caused by the transient effects of propofol-induced TGF-β1 on the activation of NF-κB [172]. However, TGF-β1 is a multifunctional cytokine, reported to decrease the activity of NF-κB with or without LPS stimulation [173, 174]. Thus, the controversial effects of propofol may be due to the ability of TGF-β1 induction. Propofol can induce the secretion of latent TGF-β1 and enhance the conversion of latent TGF-β1 into active form. Consequently, the difference in the dosages, the types of target cells, and the cell-cultured conditions will affect the synergistic effect of propofol-induced TGF-β1 to result in different experimental outcomes.






The secondary controversial phenomenon is the propofol effect on the activities of immune cell. Although many pro-inflammatory cytokines such as TNF-α which can increase the activities of granulocytes and monocytes are induced by propofol [15], propofol treatments in many laboratory studies or clinical observations are believed to down-regulate the activities of many human immune cells [52], including leukocytes [46], lymphocytes [175], monocytes [18], macrophages [37] and neutrophils [39]. Our finding could provide possible explanations to these studies. The propofol-induced latent and active TGF-β1 exerts its antagonistic effect [176-178] with pro-inflammatory cytokine which are also induced by propofol infusion. Later, sustained propofol in the

sera may decrease TNF-α production by decreasing the activity of NF-κB. Thus, sustained propofol will induce endothelial cells to produce latent TGF-β1 and enhance PBMC to be converted into the active form. Continuous provision of active TGF-β1 shall suppress the activities of TNF-α which amounts are progressively decreasing. Finally, it will cause the effect of propofol to be suppressive on immunological activities among surgical or care patients.

Thus, our results in this study should be valued for patients who are using propofol treatment during medical operations. Their innate immunity would be affected because of propofol-induced active TGF-β1. On the disadvantageous side, these patients should keep away from opportunistic infections of certain pathogens like *Staphylococcus aureus* because propofol will suppress patients' immunity by TGF-β1. Advantageously, these patients would have a lower opportunity to suffer from endotoxemia- caused death by antagonizing TNF-α effects [13].

In addition to immune regulation, TGF-β1 also plays a crucial role in a range of biological processes, including regulation of tissue repair, extracellular matrix accumulation, angiogenesis and fibrosis progression [140, 179]. Evidences indicated that TGF-β pathway involved Smad3 [180] and Smad2 [181] is pivotal in progressive fibrosis and has effects on the induction of myofibroblasts, enhancement of matrix synthesis, and inhibition of collagen breakdown. Thus, it should be noted that the

long-term sedation by propofol in intensive care patients might be at high risk of TGF-β mediated fibrosis.

In contrast, the propofol-induced TGF-β1 could be considered as a good aspect in post-operation. It is clear from studies in TGF-β1 deficient mice that TGF-β1 is necessary for wound healing [148] and also reported acceleration in healing. Such findings imply that clinical dosages of propofol could promote the recovery of trauma patients. In fact, propofol anesthesia, as compared to the awaken state, has the potential of offering a certain degree of protection against neuron damage [182] and burn injury [58]. Therefore, further studies are needed to investigate the advantages of propofol use in surgery and intensive care.

In this study, we demonstrated that clinical dosages of propofol induced both human latent and active TGF-β *in vivo* and the propofol-induced TGF-β1 had the immunosuppressive activity to monocytes. According to our results, it is worthy to investigate the effects of propofol to induce TGF-β1 in future research. The advanced results will prevent the side-effects of propofol and strengthen its medical effects for different patients during medication.

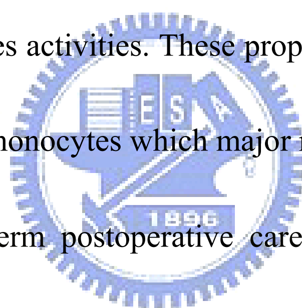
As everyone knows, TGF-β was a multi-function protein involved many biological regulations. We also found that propofol might have potential to induced IL-8 secretion from THP-1 (Fig21) although there were no differences within clinical dosage. Because

both IL-8 and TGF-β were important regulators to enhance the process of wound healing, propofol might have benefits in wound healing of trauma patients.

In this study, we investigated the effects of clinical dosage propofol on human TGF-β expressions by different types of cells and discovered the biological importance of propofol-induced TGF-β secretion and conversion *ex vivo* (Fig22). We provided the possible reasons that propofol could exert a protect effects and also might be beneficial to the inflammatory change in sepsis.

# Chapter 11 Conclusions

Although pentobarbital and propofol both have inhibitory effect on inflammatory or other immune responses, they indeed act through quite different mechanisms. Pentobarbital suppresses the expression of p38 MAPK and the activities of NF-κB and AP-1 in the presence of LPS. Changes in the intracellular signaling pathway should be responsible for the decrease in TNF-α mRNA and the reduction of TNF-α protein expression caused by pentobarbital. However, propofol could trigger endothelial cells to express latent TGF-β1 which would be converted into the active form through monocytes or lymphocytes activities. These propofol-induced TGF-β1s had activities to suppress endocytosis of monocytes which major responsive cells of innate immunity



Furthermore, long-term postoperative care after surgery is a common ways to promote the health of patients. It is cautious to use pentobarbital, propofol and other anesthetic agents which affect immune balance (Table 4). For bad respect, these immune-suppressive patients should be taken care of opportunity infections, the hazardous causes of death in hospital. For good respect, not only pentobarbital and propofol, many anesthetics like morphine and lidocaine also shown protected function during sepsis or other damage. They have proved successful in the treatment of burn injuries, interstitial cystitis, ulcerative proctitis, arthritis and herpes simplex infections [14].

In conclusion, it should be paid attention to use anesthetic agents in research or in hospital. Furthermore, based on current opinions, our current findings, and future insights into the mechanisms responsible for the wide range of inhibitory effects by the anesthetic agents on the inflammatory responses, may provide a platform for the creation of future drugs or treatments of inflammation.

# Chapter12 Tables

| **Dosage of propofol** | **Target** | **Endotoxin stimulation** | **Descriptions** | **Ref.** |
|---|---|---|---|---|
| Clinical dose and ten- fold | Cultured human whole blood from health donor | 1 μg/ml LPS | Propofol enhance LPS-stimulated TNF-α and IL-1β expression but reduced the LPS-stimulated IL-8 secretion. | [18, 19, 21] |
| Clinical dose and ten- fold | Monocytes isolated from health donor | 1 μg/ml LPS | Propofol reduce the expression density of LPS-stimulated CD14 whereas unaffect HLA-DR. | [18] |
| Clinical dose and ten- fold | Neutrophils and leukocytes from health donor | None | Propofol inhibits immunological activities of neeutrophils and the migration activity of human leukocytes through endothelial cell monolayer. | [39, 46] |

| Clinical investigation | Alveolar macrophages isolated from patients | None | Propofol decrease the phagocytosis and microbicidal activity but increased the aggregation of patient alveolar macrophages. | [36, 37] |
|---|---|---|---|---|
| Clinical investigation | Isolated alveolar macrophages from patients | None | Propofol increase the TNF-$\alpha$ and IL-1$\beta$ gene expressions in patient alveolar macrophages. | [16] |

**Table 1: There have been different observations about immune-modulated effects of propofol in human beings.** Here we focused on the reports about the immune-regulated effects of propofol in humans. There are two different results about propofol in immune response. Some studies suggested that propofol could promote inflammation induced by LPS stimulation whereas some studies implied that propofol could exert anti-inflammation effects. In addition, due to the multi-factors involved in surgical procedures, there were also having been contradictory observations in clinical investigations.

| ID | Descriptions |
|---|---|
| SB 431542 (compound) | Inhibitor of the TGF-β type1 receptor kinase activity ; Selective inhibitor of Smad3 phosphorylation |
| Tranilast (compound) | Inhibitor of expression of TGF-β1/2 and/or its receptor types |
| Decorin (38kD recombinant protein) | Bind to and inactivate the active TGF-β. |
| TGF-β1LAP (38kD recombinant protein) | Associate with active TGF-β non-covalently and form a latent complex. |
| Neutralized antibody | Mouse anti-Human TGF-β antibody (SEROTEC, MCA797) |

**Table 2: There are many inhibitors of TGF-β pathway in current study.** Several approaches have been used successfully to block *in vivo* activity of TGF-β. Some of them were listed in the table.

| No. | Age | Sex | Department | Diagnosis | Operation | propofol |
|---|---|---|---|---|---|---|
| 1 | 45 | M | PS | Lip cancer | Free flap | + |
| 2 | 65 | M | PS | Hypopharygeal cancer | Free flap | + |
| 3 | 40 | M | PS | Oral submucous cancer | wound excision and free flap | + |
| 4 | 74 | M | CVS | Coronary artery disease | Coronary artery bypass graft | + |
| 5 | 25 | M | GS | Internal bleeding | Exploratory laparotomy B-2 | + |
| 6 | 73 | M | CVS | Coronary artery disease | Coronary artery bypass graft | + |
| 7 | 61 | M | CVS | Coronary artery disease | Coronary artery bypass graft | + |
| 8 | 74 | M | CVS | Coronary artery disease | Coronary artery bypass graft | + |
| 9 | 45 | F | NS | Right intracranial hemorrhage | 1. Removal of Intracranial hemorrhage<br>2. External ventricular drainage monitor | + |
| 10 | 41 | M | NS | Intracranial hemorrhage<br>Epidural hematoma | 1. Remove of intracranial hemorrhage<br>2. Intracranial pressure monitor | + |
| 11 | 49 | M | CVS | Coronary artery disease | Coronary artery bypass graft | + |
| 12 | 51 | F | NS | Intracranial hemorrhage | stereo static aspiration | + |
| 13 | 58 | M | CVS | Coronary artery disease | Coronary artery bypass graft | + |
| 14 | 68 | M | CVS | Coronary artery disease | Coronary artery bypass graft | + |
| 15 | 46 | M | NS | 1.Head injury<br>2.Central nervous system injury | External ventricular drainage | - |
| 16 | 53 | M | CS | Old pulmonary tuberculosis | Thoracotomy | - |
| 17 | 45 | M | GS | 1.Duodenal ulcer with acute bleeding<br>2.Empyema of right lung | Transesophageal angiography | - |
| 18 | 62 | F | CS | 1.Chest contusion<br>2.Aortic dissection aneurysm<br>3.Inferior vena cava obstruction | Embolectomy | - |
| 19 | 64 | M | CS | Esophageal cancer | | - |
| 20 | 64 | M | CVS | Congestive heart failure | Coronary artery bypass graft | - |
| 21 | 38 | M | CVS | Chemical burn corrosive injury of upper gastrointestinal tract | | - |
| 22 | 73 | F | CVS | Coronary artery disease | Coronary artery bypass graft | - |
| 23 | 82 | M | GS | Stomach cancer with liver metastasis | Exploratory laparotomy for tumor excision | - |
| 24 | 19 | M | CVS | Atrial septal defect | Repair | - |

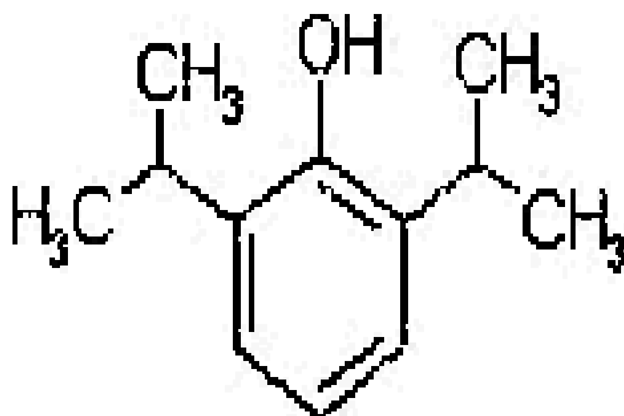
**Table 3: The information of patients used in this study.** The patients were randomly selected with different ages, sex, department, diagnosis, and operation from Tzu Chi Medicine Center Hospital, Hualien, Taiwan. "PS: plastic surgery. CVS: cardiovascular GS: general surgery. NS: neurosurgery. CS: Chest surgery." +: represented that the patient received propofol during care medication. -: represented that the patient did not receive the propofol during care medication.

| Agent | Description | Ref. |
|---|---|---|
| Ketamine | 1. Attenuate secretion of the proinflammatory cytokines IL-6 and TNF-α in human.<br>2. Inhibit TNF-α and PGE-2 of both astrocytes and microglial cells treated with LPS.<br>3. Inhibit nitric oxide synthase in LPS-treated rat alveolar macrophages. | **[183-185]** |
| Lidocaine | 1. Inhibitory effects on stimulus-induced human leukocyte metabolic activation and IL-1β secretion.<br>2. Inhibit secretion of IL-8 and IL-1β and stimulates secretion of IL-1 receptor antagonist by epithelial cells.<br>3. Inhibit secretion of IL-6 and IL-8 which responded to endotoxemia in rabbits. | **[186-188]** |
| Cocaine | 1. Induce inflammatory response in human neuronal progenitor cells.<br>2. Decrease mitogen-induced T-lymphocyte proliferation in rat whole blood.<br>3. Induce decreases in monocyte expression of proinflammatory cytokines at rest and in response to the bacterial ligand, LPS. | **[189-191]** |
| Morphine | 1. Induce defects in early response of alveolar macrophages to Streptococcus pneumoniae by modulating TLR9-NF-κB signaling.<br>2. Attenuates hemorrhagic shock-induced hyper-permeability. | **[192-194]** |
| Thiopentone | 1. Inhibit NF-κB activation in human glioma cells and experimental brain inflammation.<br>2. Impair the phagocytic activity of rat peritoneal macrophages.<br>3. Inhibit neutrophil oxidative responses to the bacterial peptide FMLP. | **[195-197]** |

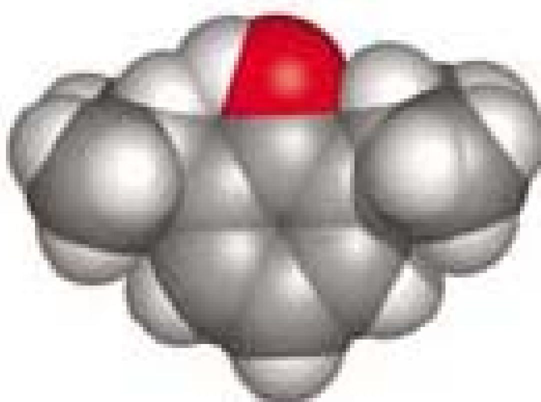
**Table 4: There are many anesthetic agents affecting immune responses in current study.** Several examples were listed in this table to show that reacting on immune systems were widespread phenomena of anesthetic agents.

## Chapter 13 Figures (2)

(A)




(B)



**Franks, N. P. (2006)**

**Fig.1 The chemical structure of propofol.** The IUPAC name of propofol is 2,6-diisopropylphenol. The chemical structure (A) and molecular models (B) of propofol was shown.

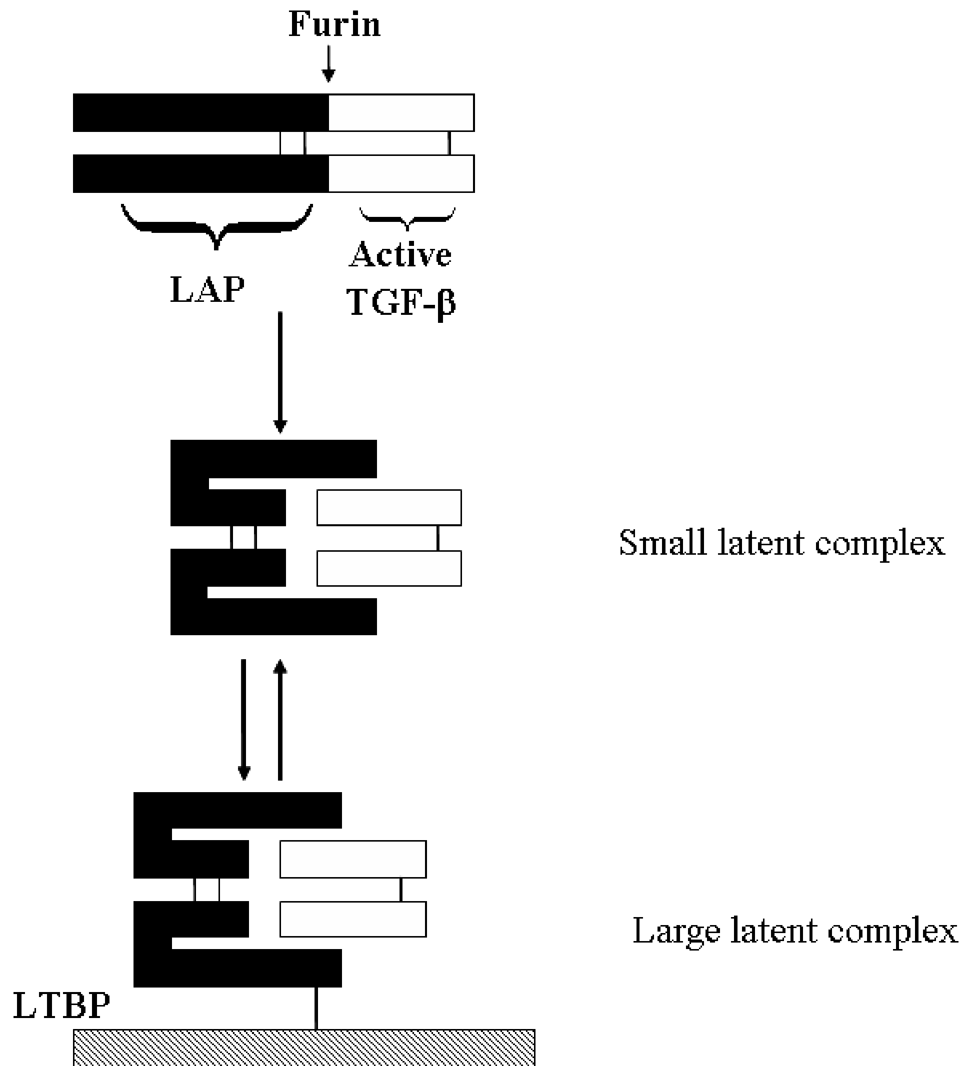
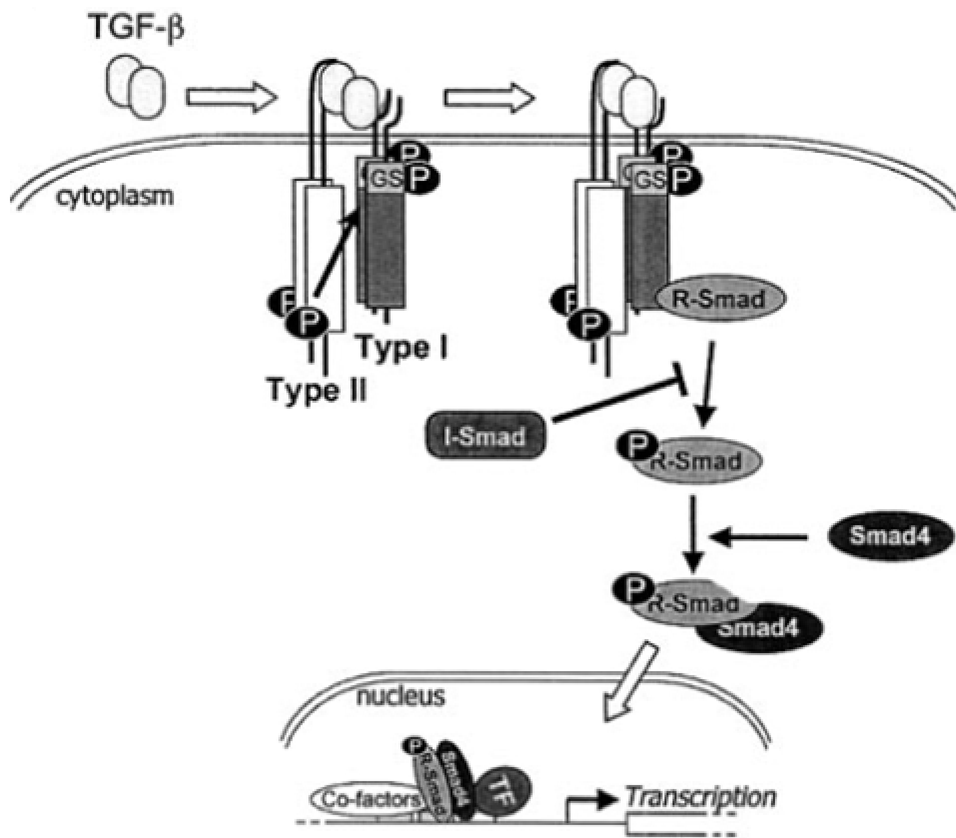



**Fig.2. Processing of TGF-β and formation of latent complex.** This figure briefly illustrated the relationship between active form and latent form. The proteolytic processing by furin results in active TGF-β (white bar) associated with LAP, latency-associated peptide (black bar) by noncovalent binding. Secreted TGF-β is either in the form of “small” (100 kDa) latent complex consisting only of LAP and mature TGF-β or a tertiary “large” (220 kDa) latent complex complex in which the LTBP, latent TGF-β binding protein (stripe bar) is covalently bound to LAP through a disulfide bond.




**Bertolino., et al. (2005)**

**Fig.3. The signaling transduction pathway of TGF-β action.** First TGF-β binds to a type II receptor and recruit type I receptor. The interactions between type II receptor and type I receptor induce phosphorylations and conformation change of type I receptor. Then, the activated type I receptor activates R-Smads by phosphorylating their C-terminus. After phosphorylation, R-Smads form heteromeric complexes with Smad4 that translocate into the nucleus. Within the nucleus, the heteromeric complexes in collaboration with transcription factors and cofactors participate in the regulation of target gene expression.

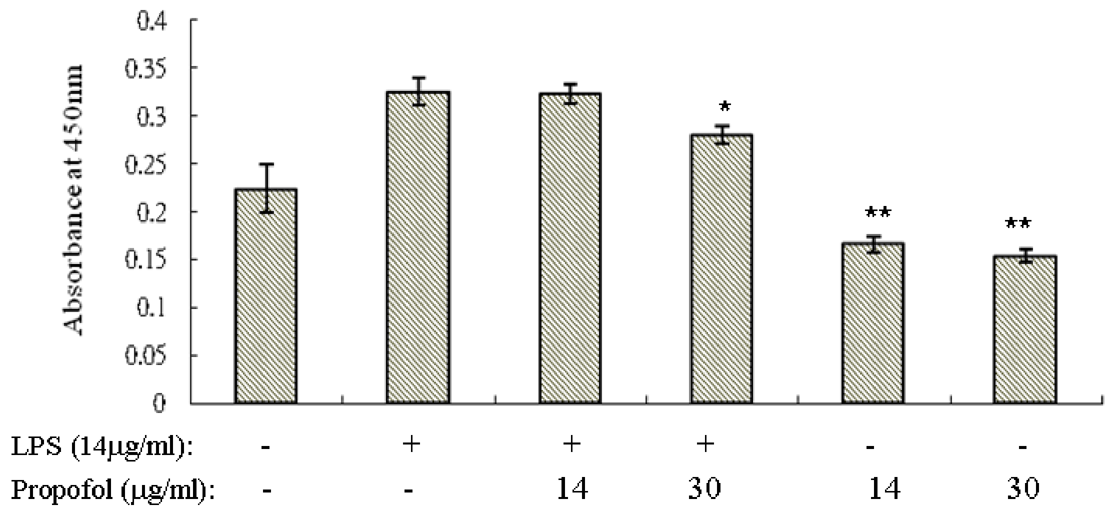



**Fig.4. The effect of propofol on IL-1β expression in mouse splenocytes.** Mouse splenocytes cultured in growth medium without LPS and propofol served as control group. In the same condition, mouse splenocytes co-incubated with LPS served as LPS group. In other group, mouse splenocytes co-incubated with different dosages of propofol or/and LPS. The amounts of IL-1β in supernatants were measured by ELISA. (*: P<0.05 indicates a significant difference compared to LPS group; **: P<0.05 indicates a significant difference compared to control group.)

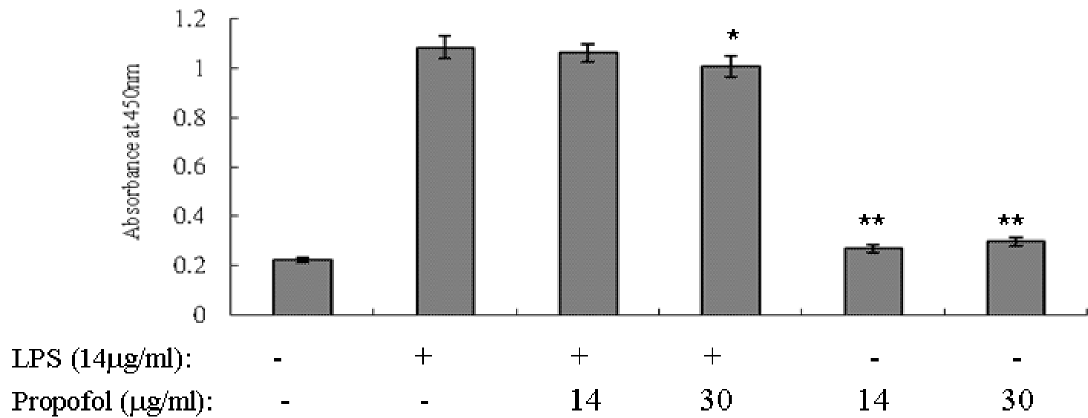



**Fig.5 The effect of propofol on TNF-α expression in mouse splenocytes.** Mouse splenocytes cultured in growth medium without LPS and propofol served as control group. In the same condition, mouse splenocytes co-incubated with LPS served as LPS group. In other group, mouse splenocytes co-incubated with different dosages of propofol or/and LPS. The amounts of TNF-α in supernatants were measured by ELISA. (*: $P<0.05$ indicates a significant difference compared to LPS group; **: $P<0.05$ indicates a significant difference compared to control group.)

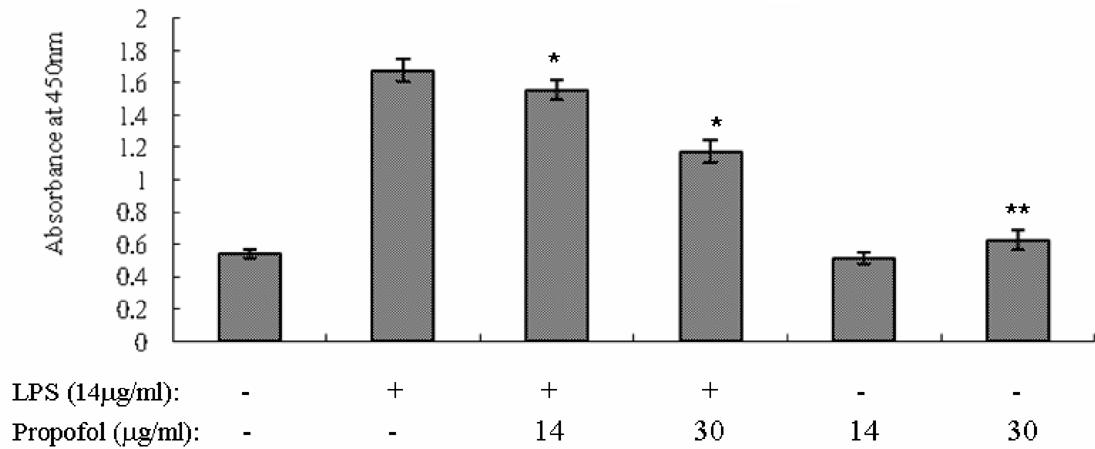



**Fig.6 The effect of propofol on IL-10 expression in mouse splenocytes.** Mouse splenocytes cultured in growth medium without LPS and propofol served as control group. In the same condition, mouse splenocytes co-incubated with LPS served as LPS group. In other group, mouse splenocytes co-incubated with different dosages of propofol or/and LPS. The amounts of IL-10 in supernatants were measured by ELISA. (*: $P<0.05$ indicates a significant difference compared to LPS group; **: $P<0.05$ indicates a significant difference compared to control group.)

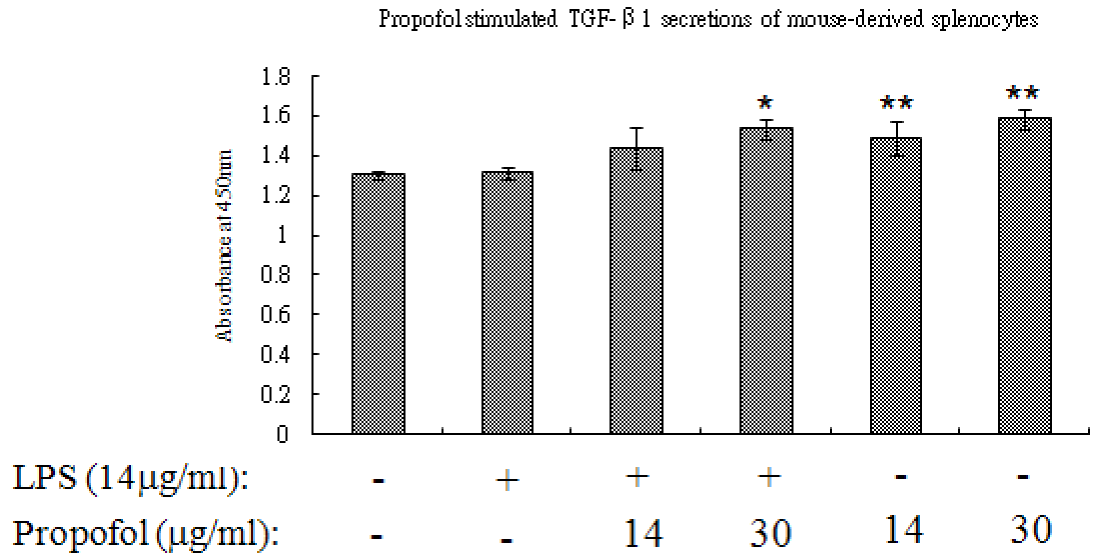



**Fig.7 Propofol induced TGF-β release of mouse splenocytes.** Mouse splenocytes cultured in growth medium without LPS and propofol served as control group. In the same condition, mouse splenocytes co-incubated with LPS served as LPS group. In other group, mouse splenocytes co-incubated with different dosages of propofol or/and LPS. (*: P<0.05 indicates a significant difference compared to LPS group; **: P<0.05 indicates a significant difference compared to control group.)

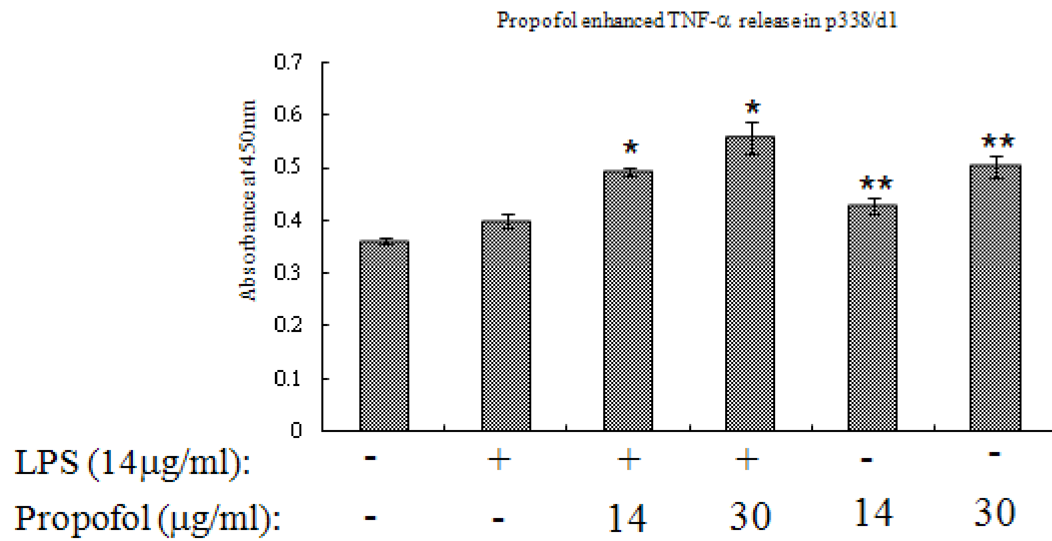
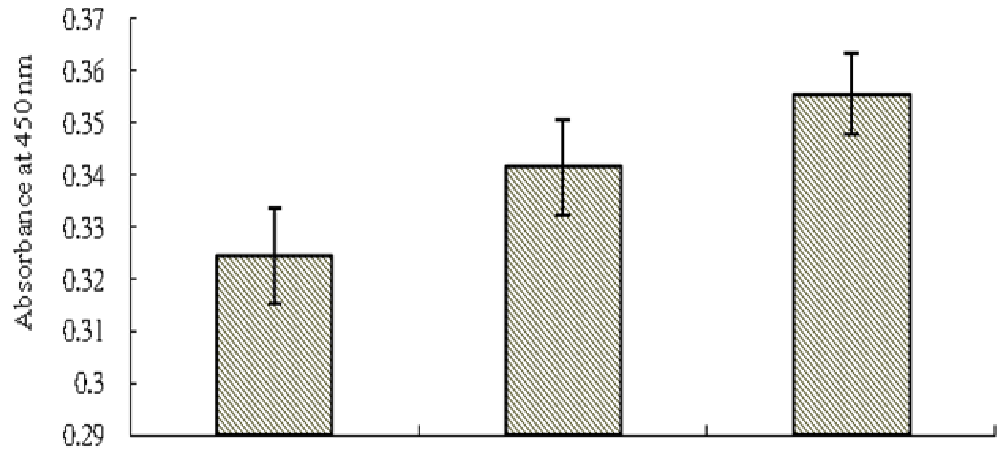



**Fig.8 Propofol enhanced TNF-α release of mouse macrophages.** P338/D1 cultured in growth medium without LPS and propofol served as control group. In the same condition, P338/D1 co-incubated with LPS served as LPS group. In other group, P338/D1 co-incubated with different dosages of propofol or/and LPS. (*: P<0.05 indicates a significant difference compared to LPS group; **: P<0.05 indicates a significant difference compared to control group.)

**(A)**

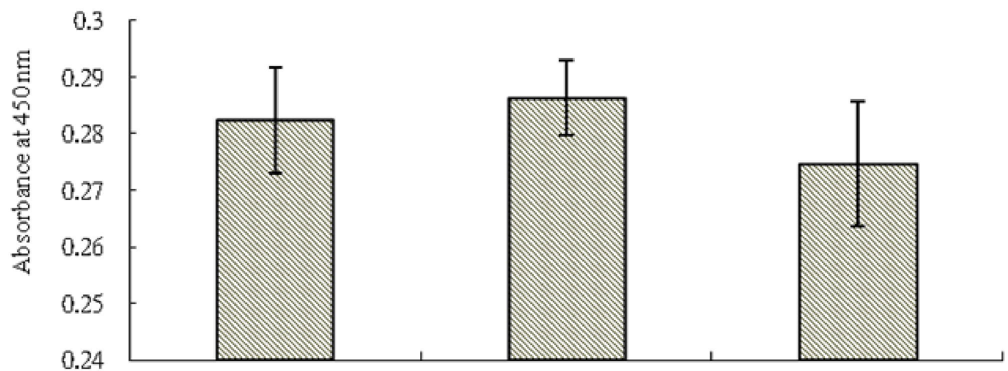
**The effect of propofol on TGF-β expression in p338/d1**




Propofol (μg/ml): 0 14 30

**(B)**

**The effect of propofol on TGF-β expression in EL-4**

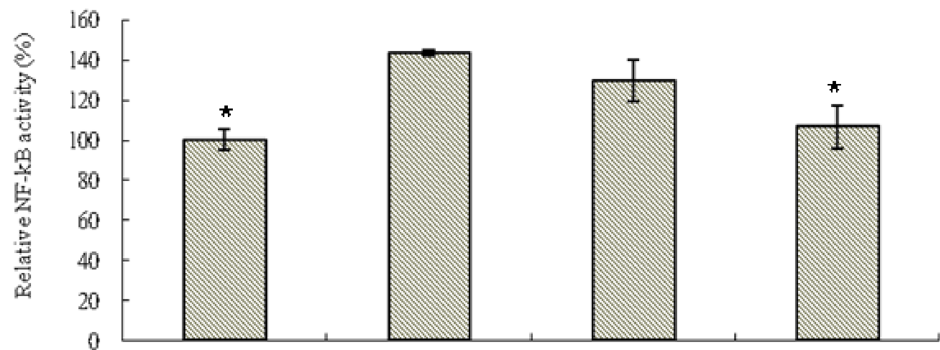



Propofol (μg/ml): 0 14 30

**Fig.9 Propofol did not significantly induce TGF-β expression in P338/D1 and EL-4.** (A) P338/D1 and (B) EL-4 cultured in growth medium without propofol served as control group. In the same condition, the cells were co-cultured with 14 or 30 μg/ml propofol and incubated with 48hrs. The total amounts of TGF-β expression in supernatants were measured by ELISA.

**(A)**

**The effect of propofol on NF−κB activity in Balb-3T3**

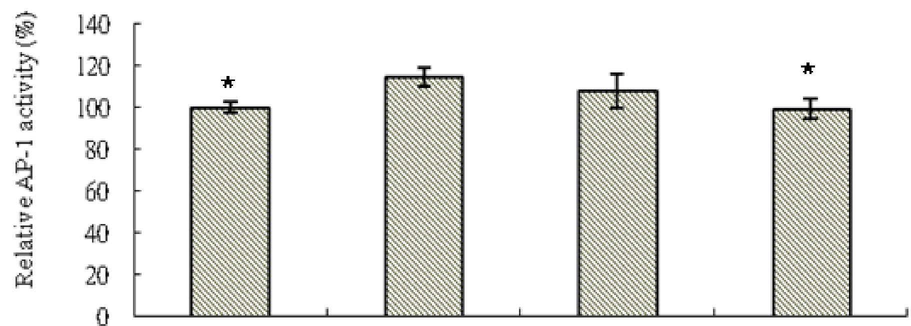



LPS (14μg/ml): - + + +

Propofol (μg/ml): - - 14 30

**(B)**

**The effect of propofol on AP-1 activity in Balb-3T3**

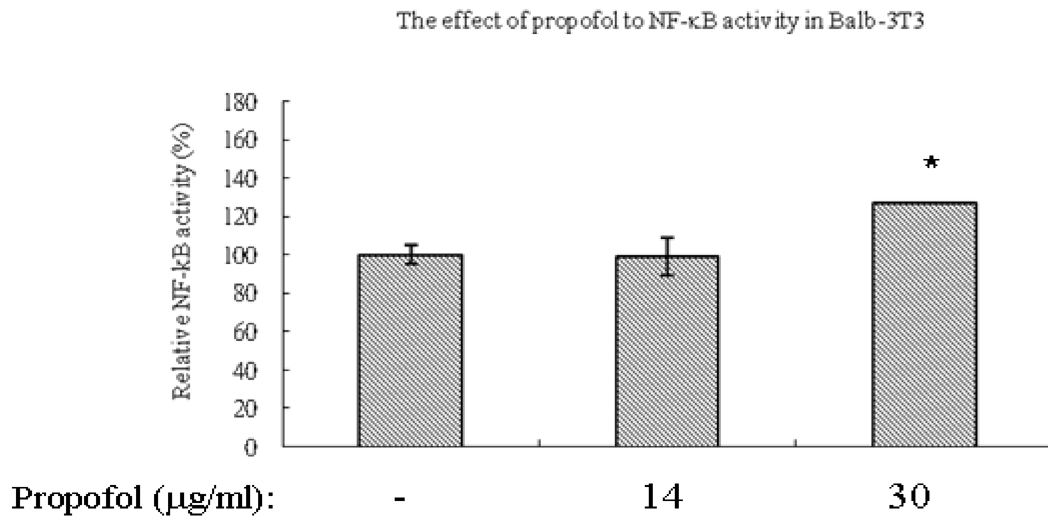



LPS (14μg/ml): - + + +

Propofol (μg/ml): - - 14 30

**Fig.10 Propofol suppressed the activities of NF-κB and AP-1 at the presence of LPS.** (A) pNF-κB/hrGFP or (B) pAP-1/hrGFP plasmids were transfected into Balb-3T3 cells. Transfectants were treated with LPS and co-incubated without or with propofol. The untreated group was the control group. (*: $p < 0.05$ indicates a significant difference compared to LPS group.).

**(A)**




**(B)**

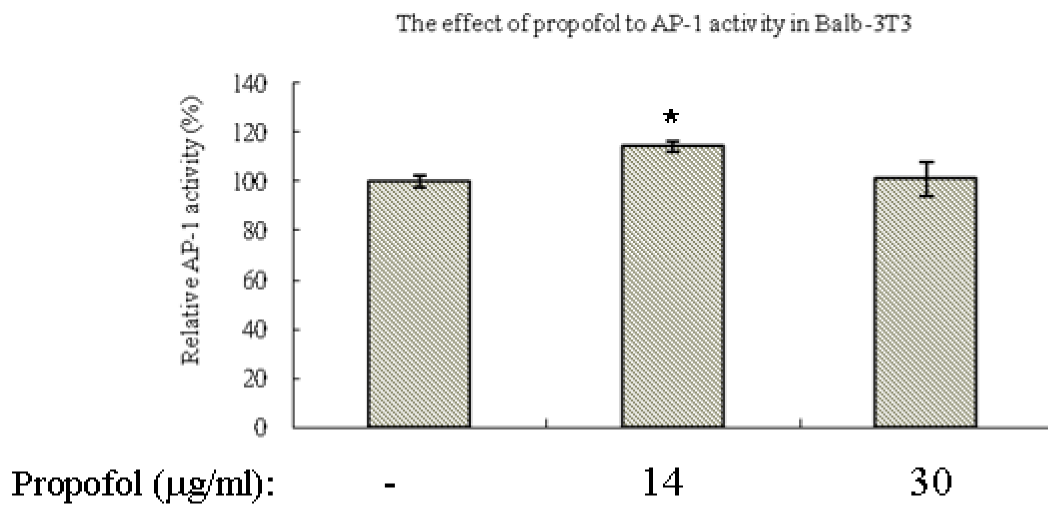
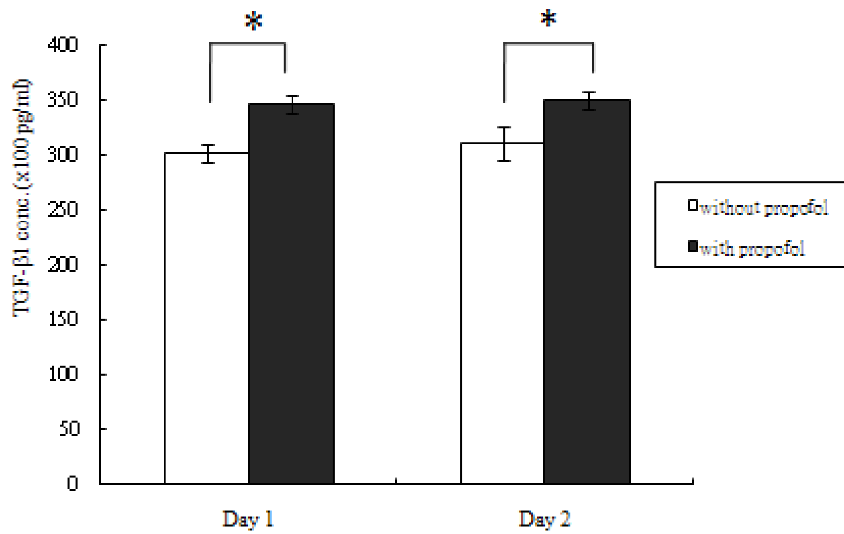



**Fig.11 Propofol raised the activities of NF-κB and AP-1.** (A) pNF-κB /hrGFP or (B) pAP-1/hrGFP plasmids were transfected into Balb-3T3 cells. Transfectants were treated with 14 or 30 μg/ml propofol. The untreated group was the control group. (*: $p < 0.05$ indicates a significant difference compared to control group.)

(A)




(B)

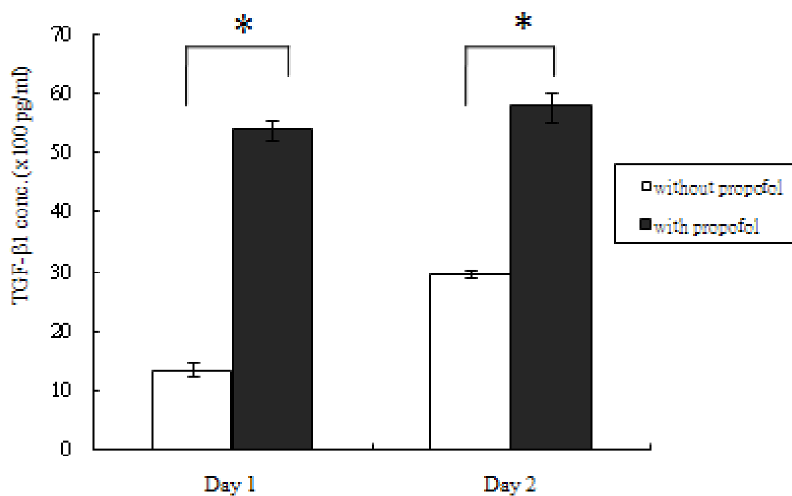



**Fig.12 Intravenous injection of propofol during medical procedure increased both total amount and active form of TGF-β1 in patient sera.** (A) The total amount TGF-β1 and (B) active form TGF-β1 in the patient sera collected daily after surgical operation within 2 day are measured by ELISA. The black and white bars represented that the patient did or did not receive propofol during surgical operation, respectively. (*: $p < 0.05$ indicates a significant difference between the two indicated groups.)

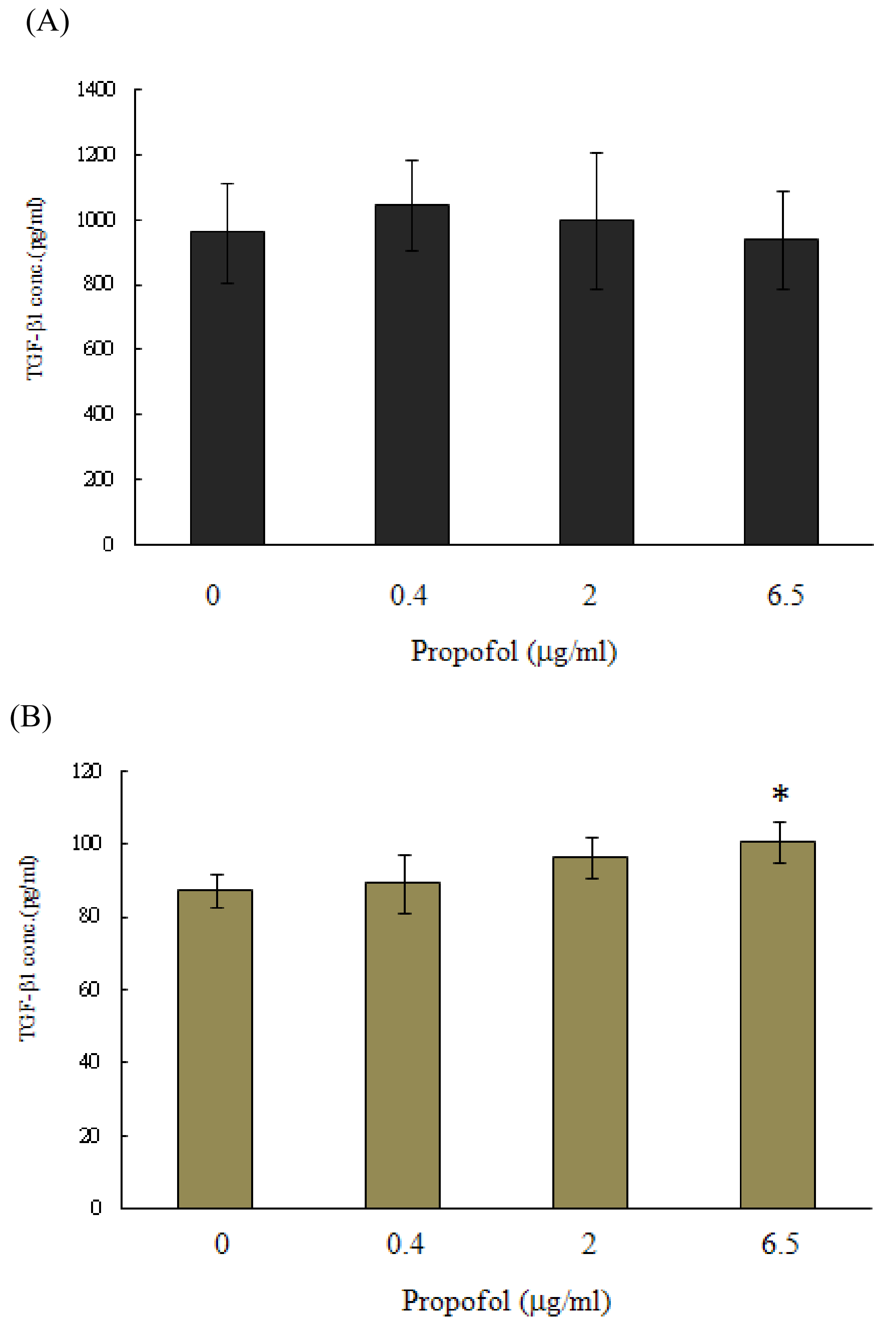
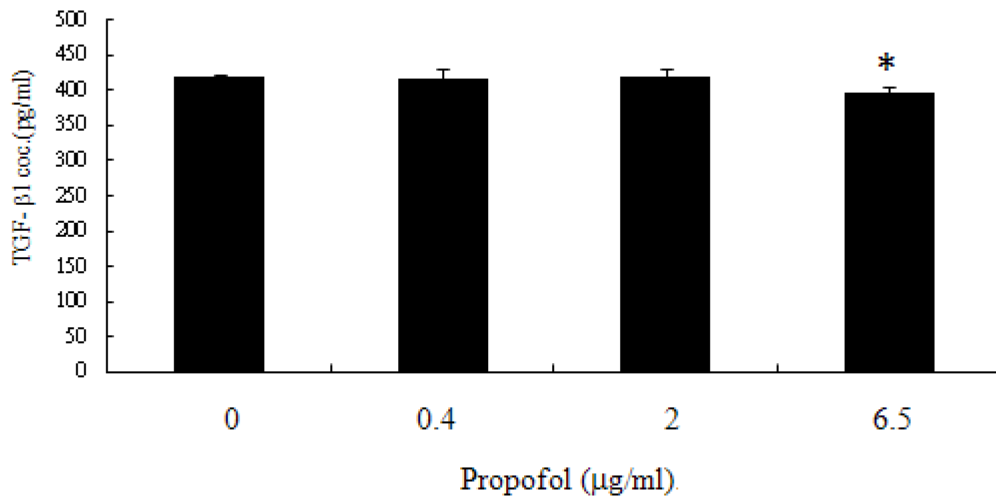



**Fig.13 Clinical dosages of propofol have no significant effect on total amounts of TGF-β1 but slightly raised the amount of active TGF-β1 in the condition mediums of human peripheral blood mononuclear cells.** (A) The total TGF- β1 and (B) active TGF-β1 in the condition mediums of isolated PBMCs co-cultured with different concentrations of propofol for 24 h were measured by ELISA. (*: $p < 0.05$ indicates a significant difference compared to the no-propofol group.)

(A)




**(B)**

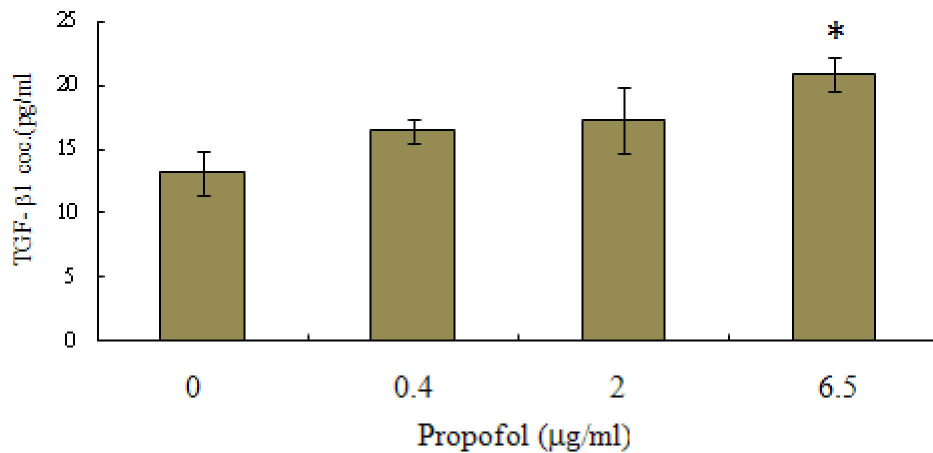
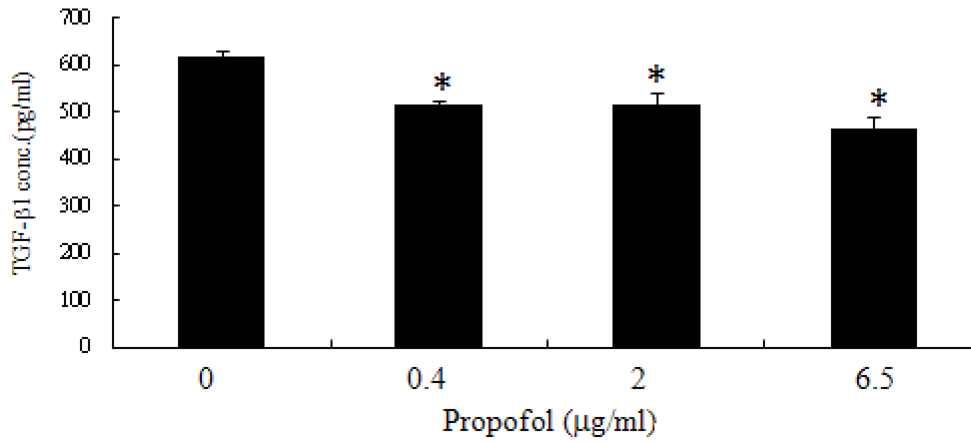



**Fig.14 Clinical dosages of propofol slightly reduced total amounts of TGF-β1 but raised the amount of active TGF-β1 in the condition mediums of human Jurkat cells.** (A) The total TGF-β1 and (B) active TGF-β1 in the condition mediums Jurkat cells co-cultured with different concentrations of propofol were measured by ELISA. (*: $p < 0.05$ indicates a significant difference compared to the no-propofol group.)

**(A)**




**(B)**

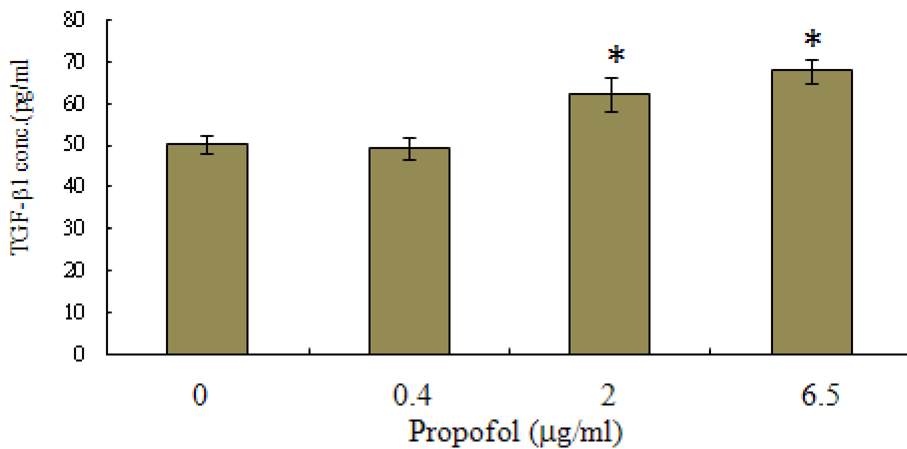



**Fig.15 Clinical dosages of propofol reduced total amounts of TGF-β1 but raised the amount of active TGF-β1 in THP-1 cells in a dose dependent manner.** (A) The total TGF-β1 and (B) active TGF-β1 in the condition mediums THP-1 cells co-cultured with different concentrations of propofol were measured by ELISA. (*: $p < 0.05$ indicates a significant difference compared to the no-propofol group.)

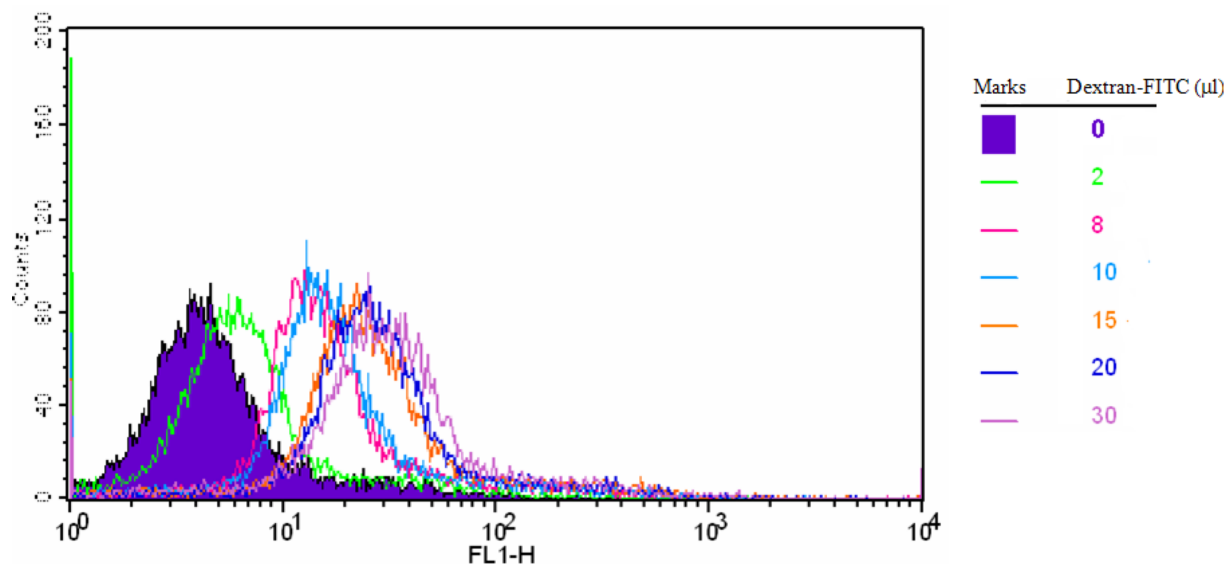



**Fig16 THP-1 uptake dextran-FITC ability was increased with dextran-FTIC concentrations.** 5x10$^5$ THP-1 cells were coincubated with several concentrations of dextran-FITC for two hours and assay the fluorescence intensity of FL-1 by flow cytometer. The total volume was fixed to 200 μl and the additions of dextran-FITC were shown with different colors. Acquisition numbers were ten thousands. The vertical axis of histogram was count numbers, and horizontal axis were fluorescence intensity of FL1 channel.

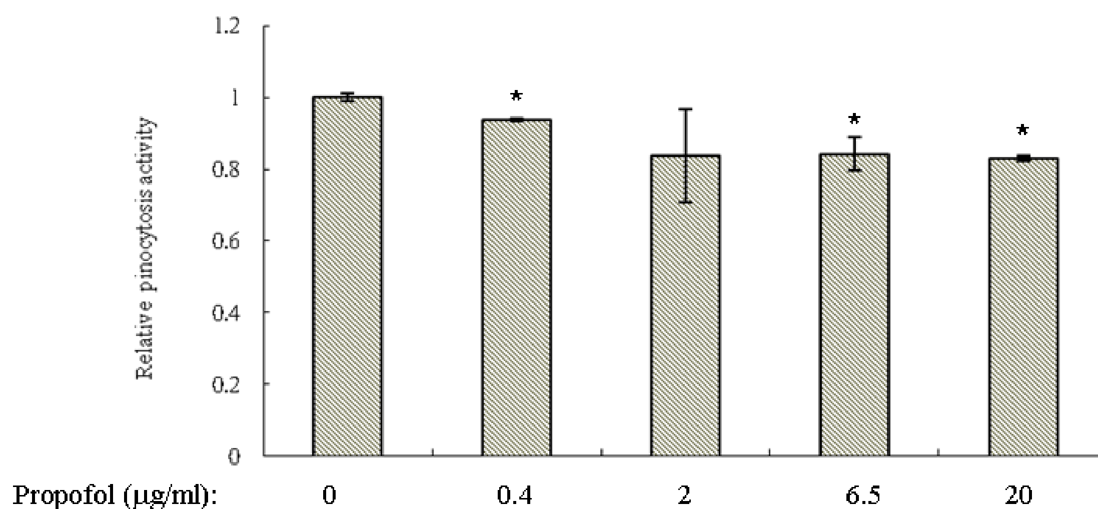
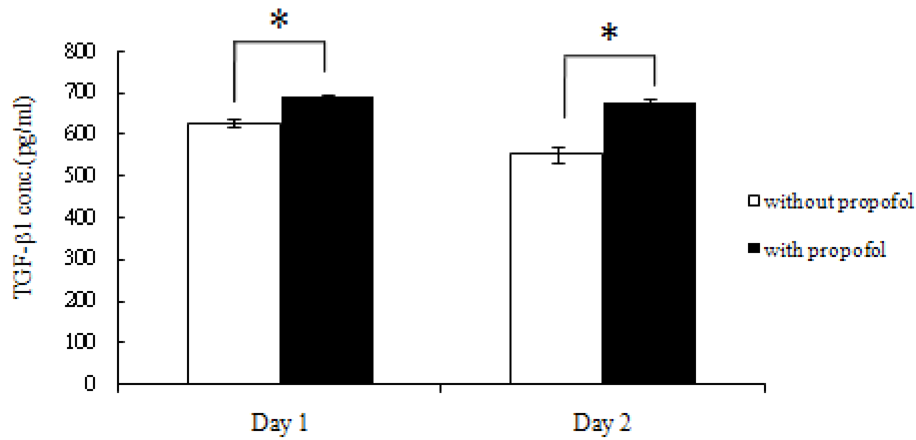



**Fig.17 Propofol inhibited endocytosis activity THP-1.** THP-1($5x10^5$, 1ml) were seeded without any treatment served as control group. In the same condition, THP-1 were treated with different concentrations of propofol. After incubated 24hrs, the same concentration of propofol as previous addition was added to each group and the cells were harvested after incubated another 24 hrs. The dextran-FITC uptake activity were assayed and the relative endocytosis activity (37℃- 0℃) = total fluorescence intensity of sample / total fluorescence intensity of control (37℃- 0℃). (*: $p < 0.05$ indicates a significant difference compared to control group.).

**(A)**




**(B)**

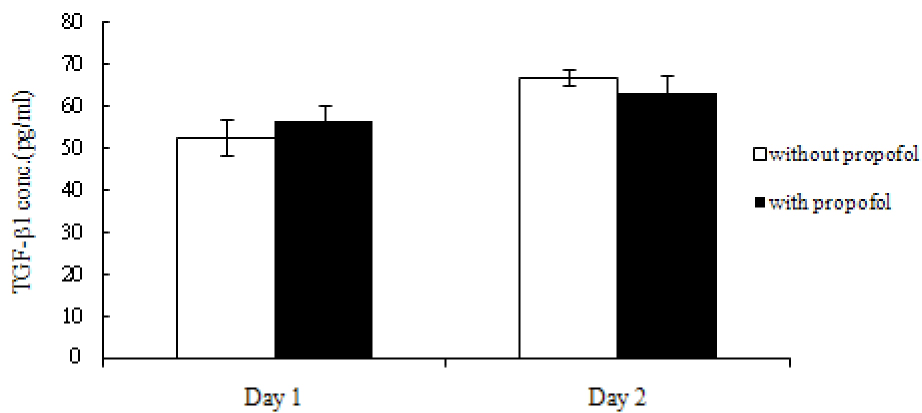



**Fig.18 Clinical dosages of propofol raised total amounts of TGF-β1 in HUVECs.** HEVEC were treated with 6.5 μg/ml propofol. After 24 h incubation, the supernatants were collected as the day1 group or the same concentration of propofol was added and the supernatants were collected after additional 24 h incubation as the day2 group. (A) The total amount TGF-β1 and (B) active form TGF-β1 are measured by ELISA. (*: $p < 0.05$ indicates a significant difference between the two indicated groups.)

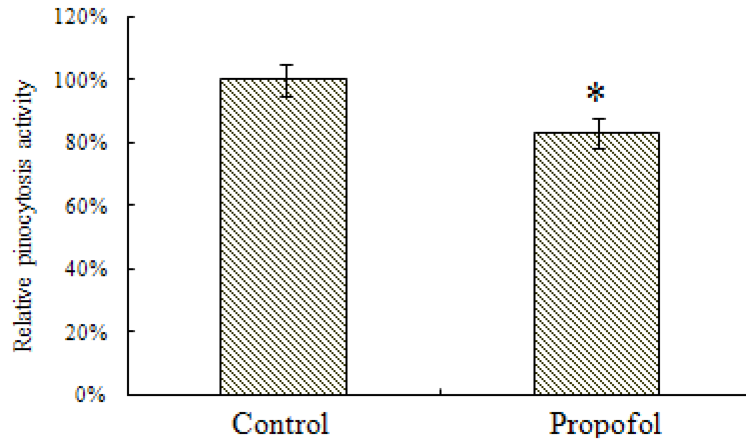
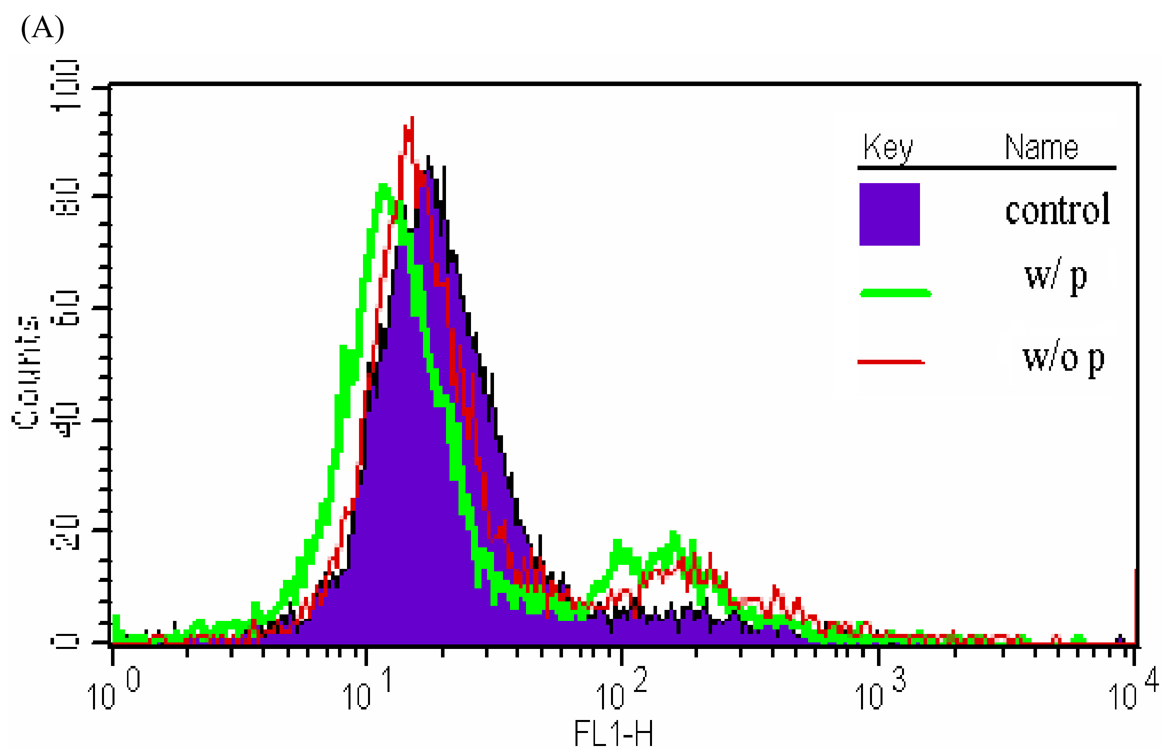
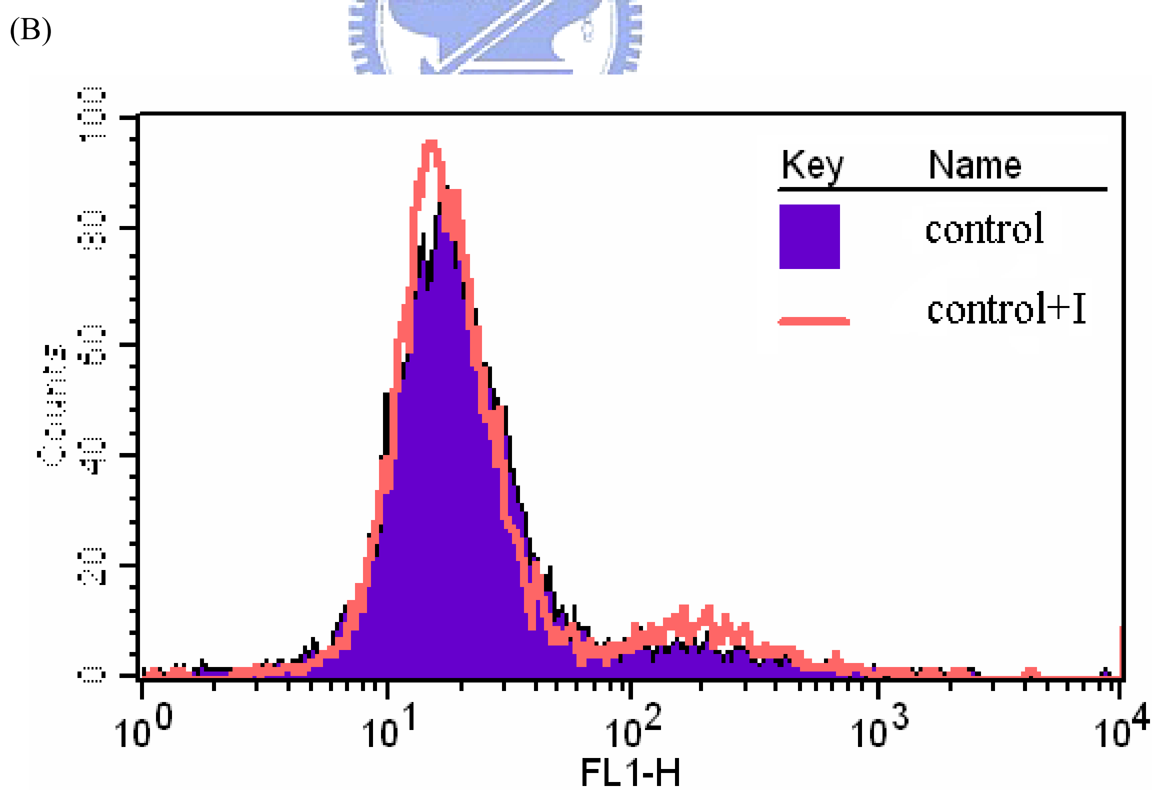



**Fig.19 The condition medium of HEVEC co-cultured with propofol inhibited endocytosis of THP-1.** THP-1 co-cultured with the condition medium of HUVEC without propofol pretreated served as the control group, and co-cultured with the condition medium of HUVEC with propofol (6.5μg/ml) pretreated served as the propofol group. The dextran-FITC uptake activities were assayed and the relative endocytosis activity = (total fluorescence intensity of sample / total fluorescence intensity of control) × 100%. (*: $p < 0.05$ indicates a significant difference compared to the control group.)

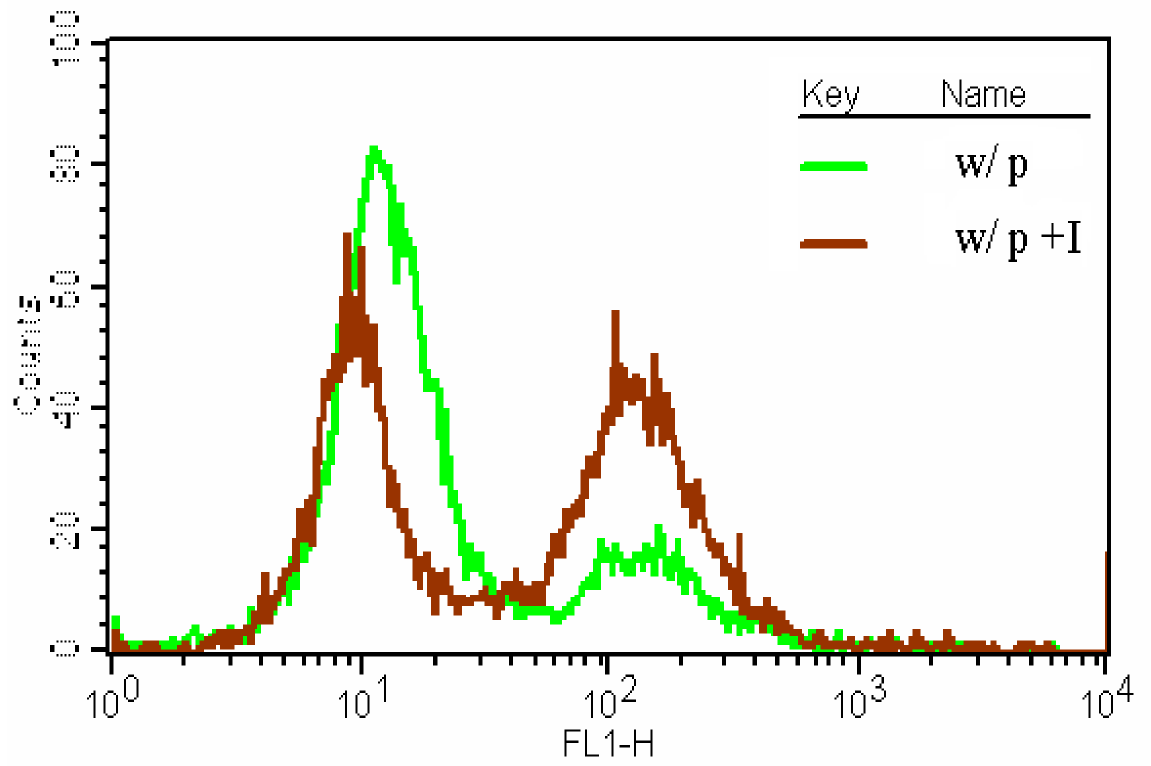
(A)



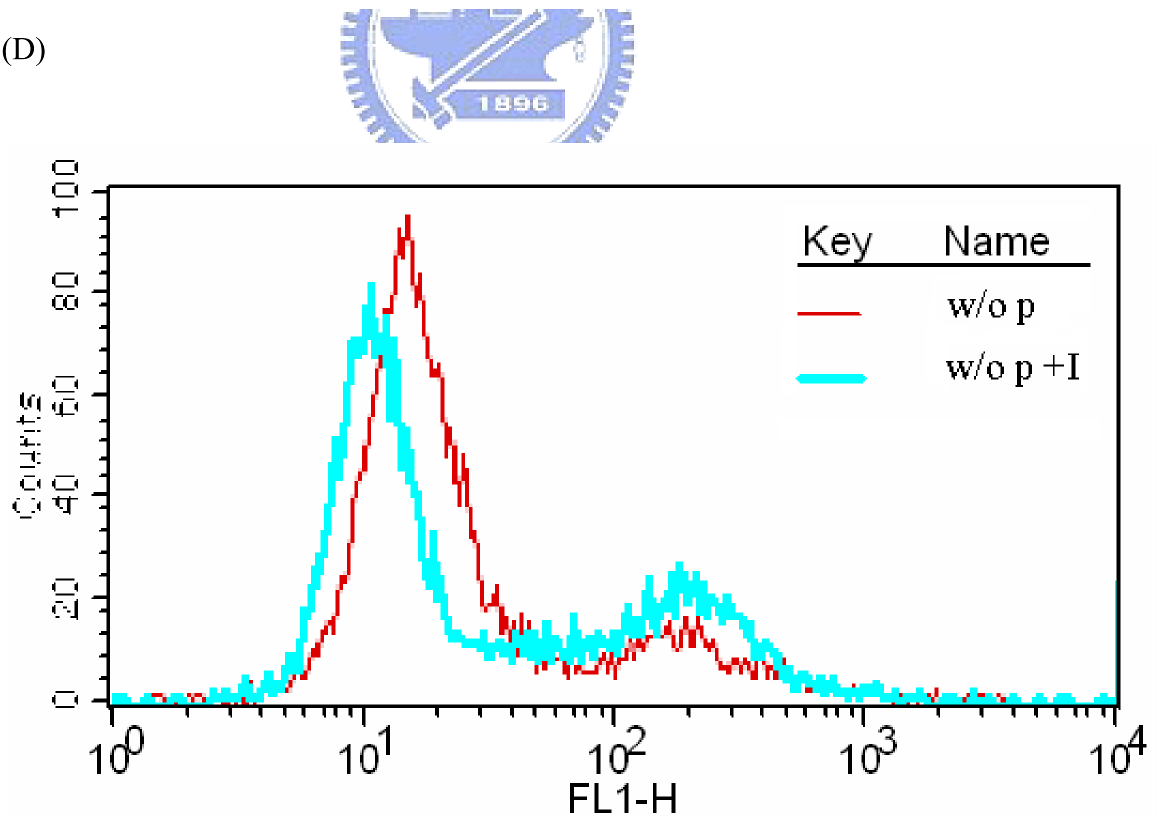
(B)



(C)




(D)



(E)

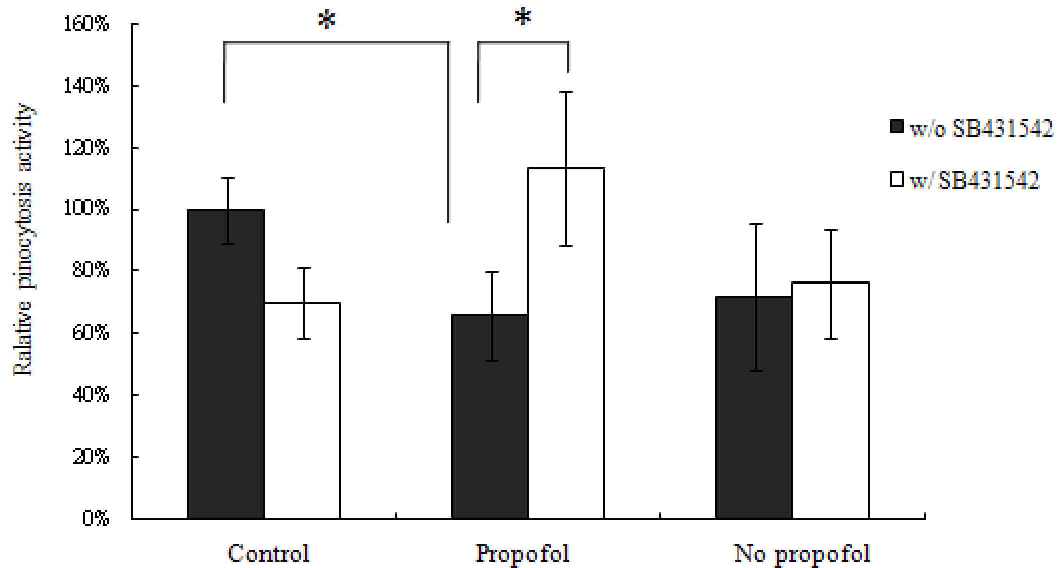



**Fig.20 The sera of patients who received propofol could inhibit the endocytosis activities of THP-1 and abrogated by TGF-β1 pathway inhibitor SB431542.** THP-1 cultured in sera-free RPMI 1640 served as the control group. THP-1 co-cultured with mixed patient sera of the propofol-received group served as propofol group; the patient sera of the no-propofol-received group were mixed and co-cultured with THP-1 served as the no-propofol group. (A-D) Representative pattern of the results from assays by flow cytometer was shown. Purple blank: THP-1 cultured in sera-free RPMI 1640 served as control group. Green line: cocultured with sera from propofol-received patients. Red line: cocultured with sera from no propofol-received patients. Pink line: cultured with SB431542 in sera-free RPMI 1640. Brown line: cocultured with SB431542 and sera from propofol-received patients. Blue line: cocultured with

SB431542 and sera from no propofol-received patients. (E) Quantifications of the fluorescence intensity were calculated as stated above. The black and white bars represented the THP-1 cultured without or with SB431542, respectively. (*: $p < 0.05$ indicates a significant difference between the two indicated groups.)

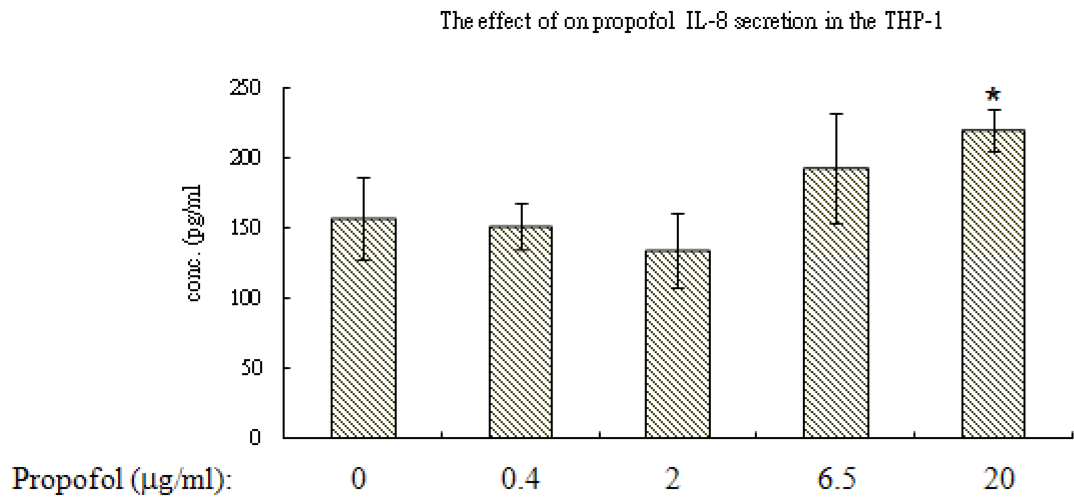



**Fig21. Continuous propofol treatments had potential to induced IL-8 secretion in THP-1.** THP-1 cultured in growth medium for 48 hrs without served as control group. In the same condition, THP-1 co-incubated with different dosages of propofol for treatment twice with a 24hr interval. The amounts of IL-8 in supernatants were measured by ELISA. (*: $P<0.05$ indicates a significant difference compared to control group).

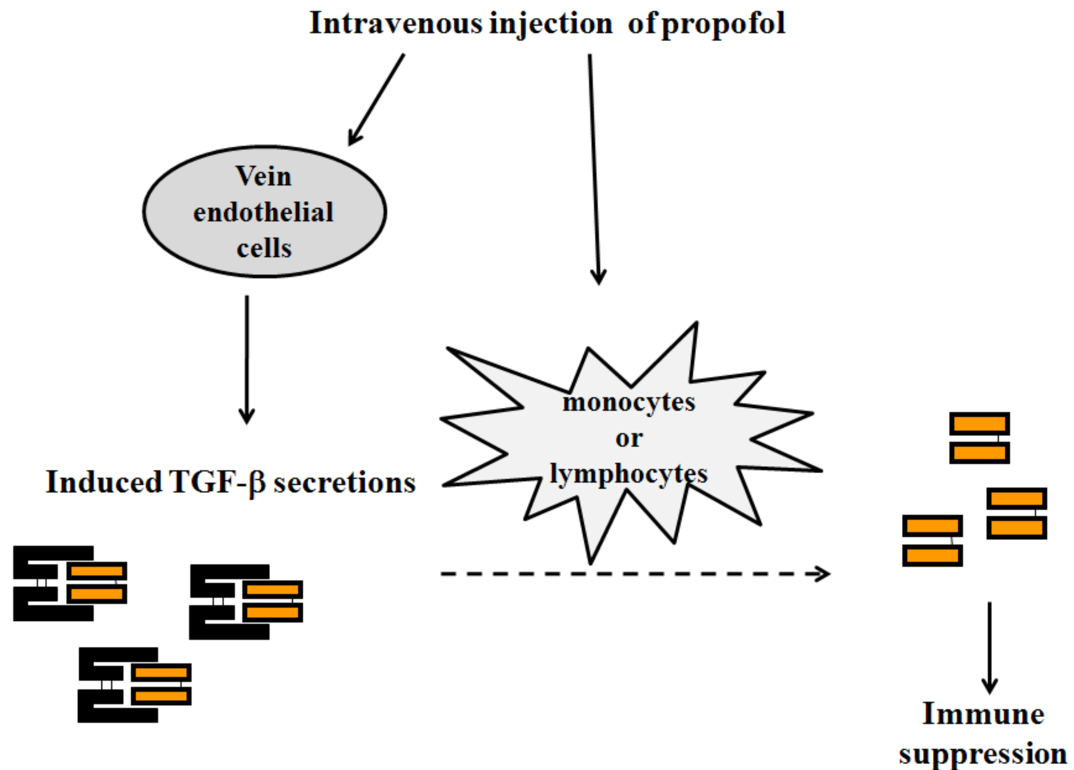



**Fig.22 Hypothesis of intravenous propofol on TGF-β expressions and activation *in vivo*.** When patients received intravenous propofol injections, the vein endothelial cells would be induced to secrete more inactive TGF-β into blood. Furthermore, propofol could induce especially macrophages to convert the inactive TGF-β into biological active form from the circumstance. After continuous circulation, propofol elevated both the total amount and active form of TGF-β in the sera. And the increased levels of active TGF-β could turn to inhibit the activity of macrophages and might result in systemic immune suppression.

## Reference


1. Kunkel, E.J., et al., *Absence of trauma-induced leukocyte rolling in mice deficient in both P-selectin and intercellular adhesion molecule 1.* J Exp Med, 1996. 183(1): p. 57-65.
2. Yao, Y.M., et al., *The inflammatory basis of trauma/shock-associated multiple organ failure.* Inflamm Res, 1998. 47(5): p. 201-10.
3. Read, M.A., et al., *Cell-free pool of CD14 mediates activation of transcription factor NF-kappa B by lipopolysaccharide in human endothelial cells.* Proc Natl Acad Sci U S A, 1993. 90(21): p. 9887-91.
4. Opal, S.M., *The host response to endotoxin, antilipopolysaccharide strategies, and the management of severe sepsis.* Int J Med Microbiol, 2007. 297(5): p. 365-77.
5. Calvano, S.E., et al., *A network-based analysis of systemic inflammation in humans.* Nature, 2005. 437(7061): p. 1032-7.
6. Tang, H., et al., *Sepsis-induced coagulation in the baboon lung is associated with decreased tissue factor pathway inhibitor.* Am J Pathol, 2007. 171(3): p. 1066-77.
7. Henson, P.M. and R.B. Johnston, Jr., *Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins.* J Clin Invest, 1987. 79(3): p. 669-74.
8. Kelbel, I. and M. Weiss, *Anaesthetics and immune function.* Curr Opin Anaesthesiol, 2001. 14(6): p. 685-91.
9. Fuentes, J.M., et al., *General anesthesia delays the inflammatory response and increases survival for mice with endotoxic shock.* Clin Vaccine Immunol, 2006. 13(2): p. 281-8.
10. Bertini, R., et al., *Urinary TNF-binding protein (TNF soluble receptor) protects mice against the lethal effect of TNF and endotoxic shock.* Eur Cytokine Netw, 1993. 4(1): p. 39-42.
11. Taniguchi, T. and K. Yamamoto, *Anti-inflammatory effects of intravenous anesthetics on endotoxemia.* Mini Rev Med Chem, 2005. 5(3): p. 241-5.
12. Choi, S.U., et al., *The effects of propofol on cardiac function after 4 hours of cold cardioplegia and reperfusion.* J Cardiothorac Vasc Anesth, 2007. 21(5): p. 678-82.
13. Yang, F.L., et al., *The reduction of tumor necrosis factor-alpha release and tissue damage by pentobarbital in the experimental endotoxemia model.* Shock, 2007. 28(3): p. 309-16.
14. Cassuto, J., R. Sinclair, and M. Bonderovic, *Anti-inflammatory properties of local anesthetics and their present and potential clinical implications.* Acta

Anaesthesiol Scand, 2006. 50(3): p. 265-82.
15. Helmy, S.A. and R.J. Al-Attiyah, *The immunomodulatory effects of prolonged intravenous infusion of propofol versus midazolam in critically ill surgical patients.* Anaesthesia, 2001. 56(1): p. 4-8.
16. Kotani, N., et al., *Expression of genes for proinflammatory cytokines in alveolar macrophages during propofol and isoflurane anesthesia.* Anesth Analg, 1999. 89(5): p. 1250-6.
17. Helmy, S.A., M.A. Wahby, and M. El-Nawaway, *The effect of anaesthesia and surgery on plasma cytokine production.* Anaesthesia, 1999. 54(8): p. 733-8.
18. Larsen, B., et al., *Effect of intravenous anesthetics on spontaneous and endotoxin-stimulated cytokine response in cultured human whole blood.* Anesthesiology, 1998. 89(5): p. 1218-27.
19. Hoff, G., et al., *Modulation of endotoxin-stimulated TNF-alpha gene expression by ketamine and propofol in cultured human whole blood.* Anaesthesist, 2001. 50(7): p. 494-9.
20. Takaono, M., et al., *Effects of intravenous anesthetics on interleukin (IL)-6 and IL-10 production by lipopolysaccharide-stimulated mononuclear cells from healthy volunteers.* Acta Anaesthesiol Scand, 2002. 46(2): p. 176-9.
21. Galley, H.F., A.M. Dubbels, and N.R. Webster, *The effect of midazolam and propofol on interleukin-8 from human polymorphonuclear leukocytes.* Anesth Analg, 1998. 86(6): p. 1289-93.
22. Lopez, A., et al., *Multiple-center, randomized, placebo-controlled, double-blind study of the nitric oxide synthase inhibitor 546C88: effect on survival in patients with septic shock.* Crit Care Med, 2004. 32(1): p. 21-30.
23. Helmer, K.S., et al., *Ketamine/xylazine attenuates LPS-induced iNOS expression in various rat tissues.* J Surg Res, 2003. 112(1): p. 70-8.
24. Helmer, K.S., et al., *Effects of ketamine/xylazine on expression of tumor necrosis factor-alpha, inducible nitric oxide synthase, and cyclo-oxygenase-2 in rat gastric mucosa during endotoxemia.* Shock, 2003. 20(1): p. 63-9.
25. Suliburk, J.W., et al., *Ketamine attenuates liver injury attributed to endotoxemia: role of cyclooxygenase-2.* Surgery, 2005. 138(2): p. 134-40.
26. Chen, R.M., et al., *Ketamine reduces nitric oxide biosynthesis in human umbilical vein endothelial cells by down-regulating endothelial nitric oxide synthase expression and intracellular calcium levels.* Crit Care Med, 2005. 33(5): p. 1044-9.
27. Castillo, C., et al., *Thiopental inhibits nitric oxide production in rat aorta.* Can J Physiol Pharmacol, 1999. 77(12): p. 958-66.

28. Yagmurdur, H., et al., *Propofol reduces nitric oxide-induced apoptosis in testicular ischemia-reperfusion injury by downregulating the expression of inducible nitric oxide synthase.* Acta Anaesthesiol Scand, 2008. 52(3): p. 350-7.
29. Chen, Y.S., et al., *Propofol-induced vascular permeability change is related to the nitric oxide signaling pathway and occludin phosphorylation.* J Biomed Sci, 2007. 14(5): p. 629-36.
30. Gonzalez-Correa, J.A., et al., *Effects of propofol on the leukocyte nitric oxide pathway: in vitro and ex vivo studies in surgical patients.* Naunyn Schmiedebergs Arch Pharmacol, 2008. 376(5): p. 331-9.
31. Tobin, J.R., et al., *Selective anesthetic inhibition of brain nitric oxide synthase.* Anesthesiology, 1994. 81(5): p. 1264-9.
32. Galley, H.F. and N.R. Webster, *Brain nitric oxide synthase activity is decreased by intravenous anesthetics.* Anesth Analg, 1996. 83(3): p. 591-4.
33. Krumholz, W., J. Endrass, and G. Hempelmann, *Inhibition of phagocytosis and killing of bacteria by anaesthetic agents in vitro.* Br J Anaesth, 1995. 75(1): p. 66-70.
34. Krumholz, W., J. Endrass, and G. Hempelmann, *Propofol inhibits phagocytosis and killing of Staphylococcus aureus and Escherichia coli by polymorphonuclear leukocytes in vitro.* Can J Anaesth, 1994. 41(5 Pt 1): p. 446-9.
35. Heller, A., et al., *Effects of intravenous anesthetics on bacterial elimination in human blood in vitro.* Acta Anaesthesiol Scand, 1998. 42(5): p. 518-26.
36. Kotani, N., et al., *Loss of alveolar macrophages during anesthesia and operation in humans.* Anesth Analg, 1995. 81(6): p. 1255-62.
37. Kotani, N., et al., *Intraoperative modulation of alveolar macrophage function during isoflurane and propofol anesthesia.* Anesthesiology, 1998. 89(5): p. 1125-32.
38. Nishina, K., et al., *The inhibitory effects of thiopental, midazolam, and ketamine on human neutrophil functions.* Anesth Analg, 1998. 86(1): p. 159-65.
39. Mikawa, K., et al., *Propofol inhibits human neutrophil functions.* Anesth Analg, 1998. 87(3): p. 695-700.
40. Heine, J., et al., *Effects of different preparations of propofol, diazepam, and etomidate on human neutrophils in vitro.* Acta Anaesthesiol Scand, 2001. 45(2): p. 213-20.
41. Nagata, T., et al., *Propofol inhibits FMLP-stimulated phosphorylation of p42 mitogen-activated protein kinase and chemotaxis in human neutrophils.* Br J

Anaesth, 2001. 86(6): p. 853-8.
42. Inada, T., et al., *Propofol depressed neutrophil hydrogen peroxide production more than midazolam, whereas adhesion molecule expression was minimally affected by both anesthetics in rats with abdominal sepsis.* Anesth Analg, 2001. 92(2): p. 437-41.
43. Thiry, J.C., et al., *Propofol scavenges reactive oxygen species and inhibits the protein nitration induced by activated polymorphonuclear neutrophils.* Eur J Pharmacol, 2004. 499(1-2): p. 29-33.
44. Hofbauer, R., et al., *Thiopental inhibits migration of human leukocytes through human endothelial cell monolayers in vitro.* Intensive Care Med, 1998. 24(9): p. 973-6.
45. Skoutelis, A., et al., *Effects of propofol and thiopentone on polymorphonuclear leukocyte functions in vitro.* Acta Anaesthesiol Scand, 1994. 38(8): p. 858-62.
46. Hofbauer, R., et al., *Propofol reduces the migration of human leukocytes through endothelial cell monolayers.* Crit Care Med, 1999. 27(9): p. 1843-7.
47. Hofbauer, R., et al., *Ketamine significantly reduces the migration of leukocytes through endothelial cell monolayers.* Crit Care Med, 1998. 26(9): p. 1545-9.
48. Weigand, M.A., et al., *Ketamine modulates the stimulated adhesion molecule expression on human neutrophils in vitro.* Anesth Analg, 2000. 90(1): p. 206-12.
49. Brand, J.M., et al., *The effects of general anesthesia on human peripheral immune cell distribution and cytokine production.* Clin Immunol Immunopathol, 1997. 83(2): p. 190-4.
50. Brand, J.M., et al., *Early alterations in the number of circulating lymphocyte subpopulations and enhanced proinflammatory immune response during opioid-based general anesthesia.* Shock, 2003. 20(3): p. 213-7.
51. Schneemilch, C.E., et al., *Effects of different anaesthetic agents on immune cell function in vitro.* Eur J Anaesthesiol, 2005. 22(8): p. 616-23.
52. Inada, T., et al., *Effect of propofol and isoflurane anaesthesia on the immune response to surgery.* Anaesthesia, 2004. 59(10): p. 954-9.
53. Helmer, K.S., J.W. Suliburk, and D.W. Mercer, *Ketamine-induced gastroprotection during endotoxemia: role of heme-oxygenase-1.* Dig Dis Sci, 2006. 51(9): p. 1571-81.
54. Wang, H.H., et al., *Propofol attenuation of renal ischemia/reperfusion injury involves heme oxygenase-1.* Acta Pharmacol Sin, 2007. 28(8): p. 1175-80.
55. McDermott, B.J., et al., *Protection of cardiomyocyte function by propofol*

*during simulated ischemia is associated with a direct action to reduce pro-oxidant activity.* J Mol Cell Cardiol, 2007. 42(3): p. 600-8.

56. Kato, R. and P. Foex, *Myocardial protection by anesthetic agents against ischemia-reperfusion injury: an update for anesthesiologists.* Can J Anaesth, 2002. 49(8): p. 777-91.
57. Yagmurdur, H., et al., *The effects of ketamine and propofol on bacterial translocation in rats after burn injury.* Acta Anaesthesiol Scand, 2005. 49(2): p. 177-82.
58. Yagmurdur, H., et al., *The effects of propofol and ketamine on gut mucosal epithelial apoptosis in rats after burn injury.* Eur J Anaesthesiol, 2007. 24(1): p. 46-52.
59. Gurfinkel, R., et al., *Ketamine improves survival in burn injury followed by sepsis in rats.* Anesth Analg, 2006. 103(2): p. 396-402, table of contents.
60. Neder Meyer, T. and A. Lazaro Da Silva, *Ketamine reduces mortality of severely burnt rats, when compared to midazolam plus fentanyl.* Burns, 2004. 30(5): p. 425-30.
61. White, M.C. and C. Karsli, *Long-term use of an intravenous ketamine infusion in a child with significant burns.* Paediatr Anaesth, 2007. 17(11): p. 1102-4.
62. Owens, V.F., et al., *Ketamine: a safe and effective agent for painful procedures in the pediatric burn patient.* J Burn Care Res, 2006. 27(2): p. 211-6; discussion 217.
63. Edrich, T., et al., *Ketamine for long-term sedation and analgesia of a burn patient.* Anesth Analg, 2004. 99(3): p. 893-5, table of contents.
64. Tosun, Z., A. Esmaoglu, and A. Coruh, *Propofol-ketamine vs propofol-fentanyl combinations for deep sedation and analgesia in pediatric patients undergoing burn dressing changes.* Paediatr Anaesth, 2008. 18(1): p. 43-7.
65. Kinoshita, H., et al., *Pentobarbital therapy for status epilepticus in children: timing of tapering.* Pediatr Neurol, 1995. 13(2): p. 164-8.
66. Holmes, G.L. and J.J. Riviello, Jr., *Midazolam and pentobarbital for refractory status epilepticus.* Pediatr Neurol, 1999. 20(4): p. 259-64.
67. Piper, R.D., et al., *Introducing Critical Appraisal to studies of animal models investigating novel therapies in sepsis.* Crit Care Med, 1996. 24(12): p. 2059-70.
68. Vincent, J.L., *New therapies in sepsis.* Chest, 1997. 112(6 Suppl): p. 330S-338S.
69. Hoffmann, J.A., et al., *Phylogenetic perspectives in innate immunity.* Science,

1999. 284(5418): p. 1313-8.

70. Youn, J.H., et al., *High Mobility Group Box 1 Protein Binding to Lipopolysaccharide Facilitates Transfer of Lipopolysaccharide to CD14 and Enhances Lipopolysaccharide-Mediated TNF-{alpha} Production in Human Monocytes.* J Immunol, 2008. 180(7): p. 5067-74.

71. Togbe, D., et al., *Toll-like receptor and tumour necrosis factor dependent endotoxin-induced acute lung injury.* Int J Exp Pathol, 2007. 88(6): p. 387-91.

72. Schnyder-Candrian, S., et al., *Dual effects of p38 MAPK on TNF-dependent bronchoconstriction and TNF-independent neutrophil recruitment in lipopolysaccharide-induced acute respiratory distress syndrome.* J Immunol, 2005. 175(1): p. 262-9.

73. Zacharowski, K., et al., *Toll-like receptor 4 plays a crucial role in the immune-adrenal response to systemic inflammatory response syndrome.* Proc Natl Acad Sci U S A, 2006. 103(16): p. 6392-7.

74. Hayashi, T., et al., *Lipopolysaccharide-induced decreased protein S expression in liver cells is mediated by MEK/ERK signaling and NFkappaB activation: involvement of membrane-bound CD14 and toll-like receptor-4.* J Thromb Haemost, 2006. 4(8): p. 1763-73.

75. Francois, S., et al., *Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-kinase/Akt and NF-kappaB signaling pathways, leading to increased levels of Mcl-1, A1, and phosphorylated Bad.* J Immunol, 2005. 174(6): p. 3633-42.

76. Jones, K.L., et al., *Activin A is a critical component of the inflammatory response, and its binding protein, follistatin, reduces mortality in endotoxemia.* Proc Natl Acad Sci U S A, 2007. 104(41): p. 16239-44.

77. Ulevitch, R.J. and P.S. Tobias, *Recognition of gram-negative bacteria and endotoxin by the innate immune system.* Curr Opin Immunol, 1999. 11(1): p. 19-22.

78. Akira, S., K. Takeda, and T. Kaisho, *Toll-like receptors: critical proteins linking innate and acquired immunity.* Nat Immunol, 2001. 2(8): p. 675-80.

79. Yokoyama, M., et al., *The effects of epidural block on the distribution of lymphocyte subsets and natural-killer cell activity in patients with and without pain.* Anesth Analg, 2001. 92(2): p. 463-9.

80. Livnat, S., et al., *Involvement of peripheral and central catecholamine systems in neural-immune interactions.* J Neuroimmunol, 1985. 10(1): p. 5-30.

81. Landmann, R., *Beta-adrenergic receptors in human leukocyte subpopulations.*

**Eur J Clin Invest, 1992. 22 Suppl 1: p. 30-6.**

82. **Yoshimura, T., et al., *Inhibition of tumor necrosis factor-alpha and interleukin-1-beta production by beta-adrenoceptor agonists from lipopolysaccharide-stimulated human peripheral blood mononuclear cells.* Pharmacology, 1997. 54(3): p. 144-52.**
83. **Weiss, M., et al., *Is inhibition of oxygen radical production of neutrophils by sympathomimetics mediated via beta-2 adrenoceptors?* J Pharmacol Exp Ther, 1996. 278(3): p. 1105-13.**
84. **Xu, H., et al., *Dose-dependent effects of propofol on renal sympathetic nerve activity, blood pressure and heart rate in urethane-anesthetized rabbits.* Eur J Pharmacol, 2000. 387(1): p. 79-85.**
85. **Greisen, J., et al., *Acute pain induces an instant increase in natural killer cell cytotoxicity in humans and this response is abolished by local anaesthesia.* Br J Anaesth, 1999. 83(2): p. 235-40.**
86. **Loeb, A.L., I. Godeny, and D.E. Longnecker, *Anesthetics alter relative contributions of NO and EDHF in rat cremaster muscle microcirculation.* Am J Physiol, 1997. 273(2 Pt 2): p. H618-27.**
87. **Nyhan, D.P., et al., *Anesthesia alters pulmonary vasoregulation by angiotensin II and captopril.* J Appl Physiol, 1992. 72(2): p. 636-42.**
88. **Chanimov, M., et al., *Substances used for local and general anaesthesia in major surgery suppress proliferative responsiveness of normal rat peripheral blood mononuclear cells in culture.* Eur J Anaesthesiol, 2000. 17(4): p. 248-55.**
89. **Chou, W.C., et al., *Expression of chimeric monomer and dimer proteins on the plasma membrane of mammalian cells.* Biotechnol Bioeng, 1999. 65(2): p. 160-9.**
90. **Lee, R.P., et al., *Physiological and chemical indicators for early and late stages of sepsis in conscious rats.* J Biomed Sci, 2002. 9(6 Pt 2): p. 613-21.**
91. **Gao, J., B. Zeng, and L. Zhou, *The protective effects of early treatment with propofol on endotoxin-induced acute lung injury in rats.* Middle East J Anesthesiol, 2003. 17(3): p. 379-401.**
92. **Gao, J., et al., *Protective effects of early treatment with propofol on endotoxin-induced acute lung injury in rats.* Br J Anaesth, 2004. 92(2): p. 277-9.**
93. **Joung, Y.H., et al., *Hypoxia activates the cyclin D1 promoter via the Jak2/STAT5b pathway in breast cancer cells.* Exp Mol Med, 2005. 37(4): p. 353-64.**
94. **Lee, M.Y., et al., *Phosphorylation and activation of STAT proteins by hypoxia***

*in breast cancer cells.* Breast, 2006. 15(2): p. 187-95.
95. Morimoto, Y., et al., *Pentobarbital inhibits apoptosis in neuronal cells.* Crit Care Med, 2000. 28(6): p. 1899-904.
96. Kotani, N., et al., *Volatile anesthetics augment expression of proinflammatory cytokines in rat alveolar macrophages during mechanical ventilation.* Anesthesiology, 1999. 91(1): p. 187-97.
97. Gotsch, U., et al., *Expression of P-selectin on endothelial cells is upregulated by LPS and TNF-alpha in vivo.* Cell Adhes Commun, 1994. 2(1): p. 7-14.
98. Shi, Q., et al., *Comparative analysis of vascular endothelial cell activation by TNF-alpha and LPS in humans and baboons.* Cell Biochem Biophys, 2004. 40(3): p. 289-303.
99. Myers, C.L., et al., *Induction of ICAM-1 by TNF-alpha, IL-1 beta, and LPS in human endothelial cells after downregulation of PKC.* Am J Physiol, 1992. 263(4 Pt 1): p. C767-72.
100. Miyabayashi, M. and K. Yasui, *Regulation of neutrophil O2- production by neutrophil-endothelial cell interaction via CD11b: its modulation by tumor necrosis factor-alpha (TNF-alpha) and lipopolysaccharide (LPS).* Int J Hematol, 1996. 65(1): p. 49-59.
101. Miller, L.S., et al., *Suppression of cytokine-induced neutrophil accumulation in rat mesenteric venules in vivo by general anesthesia.* Int J Microcirc Clin Exp, 1996. 16(3): p. 147-54.
102. Grundmann, H.J., et al., *Inhibition of endotoxin-induced macrophage tumor necrosis factor expression by a prostacyclin analogue and its beneficial effect in experimental lipopolysaccharide intoxication.* J Infect Dis, 1992. 165(3): p. 501-5.
103. Timokhov, V.S., et al., *[Plasma contents of cytokines (TNF-alpha, IL-1 beta, IL-6) and their clearance during continuous hemofiltration in patients with sepsis and multiple organ failure].* Anesteziol Reanimatol, 1997(3): p. 59-62.
104. Nick, J.A., et al., *Selective activation and functional significance of p38alpha mitogen-activated protein kinase in lipopolysaccharide-stimulated neutrophils.* J Clin Invest, 1999. 103(6): p. 851-8.
105. Chen, L.W., et al., *Thermal injury-induced priming effect of neutrophil is TNF-alpha and P38 dependent.* Shock, 2006. 26(1): p. 69-76.
106. Penner, C.G., et al., *The transcription factors NF-kappab and AP-1 are differentially regulated in skeletal muscle during sepsis.* Biochem Biophys Res Commun, 2001. 281(5): p. 1331-6.
107. Franks, N.P., *Molecular targets underlying general anaesthesia.* Br J Pharmacol, 2006. 147 Suppl 1: p. S72-81.

108. Glen, J.B. and S.C. Hunter, *Pharmacology of an emulsion formulation of ICI 35 868.* Br J Anaesth, 1984. 56(6): p. 617-26.
109. Schnider, T.W., et al., *The influence of age on propofol pharmacodynamics.* Anesthesiology, 1999. 90(6): p. 1502-16.
110. Shafer, A., et al., *Pharmacokinetics and pharmacodynamics of propofol infusions during general anesthesia.* Anesthesiology, 1988. 69(3): p. 348-56.
111. Newman, L.H., et al., *Propofol infusion for sedation in intensive care.* Anaesthesia, 1987. 42(9): p. 929-37.
112. Gan, T.J., *Propofol and postoperative nausea and vomiting.* Br J Anaesth, 1998. 81(4): p. 653.
113. Langley, M.S. and R.C. Heel, *Propofol. A review of its pharmacodynamic and pharmacokinetic properties and use as an intravenous anaesthetic.* Drugs, 1988. 35(4): p. 334-72.
114. Sebel, P.S. and J.D. Lowdon, *Propofol: a new intravenous anesthetic.* Anesthesiology, 1989. 71(2): p. 260-77.
115. Hales, T.G. and J.J. Lambert, *The actions of propofol on inhibitory amino acid receptors of bovine adrenomedullary chromaffin cells and rodent central neurones.* Br J Pharmacol, 1991. 104(3): p. 619-28.
116. Rabow, L.E., S.J. Russek, and D.H. Farb, *From ion currents to genomic analysis: recent advances in GABAA receptor research.* Synapse, 1995. 21(3): p. 189-274.
117. Krasowski, M.D. and N.L. Harrison, *General anaesthetic actions on ligand-gated ion channels.* Cell Mol Life Sci, 1999. 55(10): p. 1278-303.
118. Engblom, A.C., et al., *Point mutation in the first transmembrane region of the beta 2 subunit of the gamma--aminobutyric acid type A receptor alters desensitization kinetics of gamma--aminobutyric acid- and anesthetic-induced channel gating.* J Biol Chem, 2002. 277(20): p. 17438-47.
119. Bai, D., et al., *The general anesthetic propofol slows deactivation and desensitization of GABA(A) receptors.* J Neurosci, 1999. 19(24): p. 10635-46.
120. Hara, M., Y. Kai, and Y. Ikemoto, *Propofol activates GABAA receptor-chloride ionophore complex in dissociated hippocampal pyramidal neurons of the rat.* Anesthesiology, 1993. 79(4): p. 781-8.
121. Chen, R.M., et al., *Propofol suppresses macrophage functions and modulates mitochondrial membrane potential and cellular adenosine triphosphate synthesis.* Anesthesiology, 2003. 98(5): p. 1178-85.
122. Chen, R.M., et al., *Anti-inflammatory and antioxidative effects of propofol on lipopolysaccharide-activated macrophages.* Ann N Y Acad Sci, 2005. 1042: p. 262-71.

123. Takemoto, Y., *Dose effects of propofol on hemodynamic and cytokine responses to endotoxemia in rats.* J Anesth, 2005. 19(1): p. 40-4.
124. Chen, R.M., et al., *Propofol reduces nitric oxide biosynthesis in lipopolysaccharide-activated macrophages by downregulating the expression of inducible nitric oxide synthase.* Arch Toxicol, 2003. 77(7): p. 418-23.
125. Wang, B., et al., *Propofol reduces apoptosis and up-regulates endothelial nitric oxide synthase protein expression in hydrogen peroxide-stimulated human umbilical vein endothelial cells.* Anesth Analg, 2007. 105(4): p. 1027-33, table of contents.
126. Votta-Velis, E.G., et al., *Propofol attenuates endotoxin-induced endothelial cell injury, angiotensin-converting enzyme shedding, and lung edema.* Anesth Analg, 2007. 105(5): p. 1363-70, table of contents.
127. Gao, J., et al., *Protective effects of propofol on lipopolysaccharide-activated endothelial cell barrier dysfunction.* Inflamm Res, 2006. 55(9): p. 385-92.
128. Mathy-Hartert, M., et al., *Protective activity of propofol, Diprivan and intralipid against active oxygen species.* Mediators Inflamm, 1998. 7(5): p. 327-33.
129. Blanchette, F., et al., *Involvement of Smads in TGFbeta1-induced furin (fur) transcription.* J Cell Physiol, 2001. 188(2): p. 264-73.
130. Nunes, I., et al., *Structure and activation of the large latent transforming growth factor-Beta complex.* J Am Optom Assoc, 1998. 69(10): p. 643-8.
131. Crawford, S.E., et al., *Thrombospondin-1 is a major activator of TGF-beta1 in vivo.* Cell, 1998. 93(7): p. 1159-70.
132. Gleizes, P.E., et al., *TGF-beta latency: biological significance and mechanisms of activation.* Stem Cells, 1997. 15(3): p. 190-7.
133. Munger, J.S., et al., *The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis.* Cell, 1999. 96(3): p. 319-28.
134. Letterio, J.J. and A.B. Roberts, *Regulation of immune responses by TGF-beta.* Annu Rev Immunol, 1998. 16: p. 137-61.
135. Letterio, J.J., et al., *Invasive candidiasis stimulates hepatocyte and monocyte production of active transforming growth factor beta.* Infect Immun, 2001. 69(8): p. 5115-20.
136. Abbott, B.D. and L.S. Birnbaum, *Retinoic acid-induced alterations in the expression of growth factors in embryonic mouse palatal shelves.* Teratology, 1990. 42(6): p. 597-610.
137. Comerci, J.T., Jr., et al., *Induction of transforming growth factor beta-1 in cervical intraepithelial neoplasia in vivo after treatment with beta-carotene.*

Clin Cancer Res, 1997. 3(2): p. 157-60.

138. Castanares, C., et al., *Signaling by ALK5 mediates TGF-beta-induced ET-1 expression in endothelial cells: a role for migration and proliferation.* J Cell Sci, 2007. 120(Pt 7): p. 1256-66.

139. Bertolino, P., et al., *Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders.* Chest, 2005. 128(6 Suppl): p. 585S-590S.

140. Singh, N.N. and D.P. Ramji, *The role of transforming growth factor-beta in atherosclerosis.* Cytokine Growth Factor Rev, 2006. 17(6): p. 487-99.

141. Larisch, S., et al., *A novel mitochondrial septin-like protein, ARTS, mediates apoptosis dependent on its P-loop motif.* Nat Cell Biol, 2000. 2(12): p. 915-21.

142. Ignotz, R.A. and J. Massague, *Cell adhesion protein receptors as targets for transforming growth factor-beta action.* Cell, 1987. 51(2): p. 189-97.

143. Kim, E.S., M.S. Kim, and A. Moon, *Transforming growth factor (TGF)-beta in conjunction with H-ras activation promotes malignant progression of MCF10A breast epithelial cells.* Cytokine, 2005. 29(2): p. 84-91.

144. Kim, E.S., Y.W. Sohn, and A. Moon, *TGF-beta-induced transcriptional activation of MMP-2 is mediated by activating transcription factor (ATF)2 in human breast epithelial cells.* Cancer Lett, 2007. 252(1): p. 147-56.

145. Seiki, M., *Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion.* Cancer Lett, 2003. 194(1): p. 1-11.

146. Jakowlew, S.B., *Transforming growth factor-beta in cancer and metastasis.* Cancer Metastasis Rev, 2006. 25(3): p. 435-57.

147. Welch, D.R., A. Fabra, and M. Nakajima, *Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential.* Proc Natl Acad Sci U S A, 1990. 87(19): p. 7678-82.

148. O'Kane, S. and M.W. Ferguson, *Transforming growth factor beta s and wound healing.* Int J Biochem Cell Biol, 1997. 29(1): p. 63-78.

149. Bellone, G., et al., *Regulation of NK cell functions by TGF-beta 1.* J Immunol, 1995. 155(3): p. 1066-73.

150. Tridandapani, S., et al., *TGF-beta 1 suppresses [correction of supresses] myeloid Fc gamma receptor function by regulating the expression and function of the common gamma-subunit.* J Immunol, 2003. 170(9): p. 4572-7.

151. Takeuchi, M., P. Alard, and J.W. Streilein, *TGF-beta promotes immune deviation by altering accessory signals of antigen-presenting cells.* J Immunol, 1998. 160(4): p. 1589-97.

152. Du, C. and S. Sriram, *Mechanism of inhibition of LPS-induced IL-12p40 production by IL-10 and TGF-beta in ANA-1 cells.* J Leukoc Biol, 1998. 64(1):

p. 92-7.

153. Takaki, H., et al., *TGF-beta1 suppresses IFN-gamma-induced NO production in macrophages by suppressing STAT1 activation and accelerating iNOS protein degradation.* Genes Cells, 2006. 11(8): p. 871-82.

154. Imai, K., A. Takeshita, and S. Hanazawa, *Transforming growth factor-beta inhibits lipopolysaccharide-stimulated expression of inflammatory cytokines in mouse macrophages through downregulation of activation protein 1 and CD14 receptor expression.* Infect Immun, 2000. 68(5): p. 2418-23.

155. McCartney-Francis, N., W. Jin, and S.M. Wahl, *Aberrant Toll receptor expression and endotoxin hypersensitivity in mice lacking a functional TGF-beta 1 signaling pathway.* J Immunol, 2004. 172(6): p. 3814-21.

156. Perrella, M.A., et al., *Arrest of endotoxin-induced hypotension by transforming growth factor beta1.* Proc Natl Acad Sci U S A, 1996. 93(5): p. 2054-9.

157. Naiki, Y., et al., *Transforming growth factor-beta differentially inhibits MyD88-dependent, but not TRAM- and TRIF-dependent, lipopolysaccharide-induced TLR4 signaling.* J Biol Chem, 2005. 280(7): p. 5491-5.

158. Strobl, H. and W. Knapp, *TGF-beta1 regulation of dendritic cells.* Microbes Infect, 1999. 1(15): p. 1283-90.

159. Robinson, R.T. and J.D. Gorham, *TGF-beta 1 regulates antigen-specific CD4+ T cell responses in the periphery.* J Immunol, 2007. 179(1): p. 71-9.

160. Leveen, P., et al., *TGF-beta type II receptor-deficient thymocytes develop normally but demonstrate increased CD8+ proliferation in vivo.* Blood, 2005. 106(13): p. 4234-40.

161. Zhang, L., et al., *Transforming growth factor-beta: an important role in CD4+CD25+ regulatory T cells and immune tolerance.* Autoimmunity, 2006. 39(4): p. 269-76.

162. Nakamura, K., A. Kitani, and W. Strober, *Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta.* J Exp Med, 2001. 194(5): p. 629-44.

163. Liu, V.C., et al., *Tumor evasion of the immune system by converting CD4+CD25- T cells into CD4+CD25+ T regulatory cells: role of tumor-derived TGF-beta.* J Immunol, 2007. 178(5): p. 2883-92.

164. Holder, M.J., K. Knox, and J. Gordon, *Factors modifying survival pathways of germinal center B cells. Glucocorticoids and transforming growth factor-beta, but not cyclosporin A or anti-CD19, block surface*

*immunoglobulin-mediated rescue from apoptosis.* Eur J Immunol, 1992. 22(10): p. 2725-8.

165. Lomo, J., et al., *TGF-beta 1 and cyclic AMP promote apoptosis in resting human B lymphocytes.* J Immunol, 1995. 154(4): p. 1634-43.

166. Cazac, B.B. and J. Roes, *TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo.* Immunity, 2000. 13(4): p. 443-51.

167. van Vlasselaer, P., J. Punnonen, and J.E. de Vries, *Transforming growth factor-beta directs IgA switching in human B cells.* J Immunol, 1992. 148(7): p. 2062-7.

168. Purkerson, J. and P. Isakson, *A two-signal model for regulation of immunoglobulin isotype switching.* Faseb J, 1992. 6(14): p. 3245-52.

169. Li, M.O., et al., *Transforming growth factor-beta regulation of immune responses.* Annu Rev Immunol, 2006. 24: p. 99-146.

170. Suberville, S., et al., *Transforming growth factor-beta inhibits interleukin-10 synthesis by human monocytic cells.* Eur Cytokine Netw, 2001. 12(1): p. 141-6.

171. Seth, R.B. and Z.J. Chen, *Smads keep TABs on inflammation.* Nat Immunol, 2007. 8(5): p. 477-8.

172. Arsura, M., et al., *Transient activation of NF-kappaB through a TAK1/IKK kinase pathway by TGF-beta1 inhibits AP-1/SMAD signaling and apoptosis: implications in liver tumor formation.* Oncogene, 2003. 22(3): p. 412-25.

173. Haller, D., et al., *Transforming growth factor-beta 1 inhibits non-pathogenic Gram negative bacteria-induced NF-kappa B recruitment to the interleukin-6 gene promoter in intestinal epithelial cells through modulation of histone acetylation.* J Biol Chem, 2003. 278(26): p. 23851-60.

174. Monteleone, G., et al., *A failure of transforming growth factor-beta1 negative regulation maintains sustained NF-kappaB activation in gut inflammation.* J Biol Chem, 2004. 279(6): p. 3925-32.

175. Pirttikangas, C.O., J. Perttila, and M. Salo, *Propofol emulsion reduces proliferative responses of lymphocytes from intensive care patients.* Intensive Care Med, 1993. 19(5): p. 299-302.

176. Alevizopoulos, A. and N. Mermod, *Antagonistic regulation of a proline-rich transcription factor by transforming growth factor beta and tumor necrosis factor alpha.* J Biol Chem, 1996. 271(47): p. 29672-81.

177. Hsiao, Y.W., et al., *Interactions of host IL-6 and IFN-gamma and cancer-derived TGF-beta1 on MHC molecule expression during tumor spontaneous regression.* Cancer Immunol Immunother, 2008.

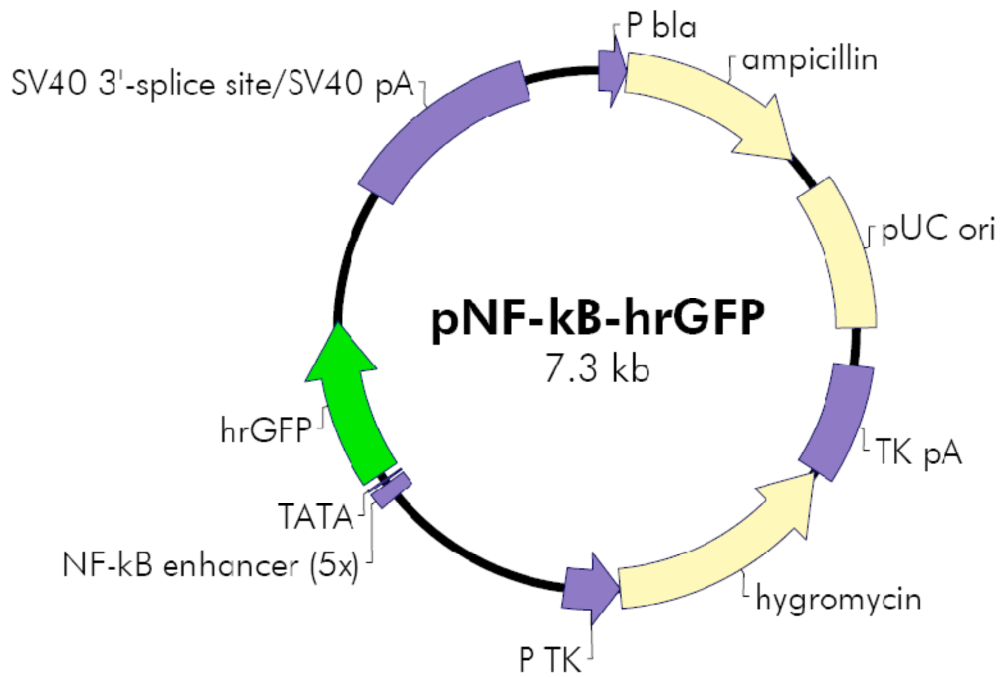
178. Hsiao, Y.W., et al., *Tumor-infiltrating lymphocyte secretion of IL-6*

***antagonizes tumor-derived TGF-beta 1 and restores the lymphokine-activated killing activity.*** **J Immunol, 2004. 172(3): p. 1508-14.**
179. **Bonniaud, P., et al.,** ***TGF-beta and Smad3 signaling link inflammation to chronic fibrogenesis.*** **J Immunol, 2005. 175(8): p. 5390-5.**
180. **Bonniaud, P., et al.,** ***Smad3 null mice develop airspace enlargement and are resistant to TGF-beta-mediated pulmonary fibrosis.*** **J Immunol, 2004. 173(3): p. 2099-108.**
181. **Heeg, M.H., et al.,** ***The antifibrotic effects of relaxin in human renal fibroblasts are mediated in part by inhibition of the Smad2 pathway.*** **Kidney Int, 2005. 68(1): p. 96-109.**
182. **Adembri, C., L. Venturi, and D.E. Pellegrini-Giampietro,** ***Neuroprotective effects of propofol in acute cerebral injury.*** **CNS Drug Rev, 2007. 13(3): p. 333-51.**
183. **Beilin, B., et al.,** ***Low-dose ketamine affects immune responses in humans during the early postoperative period.*** **Br J Anaesth, 2007. 99(4): p. 522-7.**
184. **Shibakawa, Y.S., et al.,** ***Effects of ketamine and propofol on inflammatory responses of primary glial cell cultures stimulated with lipopolysaccharide.*** **Br J Anaesth, 2005. 95(6): p. 803-10.**
185. **Li, C.Y., et al.,** ***Ketamine inhibits nitric oxide synthase in lipopolysaccharide-treated rat alveolar macrophages.*** **Can J Anaesth, 1997. 44(9): p. 989-95.**
186. **Sinclair, R., et al.,** ***Inhibitory effects of amide local anaesthetics on stimulus-induced human leukocyte metabolic activation, LTB4 release and IL-1 secretion in vitro.*** **Acta Anaesthesiol Scand, 1993. 37(2): p. 159-65.**
187. **Lahav, M., et al.,** ***Lidocaine inhibits secretion of IL-8 and IL-1beta and stimulates secretion of IL-1 receptor antagonist by epithelial cells.*** **Clin Exp Immunol, 2002. 127(2): p. 226-33.**
188. **Taniguchi, T., et al.,** ***Effects of lidocaine administration on hemodynamics and cytokine responses to endotoxemia in rabbits.*** **Crit Care Med, 2000. 28(3): p. 755-9.**
189. **Crawford, F.C., et al.,** ***Cocaine induced inflammatory response in human neuronal progenitor cells.*** **J Neurochem, 2006. 97(3): p. 662-74.**
190. **Pellegrino, T.C., K.L. Dunn, and B.M. Bayer,** ***Mechanisms of cocaine-induced decreases in immune cell function.*** **Int Immunopharmacol, 2001. 1(4): p. 665-75.**
191. **Irwin, M.R., et al.,** ***Cocaine dependence and acute cocaine induce decreases of monocyte proinflammatory cytokine expression across the diurnal period: autonomic mechanisms.*** **J Pharmacol Exp Ther, 2007. 320(2): p. 507-15.**

192. Wang, J., et al., *Morphine Induces Defects in Early Response of Alveolar Macrophages to Streptococcus pneumoniae by Modulating TLR9-NF-{kappa}B Signaling.* J Immunol, 2008. 180(5): p. 3594-600.
193. Wang, J., et al., *Morphine impairs host innate immune response and increases susceptibility to Streptococcus pneumoniae lung infection.* J Immunol, 2005. 174(1): p. 426-34.
194. Charleston, C., et al., *Morphine sulfate attenuates hemorrhagic shock-induced hyperpermeability.* Anesth Analg, 2006. 103(1): p. 156-61, table of contents.
195. Ichiyama, T., et al., *Thiopental inhibits NF-kappaB activation in human glioma cells and experimental brain inflammation.* Brain Res, 2001. 911(1): p. 56-61.
196. Salman, H., et al., *Effect of sodium thiopentone anesthesia on the phagocytic activity of rat peritoneal macrophages.* Life Sci, 1998. 63(25): p. 2221-6.
197. Frohlich, D., et al., *Thiopentone and propofol, but not methohexitone nor midazolam, inhibit neutrophil oxidative responses to the bacterial peptide FMLP.* Eur J Anaesthesiol, 1996. 13(6): p. 582-8.

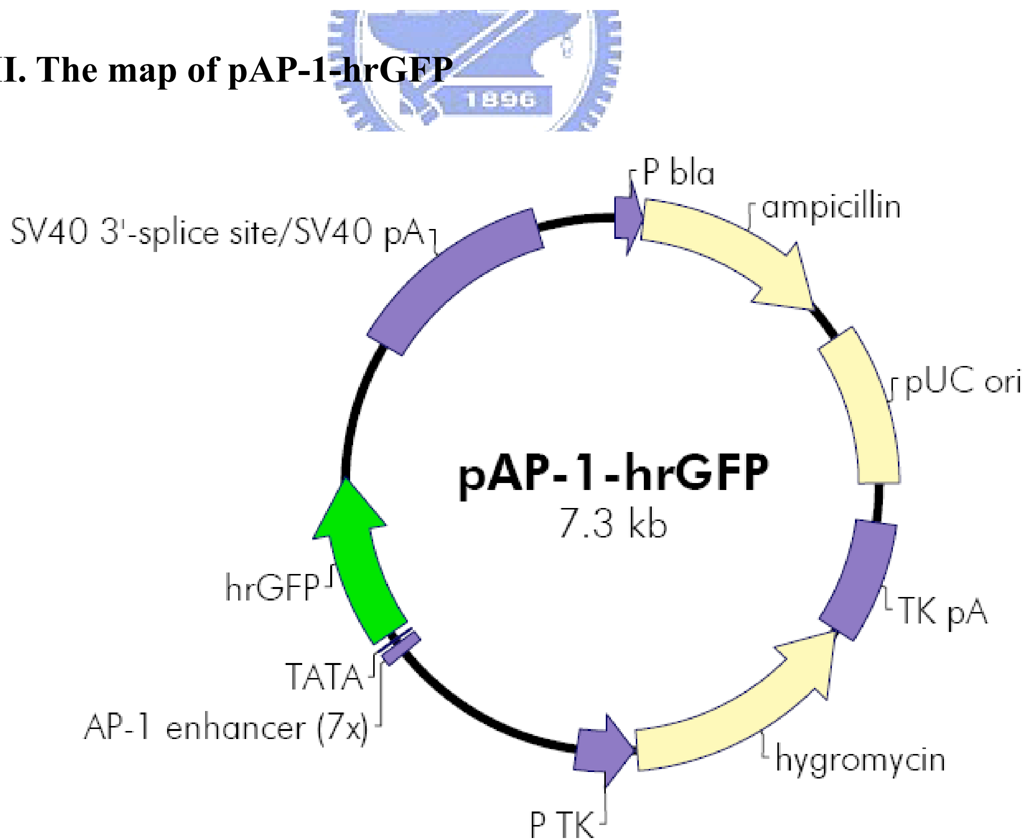
# Appendix

## I. The map of pNF-κB-hrGFP




## II. The map of pAP-1-hrGFP

1896



## III. Publishing

1. Yang FL, **Li CH**, Hsu BG, Tsai NM, Lin SZ, Harn HJ, Chen HI, Liao KW, Lee RP. Pentobarbital reducing TNF-alpha release and the tissues damage in experimental endotoxemia model. *Shock. 2007 Sep;28(3):309-16.* (Equal contributed to Yang FL)

2.Shih-Han Kao, Ching-Yi Lin, Cheng-Yu Chen, **Chi-Han Li**, Chueh-Jen Tsai, Yuan-Ting Hsieh, Shang-Chih Yang, Chang-Jer Wu, Wei-Sheng Chung, Ru-Ping Lee, Kuang-Wen Liao. The Enhancement of Anti-tumor Immunity by a SARS Fragment Fusion to a Low-immunogenic Tumor-derived Peptide. *J Gene Med (Submitted)*

3. **Chi Han Li**; Fwu Lin Yang; Ching Yi Lin; Bang Gee Hsu; Tseng Feng Jen; Yu Cheng Chen; Ru Ping Lee; Kuang Wen Liao. The Treatment of Propofol Induced the TGF-β1 Expression in Trauma Patients Sera: Propofol-Induced TGF-β1 in Human Endothelial Cells Suppress Endocytosis Activities of Monocytes in vitro. *Manuscript in preparation.* (Equal contributed to Yang FL)

**SHOCK,** Vol. 28, No. 3, pp. 309–316, 2007

# THE REDUCTION OF TUMOR NECROSIS FACTOR-$\alpha$ RELEASE AND TISSUE DAMAGE BY PENTOBARBITAL IN THE EXPERIMENTAL ENDOTOXEMIA MODEL

**Fwu Lin Yang,*†‡ Chi Han Li,§ Bang Gee Hsu,‡‖ Nu-Man Tsai,¶ Shinn Zong Lin,*† Horng Jyh Harn,*† Hsing I. Chen,* Kuang Wen Liao,§ and Ru Ping Lee*****

**Institute of Medical Sciences, Tzu Chi University; †Neural Medicine Science Center and ‡Division of Surgical Critical Care Unit, Tzu Chi Hospital, Hualien; §Department of Biological Science and Technology, National Chiao Tung University, Hsin-Chu; ‖Department of Nephrology, Tzu Chi Medicine Center Hospital, Hualien; ¶School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung; and **Department of Nursing, Tzu Chi University, Hualien, Taiwan, Republic of China*

Received 17 Jul 2006; first review completed 9 Oct 2006; accepted in final form 18 Dec 2006

ABSTRACT—Sepsis is the leading cause of death for intensive care patients. Lipopolysaccharide (LPS) administration to animals under anesthesia is a strategy for the study of uncontrolled release of proinflammatory cytokines. Anesthetics have been indicated that they can specially affect immune responses, such as the inflammatory response. Pentobarbital is an anesthetic used mainly in animal studies. Thus, the effect of pentobarbital on tumor necrosis factor-$\alpha$ (TNF-$\alpha$) release was determined. The results revealed that pentobarbital suppressed the expression of TNF-$\alpha$ mRNA and its proteins, which may result from the decrease in the activities of nuclear factor-κB and activator protein 1 and the reduction of the expression of p38 mitogen-activated protein kinase by pentobarbital. After the inhibitory activity of the pentobarbital for TNF-$\alpha$ release was proven *in vivo*, the cytotoxic effects of LPS were examined *in vivo* with or without pentobarbital treatments. *In vivo* results indicated that plasma levels of alanine aminotransferase, aspartate aminotransferase, lactic dehydrogenase, creatine kinase, serum urea nitrogen, and amylase decreased dramatically in the anesthetic group with pentobarbital administration. Finally, the effect of pentobarbital on TNF-$\alpha$–related cell death was monitored *in vitro*, and the results indicated that pentobarbital could directly enhance the viabilities of cells under the treatment of TNF-$\alpha$ and protected cells from apoptosis induced by deferoxamine mesylate–induced hypoxia. These results suggest that pentobarbital significantly influences the LPS-induced inflammatory responses and protects cells from death directly and indirectly induced by TNF-$\alpha$. The information provides a perspective to re-evaluate the results of the experiments in which animals were anesthetized with pentobarbital. The anti-inflammatory effects of the drugs may have been caused by the synergistic effect of pentobarbital.

KEYWORDS—Pentobarbital, LPS, conscious rats, organ injury, TNF-$\alpha$

## INTRODUCTION

Sepsis is the leading cause of death in intensive care patients and it can cause persistent and uncontrolled release of proinflammatory cytokines (1, 2). This severe immune response induces multiple organ failure. Lipopolysaccharide (LPS) administration to animals under anesthesia is a strategy for inducing an inflammatory response (1). However, the anesthesia model has its drawbacks. First, the exposure of laboratory animals to the anesthetic agents might change their immune function (3, 4), including the production of cytokines (5–10) and the reduction in the activity of natural killer cells (10–12). Second, hemodynamic changes after anesthesia enhance coagulation (10, 13, 14). Because most animal studies were performed under anesthesia, their conditions are different from the clinical cases in which patients are in a conscious state. Therefore, the results obtained from anesthetized animals need to be re-examined. It has been demonstrated that such anesthetics may influence the immune response (3, 4, 15–17), but pentobarbital has not been discussed yet, which is an anesthetic used mainly in animal studies. In this present report, we compared the results of endotoxemia between pentobarbital-anesthetized and conscious animals. Surprisingly, the results suggest that pentobarbital not only reduces systemic tumor necrosis factor-$\alpha$ (TNF-$\alpha$) release, but also decreases the degree of tissue damage under LPS administration. These results indicate that the medical effects of certain drugs, which were performed on pentobarbital-anesthetized animals, might have resulted from the synergistic effect of pentobarbital. This study provides a view to probe into the medical effects of anesthetics besides their anesthetic effects.

## MATERIALS AND METHODS

### *Plasmid*

The phosphorylated nuclear factor-κB (pNF-κB)/human recombinant green fluorescent protein (hrGFP) and phosphorylated activator protein 1 (pAP-1)/hrGFP plasmids containing the NF-κB and AP-1 transcription binding site, respectively, followed by a hrGFP reporter gene, were purchased from Stratagene, USA. The higher the activity of transcription factors in a cell is, the higher the expression of hrGFP is.

### *Cell lines*

The HEK 293 cell line and P338D1 cell line were obtained from the Biosource Collection and Research Center (Food Industry Research and Development Institute, Taiwan, China) and cultured with Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum.

Address reprint requests to Ru Ping Lee, PhD, Laboratory of Physiological Nursing Research, Department of Nursing, Tzu Chi University, No. 701, Section 3, Chung Yang Rd, 97004 Hualien, Taiwan. E-mail: fish@mail.tcu.edu.tw.

Supported in part by the National Science Council (grant no. 93-2314-B-320-010) and Mike Biological Technologies (Hualien, Taiwan) Ins. for assistance of equipment.

Dr. Fwu Lin Yang and Chi Han Li contributed equally to this study. Dr. Kuang Wen Liao and Dr. Ru Ping Lee contributed equally to this study.

DOI: 10.1097/shk.0b013e31803dd04d

Copyright © 2007 by the Shock Society



Copyright © 2007 by the Shock Society. Unauthorized reproduction of this article is prohibited.

### Mice

Animals were purchased from the National Laboratory Animal Center (Taipei, Taiwan, China). C57BL/6JNarl mice were 6-week female mice. Wistar-Kyoto rats were 16-week male rats. The study was approved by our Institutional Animal Care and Use Committee.

### Pentobarbital

It was purchased from MTC Incorporation (Cambridge, Ontario, Canada), whose trade name is Somnotol.

### Spleen preparation and culture

C57BL/6JNarl mice were killed by carbon dioxide asphyxiation. The spleen was taken, minced with Dulbecco modified Eagle medium, and filtered with a mesh. Soup was centrifuged at 1,200 rpm at 12°C for 5 min. The supernatant was removed and 5 mL ACK buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM Na2–EDTA) was added. After 5-min incubation, the mixture was centrifuged (at 1,200 rpm at 12°C for 5 min) and washed twice with phosphate-buffered saline (PBS) to remove ACK buffer. Five milliliters of RPMI-1640 was added to resuspend the cells. A total of $2 \times 10^6$ cells were then incubated in four conditions, as follows: (1) with RPMI-1640 (containing 10% fetal bovine serum and 1% phosphatidylserine) (the control group), (2) with RPMI-1640 and 14 $\mu$g/mL of LPS (Sigma Chemical Co, St Louis, Mo) (the LPS group), (3) with RPMI-1640 and pentobarbital (the pentobarbital group), (4) with RPMI-1640, LPS, and pentobarbital (the LPS + pentobarbital group). Each mixture was collected after 48 h and stored at −80°C.

### Cytokine measurement

One hundred microliters of capture antibody (0.8 $\mu$g/mL) was added into each well of an enzyme-linked immunosorbent assay (ELISA) plate (Costar, USA), and the plate was incubated overnight. Wash buffer (0.05% Tween 20 in PBS, pH 7.2~7.4) was applied three times. Three hundred microliters of block buffer was added, and the plate was incubated for 1 h at room temperature. Wash buffer was applied three times. One hundred microliters of samples were added into each well and incubated at room temperature for 2 h. The plate was then washed with wash buffer three times. One hundred microliters of detection antibody (150 ng/mL) was added into each well. Samples were incubated at room temperature for 2 h and then washed three times with wash buffer. One hundred microliters of tetramethylbenzidine substrate (Clinical, USA) was added into each well, and the plate was incubated at room temperature for 20 min. To stop the reaction, 50 $\mu$L of stop solution (1N HCl) was added and the quantification was determined by the ELISA reader (Sunrise, Switzerland) at the absorbance wavelength of 450 nm.

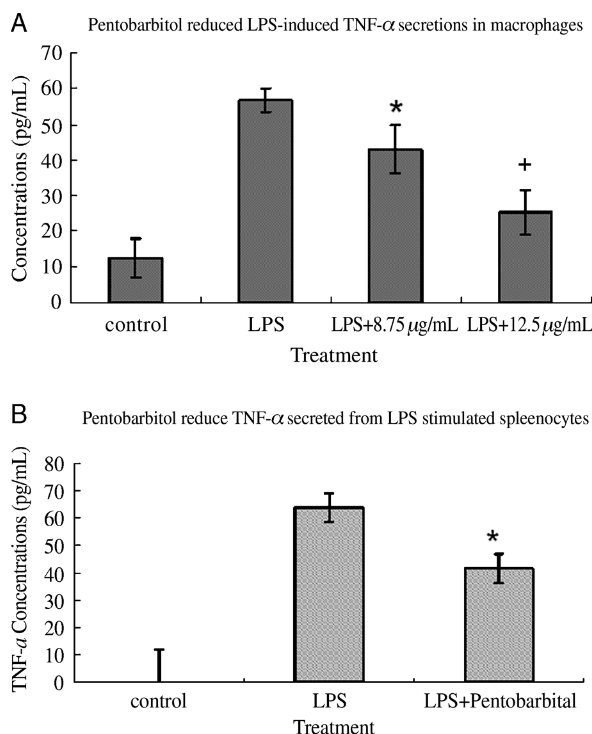



Fig. 1. **The reduction of LPS-induced TNF-$\alpha$ from immune cells by pentobarbital.** A, P338D1 cells were treated with LPS (the LPS group). In the same condition, P338D1 cells were coincubated with pentobarbital (the LPS + 8.75 $\mu$g/mL and the LPS + 12.5 $\mu$g/mL groups). The growth medium of untreated P338D1 cells served as the control group. $^*P < 0.05$ indicates a significant difference between the LPS plus 8.75 $\mu$g/mL group and the LPS group. $^\dagger P < 0.01$ indicates a significant difference between the LPS plus 12.5 $\mu$g/mL group the LPS group. B, Splenocytes were treated with LPS (the LPS group). In the same condition, splenocytes were coincubated with pentobarbital (the LPS + 12.5 $\mu$g/mL groups). The growth medium of untreated splenocytes served as the control group. $^*P < 0.05$ indicates a significant difference between the LPS plus Pento group and the LPS group.

### Tumor necrosis factor-$\alpha$ mRNA expression assay

P338D1 cells ($10^7$) were cultured and treated with LPS, and coincubated without or with pentobarbital (final concentration, 12.5 $\mu$g/mL) for 6 h. The treated cells were harvested, and total RNAs were extracted by phenol/chloroform method as described (18). Complementary DNAs were reverse-transcribed from total RNA by SuperScript First-Strand Synthesis SuperMix kit (Invitrogen, USA), and the TNF-$\alpha$ complementary DNA was amplified by polymerase chain reaction (PCR) with the primer pairs (mouse TNF-alpha 5′: ATgAgCACAgAAAgCATgATCCgCgA; mouse TNF-alpha 3′: TCACAgAgCAATgACTCCAAAgTAgAC). The products of reverse transcription–PCR were analyzed by agarose electrophoresis, and the results were photographed.

### Transcription factor activity assay

According to the manufacturer's instruction, pNF-κB/hrGFP and pAP-1/hrGFP were transfected by Lipofectamine 2000 (Invitrogen) into Balb/3T3 cells seeded in the 6-well plate, respectively. Twenty-four hours later, cells were passaged by versene (0.2 g EDTA–4 Na/L in PBS) and seeded into a 24-well plate. The transfectants were treated with LPS (14 $\mu$g/mL) and coincubated without or with pentobarbital (12.5 $\mu$g/mL) for 16 h, respectively. The transfectants were harvested and analyzed by flow cytometer. Specific FL-1 fluorescent intensities, representing the activities of the transcriptional factors, were calculated. In each plate, control plasmid phosphorylated cytomegalovirus/hrGFP was transfected into the target cells to measure the transfection efficiency, which was approximately 60%.

### p38 Mitogen-activated protein kinase expression assay

As previously described, P338D1 cell lines were treated with LPS and coincubated with or without pentobarbital. The cells were harvested and mixed with the sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 5% $\beta$-mercaptoethnol, 10% glycerol, 0.01% bromophenol blue). After the samples boiled, sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed, and the products were transferred to a polyvinylidene fluoride membrane. The samples were probed with rabbit antimouse p38 polyclonal antibody (Santa Cruz, Europe) or mouse anti–$\beta$-actin monoclonal antibody (Biovision, USA). After washes, the membranes were reprobed with goat antirabbit immunoglobulin G, horseradish peroxidase conjugated (MP Biomedicals, USA) or rabbit anti-mouse immunoglobulins/horseradish peroxidase polyclonal antibody (DakoCytomation, Ely, Denmark). Finally, the blots were washed, developed, and visualized by enhanced chemiluminescence detection according to the manufacturer's instructions (Pierce, USA).

### Tumor necrosis factor-$\alpha$ cytotoxicity assay

Target cells were seeded into the 96-well plate. Twelve hours later, pentobarbital at different concentrations was applied or not applied, with or without 2,500 pg/mL TNF-$\alpha$ (the control group, the TNF-$\alpha$ group, the pentobarbital group, the TNF-$\alpha$ + pentobarbital group). The supernatant was removed after 16 h. One hundred microliters of fresh medium was added with 20 $\mu$L MTS (CellTiter 96 Aqueous One Solution cell proliferation assay, Promega, USA). Cells were cultured in a carbon dioxide incubator at 37°C for 4 h. The absorbance was detected at 492 nm wavelength by an ELISA reader (Sunrise). Relative cell survival (%) = Sample absorbance/Control absorbance × 100%. The control group was cultured in normal growth medium, and its relative cell survival is equal to 100%.

### Cell apoptosis assay

A total of 293 cells ($2 \times 10^6$) in 3 mL growth medium were treated with 10 mM deferoxamine mesylate (DFO; Sigma) for 16 h. The cells were harvested and suspended into 100 $\mu$L staining solutions (20 $\mu$L Annexin V–fluorescein isothiocyanate (FITC) labeling reagent and 20 $\mu$L propidium iodide (PI) in 1 mL binding buffer). The mixture was incubated for 15 min and analyzed by flow cytometer. FL-1 represents Annexin V–FITC staining (apoptosis), and FL-3 represents PI staining (dead cells). The relative apoptosis index = the fluorescent intensity of samples/the fluorescent average of the negative control × 100%.

### Preparation of animals

Sixteen-week-old male Wistar-Kyoto rats were purchased from the National Animal Center and housed in the university animal rooms under a 12-h light/dark cycle. Food and water were provided *ad libitum*. Animals were anesthetized with ether inhalation for about 10 min. During the period of anesthesia, a femoral artery

Copyright © 2007 by the Shock Society. Unauthorized reproduction of this article is prohibited.

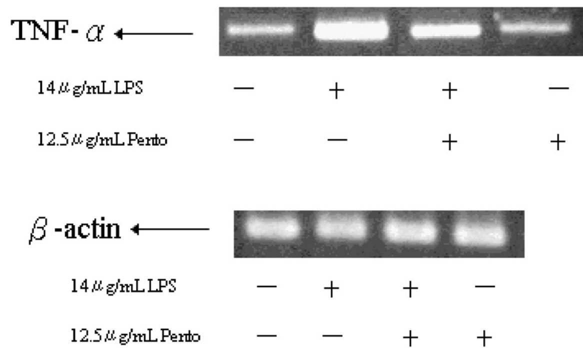



FIG. 2. **The effect of pentobarbital on the expression of TNF-α mRNA.** P338D1 cells were treated with or without LPS and were coincubated with or without pentobarbital. The mRNA levels of TNF-α were shown by reverse transcription–PCR. β-Actin mRNA levels served as an internal control to normalize the sample loading.

was cannulated and connected to a pressure transducer to record the arterial pressure and the heart rate on a polygraph recorder (PowerLab, AD Instruments Co, Mountain View, Calif). A femoral vein was catheterized for the i.v. administration of drugs. The operation procedure was completed within 15 min, and the section wound was smaller than 0.5 $cm^2$. After the operation, the animal was placed on a metabolic cage (17). The rat awoke soon after the operation. During the experiment, the body temperature was measured rectally by a digital thermometer (HR 1300 thermometer, Yokogawa, Japan) for every minute.

### Lipopolysaccharide shock

Lipopolysaccharide shock was induced by slow i.v. infusion of 10 mg/kg of LPS (Sigma) in 20 min. The infusion started 24 h after the operation. The drug was dissolved in sterile physiological saline solution immediately before use. All invasive procedures were performed under aseptic conditions. After LPS administration, animals were observed for 48 h (19).

### Experimental design

Animals were divided into the NS, LPS, and Pento groups (n = 8). The NS group received a 1-mL injection of isotonic sodium chloride solution. The LPS group received 10 mg/kg of LPS (diluted in 1 mL) infusion. The Pento group received continuous infusion of pentobarbital at 10 mg/kg per h after LPS. The blood samples were collected before isotonic sodium chloride solution and LPS and at 0.5, 1, 3, 6, 9, 12, 18, 24, 36, and 48 h after the administration of saline or the drug.

### Blood sample analyses

Blood samples for the measurement of white blood cells, lymphocytes, and platelets (Micro OT, Roche Co, Mannheim, Germany) were taken and immediately centrifuged at 3,000*g* for 10 min. The supernatant was collected for nitrate/nitrite measurement with high-performance liquid chromatography (ENO-20, AD Instruments Co, Mountain View, Calif). Enzyme-linked immunosorbent assay was performed for TNF-α measurement.

### Blood biochemical analyses

The plasma samples were diluted by 1:100 with distilled water before measurements. Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactic dehydrogenase (LDH), creatine kinase (CK), serum urea nitrogen (SUN), and amylase were measured with an autoanalyzer (Vitros 750, Johnson-Johnson Co, Rochester, NY) for evaluating various organ functions. The ALT and AST are for the liver function, the LDH and CK are for the heart and other possible organ (such as muscle) functions, the SUN is for the renal function, and the amylase is for the pancreatic function.

### Statistical analysis

Data of *in vivo* experiments are expressed as mean ± SE. Multiple analysis of variance and Scheffé test were used to compare the difference between and among groups (n = 8 in each group). $P < 0.05$ was considered to be significant. All *in vitro* data were compared by Student *t* test, and $P < 0.05$ was considered to be significant. The data of the transcriptional factor activity assay were obtained from three independent experiments and duplicated in each group (n = 6). The data of the *in vitro* cytokine assay were obtained from three independent experiments and

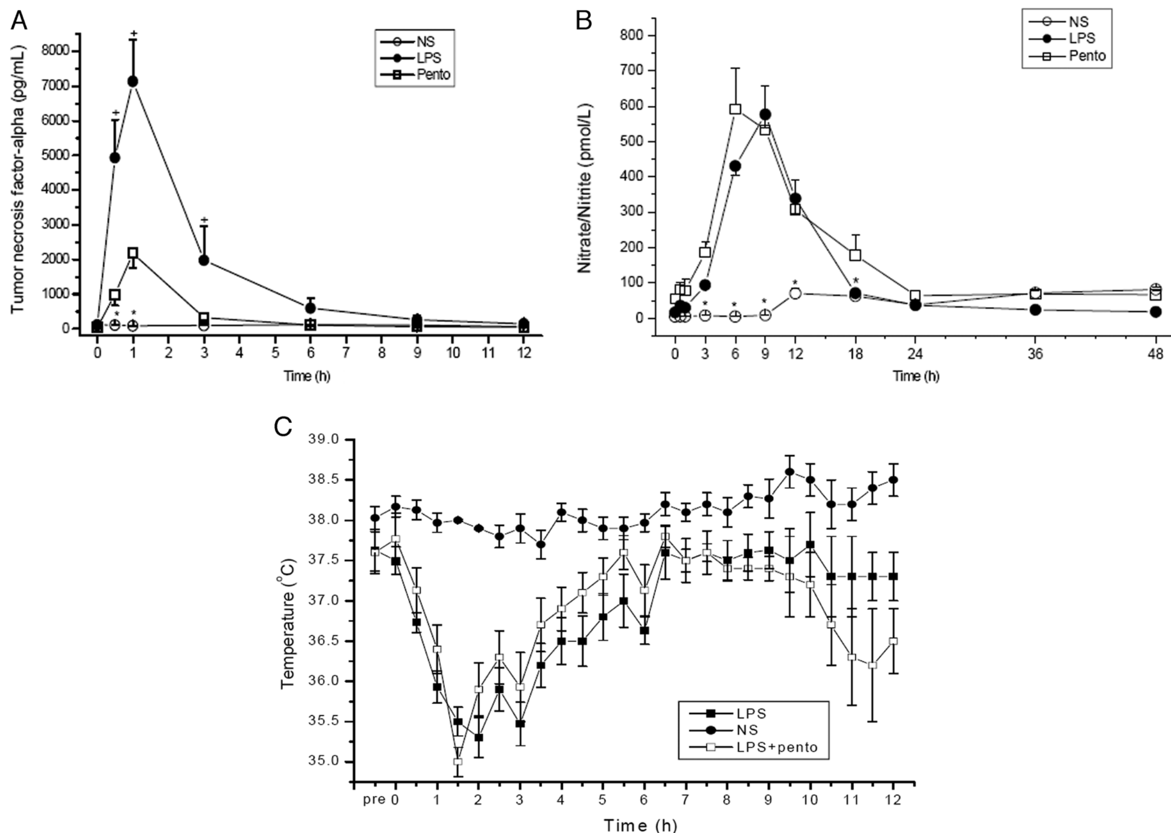



FIG. 3. **The effect of pentobarbital on the production of TNF-α *in vivo*.** After LPS infusion, the levels of TNF-α (A) and NO (B) in sera of the mice were measured. The untreated mice served as the negative control (the NS group). $^*P < 0.05$ indicates a significant difference between the Pento group and the NS group. $^{\dagger}P < 0.05$ indicates a significant difference between the Pento group and the LPS group. The body temperature (C) of the rats was measured rectally by a digital thermometer. The untreated mice served as the negative control (the NS group).

Copyright © 2007 by the Shock Society. Unauthorized reproduction of this article is prohibited.

duplicated in each group (n = 6). The data of TNF-$\alpha$ cytotoxic assay were obtained from four independent experiments (n = 4 each group). The data of the apoptosis assay were obtained from three independent experiments and duplicated in each group (n = 6).

## RESULTS

### *Pentobarbital lowers the TNF-$\alpha$ concentration in serum in the presence of LPS* in vitro *and* in vivo

Pentobarbital was assumed to be able to modify the inflammatory effects of LPS in endotoxemia. The results showed that pentobarbital significantly lowered TNF-$\alpha$ release from P338D1 cells (mouse macrophage cells) under LPS stimulation (Fig. 1A). Moreover, pentobarbital also decreased TNF-$\alpha$ expressions of splenocytes in the presence of LPS (Fig. 1B). In addition, the expression of TNF-$\alpha$ mRNA was reduced after pentobarbital treatment in the presence of LPS (Fig. 2). These results indicate that pentobarbital has anti-inflammatory ability *in vitro*.

To study the effect of pentobarbital *in vivo*, an animal model of conscious rats with LPS treatment was established and used. After LPS treatment with or without pentobarbital administration, blood samples in each group were collected to measure the levels of inflammatory substances. The LPS infusion caused a dramatic increase in TNF-$\alpha$ in sera of conscious rats *in vivo*. However, pentobarbital treatment reduced the serum concentration of TNF-$\alpha$ in the presence of LPS (Fig. 3A). The difference was observed within 12 h after LPS treatment, but no difference was detectable after 12 h. The other indicator of LPS-induced inflammatory response, nitric oxide (NO), increased in the conscious rat model after LPS treatment. In contrast, pentobarbital did not affect the expression of NO in sera (Fig. 3B). *In vitro* and *in vivo* results indicate that pentobarbital has the ability to reduce the TNF-$\alpha$ release from immune cells. Because the effects could be mediated through the reduction of the body temperature, it was measured after different treatments. The body temperature of the animals in the LPS group (n = 6) and the LPS plus Pento group (n = 6) decreased (Fig. 3C). The decrease in the body temperature should be caused by the LPS administration. The data of the two groups were not significantly different. Within 9 h after the LPS administration, a conspicuous inhibitory effect of pentobarbital on TNF-$\alpha$ release was observed. However, the body temperature was not significantly different at this stage between the two groups. Therefore, a decrease in TNF-$\alpha$ release by pentobarbital should not be caused by the change in the body temperature.

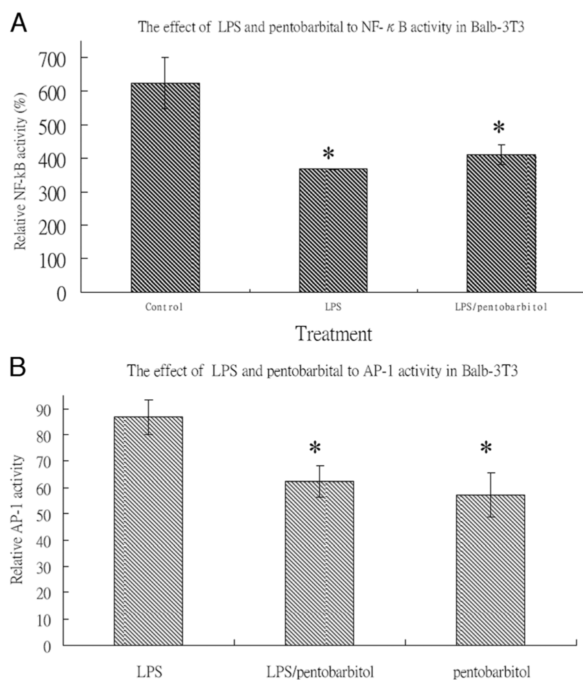



FIG. 4. **The effect of pentobarbital on the activities of NF-κB or AP-1 after LPS treatment.** The pNF-κB/hrGFP (A) or pAP-1/hrGFP (B) plasmids were transfected into Balb-3T3 cells. The untreated group was the control group. Transfectants were treated with LPS and coincubated with (the LPS/pentobarbital group) or without (the LPS group) 12.5 $\mu$g/mL pentobarbital. Transfectants were also incubated with growth medium containing 12.5 $\mu$g/mL pentobarbital without LPS (the pentobarbital group). $*P < 0.05$ indicates a significant difference between the LPS plus Pento or the Pento group and the LPS group.

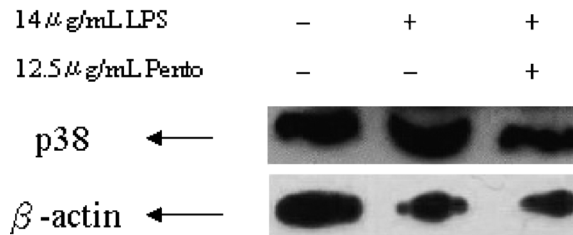



FIG. 5. **The p38 MAPK expression.** P338D1 cells with or without LPS treatment and coincubated with or without pentobarbital. After treatment, the cells were probed to determine the expressions of p38 MAPK protein (A) and $\beta$-actin protein (B).

### *Pentobarbital suppresses the activities of NF-κB and AP-1 in the presence of LPS*

Previous literatures have reported that LPS activates NF-κB and AP-1 pathways to enhance the TNF-$\alpha$ expression and release. Therefore, Balb/3T3 cells were transfected with plasmids containing the enhanced GFP reporter gene under minipromoter control (the minipromoters were composed of several copies of NF-κB or AP-1 transcriptional factor binding sites) to determine the effects of pentobarbital on these signaling pathways. In our experiments, LPS increased the activities of NF-κB in cells (Fig. 4A) and slightly enhanced the activities of AP-1 (Fig. 4B). However, pentobarbital reduced the activities of NF-κB and AP-1 in the presence of LPS (Fig. 4). The result also showed that pentobarbital alone decreased the activities of NF-κB and AP-1 in cells at a concentration of 12.5 $\mu$g/mL. In addition, because LPS-induced TNF-$\alpha$ release involves p38 mitogen-activated protein kinase (MAPK) signaling pathway, we tested whether pentobarbital could interfere with it. Our result indicated that pentobarbital could reduce the amount of p38 MAPK in the presence of LPS (Fig. 5).

### *Pentobarbital reduces tissue damages*

Tumor necrosis factor-$\alpha$ is a potent cytotoxic cytokine that results in tissue damages. As indicated in the previous results, pentobarbital decreases the TNF-$\alpha$ expression *in vitro* and *in vivo*. Whether pentobarbital could protect tissues from LPS-induced tissue damages was analyzed in the animal model of conscious rats *in vivo*. The results showed that LPS injection caused dramatic increases in ALT, AST, LDH, CK,

Copyright © 2007 by the Shock Society. Unauthorized reproduction of this article is prohibited.

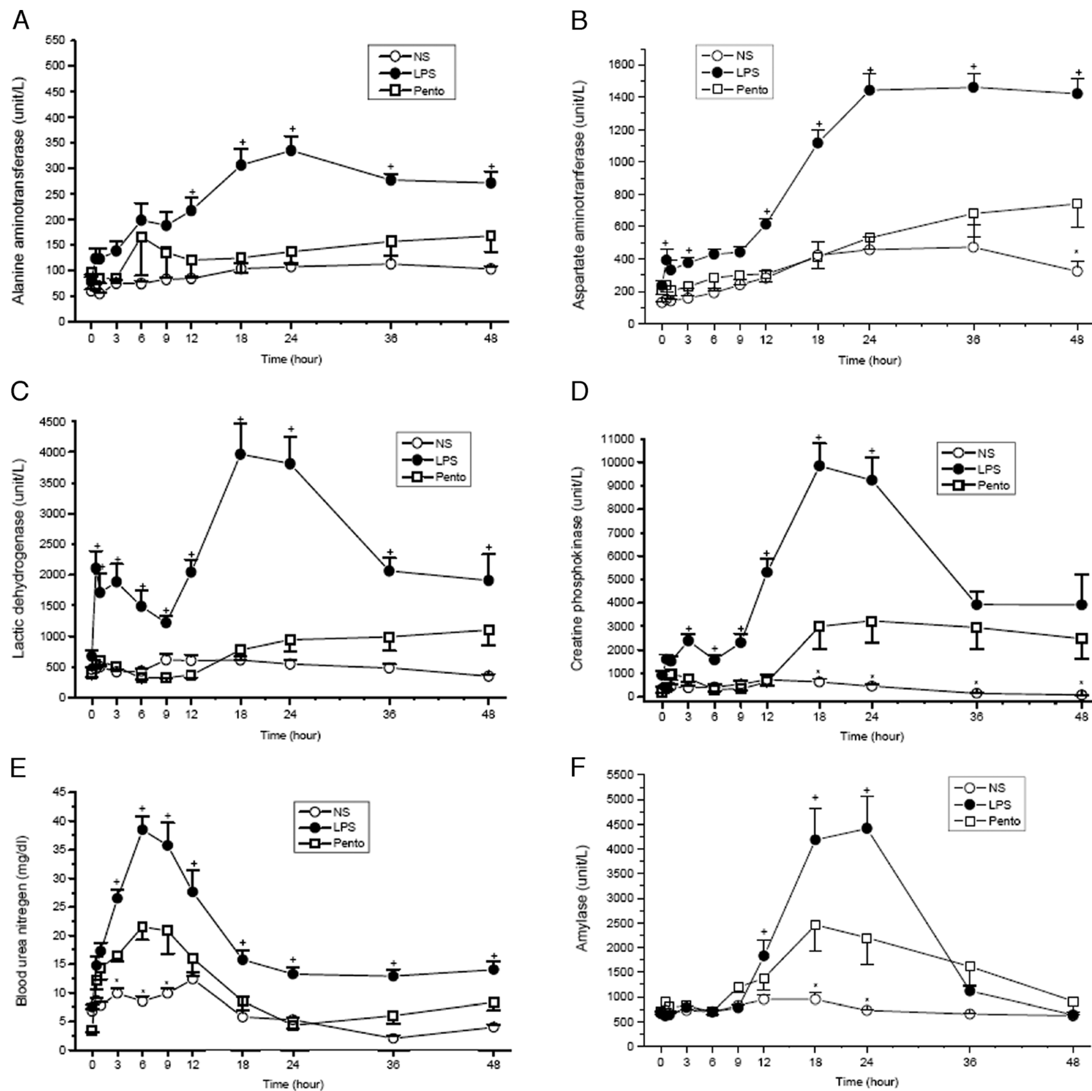



FIG. 6. **Improvement of blood biochemical factors reflecting multiple organ functions under pentobarbital anesthesia.** Plasma levels of ALT (A), AST (B), LDH (C), CK (D), BUN (E), and amylase (F) levels of rats in the Pento group, the LPS group, and the negative control group were measured. *$P < 0.05$ indicates a significant difference between the concentration of the Pento group and that of the NS group. $^{\dagger}P < 0.05$ indicates a significant difference between the concentration of the Pento group and that of the LPS group.

BUN, and amylase in sera (Fig. 6) when compared with those of the normal control group, indicating that LPS injection deteriorated the hepatic (Fig. 6, A and B), the heart or skeletal muscle (Fig. 6, C and D), the renal (Fig. 6E), and the pancreatic functions (Fig. 6F), as reflected by the changes of blood biochemical substances. However, pentobarbital infusion suppressed the increases in all bioindicators in the presence of LPS (Fig. 6), indicating that pentobarbital could protect tissue damages from LPS-induced cytotoxic effects.

### *Pentobarbital protects cells from apoptosis*

Besides the reduction of TNF-$\alpha$ release, the protective effects of pentobarbital on the survival of TNF-$\alpha$ target cells also needed to be determined. The HEK 293 human kidney cells are susceptive to TNF-$\alpha$. Our results showed that the survival rates of HEK 293 cells were less than 40% under 2.5 ng/mL of TNF-$\alpha$ treatment for 16 h (Fig. 7). Different concentrations of pentobarbital all increased the viabilities of cells in the presence of TNF-$\alpha$, whereas pentobarbital alone did not significantly affect the survival rates of HEK 293 cells (Fig. 7).

Tumor necrosis factor-$\alpha$ can increase the expression of adhesion molecules on the surface of immune cells and endothelial cells to cause the stagnant blood capillary effect and result in tissue hypoxia. When such hypoxia occurs, cells will undergo apoptosis. Deferoxamine mesylate has been shown to be able to induce apoptosis by the same mechanism as hypoxia, and therefore it was used to cause cell apoptosis in our *in vitro* experiment. We found that DFO alone increased the proportions of apoptosis of HEK 293 cells (Fig. 8). Nevertheless, pentobarbital protected the cells from apoptosis in DFO-induced hypoxia (Fig. 8).

## DISCUSSION

In this study, the results suggest that pentobarbital infusion attenuates the multiple organ dysfunctions induced by LPS (Fig. 6). First, pentobarbital reduces the expression of TNF-$\alpha$ in the presence of LPS (Figs. 1 and 2). Gao et al. (20, 21) have

Copyright © 2007 by the Shock Society. Unauthorized reproduction of this article is prohibited.

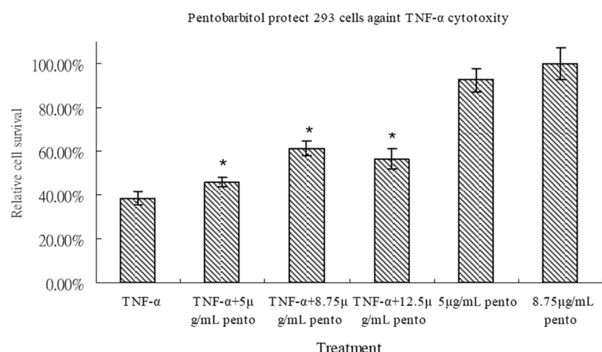



Fig. 7. **The protection of HEK 293 cells against TNF-$\alpha$ cytotoxicity by pentobarbital.** HEK 293 cells in the normal growth medium served as the control group. HEK 293 cells were treated with or without TNF-$\alpha$ and coincubated without or with different dosages of pentobarbital. *$P < 0.05$ indicates that the relative survival significantly differed from that of the TNF-$\alpha$–treated group.

demonstrated that LPS injection produces a large increase in the plasma TNF-$\alpha$. In general, TNF-$\alpha$ is considered to be a principal mediator of endotoxemia and organ failure (22). The TNF-$\alpha$ has been implicated as an important mediator of the lethal effect of endotoxin, which can cause hepatic failure, and so on. Several literatures have shown that inhibitors for reducing the activity or the expression of TNF-$\alpha$ significantly decrease the endotoxin-induced damages (22–24). In addition, numerous studies have shown that the function of blood capillary is impaired and adhesion molecules aggregate to vessel walls under the septic progress (1, 8, 19, 25). The TNF-$\alpha$ strongly induces the expression of intercellular adhesion molecule, vascular cell adhesion molecule, and P-selectin in endothelial cells (26–28), which causes blood cells to adhere to endothelial cells (29). The amount of TNF-$\alpha$ in serum can be associated with the degree of tissue damage because of the stagnant blood capillary. In fact, several anesthetic agents, including pentobarbital, have been demonstrated that they markedly suppress the TNF-$\alpha$–induced neutrophil-venule adhesion (30). For these reasons, the protective effect of pentobarbital might be caused by the suppression of the systemic release of TNF-$\alpha$.

In the second protective mechanism, pentobarbital directly protects tissue cells from the cytotoxic effect of TNF-$\alpha$, which is a well-known cytotoxic cytokine for certain tissue cells (Figs. 6 and 7). The LPS raised the levels of several biophysical indicators (ALT, AST, LDH, CK, BUN, and amylase) in sera (Fig. 6), which reflected the LPS-inducing damages of organ tissues. Pentobarbital increased the viability of the cells at the presentation of TNF-$\alpha$, demonstrating its role in cell protection (Fig. 7). Lipopolysaccharide stimulation can cause the stagnant blood capillary effect, resulting in hypoxia in tissues. When such hypoxia occurs, cells will undergo apoptosis. Deferoxamine mesylate can induce the same apoptosis as hypoxia and has been used to cause cell apoptosis *in vitro* (31, 32). Pentobarbital can decrease the percentage of cells undergoing apoptosis in the presence of DFO treatment (Fig. 8). A similar phenomenon has been reported that barbiturates have a protective effect against cerebral ischemia, and it has suggested that pentobarbital inhibits apoptosis to prevent ischemic neuronal death (33).

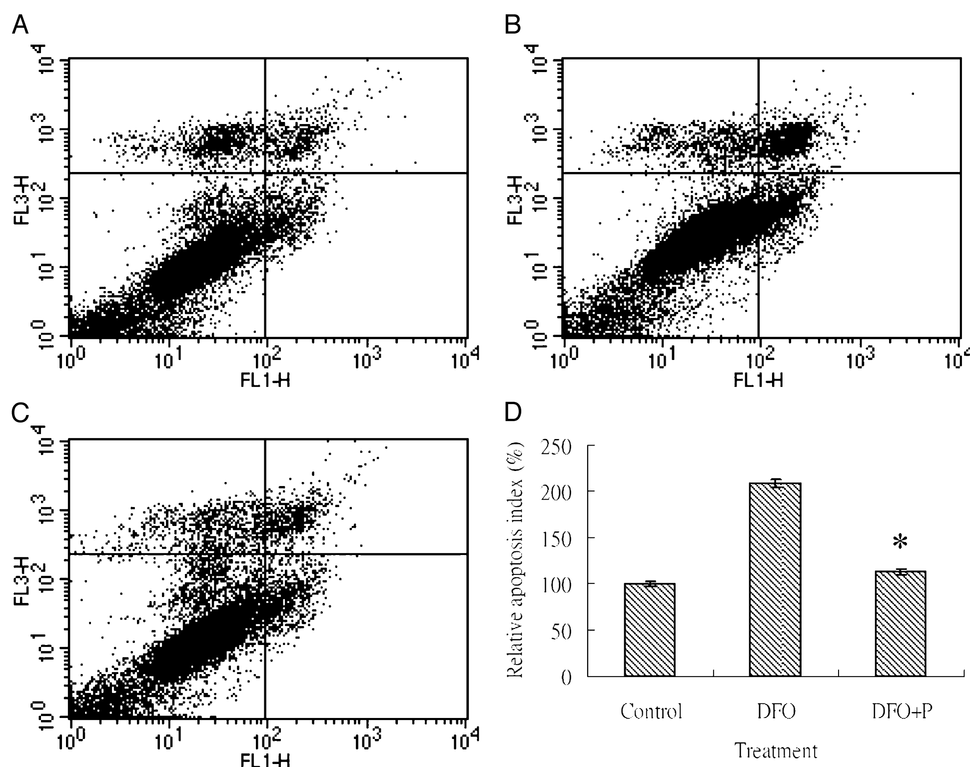



Fig. 8. **The protection of HEK 293 cells against apoptosis by pentobarbital under hypoxia.** A, The normal medium–cultured HEK 293 cells served as the control group. The HEK 293 cells were treated with 10 mM DFO and coincubated without (B) or with pentobarbital (C). The treated cells were probed with Annexin V–FITC (FL-1) and PI (FL-3), and analyzed by flow cytometer. D, The relative apoptosis index was calculated and shown. *$P < 0.05$ indicates a significant difference between the DFO plus P group and the DFO-treated group.

Copyright © 2007 by the Shock Society. Unauthorized reproduction of this article is prohibited.

Therefore, the information that pentobarbital should have the ability to protect tissue cells from the LPS directly or indirectly induced cytotoxicity of TNF-$\alpha$.

In mammals, toll-like receptor 4 on macrophages sends signals in the presence of LPS by associating with CD14 to activate its NF-$\kappa$B pathway (34). Besides NF-$\kappa$B, previous literatures have also reported that LPS-induced endotoxemia can cause an increase in the p38 MAPK expression, which is important for the LPS-induced TNF-$\alpha$ release (35, 36) and AP-1 activities in cells (37). In this study, the results reveal that pentobarbital suppresses the expression of p38 MAPK (Fig. 5) and the activities of NF-$\kappa$B and AP-1 (Fig. 4) in the presence of LPS. Changes in the intracellular signaling pathway should be responsible for the decrease in TNF-$\alpha$ mRNA (Fig. 2) and the reduction of TNF-$\alpha$ protein expression caused by pentobarbital (Fig. 1, A and B).

During endotoxemia, proinflammatory cytokines act both locally and systemically to aggravate the organ damage. Many investigations have shown that i.v. anesthetics have anti-inflammatory effects on endotoxemia both *in vitro* and *in vivo* (12, 38). According to the results in this study, pentobarbital not only has an anti-inflammatory activity, but also directly protects cells from apoptosis. Accordingly, pentobarbital may be beneficial in preventing organ dysfunction in endotoxemia or septicemia, but it should be used with caution regarding their potential immunomodulatory properties in critically ill patients.

## ACKNOWLEDGMENTS

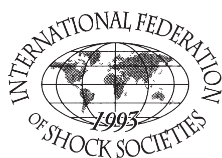
The authors thank Ke W. Yang and Yu K. Hung for technical assistance.

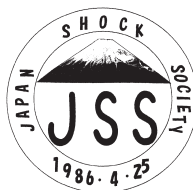
## REFERENCES


1. Piper RD, Cook DJ, Bone RC, Sibbald WJ: Introducing Critical Appraisal to studies of animal models investigating novel therapies in sepsis. *Crit Care Med* 24:2059–2070, 1996.
2. Vincent JL: New therapies in sepsis. *Chest* 112:330S–338S, 1997.
3. Kotani N, Hashimoto H, Sessler DI, Kikuchi A, Suzuki A, Takahashi S, Muraoka M, Matsuki A: Intraoperative modulation of alveolar macrophage function during isoflurane and propofol anesthesia. *Anesthesiology* 89: 1125–1132, 1998.
4. Kotani N, Hashimoto H, Sessler DI, Yasuda T, Ebina T, Muraoka M, Matsuki A: Expression of genes for proinflammatory cytokines in alveolar macrophages during propofol and isoflurane anesthesia. *Anesth Analg* 89:1250–1256, 1999.
5. Yokoyama M, Itano Y, Mizobuchi S, Nakatsuka H, Kaku R, Takashima T, Hirakawa M: The effects of epidural block on the distribution of lymphocyte subsets and natural-killer cell activity in patients with and without pain. *Anesth Analg* 92:463–469, 2001.
6. Livnat S, Felten SY, Carlson SL, Bellinger DL, Felten DL: Involvement of peripheral and central catecholamine systems in neural-immune interactions. *J Neuroimmunol* 10:5–30, 1985.
7. Landmann RM, Burgisser E, Wesp M, Buhler FR: Beta-adrenergic receptors are different in subpopulations of human circulating lymphocytes. *J Recept Res* 4:37–50, 1984.
8. Yoshimura T, Kurita C, Nagao T, Usami E, Nakao T, Watanabe S, Kobayashi J, Yamazaki F, Tanaka H, Inagaki N, et al.: Inhibition of tumor necrosis factor-alpha and interleukin-1–beta production by beta-adrenoceptor agonists from lipopolysaccharide-stimulated human peripheral blood mononuclear cells. *Pharmacology* 54:144–152, 1997.
9. Weiss M, Schneider EM, Tarnow J, Mettler S, Krone M, Teschemacher A, Lemoine H: Is inhibition of oxygen radical production of neutrophils by sympathomimetics mediated via beta-2 adrenoceptors? *J Pharmacol Exp Ther* 278:1105–1113, 1996.
10. Xu H, Aibiki M, Yokono S, Ogli K: Dose-dependent effects of propofol on renal sympathetic nerve activity, blood pressure and heart rate in urethane-anesthetized rabbits. *Eur J Pharmacol* 387:79–85, 2000.
11. Greisen J, Hokland M, Grofte T, Hansen PO, Jensen TS, Vilstrup H, Tonnesen E: Acute pain induces an instant increase in natural killer cell cytotoxicity in humans and this response is abolished by local anaesthesia. *Br J Anaesth* 83:235–240, 1999.
12. Fuentes JM, Talamini MA, Fulton WB, Hanly EJ, Aurora AR, De Maio A: General anesthesia delays the inflammatory response and increases survival for mice with endotoxic shock. *Clin Vaccine Immunol* 13:281–288, 2006.
13. Loeb AL, Godeny I, Longnecker DE: Anesthetics alter relative contributions of NO and EDHF in rat cremaster muscle microcirculation. *Am J Physiol* 273:H618–H627, 1997.
14. Nyhan DP, Chen BB, Fehr DM, Rock P, Murray PA: Anesthesia alters pulmonary vasoregulation by angiotensin II and captopril. *J Appl Physiol* 72:636–642, 1992.
15. Mikawa K, Akamatsu H, Nishina K, Shiga M, Maekawa N, Obara H, Niwa Y: Propofol inhibits human neutrophil functions. *Anesth Analg* 87:695–700, 1998.
16. Galley HF, Dubbels AM, Webster NR: The effect of midazolam and propofol on interleukin-8 from human polymorphonuclear leukocytes. *Anesth Analg* 86:1289–1293, 1998.
17. Chanimov M, Berman S, Weissgarten J, Averbukh Z, Cohen M, Grinshpun Y, Bahar M: Substances used for local and general anaesthesia in major surgery suppress proliferative responsiveness of normal rat peripheral blood mononuclear cells in culture. *Eur J Anaesthesiol* 17:248–255, 2000.
18. Chou WC, Liao KW, Lo YC, Jiang SY, Yeh MY, Roffler SR: Expression of chimeric monomer and dimer proteins on the plasma membrane of mammalian cells. *Biotechnol Bioeng* 65:160–169, 1999.
19. Lee RP, Wang D, Lin NT, Chen HI: Physiological and chemical indicators for early and late stages of sepsis in conscious rats. *J Biomed Sci* 9:613–621, 2002.
20. Gao J, Zeng B, Zhou L: The protective effects of early treatment with propofol on endotoxin-induced acute lung injury in rats. *Middle East J Anesthesiol* 17:379–401, 2003.
21. Gao J, Zeng BX, Zhou LJ, Yuan SY: Protective effects of early treatment with propofol on endotoxin-induced acute lung injury in rats. *Br J Anaesth* 92:277–279, 2004.
22. Joung YH, Lim EJ, Lee MY, Park JH, Ye SK, Park EU, Kim SY, Zhang Z, Lee KJ, Park DK, et al.: Hypoxia activates the cyclin D1 promoter via the Jak2/STAT5b pathway in breast cancer cells. *Exp Mol Med* 37:353–364, 2005.
23. Lee MY, Hee Joung Y, Joung Lim E, Park JH, Ye SK, Park T, Zhang Z, Ki Park D, Jeon Lee K, Yang YM: Phosphorylation and activation of STAT proteins by hypoxia in breast cancer cells. *Breast* 15:187–195, 2005.
24. Morimoto Y, Nishihira J, Kemmotsu O, Shibano T, Gando S, Shikama H: Pentobarbital inhibits apoptosis in neuronal cells. *Crit Care Med* 28: 1899–1904, 2000.
25. Kotani N, Takahashi S, Sessler DI, Hashiba E, Kubota T, Hashimoto H, Matsuki A: Volatile anesthetics augment expression of proinflammatory cytokines in rat alveolar macrophages during mechanical ventilation. *Anesthesiology* 91:187–197, 1999.
26. Gotsch U, Jager U, Dominis M, Vestweber D: Expression of P-selectin on endothelial cells is upregulated by LPS and TNF-alpha in vivo. *Cell Adhes Commun* 2:7–14, 1994.
27. Shi Q, Wang J, Wang XL, VandeBerg JL: Comparative analysis of vascular endothelial cell activation by TNF-alpha and LPS in humans and baboons. *Cell Biochem Biophys* 40:289–304, 2004.
28. Myers CL, Wertheimer SJ, Schembri-King J, Parks T, Wallace RW: Induction of ICAM-1 by TNF-alpha, IL-1 beta, and LPS in human endothelial cells after downregulation of PKC. *Am J Physiol* 263:C767–C772, 1992.
29. Miyabayashi M, Yasui K: Regulation of neutrophil $O2^-$ production by neutrophil–endothelial cell interaction via CD11b: its modulation by tumor necrosis factor-alpha (TNF-alpha) and lipopolysaccharide (LPS). *Int J Hematol* 65:49–59, 1996.
30. Miller LS, Morita Y, Rangan U, Kondo S, Clemens MG, Bulkley GB: Suppression of cytokine-induced neutrophil accumulation in rat mesenteric venules in vivo by general anesthesia. *Int J Microcirc Clin Exp* 16:147–154, 1996.
31. Timokhov VS, Iakovleva II, Kalashnikova EA, Ipat'eva EI: Plasma contents of cytokines (TNF-alpha, IL-1 beta, IL-6) and their clearence during continuous hemofiltration in patients with sepsis and multiple organ failure. *Anesteziol Reanimatol* 3:59–62, 1997. [in Russian].
32. Grundmann HJ, Hahnle U, Hegenscheid B, Sahlmuller G, Bienzle U, Blitstein-Willinger E: Inhibition of endotoxin-induced macrophage tumor necrosis factor expression by a prostacyclin analogue and its beneficial effect in experimental lipopolysaccharide intoxication. *J Infect Dis* 165:569–573, 1992.
33. Bertini R, Delgado R, Faggioni R, Gascon MP, Ythier A, Ghezzi P: Urinary TNF-binding protein (TNF soluble receptor) protects mice against the lethal effect of TNF and endotoxic shock. *Eur Cytokine Netw* 4:39–42, 1993.


Copyright © 2007 by the Shock Society. Unauthorized reproduction of this article is prohibited.

34. Read MA, Cordle SR, Veach RA, Carlisle CD, Hawiger J: Cell-free pool of CD14 mediates activation of transcription factor NF-kappa B by lipopolysaccharide in human endothelial cells. *Proc Natl Acad Sci U S A* 90:9887–9891, 1993.
35. Nick JA, Avdi NJ, Young SK, Lehman LA, McDonald PP, Frasch SC, Billstrom MA, Henson PM, Johnson GL, Worthen GS: Selective activation and functional significance of p38alpha mitogen-activated protein kinase in lipopolysaccharide-stimulated neutrophils. *J Clin Invest* 103:851–858, 1999.
36. Chen LW, Huang HL, Lee IT, Hsu CM, Lu PJ: Thermal injury-induced priming effect of neutrophil is TNF-alpha and P38 dependent. *Shock* 26:69–76, 2006.
37. Penner CG, Gang G, Wray C, Fischer JE, Hasselgren PO: The transcription factors NF-kappab and AP-1 are differentially regulated in skeletal muscle during sepsis. *Biochem Biophys Res Commun* 281:1331–1336, 2001.
38. Taniguchi T, Yamamoto K: Anti-inflammatory effects of intravenous anesthetics on endotoxemia. *Mini Rev Med Chem* 5:241–245, 2005.





Copyright © 2007 by the Shock Society. Unauthorized reproduction of this article is prohibited.