行政院國家科學委員會專題研究計畫 期末報告

克雷白氏肺炎桿菌經由腸道上皮入侵細胞的感染致病機轉研究

計 畫 類 別 ：個別型
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執 行 期 間 ：101 年 08 月 01 日至 102 年 07 月 31 日
執 行 單 位 ：中山醫學大學醫學系微生物及免疫學科

計 畫 主 持 人：盧敏吉
共 同 主 持 人：黃志揚、賴怡琪
計畫參與人員：博士班研究生-兼任助理人員：林怡均

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 102 年 11 月 19 日

中 文 摘 要 ： 在近三十年來，克雷伯氏肺炎桿菌引起的社區性肝膿瘍感染廣泛的流行，尤其是在台灣。類似的肝膿瘍病例也在世界各地陸續的被報導。克雷伯氏肺炎桿菌引起的肝膿瘍尤其好發於糖尿病病人。目前雖有許多研究描述眾多與克雷伯氏肺炎桿菌引起的肝膿瘍有關的毒力因子，可是對於此菌所引發的肝細胞損傷的相關機制仍不清楚。在本實驗室所建立的克雷伯氏肺炎桿菌引起的肝膿瘍動物模式中發現肝細胞的Interferon-γ(IFN-γ)有明顯增加的情形。IFN-γ 已知能引發肝細胞的凋亡或抑制細胞生長週期並進而影響肝臟再生，可見其在肝臟疾病上的重要性。因此，在後續的研究中，帶有冷光基因的克雷伯氏肺炎桿菌也同樣感染糖尿病和正常血糖的控制組小鼠以研究其與 IFN-γ 訊息傳遞路徑的相關性。更進一步，利用非侵入式活體分子影像系統監測細菌在小鼠體內感染分布的狀況。實驗結果顯示：克雷伯氏肺炎桿菌所引起的肝膿瘍感染能活化肝細胞的 IFN-γ/signal transducers and activators of transcription (STAT)/IFN regulatory factor-1 (IRF-1)訊息傳遞路徑。而克雷伯氏肺炎桿菌在糖尿病小鼠所造成較嚴重的肝細胞損傷，可能與 Interleukin-1 (IL-1) 和 Macrophage inflammatory protein-2 (MIP-2)的延遲增加導致過量的嗜中性白血球蓄積造成更嚴重的發炎，以及內質網壓力上升造成肝細胞的凋亡增加有關。

中文關鍵詞： 克雷伯氏肺炎桿菌，糖尿病，肝膿瘍， γ 型干擾素

英 文 摘 要 ： Klebsiella pneumoniae-caused liver abscess (KLA) has become a health problem in Taiwan and is continually reported in other countries. Diabetes mellitus, the most common metabolic disorder, underlies half of the KLA patients in Taiwan. The clinical impact of KLA has been well-documented. Nevertheless, the molecular basis regarding how K. pneumoniae causes liver infection, particularly in diabetic individuals, remains unclear. Auto-bioluminescence-expressing K. pneumoniae was inoculated into diabetic mice and age-match na&iuml；ve control. With the use of in vivo imaging system, translocation of the bioluminescenceexpressing K. pneumoniae from intestine to extraintestinal organs, mainly the liver, was noted in 80% of the diabetic mice, whereas the same bacteria causes extraintestinal infections in only 31% of na&iuml；ve mice. Besides increased

morbidity, the severity of hepatic tissue injury was also enhanced in the K. pneumoniae-infected diabetic mice. Upon K. pneumoniae infection, IFN-γ production was significantly evoked in the liver. To mediate IFN-γ signal, STAT (signal transducers and activators of transcription) 1 and 3 were activated in hepatocytes, and so was the expression of IRF (interferon regulatory factor)-1. Moreover, accumulation of neutrophils which was triggered by prolonged production of IL-1β and MIP-2, and significant increases in the level of active caspase 3 and phospho-eIF2α, were exclusively revealed in the K. pneumoniae-infected diabetic mice. The activation of IFN-γ/STAT/IRF-1 signaling demonstrated by this work emphasizes the role of IFN-γ for mediating the hepatic response to K. pneumoniae infection.

英文關鍵詞： Klebsiella pneumoniae, diabetes, liver abscess, IFN-γ

Research project's background and goals

Klebsiella pneumoniae is a Gram-negative enterobacterium ubiquitous in nature with two classical habitats, the environment and the mucosal surfaces of mammals. As behaving like a saprophyte resided in nasopharyngeal or intestinal mucosa of humans, *K. pneumoniae* frequently involves in a wide range of clinical illnesses, such as pneumonia, urinary tract infections, suppurative infections, bacteremia, meningitis, and septicemia. Without immediate treatment, infections caused by this bacterium have a significantly high rate of mortality [1]. During 1990s, *K. pneumoniae* has been noticed as the primary pathogen responsible for community-acquired pyogenic liver abscess (PLA) in Taiwan [2]. Despite *K. pneumoniae*-caused liver abscess (KLA) was initially thought as a disease of regional distribution, it has now been continually reported from other Asian and Western countries and is considered an emerging disease worldwide [3]. Distinct from *Escherichia coli*-associated liver abscess, KLA is generically cryptogenic without underlying hepatobiliary disorders and is frequently complicated in up to 10% of cases with septic metastatic lesions to other organs [2, 4-7]. By virtue of its primary and invasive nature, KLA represents one of the most severe infections caused by *K. pneumoniae* [8-11].

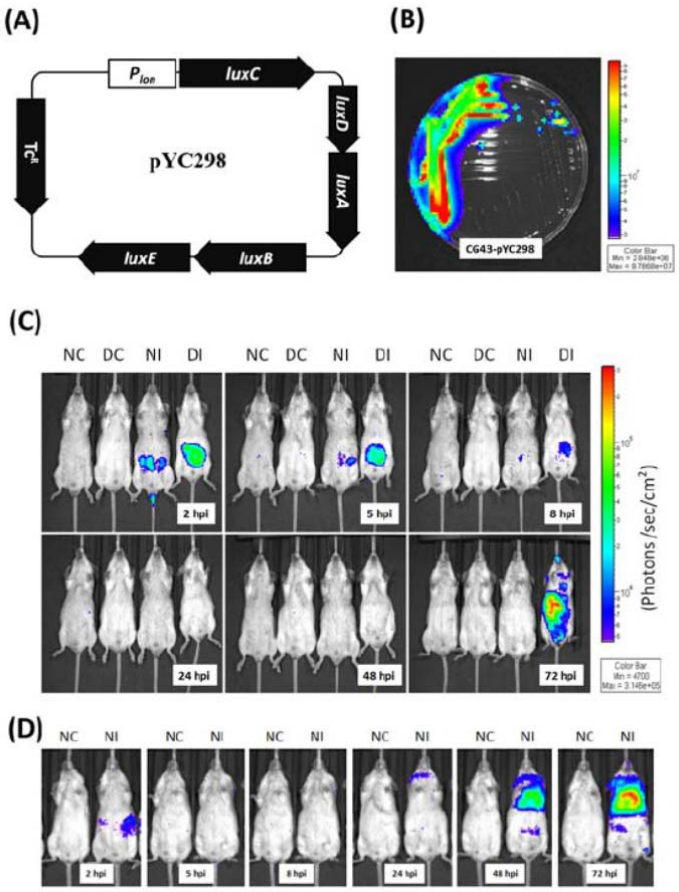
The clinical impact of KLA has been well-documented; however, our knowledge regarding the basis of how *K. pneumoniae* causes an infection particularly in the liver is rather restricted. To address this issue, we have established a KLA model with BALB/c mice in which 28 genetic loci of *K. pneumoniae* have been identified as KLA-related [12]. The progression of KLA in this model can be divided into 4 stages: intestinal colonization, extraintestinal dissemination, hepatosplenic replication, and septic metastasis. Interestingly, the failure to develop KLA for more than two thirds of the KLA-attenuated mutants was attributed to their incapability to disseminate from the intestines, suggesting that a replicating pool of *K. pneumoniae* has to be established prior to invading the internal organs. This intestine-spleen-liver infectious pathway of *K. pneumoniae* is reminiscent of the dissemination route that has been reported for *Yersinia enterocolitica* and *Salmonella enterica* serovar Typhimurium [13]. The oral-inoculated *Yersinia* resides in lymphoid Peyer's patches (PP) and the mesenteric lymph nodes (MLN) within 24 h and then appears in the liver and spleen between 48-72 h [14]. The ordered spread from the intestinal lumen into the PP and MLN then to the spleen and liver is also the presumed model of *Salmonella* dissemination. However, the existence of pathways bypassing the PP and MLN is recently reported for the intestinal translocation of *Y.*

pseudotuberculosis [15] and *Salmonella* [16]. There are at least three possible translocation mechanisms that bypass the PP-MLN. First, local micro-damages in the intestinal epithelium which may be caused by host or bacteria provide sites for translocation. The release of *Yersinia* effectors through a type III secretion system may break down the tight junctions between intestinal epithelial cells and may also cause apoptotic occurring at the top of intestinal villi, permitting translocation of bacteria [17]. Second, villous-associated M cells that allow sampling intestinal contents are recently identified to be served as portals across the [18]. Finally, phagocytic or dendritic cells (DC) interdigitated within the intestinal epithelium that capture luminal bacteria may provide a phagocytic route for translocation, as evident for *S.* Typhimurium. Upon the expression of chemokine receptor CXC3CR1, the transepithelial extensions of dendritic cells facilitate sampling of intestinal bacteria that in turn provide as sites for attachment by intestinal pathogens [19]. Even these processes that lead to translocation across the intestine may not be particularly efficient though, the loads of bacteria in systemic infection can be derived from as few as one intestinal clone [15]; therefore, enteropathogens still hold the key to develop a successful systemic infection with the multiple strategies provided for initiating dissemination.

This is a continuing project of NSC 100-2320-B-040 -016. In general, we successfully established *K. pneumoniae* infections in diabetic mice this year. The progress is reported as the following.

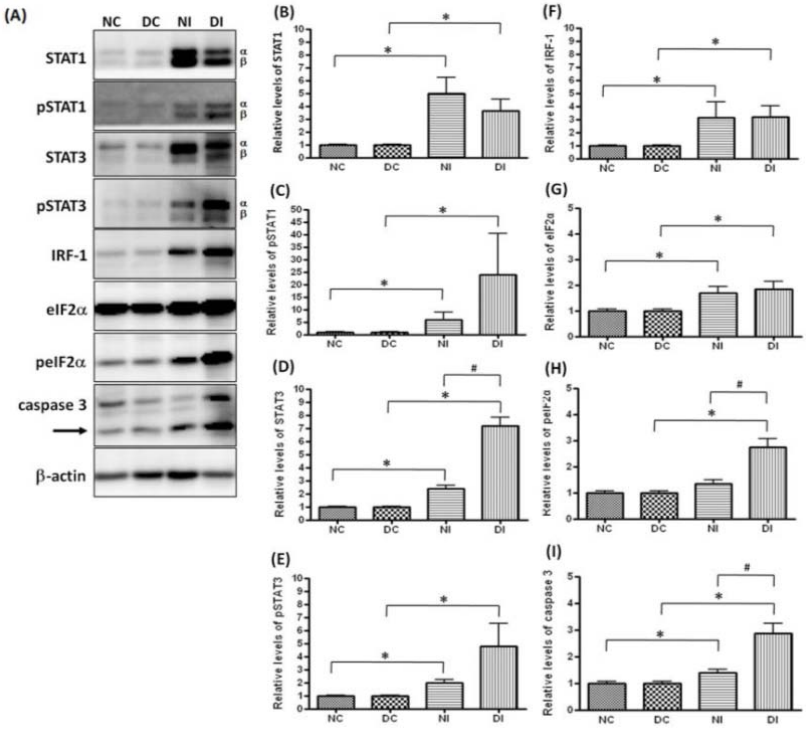
Results and discussion

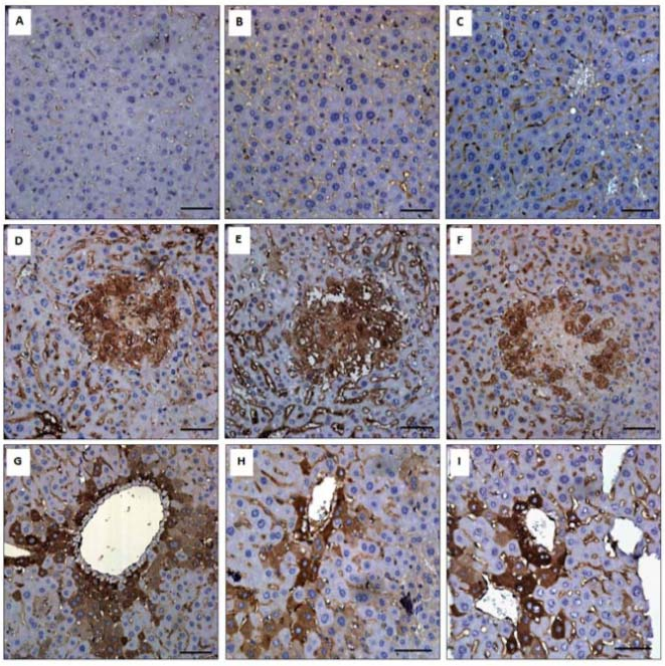
1 ***In vivo* imaging dissemination of auto-bioluminescence-expressing *K. pneumoniae*.** To monitor *K. pneumoniae* infection comparatively in diabetic and naïve mice, auto-bioluminescence-expressing *K. pneumoniae* was generated by transformation with pYC298 (Fig. 1A). With the use of Xenogen IVIS Imaging System, the auto-bioluminescence-expressing *K. pneumoniae* (Fig. 1B) was handily detected by a minimum limit of 1×10^4 CFU/ml in LB culture. Given that intestinal colonization with *K. pneumoniae* is considered the first step of KLA [20, 21], suspension of 3×10^8 CFU of auto-bioluminescent *K. pneumoniae* was inoculated into groups of diabetic and age-matched naïve mice via an oral route. As shown in Fig. 1C, bioluminescence signals were detected primarily in the abdomen of *K. pneumoniae*-infected mice (NI and DI) at 2 hpi (hour post-inoculation), and were continually reduced with a more accelerated rate in the naïve group, suggesting that the inoculums of *K. pneumoniae* were mostly shed through the feces. Although the bioluminescence signal was under the



limit of detection by the Xenogen IVIS system at 8, 24, and 48 hpi, small amounts of intestinal *K. pneumoniae* were enough to initiate an extraintestinal infection. As shown in Fig. 1C at 72 hpi, the location of the strongest bioluminescent intensity spots (as red color) coincided with the approximate location of liver in the *K. pneumoniae*-infected diabetic mice (DI), whereas no signal was detected in the naïve mice that successfully conquered *K. pneumoniae* invasion (NI). However, once *K. pneumoniae* penetrated the intestinal barrier of naïve mice, it also developed severe extraintestinal infections at 72 hpi (Fig. 1D; NC *vs*. NI).

2 **Activation of IFN-γ/STAT/IRF-1 signaling in response to *K. pneumoniae* infection.** IFN-γ was critical for the innate responses against pulmonary *K. pneumoniae* infections [22]. However, excessive production of IFN-γ impaired host resistance to bacteria, as a significant number of liver-specific IFN-γ transgenic mice died from enteric bacteremia [23]. The production of hepatic IFN-γ was significantly increased in response to *K. pneumoniae* infection in both the diabetic and naïve mice. To address the role

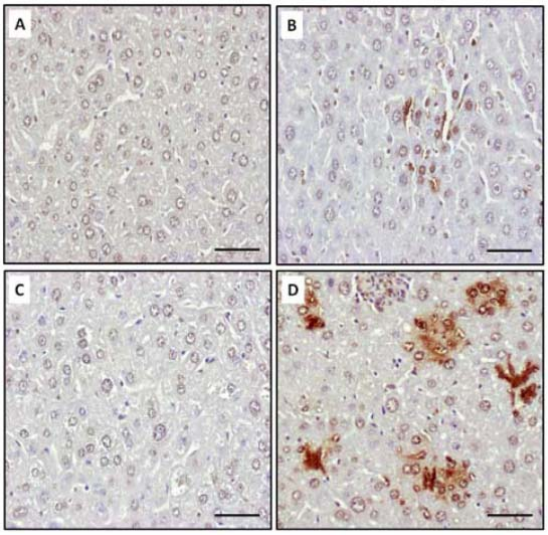




of IFN-γ in hepatic responses to *K. pneumoniae* infection, activation of IFN-γ/STAT/IRF-1 signaling was examined. As shown in Fig. 2A-E, upon *K. pneumoniae* infections, the production of IFN-γ significantly elevated the level of expression and phosphorylation of STAT1 and STAT3 in the liver for both the diabetic and naïve mice. The formation of phospho-STAT1/STAT3 homo- and hetero-dimers transactivated the expression of downstream genes. The protein level of IRF-1 was elevated to 3-fold in response to *K. pneumoniae*-caused liver infection (Fig. 2A and F). The activation of IFN-γ/STAT/IRF-1 pathway was confirmed by immunohistochemistry analyses. When compared with the PBS control (Fig. 3A-C), positive signals for phospho-STAT1 (Fig. 3D), phospho-STAT3 (Fig. 3E), and IRF-1 (Fig. 3F) were detected mainly within the foci of microabscess in the diabetic mice and were observed inside the liver parenchyma cells of *K. pneumoniae*-infected naïve mice (Fig. 3G-H). The results demonstrated the activation of IFN-γ/STAT/IRF-1 pathway in hepatic response to *K. pneumoniae* infection.

3 **Significant increases in the level of phospho-eIF2α and active caspase 3 in *K. pneumoniae*-infected diabetic mice.** IFN-γ elicits apoptosis in a number of normal cells, including hepatocytes. Among the multiple pathways involved, ER stress has been shown to play a critical role in the signaling of IFN-γ induced apoptosis of primary hepatocytes [24]. Given that several apoptotic characteristics were noted in the *K. pneumoniae*-infected diabetic mice, the involvement of ER stress was investigated. The level of phosphorylated eukaryotic initiation factor 2-alpha (peIF2α) (Fig. 2A, G, and H), induced by PKR-like ER-localized eIF2α kinase (PERK) due to the ER protein load, was elevated exclusively in the diabetic mice upon *K. pneumoniae* infection. Moreover, protein level of p20 subunit of the activated caspase 3 that was proteolytically generated during apoptosis (Fig. 2A; indicated by an arrow) was significantly increased in the *K. pneumoniae*-infected naïve and diabetic mice in comparison with the

uninfected control (Fig. 2I; * $P < 0.05$). To ascertain that the activation of caspase 3 was due to apoptosis, the liver sections retrieved from the naïve and diabetic mice which had *K. pneumoniae*-caused liver infection at 72 hpi were subjected to TUNEL assay. Apoptosis in a number of hepatic cells was induced by *K. pneumoniae* infection in both the diabetic and naïve mice (Fig. 4). However, the significantly two-fold higher increase in the level of active caspase 3 in *K. pneumoniae*-infected diabetic mice in comparison with the naïve group (Fig. 2I; $^{\#} P < 0.05$) suggested that *K. pneumoniae*-induced apoptosis was enhanced in mice with diabetes.



4 Taken together, the activation of IFN-γ/STAT/IRF-1 signaling in the hepatic response to *K. pneumoniae* demonstrated by our previous work emphasizes the role of IFN-γ for mediating innate immunological responses, including macrophage activation, infiltrates of neutrophils, inflammatory tissue injury, and hepatic apoptosis. Prolonged production of IL-1β and MIP2, induction of ER stress, and increased apoptosis might contribute to *K. pneumoniae*-related hepatic damage in mice with diabetes.

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國科會補助計畫衍生研發成果推廣資料表

日期:2013/11/19

國科會補助計畫	計畫名稱: 克雷白氏肺炎桿菌經由腸道上皮入侵細胞的感染致病機轉研究
	計畫主持人: 盧敏吉
	計畫編號: 101-2314-B-040-009- 學門領域: 血液科腫瘤科風濕免疫及感染

無研發成果推廣資料

101 年度專題研究計畫研究成果彙整表

計畫主持人：盧敏吉	計畫編號：101-2314-B-040-009-
計畫名稱：克雷白氏肺炎桿菌經由腸道上皮入侵細胞的感染致病機轉研究	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	1	1	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	1	1	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

Yi-Chun Lin, Min-Chi Lu, Chingju Lin, Ming-Ko Chiang, Ming-Shiou Jan, Hui-Ling Tang, Hsu-Chung Liu, Wea-Lung Lin, Chih-Yang Huang, Chuan-Mu Chen and Yi-Chyi Lai. (2013) Activation of IFN-γ/STAT/IRF-1 in hepatic responses to Klebsiella pneumoniae infection. PLoS one 8: 11. (SCI, IF = 3.73, Multidisciplinary sciences, 7/56 = 12.5%)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

The activation of IFN-γ/STAT/IRF-1 signaling in the hepatic response to K. pneumoniae demonstrated by this work emphasizes the role of IFN-γ as a pivotal cytokine for mediating innate immunological responses, including macrophage activation, infiltrates of neutrophils, inflammatory tissue injury, and hepatic apoptosis. Based on the finding that the hepatic injury of diabetic mice was deteriorated by K. pneumoniae-induced ER stress, the use of small molecule modulators of ER stress has therapeutic potential for alleviating the symptom of diabetic patients who suffer from KLA disease.