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碩士學位論文

探討 Src 在 poly(I:C)刺激巨噬細胞產生 IFN-β 的角色扮演

Role of Src in poly(I:C)-induced interferon-beta (IFN-β)
production in macrophages

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致謝

實驗的壓力下，互相給彼此好的意見。最後，我的父母，也是我最想

感謝的人，給予我充足的補充，讓我能夠完整的放心在碩士的路途

上。

Abstract

By recognizing double stranded RNA (dsRNA), a product of viral infection, TLR3 can produce type I interferons such interferon alpha and beta (i.e. IFNα/β) that possess anti-viral and immune modulating functions. As the synthetic analogue of dsRNA, polyinosinic-polycytidylic acid (poly(I:C)) can effectively induce the production of IFNα/β in macrophages. In this study, we investigated the signal transduction pathways in IFN-β secretion elicited by poly(I:C). We observed that poly(I:C)-mediated IFN-β generation was PP2 (an inhibitor for Src family kinases(SFKs)) sensitive in murine RAW264.7 cells. Pharmacological blockade of AG (an iNOS inhibitor) and ODQ (a sGC inhibitor) suppressed poly(I:C)-evoked IFN-β mRNA expression. Either SNAP (a NO donor) or 8-br-cGMP (a cGMP analogue) could rescue these defects. We found that the increment of IFN-β could be attributable to their increased transcripts. Interestingly, knockout of iNOS suppressed poly(I:C)-evoked IFN-β secretion and Src induction. Notably, attenuation of Src by *src*-specific siRNA reduced poly(I:C)-, and SNAP-enhanced IFN-β production in macrophages, and reintroduction of avain Src could rescue this defect. With these results, we concluded that iNOS was critical for poly(I:C)-mediated IFN-β production and Src played a pivotal role in this process.

Key words: poly(I:C); TLR3; Src; macrophages; interferon-β; iNOS

摘要

Toll-like receptor 3 (TLR3)藉著辨識病毒產物雙股 RNA (dsRNA)，會產生干擾素 α 和 β，而兩者皆屬於第一型干擾素。而第一型干擾素具有對抗病毒入侵和調控免疫反應的功能。現在已知人工合成的 polyinosinic-polycytidylic acid (poly(I:C))能夠有效的刺激巨噬細胞,產生 type I interferons。在本篇的研究,我們將探討有什麼訊息傳遞路徑參與在 poly(I:C)刺激巨噬細胞所產生的 IFN-β 的過程中。我們發現 PP2 (Src family kinases 的抑制劑)可以抑制 poly(I:C)刺激老鼠的巨噬細胞 RAW264.7 所產生的 IFN-β。PP2 會抑制 poly(I:C)刺激下所產生的 IFN-β 且其 mRNA 也顯著降低。另外一方面,SNAP (a NO donor) 或 8-br-cGMP (a cGMP analogue)皆增加 IFN-β mRNA。故 IFN-β 釋放量的增加,可由其 mRNA 量的增加來反應。有趣的是,基因剔除 iNOS 的表現確實可以使的 poly(I:C) 刺激巨噬細胞產生 IFN-β 的釋放量及 Src 表現量降低。使用了弱化 Src 的 src-specific siRNA 細胞會使的 poly(I:C)和 SNAP 所刺激的 IFN-β 釋放量下降,不過使用了放回 ectopic Src 的細胞則又能恢復 IFN-β 的產生。根據這些結果,我們可以得到一個結論,那就是 iNOS 參與 poly(I:C)-induced IFN-β production,

而且 Src 在這個過程中也扮演重要的角色。

Introduction

Viral infection

Viruses are the smallest organisms that cause infection in our body, much smaller even than other microbes. Viruses consist of one or more genetic materials of DNA or RNA, which contain their genes in the protein coat. In order to survive, virus must attach and invade to living cells. When viruses invade into the cell, they may kill or alter the cell's functions by replicating their genomes to generate new viruses. Newly formed viruses will result in further infection. Most viruses do cause diseases when they invade normal, living cells, through different ways like inhalation, swallowing, touching or unsafe sex. Culture and eating habits also increase the risk of viral infections.

Various human viruses can attack specific cells in particular parts of our body's such as respiratory system, digestive system, liver or blood.   The main types of viral infection include common cold and influenza (flu). These diseases are frequently caused by viral infection of nose, throat and have some unpleasant symptoms such as stuffy nose, sneezing, sore throat, coughing, body chills, fever, headache, and muscle ache. The skin can also be infected by viral attack. The prominent example is chickenpox. Chickenpox is an infectious disease and spreads very easily in highly populated areas. Furthermore, many serious lethal diseases such

as ebola, avian influenza, SARS and AIDS are also transmitted by viral attack and have symptoms like hemorrhagic fever, dehydration, acute respiratory syndrome. Until 2009, an estimated 1.1 million persons are currently living with HIV/AIDS in the US [1]. Recently, new influenza A is a global epidemiological disease by H1N1 virus infection. WHO raised the level from phase 5 to phase 6, 74 countries in 5 WHO regions have been affected [2]. These highly lethal viral pathogens are the world's worst epidemic threat.

Immune system and Macrophage

Encounter to viral infections, the immune system offers the main defense in our body. The immune system is composed of organs, tissues and cells that interact to protect the body from external invaders. Several levels are in the two subdivisions of the immune system. Mechanical barriers include skin and mucous membrane. Physical and chemical barriers include coughing, sneezing, vomiting, diarrhea, tears, saliva, and perspiration. Mechanical, physical and chemical barriers are the first lines of defense that belong to non-specific immunity. When they are invalid the second line of defense in innate (non-specific) immunity comes into play. The defensive measures include phagocytosis by granulocytes (the white blood cells called neutrophils, eosinophils and basophils) and monocytes/macrophages, secretion of protective proteins like cytokines and complement proteins. The last lines of defense are cell-mediated (T lymphocytes) and humoral (B lymphocytes) immunity that are pathogen-specific.

The innate immune responses are rapid than the adaptive responses, when viral infection occur macrophages and dentritic cells (DCs) can start the innate immune responses. The myeloid cells in innate immunity are derived from pluripotent hematoietic stem cells in the bone marrow that perform various immune tasks. Macrophages are derived from monocytes that circulate in blood and migrate to tissues where they become resident. Macrophages play an essential role for in innate immunity and have

different names dependent on the tissues they reside. For example, Kupper cells in the liver, Microglia in the central nerve system. Macrophages are important in fighting viruses and bacteria, with digestive enzymes to destroy invading microorganisms. Macrophages detect microorganisms through pattern-recognition receptors (PRRs) like Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors (RLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs) [3]. Through these receptors, macrophages detect and phagocytose invaders to present antigens and releas several cytokines (i.e. tumor necrosis factor α, interleukins and interferons) to activate adaptive immune responses.
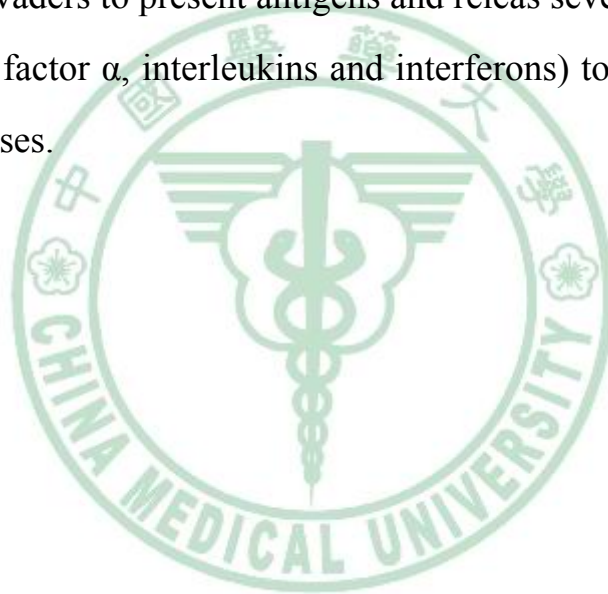
Interferons (IFNs) and Toll-like receptor 3 (TLR3)

Interferons (IFNs) are widely expressed cytokines, was the first to be described by Isaacs and Lindenmann in 1957. It was designated due to its ability " interfere " viral replication activities [4]. IFNs have several biological functions. Among them, antiviral and immunomodulatory, are the major IFNs-exerted effects [5]. These cytokines are first line of defense in the innate immunity against viruses, bacteria infections and have important roles to activate antigen-presenting cells and natural killer (NK) cells in the innate immune system. The IFN family has two types: type I interferons and type II interferons. In human, there are many type I interferons include IFN-α and its subtypes [6], IFN-β, IFN-ε, IFN-κ, and IFN-ω [7]. By contrast, there is only one type II interferon, IFN-γ. In the antiviral host immune response, protein kinase R (PKR), 2'-5'-oligoadenylate synthases and RNaseL are important antiviral mediators. These IFNs-induced proteins are believed to affect viral multiplication through inhibition of the translation initiation and degradation of viral RNA [8]. IFNs also link the innate responses and the adaptive immune responses. Viral recognition by PRRs in the innate immunity, initiates the activation of the signaling pathway that leads to type I interferons and inflammatory cytokines production that eliminate viruses.

Type I interferons (IFN-β) induction can be triggered by TLRs in immune cell such as macrophages and DCs. In 1985, toll was initially identified to play role in Drosophila development. Later in 1996, the important of toll

in Drosophila immunity was described. One year later, a human homologue of the drosophila toll protein (TLR4) was identified that can induce expression of some inflammatory genes [9, 10]. Mammalian TLRs family consists 13 members with different cellular localization that can be recognize by specific pathogen-associated molecular patterns (PAMPs). As TLR1, TLR2, TLR4, TLR5, TLR6 localize to the cell surface and recognize lipid and protein PAMPs, TLR3, TLR7, TLR9 are expressed in intracellular compartments such as endosome to detect viral nucleic acid PAMPs. Each TLR has its own signaling pathway and induces specific biological responses. By utilizing different adaptors, TLRs can trigger several signaling pathways. While most TLRs utilize MyD88-dependent pathway like TLR4, TLR2 can utilize myeloid differentiation factor 88 (MyD88), TLR3 triggers signaling pathways in a MyD88-independent manner.

TLR3 detects viral dsRNAs, that can be mimicked by the synthetic analog polyinosinic acid-cytidylic acid (Poly(I:C)). TLR3-deficient mice showed reduced production type I IFNs and proinflammatory cytokines [11]. TLR3 signals only via the TIR domain-containing adapter inducing interferon-β (TRIF) that is also called TIR domain–containing adapter molecule-1 (TICAM-1) [12]. Generation of TRIF-deficient mice hampered TLR3-mediated activation of IFN-β and IFN-β inducible genes production [13]. TRIF interacts with TRAF3, TBK1 and inducible IκB kinase (IKK-i). TBK1 and IKKi mediate phosphorylation of interferon-regulatory factors, IRF3 and IRF7. Phosphorylated IRF3 and

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IRF7 dimerizes and translocated to the nucleus to activate the expression of type I IFN and IFN-inducible genes [14].

Src kinase

The viral *src* (v-*src*) was the first indentified oncogene in Rous sarcoma virus (RSV) by Peyton Rous in 1911. Until 1976, this oncogene genetic and physical was indentified. As a counterpart of v-src, cellular src (c-src) was identified as a proto-oncogene in normal cells. Mounting evidence indicates that c-src (the protein encoded by c-src) play an important role in normal cellular functions [15].

Src is a non-receptor tyrosine kinase that contains several structural features. There are several domains in this Src protein. Form N to C terminus, there are Src Homology 4 (SH4), SH3, SH2 and catalytic/regulatory domains. The N-terminus of c-Src is myristoylated at Gly2, and becomes membrane-associated. As SH3 domain can interact with proline-rich sequences. SH2 domain can interact with phosphorylated tyrosine. Therefore, both domains can mediate protein-protein interaction. The catalytic and regulatory domains are important to regulate Src activity. Tyr-416 and Tyr-527 are two major sites whose phosphorylation can modulate Src activity. While phosphorylation of Tyr-416 resultes in Src activation, phosphorylation of Tyr-527 decrease its activity. This is become phosphorylated Tyr-527 (Pi-Tyr527) will interact with SH2 domain, renders Src in an inactive conformation. However, dephosphorylated Tyr-527 and Pi-Tyr416 will render in an active Src conformation. In this report, we use the level of Pi-Tyr416 as an indicator Src activation.

Since the discovery of Src, Yes, Fgr, Fyn, Lck, Lyn, Blk, and Hck have been identified as membranes of Src family kinases (SFKs) [15]. Despite Src family members are expressed in a wide variety of tissues, Lyn, Fgr, Hck, Lck, and Blk are predominantly expressed in hematopoietic cells. Accumulated evidence indicates the participation of tyrosine kinases in LPS-stimulated macrophages. Through Lyn, Fgr and Hck are largely expressed in monocytes and macrophages, macrophages derived from $lyn^{-/-}fgr^{-/-}hck^{-/-}$ triple knockout mice still retained full LPS responsiveness [16]. This implicates the possible involvement of an Src family kinase in LPS-induced macrophage activation. Interestingly, we previously reported, found Src up-regulation in LPS-stimulated macrophages both in vitro and in vivo [17]. In this report we present evidences demonstrating the induction of c-Src in RAW264.7 and peritoneal macrophages in response to poly(I:C)-mediated IFN-β production.

iNOS (inducible nitric oxide synthase)

Nitric oxide (NO) has been identified as an important signaling molecular involved in many biological functions, immune, vasodilatation. NO synthesis has been found in a variety of different cell types. With the enzyme nitric oxide synthase (NOS), NO direct formation and release. There are three distinct isoforms of NOSs, neuronal and endothelial NOS that are constitutively expressed whereas inducible NOS induced by cell-mediated immunity and inflammation. Unlike nNOS and eNOS, iNOS is independent of Ca^{+2} /calmodulin regulation.

In the immune system, NO and its derivatives are important to the antimicrobial activity especially when macrophages activation. Macrophages generate NO from L-arginine through the inducible isoform of nitric oxide synthase (iNOS) by LPS stimuli. iNOS expressing in several cells including endothelium, hepatocytes, monocytes, mast cells, macrophages and smooth muscle cells participates in many signaling pathway. Depending on the cytokines or TLRs ligands stimulus in the immune cells (macrophages and DCs) increase iNOS expressing causes NO production, than following activation of sGC results cGMP production. NO activates NF-κB and up-regulates iNOS (positive feedback). NO and cGMP are second messenger molecules allow smooth muscle to relax and immune cells activation, enhance anti-microorganisms activity.

Furthermore, in 2008 we demonstrated lipopolysaccharide-mediated Src

induction and macrophages migration was inducible nitric-oxide synthase (iNOS) [18]. Later, similar study indicated Src kinase-mediated phosphorylation stabilizes iNOS in normal cells and cancer cells [19]. In this study, we demonstrated that iNOS was required in poly(I:C)-mediated IFN-β production which was PP2-sensitive. And attenuation of Src by *src*-specific siRNA reduced poly(I:C)-, SNAP-, enhanced IFN-β production in macrophages. Interestingly, pharmacological blockade like AG (a iNOS inhibitor), ODQ (a sGC inhibitor) or knockout of iNOS suppressed poly(I:C)-evoked type I interferons (IFN-β) secretion. By RT-PCR, we found that the increment of IFN-β could be attributable to their increased transcripts. In doing this we have evaluated the role of IFN-β is correlated with the activation of Src and IRF proteins that depends on iNOS.
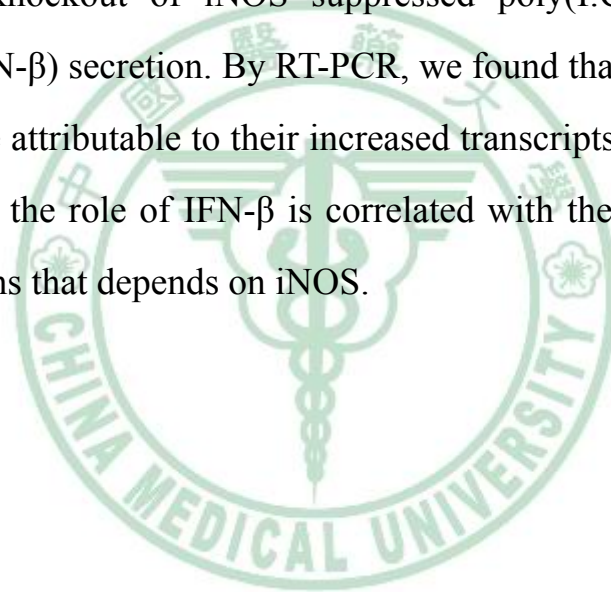
Aim

Macrophages play a critical role in inflammatory responses. Macrophages have antigen presentation, phagocytosis, and immunomodulation through production of various cytokines and growth factors. Previously, we demonstrated poly(I:C)-mediated macrophage migration. Poly(I:C) used to mimic viral dsRNA, is recognized by TLR3. Poly(I:C), a byproduct of viral infection can promote macrophage migration and anti-viral cytokines production in inflammatory processes. TLR3 mediates a more potent antiviral response than other TLRs [20]. This is why we study TLR3 pathway investigated downstream signaling activation mediated anti-viral cytokines IFN-β expression. Previous research indicates c-Src tyrosine kinase associates with initiate TLR3 antiviral signaling in human monocyte-derived dendritic cell [21]. In this report we want to know the induction of c-Src in RAW264.7 and peritoneal macrophages in response to poly(I:C)-mediated IFN-β production.

Materials and Methods

Cell Culture

RAW264.7 (American Type Culture Collection, murine macrophage cell line), was cultured and propagated in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (JRH Biosource), 100unit/ml-100μg/ml Penicillin-streptomycin (Sigma) and 2mM L-glutamine(Sigma) at 37 °C in humidified atmosphere of 5% CO_2 and air.

Preparation of mouse peritoneal macrophages (PEMs)

C57BL/6JNarl male mice were purchased from National Laboratory Animal Center in Taiwan and maintained in the animal center of China Medical University at Taichung. The animal room was maintained on 12 hour light and 12 hour dark cycle with a standard temperature and humidity. All mice were grown at 8-10 weeks old, sacrificed under anesthesia, and used to obtain cells from peritoneal exudates. All procedures adhered to the "Guide for Care and Use of Laboratory Animals" (National Research Council, USA) and were approved by the animal experiment committee of China Medical University. Mouse peritoneal macrophages (PEMs) were obtained from wild-type and iNOS-null mice by lavage with 10ml of cold PBS per mouse at 3 days after intraperitoneal injection of 3% 1ml thioglycollate (MERCK) in PBS.

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The PEMs were seeded in culture plates and incubated at 37 °C in humidified 5% CO_2 and 95% air to allow macrophages adherence. After 6 hours, the non-adherent cells were removed by washing with warm PBS and remaining cells were used for experiment.

Generation of RAW264.7 mcrophages expressing *src* siRNA

To generating the cells expressing nonspecific siRNA (Ctrl-1, Ctrl-2) or *src* siRNA (siRNA), RAW264.7 mcrophages were transfected with plasmid DNA pLKO.1-msrc (puro) (targeted sequence: 5'--CGAGCCGCCAATATCCTAGTA-3' or the negative control pSilencer plasmid (not present in mouse genome and provided by Ambion Inc.) by the Lipofectamine followed by puromycin or hygromycin selection. RAW264.7 mcrophages expressing src siRNA were cotransfected with pBabe (hygro) DNA and a plasmid DNA encoding avian c-Src [22] to generate Src-attenuated cells expressing ectopic Src (siRNA-1/Src6, siRNA-1/Src15).

Reagents and Antibodies

Polyinosinic-polycytidylic acid (poly(I:C)), aminoguanidine hemisulfate (AG), 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ), S-nitroso-N-acetylpenicillamine (SNAP), 8-bromo-cyclic guanosine monophosphate (8-Br-cGMP) were obtained from Sigma. Thioglycollate and 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo-[3,4-d]pyrimidine

(PP2) were obtained from MERCK. The primary antibodies used were actin (Sigma); FAK and iNOS (Upstate); Pi-Y861 FAK and Pi-Y416 Src (BIOSOURCE International); Lyn, Fgr and Hck (Santa Cruz); IRF3, IRF7 and PCNA (Santa Cruz); HRP-goat anti-mouse antibody and HRP-goat anti-rabbit antibody (Jackson). Src-specific mouse monoclonal antibody are provided by Dr. Leu in National Cheng Kung University, Tainan.

ELISA for mouse IFN-β

RAW264.7 and its based, Ctrl-1, Ctrl-2, siRNA, siRNA-1/Src6, and siRNA-1/Src15 cells were induced with poly(I:C) for 48 hours. The culture supernatants were examined by enzyme-linked immunosorbent assay (ELISA). The IFN-β ELISA kit was purchased from PBL Biomedical Laboratories. Murine IFN-β production was detected by ELISA according to the manufacturer's procedure (PBL Biomedical Laboratories) [23]. Produces are following:

Prepare Wash Buffer as follows: Dilute the 50 ml of 10x wash concentrate with 450 ml of distilled or deionized water. The Final wash solution should be stored at 2-8°C and mixed thoroughly before use. All the wash steps should be performed at room temperature (22-25°C). Wash steps can be performed manually with a squirt bottle or by using an automated plate washer.

Prepare a 1:10 working stock of the Mouse IFN-β standard by pipetting

10 μl of the Mouse IFN-β Standard in 90 μl of sample diluent. Prepare fresh dilutions of the standard curve for each assay run.

Determine the number of microplate wells required to test the desired number of samples and standard curve. Place precisely 100 μl of the interferon samples and standard curve prepared in Step 2 in individual wells of the microtiter plate, at least in duplicate. Remaining strips should be resealed with desiccant in the foil pouch and stored at 2-8°C for future use.

Cover the microtiter plate with one of the enclosed plastic plate sealers and incubate for 60 minutes in a closed chamber at 22-25°C. Optionally, the incubations can be carried out at room temperature, keeping the plate away from drafts and other temperature fluctuations. After the 60 minutes incubation, wash three times (3x) with prepared wash solution (at least 250 μl/well). Washes can be performed manually with a squirt bottle or by utilizing an automated plate washer.

During the incubation period, prepare the Antibody Solution for use in step 6. Dilute the Antibody Concentrate with Concentrate Diluent. Refer to the lot specific Certificate of Analysis (COA) for the correct amounts of Antibody Solution to prepare. Store used undiluted antibody concentrate at 2-8°C for future use.

Add 100 μl of the Antibody Solution prepared using the COA, to each well. Cover the microtiter plate with one of the enclosed plastic plate sealers and incubate for 60 minutes in a closed chamber at 22-25°C. After the 60 minutes incubation, wash three times (3x) with prepared wash solution (at least 250 μl /well).

During the incubation period, prepare the HRP solution for use in step 8. Dilute the HRP Conjugate Concentrate with HRP Conjugate Diluent. Refer to the lot specific Certificate of Analysis (COA) for the correct amounts of HRP Solution to prepare.

Add 100 μl of the HRP solution prepared using the COA, to each well. Cover the microtiter plate with one of the enclosed plastic plate sealers and incubate for 60 minutes in a closed chamber at 22-25°C. After the 60 minutes incubation, wash three times (3x) with prepared wash solution (at least 250 μl/well).

Add 100 μl of the TMB Substrate Solution to each well. Incubate for 15 minutes in a closed chamber at 22-25°C.

Add 100 μl of Stop Solution to each well. Mix gently. Using a microplate reader, determine the absorbance at 450 nm within 5 minutes after the addition of the stop solution.

RT-PCR for IFN-β

Total RNA was isolated from RAW264.7 mcrophages, Ctrl-1, siRNA, siRNA-1/Src6, and siRNA-1/Src15 cells by utilizing the Trizol reagent (REzol, Protech, Taiwan) according to the manufacturer's protocols. Transfer these cell lysate to a microfuge tube, and incubate the homogenate for 5 minutes at room temperature. Add 0.2 mL of chloroform (Sigma) per 1 mL of REzolTM C & T. Cover the samples tights and shake vigorously for 15 seconds and incubate them at room temperature for 2 minutes. Centrifuge the samples at 14000 xg for 15

minutes at 4°C. After centrifugation, the homogenate form two phase: the lower red phenol-chloroform phase and the colorless upper aqueous phase. The RNA remains exclusively in the aqueous phase (with a volume of approximately 0.6 mL), whereas the DNA and proteins remain in the interphase and organic phase. Carefully transfer the aqueous phase (0.5 mL) to a new microfuge tube without disturbing or touching the interphase. Add an equal volume (0.5 mL) of isopropanol (Sigma) and mix gently. Incubate the samples for 10 minutes at room temperature and centrifuge them at 14000 xg for 10 minutes at 4°C. The RNA precipitate (often invisible before centrifugation) forms a white pellet at the bottom of the tube. Carefully remove the supernatant, wash the RNA pellet in 1 mL of 75% ethanol (in DEPC-treated water) by vortex-mixing and subsequence centrifugation at 14000 xg for 5 minutes at 4°C. Carefully remove the ethanol and dry the pellet by air or under vacuum. Do not dry the RNA by vacuum. It is important not to over-dry the pellet because this makes it harder to dissolve the RNA. Dissolve the RNA pellet in 50 μl of DEPC-treated water. The RNA was quantified and checked for purity and condition by spectrophotometry at a wavelength of 260 nm. RNA concentration (μg/ml) = Dilution factor × RNA (OD260) × (40μg/ml/OD260). The RNA samples of 1 μg were then subjected to reverse ranscription-polymerase chain reaction (RT-PCR) by one tube reaction system-Fast Run™ HotStart RT-PCR kit (Protech technology, Taiwan) to determine the mRNA level of IFN-β. The sequences of primers used for murine *ifnβ* transcript were CCACAGCCCTCTCCATCAACTATAAGC (sense) and

AGCTCTTCAACTGGAGAGCAGTTGAGG   (antisense).   The
amplification cycles included 94 °C for 60 s, 60 °C for 60 s, and 72 °C
for 120 s. After 30 cycles of amplification, the PCR products were
subjected to electrophoresis on a 0.5% agarose gel and then visualized by
ethidium bromide staining. The mRNA of *gapdh* served as control for the
sample integrity and loading.

Western blotting

Cells were washed three times with PBS and re-suspended in lysis buffer
(4.38 g NaCl, 3.0285 g Tris-base, 1.25 g deoxycholate, 5 ml IGEPAL
CA-630), with proteinase inhibitor (sodium orthovannadate, EGTA,
aprotinin, and PMSF). The protein concentration was determined by
colorimetric assay (Bio-Rad). Sample containing equal protein (50 μg)
were separated by 8% SDS-PAGE and transferred onto polyvinylidene
difluoride membranes (Millipore, Belford, MA). The membranes were
blocked with blotting buffer containing 0.05% Tween 20 and 3% BSA in
a shaker for 1 hour and then incubated overnight with first antibodies at
4°C or incubated with first antibodies 3 hours at room temperature. The
membranes were washed with TBST three times and then incubated with
Horseradish peroxidase conjugated secondary antibody (Jackson). The
membranes were washed with TBST three times. The proteins of interest
were detected by exposure the autoradiograph to X-ray films (Kodak).

Nuclear Extraction

Cells were washed three times with PBS and re-suspended with 200 μl hypotonic buffer containing 10 mM HEPES, pH 7.9; 10 mM KCL; 0.1 mM EDTA; 0.1 Mm EGTA; DDH_2O and proteinase inhibitor 10X (10 mM DTT, 10 mM PMSF, 10 mM NaF, 10 mM Na3VO4, DDH_20) and incubated 5 minutes on ice to allow cells to swell. Centrifuge the homogenate at 13200 g at 4°C for 5 minutes. The cytoplasmic fraction was carefully aspirated. The pellet was washed three times with PBS and re-suspended with extraction buffer containing 20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM EGTA; DDH_2O and proteinase inhibitor 10X and vigorously votexed. Centrifuge the homogenate at 13200 g at 4°C for 15 minutes. Collect the supernatants (nuclear extracts). The protein concentration was determined by colorimetric assay (Bio-Rad).

Statistical analysis

All experiments were repeated at least three times using independent culture preparations. Values are shown as mean±SD. Student's t test was used to calculate the statistical significance of the experiments. A value of $P<0.05$ was considered statistically significant.

Result

1. Poly(I:C) induced interferon-β (IFN-β) production in murine RAW264.7 macrophages.

Each TLR has its own signaling pathway and induces specific biological responses. TLR3, which localizes to endosomes can recognize viral dsRNA. Unlike the other endosomal TLRs, TLR3 induces its signals through MyD88-independent pathway. The TLR3-TRIF signaling pathway can activate subsequent IRFs (interferon regulatory factors), MAPKs and NF-κB to increase production of type I interferons and pro-inflammatory cytokines. In this study, we use polyinosinic acid-cytidylic acid (Poly(I:C)) to mimic viral dsRNA. Murine RAW264.7 macrophages were incubated with poly(I:C) (20 μg/ml) for 24, 48 and 72 hours. Conditioned medium were harvested from each time point and the amount of IFN-β was examined by ELISA. As demonstrated in Figure 1, increased IFN-β was detected in poly(I:C)-treated macrophages as compared to non-treated ones.

2. Src family kinase was required for IFN-β production in macrophages in response to poly(I:C).

Previous study indicated LPS stimulates murine macrophages activation can increase protein tyrosine phosphorylation [24]. Two years later, others demonstrated Src family kinases Lyn, Fgr, Hck play an important role in LPS stimulates macrophages activation [25, 26]. We want to know

whether poly(I:C) (20 μg/ml) treatment will increase protein tyrosine phosphorylation, Previously, we demonstrated that show poly(I:C) (20 μg/ml) increased the whole protein tyrosine phosphorylation (pTyr) in macrophages. And PP2 treatment greatly reduced this poly(I:C)–mediated phosphotyrosine enhancement, suggesting the involvement of SFKs in this process (Supplementary Figure 1).

To confirm this point, RAW264.7 cells were pretreated with PP2 (10 μM), and then stimulated with poly(I:C) (20 μg/ml) for 48 hours. As demonstrated in Figure 2A, PP2 (a SFK inhibitor) greatly suppressed the production of IFN-β in poly(I:C)-stimulated macrophages. Consistently, PP2 almost wiped out *ifn-β* transcript (Figure 2B). These findings pointed out Src family kinases were involved in poly(I:C)-elicited increased of the level of transcript and protein expression of ifn-β gene.

3. Enhanced Src expression in RAW264.7 macrophages and peritoneal macrophages (PEMs) in response to poly(I:C).

Given that SFKs were crucial in poly(I:C)-induced IFN-β production (Figure 2), therefore , we examined the expression Src and its myeloid relatives (i.e. Lyn, Fgr, and Hck) in poly(I:C)-treated RAW264.7. As exhibited in Supplementary Figure 3, unlike the most constant expression of Lyn, Fgr, and Hck, the amount of Src was greatly induced in poly(I:C)-treated RAW264.7. Notably, similar phenomenon was also observed in PEMs in response to poly(I:C).

4. **Src attenuation reduced poly(I:C)-elicited IFN-β production in macrophages.**

Since Src is the only SFK whose expression can be induced by poly(I:C), therefore its involvement in poly(I:C)-mediated IFN-β production in macrophages was attempt. To answer this question, nonspecific and *src*-specific siRNA–bearing plasmids were transfected into RAW264.7 macrophages to obtain the control (Ctrl-1, Ctrl-2) and Src-attenuated (siRNA-1) cells. As shown in Figure 3, Src induction was observed in RAW264.7 and control (Ctrl-1, Ctrl-2) cells but not in Src attenuated (siRNA-1) cells following poly(I:C) stimulation. Interestingly, the expression of Lyn was not significantly altered in the presence or absence of poly(I:C) in RAW264.7, control and siRNA cells. Further analysis of IFN-β production in these cells revealed that poly(I:C)-induced IFN-β production was significantly suppressed in Src attenuated (siRNA-1) cells as compared to RAW264.7 and control cells following poly(I:C) treatment. To confirm the contribution of Src, we checked whether Src-attenuated cells ectopic Src (siRNA-1/Src6, siRNA-1/Src15) could rescue this event. As shown in Figure3, suppressed IFN-β production by attenuated Src could be restored by ectopic Src. Thus, the pivotal role of Src in poly(I:C)-induced IFN-β production was corroborated.

5. **NO/cGMP mediated IFN-β production.**

Our previous study demonstrated that poly(I:C)-mediated macrophage

migration was iNOS-dependent. Due to Src expression was NO-inducible, therefore we wonder the involvement of iNOS in poly(I:C)-induced IFN-β production in macrophages. To answer this question, we pretreated RAW264.7 with AG (an iNOS inhibitor) (2mM) or ODQ (a sGC inhibitor) (100 μM) for 30 minutes, then cells were stimulated without and with poly(I:C) (20 μg/ml). Total RNAs extracted from each sample were analyzed by reverse transcription polymerase chain reaction (RT-PCR). As demonstrated Figure 4A, AG and ODQ abrogated poly(I:C)-elicited increase of ifn-β transcript. Notably, SNAP (100 μM) and 8-Br-cGMP (cGMP analogue, 100 μM) significantly increased the amount of ifn-β transcript (Figure 4B).

To confirm the importance of iNOS in IFN-β production in macrophages by poly(I:C), the IFN-β expression of PEMs derived from WT and iNOS$^{-/-}$ mice treated with or without poly(I:C) was analyzed. As shown in Figure 5, compared with WT PEMs, reduced IFN-β production by poly(I:C) treatment was observed in iNOS null PEMs. These findings indicate the participation of the NO/cGMP pathway in poly(I:C)-mediated IFN-β production.

## 6. Src participated in SNAP-mediated IFN-β production in macrophages.

As shown in Figure 5, we demonstrated that iNOS played an important role in poly(I:C)-mediated IFN-β production in macrophages.

Interestingly, we also found that poly(I:C)-induced Src expression was abrogated in iNOS knockout macrophages (Supplementary Figure 4). Since Src expression was NO-inducible, therefore we wondered that Src was critical in NO-elicited IFN-β production in macrophages. To confirm this speculation, RAW264.7 and its derived control (Ctrl-1, Ctrl-2) and siRNA (siRNA-1) cells were stimulated without or with SNAP (NO donor) (100 μM) for 48 hours and the secretion of IFN-β in these cells was determined. As shown in Figure 6, we observed that SNAP-mediated Src expression and IFN-β production were significantly suppressed in Src attenuated (siRNA-1) cells as compared to RAW264.7 and Ctrl-1 and -2 cells following SNAP treatment. Interestingly, ectopic src could rescue these events. From these results, we confirmed that by induction of Src, iNOS was critical in IFN-β production.

7. Src-mediated IRF7 nuclear translocation was required for IFN-β production in poly(I:C)-treated macrophages.

Poly(I:C)-mediated IFN-β production required the participation of Src in macrophages (Figure 3). Since IRF3 and IRF7 were reported to be pivotal in ifn-b gene transcription, thereby we wondered whether Src affected nuclear translocation of IRF3 and IRF7. As exhibited in Figure 7A, poly(I:C)-mediated nuclear translocation of IRF3 and IRF7 was PP2-sensitive. To study the role of Src in poly(I:C)-mediated nuclear translocation of IRF3 and IRF7, nuclear extracts derived from RAW264.7, Ctrl-1, siRNA-1, siRNA-1/Src6 and siRNA-1/Src15 cells following

poly(I:C) stimulation were analyzed. As shown in Figure 7B, that poly(I:C)-mediated increase of nuclear IRF7 was significantly suppressed in Src attenuated (siRNA-1) cells as compared to Ctrl-1 cells following 48 hours poly(I:C) treatment. Notably, ectopic Src rescue these events. Due to the amount of poly(I:C)-induced nuclear IRF3 was not suppressed by Src attenuation. Since, IRF3 was preceded of IRF7, so we chose 12 hour instead of 48 hour poly(I:C) treated cells to analyze the translocation of IRF3 and IRF7 to nucleus. As demonstrated in Figure 7C, the amount of poly(I:C)-enhanced nuclear IRF7 was suppressed in Src attenuated (siRNA-1) cells, but the amount of nuclear IRF3 was not changed in siRNA-1 cells. These results indicated that by augmenting the amount of IRF7, Src was required for poly(I:C)-mediated IFN-β production in macrophages.
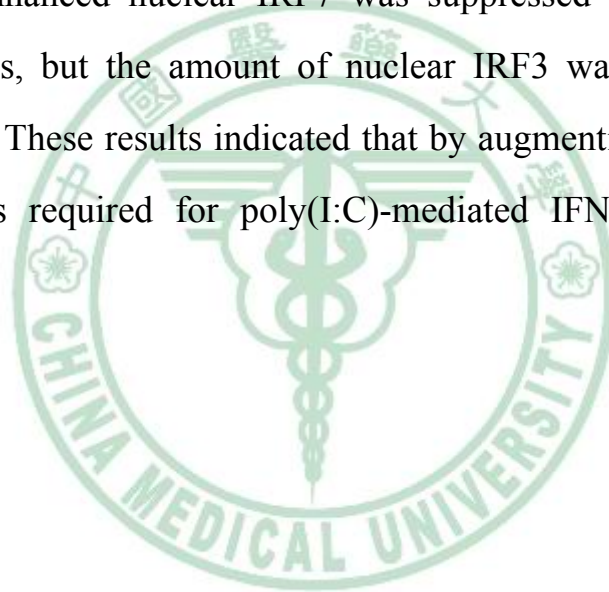
Discussion

In this study, we found that iNOS and Src play critical roles in poly(I:C)-mediated IFN-β production in macrophages in vitro (Figure 6) and in vivo (Figure 5, Supplementary Figure 4). The function of type I IFNs is well characterized and they are known to be essential for a powerful host response against viral infection. There are four effector pathways of the IFN-mediated antiviral responses: the ISG15 ubiquitin-like pathway, the Mx GTPase pathway, the 2', 5'-oligoadenylate-synthetase-directed ribonuclease L pathway, and the protein kinase R pathway. These pathways degrade viral RNA, block viral transcription, inhibit translation and modify protein function to control viral replication.

ISG15 functions to prevent virus-mediated degradation of IRF3, and increases the induction of IFN-β to against viral infection [27]. ISG15 target antiviral effector proteins such as Mx, PKR and RNaseL [28]. Following viral infection, Mx proteins produce from an IFN-stimulated response element (ISRE) to block viral gene transcription [29]. Following activation by viral double-stranded RNA (dsRNA), 2', 5'-oligoadenylate-synthetase expression is upregulated by type I IFNs [30], and activated the constitutively expressed inactive ribonuclease L (RNaseL). The binding of 2', 5'-oligoadenylate-synthetase to RNaseL enables RNaseL activation and be capable to cleave virals RNA. Protein kinase R (PKR) expression is also upregulated by type I IFNs. PKR is

activated directly by viral RNA, and can suppress viral proteins translation ability [31] by phosphorylation of eukaryotic translation initiation factor 2α.

IFN-β production is one of the means of innate immune system to against viral infection. Innate immune cells include dendritic cells (DCs), macrophages, neutrophils, and others. In host defense, the innate immune cells recognize microbial pathogens through distinct pattern recognition receptors (PRRs). Through induction of costimulatory molecules and cytokines to activation and differentiation of T lymphocytes, TLRs can trigger adaptive immune responses. TLRs detect microbial nucleic acids, such as double-stranded RNA (dsRNA) (TLR3), single-stranded RNA (ssRNA) (TLR7), and dsDNA (TLR9). TLR3 mainly detect viruses like reovirus, rotavirus in the gastrointestianl system, and Influenza A virus from infected cells but not in the direct viruses themselves [32]. TLR3 can activate its downstream signaling pathways to produce type I IFNs.

To mimic viral infection, we use poly(I:C), which is a synthetic analog of double-stranded RNA (dsRNA), a molecular pattern associated with viral infection and virus replication by-product recognized by TLR3 [33]. TLR3 is activated by extracellular dsRNA and endogenous mRNA [34], which escaping from damaged tissue or in endocytosed cells. TLR3 can not only detect exogenous danger signals, but also sense distress from damaged or stressed tissue. TLR3-mediated type I IFN signaling is only one of TLR3-mediated signaling pathway. All of TLR family membranes,

only TLR3 use MyD88-independent adaptor protein TRIF/ Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule TICAM-1 [13, 35]. Through TRIF/ TICAM-1-mediated activation of transcription factors (i.e. NF-κB, AP-1 and IRF3/7), transcription of ifn-b and proinflammatory cytokine encoding genes can be induced [36]. In this study, we demonstrated the non-recetpor tyrosine kinase Src participated in TLR3-mediated IFN-β production in macrophage. Interestingly, c-Src is required for TLR3-mediated activation in dentritic cells [21]. Previously, Src Family Kinases (SFKs) were documented to be involved in inflammatory cytokines production in human macrophages response to HIV-1 envelope protein SFKs were involved [37]. Recently mounting evidence indicated encephalomyocarditis virus-mediated COX2 expression in macrophages is regulated by SFKs [38]. The ability of PP2 and Src attenuation to reduce poly(I:C)-induced IFN-β expression (Figure 2B, Figure 3) and nuclear translocation of IRF7 (Figure 7), suggests that Src participates in poly(I:C)-mediated downstream signaling in the innate immune cells. Furthermore, we also found Src was involved in poly(I:C)-induced proinflammtory cytokines TNFα production in macrophages (data not shown).

Previously, we have demonstrated that LPS-induced Src induction and macrophage migration are required for NO/cGMP [18]. Expression of iNOS and NO production is well known to be an important effector in macrophages in response to viral infection [39, 40]. It is known that iNOS and NO inhibit viral replication and play a role in the host defense against

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viruses [41, 42]. Using poly(I:C) to mimic viral infection, we also demonstrated suppressed reduced migration in iNOS null PEMs as compared to WT PEMs (Appendix 2), indicating iNOS expression plays a critical role in macrophages activation including migration, cytokines production. In this study, we use pharmacological blockade, AG (iNOS inhibitor) and ODQ (sGC inhibitor), suppressed poly(I:C)-evoked increment of ifn-b mRNA (Figure 4A), and SNAP (a NO donor) or 8-br-cGMP (a cGMP analogue) could increase the abundance of ifn-b transcript (Figure 4B). With these findings, we conclude that iNOS also plays a critical role in antiviral cytokines production. Since exogenous IFN-β can accelerate the induction of iNOS [43], and iNOS is involved in IFN-β production, therefore there might exist a loop to amplify poly(I:C)-mediated effects. At the same time, we also found Src induction is dependent on iNOS (Supplementary Figure 4).

According to our results, we demonstrate that poly(I:C)-mediated IFN-β production, which is the requirement of iNOS in the innate immune cells macrophages. We suggest that Src seemed to be one of the downstream targets in this process and induction by iNOS. In the immune system, macrophages are important for our body to against infection agents. We clear up iNOS and Src playing important roles in poly(I:C)-mediated IFN-β production in macrophages .

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Chem, 2001. **276**(51): p. 47950-7.

Figure
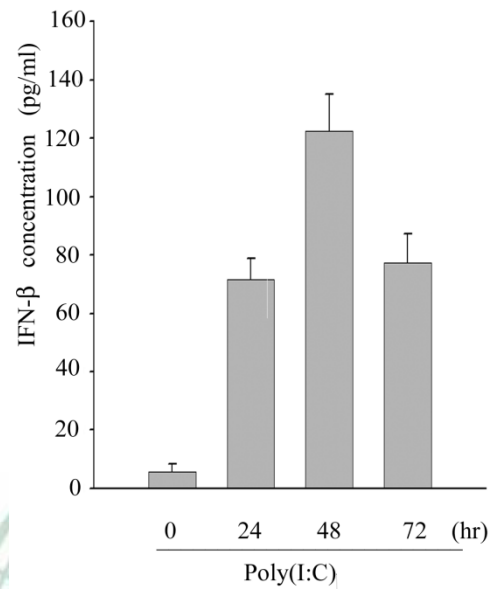
Figure 1.   Poly(I:C) treatment induces IFN-β production in murine RAW264.7 macrophages.

RAW264.7 cells were treated with poly(I:C) (20 μg/ml) for various times points as indicated. The concentration of IFN-β in culture medium of each sample was determined by ELISA.
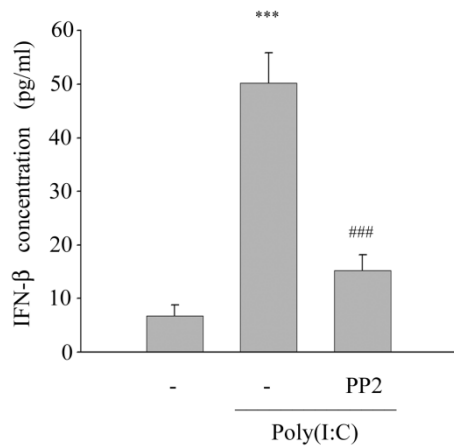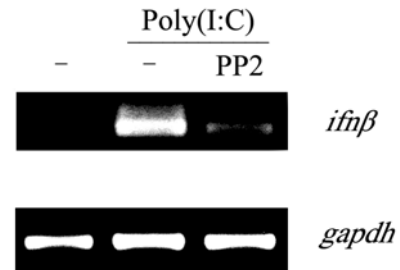
Figure 2. Poly(I:C)-induced IFN-β was PP2 sensitive.

(A) RAW264.7 cells were incubated with PP2 (10 μM) after 30 minutes poly(I:C) (20 μg/ml) stimulation. The concentration of IFN-β in culture medium of each group was determined by ELISA. *** and ###, P<0.001.

(B) RAW264.7 cells were incubated with PP2 (10 μM) after 30 minutes poly(I:C) (20 μg/ml) stimulation. The amount of *ifn-β* transcript was analysis by RT-PCR. *gapdh* was utilized as an internal control for amplification efficiency.
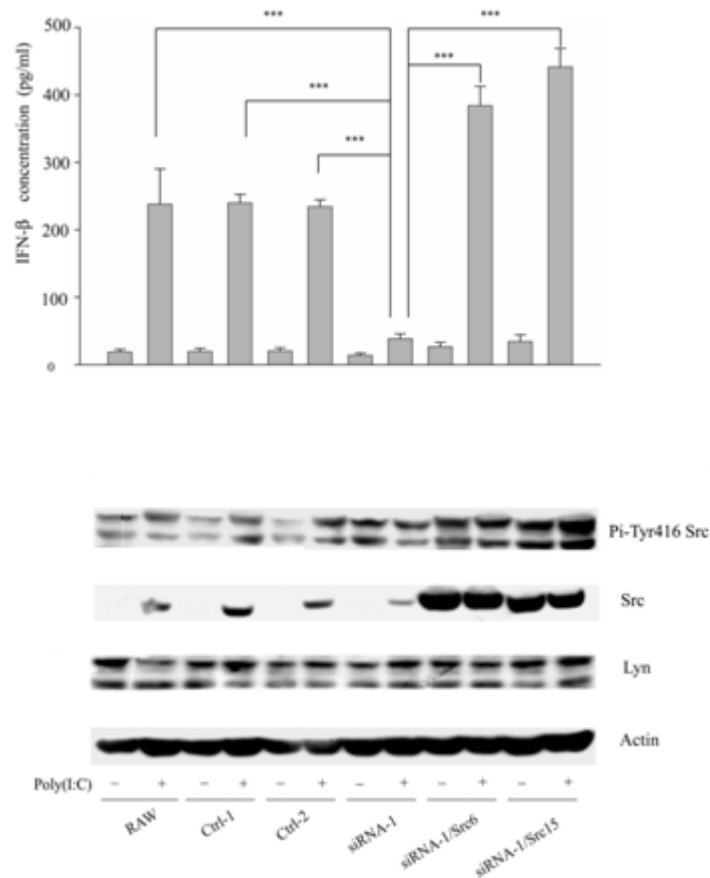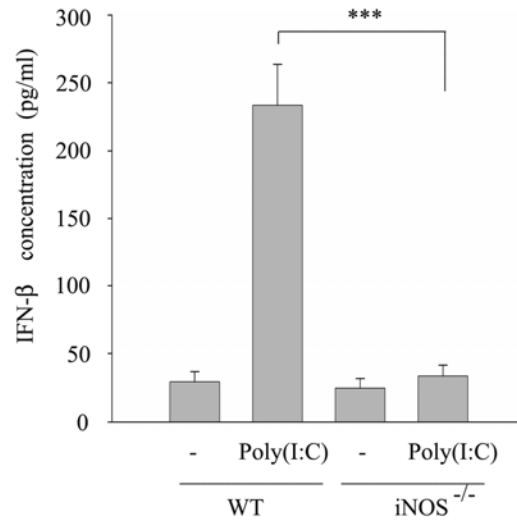
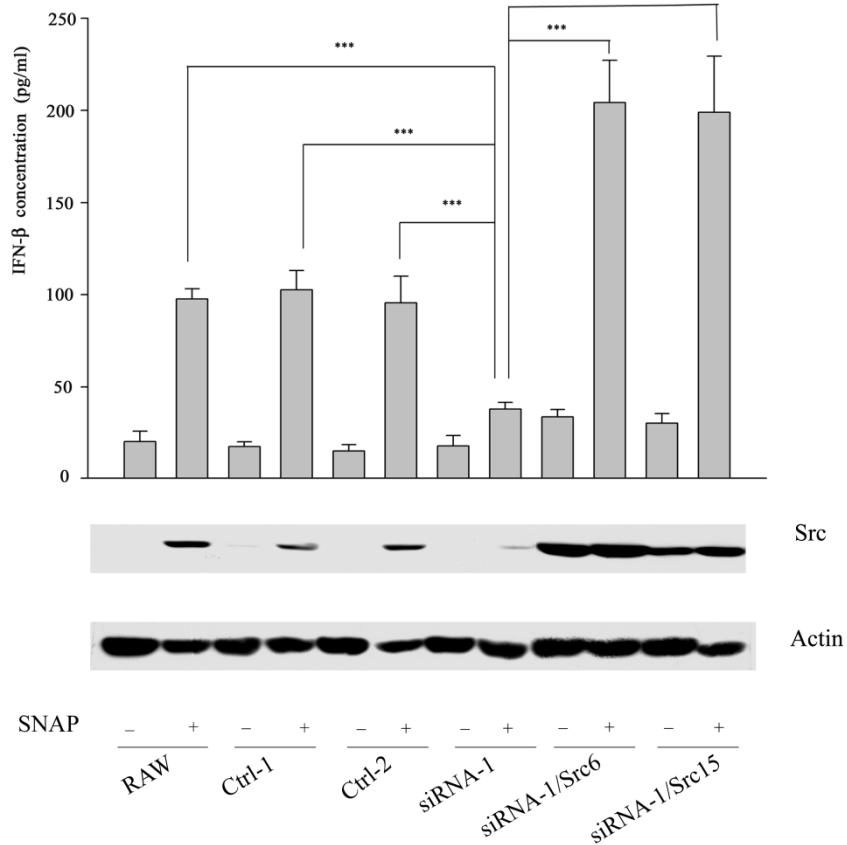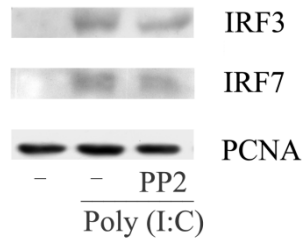**Figure 3. Poly(I:C)-mediated IFN-β secretion was inhibited by src-specific siRNA, which could be reversed by ectopic Src.**

RAW264.7 cell and its derived control (ctrl-1, -2), Src attenuated cells (siRNA-1, -2) and Src attenuated cells harboring plasmid encoding avian Src (siRNA/Src6, siRNA/Src15) were stimulated without or with poly(I:C) (20 μg/ml) for 48 hours. Equal amounts of lysates (80 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. The concentration of IFN-β in culture medium of each group was determined by ELISA. The arrow indicates the position of Src. ***, P<0.001.

(A)
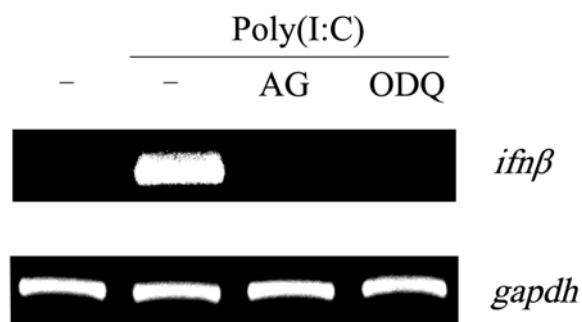
(B)
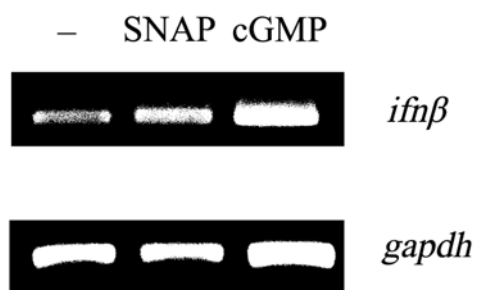
Figure 4. Poly(I:C)-induced *ifn-β* transcript was sensitive to inhibitors of inducible nitric oxide synthase (iNOS) and soluble guanylyl cyclase (sGC).

(A) RAW264.7 cells were pretreated without or with AG (2 mM), ODQ (100 μM) for 30 mins , and then cells were stimulated without and with poly(I:C). (B) RAW264.7 cells were stimulated without and with SNAP (100 μM), 8-br-cGMP (cGMP analogue, 100 μM) for 48 hours. The amount of *ifn-β* transcript was analysis by RT-PCR. *gapdh* was utilized as an internal control for amplification efficiency.

Figure 5. iNOS was involved in poly(I:C)-mediated IFN-β production.

Primary peritoneal macrophages from wild type (WT) and iNOS$^{-/-}$ mice were treated without and with poly(I:C) (20 μg/ml) for 48 hours. The concentration of IFN-β in culture medium of each sample was determined by ELISA. ***, P<0.001.
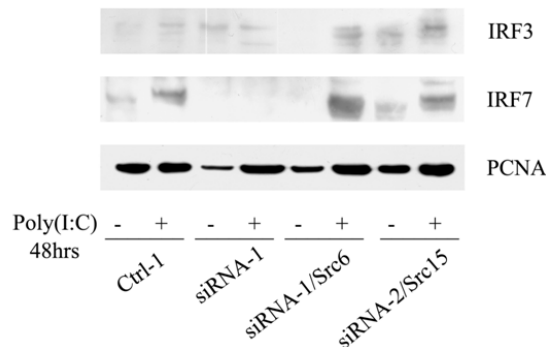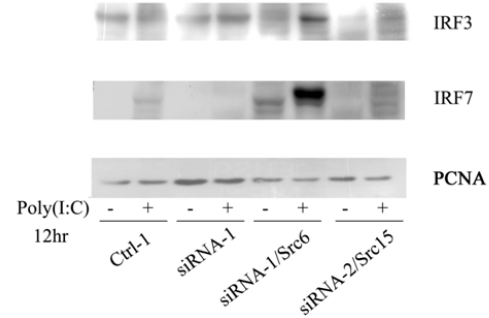
Figure 6. SNAP-mediated IFN-β secretion was inhibited by *src*-specific siRNA, which could be reversed by ectopic Src.

RAW264.7 cell and its derived control (ctrl-1, -2), Src attenuated cells (siRNA-1, -2) and Src attenuated cells harboring plasmid encoding avian Src (siRNA/Src6, siRNA/Src15) were stimulated without or with SNAP for 48 hours. Equal amounts of lysates (80 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. The concentration of IFN-β in culture medium of each group was determined by ELISA. The arrow indicates the position of Src. ***, P<0.001.

(A)

(B)   (C)

Figure 7. Poly(I:C)-mediated IRF7 nuclear translocation was inhibited by *src*-specific siRNA, which could be reversed by ectopic Src.

(A) RAW 264.7 cells were incubated with PP2 (10 μM) after 30 minutes poly(I:C) (20 μg/ml) stimulation. The amount of nuclear IRF3 and IRF7 were analysis by nuclear extraction. (B)(C)The RAW264.7 expressing nonspecific siRNA (Ctrl-1), Src attenuated cells (siRNA-1) and Src attenuated cells harboring plasmid encoding avian Src (siRNA/Src6, siRNA/Src15) were stimulated without or with poly(I:C) (20 μg/ml) for 48 hours and 12 hours. The amount of nuclear IRF3 and IRF7 were analysis by nuclear extraction.
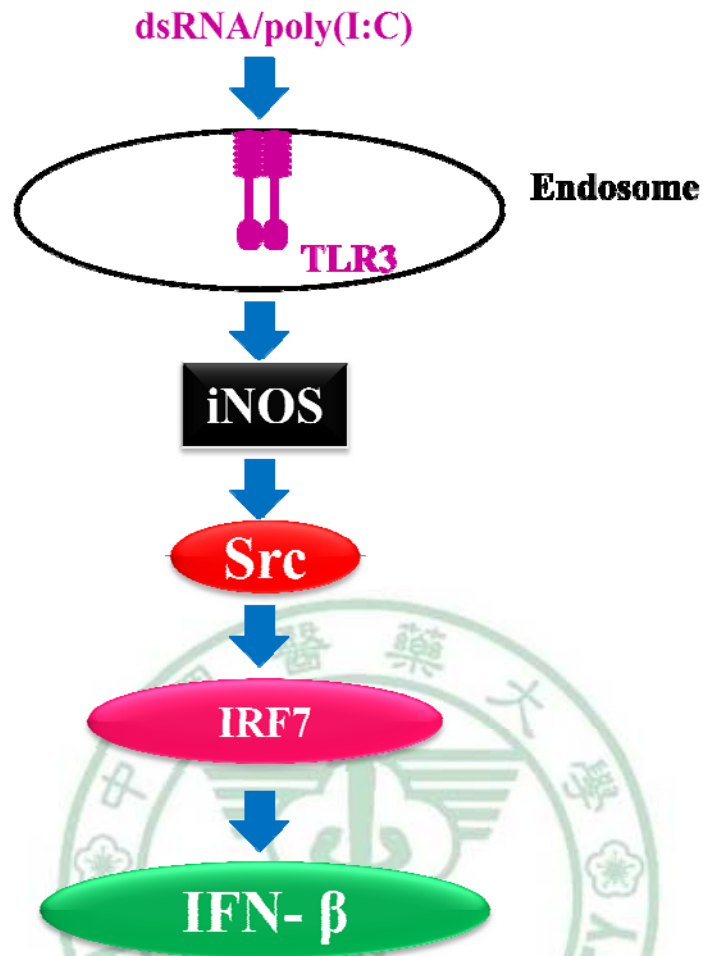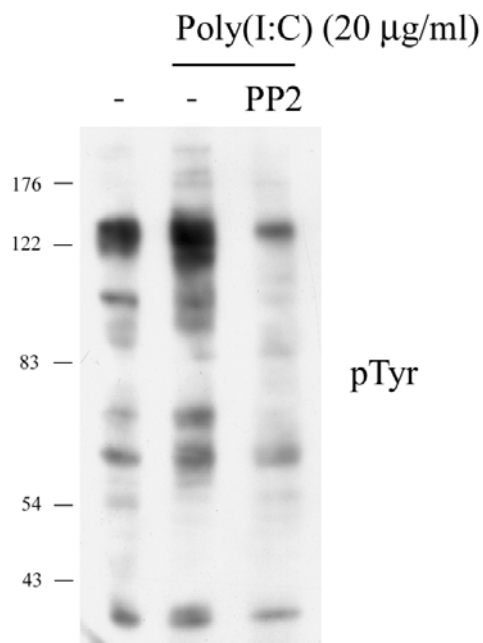
Figure 8. The signaling pathway for IFN-β secretion in poly(I:C)-stimulated macrophages.

Following poly(I:C) treatment, the signal is transduced into macrophages via Toll-like receptor 3, increases expression of iNOS. NO produced by iNOS contribures to Src induction. And Src activation effect the amount of nuclear IRF7 cause IFN-β production.
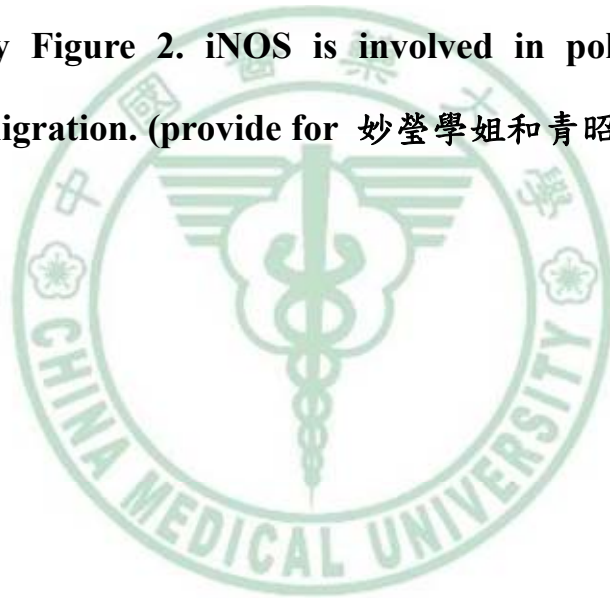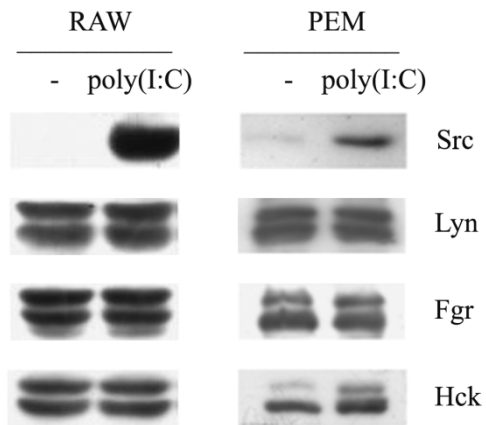
Appendix

Supplementary Figure 1. The inhibitory effect of PP2 on poly(I:C)-induced protein tyrosine phosphorylation. (provide for 妙瑩學姐和青昭學姐)
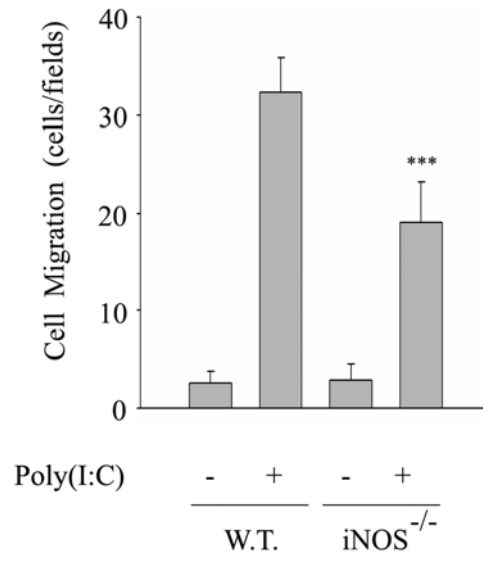
Supplementary Figure 2. iNOS is involved in poly(I:C)-mediated macrophage migration. (provide for 妙瑩學姐和青昭學姐)
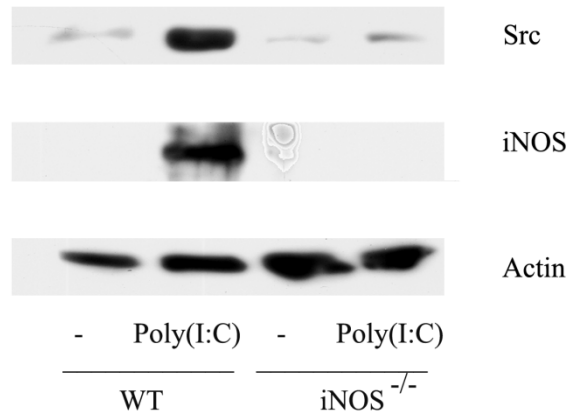
Supplementary Figure 3. Poly(I:C) induced Src expression in murine RAW264.7 macrophages and rat peritoneal macrophages (PEMs). (provide for 妙瑩學姐和青昭學姐)

| | - | Poly(I:C) | - | Poly(I:C) |
| | | WT | | iNOS$^{-/-}$ |

Supplementary Figure 4. Poly(I:C) induced Src expression is dependent on iNOS. (provide for 妙瑩學姐和青昭學姐**)**