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MyD88-dependent protective mechanism of alveolar macrophages induced by *Bacillus subtilis* spore in mice infected with respiratory syncytial virus A2

호흡기성 융합 바이러스 감염 마우스에서 *Bacillus subtilis* 포자에 의해 유도된 폐포 대식세포의 MyD88 신호전달 의존적 방어기전

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농 학 석 사 학 위 논 문

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홍 지 은

홍지은의 석사학위논문을 인준함

2015 년 08 월

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Summary

Respiratory syncytial virus (RSV) is one of the most common respiratory diseases in children and elderly who are immune-compromised. Although induction of successful innate immunity is critical for the protection against viral infection, specific role and defense mechanism of alveolar macrophages (AMs) in RSV infection are yet to be illuminated. Therefore, the objective of the present study was to elucidate the exact role of AMs activated with *Bacillus subtilis* spore in mice infected with RSV.

The results showed that AMs played a pivotal role in the protection during the initial stage of RSV infection and that the administration of spore derived from *Bacillus subtilis* induced activation of AM population coincident with enhancing antiviral effector molecules, GM-CSF and classically activated macrophages (M1 macrophage)-related genes. Furthermore, these protective immune responses were dependent on MyD88 signaling pathway in the AMs.

Pre-treatment with spore through intranasal route induced protective immunity in mice infected with RSV as shown by significantly low viral load at 4 days post infection. It was noting that spore-treated mice displayed the increase of AMs, but not neutrophils or inflammatory monocytes after the infection. Also, these mice showed notably increased level of IFN-β and IL-12p40. When AMs were depleted, mice became intensified the disease severity as shown by persisted high level of viral load with increased pathology scores of pulmonary in the lung resulting a very weak protective efficiency against RSV infection. These results suggest that AMs

treated with spore are indispensable for the effective protection against RSV infection. Furthermore, MyD88$^{-/-}$ mice were unable to induce protective responses regardless of spore treatment, suggesting that the protection by spore-treated AMs was mediated through MyD88-dependent signaling pathway.

In conclusion, I revealed that administration of spore via intranasal route led to the early activation of AMs via MyD88-dependent pathway is responsible for the protective immunity in mice infected with RSV.

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List of Abbreviations

AMs: alveolar macrophages

BAL: bronchoalveolar lavage

BMMs: bone marrow-derived macrophages

CFU: colony forming unit

Clod: clodronate-encapsulated liposome

Cont: control liposome

DPI: day post infection

ELISA: enzyme-linked immunosorbent assay

GM-CSF: granulocyte-macrophage colony-stimulating factor

H&E: Hematoxylin and Eosin

IFN: interferon

i.n.: intranasal

IL: interleukin

i.t.: intratracheal

KO: knock out

MOI: multiplicity of infection

MyD88: myeloid differentiation primary response gene

M1 macrophage: classically activated macrophage

M2 macrophage: alternatively activated macrophage

PFU: plaque-forming unit

qRT-PCR: quantitative real time polymerase chain reaction

RSV: respiratory syncytial virus

TLR: toll-like receptor

TNF: tumor necrosis factor

TSB: tryptic soy broth

WT: wild type

YE: yeast extract

I. Introduction

Respiratory syncytial virus (RSV) causes a common viral infectious disease, especially for the infants and elderly, whose symptom is accompanied by a serious bronchiolitis resulting in up to 200,000 annual deaths worldwide (Nair et al., 2010). Despite the numerous efforts, we have no effective ways to prevent RSV infection until now. RSV non-structural-1 and -2 proteins hinder both innate and adaptive immunities (Meng et al., 2014) causing malfunction of overall immune protection. It has been suggested that the main protection against RSV is mediated via TLR2 and 4 (Kurt-Jones et al., 2000, Murawski et al., 2009) Indeed, poor innate defense mechanism at the initial stage of the infection brought about the serious consequences in RSV infection, and thus several attempts have been made to improve innate immunity.

One of the primary innate cells in the lung is alveolar macrophages (AMs), which play a pivotal role to maintain homeostasis and induce effective defense mechanism. AMs have unique properties compared to macrophages in other tissue as they are in direct contact with external environment that could allow rapid recognition of antigenic molecules and participate in the immediate initiation of host defense (Herold et al., 2011, Hashimoto et al., 2011). Recent studies have shown that collapse of early AM-mediated defense responses caused insufficient protection against various respiratory diseases and led to improper recruitment of immune cells coincident with failing the control of lung homeostasis (Maus et al., 2002, Snelgrove et al., 2008). Despite the importance of AMs in various respiratory

viral diseases, the precise role and protective mechanisms of AMs in RSV infection are yet to be illuminated.

Several attempts using probiotics have gained an insight for the priming of innate immune system where the administration of probiotics induced a rapid activation of innate immunity that acquired enhanced protective property in respiratory viral infections (Park et al., 2013, Garcia-Crespo et al., 2013). Furthermore, spore derived from probiotics is one of the alternatives not only for inducing enhanced innate immune responses but also for better safety and stability than whole bacteria. Hence, in this study, I investigated whether and how spore derived from *Bacillus subtilis* would protect mice from RSV infection.

III. Materials and Methods

Mice

Female BALB/c mice, 6 - 8 week-old, were purchased from Orient Bio Inc., Korea. MyD88$^{-/-}$ mice were purchased from Jackson laboratory (Bar harbor, ME, USA). All the experimental procedures using mice were approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University (Approval NO.: SNU-130527-5), Korea.

Preparation and isolation of Bacillus subtilis spore

Bacillus subtilis was spread in an agar plate containing 3% Trypticase soy broth (TSB), 0.5% Yeast extract (YE) and 1.5% Bacto Agar (all from BD Biosciences, San Diego, CA, USA) and incubated at 37℃ for 9 hours. One colony was picked and inoculated in 25 ml of 3% TSB and 0.5% YE liquid media. Then it was incubated for 5 hours in the shacking incubator at 150 rpm at 37℃ until the OD value reached between 0.45 - 0.6. For sporulation, culture was transferred to 500 ml of the autoclaved media which containing 5 ml of 10% KCl, 5 ml of 1.2% $MgSO_4 \cdot 7H_2O$ (pH 7.6), 0.5 ml of 1 M $Ca(NO_2)_3$, 0.01 M $MnCl_2$, and 1 mM $FeSO_4$. The culture was incubated at 37℃ for 48 hours with shaking at 150 rpm. The cells were collected by centrifugation at 5516g for 10 minutes, resuspended in distilled water, and incubated at 4℃ for 48 hours on the rocker. Then, the cells were sonicated at 35% amplitude (1 watt) for 90 seconds with 0.5 second pulse. Spore

loaded on the layers of 35%, 25%, 15% OptiPrep Density gradient (Sigma-Aldrich, Wt. Louis, MO, USA) was centrifuged at 10,000g for 40 minutes at 25℃ without break for the purification. The spore was washed 3 times with distilled water and resuspended in 1 ml of distilled water.

Preparation and isolation of respiratory syncytial virus A2

RSV A2 strain was amplified as following; HEp-2 cells (ATCC, Manassas, VA, USA) were grown in MEM containing 10% of FBS and 1% of antibiotics. When the cells reached at approximately 80% confluence, the cells were washed and inoculated with 0.01 - 0.05 MOI of virus in MEM containing 1% of antibiotics and 25 mM HEPES (Gibco). The cells were incubated at 37℃ for 2 hours, added MEM containing 6% of FBS only, and then incubated for additional 72 - 96 hours at 37℃. The cells were scraped and combined into the conical tubes on ice and centrifuged at 1,400 rpm for 4 minutes at 4℃. The cell pellets were collected into conical tubes, resuspended with cold conditioned media with 60% sorbitol, sonicated for 15 minutes in slurry ice and centrifuged at 4,000 – 5,000 rpm for 10 minutes at 4℃, and then the sonication and centrifugation step was repeated in the same condition. The supernatants were transferred into new tubes, centrifuged at 23,000 rpm for 1 hour at 4℃, and then discarded. The resulting whitish virus pellet was resuspended with 500 μl of cold MEM and the titer was determined by standard RSV plaque assay.

Isolation and culture of bone marrow-derived macrophages

Bone marrow was flushed from femurs and tibias of 6 – 8 week-old female BALB/c mice (Orient Bio Inc., Korea) using Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 2% FBS (Gibco). Red blood cells were lysed with ACK lysing buffer (Gibco) and whitish marrow cells were seeded in 90 x 15mm Petri dish with complete media containing 10% FBS, 1% antibiotics with 20% L-929 conditioned media (Zhang et al., 2008)(Zhang et al., 2008)(Zhang et al., 2008)(Zhang et al., 2008) and incubated at 37℃ in humidified incubator with 5% CO_2 for 7 days. On day 3, another 5 ml of fresh complete media were added to each dish. On day 7, only adherent cells were collected using non-enzymatic cell dissociation solution (Gibco).

Macrophage cell line

MH-S cells (ATCC, Manassas, VA, USA), mouse alveolar macrophages were grown in RPMI-1640 GlutaMax medium containing 10% FBS, 1.5% antibiotics (all from Gibco) in a 5% CO_2 incubator at 37℃.

RNA isolation and quantitative RT-PCR

To quantitative analyze for the expression of GM-CSF, TNF-α, IFN-γ, IL-12p40, IL-6, PPAR-γ, and TGF-β1 at mRNA level, quantitative real time polymerase chain reaction (qRT-PCR) was conducted. RNA was extracted from perfused lungs using TRIZOL (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated by adding

chloroform followed by centrifugation at 4℃, 12,000g for 15 minutes and addition of isopropanol for 10 minutes at room temperature for RNA precipitation. RNA pellet was obtained by washing with 75% ethanol and air dried for 10-15 minutes then resuspended with DEPC distilled water (Sigma-Aldrich, Wt. Louis, MO, USA) and quantified with NanoDrop (Amersham Bioscience, USA) at A_{260}. One microgram of RNA was reverse transcribed into cDNA and amplified with murine primers specific for GM-CSF (forward primer: 5'-CTGCCTTAAAGGGACCAAGAGA-3', reverse primer: 5'-TTCCGCTGTCCAAGCTGAGT-3'), TNF-α (forward primer: 5'-GCCAACGGCATGGATCTC-3', reverse primer: 5-GTGGGTGAGGAGCACGTAGTC-3'), IFN-γ (forward primer: 5'-GCCATCGGCTGACCTAGAGA-3', reverse primer: 5'-GCAGTGTGTAGCGTTCATTGTCT-3'), IL-12p40 (forward primer: 5'-GAAAGGTGCGTTCCTCGTAGA-3', reverse primer: 5'-GGAACACATGCCCACTTGCT-3'), IL-6 (forward primer: 5'-CACAGAGGATACCACTCCCAACA-3', reverse primer: 5'-TCAGAATTGCCATTGCACAAC-3'), PPAR-γ (forward primer: 5'-CAGGAGCCTGTGAGACCAACA-3', reverse primer: 5'-ATCAGTGGTTCACCGCTTCTTT-3'), TGF-β1 (forward primer: 5'-TCGTCTGCATTGCACTTATGC-3', reverse primer: 5'-GTGGTGCCCTCTGAAATGAAA-3') and GAPDH (forward primer: 5'-CTCCACTCACGGCAAATTCA-3', reverse primer: 5'-GCCTCACCCCATTTGATGTT-3'). Real-time PCR was performed using Power

SYBR Green PCR master mix (Applied Biosystem, Waltham, MA, USA) and analysis of the data was performed by One-step RT PCR (Applied Biosystem). Target gene expression was normalized to GAPDH expression.

Measurement of cytokine production

Bronchoalveolar lavage (BAL) samples were collected via tracheotomy using 600 μl of PBS and the cells were separated from the BAL fluid by centrifugation at 1400 rpm for 5 minutes at 4℃. For *in vitro* experiments, supernatants from bone marrow-derived macrophages or MH-S after virus infection were carefully collected. Production of IFN-β (BioLegend, San Diego, CA, USA), IL-12p40, GM-CSF and TNF-α (R&D System, Minneapolis, MN, USA) was examined using ELISA kit.

Phenotypic characterization of the cells

To examine the absolute number of AMs and other innate immune cells, BAL cells were collected as above and perfused lungs were isolated and minced through 70 μm cell strainer using MEM. The cells were stained with FITC-conjugated anti-CD11c (HL3), PE-conjugated anti-Siglec-F (E50-2440), PerCP-conjugated anti-Ly6C (AL-21), PE-Cy7-conjugated anti-Ly6G (1A8), APC-conjugated anti-CD11b (M1-70) or APC-conjugated anti-F4/80 (BM8), and APC-Cy7-conjugated anti-CD45 (30-F11) (all from BD Biosciences except anti-F4/80 from BioLegend). The cells were acquired using FACS LSR II and flow cytometric data were analyzed by using FlowJo software (Tree Star, San Carlos, CA, USA). We defined

$CD45^+Ly6C^-Ly6G^-CD11c^+Sigleg\text{-}F^+F4/80^+$ as AMs, $CD45^+CD11b^+Ly6C^+Ly6G^-$ for inflammatory monocytes, and $CD45^+CD11b^+Ly6G^+Ly6C^-$ for neutrophils.

Selective depletion and adoptive transfer of alveolar macrophages

To selectively deplete AMs, 350 mg per mouse of clodronate-encapsulated liposome (FormuMax Scientific Inc, CA, USA) were given i.t. in a volume of 50 µl at 1 and 3 days before the challenge with or without spore treatment. To verify the depletion of AMs, naïve mice were given control liposome via i.t. The data were acquired using FACS LSR II and acquired data were analyzed by using FlowJo software.

Virus titration in the lung

To determine the viral titers, lungs from RSV-infected mice were isolated at day 4 post-infection. The lungs were minced through 70-µm cell strainer using cold MEM. Cell lysates were collected and RSV titers were determined by plaque assay using HEp-2 cells. The virus titers in the whole lung were normalized to weight of the lung tissue and indicated as PFU/g.

Lung histology and pathology scoring

For histology studies, mice were administered with spore and/or clodronate encapsulated liposome prior to RSV infection. For control, mice were administered with PBS or control liposome. At 4 days post infection, blood perfused lungs were

fixed with 4% paraformaldehyde and embedded in paraffin. Lung sections were produced and stained with Hematoxylin and Eosin to examine the abnormalities. Four inflammatory parameters were scored independently from 0 to 5 for each section: alveolitis (inflammatory cells within alveolar spaces), interstitial pneumonitis (increased thickness of alveolar walls associated with inflammatory cells), peribronchiolitis (inflammatory cells surrounding a bronchiole), and perivasculitis (inflammatory cells surrounding a blood vessel). Slides were randomized, read blindly, and scored for each parameter.

Spore administration and infection in mice

Each mouse was administered with either 1 x 10^9 CFU of spore or, as a control PBS i.n. in a volume of 20 μl at 5 days before the infection. Then, the mice were infected i.t. with 2 x 10^6 PFU of live RSV A2.

Statistical analysis

Statistical significance was analyzed with Anova test and considered statistically significant at *P* value less than 0.05.

III. Results

1) Delivery of spore through intranasal route induces protective immunity in mice infected with RSV

It has been suggested that administration of probiotics or probiotics-derived biomaterials for the induction of innate immunity showed a certain level of protection against respiratory viral infection (Kiso et al., 2013, Lee et al., 2013)(Kiso et al., 2013, Lee et al., 2013)(Kiso et al., 2013, Lee et al., 2013) (Kiso et al., 2013, Lee et al., 2013). This raises a question whether pre-treatment of spore derived from *Bacillus subtilis* via intranasal (i.n.) route would alter the outcome of RSV infection. To address this, a group of mice was administered i.n. with spore or PBS at 5 days prior to RSV infection. Mice pre-administered with spore did not cause a significant change of body weight (Fig. 1A). The striking finding was, however, that viral counts in the lung at 4 days post-infection (DPI) were considerably lower in spore-treated mice than PBS-treated mice (Fig. 1B). These results suggest that i.n. pre-delivery of spore would enhance a anti-viral immunity against RSV infection.

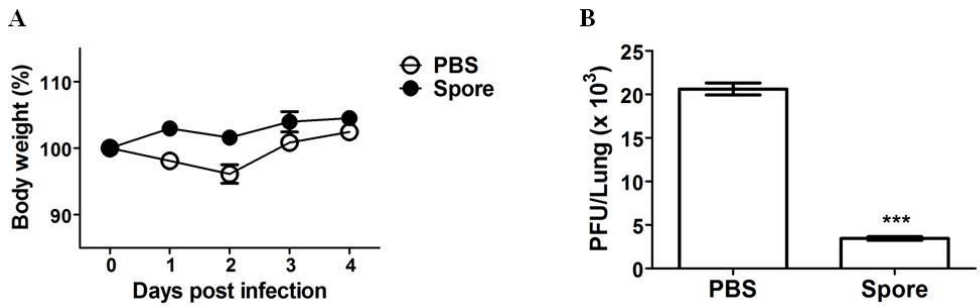


Figure 1. Pre-treatment with spore through intranasal route reduces the disease severity following RSV infection. Mice were administered with 1 x 10^9 CFU of spore via i.n. route at 5 days prior to RSV infection with 2 x 10^6 PFU per mouse. (A) Body weight was monitored daily after the infection and (B) viral load in the lungs was analyzed by plaque assay at 4 days post-infection. Data are expressed as mean ± S.E.M. for the group (n=5). *** indicates that the values are significantly different at $P < 0.001$ compared to the control.

2) Administration of spore via intranasal route increases the number of alveolar macrophages

I found that mice pre-treated with spore induced the unique change of innate immune cell population, especially alveolar macrophages (AMs) in the lung and bronchoalveolar lavage (BAL). Delivery of spore via i.n. changed GM-CSF and M1 macrophage-related cytokines (Murray et al., 2014)(Murray et al., 2014)(Murray et al., 2014)(Murray et al., 2014) including TNF-α, IFN-γ, IL-12p40 and IL-6 both at mRNA (Supplementary Fig. 1A) and protein levels with high concentration throughout the experimental period (Supplementary Fig. 1B). GM-CSF was shown to influence the ability of AMs for their survival, local differentiation, replenishment and of host defense (Shibata et al., 2001, Steinwede et al., 2011)(Shibata et al., 2001, Steinwede et al., 2011)(Shibata et al., 2001, Steinwede et al., 2011)(Shibata et al., 2001, Steinwede et al., 2011). On the contrary, the expression of TGF-β1 which is the typical M2 macrophage-related gene showed no significant changes regardless of spore pre-treatment (Supplementary Fig. 1A). Interestingly, in line with enhanced expression of M1 macrophage-related cytokines, spore pre-treated mice after RSV infection showed significantly high number of AMs in both lung (Fig. 2A and Supplementary Fig. 2A) and BAL (Fig. 2B and Supplementary Fig. 2B), together with increased levels of IFN-β and IL-12p40 (Fig. 2C). These results imply that intranasal administration of spore induced significantly high number of AMs, and augmented expression of GM-CSF and cytokines characteristics of M1 macrophage differentiation.

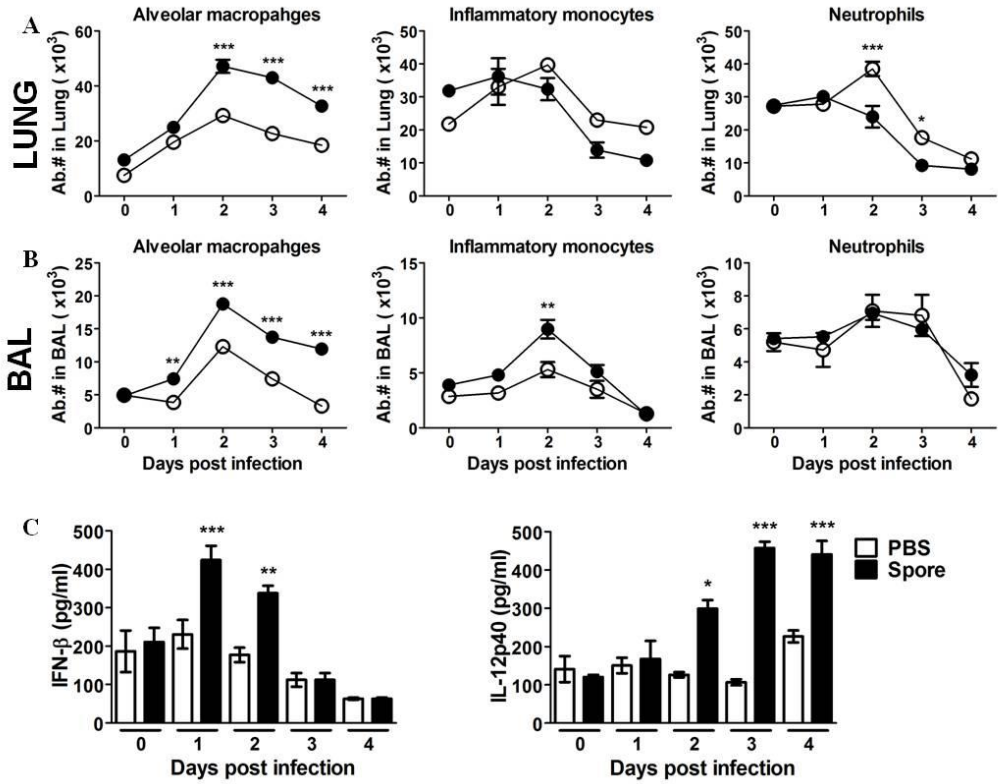


Figure 2. Pre-treatment with spore in mice infected with RSV induces the population change of alveolar macrophages and enhances antiviral effector molecules. Mice were administered with spore via i.n. route at 5 days prior to RSV infection. Then, (A) change of various innate immune cells in the post-lavaged lung and (B) BAL fluid was analyzed by flow cytometry at 0 to 4 DPI. Empty and filled circles indicate PBS and spore pre-treated mice, respectively. (C) IFN-β and IL-12p40 in BAL fluid were measured by ELISA. Data are expressed as mean ± S.E.M. for the group (n=5). Significant differences from results with the PBS control are *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, respectively.

3) Depletion of alveolar macrophages aggravates disease severity in mice infected with RSV

Absence of AMs, by depletion or viral infection, during the early phase of infection aggravates disease severity (Schneider et al., 2014a)(Schneider et al., 2014a)(Schneider et al., 2014a)(Schneider et al., 2014a) because of alarming failure with no scavenger activity to infected cells (Pribul et al., 2008, Kumagai et al., 2007)(Pribul et al., 2008, Kumagai et al., 2007)(Pribul et al., 2008, Kumagai et al., 2007) (Pribul et al., 2008, Kumagai et al., 2007). Moreover, mice depleted AMs are unable to deliver the antigen to dendritic cells for the antigen presentation (Ugonna et al., 2014)(Ugonna et al., 2014)(Ugonna et al., 2014)(Ugonna et al., 2014) and strengthen the immune response. To determine the particular role of AMs in RSV infection, I conducted the depletion of AMs by injection of clodronate-encapsulated liposome (Clod) twice through the intratracheal (i.t.) route at days 1 and 3. As a result, absolute number of AMs in the lungs and BAL from Clod-treated mice declined by approximately 85 percent compared to that in control (Supplementary Fig. 3A) without significant histological changes (Fig. 3C, left). At 1 DPI, AM-depleted mice showed a shape decline in body weight (Fig. 3A) and at 4 DPI, significantly higher lung viral counts than those of control (Fig. 3B). The pathological results displayed thickened alveolar epithelium, destruction of epithelial walls, overall alveolar swelling and the accumulation of immune cells in the lung compared to the control group which showed partial and slight destruction of epithelium (Fig. 3C, right and D). Furthermore, the accumulation of inflammatory cells in the interstitial space, bronchi and vessel was apparent in

lungs from AM-depleted mice (Fig. 3D and Supplementary Fig. 4A). Taken together, the depletion of AMs at the initial phase of infection leads to the exacerbated disease severity coincide with increased pulmonary inflammation.

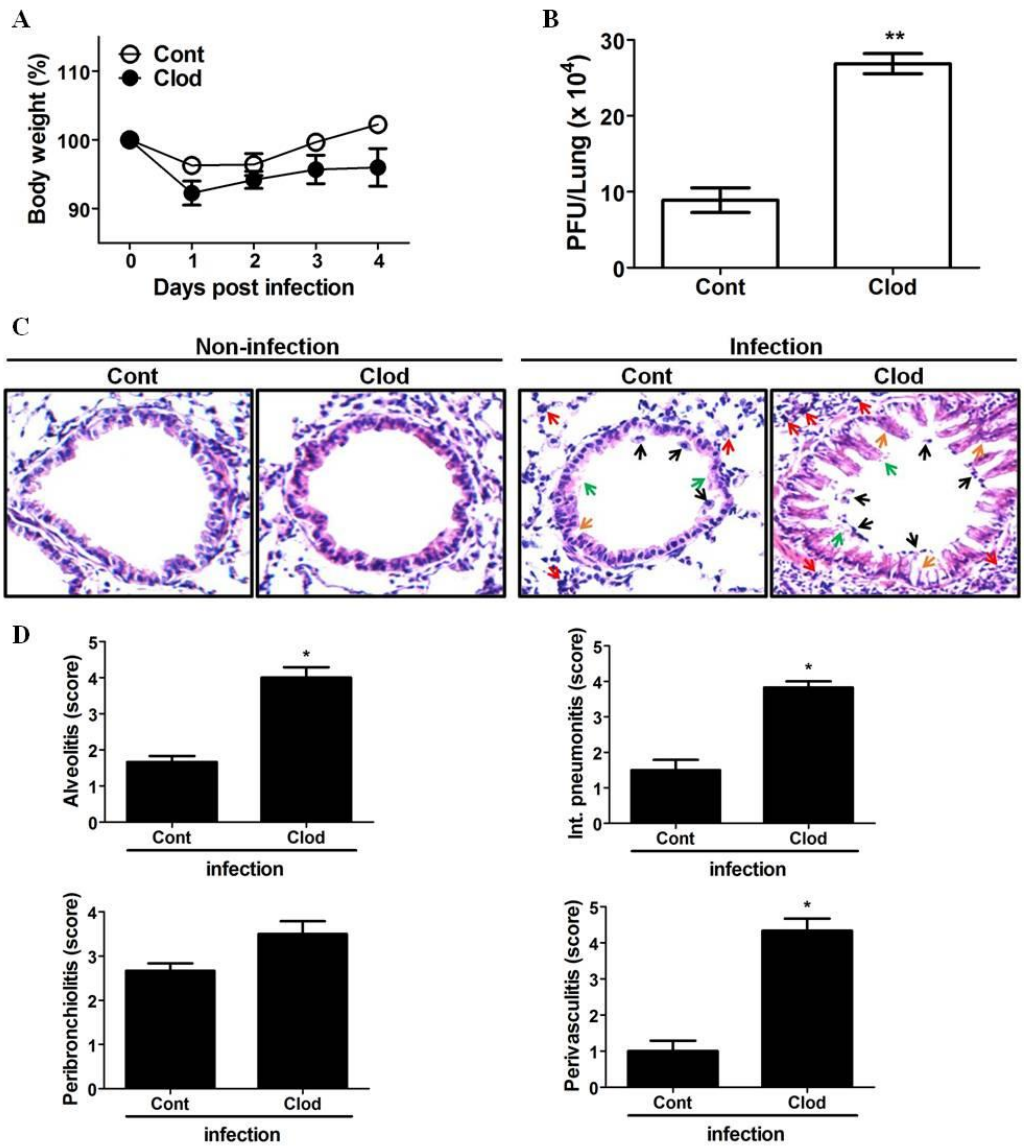


Figure 3. Mice selectively depleted with alveolar macrophages fail to protect RSV infection. Mice were injected i.t. with clodronate-encapsulated liposome twice on days 3 and 1 prior to RSV infection. (A) Body weight was monitored daily after the infection and (B) viral load in the lungs was analyzed by plaque assay at 4 DPI. At 4 DPI, perfused lungs were stained with H&E (C) for histological examination by microscopy at 200 x magnifications and (D) scored for histopathology. 'Cont' indicates the mice injected with control liposome and 'Clod' indicates the mice injected with clodronate-encapsulated liposome. Arrows indicated as follows; orange: epithelium thickness and destruction, green: pulmonary edema, red: inflammatory cells, and black: cell death. Data are expressed as mean ± S.E.M. for the group (n=5). * and ** indicate significant differences at $P < 0.05$ and, $P < 0.01$, respectively.

4) Spore directly enhances the antiviral function of alveolar macrophages

To explore direct effect of spore on AMs, MH-S, AM cell line originated from BALB/c mice was used. The cells were treated with spore for 24 hours and then infected with RSV. The number of plaques was significantly reduced when the AM cell line was incubated with spore in a dose-dependent manner (Fig. 4A and Supplementary Fig. 5A). Inflammatory cytokines, IL-12p40 and IL-6, in the supernatant from the cells treated with spore followed by RSV infection were measured as an initial assessment for their antiviral activity (Puddu et al., 1997)(Puddu et al., 1997)(Puddu et al., 1997)(Puddu et al., 1997). The results showed that the spore treatment led to substantial increase of IL-6 and, to a lesser extent, IL-12p40 (Fig. 4B and Supplementary Fig 5B). These results demonstrated that spore directly promotes the antiviral activity of AMs, especially at the early time point after the infection, in a spore dose-dependent manner.

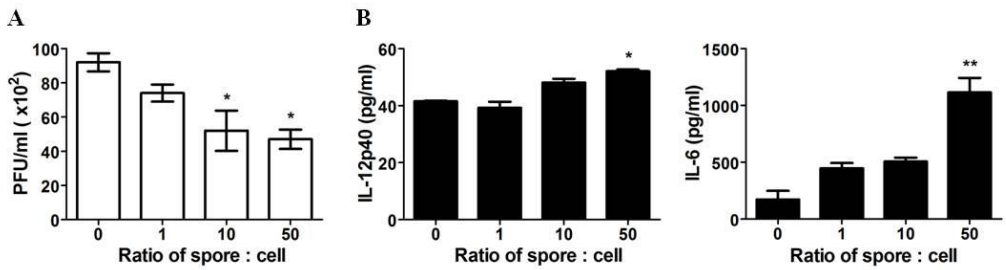


Figure 4. Alveolar macrophages pre-treated with spore extend their antiviral effects. MH-S cells were stimulated with spore for 24 hours, with a ratio of MH-S:Spore at = 1:0, 1:1, 1:10, and 1:50. Then, the cells were washed and infected with 1 MOI virus for 12 hours. (A) The viral load was analyzed by using plaque assay and (B) the production of IL-12p40 and IL-6 was measured by ELISA. Data are expressed as mean ± S.E.M. of three replicates. * and ** indicate significant differences at $P < 0.05$ and, $P < 0.01$, respectively.

5) Alveolar macrophages activated by spore have a pivotal protective role in mice infected with RSV

To validate direct involvement of spore-activated AMs for the protection from RSV infection *in vivo*, spore pre-administered mice were depleted for AMs before RSV infection. It was noting that mice treated with spore did not have any significant weight loss (Fig. 5A), in line with the ability of spore-treated mice to better clear the virus (Fig. 5B). However, spore-treated mice with AMs depleted showed a moderate decrease of body weight (Fig. 5A) and led to viral counts be increased in the lung compared to those without AMs depletion (Fig. 5B), suggesting that AMs play an important role in the protection against RSV infection. To note, mice infected with RSV showed a severe weight loss especially at the early stage after the infection as expected. Severe destruction of epithelium and infiltration of immune cells in alveolus (Fig. 5C) and bronchi (Supplementary Fig. 6) were observed in lungs from the infected mice without spore treatment, resulting in high pathological score (Fig. 5D). It was obvious that the protection efficacy of spore become less effective in mice when depleted AMs. Taken together, these results support the hypothesis that the spore enhances host protection against RSV infection by enhancing the activation of AMs.

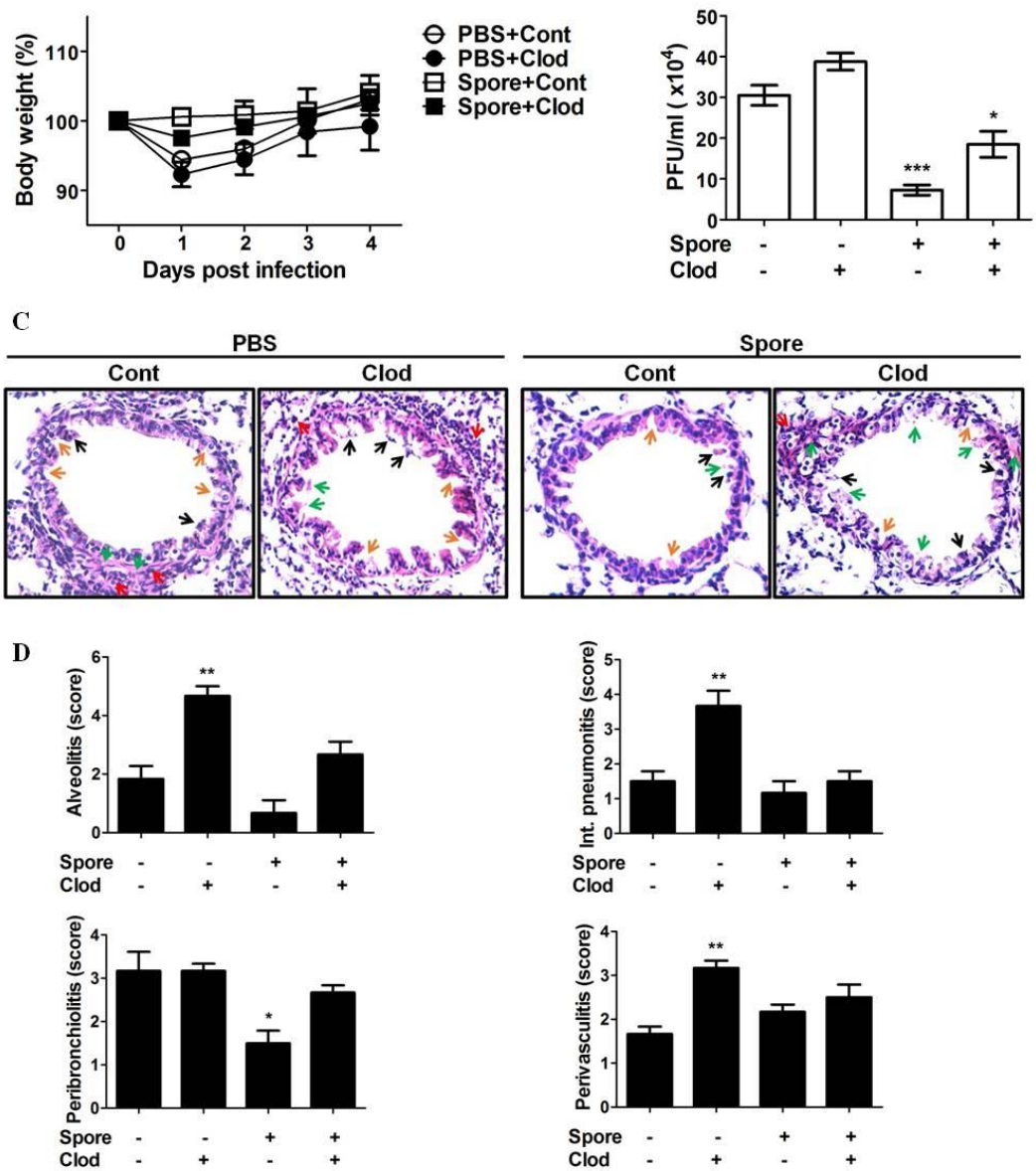
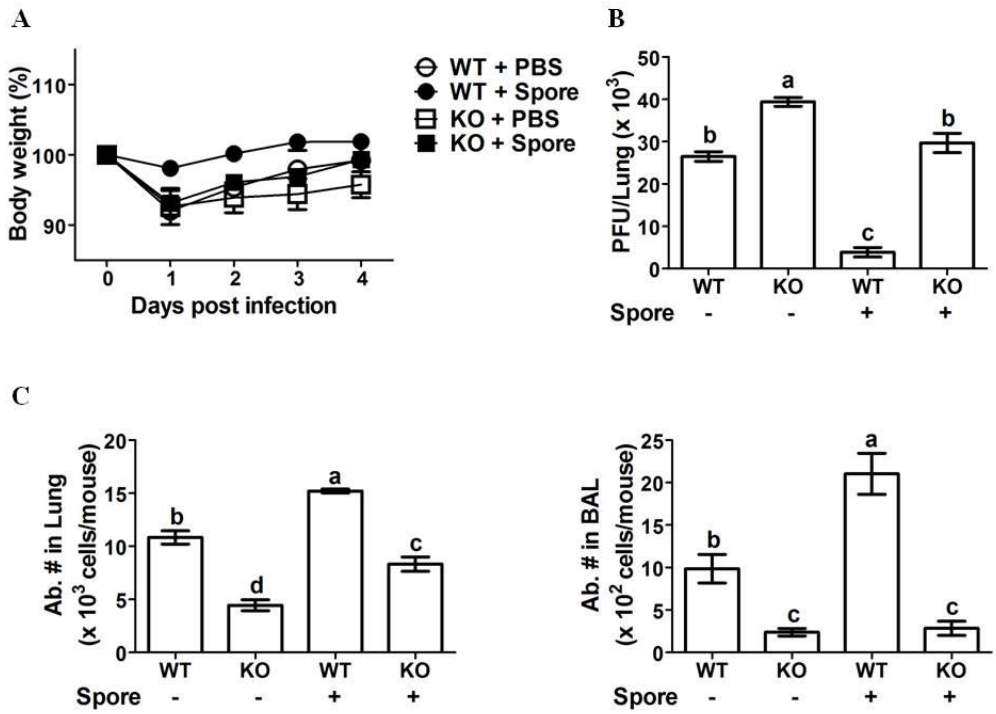


Figure 5. Alveolar macrophages in mice intranasally administered with spore play a key role in RSV infection. Mice were intranasally administered with spore at 5 days prior to RSV infection and injected with control or clodronate-

encapsulated liposome through intratracheal on days 3 and 1 before the infection and sacrifice on 4 DPI. (A) Body weight was monitored daily after the infection and (B) viral load in the lung was examined at 4 DPI, respectively. (C) At 4 DPI, perfused lungs were stained with H&E for histological examination by microscopy at 200 x magnification and (D) scored for histopathology. Arrows indicated are as follows; orange: epithelium thickness and destruction, green: pulmonary edema, red: inflammatory cells, and black: cell death. Data are expressed as mean ± S.E.M. for the group (n=5). *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

6) Protection of mice infected with RSV is enhanced by spore treatment through MyD88-dependent manner

The next question was the signaling pathways involved in protection and antiviral functions in AMs treated with spore. Since viral titer was prolonged in TLR2 or TLR4 knockout mouse (Kurt-Jones et al., 2000, Murawski et al., 2009)(Kurt-Jones et al., 2000, Murawski et al., 2009)(Kurt-Jones et al., 2000, Murawski et al., 2009)(Kurt-Jones et al., 2000, Murawski et al., 2009), I raised a question whether MyD88 downstream signal of TLRs in AMs is necessary against RSV infection. Interestingly, MyD88$^{-/-}$ mice were unable to induce the protective response showing the reduction of body weight (Fig. 6A) with significantly high viral load (Fig. 6B) regardless of spore pre-treatment. Consistent with these, infiltration of AMs in the lungs and BAL (Fig. 6C) was indistinguishable and inflammation with cell death and severe destruction of epithelium in alveolus (Fig. 6D, E) and bronchi (Supplementary Fig. 7) were found in MyD88$^{-/-}$ mice. In addition, the disease severity was far worsen in MyD88$^{-/-}$ mice than wild-type mice and no rescuable by spore treatment, as illustrated by decline of body weight, high level of viral titer, the failure of AMs infiltration, and the increase of AMs number, and the severe pathological results. Taken together, these results suggest that spore may serve as TLR-like ligands and act directly on AMs probably through the activation of MyD88 signal, which in turn induce expression of genes that exert antiviral functions in mice infected with RSV.


A
Body weight (%)
110
100
90
0
1
2
3
4
Days post infection
WT + PBS
WT + Spore
KO + PBS
KO + Spore
B
PFU/Lung (x 10^3)
50
40
30
20
10
0
a
b
c
b
WT
KO
WT
KO
Spore
-
-
+
+
C
Ab. # in Lung
(x 10^3 cells/mouse)
20
15
10
5
0
b
d
a
c
WT
KO
WT
KO
Spore
-
-
+
+
Ab. # in BAL
(x 10^2 cells/mouse)
25
20
15
10
5
0
b
c
a
c
WT
KO
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KO
Spore
-
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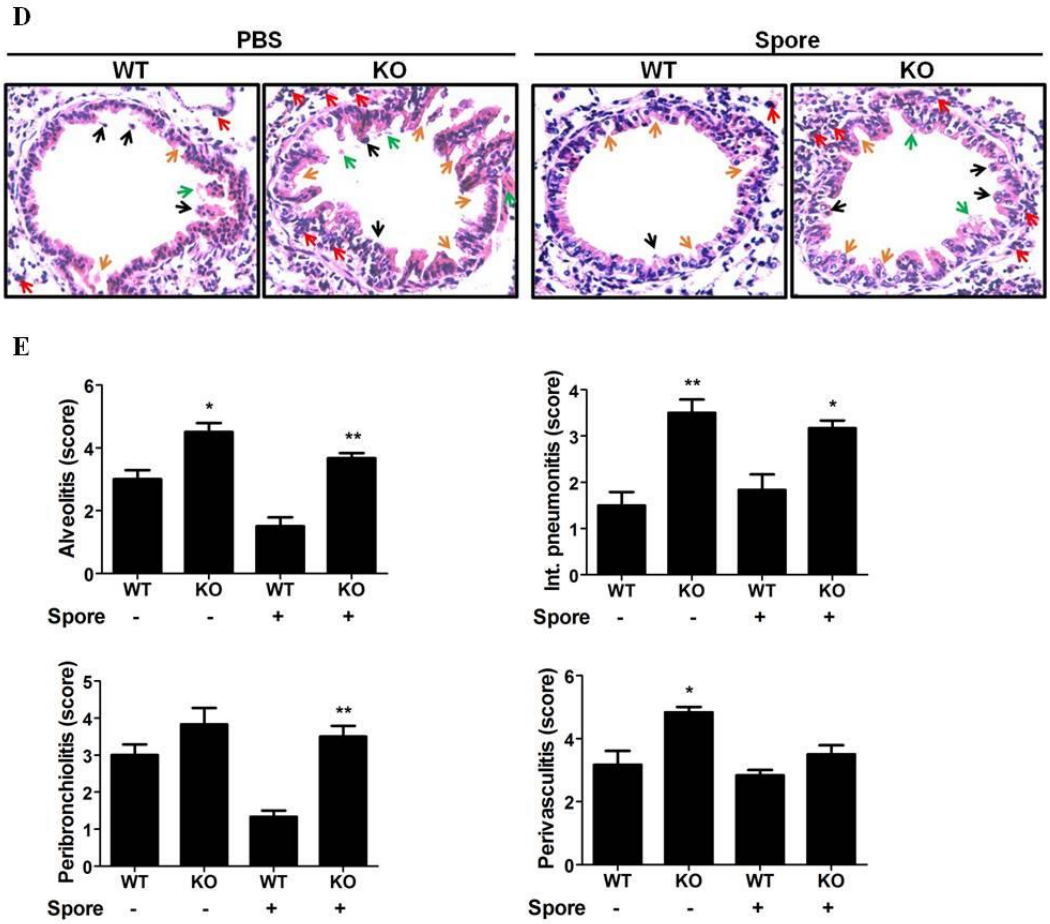

Figure 6. Protective mechanisms of spore pre-treated mice infected with RSV are dependent on MyD88 signaling in alveolar macrophages. Wild type or MyD88 knockout BALB/c mice were administered i.n. with spore at 5 days prior to RSV infection. (A) Body weight of wild type or MyD88 knockout mice was monitored daily after the infection. At 4 DPI, (B) viral load in the lungs was analyzed by plaque assay and (C) absolute number of AMs in post lavaged lungs and BAL cells were acquired by flow cytometry. (D) Blood-perfused lungs were stained with H&E for histological examination by microscopy at 200X

magnification and (E) scored for histopathology at 4 DPI. Arrows indicated are as follow; orange: epithelium thickness and destruction, green: pulmonary edema, red: inflammatory cells, and black: cell death. Data are expressed as mean ± S.E.M. for the group (n=5) or 3 independent experiments. * and ** indicate significant differences at $P < 0.05$ and, $P < 0.01$, respectively.

7) Improvement of antiviral activity of macrophages treated with spore is dependent on MyD88 signaling pathway

To confirm the necessity of MyD88-signaling in macrophages activated with spore, I have used bone marrow-derived macrophages (BMMs) from wild type or MyD88 knockout mouse. After the spore treatment and RSV infection, the viral titer in the culture supernatant was examined. Significantly high viral counts from MyD88-deficient BMMs were observed when compared to those of wild-type BMMs (Fig. 7A and Supplementary Fig. 8A) and spore-treated BMMs showed a meaningful reduction of viral load in a dose-dependent manner both MyD88-deficient and wild-type BMMs.. Furthermore, the ability to produce IL-12p40, best known to induce IFN-γ, was markedly augmented by spore treatment and substantially higher in wild-type BMMs than MyD88-deficient BMMs (Fig. 7B and Supplementary Fig. 8B). Hence, these results strongly support the idea that protective mechanism of macrophages by spore was indeed mediated via MyD88-dependent signaling.

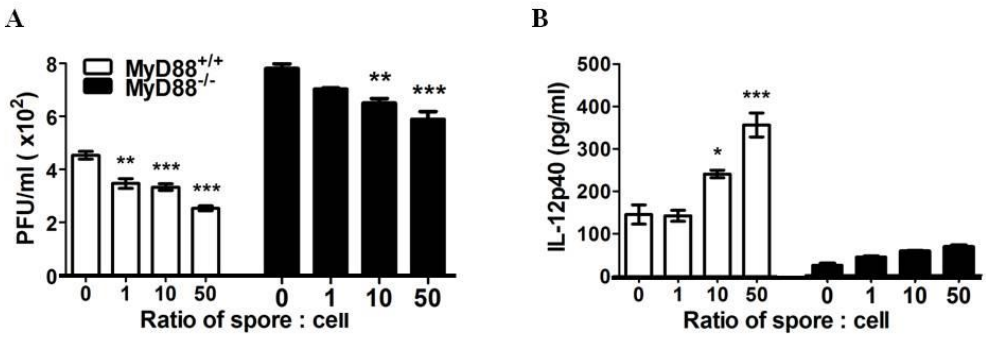

Figure 7. Bone marrow-derived macrophages pre-treated with spore develop their antiviral effects through MyD88-dependent pathway. Wild type or MyD88 knockout BMMs were treated with spore for 24 hour at ratio of spore per number of cells for 0, 1, 10, and 50. Then, the cells were washed and infected with 1 MOI virus for additional 12 hours. (A) Viral titers from wild type or MyD88 knockout BMMs were measured by plaque assay and (B) the production of IL-12p40 was measured by ELISA. Data are expressed as mean ± S.E.M. for 3 independent experiments. *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

IV. Supplementary results

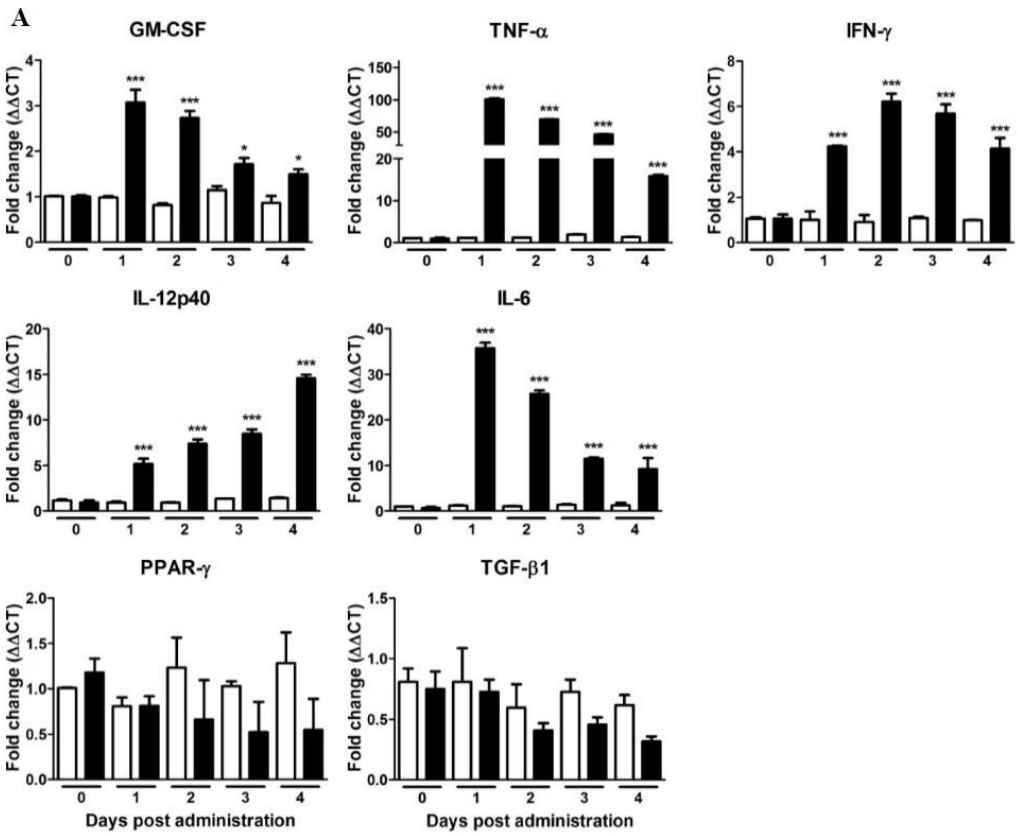

Figure S1. Intranasal administration with spore induces differentiation of M1 alveolar macrophages and expression of GM-CSF. Mice were administered with spore via intranasal route and then, blood perfused lungs were taken every day for 4 days. (A) Expression of genes associated with differentiation of M1 (TNF-α, IFN-γ, IL-12p40 and IL-6) or M2 (TGF-β) macrophages, GM-CSF, PPAR-γ were analyzed by quantitative real-time PCR. (B) Protein levels of GM-CSF and TNF-α were measured by ELISA. Empty and filled bars indicate PBS and Spore pre-treated mice, respectively. Data are presented as means ± S.E.M. (N=3). *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

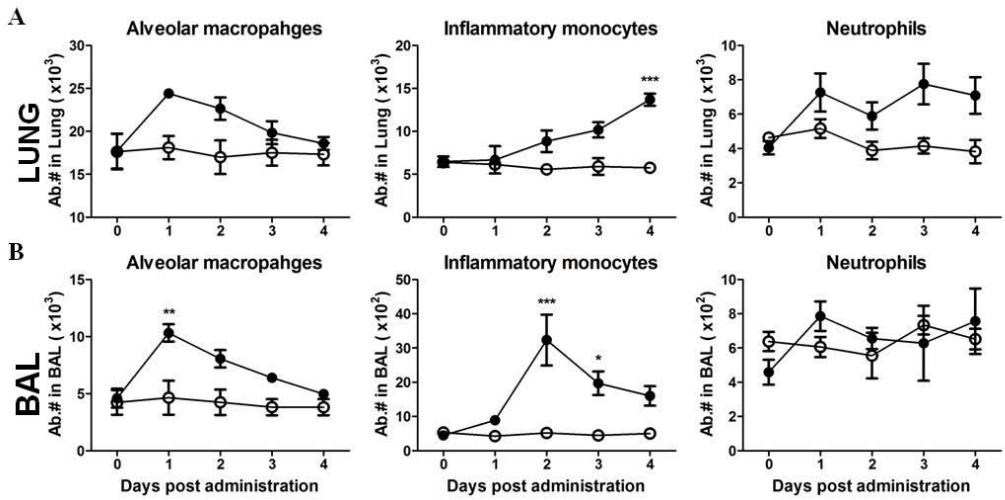

Figure S2. Intranasal administration with spore increases the number of alveolar macrophages. Mice were administered with spore via intranasal route and then, blood perfused lungs were taken every day for 4 days. (A) Change of various innate immune cells in the post-lavaged lung and (B) BAL fluid was analyzed by flow cytometry at 0 to 4 days post administration. Empty and filled circles indicate PBS and Spore pre-treated mice, respectively. Data are presented as means ± S.E.M. (N=3). *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

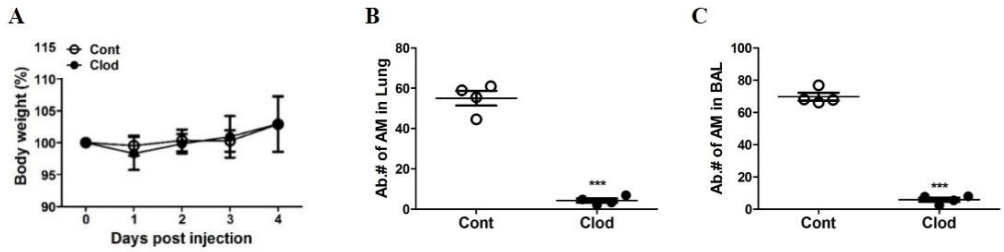

Figure S3. Intratracheal injection of clodronate-encapsulated liposome leads to the efficient depletion of alveolar macrophages. Mice were injected with clodronate-encapsulated liposome via intratracheal route at days 1 and 3 before sacrifice. (A) Body weight was monitored daily after the injection, and absolute number of alveolar macrophages in the (B) lung and (C) BAL was analyzed at day 4 post administration. 'Cont' and 'Clod' indicate the mice injected with control liposome and clodronate-encapsulated liposome, respectively. Data are expressed as mean ± S.E.M. for the group (n=5). *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

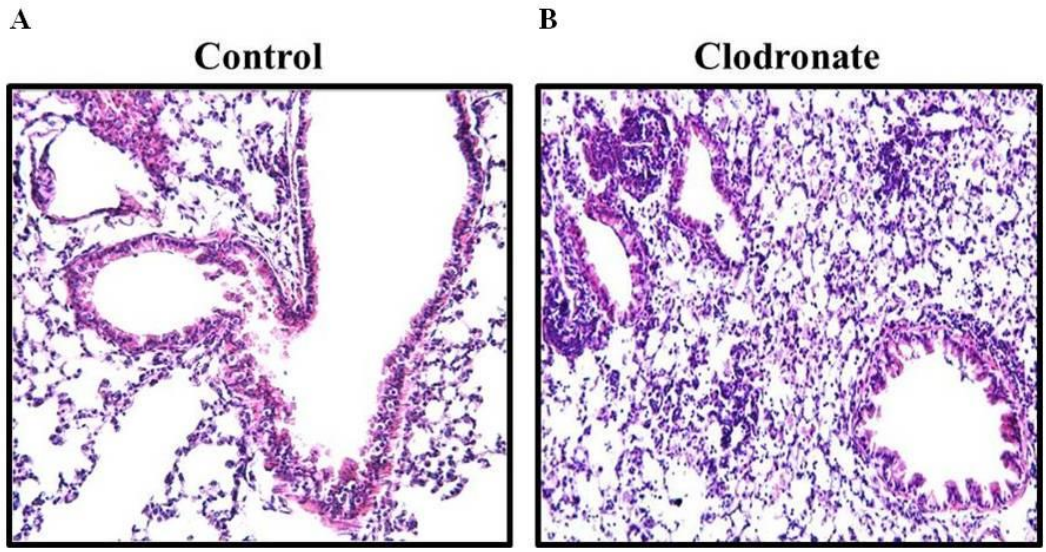

Figure S4. Depletion of alveolar macrophages exacerbates the pathology in RSV infected mice. Mice were injected with clodronate-encapsulated liposome through intra tracheal route on days 1 and 3 before RSV infection. At 4 DPI, perfused lungs were stained with H&E for histological examination by microscopy at 100 x magnification. Bronchus and blood vessel of (A) control and (B) clodronate-encapsulated liposome injected mice are shown.

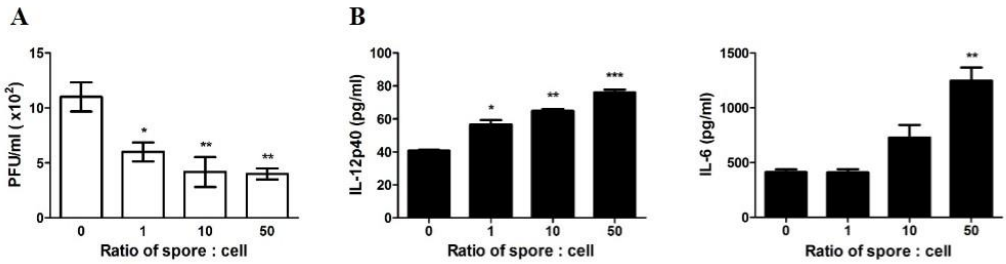

Figure S5. Alveolar macrophages cell line pre-treated with spore enhances antiviral effects. MH-S cells were stimulated with spore for 24 hours at the ratio of spore per number of cells for 0, 1, 10, and 50. Then, the cells were washed and infected with 1 MOI virus for 24 hours. (A) Viral load was analyzed by using plaque assay and (B) the production of IL-12p40 and IL-6 were measured by ELISA at 24 hours post infection. Data are expressed as mean ± S.E.M. for 2 independent experiments. *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively compared with the control.

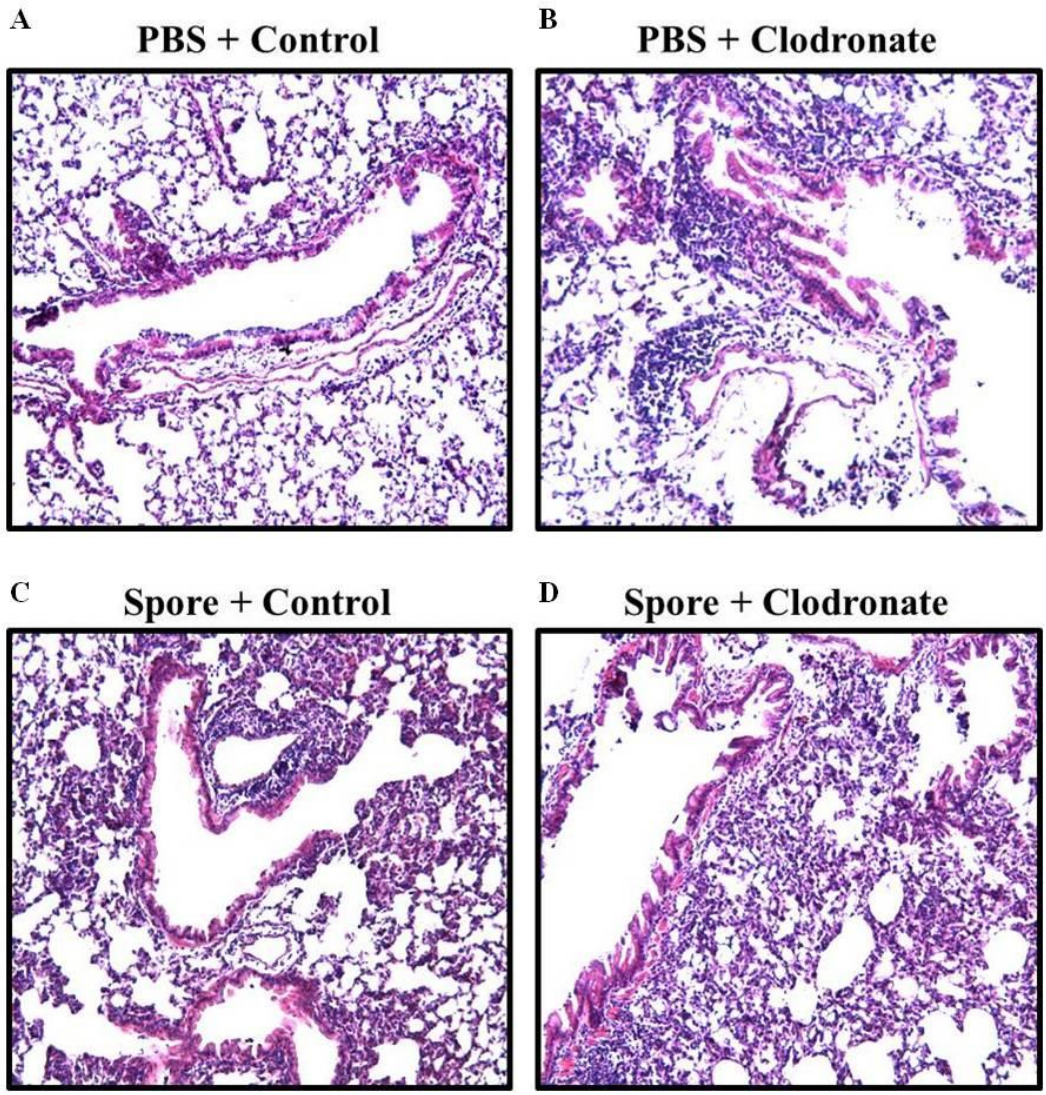

Figure S6. Alveolar macrophages are indispensable for the protection in mice infected with RSV. Mice were administered with spore via intranasal route at 5 days prior to RSV infection. Then the mice were injected with control or clodronate-encapsulated liposome through intratracheal route on days 1 and 3 before the infection. At 4 DPI, perfused lungs were stained with H&E for

histological examination by microscopy at 100 x magnification. Bronchus and blood vessel of mice treated with (A) PBS / control-liposome, (B) PBS / clodronate-encapsulated liposome, (C) spore / control liposome, and (D) spore / clodronate-encapsulated liposome are shown.

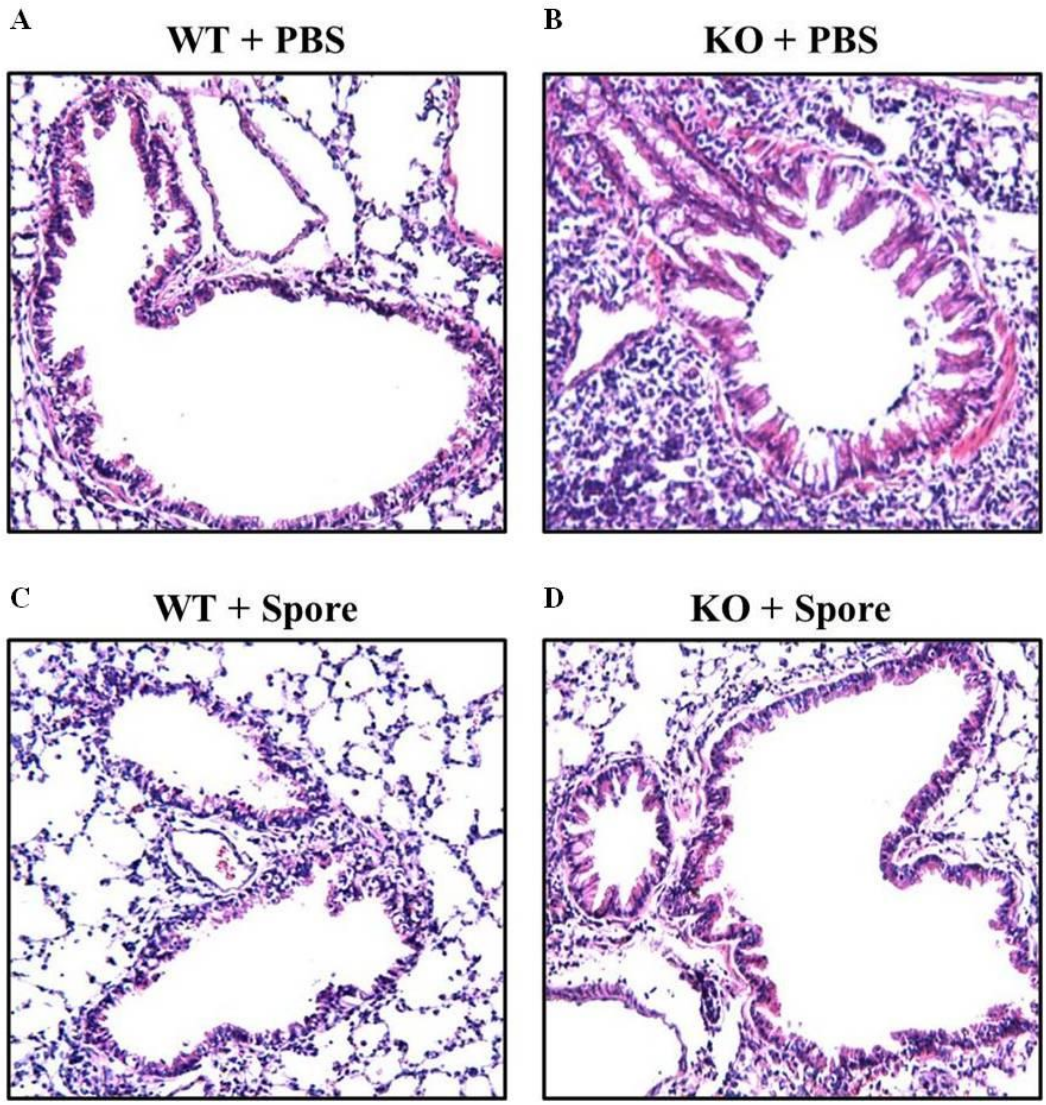

Figure S7. MyD88 signaling plays a crucial role to alleviate histopathology of mice infected with RSV. Wild type or MyD88 knockout mice were administered with spore via intranasal route on 5 days prior to RSV infection. At 4 DPI, perfused lungs were stained with H&E for histological examination by microscopy at 100 x magnification. Bronchus and blood vessel on the (A) PBS pre-treated wild type

mice, (B) PBS pre-treated knockout mice, (C) spore pre-treated wild type mice, and (D) spore pre-treated knockout mice are shown.

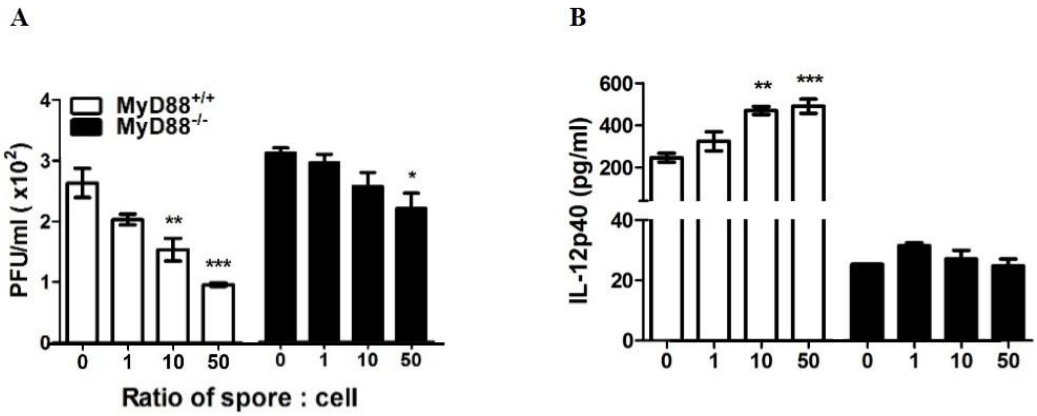

Figure S8. Bone marrow-derived macrophages pre-treated with spore enhance their antiviral effects via MyD88-dependent manner. BMMs were stimulated for 24 hours with spore at ratio of spore per number of cells was 0, 1, 10, and 50. Then, the cells were washed and infected with 1 MOI virus for 24 hours. (A) Viral load was analyzed by using plaque assay and (B) the production of IL-12p40 was measured by ELISA at 24 hours post infection. Data are expressed as mean ± S.E.M. for 3 independent experiments. ** and *** indicate significant differences at $P < 0.01$ and $P < 0.001$, respectively compared with the control.

V. Discussion

Importance of alveolar macrophages (AMs) for host defense mechanism during early viral infection in respiratory tract is well established, however the precise role of AMs, in the RSV infected mice is less clear.

In the present study, I focused on the specific role of AMs treated with *Bacillus subtilis* spore in RSV infected mice for their protection.

Notable findings were as following; (1) AMs have a pivotal role for protection during the initial stage of RSV infection; (2) Administration of spore derived from *Bacillus subtilis* induces the activation of AMs coincident with up-regulation of GM-CSF and classically activated macrophages (M1 macrophages)-related cytokines; (3) Nasal delivery of spore induces the increase of antiviral effector molecules against RSV infection; and (4) MyD88 signals in the AMs are critical for the protection against RSV.

Induction of innate immune response in the early phase of viral infection is necessary for host immunity (Copenhaver et al., 2014, McGill et al., 2009), and thus over the years various attempts have been tried to improve the innate immunity in order to improve the protection against viral infection. Delivery of probiotics and their related materials is one of the strategies (Yaqoob, 2014, Kiso et al., 2013) and in the present study I have sought a potential ability of spore to enhance innate immunity against RSV in line with other studies (McKenney et al., 2013, Hong et al., 2008).

It has been well demonstrated on the importance of AMs in diverse respiratory infections such as a primary recognition of antigens and scavenger to infected cells

(Tate et al., 2010) but the precise role and protective mechanism of AMs in RSV infected mice are yet to be illuminated.

The results showed that mice pre-treated spore through i.n. route rapidly up-regulated GM-CSF and classically activated macrophages (M1 macrophage)-related genes such as TNF-α, IFN-γ, IL-6, and IL-12p40. GM-CSF is well known an essential factor for the local differentiation (Guilliams et al., 2013), survival (Shibata et al., 2001), replenishment (Steinwede et al., 2011) and the ability of host defense (Ghoneim et al., 2013) of macrophages. Also, IFN-γ could lead monocytes differentiate into classically activated macrophages (M1 macrophages). This polarization is important in limiting tissue damage (Barros et al., 2013) and induces the increase of cytokines that enhance the ability of AMs for the killing of intracellular pathogens.-related genes enhance the ability of AMs for the killing of intracellular pathogens (Barros et al., 2013, Martinez and Gordon, 2014). These results imply that pre-treatment of spore could enhance the viability of macrophages that promotes their antiviral functions. On the other hand, pre-treatment with spore fail to induce TGF-β and PPAR-γ which keeps close link with alternatively activated macrophages (M2 macrophages) differentiation. (Ginhoux, 2014, Schneider et al., 2014), suggesting AMs, found increased in the present study, were not differentiated but most likely infiltrated to the lung area.

Consistent with these, mice pre-treated with spore before the RSV infection displayed a certain protective immunity as shown by significantly low level of viral titer and maintenance of body weight. Interestingly, mice pre-treated with spore showed a rapid increase of AM population after the infection. It was reported that

AMs play a reservoirs role for inhaled antigens (Copenhaver et al., 2014), and thus, the rapid increase of AMs could give an efficient protective effect. Furthermore, spore treatment induced inflammatory monocytes with Ly6C high phenotype, known to regulate type I IFN signaling during acute viral pneumonia in mice (Seo et al., 2011) with ability to differentiate into macrophages (Geissmann et al., 2010). Indeed spore pre-treated mice showed remarkably high concentration of IFN-β. Recent study showed that AMs are the major source of type I IFNs during RSV infection and an underappreciated facet of type I IFNs-dependent resistance lead to a cell-extrinsic response through rapid recruitment of antiviral inflammatory monocytes to the site of infection (Goritzka et al., 2015).

It has been suggested that the absence of AM-mediated defense mechanism led to the failure of protective immune responses, the exacerbation of pathology, and the accumulation of debris following pulmonary infection (Kolli et al., 2014). Direct evidence showing important role of AMs in the present study came from the *in vivo* experiment with depletion of AMs. The results showed that the AM-depleted mice suffered from high viral load and intense pathological episode as shown by the accumulation of dead cells and debris especially in bronchi and blood vessels, indicative of early pulmonary inflammation. All these findings clearly support the critical role of AM in controlling disease severity of mice infected with RSV. With regard to the effect of spore on AMs, AM treated with spore rendered less susceptible to RSV infection and more importantly, anti-viral function of spore was markedly reduced upon depletion of AMs, implying AM as a major target for spore as well as a key player for the protection.

TLR2 and TLR4 signaling pathways, in which MyD88 serves as downstream molecules, are essential for the protection in RSV infection (Shirey et al., 2010, Murawski et al., 2009). In the present study, MyD88$^{-/-}$ mice showed persistence of high viral counts in parallel with impaired body weight gain after the infection suggesting that MyD88 is indispensable for the protection against RSV infection. Furthermore, it was evident that MyD88$^{-/-}$ mice fail to recruit AMs into the lung region, irrespective of a spore administration, suggesting that spore-induced infiltration of AMs in the lung and BAL was dependent on MyD88. MyD88$^{-/-}$ mice also displayed severe pathological signs in the lung compared to wild type mice. These results imply that MyD88-dependent signaling might be the key mechanism by which AMs treated with spore contribute to protection of mice from RSV infection.

Spore could promote the GM-CSF and classically activated macrophages (M1 macrophages)-related cytokines to enhance the viability and M1 macrophages prone signatures including IFN-γ and TNF-α, which in turn induce the infiltration of AMs via MyD88 pathway and/or directly influence antiviral function of AMs (Fig 8). Under the infected circumstance, AMs treated with and activated by spore are enforced to produce IFN-β and IL-12p40, which may serve as an alarm signal and facilitate IFN-γ production. This would likely to trigger efficient adaptive immunity for fighting against RSV infection and protect hosts from severe lung injury (Fig 9). When AMs were depleted and infected with RSV, the mice were unable to control the viral replication and thus succumbed to the aggressive lung

injury as shown by the failure of viral clearance and the severe destruction of epithelium (Fig 10).

Collectively, the present study provokes a key function of AMs induced by *Bacillus subitilis* spore in antiviral activity and protection of lung environment against RSV-induced damage in mice infected with RSV via MyD88 signal.

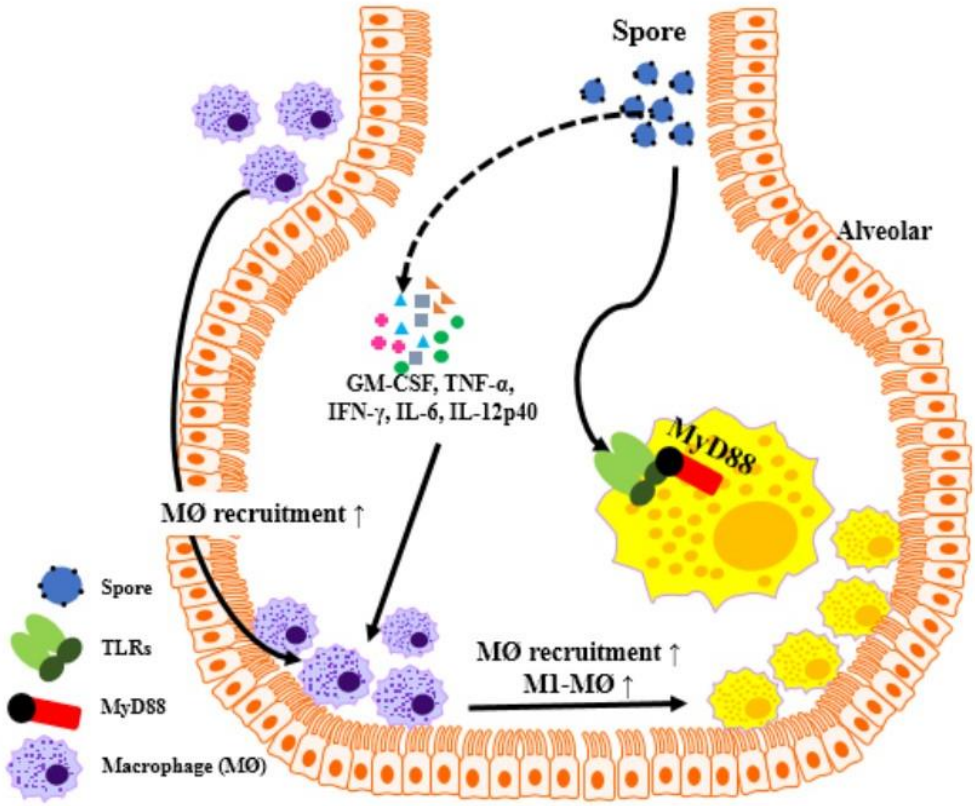

Figure 8. The administration of spore through intranasal route upregulates GM-CSF and classically activated macrophages (M1)-related cytokines coincident with recruitment of alveolar macrophages.

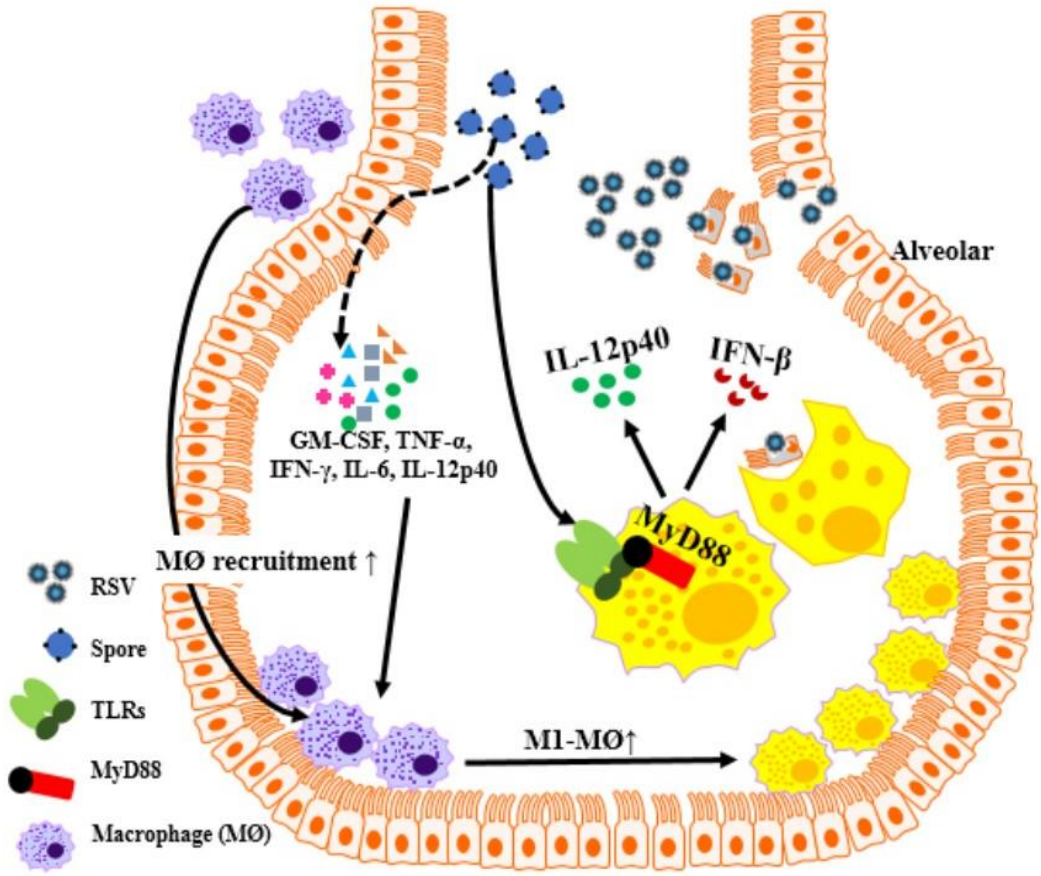

Figure 9. Intranasal pre-treatment of spore in mouse infected with RSV induces protective immunity through MyD88 signaling.

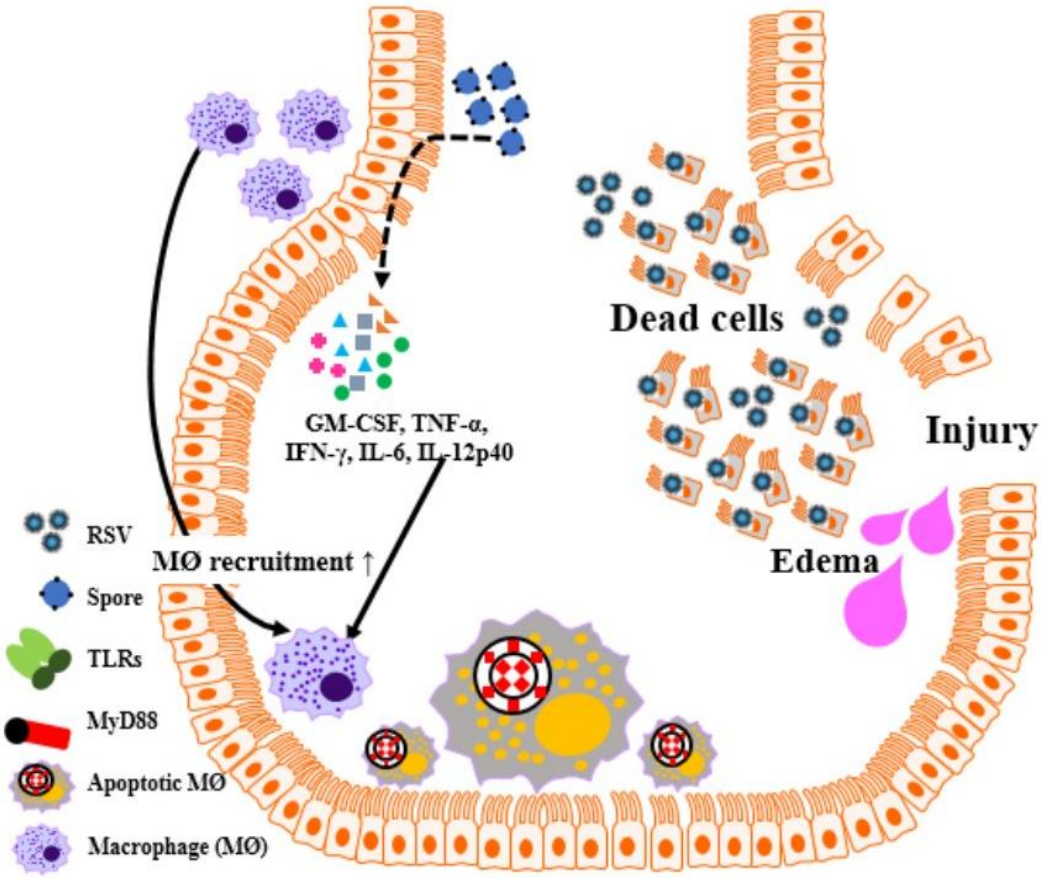

Figure 10. The absence of alveolar macrophage aggravates the disease severity following RSV infection.

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VII. Summary in Korean

호흡기성 융합 바이러스 (respiratory syncytial virus A2, RSV) 감염은 면역체계가 약화된 영·유아 그리고 노인들에게서 흔히 발생하는 호흡기성 질환이다. 다양한 감염성 호흡기 질환에서 성공적인 초기 면역 반응의 유도가 질병 대항에 매우 중요하다는 것은 익히 알려졌지만, 초기 면역 반응을 구성하는 대표적인 내재 면역 세포인 폐포 대식세포의 호흡기성 융합 바이러스 감염 시 구체적인 방어기전은 아직 자세히 밝혀져 있지 않다. 따라서 본 연구는 *Bacillus subtilis* 포자에 의해 활성화된 폐포 대식세포가 호흡기성 융합 바이러스 감염에 방어하는 기전을 마우스 모델에서 규명하였다.

본 연구 결과는 호흡기성 융합 바이러스의 감염 초기 단계에서 폐포 대식세포가 중요하게 작용함을 보여주었으며, *Bacillus subtilis* 포자의 비강 투여는 폐포 대식세포의 활성화를 유도함과 함께 항 바이러스성 효과 물질인 IFN-β 와 IL-12p40 과 GM-CSF, 그리고 M1-마크로파지 관련 유전자를 유도함을 밝혔다. 더욱이, 이러한 방어기전은 폐포 대식세포의 MyD88 시그널에 의존적임을 증명하였다. 비강 투여를 통한 *Bacillus subtilis* 포자의 전 처리는 호흡기성 융합 바이러스 감염 마우스에서 효과적인 방어 면역반응을 유도하여 감염 후 4 일 뒤 채취한 마우스 폐의 바이러스 농도가 유의적으로 감소함을 보였다. 이는 일반적으로 나타나는 호중구와 염증성 단핵구 증감이 아닌 폐포

대식세포의 증가라는 특이적인 현상과 관련이 있음을 확인할 수 있었다. 또한 폐포 대식세포가 제거된 마우스에서는, 감염 후 폐의 바이러스 농도가 매우 높은 수준으로 측정되는 결과를 얻었으며, 동시에 병리학적 질병 심화가 유도됨을 알 수 있었다. 이러한 결과로부터 *Bacillus subtilis* 포자로 활성화된 폐포 대식세포는 호흡기성 융합 바이러스 감염의 방어에서 필수 불가결한 구실을 함을 확인할 수 있었다. 더불어 MyD88 신호전달이 결여된 MyD88 유전자 결핍 마우스는 *Bacillus subtilis* 포자의 처리와 무관하게 호흡기성 융합 바이러스 감염에 감수성이 매우 높은 것으로 보아 효과적인 방어기전에는 폐포 대식세포의 MyD88 신호전달이 필수적임을 알 수 있었다.

결론적으로, 본인은 이 연구를 통하여 *Bacillus subtilis* 포자의 비강 투여가 MyD88 신호전달 의존적으로 초기 폐포 대식세포의 활성을 유도하며, 이 과정은 호흡기성 융합 바이러스 감염에 대항하기 위해 필수적임을 밝혔다.

A Thesis

for the Degree of Master of Science

MyD88-dependent protective mechanism of alveolar macrophages induced by *Bacillus subtilis* spore in mice infected with respiratory syncytial virus A2

호흡기성 융합 바이러스 감염 마우스에서 *Bacillus subtilis* 포자에 의해 유도된 폐포 대식세포의 MyD88 신호전달 의존적 방어기전

August 2015

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농 학 석 사 학 위 논 문

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2015 년 08 월

서울대학교 대학원

농생명공학부

홍 지 은

홍지은의 석사학위논문을 인준함

2015 년 08 월

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Summary

Respiratory syncytial virus (RSV) is one of the most common respiratory diseases in children and elderly who are immune-compromised. Although induction of successful innate immunity is critical for the protection against viral infection, specific role and defense mechanism of alveolar macrophages (AMs) in RSV infection are yet to be illuminated. Therefore, the objective of the present study was to elucidate the exact role of AMs activated with *Bacillus subtilis* spore in mice infected with RSV.

The results showed that AMs played a pivotal role in the protection during the initial stage of RSV infection and that the administration of spore derived from *Bacillus subtilis* induced activation of AM population coincident with enhancing antiviral effector molecules, GM-CSF and classically activated macrophages (M1 macrophage)-related genes. Furthermore, these protective immune responses were dependent on MyD88 signaling pathway in the AMs.

Pre-treatment with spore through intranasal route induced protective immunity in mice infected with RSV as shown by significantly low viral load at 4 days post infection. It was noting that spore-treated mice displayed the increase of AMs, but not neutrophils or inflammatory monocytes after the infection. Also, these mice showed notably increased level of IFN-β and IL-12p40. When AMs were depleted, mice became intensified the disease severity as shown by persisted high level of viral load with increased pathology scores of pulmonary in the lung resulting a very weak protective efficiency against RSV infection. These results suggest that AMs

treated with spore are indispensable for the effective protection against RSV infection. Furthermore, MyD88$^{-/-}$ mice were unable to induce protective responses regardless of spore treatment, suggesting that the protection by spore-treated AMs was mediated through MyD88-dependent signaling pathway.

In conclusion, I revealed that administration of spore via intranasal route led to the early activation of AMs via MyD88-dependent pathway is responsible for the protective immunity in mice infected with RSV.

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List of Abbreviations

AMs: alveolar macrophages

BAL: bronchoalveolar lavage

BMMs: bone marrow-derived macrophages

CFU: colony forming unit

Clod: clodronate-encapsulated liposome

Cont: control liposome

DPI: day post infection

ELISA: enzyme-linked immunosorbent assay

GM-CSF: granulocyte-macrophage colony-stimulating factor

H&E: Hematoxylin and Eosin

IFN: interferon

i.n.: intranasal

IL: interleukin

i.t.: intratracheal

KO: knock out

MOI: multiplicity of infection

MyD88: myeloid differentiation primary response gene

M1 macrophage: classically activated macrophage

M2 macrophage: alternatively activated macrophage

PFU: plaque-forming unit

qRT-PCR: quantitative real time polymerase chain reaction

RSV: respiratory syncytial virus

TLR: toll-like receptor

TNF: tumor necrosis factor

TSB: tryptic soy broth

WT: wild type

YE: yeast extract

I. Introduction

Respiratory syncytial virus (RSV) causes a common viral infectious disease, especially for the infants and elderly, whose symptom is accompanied by a serious bronchiolitis resulting in up to 200,000 annual deaths worldwide (Nair et al., 2010). Despite the numerous efforts, we have no effective ways to prevent RSV infection until now. RSV non-structural-1 and -2 proteins hinder both innate and adaptive immunities (Meng et al., 2014) causing malfunction of overall immune protection. It has been suggested that the main protection against RSV is mediated via TLR2 and 4 (Kurt-Jones et al., 2000, Murawski et al., 2009) Indeed, poor innate defense mechanism at the initial stage of the infection brought about the serious consequences in RSV infection, and thus several attempts have been made to improve innate immunity.

One of the primary innate cells in the lung is alveolar macrophages (AMs), which play a pivotal role to maintain homeostasis and induce effective defense mechanism. AMs have unique properties compared to macrophages in other tissue as they are in direct contact with external environment that could allow rapid recognition of antigenic molecules and participate in the immediate initiation of host defense (Herold et al., 2011, Hashimoto et al., 2011). Recent studies have shown that collapse of early AM-mediated defense responses caused insufficient protection against various respiratory diseases and led to improper recruitment of immune cells coincident with failing the control of lung homeostasis (Maus et al., 2002, Snelgrove et al., 2008). Despite the importance of AMs in various respiratory

viral diseases, the precise role and protective mechanisms of AMs in RSV infection are yet to be illuminated.

Several attempts using probiotics have gained an insight for the priming of innate immune system where the administration of probiotics induced a rapid activation of innate immunity that acquired enhanced protective property in respiratory viral infections (Park et al., 2013, Garcia-Crespo et al., 2013). Furthermore, spore derived from probiotics is one of the alternatives not only for inducing enhanced innate immune responses but also for better safety and stability than whole bacteria. Hence, in this study, I investigated whether and how spore derived from *Bacillus subtilis* would protect mice from RSV infection.

III. Materials and Methods

Mice

Female BALB/c mice, 6 - 8 week-old, were purchased from Orient Bio Inc., Korea. MyD88$^{-/-}$ mice were purchased from Jackson laboratory (Bar harbor, ME, USA). All the experimental procedures using mice were approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University (Approval NO.: SNU-130527-5), Korea.

Preparation and isolation of Bacillus subtilis spore

Bacillus subtilis was spread in an agar plate containing 3% Trypticase soy broth (TSB), 0.5% Yeast extract (YE) and 1.5% Bacto Agar (all from BD Biosciences, San Diego, CA, USA) and incubated at 37℃ for 9 hours. One colony was picked and inoculated in 25 ml of 3% TSB and 0.5% YE liquid media. Then it was incubated for 5 hours in the shacking incubator at 150 rpm at 37℃ until the OD value reached between 0.45 - 0.6. For sporulation, culture was transferred to 500 ml of the autoclaved media which containing 5 ml of 10% KCl, 5 ml of 1.2% $MgSO_4 \cdot 7H_2O$ (pH 7.6), 0.5 ml of 1 M $Ca(NO_2)_3$, 0.01 M $MnCl_2$, and 1 mM $FeSO_4$. The culture was incubated at 37℃ for 48 hours with shaking at 150 rpm. The cells were collected by centrifugation at 5516g for 10 minutes, resuspended in distilled water, and incubated at 4℃ for 48 hours on the rocker. Then, the cells were sonicated at 35% amplitude (1 watt) for 90 seconds with 0.5 second pulse. Spore

loaded on the layers of 35%, 25%, 15% OptiPrep Density gradient (Sigma-Aldrich, Wt. Louis, MO, USA) was centrifuged at 10,000g for 40 minutes at 25℃ without break for the purification. The spore was washed 3 times with distilled water and resuspended in 1 ml of distilled water.

Preparation and isolation of respiratory syncytial virus A2

RSV A2 strain was amplified as following; HEp-2 cells (ATCC, Manassas, VA, USA) were grown in MEM containing 10% of FBS and 1% of antibiotics. When the cells reached at approximately 80% confluence, the cells were washed and inoculated with 0.01 - 0.05 MOI of virus in MEM containing 1% of antibiotics and 25 mM HEPES (Gibco). The cells were incubated at 37℃ for 2 hours, added MEM containing 6% of FBS only, and then incubated for additional 72 - 96 hours at 37℃. The cells were scraped and combined into the conical tubes on ice and centrifuged at 1,400 rpm for 4 minutes at 4℃. The cell pellets were collected into conical tubes, resuspended with cold conditioned media with 60% sorbitol, sonicated for 15 minutes in slurry ice and centrifuged at 4,000 – 5,000 rpm for 10 minutes at 4℃, and then the sonication and centrifugation step was repeated in the same condition. The supernatants were transferred into new tubes, centrifuged at 23,000 rpm for 1 hour at 4℃, and then discarded. The resulting whitish virus pellet was resuspended with 500 μl of cold MEM and the titer was determined by standard RSV plaque assay.

Isolation and culture of bone marrow-derived macrophages

Bone marrow was flushed from femurs and tibias of 6 – 8 week-old female BALB/c mice (Orient Bio Inc., Korea) using Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 2% FBS (Gibco). Red blood cells were lysed with ACK lysing buffer (Gibco) and whitish marrow cells were seeded in 90 x 15mm Petri dish with complete media containing 10% FBS, 1% antibiotics with 20% L-929 conditioned media (Zhang et al., 2008)(Zhang et al., 2008)(Zhang et al., 2008)(Zhang et al., 2008) and incubated at 37℃ in humidified incubator with 5% CO_2 for 7 days. On day 3, another 5 ml of fresh complete media were added to each dish. On day 7, only adherent cells were collected using non-enzymatic cell dissociation solution (Gibco).

Macrophage cell line

MH-S cells (ATCC, Manassas, VA, USA), mouse alveolar macrophages were grown in RPMI-1640 GlutaMax medium containing 10% FBS, 1.5% antibiotics (all from Gibco) in a 5% CO_2 incubator at 37℃.

RNA isolation and quantitative RT-PCR

To quantitative analyze for the expression of GM-CSF, TNF-α, IFN-γ, IL-12p40, IL-6, PPAR-γ, and TGF-β1 at mRNA level, quantitative real time polymerase chain reaction (qRT-PCR) was conducted. RNA was extracted from perfused lungs using TRIZOL (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated by adding

chloroform followed by centrifugation at 4℃, 12,000g for 15 minutes and addition of isopropanol for 10 minutes at room temperature for RNA precipitation. RNA pellet was obtained by washing with 75% ethanol and air dried for 10-15 minutes then resuspended with DEPC distilled water (Sigma-Aldrich, Wt. Louis, MO, USA) and quantified with NanoDrop (Amersham Bioscience, USA) at A_{260}. One microgram of RNA was reverse transcribed into cDNA and amplified with murine primers specific for GM-CSF (forward primer: 5'-CTGCCTTAAAGGGACCAAGAGA-3', reverse primer: 5'-TTCCGCTGTCCAAGCTGAGT-3'), TNF-α (forward primer: 5'-GCCAACGGCATGGATCTC-3', reverse primer: 5-GTGGGTGAGGAGCACGTAGTC-3'), IFN-γ (forward primer: 5'-GCCATCGGCTGACCTAGAGA-3', reverse primer: 5'-GCAGTGTGTAGCGTTCATTGTCT-3'), IL-12p40 (forward primer: 5'-GAAAGGTGCGTTCCTCGTAGA-3', reverse primer: 5'-GGAACACATGCCCACTTGCT-3'), IL-6 (forward primer: 5'-CACAGAGGATACCACTCCCAACA-3', reverse primer: 5'-TCAGAATTGCCATTGCACAAC-3'), PPAR-γ (forward primer: 5'-CAGGAGCCTGTGAGACCAACA-3', reverse primer: 5'-ATCAGTGGTTCACCGCTTCTTT-3'), TGF-β1 (forward primer: 5'-TCGTCTGCATTGCACTTATGC-3', reverse primer: 5'-GTGGTGCCCTCTGAAATGAAA-3') and GAPDH (forward primer: 5'-CTCCACTCACGGCAAATTCA-3', reverse primer: 5'-GCCTCACCCCATTTGATGTT-3'). Real-time PCR was performed using Power

SYBR Green PCR master mix (Applied Biosystem, Waltham, MA, USA) and analysis of the data was performed by One-step RT PCR (Applied Biosystem). Target gene expression was normalized to GAPDH expression.

Measurement of cytokine production

Bronchoalveolar lavage (BAL) samples were collected via tracheotomy using 600 μl of PBS and the cells were separated from the BAL fluid by centrifugation at 1400 rpm for 5 minutes at 4℃. For *in vitro* experiments, supernatants from bone marrow-derived macrophages or MH-S after virus infection were carefully collected. Production of IFN-β (BioLegend, San Diego, CA, USA), IL-12p40, GM-CSF and TNF-α (R&D System, Minneapolis, MN, USA) was examined using ELISA kit.

Phenotypic characterization of the cells

To examine the absolute number of AMs and other innate immune cells, BAL cells were collected as above and perfused lungs were isolated and minced through 70 μm cell strainer using MEM. The cells were stained with FITC-conjugated anti-CD11c (HL3), PE-conjugated anti-Siglec-F (E50-2440), PerCP-conjugated anti-Ly6C (AL-21), PE-Cy7-conjugated anti-Ly6G (1A8), APC-conjugated anti-CD11b (M1-70) or APC-conjugated anti-F4/80 (BM8), and APC-Cy7-conjugated anti-CD45 (30-F11) (all from BD Biosciences except anti-F4/80 from BioLegend). The cells were acquired using FACS LSR II and flow cytometric data were analyzed by using FlowJo software (Tree Star, San Carlos, CA, USA). We defined

$CD45^+Ly6C^-Ly6G^-CD11c^+Sigleg\text{-}F^+F4/80^+$ as AMs, $CD45^+CD11b^+Ly6C^+Ly6G^-$ for inflammatory monocytes, and $CD45^+CD11b^+Ly6G^+Ly6C^-$ for neutrophils.

Selective depletion and adoptive transfer of alveolar macrophages

To selectively deplete AMs, 350 mg per mouse of clodronate-encapsulated liposome (FormuMax Scientific Inc, CA, USA) were given i.t. in a volume of 50 µl at 1 and 3 days before the challenge with or without spore treatment. To verify the depletion of AMs, naïve mice were given control liposome via i.t. The data were acquired using FACS LSR II and acquired data were analyzed by using FlowJo software.

Virus titration in the lung

To determine the viral titers, lungs from RSV-infected mice were isolated at day 4 post-infection. The lungs were minced through 70-µm cell strainer using cold MEM. Cell lysates were collected and RSV titers were determined by plaque assay using HEp-2 cells. The virus titers in the whole lung were normalized to weight of the lung tissue and indicated as PFU/g.

Lung histology and pathology scoring

For histology studies, mice were administered with spore and/or clodronate encapsulated liposome prior to RSV infection. For control, mice were administered with PBS or control liposome. At 4 days post infection, blood perfused lungs were

fixed with 4% paraformaldehyde and embedded in paraffin. Lung sections were produced and stained with Hematoxylin and Eosin to examine the abnormalities. Four inflammatory parameters were scored independently from 0 to 5 for each section: alveolitis (inflammatory cells within alveolar spaces), interstitial pneumonitis (increased thickness of alveolar walls associated with inflammatory cells), peribronchiolitis (inflammatory cells surrounding a bronchiole), and perivasculitis (inflammatory cells surrounding a blood vessel). Slides were randomized, read blindly, and scored for each parameter.

Spore administration and infection in mice

Each mouse was administered with either 1 x 10^9 CFU of spore or, as a control PBS i.n. in a volume of 20 μl at 5 days before the infection. Then, the mice were infected i.t. with 2 x 10^6 PFU of live RSV A2.

Statistical analysis

Statistical significance was analyzed with Anova test and considered statistically significant at *P* value less than 0.05.

III. Results

1) Delivery of spore through intranasal route induces protective immunity in mice infected with RSV

It has been suggested that administration of probiotics or probiotics-derived biomaterials for the induction of innate immunity showed a certain level of protection against respiratory viral infection (Kiso et al., 2013, Lee et al., 2013)(Kiso et al., 2013, Lee et al., 2013)(Kiso et al., 2013, Lee et al., 2013) (Kiso et al., 2013, Lee et al., 2013). This raises a question whether pre-treatment of spore derived from *Bacillus subtilis* via intranasal (i.n.) route would alter the outcome of RSV infection. To address this, a group of mice was administered i.n. with spore or PBS at 5 days prior to RSV infection. Mice pre-administered with spore did not cause a significant change of body weight (Fig. 1A). The striking finding was, however, that viral counts in the lung at 4 days post-infection (DPI) were considerably lower in spore-treated mice than PBS-treated mice (Fig. 1B). These results suggest that i.n. pre-delivery of spore would enhance a anti-viral immunity against RSV infection.

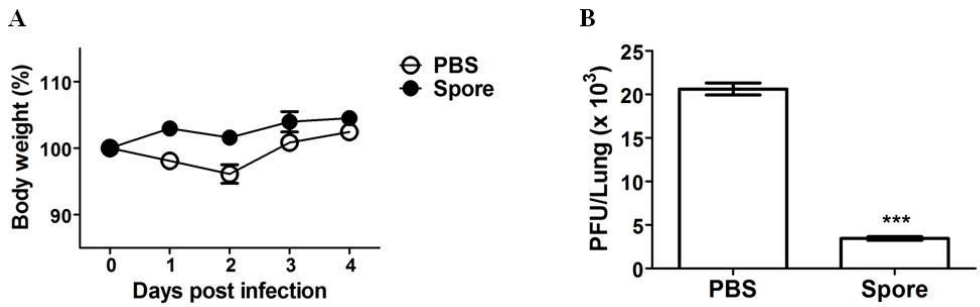


Figure 1. Pre-treatment with spore through intranasal route reduces the disease severity following RSV infection. Mice were administered with 1 x 10^9 CFU of spore via i.n. route at 5 days prior to RSV infection with 2 x 10^6 PFU per mouse. (A) Body weight was monitored daily after the infection and (B) viral load in the lungs was analyzed by plaque assay at 4 days post-infection. Data are expressed as mean ± S.E.M. for the group (n=5). *** indicates that the values are significantly different at $P < 0.001$ compared to the control.

2) Administration of spore via intranasal route increases the number of alveolar macrophages

I found that mice pre-treated with spore induced the unique change of innate immune cell population, especially alveolar macrophages (AMs) in the lung and bronchoalveolar lavage (BAL). Delivery of spore via i.n. changed GM-CSF and M1 macrophage-related cytokines (Murray et al., 2014)(Murray et al., 2014)(Murray et al., 2014)(Murray et al., 2014) including TNF-α, IFN-γ, IL-12p40 and IL-6 both at mRNA (Supplementary Fig. 1A) and protein levels with high concentration throughout the experimental period (Supplementary Fig. 1B). GM-CSF was shown to influence the ability of AMs for their survival, local differentiation, replenishment and of host defense (Shibata et al., 2001, Steinwede et al., 2011)(Shibata et al., 2001, Steinwede et al., 2011)(Shibata et al., 2001, Steinwede et al., 2011)(Shibata et al., 2001, Steinwede et al., 2011). On the contrary, the expression of TGF-β1 which is the typical M2 macrophage-related gene showed no significant changes regardless of spore pre-treatment (Supplementary Fig. 1A). Interestingly, in line with enhanced expression of M1 macrophage-related cytokines, spore pre-treated mice after RSV infection showed significantly high number of AMs in both lung (Fig. 2A and Supplementary Fig. 2A) and BAL (Fig. 2B and Supplementary Fig. 2B), together with increased levels of IFN-β and IL-12p40 (Fig. 2C). These results imply that intranasal administration of spore induced significantly high number of AMs, and augmented expression of GM-CSF and cytokines characteristics of M1 macrophage differentiation.

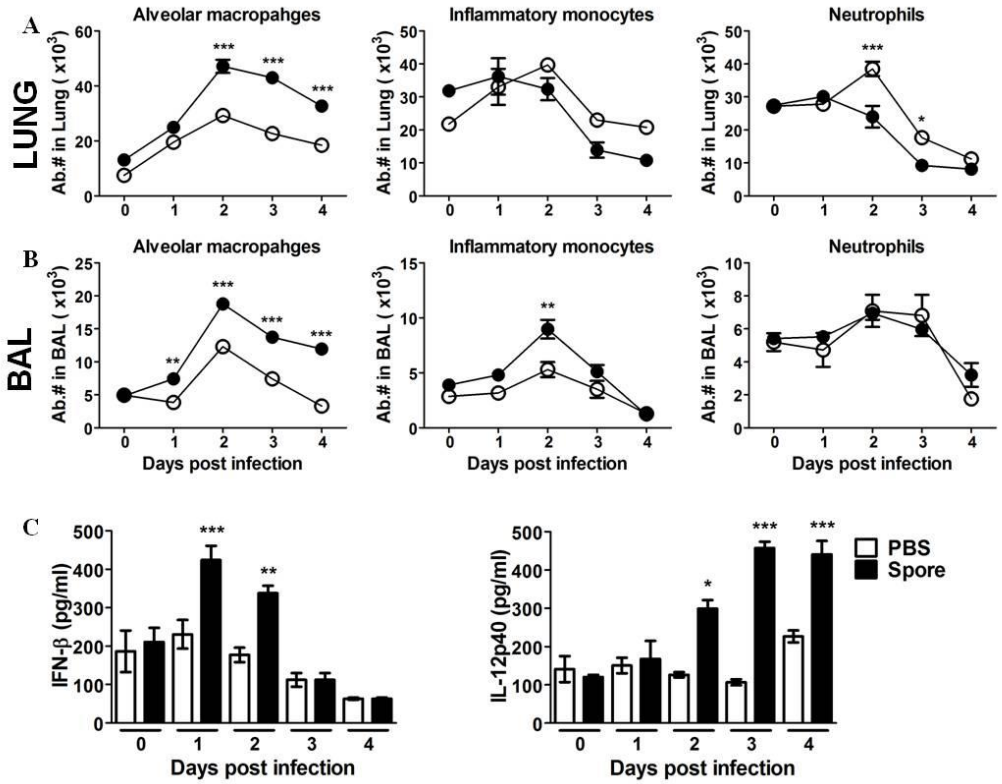


Figure 2. Pre-treatment with spore in mice infected with RSV induces the population change of alveolar macrophages and enhances antiviral effector molecules. Mice were administered with spore via i.n. route at 5 days prior to RSV infection. Then, (A) change of various innate immune cells in the post-lavaged lung and (B) BAL fluid was analyzed by flow cytometry at 0 to 4 DPI. Empty and filled circles indicate PBS and spore pre-treated mice, respectively. (C) IFN-β and IL-12p40 in BAL fluid were measured by ELISA. Data are expressed as mean ± S.E.M. for the group (n=5). Significant differences from results with the PBS control are *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, respectively.

3) Depletion of alveolar macrophages aggravates disease severity in mice infected with RSV

Absence of AMs, by depletion or viral infection, during the early phase of infection aggravates disease severity (Schneider et al., 2014a)(Schneider et al., 2014a)(Schneider et al., 2014a)(Schneider et al., 2014a) because of alarming failure with no scavenger activity to infected cells (Pribul et al., 2008, Kumagai et al., 2007)(Pribul et al., 2008, Kumagai et al., 2007)(Pribul et al., 2008, Kumagai et al., 2007) (Pribul et al., 2008, Kumagai et al., 2007). Moreover, mice depleted AMs are unable to deliver the antigen to dendritic cells for the antigen presentation (Ugonna et al., 2014)(Ugonna et al., 2014)(Ugonna et al., 2014)(Ugonna et al., 2014) and strengthen the immune response. To determine the particular role of AMs in RSV infection, I conducted the depletion of AMs by injection of clodronate-encapsulated liposome (Clod) twice through the intratracheal (i.t.) route at days 1 and 3. As a result, absolute number of AMs in the lungs and BAL from Clod-treated mice declined by approximately 85 percent compared to that in control (Supplementary Fig. 3A) without significant histological changes (Fig. 3C, left). At 1 DPI, AM-depleted mice showed a shape decline in body weight (Fig. 3A) and at 4 DPI, significantly higher lung viral counts than those of control (Fig. 3B). The pathological results displayed thickened alveolar epithelium, destruction of epithelial walls, overall alveolar swelling and the accumulation of immune cells in the lung compared to the control group which showed partial and slight destruction of epithelium (Fig. 3C, right and D). Furthermore, the accumulation of inflammatory cells in the interstitial space, bronchi and vessel was apparent in

lungs from AM-depleted mice (Fig. 3D and Supplementary Fig. 4A). Taken together, the depletion of AMs at the initial phase of infection leads to the exacerbated disease severity coincide with increased pulmonary inflammation.

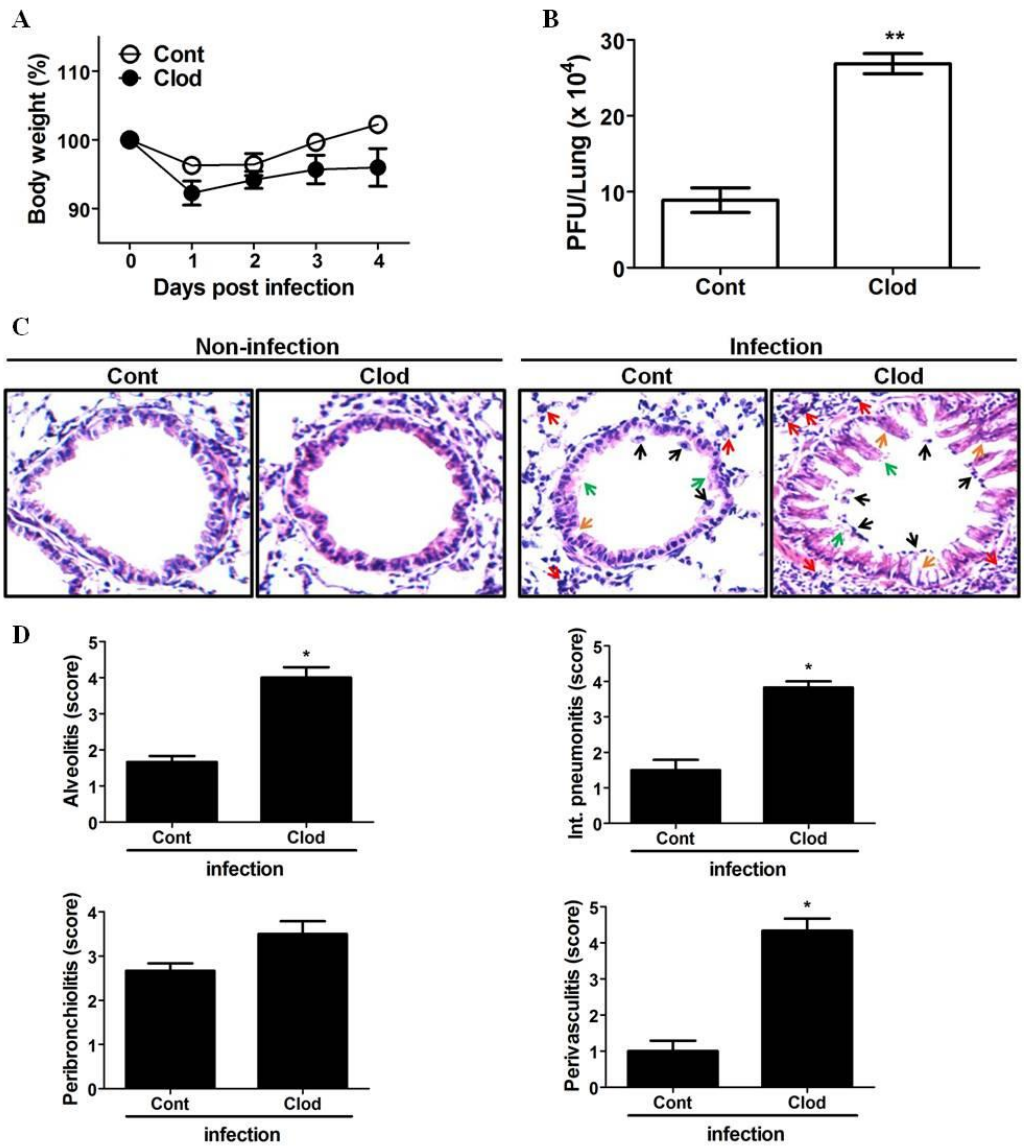


Figure 3. Mice selectively depleted with alveolar macrophages fail to protect RSV infection. Mice were injected i.t. with clodronate-encapsulated liposome twice on days 3 and 1 prior to RSV infection. (A) Body weight was monitored daily after the infection and (B) viral load in the lungs was analyzed by plaque assay at 4 DPI. At 4 DPI, perfused lungs were stained with H&E (C) for histological examination by microscopy at 200 x magnifications and (D) scored for histopathology. 'Cont' indicates the mice injected with control liposome and 'Clod' indicates the mice injected with clodronate-encapsulated liposome. Arrows indicated as follows; orange: epithelium thickness and destruction, green: pulmonary edema, red: inflammatory cells, and black: cell death. Data are expressed as mean ± S.E.M. for the group (n=5). * and ** indicate significant differences at $P < 0.05$ and, $P < 0.01$, respectively.

4) Spore directly enhances the antiviral function of alveolar macrophages

To explore direct effect of spore on AMs, MH-S, AM cell line originated from BALB/c mice was used. The cells were treated with spore for 24 hours and then infected with RSV. The number of plaques was significantly reduced when the AM cell line was incubated with spore in a dose-dependent manner (Fig. 4A and Supplementary Fig. 5A). Inflammatory cytokines, IL-12p40 and IL-6, in the supernatant from the cells treated with spore followed by RSV infection were measured as an initial assessment for their antiviral activity (Puddu et al., 1997)(Puddu et al., 1997)(Puddu et al., 1997)(Puddu et al., 1997). The results showed that the spore treatment led to substantial increase of IL-6 and, to a lesser extent, IL-12p40 (Fig. 4B and Supplementary Fig 5B). These results demonstrated that spore directly promotes the antiviral activity of AMs, especially at the early time point after the infection, in a spore dose-dependent manner.

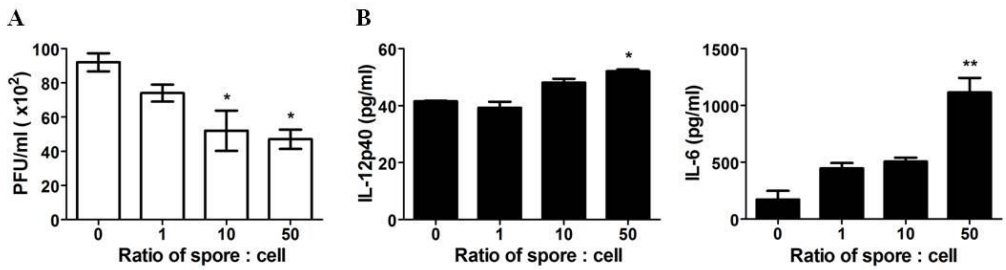


Figure 4. Alveolar macrophages pre-treated with spore extend their antiviral effects. MH-S cells were stimulated with spore for 24 hours, with a ratio of MH-S:Spore at = 1:0, 1:1, 1:10, and 1:50. Then, the cells were washed and infected with 1 MOI virus for 12 hours. (A) The viral load was analyzed by using plaque assay and (B) the production of IL-12p40 and IL-6 was measured by ELISA. Data are expressed as mean ± S.E.M. of three replicates. * and ** indicate significant differences at $P < 0.05$ and, $P < 0.01$, respectively.

5) Alveolar macrophages activated by spore have a pivotal protective role in mice infected with RSV

To validate direct involvement of spore-activated AMs for the protection from RSV infection *in vivo*, spore pre-administered mice were depleted for AMs before RSV infection. It was noting that mice treated with spore did not have any significant weight loss (Fig. 5A), in line with the ability of spore-treated mice to better clear the virus (Fig. 5B). However, spore-treated mice with AMs depleted showed a moderate decrease of body weight (Fig. 5A) and led to viral counts be increased in the lung compared to those without AMs depletion (Fig. 5B), suggesting that AMs play an important role in the protection against RSV infection. To note, mice infected with RSV showed a severe weight loss especially at the early stage after the infection as expected. Severe destruction of epithelium and infiltration of immune cells in alveolus (Fig. 5C) and bronchi (Supplementary Fig. 6) were observed in lungs from the infected mice without spore treatment, resulting in high pathological score (Fig. 5D). It was obvious that the protection efficacy of spore become less effective in mice when depleted AMs. Taken together, these results support the hypothesis that the spore enhances host protection against RSV infection by enhancing the activation of AMs.

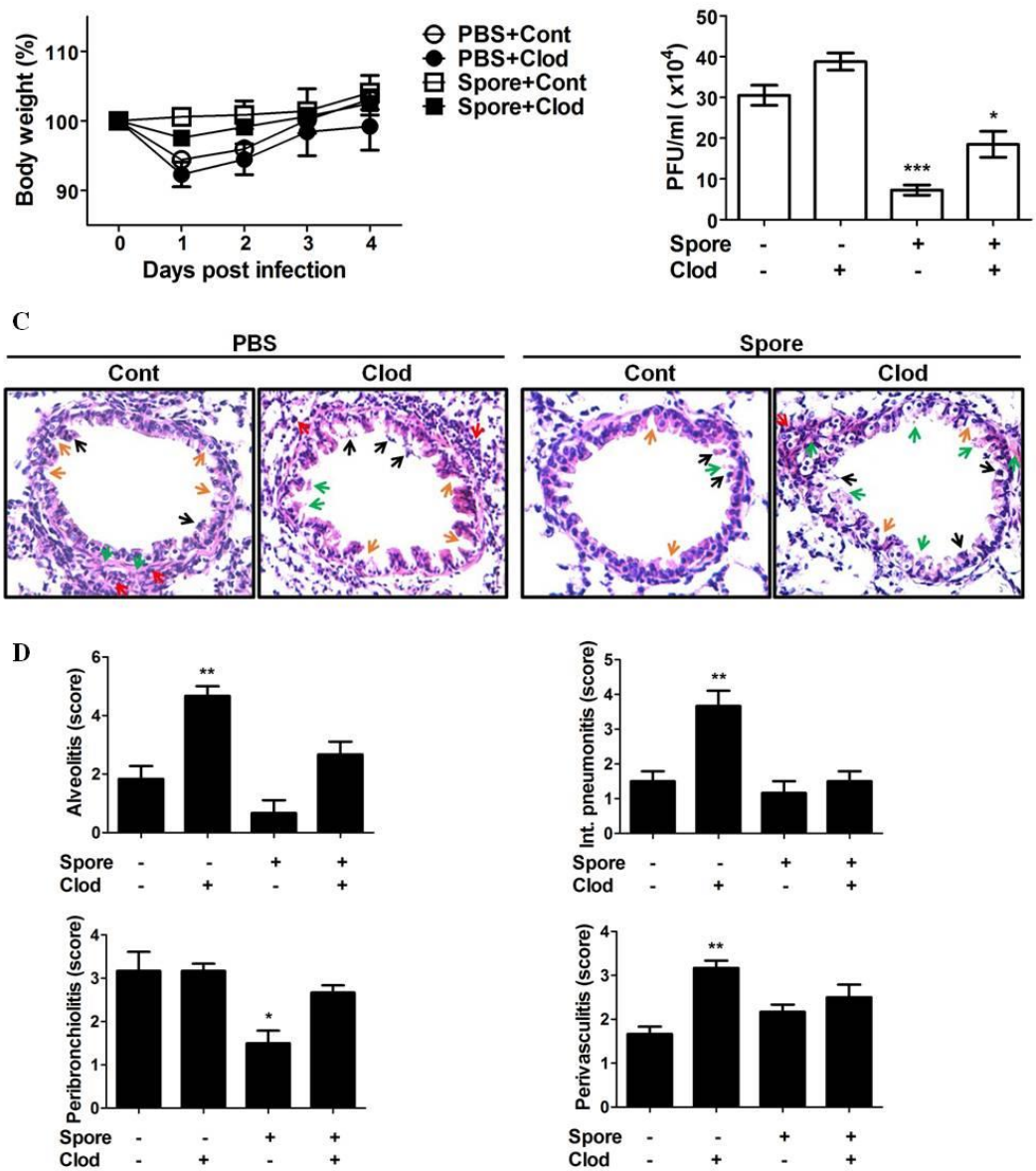
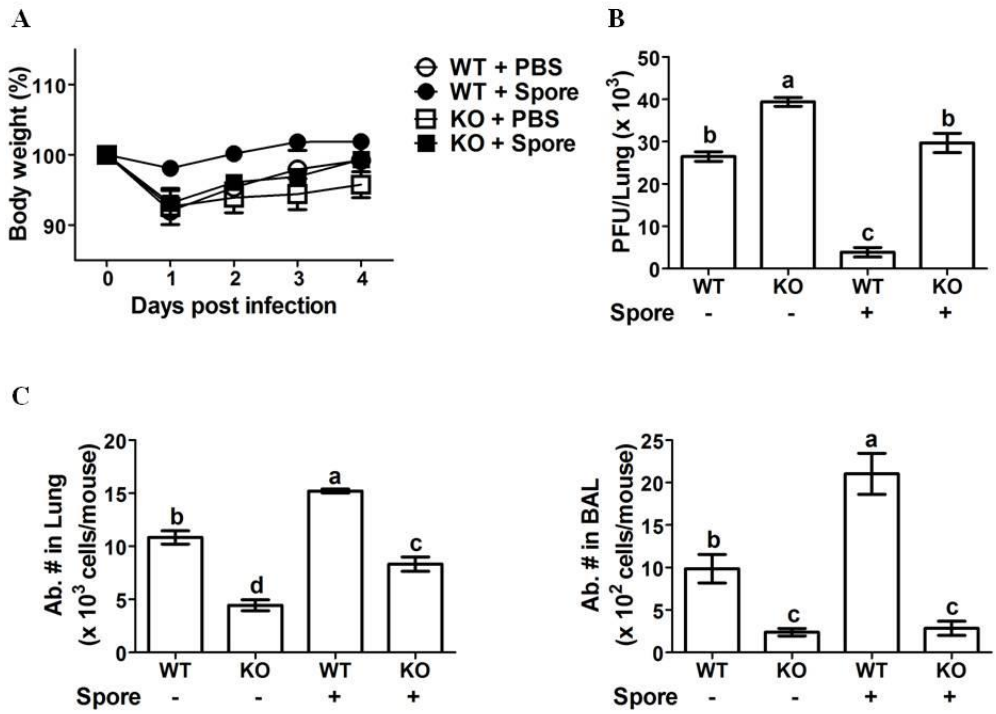


Figure 5. Alveolar macrophages in mice intranasally administered with spore play a key role in RSV infection. Mice were intranasally administered with spore at 5 days prior to RSV infection and injected with control or clodronate-

encapsulated liposome through intratracheal on days 3 and 1 before the infection and sacrifice on 4 DPI. (A) Body weight was monitored daily after the infection and (B) viral load in the lung was examined at 4 DPI, respectively. (C) At 4 DPI, perfused lungs were stained with H&E for histological examination by microscopy at 200 x magnification and (D) scored for histopathology. Arrows indicated are as follows; orange: epithelium thickness and destruction, green: pulmonary edema, red: inflammatory cells, and black: cell death. Data are expressed as mean ± S.E.M. for the group (n=5). *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

6) Protection of mice infected with RSV is enhanced by spore treatment through MyD88-dependent manner

The next question was the signaling pathways involved in protection and antiviral functions in AMs treated with spore. Since viral titer was prolonged in TLR2 or TLR4 knockout mouse (Kurt-Jones et al., 2000, Murawski et al., 2009)(Kurt-Jones et al., 2000, Murawski et al., 2009)(Kurt-Jones et al., 2000, Murawski et al., 2009)(Kurt-Jones et al., 2000, Murawski et al., 2009), I raised a question whether MyD88 downstream signal of TLRs in AMs is necessary against RSV infection. Interestingly, MyD88$^{-/-}$ mice were unable to induce the protective response showing the reduction of body weight (Fig. 6A) with significantly high viral load (Fig. 6B) regardless of spore pre-treatment. Consistent with these, infiltration of AMs in the lungs and BAL (Fig. 6C) was indistinguishable and inflammation with cell death and severe destruction of epithelium in alveolus (Fig. 6D, E) and bronchi (Supplementary Fig. 7) were found in MyD88$^{-/-}$ mice. In addition, the disease severity was far worsen in MyD88$^{-/-}$ mice than wild-type mice and no rescuable by spore treatment, as illustrated by decline of body weight, high level of viral titer, the failure of AMs infiltration, and the increase of AMs number, and the severe pathological results. Taken together, these results suggest that spore may serve as TLR-like ligands and act directly on AMs probably through the activation of MyD88 signal, which in turn induce expression of genes that exert antiviral functions in mice infected with RSV.


A
Body weight (%)
110
100
90
0
1
2
3
4
Days post infection
WT + PBS
WT + Spore
KO + PBS
KO + Spore
B
PFU/Lung (x 10^3)
50
40
30
20
10
0
a
b
c
b
WT
KO
WT
KO
Spore
-
-
+
+
C
Ab. # in Lung
(x 10^3 cells/mouse)
20
15
10
5
0
b
d
a
c
WT
KO
WT
KO
Spore
-
-
+
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Ab. # in BAL
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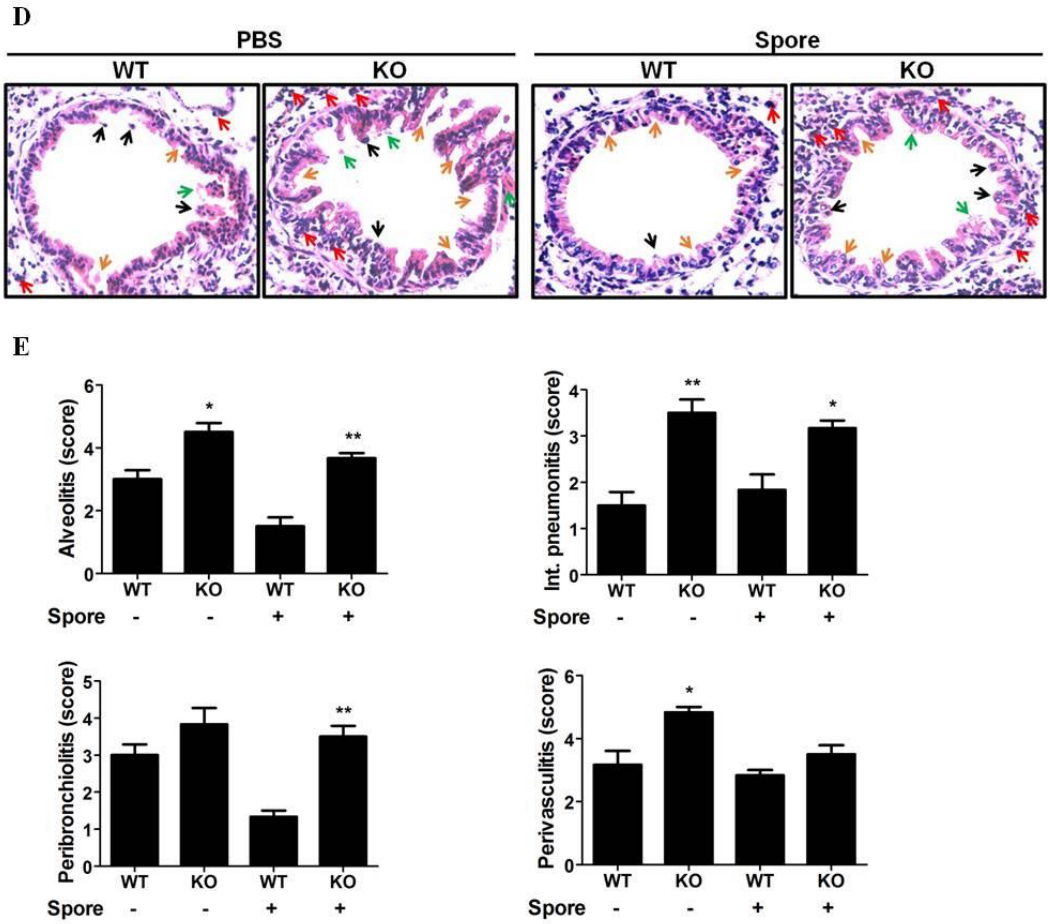

Figure 6. Protective mechanisms of spore pre-treated mice infected with RSV are dependent on MyD88 signaling in alveolar macrophages. Wild type or MyD88 knockout BALB/c mice were administered i.n. with spore at 5 days prior to RSV infection. (A) Body weight of wild type or MyD88 knockout mice was monitored daily after the infection. At 4 DPI, (B) viral load in the lungs was analyzed by plaque assay and (C) absolute number of AMs in post lavaged lungs and BAL cells were acquired by flow cytometry. (D) Blood-perfused lungs were stained with H&E for histological examination by microscopy at 200X

magnification and (E) scored for histopathology at 4 DPI. Arrows indicated are as follow; orange: epithelium thickness and destruction, green: pulmonary edema, red: inflammatory cells, and black: cell death. Data are expressed as mean ± S.E.M. for the group (n=5) or 3 independent experiments. * and ** indicate significant differences at $P < 0.05$ and, $P < 0.01$, respectively.

7) Improvement of antiviral activity of macrophages treated with spore is dependent on MyD88 signaling pathway

To confirm the necessity of MyD88-signaling in macrophages activated with spore, I have used bone marrow-derived macrophages (BMMs) from wild type or MyD88 knockout mouse. After the spore treatment and RSV infection, the viral titer in the culture supernatant was examined. Significantly high viral counts from MyD88-deficient BMMs were observed when compared to those of wild-type BMMs (Fig. 7A and Supplementary Fig. 8A) and spore-treated BMMs showed a meaningful reduction of viral load in a dose-dependent manner both MyD88-deficient and wild-type BMMs.. Furthermore, the ability to produce IL-12p40, best known to induce IFN-γ, was markedly augmented by spore treatment and substantially higher in wild-type BMMs than MyD88-deficient BMMs (Fig. 7B and Supplementary Fig. 8B). Hence, these results strongly support the idea that protective mechanism of macrophages by spore was indeed mediated via MyD88-dependent signaling.

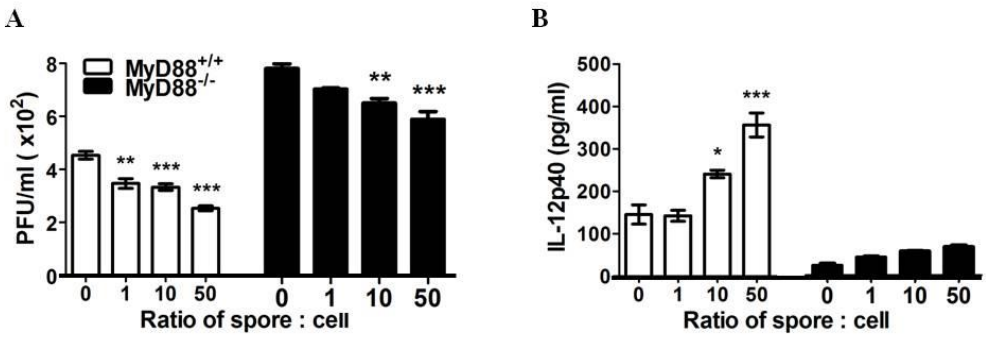

Figure 7. Bone marrow-derived macrophages pre-treated with spore develop their antiviral effects through MyD88-dependent pathway. Wild type or MyD88 knockout BMMs were treated with spore for 24 hour at ratio of spore per number of cells for 0, 1, 10, and 50. Then, the cells were washed and infected with 1 MOI virus for additional 12 hours. (A) Viral titers from wild type or MyD88 knockout BMMs were measured by plaque assay and (B) the production of IL-12p40 was measured by ELISA. Data are expressed as mean ± S.E.M. for 3 independent experiments. *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

IV. Supplementary results

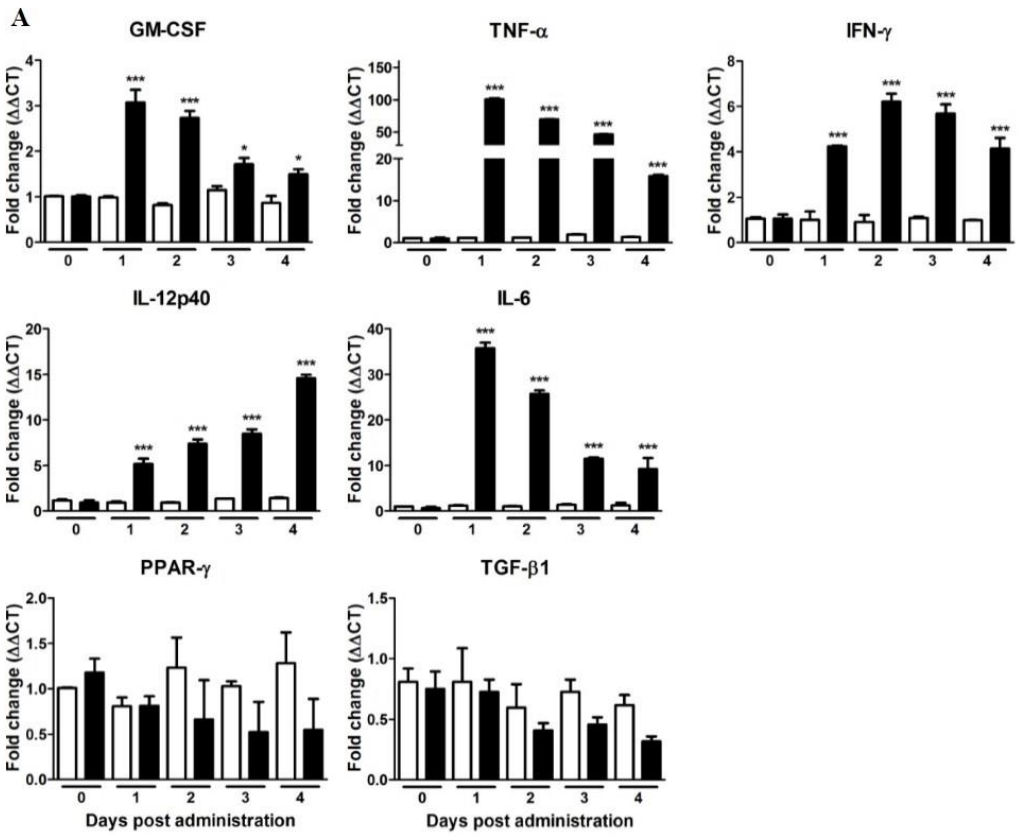

Figure S1. Intranasal administration with spore induces differentiation of M1 alveolar macrophages and expression of GM-CSF. Mice were administered with spore via intranasal route and then, blood perfused lungs were taken every day for 4 days. (A) Expression of genes associated with differentiation of M1 (TNF-α, IFN-γ, IL-12p40 and IL-6) or M2 (TGF-β) macrophages, GM-CSF, PPAR-γ were analyzed by quantitative real-time PCR. (B) Protein levels of GM-CSF and TNF-α were measured by ELISA. Empty and filled bars indicate PBS and Spore pre-treated mice, respectively. Data are presented as means ± S.E.M. (N=3). *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

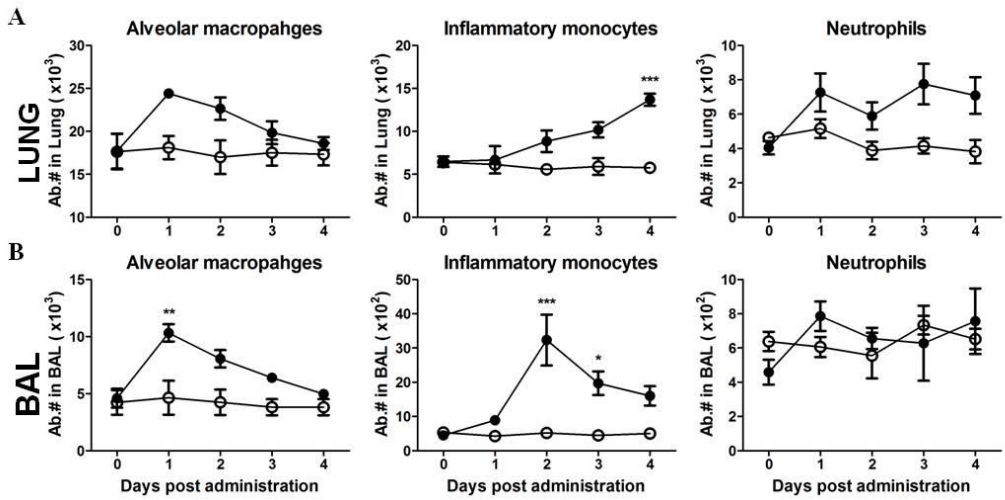

Figure S2. Intranasal administration with spore increases the number of alveolar macrophages. Mice were administered with spore via intranasal route and then, blood perfused lungs were taken every day for 4 days. (A) Change of various innate immune cells in the post-lavaged lung and (B) BAL fluid was analyzed by flow cytometry at 0 to 4 days post administration. Empty and filled circles indicate PBS and Spore pre-treated mice, respectively. Data are presented as means ± S.E.M. (N=3). *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

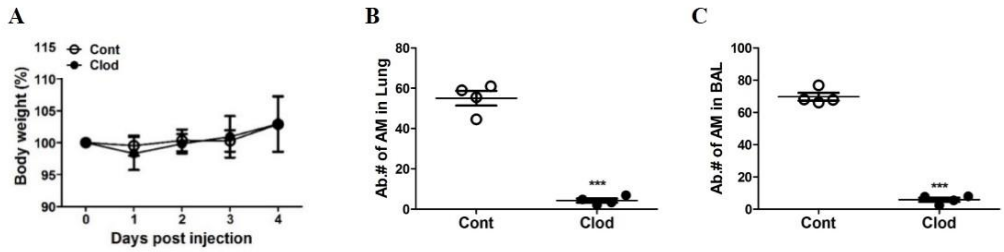

Figure S3. Intratracheal injection of clodronate-encapsulated liposome leads to the efficient depletion of alveolar macrophages. Mice were injected with clodronate-encapsulated liposome via intratracheal route at days 1 and 3 before sacrifice. (A) Body weight was monitored daily after the injection, and absolute number of alveolar macrophages in the (B) lung and (C) BAL was analyzed at day 4 post administration. 'Cont' and 'Clod' indicate the mice injected with control liposome and clodronate-encapsulated liposome, respectively. Data are expressed as mean ± S.E.M. for the group (n=5). *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

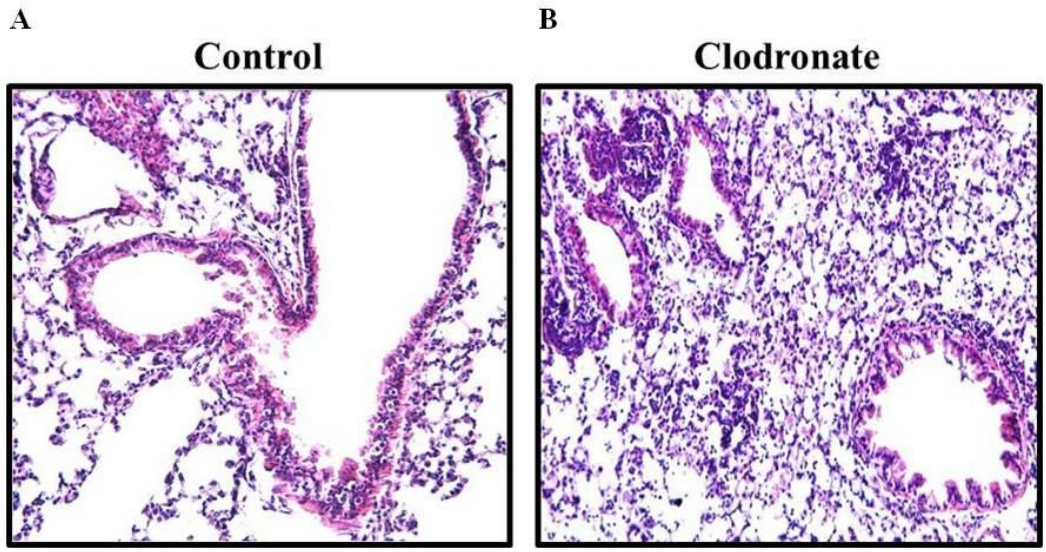

Figure S4. Depletion of alveolar macrophages exacerbates the pathology in RSV infected mice. Mice were injected with clodronate-encapsulated liposome through intra tracheal route on days 1 and 3 before RSV infection. At 4 DPI, perfused lungs were stained with H&E for histological examination by microscopy at 100 x magnification. Bronchus and blood vessel of (A) control and (B) clodronate-encapsulated liposome injected mice are shown.

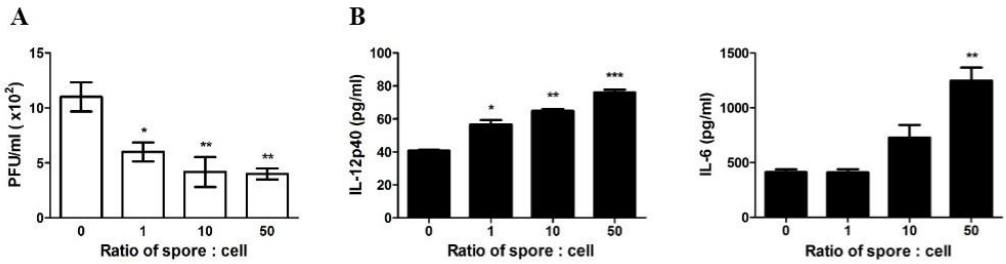

Figure S5. Alveolar macrophages cell line pre-treated with spore enhances antiviral effects. MH-S cells were stimulated with spore for 24 hours at the ratio of spore per number of cells for 0, 1, 10, and 50. Then, the cells were washed and infected with 1 MOI virus for 24 hours. (A) Viral load was analyzed by using plaque assay and (B) the production of IL-12p40 and IL-6 were measured by ELISA at 24 hours post infection. Data are expressed as mean ± S.E.M. for 2 independent experiments. *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively compared with the control.

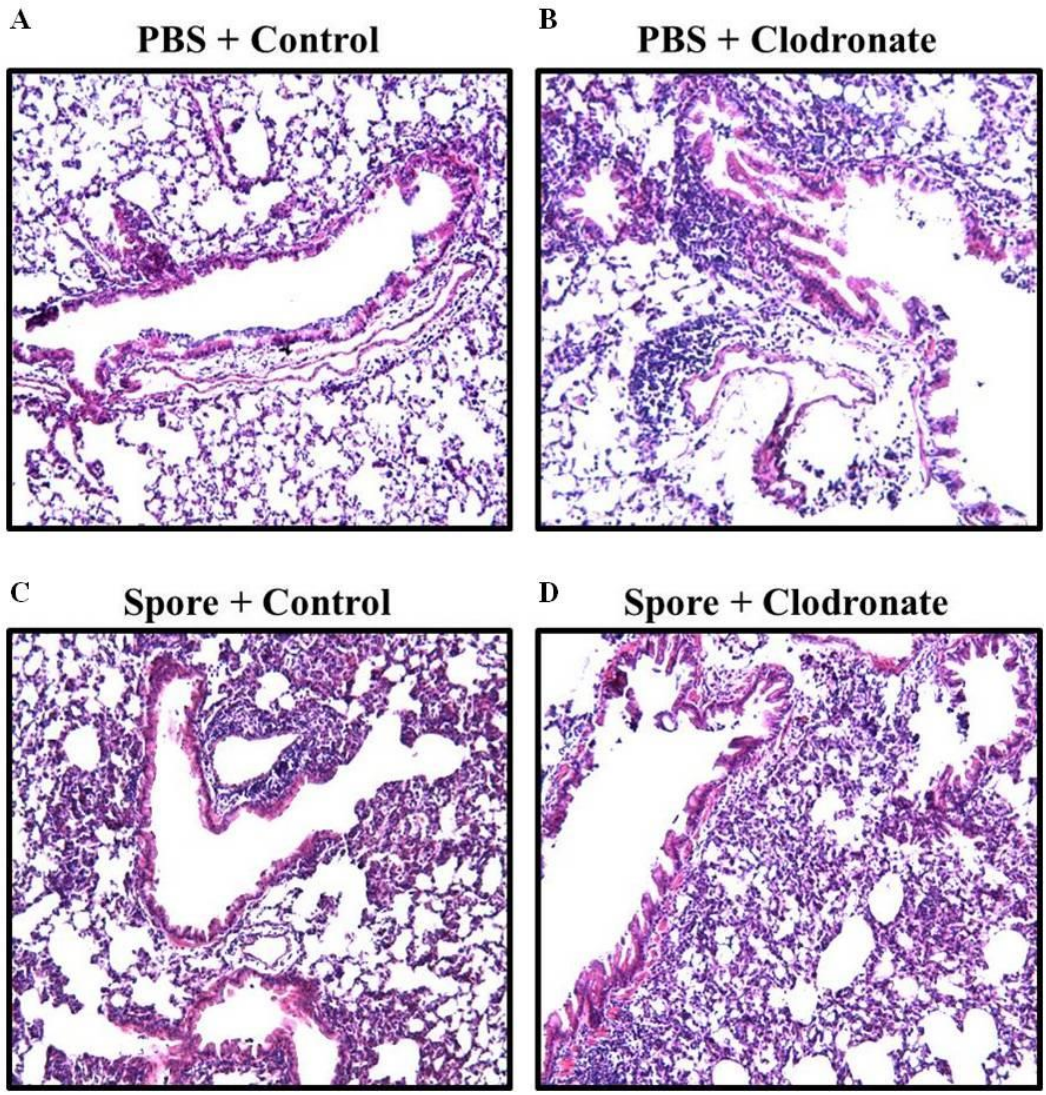

Figure S6. Alveolar macrophages are indispensable for the protection in mice infected with RSV. Mice were administered with spore via intranasal route at 5 days prior to RSV infection. Then the mice were injected with control or clodronate-encapsulated liposome through intratracheal route on days 1 and 3 before the infection. At 4 DPI, perfused lungs were stained with H&E for

histological examination by microscopy at 100 x magnification. Bronchus and blood vessel of mice treated with (A) PBS / control-liposome, (B) PBS / clodronate-encapsulated liposome, (C) spore / control liposome, and (D) spore / clodronate-encapsulated liposome are shown.

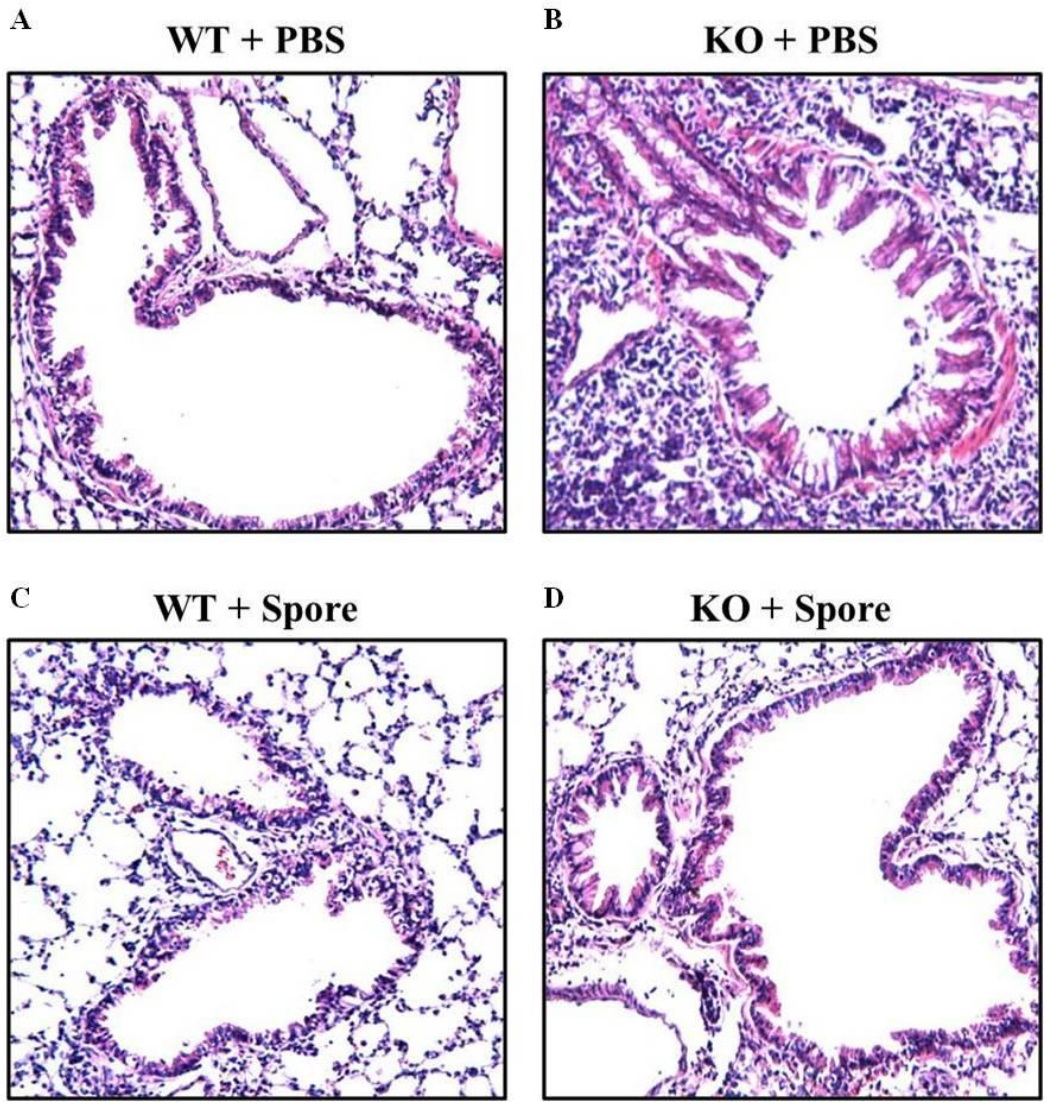

Figure S7. MyD88 signaling plays a crucial role to alleviate histopathology of mice infected with RSV. Wild type or MyD88 knockout mice were administered with spore via intranasal route on 5 days prior to RSV infection. At 4 DPI, perfused lungs were stained with H&E for histological examination by microscopy at 100 x magnification. Bronchus and blood vessel on the (A) PBS pre-treated wild type

mice, (B) PBS pre-treated knockout mice, (C) spore pre-treated wild type mice, and (D) spore pre-treated knockout mice are shown.

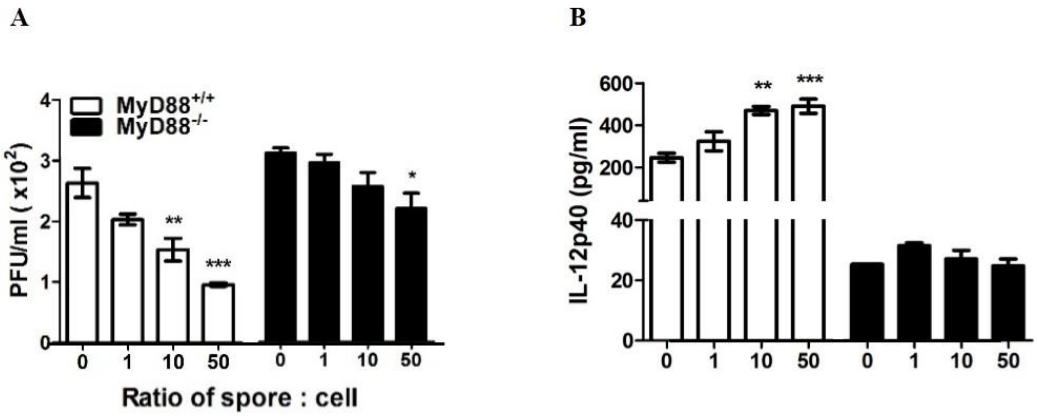

Figure S8. Bone marrow-derived macrophages pre-treated with spore enhance their antiviral effects via MyD88-dependent manner. BMMs were stimulated for 24 hours with spore at ratio of spore per number of cells was 0, 1, 10, and 50. Then, the cells were washed and infected with 1 MOI virus for 24 hours. (A) Viral load was analyzed by using plaque assay and (B) the production of IL-12p40 was measured by ELISA at 24 hours post infection. Data are expressed as mean ± S.E.M. for 3 independent experiments. ** and *** indicate significant differences at $P < 0.01$ and $P < 0.001$, respectively compared with the control.

V. Discussion

Importance of alveolar macrophages (AMs) for host defense mechanism during early viral infection in respiratory tract is well established, however the precise role of AMs, in the RSV infected mice is less clear.

In the present study, I focused on the specific role of AMs treated with *Bacillus subtilis* spore in RSV infected mice for their protection.

Notable findings were as following; (1) AMs have a pivotal role for protection during the initial stage of RSV infection; (2) Administration of spore derived from *Bacillus subtilis* induces the activation of AMs coincident with up-regulation of GM-CSF and classically activated macrophages (M1 macrophages)-related cytokines; (3) Nasal delivery of spore induces the increase of antiviral effector molecules against RSV infection; and (4) MyD88 signals in the AMs are critical for the protection against RSV.

Induction of innate immune response in the early phase of viral infection is necessary for host immunity (Copenhaver et al., 2014, McGill et al., 2009), and thus over the years various attempts have been tried to improve the innate immunity in order to improve the protection against viral infection. Delivery of probiotics and their related materials is one of the strategies (Yaqoob, 2014, Kiso et al., 2013) and in the present study I have sought a potential ability of spore to enhance innate immunity against RSV in line with other studies (McKenney et al., 2013, Hong et al., 2008).

It has been well demonstrated on the importance of AMs in diverse respiratory infections such as a primary recognition of antigens and scavenger to infected cells

(Tate et al., 2010) but the precise role and protective mechanism of AMs in RSV infected mice are yet to be illuminated.

The results showed that mice pre-treated spore through i.n. route rapidly up-regulated GM-CSF and classically activated macrophages (M1 macrophage)-related genes such as TNF-α, IFN-γ, IL-6, and IL-12p40. GM-CSF is well known an essential factor for the local differentiation (Guilliams et al., 2013), survival (Shibata et al., 2001), replenishment (Steinwede et al., 2011) and the ability of host defense (Ghoneim et al., 2013) of macrophages. Also, IFN-γ could lead monocytes differentiate into classically activated macrophages (M1 macrophages). This polarization is important in limiting tissue damage (Barros et al., 2013) and induces the increase of cytokines that enhance the ability of AMs for the killing of intracellular pathogens.-related genes enhance the ability of AMs for the killing of intracellular pathogens (Barros et al., 2013, Martinez and Gordon, 2014). These results imply that pre-treatment of spore could enhance the viability of macrophages that promotes their antiviral functions. On the other hand, pre-treatment with spore fail to induce TGF-β and PPAR-γ which keeps close link with alternatively activated macrophages (M2 macrophages) differentiation. (Ginhoux, 2014, Schneider et al., 2014), suggesting AMs, found increased in the present study, were not differentiated but most likely infiltrated to the lung area.

Consistent with these, mice pre-treated with spore before the RSV infection displayed a certain protective immunity as shown by significantly low level of viral titer and maintenance of body weight. Interestingly, mice pre-treated with spore showed a rapid increase of AM population after the infection. It was reported that

AMs play a reservoirs role for inhaled antigens (Copenhaver et al., 2014), and thus, the rapid increase of AMs could give an efficient protective effect. Furthermore, spore treatment induced inflammatory monocytes with Ly6C high phenotype, known to regulate type I IFN signaling during acute viral pneumonia in mice (Seo et al., 2011) with ability to differentiate into macrophages (Geissmann et al., 2010). Indeed spore pre-treated mice showed remarkably high concentration of IFN-β. Recent study showed that AMs are the major source of type I IFNs during RSV infection and an underappreciated facet of type I IFNs-dependent resistance lead to a cell-extrinsic response through rapid recruitment of antiviral inflammatory monocytes to the site of infection (Goritzka et al., 2015).

It has been suggested that the absence of AM-mediated defense mechanism led to the failure of protective immune responses, the exacerbation of pathology, and the accumulation of debris following pulmonary infection (Kolli et al., 2014). Direct evidence showing important role of AMs in the present study came from the *in vivo* experiment with depletion of AMs. The results showed that the AM-depleted mice suffered from high viral load and intense pathological episode as shown by the accumulation of dead cells and debris especially in bronchi and blood vessels, indicative of early pulmonary inflammation. All these findings clearly support the critical role of AM in controlling disease severity of mice infected with RSV. With regard to the effect of spore on AMs, AM treated with spore rendered less susceptible to RSV infection and more importantly, anti-viral function of spore was markedly reduced upon depletion of AMs, implying AM as a major target for spore as well as a key player for the protection.

TLR2 and TLR4 signaling pathways, in which MyD88 serves as downstream molecules, are essential for the protection in RSV infection (Shirey et al., 2010, Murawski et al., 2009). In the present study, MyD88$^{-/-}$ mice showed persistence of high viral counts in parallel with impaired body weight gain after the infection suggesting that MyD88 is indispensable for the protection against RSV infection. Furthermore, it was evident that MyD88$^{-/-}$ mice fail to recruit AMs into the lung region, irrespective of a spore administration, suggesting that spore-induced infiltration of AMs in the lung and BAL was dependent on MyD88. MyD88$^{-/-}$ mice also displayed severe pathological signs in the lung compared to wild type mice. These results imply that MyD88-dependent signaling might be the key mechanism by which AMs treated with spore contribute to protection of mice from RSV infection.

Spore could promote the GM-CSF and classically activated macrophages (M1 macrophages)-related cytokines to enhance the viability and M1 macrophages prone signatures including IFN-γ and TNF-α, which in turn induce the infiltration of AMs via MyD88 pathway and/or directly influence antiviral function of AMs (Fig 8). Under the infected circumstance, AMs treated with and activated by spore are enforced to produce IFN-β and IL-12p40, which may serve as an alarm signal and facilitate IFN-γ production. This would likely to trigger efficient adaptive immunity for fighting against RSV infection and protect hosts from severe lung injury (Fig 9). When AMs were depleted and infected with RSV, the mice were unable to control the viral replication and thus succumbed to the aggressive lung

injury as shown by the failure of viral clearance and the severe destruction of epithelium (Fig 10).

Collectively, the present study provokes a key function of AMs induced by *Bacillus subitilis* spore in antiviral activity and protection of lung environment against RSV-induced damage in mice infected with RSV via MyD88 signal.

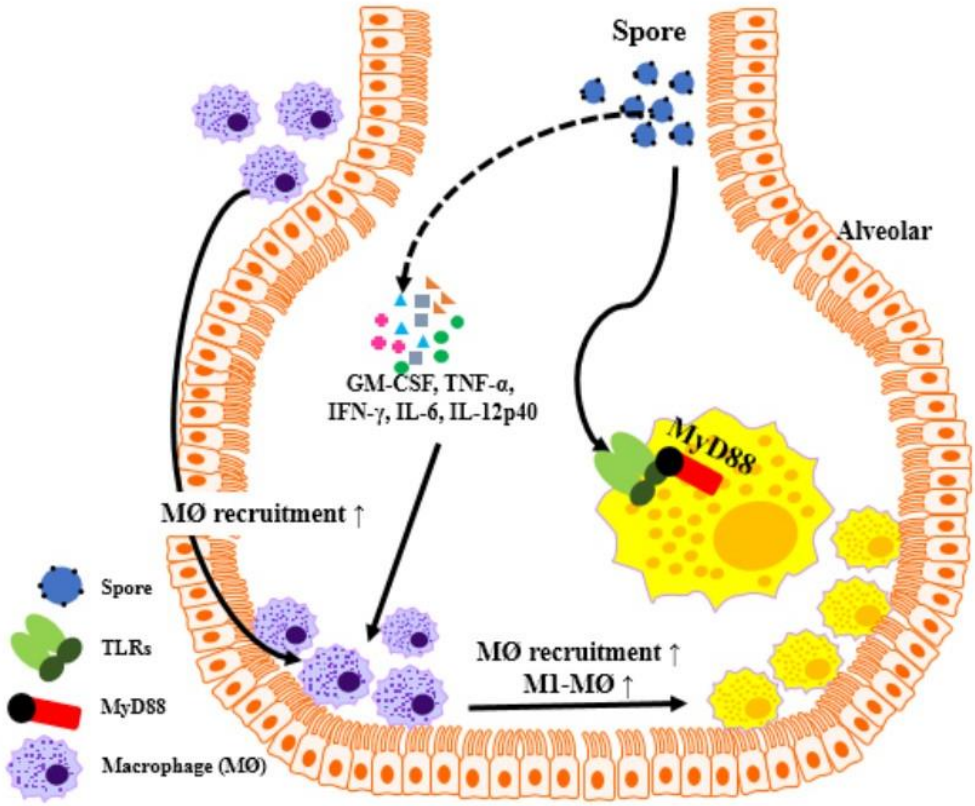

Figure 8. The administration of spore through intranasal route upregulates GM-CSF and classically activated macrophages (M1)-related cytokines coincident with recruitment of alveolar macrophages.

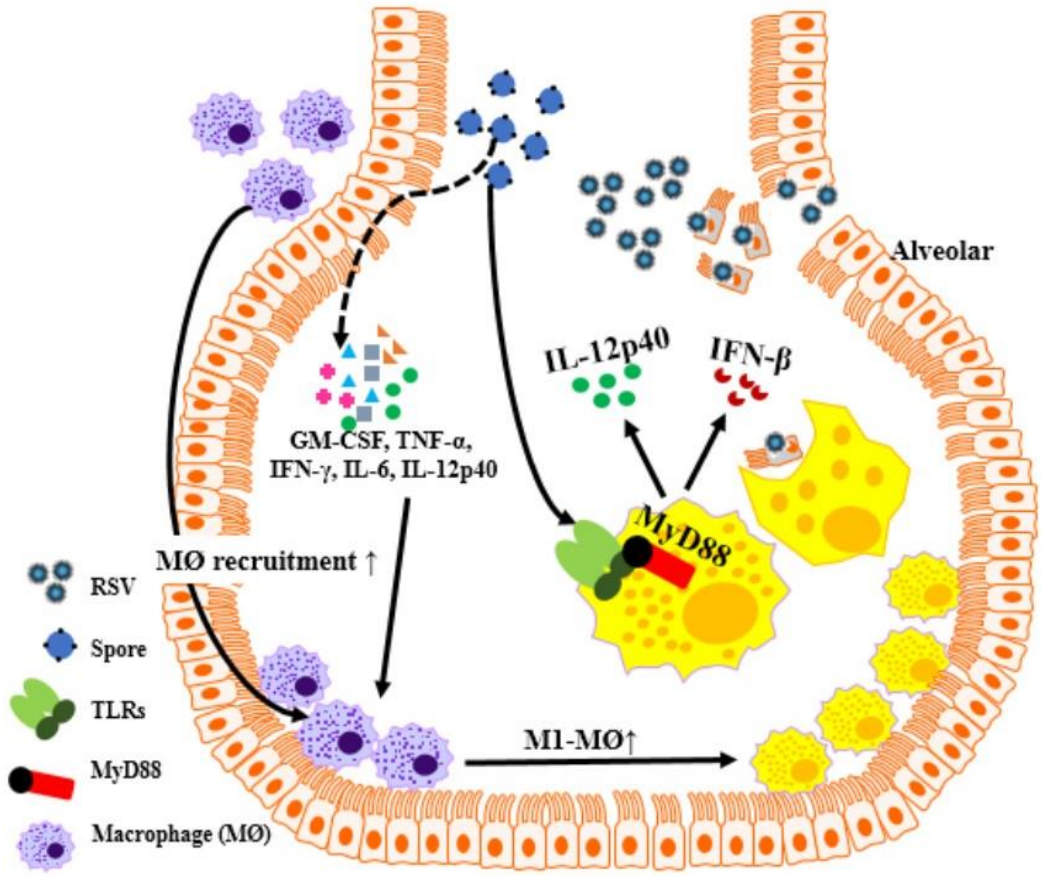

Figure 9. Intranasal pre-treatment of spore in mouse infected with RSV induces protective immunity through MyD88 signaling.

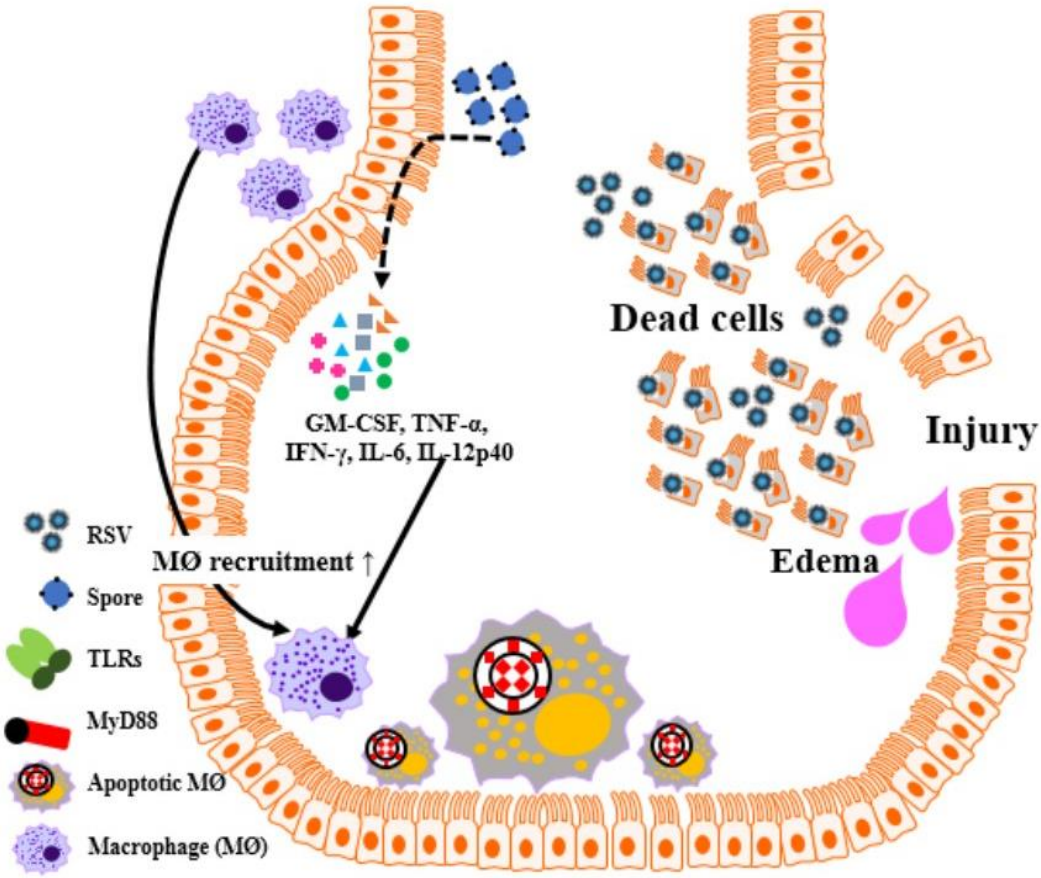

Figure 10. The absence of alveolar macrophage aggravates the disease severity following RSV infection.

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VII. Summary in Korean

호흡기성 융합 바이러스 (respiratory syncytial virus A2, RSV) 감염은 면역체계가 약화된 영·유아 그리고 노인들에게서 흔히 발생하는 호흡기성 질환이다. 다양한 감염성 호흡기 질환에서 성공적인 초기 면역 반응의 유도가 질병 대항에 매우 중요하다는 것은 익히 알려졌지만, 초기 면역 반응을 구성하는 대표적인 내재 면역 세포인 폐포 대식세포의 호흡기성 융합 바이러스 감염 시 구체적인 방어기전은 아직 자세히 밝혀져 있지 않다. 따라서 본 연구는 *Bacillus subtilis* 포자에 의해 활성화된 폐포 대식세포가 호흡기성 융합 바이러스 감염에 방어하는 기전을 마우스 모델에서 규명하였다.

본 연구 결과는 호흡기성 융합 바이러스의 감염 초기 단계에서 폐포 대식세포가 중요하게 작용함을 보여주었으며, *Bacillus subtilis* 포자의 비강 투여는 폐포 대식세포의 활성화를 유도함과 함께 항 바이러스성 효과 물질인 IFN-β 와 IL-12p40 과 GM-CSF, 그리고 M1-마크로파지 관련 유전자를 유도함을 밝혔다. 더욱이, 이러한 방어기전은 폐포 대식세포의 MyD88 시그널에 의존적임을 증명하였다. 비강 투여를 통한 *Bacillus subtilis* 포자의 전 처리는 호흡기성 융합 바이러스 감염 마우스에서 효과적인 방어 면역반응을 유도하여 감염 후 4 일 뒤 채취한 마우스 폐의 바이러스 농도가 유의적으로 감소함을 보였다. 이는 일반적으로 나타나는 호중구와 염증성 단핵구 증감이 아닌 폐포

대식세포의 증가라는 특이적인 현상과 관련이 있음을 확인할 수 있었다. 또한 폐포 대식세포가 제거된 마우스에서는, 감염 후 폐의 바이러스 농도가 매우 높은 수준으로 측정되는 결과를 얻었으며, 동시에 병리학적 질병 심화가 유도됨을 알 수 있었다. 이러한 결과로부터 *Bacillus subtilis* 포자로 활성화된 폐포 대식세포는 호흡기성 융합 바이러스 감염의 방어에서 필수 불가결한 구실을 함을 확인할 수 있었다. 더불어 MyD88 신호전달이 결여된 MyD88 유전자 결핍 마우스는 *Bacillus subtilis* 포자의 처리와 무관하게 호흡기성 융합 바이러스 감염에 감수성이 매우 높은 것으로 보아 효과적인 방어기전에는 폐포 대식세포의 MyD88 신호전달이 필수적임을 알 수 있었다.

결론적으로, 본인은 이 연구를 통하여 *Bacillus subtilis* 포자의 비강 투여가 MyD88 신호전달 의존적으로 초기 폐포 대식세포의 활성을 유도하며, 이 과정은 호흡기성 융합 바이러스 감염에 대항하기 위해 필수적임을 밝혔다.