SHOCK, Vol. 40, No. 4, pp. 312–319, 2013

INHIBITION OF NOTCH SIGNALING PROTECTS MOUSE LUNG AGAINST ZYMOSAN-INDUCED INJURY

Han Han,* Gu Gong,† Xiaoguang Bai,* Yen-Chang Lin,‡§ Jing Sun,* Wenyong Wang,‖ Yiling Zhao,‖ Lifang Yang,* Xiaoxia Wang,* Zexin Zhang,* Hailong Dong,* Lichao Hou,¶ and Lize Xiong*

**Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi Province; and †Department of Anesthesiology, General Hospital of Chengdu Military Region, Chengdu, Sichuan Province, People's Republic of China; ‡Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia; §Graduate Institute of Biotechnology, Chinese Culture University, Yang-Ming-Shan, Taipei, Taiwan, Republic of China; and ‖Department of Pathology and Pathophysiology, Fourth Military Medical University, and ¶Surgical ICU, Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi Province, People's Republic of China*

Received 31 Dec 2012; first review completed 18 Jan 2013; accepted in final form 18 Jun 2013

ABSTRACT—Notch signaling, a critical pathway in cell fate determination, is well known to be involved in immune and inflammatory reactions, whereas its role in acute lung injury (ALI) remains unclear. Here, we report that notch signal activity is upregulated in lung tissue harvested from an ALI mouse model (induced by zymosan). We showed that notch signal activity in lung tissue was increased 6 h after zymosan injection and peaked at 24 h. Inhibition of notch signaling by either pre– or post–zymosan treatment with N-[N-(3,5-difluorophenacetyl)-l-alanyl]-(S)-phenylglycine t-butyl ester (DAPT) significantly reduced lung injury, characterized by improvement in lung histopathology, lung permeability (protein concentration in bronchoalveolar lavage fluid and lung wet-to-dry weight ratio), lung inflammation (bronchoalveolar lavage fluid cell count, lung myeloperoxidase, and tumor necrosis factor α), and also alleviated systemic inflammation and tissue damage, thus increasing the 7-day survival rate in zymosan-challenged mice. In conclusion, the role of notch signaling is functionally significant in the development of ALI. Inhibition of notch signaling by pretreatment or posttreatment with DAPT likely exerts its effects in part by mediating the expression of proinflammatory and anti-inflammatory cytokines and influencing tissue neutrophil recruitment. These results also imply that notch inhibitors may help attenuate local inflammatory lung damage.

KEYWORDS—Zymosan, acute lung injury, notch signaling, inflammation

ABBREVIATIONS—ALI — acute lung injury; BALF — bronchoalveolar lavage fluid; CRP — C-reactive protein; DMSO — dimethyl sulfoxide; IL-10 — interleukin 10; LDH — lactate dehydrogenase; MODS — multiple organ dysfunction syndrome; NICD — notch intracellular domain; NS — normal saline; SIRS — systemic inflammatory response syndrome; TNF-α — tumor necrosis factor α; WBC — white blood cell

INTRODUCTION

Multiple organ dysfunction syndrome (MODS) is the leading cause of morbidity and mortality in current intensive care unit practice (1). Lung is the most common primary organ injured, manifested by pulmonary dysfunction, which occurs before any other organ dysfunction. Although it is generally acknowledged that systemic inflammatory response syndrome (SIRS) plays a major role in the pathogenesis and pathophysiology of sepsis/MODS (2), and SIRS is considered a common pathway from critical diseases to MODS/multiple organ failure (3), the underlying mechanisms behind these afflictions remain incompletely understood (4, 5).

The notch signaling pathway regulates cell differentiation, proliferation, survival, and development, as well as inflammatory diseases (6, 7). The notch pathway comprises a family of transmembrane receptors, ligands, negative and positive modifiers, and transcription factors. Ligand binding induces proteolytic cleavage including S2 cleavage mediated by metalloprotease and S3 cleavage mediated by γ-secretase. S3 cleavage is considered more important as it causes notch receptor cleavage, leading to release of the notch intracellular domain (NICD) from the plasma membrane. This subsequently allows translocation into the nucleus where notch binds to the DNA-binding protein CBF1/RBPj/Su (H)/Lag-1 (CSL) through RAM domains that mediate transcriptional activation of target genes, which in turn play a key role in notch signal transduction (8, 9). N-[N-(3,5-difluorophenacetyl)-l-alanyl]-(S)-phenylglycine t-butyl ester (DAPT) is a γ-secretase inhibitor, which represses S3 cleavage, hampers the release and translocation of NICD, and therefore inhibits the activation of notch signaling (10, 11). It has been demonstrated that Toll-like receptor pathways activate notch target genes without notch signal activation, whereas notch signals increase the production of Toll-like receptor–induced cytokines in macrophages (6, 12). Notch signals also induce activation of the nuclear factor κB pathway, which is a vital pathway in the development of inflammation (13, 14). In addition, notch

Address reprint requests to Lichao Hou, PhD, MD, Surgical ICU, Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi Province, People's Republic of China. E-mail: Lichao.Hou@gmail.com.

Co-correspondence: Lize Xiong, PhD, MD, Surgical ICU, Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi Province, People's Republic of China. E-mail: mzkxlz@126.com.

H.H., G.G., X.B., and Y.-C.L. contributed equally to this work.

This work was supported by research grants from the National Natural Science Foundation of China (30672041 to L.H. and 30725039 to L.X.), Shaanxi Province (2004K17-G15 to L.H.), and PLA (06G086 to L.X.).

The authors declare that there is no conflict of interest.

DOI: 10.1097/SHK.0b013e3182a102e5

signaling has been shown to play an essential role in airway inflammation (15). Recently, Tsao et al. (16) observed a synergistic effect between notch signaling and the lipopolysaccharide (LPS) pathway, both *in vitro* and *in vivo*. Here, we hypothesized that the notch signaling pathway might be involved in the emergence and development of inflammation-associated lung injury.

Previous studies have shown that zymosan (ZY), a substance derived from the cell wall of the yeast *Saccharomyces cerevisiae*, induces systemic inflammation by activating a wide range of inflammatory mediators when injected in animals (17). It has been demonstrated that i.p. injection of ZY induces a SIRS/MODS model in rats or mice, and the ZY-induced generalized inflammation (ZIGI) model is a widely accepted animal model of MODS, which has been used in many previous studies (18–20). Our previous work has also reported that i.p. injection of ZY (1.0 g/kg body weight) successfully induces a murine acute lung injury (ALI) as well as a SIRS/MODS model (21, 22). The present study demonstrates that pharmacological blockade of notch activation attenuates lung injury, alleviates systemic inflammation, and improves the survival rate of ZY-challenged mice.

MATERIALS AND METHODS

Animals

Male Kunming mice (specific pathogen-free), a kind of Swiss mice, 6 to 8 weeks old, 20 to 25 g in weight, were purchased from the Laboratory Animal Center of the Fourth Military Medical University. All animals were housed in plastic boxes with free access to food and water, at 20°C to 22°C with a constant 12-h light-dark cycle. All experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University and were handled in accordance with the National Institutes of Health guidelines.

ZIGI model

Zymosan (Sigma Chemical Co, St Louis, Mo) was dissolved in normal saline (NS) to a final concentration of 25 mg/mL and sterilized at 100°C for 80 min. All suspensions were made fresh before use. A generalized inflammation model was induced by aseptic i.p. injection of ZY at a dose of 1 g/kg body weight (21, 23). For the control, an equal volume of NS was administered by the same route.

Pretreatment or posttreament with DAPT

A notch signal inhibitor, DAPT (40 mg/kg body weight) (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), dissolved in 10% dimethyl sulfoxide (DMSO) was administered intravenously in mice 30 min before or 60 min after the administration of ZY or an equal volume of NS. Equal volumes of DMSO were used as controls (10).

Western blot analysis

Lung tissues were lysed in ice-cold RIPA buffer. Lung homogenate samples (60 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride. The blots were probed with anti-NICD antibody (Abcam, Cambridge, UK), followed by anti–rabbit horseradish peroxidase–conjugated antibody (Cell Signaling, Beverly, Mass). Immunostained proteins were detected by ECL (CWBIO, Peking, China).

Lung histological observations

Lungs were obtained from different groups at desired time points. Tissues were fixed with 10% formalin at room temperature for 48 h, embedded in paraffin, and sectioned into 4- to 6-μm slices. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin. Evaluation of lung injury was performed by two pathologists who were blinded to experimental groups based on the scoring standard described below. Normal lung histological findings were scored as 1. Edema, congestion, slightly alveolar, macrophage infiltration, or small area hemorrhage was scored as 2. Large area hemorrhage, severe macrophage infiltration and proliferation, or consolidation involving half of the lung was scored as 3. Severe hemorrhage and consolidation involving virtually the whole lung were scored as 4.

Wet-to-dry weight ratio of lung tissue

Lung weight ratio was measured to evaluate the magnitude of pulmonary edema. The left lung was harvested and weighed and then heated in an oven at 95°C for 24 h. The weight of the left lung was again obtained after heating, and the wet-to-dry (W/D) ratio was recorded.

Cell count and total protein assay in bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was obtained by cannulating the trachea with a 20-gauge catheter. Animals were anesthetized, and the trachea and lung were exposed by thoracotomy. A cannula was inserted into the trachea and was ligated by using a thread. Phosphate-buffered saline (pH 7.4) was injected into the lung via a syringe fitted with the tracheal cannula. The phosphate-buffered saline was allowed to stay in the lung for 30 s and was then instilled with a syringe, which was repeated three times with the same solution (0.7 mL per lavage). The total return volume of BALF was 1.6 to 1.9 mL. Lavage samples were prepared for white cell count with a hemocytometer (3536A, BILON) followed by centrifugation (1,500*g*, 10 min, 4°C). The supernatant was stored at 20°C. Neutrophil counts were performed on cytocentrifuge preparations (Cytospin 3; Shandon Scientific, Cheshire, UK) stained with Diff-Quik stain (Baxter Diagnostics, McGaw Park, Ill). Total protein was measured in the cell-free supernatant using the Lowry method. Bovine serum albumin was used as a standard (24).

Measurement of lung myeloperoxidase activity

Activity of myeloperoxidase (MPO), an indicator of neutrophil infiltration in lung tissue, was detected in lung supernatant homogenates and was measured as previously reported (25). Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 37°C and was expressed in units per milligram weight of wet tissue (U/mg tissue). The change in absorbance was measured at 590 nm with a spectrophotometer (Nano Quant Infinite M200; Tecan, Männedorf, Switzerland). The activity of MPO was measured using commercial kits purchased from Cayman Chemical Company (Ann Arbor, Mich).

Detection of tumor necrosis factor α in serum and lung, and interleukin 10, C-reactive protein, and lactate dehydrogenase in serum

At predetermined time points, animals were anesthetized with pentobarbital (50 mg/kg), and blood samples were collected by cardiac puncture, followed by clotting for 30 min at 25°C. The serum was separated by centrifugation at 3,000*g* for 10 min at 4°C, aliquoted, and stored at −80°C until assayed. After the blood had been sampled, the lung was immediately removed. Lung tissue homogenates were prepared in prechilled phosphate buffer (0.1 M, pH 7.4) and were centrifuged at 10,000*g* at 4°C for 10 min. The supernatants were collected, aliquoted, and stored at −80°C until assayed.

The levels of serum interleukin 10 (IL-10), C-reactive protein (CRP), and lactate dehydrogenase (LDH), as well as serum and lung tumor necrosis factor α (TNF-α), were determined using specific enzyme-linked immunosorbent assay kits (TNF-α, IL-10, CRP, LDH; R&D Systems Inc, Minneapolis, Minn) with a microplate reader (Nano Quant Infinite M200; Tecan). All standards and samples were run in triplicate.

Statistical analysis

Survival rates are expressed as a percentage, and the histopathological scores are expressed as a median (range) value. The measurement data are expressed as a mean (SD). Survival data were calculated by log-rank test. The intergroup differences in histopathological scores were tested using the Kruskal-Wallis *H* method, followed by Nemenyi test for multiple comparisons. The intergroup differences in biochemical parameters, inflammatory cytokines, BALF cell count and total protein, and lung W/D ratio were tested using one-way analysis of variance followed by a least significant difference test for multiple comparisons. The statistical analysis was performed with SPSS 16.0 software (SPSS Inc, Chicago, Ill). In all tests, $P < 0.05$ was considered statistically significant.

RESULTS

Zymosan increased lung NICD expression

The new mobile cytoplasmic subunit (NICD) is considered a critical molecule in the notch pathway (26). Therefore, we first examined expression levels of NICD in lung tissue after ZY administration. Lung tissues were collected at 0, 1, 6, 12, 24, 48, and 72 h after ZY injection (1 g/kg, i.p.). As shown in Figure 1, lung NICD expression level significantly increased upon ZY

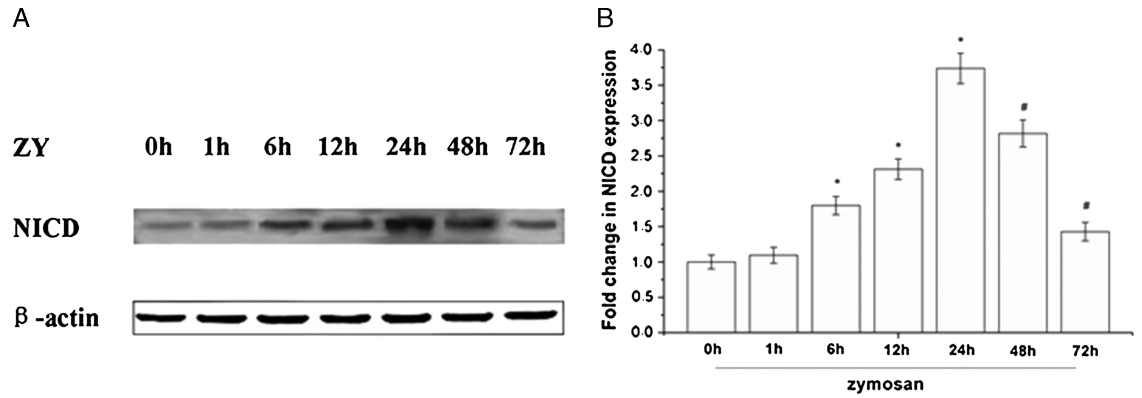

FIG. 1. **Zymosan increased notch signal in lung**. Animals received ZY injection (1 g/kg, i.p.). Lung tissues were collected 0, 1, 6, 12, 24, 48, and 72 h after ZY administration. The expression of NICD was determined by Western blot. $^{*}P < 0.05$ vs. 0 h after ZY administration; $^{\#}P < 0.05$ vs. 24 h after ZY administration.

stimulation at 6 h, peaked at 24 h, and declined after 48 h, as shown by Western blot. These results demonstrated that ZY administration induced an increase in NICD expression in the mouse lung.

DAPT inhibition of notch signaling prevented the increase in ZY-induced NICD expression

As a γ-secretase inhibitor, DAPT can repress the generation of NICD (11). To confirm this effect, we monitored lung NICD expression in animals treated with DAPT. As shown in Figure 2, NICD expression was increased in the ZY + pre-DMSO and ZY + post-DMSO groups compared with those in the NS + DMSO group. These results were reversed upon inhibition of notch signaling via DAPT administration before or after ZY injection (Fig. 2). The results indicated that either pretreatment or posttreatment with DAPT prevented the previously observed increase in NICD expression induced by ZY.

DAPT inhibition of notch signaling reduced ZY-induced lung injury in mice

To test whether notch signaling is required for ZIGI *in vivo*, we investigated the effects of the notch signal inhibitor DAPT on inflammation, tissue damage, animal survival, and lung injury in ZY-stimulated mice. As the lung is the most frequent target organ in MODS, we closely examined lung injury by assessing lung histopathology, lung permeability (protein concentration in BALF, and lung W/D weight ratio), as well as lung inflammation (BALF cell count, MPO, TNF-α, and IL-6 in lung homogenate).

As shown in Figures 3 to 5, no significant lung injuries were found in either the NS + DMSO or NS + DAPT groups. Zymosan stimulation induced lung injury in the ZY + pre-DMSO and ZY + post-DMSO groups, which was partially reversed by administration of DAPT before or after ZY injection.

No significant histopathological changes were observed in the NS + DMSO and NS + DAPT groups (Fig. 3, A and B). In animals that underwent ZY challenge, we observed significant histopathological changes in the lung characterized by alveolar wall thickening, neutrophil infiltration into the interstitium and alveolar space, and alveolar hemorrhage in lung tissue sections (Fig. 3, C and D). Either pretreatment or posttreatment with DAPT alleviated these changes (Fig. 3, E and F).

As shown in Table 1, the lung histopathological score in the NS + DMSO group was 1. In the ZY + pre-DMSO and

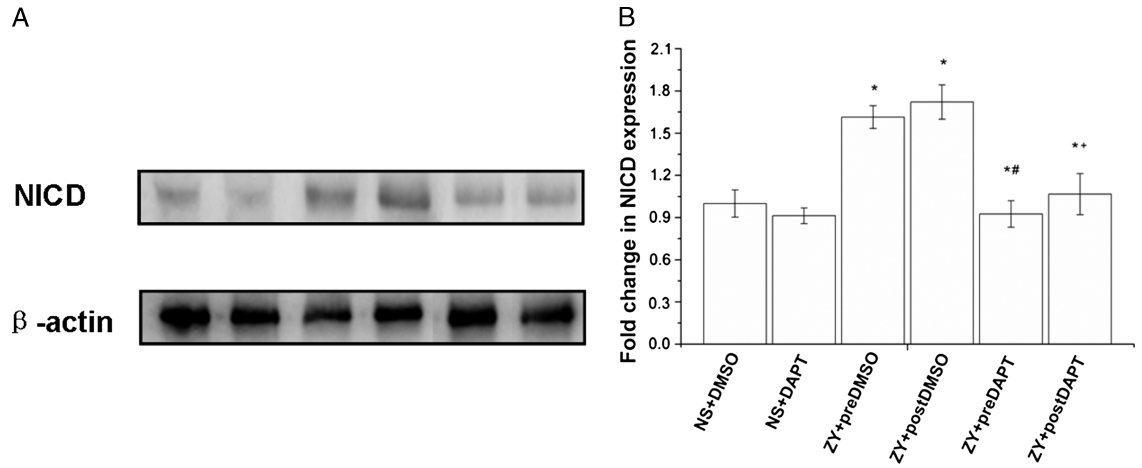

FIG. 2. **Inhibition of notch signaling by either pretreatment or posttreatment with DAPT prevented the increase in ZY-induced NICD expression**. Animals were randomly divided into six groups (n = 6 per group): NS + DMSO, NS + DAPT, ZY + pre-DMSO, ZY + post-DMSO, ZY + pre-DAPT, and ZY + post-DAPT groups. In the ZY + pre-DMSO, ZY + post-DMSO, ZY + pre-DAPT, and ZY + post-DAPT groups (pre: drugs were administered in mice 30 min before the administration of ZY or equal volume of NS; post: drugs were administered in mice 60 min after the administration of ZY or equal volume of NS), the generalized inflammation was induced by i.p. injection of ZY. In the NS + DMSO and NS + DAPT groups, the same volume of NS was used as a control. Lung tissues were collected 24 h after ZY or NS injection. The NICD expression was determined by Western blot. $^{*}P < 0.05$ vs. NS + DMSO group; $^{\#}P < 0.05$ vs. ZY + pre-DMSO group; $^{+}P < 0.05$ vs. ZY + post-DMSO group.

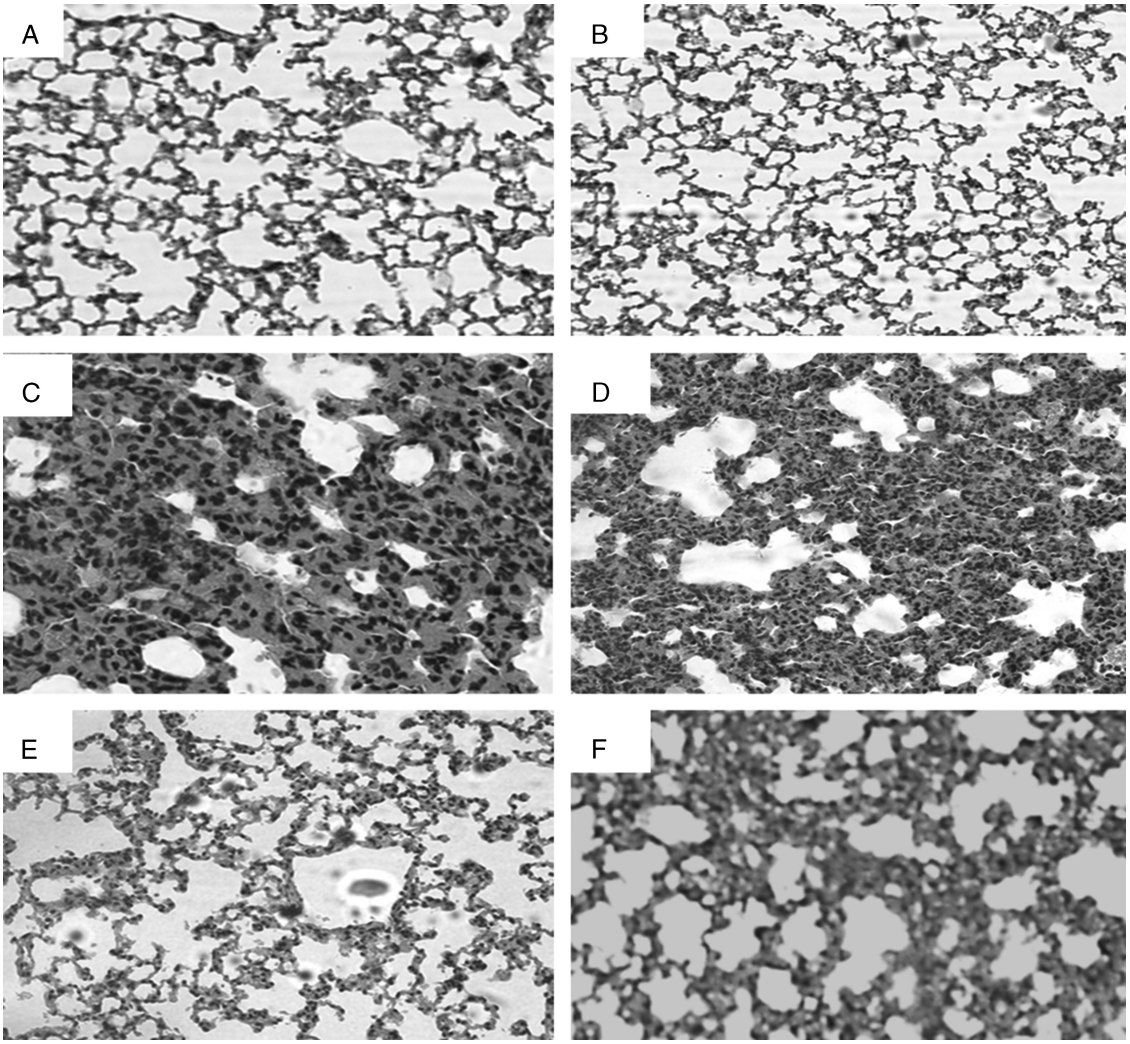

Fig. 3. **Inhibition of notch signaling reduced lung histopathological injury in ZY-challenged mice**. The lungs were stained with hematoxylin-eosin (original magnification ×40), respectively. The grouping methods and experimental protocols were described as before. A, NS + DMSO group. B, NS + DAPT group. C, ZY + pre-DMSO group. D, ZY + post-DMSO group. E, ZY + pre-DAPT group. F, ZY + post-DAPT group.

ZY + post-DMSO groups, lung histopathological scores were 3.5, significantly higher than the NS + DMSO group ($P < 0.05$, n = 6 per group; Table 1). This phenotype was partially reversed by administration of DAPT before or after ZY injection ($P < 0.05$, n = 6 per group; Table 1).

As shown in Figure 4, A and B, in the NS + DMSO group, protein concentration in BALF was 0.07 ± 0.006 mg/mL, and lung W/D weight ratio was 3.8 ± 0.31. Zymosan administration caused statistically significant increases in BALF protein concentration in both the ZY + pre-DMSO group ($P < 0.05$ vs. NS + DMSO

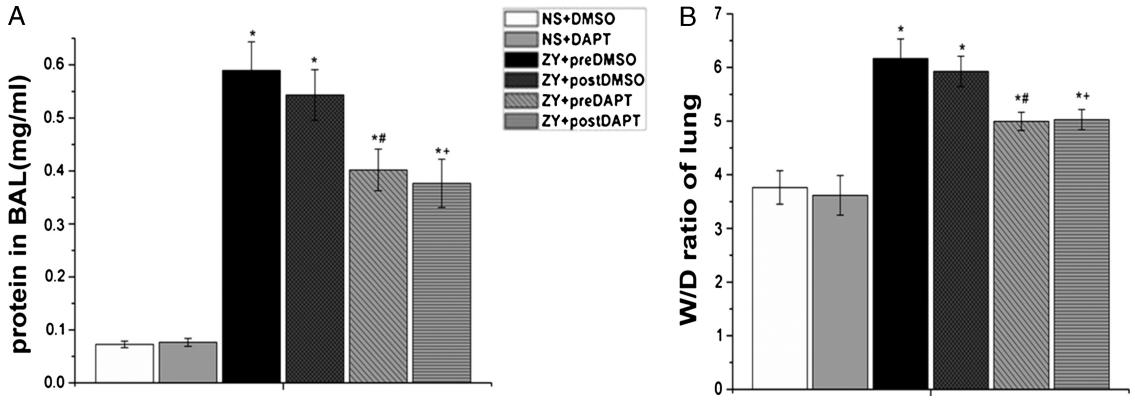

Fig. 4. **Inhibition of notch signaling prevented an increase in lung permeability in ZY-challenged mice**. A, Total protein in BALF (n = 6 for each group). B, Lung W/D weight ratio (n = 6 for each group). The grouping methods and experimental protocols were described as before. The values are expressed as mean ± SD. $^{*}P < 0.05$ vs. NS + DMSO group; $^{\#}P < 0.05$ vs. ZY + pre-DMSO group; $^{+}P < 0.05$ vs. ZY + post-DMSO group.

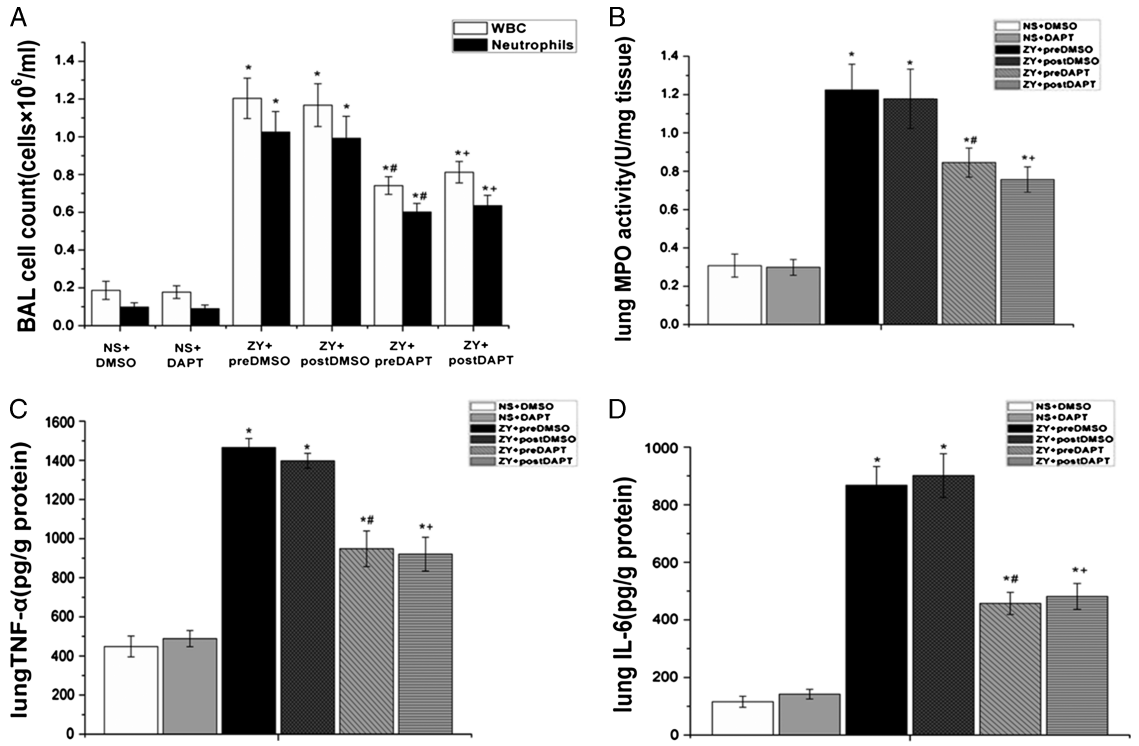

FIG. 5. **Inhibition of notch signaling relieved lung inflammation in ZY-challenged mice**. A, Bronchoalveolar lavage fluid cell counts (n = 6 for each group). B, Levels of MPO in lung homogenate (n = 6 for each group). C, Levels of TNF-α in lung homogenate (n = 6 for each group). D, Levels of IL-6 in lung homogenate (n = 6 for each group). The grouping methods and experimental protocols were described as before. The values are expressed as mean ± SD. $^{*}P < 0.05$ vs. NS + DMSO group; $^{\#}P < 0.05$ vs. ZY + pre-DMSO group; $^{+}P < 0.05$ vs. ZY + post-DMSO group.

group, n = 6 per group) and ZY + post-DMSO group ($P < 0.05$ vs. NS + DMSO group, n = 6 per group). These increases were partially reversed by administration of DAPT before or after ZY injection ($P < 0.05$, ZY + pre-DAPT group vs. ZY + pre-DMSO group, ZY + post-DAPT group vs. ZY + post-DMSO group, n = 6 per group; Fig. 4, A and B). Zymosan injection also caused statistically significant increases in lung W/D weight ratio in both the ZY + pre-DMSO group ($P < 0.05$ vs. NS + DMSO group, n = 6 per group) and ZY + post-DMSO group ($P < 0.05$ vs. NS + DMSO group, n = 6 per group). Again, these increases were partially reversed by administration of DAPT before or after ZY injection ($P < 0.05$, ZY + pre-DAPT group vs. ZY + pre-DMSO group, ZY + post-DAPT group vs. ZY + post-DMSO group, n = 6 per group; Fig. 4, A and B).

As shown in Figure 5, in the NS + DMSO group, white blood cell (WBC) counts in BALF were 1.9 ± 0.4 × 10^5/mL, and neutrophil counts in BALF were 1.0 ± 0.2 × 10^5/mL. Mice demonstrated a significant increase in BALF WBC counts (1.2 ± 0.1 × 10^6/mL, 1.16 ± 0.1 × 10^6/mL) and neutrophil counts (1.02 ± 0.1 × 10^6/mL, 0.99 ± 0.1 × 10^6/mL) in both ZY + pre-DMSO and ZY + post-DMSO groups, respectively ($P < 0.05$ vs. NS + DMSO group, n = 6 per group; Fig. 5A). This increase was also partially reversed in both the ZY + pre-DAPT and ZY + post-DAPT groups ($P < 0.05$ vs. ZY + pre-DMSO and ZY + post-DMSO groups, n = 6 per group; Fig. 5).

In the NS + DMSO group, lung MPO activity was 0.31 ± 0.06 U/mg tissue, whereas in the ZY + pre-DMSO and ZY + post-DMSO groups lung MPO activity was markedly increased ($P < 0.05$; Fig. 5B). This increase was partially reversed in both the ZY + pre-DAPT and ZY + post-DAPT groups ($P < 0.05$ vs. ZY + pre-DMSO and ZY + post-DMSO groups; n = 6 per group; Fig. 5B).

In the NS + DMSO group, lung TNF-α levels were 448.6 ± 53.3 pg/g protein. In the ZY + pre-DMSO and ZY + post-DMSO groups, lung TNF-α levels were increased markedly ($P < 0.05$; Fig. 5C). Again, this was partially reversed in both the ZY + pre-DAPT and ZY + post-DAPT groups ($P < 0.05$ vs. ZY + pre-DMSO and ZY + post-DMSO groups; n = 6 per group; Fig. 5C).

In the NS + DMSO group, lung IL-6 levels were 115.6 ± 18.9 pg/g protein. In the ZY + pre-DMSO and ZY + post-DMSO groups, lung IL-6 levels were significantly increased ($P < 0.05$; Fig. 5D), and this increase was partially reversed in both the ZY + pre-DAPT and ZY + post-DAPT groups ($P < 0.05$ vs. ZY + pre-DMSO and ZY + post-DMSO groups; n = 6 per group; Fig. 5D).

There were no statistically significant differences in BALF WBC and neutrophil counts, lung MPO activity, or lung TNF-α

TABLE 1. **Effect of notch signal inhibitor DAPT on lung histopathological scores in ZY-stimulated mice**

	Group					
Organ	NS + DMSO	NS + DAPT	ZY + pre-DMSO	ZY + post-DMSO	ZY + pre-DAPT	ZY + post-DAPT
Lung	1.0 (1–1)	1.0 (1–1)	3.5 (3–4)*	3.5 (3–4)*	2.0 (1–3)*†	2.0 (1–3)*‡

The grouping methods and experimental protocols were the same as before. The values are expressed as median (range) (n = 6 for each group).
*$P < 0.05$ vs. NS + DMSO group.
†$P < 0.05$ vs. ZY + pre-DMSO group.
‡$P < 0.05$ vs. ZY + post-DMSO group.

or IL-6 levels between the NS + DMSO and NS + DAPT groups ($P > 0.05$).

The above results suggest that inhibition of notch signaling by either pretreatment or posttreatment with DAPT reduced ZY-induced lung injury in mice by attenuating abnormal changes in lung histopathology and lung permeability (protein concentration in BALF and lung W/D weight ratio), as well as lung inflammation (BALF cell count and MPO, TNF-α, IL-6 in lung homogenate).

Inhibition of notch signaling demonstrated by either pretreatment or posttreatment with DAPT improved survival rate in ZY-challenged mice

According to the above results, blocking notch signaling may benefit ZY-challenged mice. We therefore observed the effects of pretreatment or posttreatment with DAPT on the survival rate of ZY-challenged mice. As shown in Figure 6, in the NS + DMSO group, all animals survived through the entire 7-day observation period. The 7-day survival rate was decreased to 15% in both the ZY + pre-DMSO and ZY + post-DMSO groups ($P < 0.05$ vs. NS + DMSO group, n = 40 per group) and was increased to 50% in both the ZY + pre-DAPT and ZY + post-DMSO groups ($P < 0.01$ vs. ZY + post-DMSO group, n = 40 per group). No statistically significant difference was observed between the ZY + pre-DAPT and ZY + post-DAPT groups ($P > 0.05$, Fig. 6). These results suggest that inhibition of notch signaling using either pretreatment or posttreatment with DAPT improved the survival of ZY-challenged mice.

Inhibition of notch signaling alleviated systemic inflammation and tissue damage induced by ZY stimulation

Systemic inflammatory response syndrome plays a major role in the pathogenesis and pathophysiology of sepsis/MODS (2). Tumor necrosis factor α is a critical mediator in the pathogenesis of SIRS/MODS, and therapeutic interventions that suppress TNF-α levels or its activity have been used to improve survival (27, 28). Interleukin 10 is a potent anti-inflammatory cytokine primarily acting on antigen-presenting cells such as macrophages and dendritic cells and is upregulated in the ZY-induced murine model of MODS (16, 29). C-reactive protein levels are used to confirm the presence of SIRS with progression to sepsis even when clinical features are incomplete or equivocal (30). To evaluate the effects of DAPT on ZY-induced systemic inflammation and tissue damage, we determined serum TNF-α, IL-6, IL-10, CRP, and LDH levels 24 h after ZY or NS injection—when NICD expression achieved its peak value in the lung.

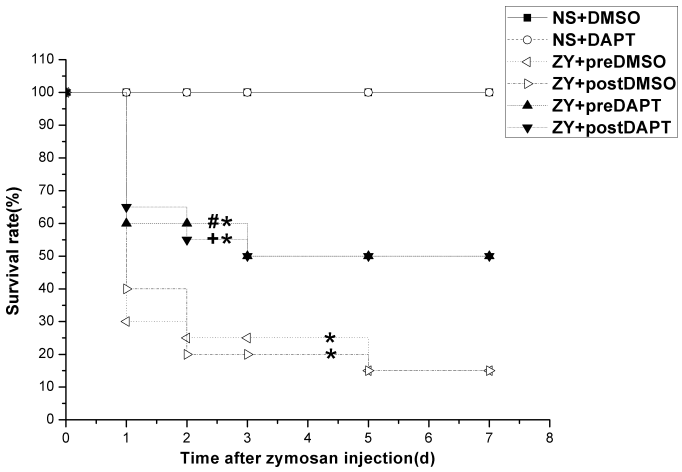

FIG. 6. **Inhibition of notch signaling improved survival rates in ZY-challenged mice**. The grouping methods and experimental protocols were described as before. The values are expressed as survival percentage (n = 40 for each group). $^{*}P < 0.05$ vs. NS + DMSO group; $^{\#}P < 0.05$ vs. ZY + pre-DMSO group; $^{+}P < 0.05$ vs. ZY + post-DMSO group.

As shown in Figure 7, in the NS + DMSO group, the levels of serum TNF-α, IL-6, IL-10, CRP, and LDH were 42.4 ± 2.7 pg/mL, 17.8 ± 1.9 pg/mL, 4.9 ± 0.9 pg/mL, 13.3 ± 1.5 ng/mL, and 512.1 ± 37.9 U/L, respectively. Zymosan injection caused significant increases in serum TNF-α (334.3 ± 18.7 and 321.1 ± 16.4 pg/mL), IL-6 (446.3 ± 27.7 and 398.5 ± 33.2 pg/mL), IL-10 (122.9 ± 16.9 and 132.3 ± 12.7 pg/mL), CRP (193.2 ± 14.2 and 203.3 ± 18.9 ng/mL), and LDH (1,516.9 ± 70 and 1,605.6 ± 73.5 U/L) levels in both the ZY + pre-DMSO and ZY + post-DMSO groups ($P < 0.05$ vs. NS + DMSO group, n = 6 per group). In the ZY + pre-DAPT and ZY + post-DAPT groups, serum TNF-α, IL-6, CRP, and LDH levels were markedly decreased, whereas serum IL-10 levels were further increased compared with those in the ZY + pre-DMSO and ZY + post-DMSO groups ($P < 0.05$, n = 6 per group).

No statistically significant differences were observed in serum TNF-α, IL-6, CRP, IL-10, and LDH levels between the ZY + pre-DAPT and ZY + post-DAPT groups ($P > 0.05$) or between the NS + DAPT and NS + DMSO groups ($P > 0.05$).

These results suggest that inhibition of notch signaling through either pretreatment or posttreatment with DAPT alleviated systemic inflammation and tissue damage in ZY-challenged mice.

DISCUSSION

The present study showed that ZY administration induced an increase in NICD expression in lung. Inhibition of notch signaling by either pretreatment or posttreatment with DAPT reduced lung injury, as determined by lung histopathology, lung permeability (protein concentration in BALF and lung W/D weight ratio), and lung inflammation (BALF cell count, lung MPO, TNF-α, and IL-6), and alleviated systemic inflammation and tissue damage, thus improving the survival rate of ZY-challenged mice.

Acute lung injury and acute respiratory distress syndrome are characterized by increased permeability of the alveolar-capillary barrier, resulting in lung edema with protein-rich fluid and subsequent impairment of arterial oxygenation. In the present study, we observed lung pathological changes and abnormal lung endothelial permeability (total protein concentration in BALF, lung W/D ratio), as well as abnormal lung inflammation levels (TNF-α, IL-6, MPO activity, and cell count in BALF) in the ZY-stimulated mice.

The ZIGI model is a widely accepted animal model for MODS (18–20, 31). Zymosan is a substance derived from the cell wall of the yeast *S. cerevisiae*. As ZY is not degradable, phagocytosis by macrophages results in a prolonged inflammatory response. Therefore i.p. injection of a high dose of ZY (0.8–1.0 g/kg body weight) can induce a sterile sepsis/MODS

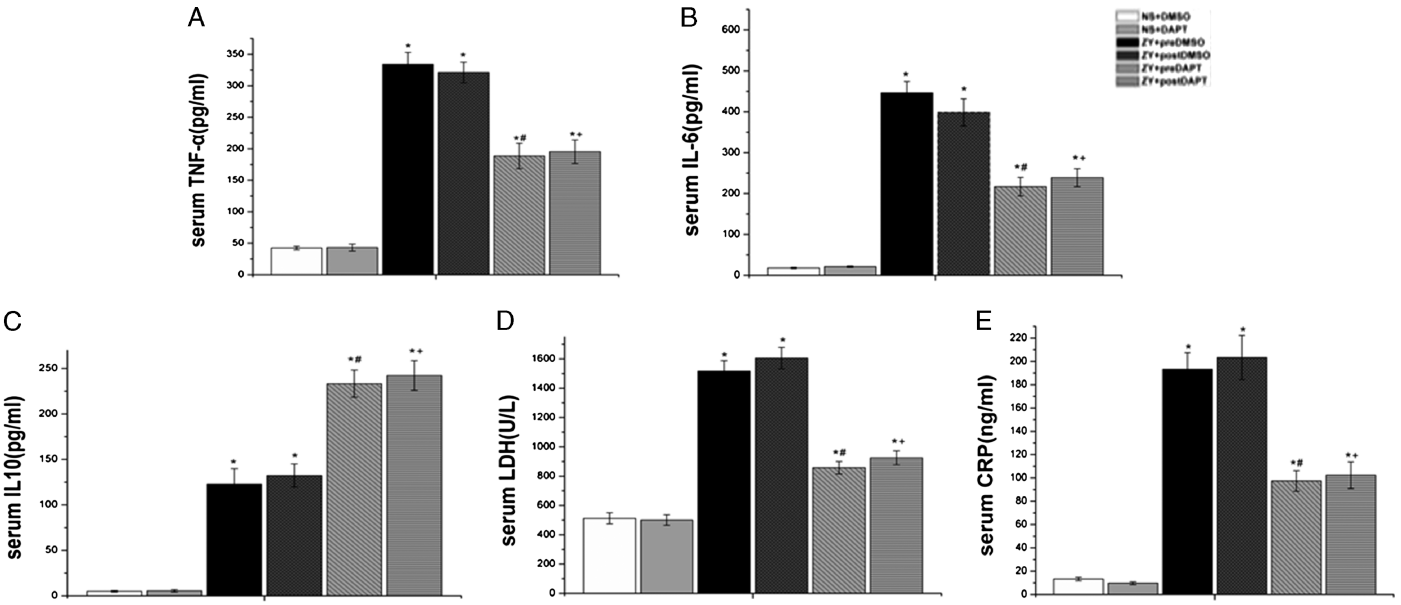

FIG. 7. **Inhibition of notch signaling reduced the increases in serum TNF-α, IL-6, LDH, and CRP levels while further increased IL-10 levels in ZY-challenged mice**. A, Serum TNF-α levels (n = 6 each group). B, Serum IL-6 levels (n=6 each group). C, Serum IL-10 levels (n = 6 each group). D, Serum LDH levels (n = 6 each group). E, Serum CRP levels (n = 6 each group). The grouping methods and experimental protocols were described as before. The values are expressed as mean ± SD. $^{*}P < 0.05$ vs. NS + DMSO group; $^{\#}P < 0.05$ vs. ZY + pre-DMSO group; $^{+}P < 0.05$ vs. ZY + post-DMSO group.

model in rats or mice (23). Our previous studies have also demonstrated that i.p. injection of ZY (1.0 g/kg body weight) successfully induces a murine ALI as well as SIRS/MODS model (21, 22). In the present study, mice receiving ZY (1.0 g/kg body weight, i.p. injection) showed a three-phase illness, which has been characterized for ZIGI, and the 7-day mortality rate was 85% (in agreement with our previous experiments). According to the previous studies, during the first 1 to 2 days after ZY administration, significant changes in lung MPO levels and lung endothelial permeability are observed, indicating the presence of ALI (32, 33), so we chose ZY-induced ALI in our study. Our previous studies also showed that severe lung injury, including large-area hemorrhage, macrophage infiltration, and proliferation, occurs 24 h after ZY injection (34).

Recently, many studies have demonstrated that notch signaling participates in the regulation of immune inflammation (6, 14). Tsao et al. (16) reported that notch receptors and ligands are upregulated within 3 h after LPS stimulation, whereas no obvious increase in the expression of NICD has been observed during the first 3 h of LPS stimulation. Here, we observed that lung NICD was increased at 6 h after ZY administration, peaked at 24 h, and then recovered to normal levels at 48 h. We further showed that inhibition of notch signaling by either pretreatment or posttreatment with DAPT effectively blocked the increase in lung NICD expression at 24 h after ZY injection, in accordance with the LPS-induced sepsis model (16). To test the specificity of the DAPT effect on notch signaling in their study, Tsao et al. (16) also used JLK6, another γ-secretase inhibitor that cannot prevent NICD formation, instead of DAPT under the same experimental conditions. The results demonstrated that JLK6 has no effect on LPS-induced cytokine production (16). These data together demonstrated that inhibition of notch signaling by either pretreatment or posttreatment with DAPT prevented activation of notch signaling in ZY-challenged mice, indicating potential preventive and therapeutic effects of the notch inhibitor DAPT. So, what is the role of notch signaling in ZIGI as well as ZY-induced lung injury? In the present study, we observed that inhibition of notch signaling through either pretreatment or posttreatment with DAPT reduced lung histopathological changes and lung permeability (protein concentration in BALF and lung W/D weight ratio) in ZY-stimulated mice. This demonstrated that inhibition of notch signaling by either posttreatment or pretreatment with DAPT is beneficial to ZY-induced ALI.

In several sepsis models, neutrophil and macrophage counts change. Previous studies have verified that total cell counts, neutrophils, and macrophages in BALF increase dramatically beginning 0.5 days after LPS injection and remain elevated 4 days after LPS administration (34). Similar results were seen in our study. It has been demonstrated that inflammatory mediators released by inflammatory cells play a significant role in shock, sepsis, and ALI (35). Our data demonstrated that inhibition of notch signaling through either pretreatment or posttreatment with DAPT partially reversed ZY-induced systemic inflammation and tissue damage, characterized by increased levels of serum TNF, IL-6, CRP, and LDH, as well as a decreased 7-day animal survival rate. These data are in accordance with previous findings (16, 21). The above results demonstrated that inhibition of notch signaling improved animal survival through reduced ZIGI and lung inflammation. In the study of Tsao et al (16), serum TNF-α did not change significantly. We presume that the difference in results between the two experiments is a result of different models and strains of mice. To our knowledge, this is the first observation indicating the potential therapeutic action of DAPT on ZY-challenged mice.

There are of course some limitations to our study. It is well known that most cells in BALF are neutrophils (36). However, we did not provide more detailed information about the roles of the particular cell types. Further studies may explain this issue.

We concluded that notch signaling is associated with the progression of ZIGI. Inhibition of notch signaling by either pretreatment or posttreatment with DAPT is beneficial to ZY-challenged mice. Treatment results in attenuation of ZIGI and lung inflammation. These results indicate a new potentially beneficial therapy for patients with critical illnesses.

REFERENCES

1. Murray MJ, Coursin DB: Multiple organ dysfunction syndrome. *Yale J Biol Med* 66(5):501–510, 1993.
2. Pinsky MR: Pathophysiology of sepsis and multiple organ failure: pro-versus anti-inflammatory aspects. *Contrib Nephrol* 144:31–43, 2004.
3. Kaneider NC, Agarwal A, Leger AJ, Kuliopulos A: Reversing systemic inflammatory response syndrome with chemokine receptor pepducins. *Nat Med* 11(6):661–665, 2005.
4. Malleo G, Mazzon E, Genovese T, Di Paola R, Caminiti R, Esposito E, Bramanti P, Cuzzocrea S: Absence of endogenous interleukin-10 enhanced organ dysfunction and mortality associated to zymosan-induced multiple organ dysfunction syndrome. *Cytokine* 41(2):136–143, 2008.
5. Hotchkiss RS, Karl IE: The pathophysiology and treatment of sepsis. *N Engl J Med* 348(2):138–150, 2003.
6. Hu X, Chung AY, Wu I, Foldi J, Chen J, Ji JD, Tateya T, Kang YJ, Han J, Gessler M, et al.: Integrated regulation of Toll-like receptor responses by notch and interferon-γ pathways. *Immunity* 29(5):691–703, 2008.
7. Radtke F, Fasnacht N, Macdonald HR: Notch signaling in the immune system. *Immunity* 32(1):14–27, 2010.
8. Bray SJ: Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* 7(9):678–689, 2006.
9. Niranjan T, Bielesz B, Gruenwald A, Ponda MP, Kopp JB, Thomas DB, Susztak K: The notch pathway in podocytes plays a role in the development of glomerular disease. *Nat Med* 14(3):290–298, 2008.
10. Arumugam TV, Chan SL, Jo DG, Yilmaz G, Tang SC, Cheng A, Gleichmann M, Okun E, Dixit VD, Chigurupati S, et al.: Gamma secretase-mediated notch signaling worsens brain damage and functional outcome in ischemic stroke. *Nat Med* 12(6):621–623, 2006.
11. Geling A, Steiner H, Willem M, Bally-Cuif L, Haass C: A gamma-secretase inhibitor blocks notch signaling *in vivo* and causes a severe neurogenic phenotype in zebrafish. *EMBO Rep* 3(7):688–694, 2002.
12. Hertzog P: A notch in the Toll belt. *Immunity* 29(5):663–665, 2008.
13. Jang MS, Miao H, Carlesso N, Shelly L, Zlobin A, Darack N, Qin JZ, Nickoloff BJ, Miele L: Notch-1 regulates cell death independent of differentiation in murine erythroleukemia cells through multiple apoptosis and cell cycle pathways. *J Cell Physiol* 199(3):418–433, 2004.
14. Wang Z, Banerjee S, Li Y, Rahman KM, Zhang Y, Sarkar FH: Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-κB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. *Cancer Res* 66(5):2778–2784, 2006.
15. Okamoto M, Takeda K, Joetham A, Ohnishi H, Matsuda H, Swasey CH, Swanson BJ, Yasutomo K, Dakhama A, Gelfand EW: Essential role of notch signaling in effector memory $CD8^+$ T cell-mediated airway hyperresponsiveness and inflammation. *J Exp Med* 205:1087–1097, 2008.
16. Tsao PN, Wei SC, Huang MT, Lee MC, Chou HC, Chen CY, Hsieh WS: Lipopolysaccharide-induced notch signaling activation through JNK-dependent pathway regulates inflammatory response. *J Biomed Sci* 18:56, 2011.
17. Fitzpatrick FW, DiCarlo FJ: Zymosan. *Ann N Y Acad Sci* 118:233–262, 1964.
18. Di Paola R, Mazzon E, Genovese T, Crisafulli C, Bramanti P, Caminiti R, Esposito E, Fink MP, Cuzzocrea S: Ethyl pyruvate reduces the development of zymosan-induced generalized inflammation in mice. *Crit Care Med* 37(1): 270–282, 2009.
19. Di Paola R, Mazzon E, Muia C, Crisafulli C, Genovese T, Di Bella P, Esposito E, Menegazzi M, Meli R, Suzuki H, et al.: Green tea polyphenol extract attenuates zymosan-induced non-septic shock in mice. *Shock* 26(4):402–409, 2006.
20. Cuzzocrea S, Genovese T, Mazzon E, Esposito E, Crisafulli C, Di Paola R, Bramanti P, Salvemini D: Fumonisin b1 reduces the development of multiple organ failure induced by zymosan in mice. *Shock* 31(2):170–177, 2009.
21. Hou L, Xie K, Li N, Qin M, Lu Y, Ma S, Ji G, Xiong L: 100% oxygen inhalation protects against zymosan-induced sterile sepsis in mice: the roles of inflammatory cytokines and antioxidant enzymes. *Shock* 32(4):451–461, 2009.
22. Pei Y, Bai X, Dong H, Han H, Zhang Z, Dong H, Hou L, Xiong L: Beta2-adrenergic receptor antagonist butoxamine partly abolishes the protection of 100% oxygen treatment against zymosan-induced generalized inflammation in mice. *Shock* 36(3):272–278, 2011.
23. Volman TJ, Hendriks T, Goris RJ: Zymosan-induced generalized inflammation: experimental studies into mechanisms leading to multiple organ dysfunction syndrome. *Shock* 23(4):291–297, 2005.
24. Aggarwal NR, D'Alessio FR, Tsushima K, Files DC, Damarla M, Sidhaye VK, Fraig MM, Polotsky VY, King LS: Moderate oxygen augments lipopolysaccharide-induced lung injury in mice. *Am J Physiol Lung Cell Mol Physiol* 298(3):371–381, 2010.
25. Chen LW, Hsu CM, Wang JS, Chen HL, Chen JS: Inhibition of inducible nitric oxide synthase (iNOS) prevents lung neutrophil deposition and damage in burned rats. *Shock* 15(2):151–156, 2001.
26. Guo S, Liu M, Gonzalez-Perez RR: Role of notch and its oncogenic signaling crosstalk in breast cancer. *Biochim Biophys Acta* 1815(2):197–213, 2011.
27. Tracey KJ, Cerami A: Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Ann Rev Med* 45:491–503, 1994.
28. Pavlov VA, Ochani M, Yang LH, Gallowitsch-Puerta M, Ochani K, Lin X, Levi J, Parrish WR, Rosas-Ballina M, Czura CJ, et al.: Selective α7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis. *Crit Care Med* 35(4):1139–1144, 2007.
29. Ferrer TJ, Webb JW, Wallace BH, Bridges CD, Palmer HE, Robertson RD, Cone JB: Interleukin-10 reduces morbidity and mortality in murine multiple organ dysfunction syndrome (MODS). *J Surg Res* 77(2):157–164, 1998.
30. Pancer G, Engelman E, Hoque F, Alam1 M, Rucinski J, Bernstein LH: C-reactive protein for the enhanced evaluation of the systemic inflammatory response syndrome (SIRS). *Open Clin Chem J* 4:1–9, 2011.
31. Millot S, Andrieu V, Letteron P, Lyoumi S, Nedelec MH, Karim Z, Thibaudeau O, Bennada S, Charrier J-L, Lasocki S, et al.: Erythropoietin stimulates spleen BMP4-dependent stress erythropoiesis and partially corrects anemia in a mouse model of generalized inflammation. *Blood* 116:6072–6081, 2010.
32. Shayevitz JR, Miller C, Johnson KJ, Rodriguez JL: Multiple organ dysfunction syndrome: end organ and systemic inflammatory response in a mouse model of nonseptic origin. *Shock* 4(6):389–396, 1995.
33. Deng X, Wang X, Andersson R: Alterations in endothelial barrier permeability in multiple organs during overactivation of macrophages in rats. *Shock* 6(2):126–133, 1996.
34. Mu J, Xie K, Hou L, Peng D, Shang L, Ji G, Li J, Lu Y, Xiong L: Subanesthetic dose of isoflurane protects against zymosan-induced generalized inflammation and its associated acute lung injury in mice. *Shock* 34(2):183–189, 2010.
35. Brown KA, Brain SD, Pearson JD, Edgeworth JD, Lewis SM, Treacher DF: Neutrophils in development of multiple organ failure in sepsis. *Lancet* 368:157–169, 2006.
36. Sun S, Sursal T, Adibnia Y, Zhao C, Zheng Y, Li H, Otterbein LE, Hauser CJ, Itagaki K: Mitochondrial DAMPs increase endothelial permeability through neutrophil dependent and independent pathways. *PLoS One* 8(3):e59989, 2013.