# Lyn inhibited BCR signal pathway mediates B-cell anergy induced by avian leukosis virus subgroup J

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**Research**

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# Abstract

Background: Immune tolerance induced by retrovirus is a prerequisite for tumorigeness. We had reported that B-cell anergy was the main reason for immune tolerance induced by avian leukosis virus subgroup J (ALV-J). However, the molecular mechanism remains unclear. Results: Initially, we found that Lyn showed down-regulation in chick embryo fibroblasts (CEF) and up-regulation in B-cells infected by ALV-J. So, we speculated that tyrosine kinase Lyn plays a key role in B-cell anergy induced by ALV-J. Confocal laser scanning microscopy (CLSM) and co-immunoprecipitation (Co-IP) results demonstrated that ALV-J indirectly regulated the expression of Lyn. To further investigate the role and regulatory mechanism of Lyn in B-cell anergy induced by ALV-J, the expression levels of Lyn and Syk at different phosphorylation site, the Ca 2+ mobilization, and the expression levels of NF-κB p65 protein in vitro and vivo were detected in B-cells. The result showed that Ca 2+ mobilization was delayed and p65 expression level was decreased in B-cells after ALV-J infection. Consistently, the retrieve of Ca 2+ mobilization, expression levels of NF-κB p65 were found after RNA interference of Lyn. Subsequently, we demonstrated that the activation of phosphorylated Lyn protein at Tyr507 site played a critical role in B-cells anergy, which were verified by the fact of the significantly up-regulation of the expression levels of phosphorylated Syk protein at Tyr525/526 site when RNA interference for Lyn were performed in B-cells. Furthermore, immunohistochemical (IHC) staining results confirmed that the expression levels of Lyn phosphorylated protein at Tyr507 site in bursal cells were increased, while the expression levels of Syk phosphorylated protein at Tyr525/526 sites were decreased. Conclusions: These findings suggested that Lyn inhibited BCR signal pathway mediates B-cell anergy under ALV-J infection which will provide a new insight for revealing the molecular mechanism of immune tolerance induced by ALV-J. Keywords: avian leukosis virus subgroup J , B-cell anergy, immune tolerance, Lyn, Syk


# Background

Avian leukosis virus subgroup J (ALV-J) is an oncogenic retrovirus, characterized by induction of immune tolerance and neoplasia [1–3]. Chickens infected with ALV-J often show persistent viremia due to the lack of effective neutralizing antibody in vivo [4, 5]. The adaptive immune system is tasked with producing antibodies that recognize and eliminate a wide scope of pathogens. Obviously, the acquired immunity system often fails to exert the role of neutralizing virus in the process of ALV-J infection [6]. Although the suppressed effect of ALV-J on immune system of chickens has been studied extensively [7, 8], little was still known about the molecular pathogenesis of immune tolerance.

It has been proved that the process of lymphocyte anergy is an important tolerance mechanism whereby cells are functionally inactivated[9]. Experimental data have shown that the cause of immune tolerance induced by some virus such as human immunodeficiency virus (HIV) could be attributed to the anergy of lymphocytes[10]. The existence and identity of anergic B-cell in animal models or human have been known for some time [11, 12], but the pathogenesis of B-cell anergy induced by some viruses has not been completely addressed. However, our previous studies has showed that the main cause of immune tolerance was associated with the B-cell anergy induced by ALV-J[13].

Previous studies have shown that the anergic B-cell sending signals through the B-cell receptors (BCR) has inherent defects, and the intracellular calcium mobilization and tyrosine kinase phosphorylation levels were also abnormal[11, 14]. In contrast, BCR signaling in normal B-cell is initiated by activation of Src family kinases(SFKs)after recognition of antigen, and then that leads to transduction and propagation of BCR signals that induce expression of activation markers and prepare the cell to interact productively with T-cells[15, 16]. It has been shown that strong BCR signal can cause Syk to be activated through Lyn dependent pathway, but the negative regulatory function of tyrosine kinases Lyn can induce B-cell anergy [17, 18].

Here, to further study the molecular pathogenesis of B-cell anergy caused by ALV-J, the differential expression proteins in host cells of ALV-J were firstly screened by proteomic analysis, and then the molecular mechanism of how key proteins regulated BCR signal transduction and thus induce B-cell anergy under ALV-J infection was further studied.

# Result

Lyn was significantly down-regulated in CEF infected with ALV-J

To screen differentially expressed proteins which plays a key role in cell signal transduction under ALV-J infection, the TMT-based proteomic analysis and hierarchical clustering method were used to visualize changes in the abundance of differentially expressed proteins in the CEF(laboratory host cells of ALV-J) between ALV-J-infected and normal groups. GO functional analysis revealed that ALV-J infection resulted in significant changes in protein expression associated with immune and developmental processes in CEF (Fig. 1a). We identified the top 19 differentially proteins associated with immune and developmental processes in CEF. Test results showed that the abundance of tyrosine protein kinase Lyn, which mediates the B-cell signal transduction, were significantly down-regulated in the CEF (Fig. 1b and 1c).We further used the quantitative real-time PCR (qPCR) and the western blot (WB) to verified the analysis results of proteomics in CEF infected with ALV-J(Fig. 1d, 1e, and 1f).

Enhanced expression of Lyn in B-cells infected with ALV-J

A series of research data showed that Lyn plays an important role in BCR signal transduction pathway which associated with the development, differentiation, maturation, or tolerance (anergy) of B-cell [19–21]. Based on the proteomic results mentioned above, we investigated the Lyn expression in B-cell infected by ALV-J. Confocal laser scanning microscopy (CLSM) analysis showed that ALV-J and Lyn were both located in the cytoplasm of chicken B-cell, indicating that ALV-J directly or indirectly affects Lyn in the cytoplasm (Fig. 2a). The mRNA level and protein level of Lyn in chicken B-cells were up-regulated after ALV-J infection tested by qPCR and WB detection, different from the proteomic data of CEF (Fig. 2b, 2c, and 2d). As another molecular switch in BCR singling, tyrosine kinase Fyn also plays the active role in BCR singling regulation [22, 23]. However, the expression levels of Fyn showed no significant difference in chicken B-cells before and after infection (Fig. 2b, 2c, and 2d). Furthermore, we also detected the expression level of Lyn in bursal B-cells in vivo. The expression levels of Lyn were increased significantly

in B-cells of bursa of Fabricius of chicken infected with ALV-J in 14 and 28 days old tested by immunohistochemistry (IHC) and WB (Fig. 2e, 2f, 2 g, and 2 h), which was consistent with the expression level of Lyn in chicken B-cells infected with ALV-J in vitro.

Enhanced phosphorylation of Lyn but decreased phosphorylation of Syk in B-cells infected with ALV-J

Studies on the molecular structure of Lyn have shown that there are many phosphorylation sites in Lyn, two of which have the opposite effect. The phosphorylation of Tyr397 site can activates the positive regulation effect of Lyn, but the phosphorylation of Tyr507 can activates the negative regulation effect of Lyn[24]. Based on this, we further detected the expression levels of the two phosphorylation site that determined whether Lyn was activated or not. As shown the detection results of flow cytometry(FCM), although the two phosphorylated Lyn expressed significantly higher than the housekeeping protein after BCR signaling pathway was activated by anti IgM antibody, the Tyr525/526 phosphorylation level of Lyn downstream direct substrate Syk decreased significantly compared with the same enhanced phosphorylation Lyn protein(Fig. 3a and 3b). These results indicate that Lyn actually plays an inhibitory role in BCR signal transduction of chicken B-cells infected with ALV-J.

Previous studies have shown that Lyn is mainly expressed in B-cells or other some blood cells except T-cells[25]. To further verify the actual effect of ALV-J on the expression levels of Lyn in vivo, the phosphorylation level of Lyn and Syk in bursal cells of chicken were measured by IHC and WB. In accordance with the results of in vitro assay, it was found that Tyr397 and Tyr507 phosphorylation levels of Lyn increased significantly in bursal cells of chicken infected with ALV-J, while the phosphorylation levels of Syk decreased significantly (Fig. 3c, 3d, 3e, and 3f). Above results suggest that ALV-J interferes the expression and regulation of Lyn in the BCR signaling pathway.

Phosphorylation levels of Syk were retrieved after Lyn interference in B-cells infected with ALV-J

To further verify the effect of ALV-J on the expression levels of Lyn in B-cells, the shRNA interference of Lyn was performed in chicken B-cells, and the expression levels of Lyn was detected. It was found that the protein levels of Lyn were significantly inhibited 24 hours after Lyn interference on chicken B-cells of normal chickens, meanwhile, the protein level of Lyn in infection group chicken B-cells significantly decreased after Lyn interference, which was equivalent of the expression levels of Lyn in normal group chicken B-cells (Fig. 4a and 4b). This finding indicated that the expression levels of Lyn in chicken B-cells were promoted by ALV-J. To verify whether Lyn inhibited the BCR signaling through the phosphorylation of Tyr507 under ALV-J infection, the phosphorylation levels of Syk in chicken B-cells were detected. The FCM results showed that expression levels of phosphorylation Tyr525/525 site of Syk were significantly retrieved (enhanced) under activated by anti-IgM antibody after 48 hours of Lyn interference (Fig. 4c and 4d). These findings further confirmed that Lyn performed actually the negative regulatory effect in BCR signal transduction through the phosphorylation of Tyr507 under ALV-J infection.

$Ca^{2+}$ flux and NF-κB p65 levels were retrieved after Lyn interference in B-cells infected with ALV-J

Data shows that many anergy B-cells are defective in intracellular calcium mobilization and transcription of nuclear transcription factors [26]. To further analyze, the effect of Lyn on BCR signaling cascades under ALV-J infection, the $Ca^{2+}$ mobilization and protein expression of NF-κB p65 were detected in chicken B-cells under different conditions. Test results showed that the response time of intracellular $Ca^{2+}$ mobilization was delayed, the intracellular $Ca^{2+}$ flux was decreased, and the protein level of NF-κBp65 were decreased in chicken B-cells infected with ALV-J. However, the levels of the $Ca^{2+}$ flux and NF-κB p65 were retrieved in chicken B-cells infected with ALV-J after Lyn interference (Fig. 5a, 5b, 5c, and 5d). These results further indicated that Lyn played a negative regulatory role in BCR signal cascades in ALV-J infected B-cells.

# Discussion

Since the phenomenon of chickens with congenital infection with ALV-J showing persistent viremia was discovered and defined as immune tolerance, the pathogenesis of immune tolerance induced by ALV-J has been studied continuously. Because the immune tolerance caused by ALV-J, HBV, and HIV has many common features in the pathological process [27, 28], it has drawn a great attraction to study the pathogenesis of immune tolerance induced by these viruses. It has been found that the damage of lymphocytes [29], the abnormal secretion of cytokines [30], the variation of viral epitopes [31], and the expression of EAV-HP gene in embryo [32] all play more or less roles in the induction of immune tolerance by ALV-J. In previous study, we demonstrated that B-cell anergy induce by ALV-J should take most of the responsibility for immune tolerance[13]. After all, the antiviral neutralizing antibodies were produced by normal B-cells. To screening the specific protein closely related to the immune tolerance induced by ALV-J, we selected primary cells (such as CEF) rather than cell lines (such as DF-1 cell lines or chicken B-cells lines) in process of proteomic analysis for investigating the practical regulation effect of ALV-J on protein expression [33]. As expected, we screened and confirmed that Lyn, a key protein initiating BCR signal transduction in B-cells[34], was significantly affected by ALV-J in both CEF cells and chicken B-cells.

Tyrosine kinase Lyn is a member of SFKs expressed in various cells except for T lymphocytes, and exerts a unique dual role acting both as a positive and a negative regulatory molecule in BCR signaling [35, 36]. The self-phosphorylation of Lyn protein triggers the assembly of the BCR signalosome and cascade reaction of BCR signal transduction[37]. In response B cells, BCR signaling is initiated by Lyn phosphorylating the ITAM, which further recruit and activate Syk[38]. However, anergic B-cells are characterized by reduced ability to proliferate and secrete antibody after antigen stimulation accompanied by reduced BCR-signaling responses which including delay of intracellular calcium mobilization and activation of inhibitory tyrosine kinase phosphorylation[39, 40]. Antigen-induced aggregation of the BCR on normal mature B-cells results in the initiation of BCR signal cascades that culminate in proliferation, the increased of the $Ca^{2+}$ influx and alterations in protein expression such as NF-κB p65. Our data showed that the response time of $Ca^{2+}$ mobilization in chicken B-cells was delayed, and as one of BCR signal activation events, the intracellular $Ca^{2+}$ influx level was also significantly decreased. Meanwhile, the protein level of NF-κB p65 was also decreased. In this context, these results

indicated that Lyn appears to function as a driver of inhibitory signaling pathways of BCR under ALV-J infection.

The unique role of Lyn in down-modulating B-cell receptor (BCR) activation mainly is mainly realized by the phosphorylation of inhibitory molecules and receptors [41]. Moreover, chronic antigen stimulation drives biased monophosphorylation of CD79 immunoreceptor tyrosine-based activation motif (ITAM) leading to recruitment of Lyn instead of Syk, which ultimately leads to B-cell anergy [42, 43]. Present study results showed that ALV-J significantly up-regulated the protein expression level of Lyn in chicken B-cells and activated the phosphorylation of Tyr507 site in Lyn, which triggered the inhibitory effect of Lyn in BCR signal transduction, as shown by the decrease of the phosphorylation level of Syk, a downstream direct substrate of Lyn. Therefore, we suggested that the inhibitory effect of Lyn on BCR signal transduction is the crucial factor for the anergy of chicken B-cells induced by ALV-J.

In this study, the up-regulation of Lyn expression in chicken B-cells infected with ALV-J eventually led to the suppression of BCR signaling and the down-regulation of the nuclear transcription factor NF-κBp65. We speculate that these anergic B-cells eventually undergo apoptosis and lead to the maintenance of viremia. Interestingly, previous studies have shown that up-regulated of programmed cell death protein 1 (PD-1) or down-regulated of anti apoptotic molecule Bcl-2 in immature B-cells in the peripheral blood of HIV patients finally made B-cells prone to apoptosis by endogenous apoptotic pathway[44, 45], which was the important pathological process of immune tolerance induced by HIV-1.Therefore, we hypothesized that ALV-J, as a chronic stimulant antigen, activated the negative regulation role of Lyn in B-cell signal transduction.

# Conclusions

These findings suggested that ALV-J activated the negative regulation role of Lyn in B-cell signal transduction which induced B-cell anergy. Previous studies on the role of Lyn in B-cell signal transduction were mainly carried out on transgenic animal models or gene knockout cells. Here, we investigated the role of Lyn in chicken B-cell in the context of virus infection, which will provide a new insight for studying the pathogenic mechanism of immune tolerance induced by retrovirology viruses. Admittedly, further studies are necessary to investigate how ALV-J regulates the expression of Lyn.

# Materials And Methods

Virus, cells and chickens

ALV-J (NX0101 strain) [46] were maintained in our laboratory and titered by TCID50 assays in CEF. The CEF and the chicken B-cells line (DT40 cells) [47] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% l-glutamine, in a 5% CO2 incubator at 37 °C. Cells were infected with ALV-J containing 10 − 4 TCID50, it was needed to add 8 μg/mL polybrene when infected chicken B-cells.

The 6-day-old embryos of Leghorn specific-pathogen-free (SPF) chickens (Jinan poultry technology company, China) were injected with ALV-J through the allantoic cavity to establish the infected group (n = 30).The control group (n = 30) was established by injecting DMEM instead of virus. The treatment of virus inoculation, chicken embryos incubation, and virus detection were all followed as our previous description[13].

Proteomics assay and bioinformatics analysis

In order to screen differentially expressed proteins that plays a key role in cell signal transduction under ALV-J infection,protein lysates from ALV-J-infected CEF and normal CEF were collected for proteomic analysis and bioinformatics analysis. The experimental process, iTRAQ labelled LC-MS/MS, and bioinformatics analysis were all followed as our previous description [48].

Confocal laser scanning microscopy

Chicken B-cells were cultured in flask and infected with ALV-J. These cells were maintained for 48 h with DMEM containing 10% neonatal calf serum at 37 °C in a 5% $CO_2$ incubator. The cells were fixed with the ice-cold methanol for 10 min, and blocked with PBS containing 10% FBS for 10 min at room temperature after washed with PBS. The cells were then incubated with the rabbitanti-chicken ALV-J primary antibodies (made in our laboratory, 1:200) and the mouse anti-chicken Lyn primary antibodies (Novus Biotech, Colorado, USA, 1:200) for 2 h at 37 °C, followed by incubation with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit secondary antibodies and the alexafluor 647 (AF647)-labeled goat anti-mouse secondary antibodies (Bioss, Beijin, China, 1:1000) for 30 min at 37 °C. After staining cell nuclei with 4', 6-diamidino-2-phenylindole (DAPI), the cells were seeded on glass bottom dish and observed by SP8 CLSM (Leica,Germany).

Quantitative real-time PCR

Total RNA was extracted from CEF cells or chicken B-cells using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). One microgram of total RNA was used as a template to synthesize cDNA using a reverse transcriptase kit (TaKaRa, Shiga, Japan) to generate first-strand cDNA. qPCR was performed by the LightCycler 96 system (Roche, Basel, Switzerland) with the diluted cDNA. The mRNA levels were analyzed using the $2^{-\Delta\Delta Ct}$ method[49].

The primers were as follows: Lyn-F: 5'-TGCGTG CGTGGTTATTATTTC-3',

Lyn-R: 5'-AATGGTGAGGTCGCTGACTGT-3';

Fyn-F: 5'-TTGTTGAAGGCAAGCATCAG-3',

Fyn-R: 5'-GAGGATAGCATCTGCCCTTT-3';

Syk-F: 5'-G TCTCCATCCACCACACCTTCT-3',

Syk-R: 5’-ACAAGCAGTCCAAGGCAGTGA-3’.

## Western blot

Tissues or cells were collected and then lysed in RIPA lysis buffer (BeyotimeBiotech, Shanghai, China). Proteins from the tissue lysates were separated by polyvinylidenedifluoride (PVDF) membranes electrophoresis. The PVDF membranes were incubated with primary antibodies after being blocked by difco skim milk (Solarbio, Beijing, China). The primary antibodies included mouse anti-chicken Lyn monoclonal antibody (Novus Biotech, Colorado, USA,1:200), rabbit anti-Fyn polyclonal antibody (Jackson, Westgrove, USA,1:800),rabbit anti-NF-κB p65 polyclonal antibody (Bioss, Beijin, China,1:200), and mouse anti-GAPDH-loading control antibody(Bioss, Beijing, China,1:5000).Finally, the membranes were exposed to a chemiluminescence instrument.

## Immunohistochemistry

At the age of 14 and 28 days, the bursa of Fabricius from infected chickens and control chickens were sampled, formalin-fixed, paraffin embedded, and sectioned (5-μm thickness) sections for IHC. All chickens were euthanized with sodium pentobarbital before the organs were removed. The perform of IHC test were followed as our previous description.[13]Negative controls were also performed with the same tissues. Primary antibodies include mouse anti-chicken Lyn monoclonal antibodies (Novus Biotech, Colorado, USA,1:200), rabbit anti-phospho-Lyn(Try397)polyclonal antibody (Bioss, Beijing, China,1:200), rabbit anti-phospho-Lyn（Try507）polyclonal antibody (Bioss, Beijing, China,1:200), rabbit anti-phospho-Syk（Tyr525/526）polyclonal antibody (Bioss, Beijing, China,1:200). Secondary antibodies were horseradish peroxidase-labelled goat anti-mouse/rabbit IgG polymer. Six randomly selected fields of positive expression in each target tissue section were analysed in Image J software to accurately calculate the positive area and the mean optical density.

## Flow cytometry for tyrosine phosphorylation

FCM analysis was performed to assess the levels of phosphorylated Lyn protein (Tyr397 or Tyr507) and phosphorylated Syk protein(Tyr525/526) in B-cells. 8 μg/mL polybrene were added into the DMEM medium containing chicken B-cells to improve the infection efficiency of ALV-J, and then the (Southern Biotech, USA, 20 μg/ml), were added to activate chicken B-cells in logarithmic growth phase. Chicken B-cells were harvested in 2, 5, 10, 30 minutes after stimulation by anti-IgM antibody, and fixed in 100% ice-cold methanol solution. Then these chicken B-cells were divided into three groups and respectively labeled with rabbit anti-phospo-Lyn (Tyr397)-FITC polyclonal antibody(Bioss, Beijing, China,1:200), rabbit anti-phospo-Lyn (Tyr507)-FITC polyclonal antibody(Bioss, Beijing, China,1:200), rabbit anti-phospo-Syk (Tyr525/526) -FITC polyclonal antibody(Bioss, Beijing, China,1:200), incubated at 4 °C in dark for 30 min, washed with PBS buffer and analyzed with BD flow cytometer (BD Biosciences,USA). The same experiment was repeated three times, and isotype control antibodies were also used. Data were analysed using FlowJo (TreeStar) software.

Short-hairpin RNA (shRNA) interference

The pgpu6 / GFP / Neo shRNA interference expression vector (three segments: Lyn-chicken-544: gcagttattctcttctgtcatcatcataa; Lyn-chicken-944: gcttcagcatgaagctagt; Lyn-chicken-1345: ggattctcctgtatatgaaaaatcg) targeted Lyn was constructed and then transfected into chicken B-cellsaccordance with the manufacturer's instructions. The transfection efficiency was observed by fluorescence microscope and cell growth after transfection with shLyn vector was determined by cell counting kit-8 (CCK-8) test. The Lyn expression after transfection was detected by WB. Then the Syk tyrosine phosphorylation was analysed by FCM as mentioned above.

Calcium mobilization measurement

For recording BCR-induced $Ca^{2+}$ flux, $10^6$ chicken B-cells were loaded with 2.5 μL Fluo4-AM (Molecular Probes) in 200 μL hanks balanced salt solution containing 1% FBS and at 37 °C for 25 min. These chicken B-cellswere washed with PBS buffer saline for three times, and then kept it at 37 °C. The intracellular calcium basal level was detected by FCM for one minute. Then, the anti-chicken IgM(Southern Biotech, USA, 2 μg/ml) was added to stimulate these chicken B-cells.The fluorescence of Fluo-4 at 516 nmwas continuously measured by the flow cytometer to determine the change of intracellular $Ca^{2+}$ flux.

Statistical analysis

Multiple sets of data comparisons were measured using one-way analysis of variance (ANOVA). The unpaired t-test was used when two groups were compared. The results were accepted as significantly different when $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$. Analysis and plotting of data were performed using GraphPad Prism 6.0 and are expressed as the means ± SEM.

# Abbreviations

ALV-J: avian leukosis virus subgroup J; AF647: alexafluor 647; BCR: B cell antigen receptor; CEF: chicken embryonic fibroblasts; Co-IP: co-immunoprecipitation; CLSM: confocal laser scanning microscopy; FCM: flow cytometry; FITC: fluoresceinisothiocyanate; HBV: hepatitis B virus; HIV: human immunodeficiency virus; iTRAQ: isobaric tags for relative and absolute quantification; IHC: immunohistochemistry; ITAM: immunoreceptor tyrosine-based activation motif; LC-MS/MS: liquid chromatography-tandem mass spectrometry; NF-κB: nuclear factor-κB; $TCID_{50}$: 50%tissue culture infective dose; PVDF: polyvinylidenedifluoride; qPCR: quantitative real-time polymoerase chain reaction; shRNA: short-hairpin RNA; SFK: Src family kinase; SPF: specific pathogen free; WB: western blot.

# Declarations


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## Authors' contributions

ZC and VN conceived and designed the research. SH participated in the design of the study, performed the research, and did the analysis and interpretation of data and the writing of the manuscript. GZ and DZ performed the research and interpretation of data, XY contributed reagents/materials/analysis tools. YY and JD participated in the interpretation of data and manuscript writing. All of the authors discussed the results.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Ethics approval and consent to participate

Approval for this study was obtained through the Committee on the Ethics of Animal Experiments of Shandong Province (Permit Number of Protocol: 20160124).

## Consent for publication

All authors consented to the publication of this manuscript. The funding agencies had no role in the decision to publish this manuscript.

## Competing interests

The authors declare that they have no competing interests.

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# Figures

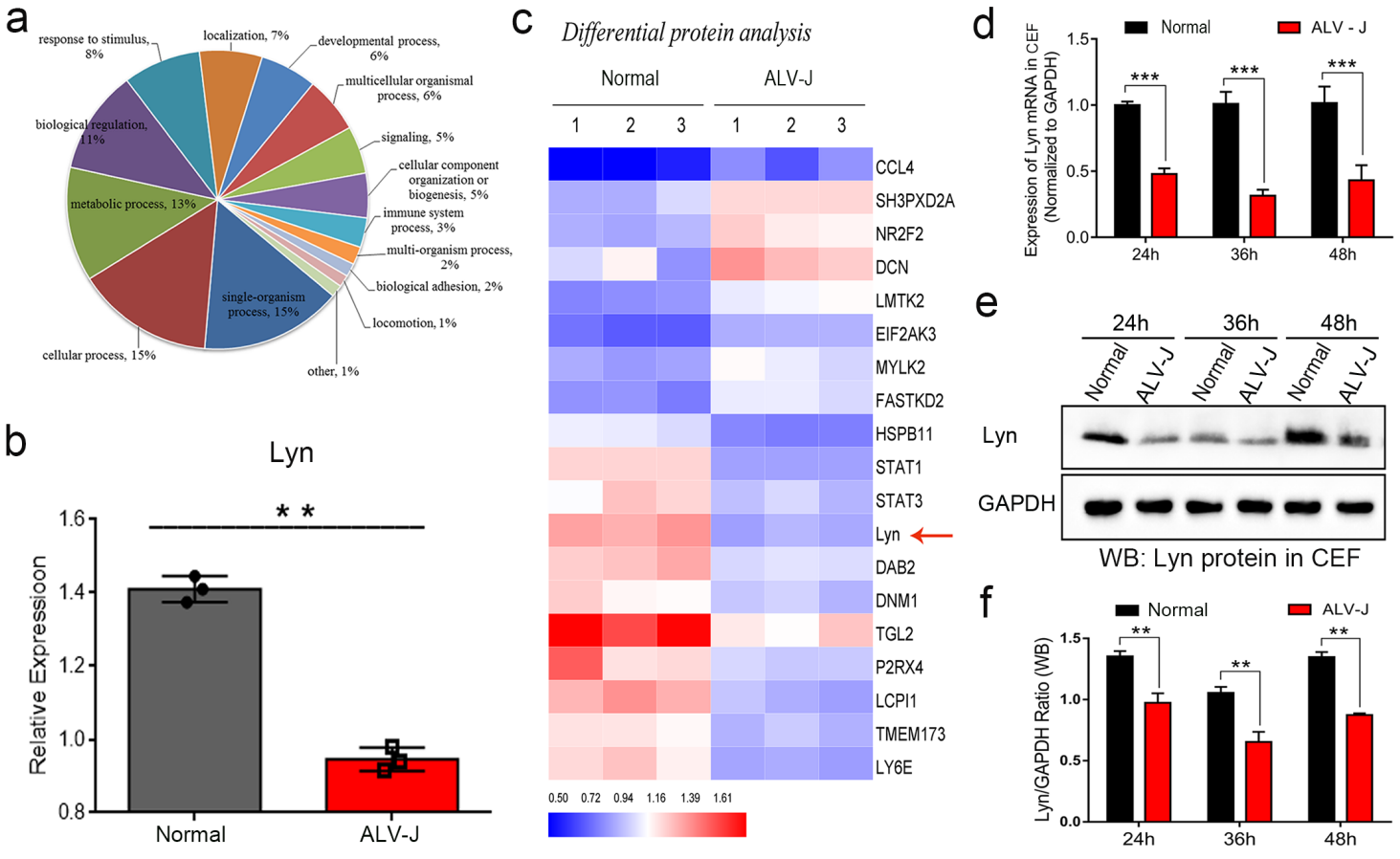
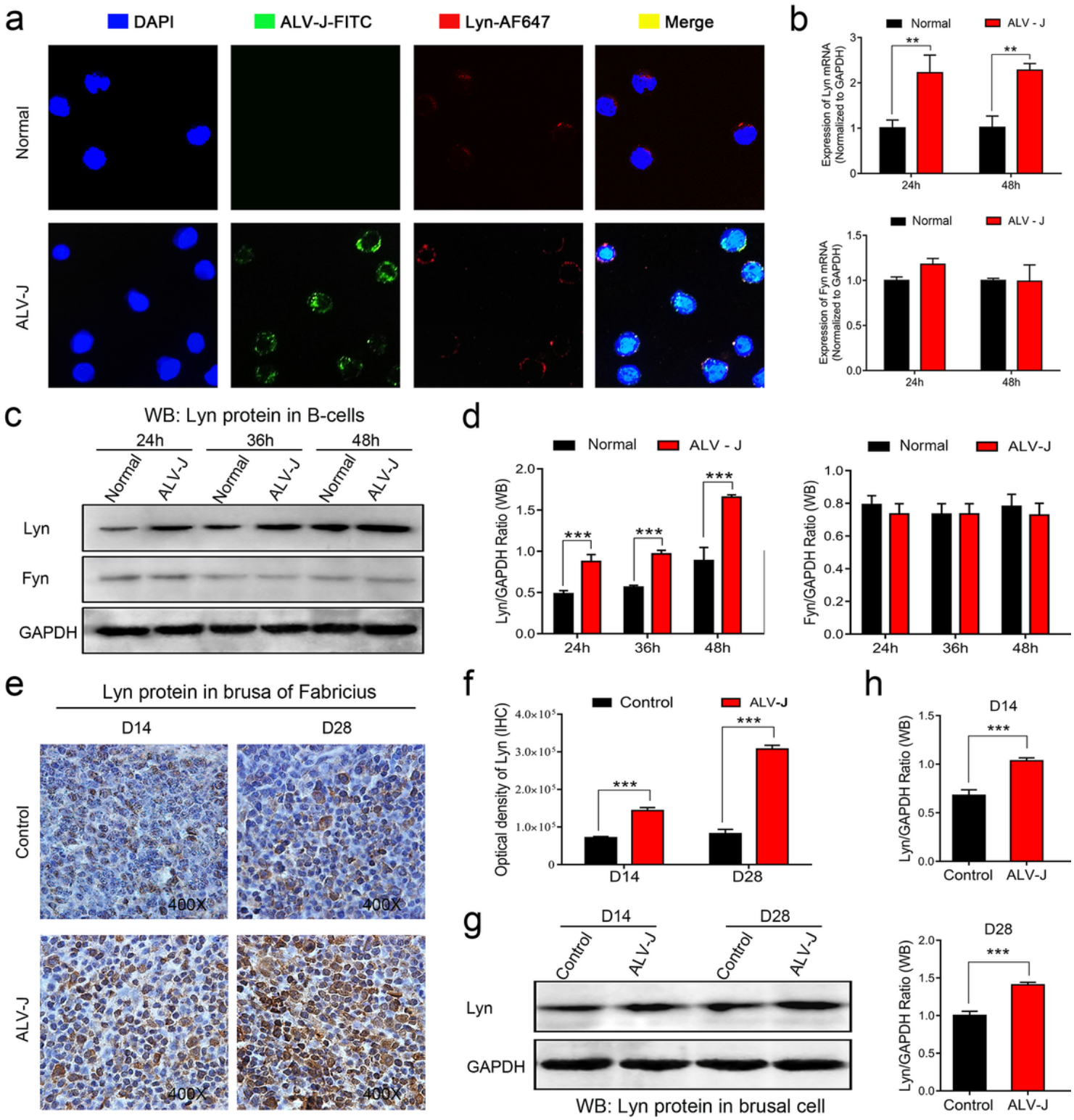



**Figure 1**

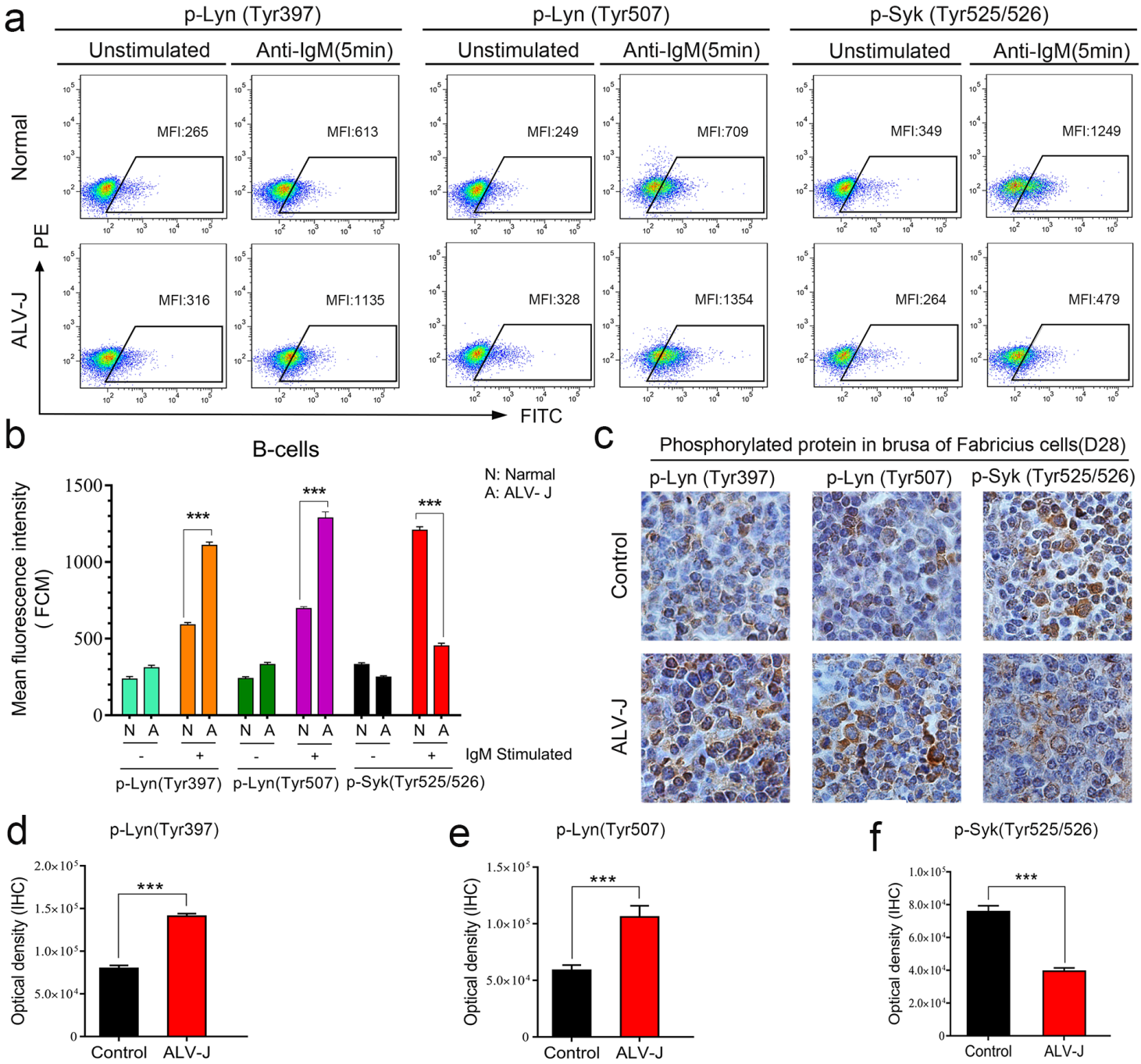
Expression levels of Lyn were significantly down-regulated in CEF infected with ALV-J. a GO functional analysis revealed that ALV-J infection resulted in significant changes in protein expression associated with immune and developmental processes in CEF. b Heat map of the top 19 differentially proteins associated with immune and developmental processes in CEF. Red color indicates up-regulated proteins, and blue color indicates down-regulated proteins. Arrow showed that the Lyn were significantly down-regulated. c Protein levels of Lyn were significantly down-regulated in ALV-J-infected CEF by LC-MS/MS analysis. d mRNA levels of Lyn were significantly down-regulated in CEF infected with ALV-J by qPCR analysis. e Protein levels of Lyn in CEF infected with ALV-J by WB analysis. f Quantification of e, protein levels of Lyn were significantly down-regulated. ** $p< 0.01$, *** $p < 0.001$, ANOVA was performed. Data are expressed as the means ± SEM.




## Figure 2

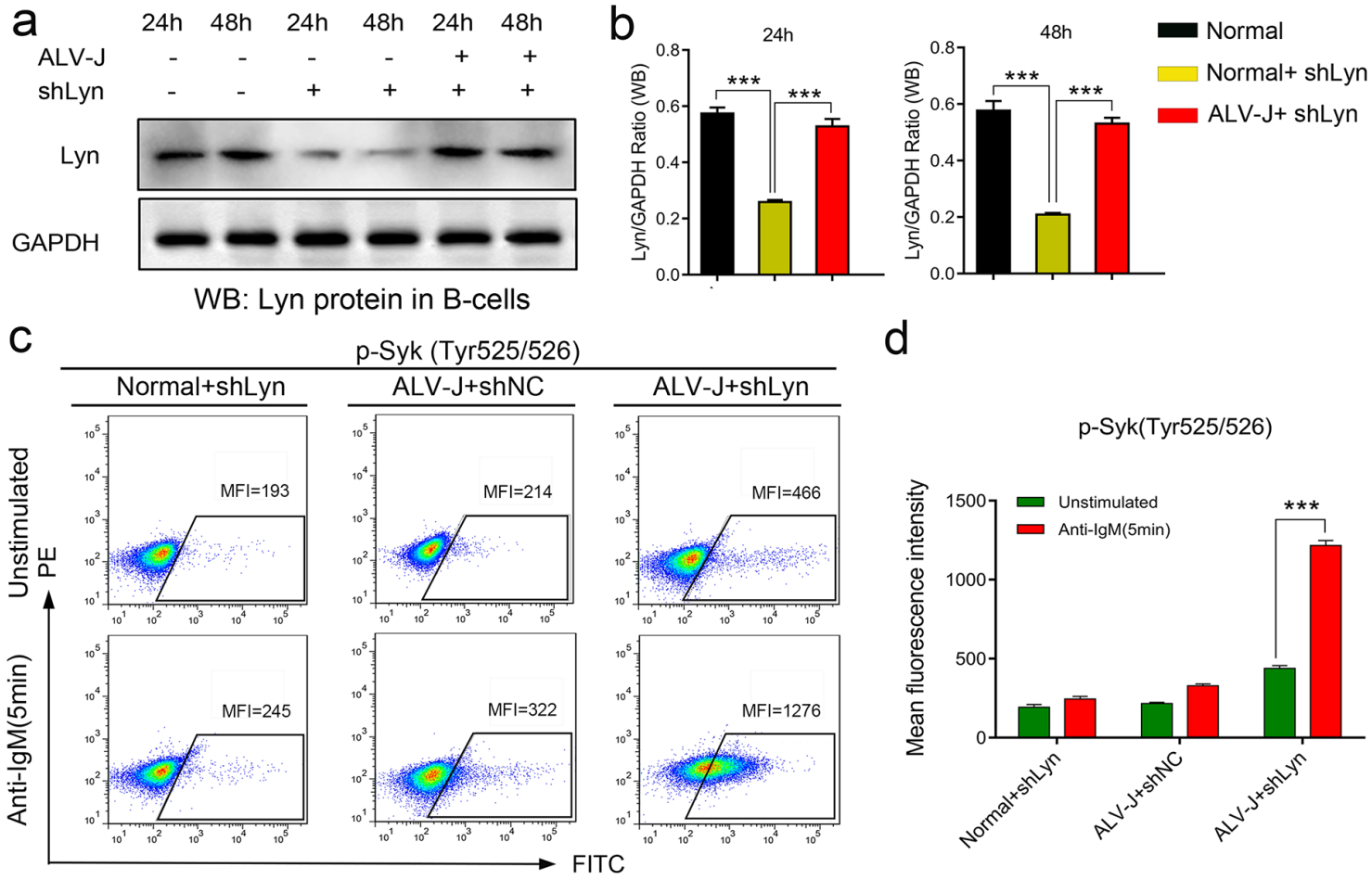
Expression of Lyn was activated in B-cell after ALV-J infection in vivo and vitro. a Immunofluorescence confocal laser microscopy showed that ALV-J (gp85) and Lyn coexist in the cytoplasm of chicken B-cells. b Relative expression levels of Lyn and Fyn in chicken B-cell by qPCR. c Protein levels of Lyn in chicken B-cells by WB analysis. d Quantification of c, protein levels of Lyn were significantly up-regulated but Fyn was indifferent under ALV-J infection. e Protein levels of Lyn in bursal cells by IHC. f Quantification of e,

protein levels of Lyn were significantly up-regulated under ALV-J infection. g Protein levels of Lyn in bursal cells by WB. h Quantification of g, protein levels of Lyn were significantly up-regulated under ALV-J infection. **p<0.01, ***p<0.001, ANOVA was performed. Data are expressed as the means ± SEM.



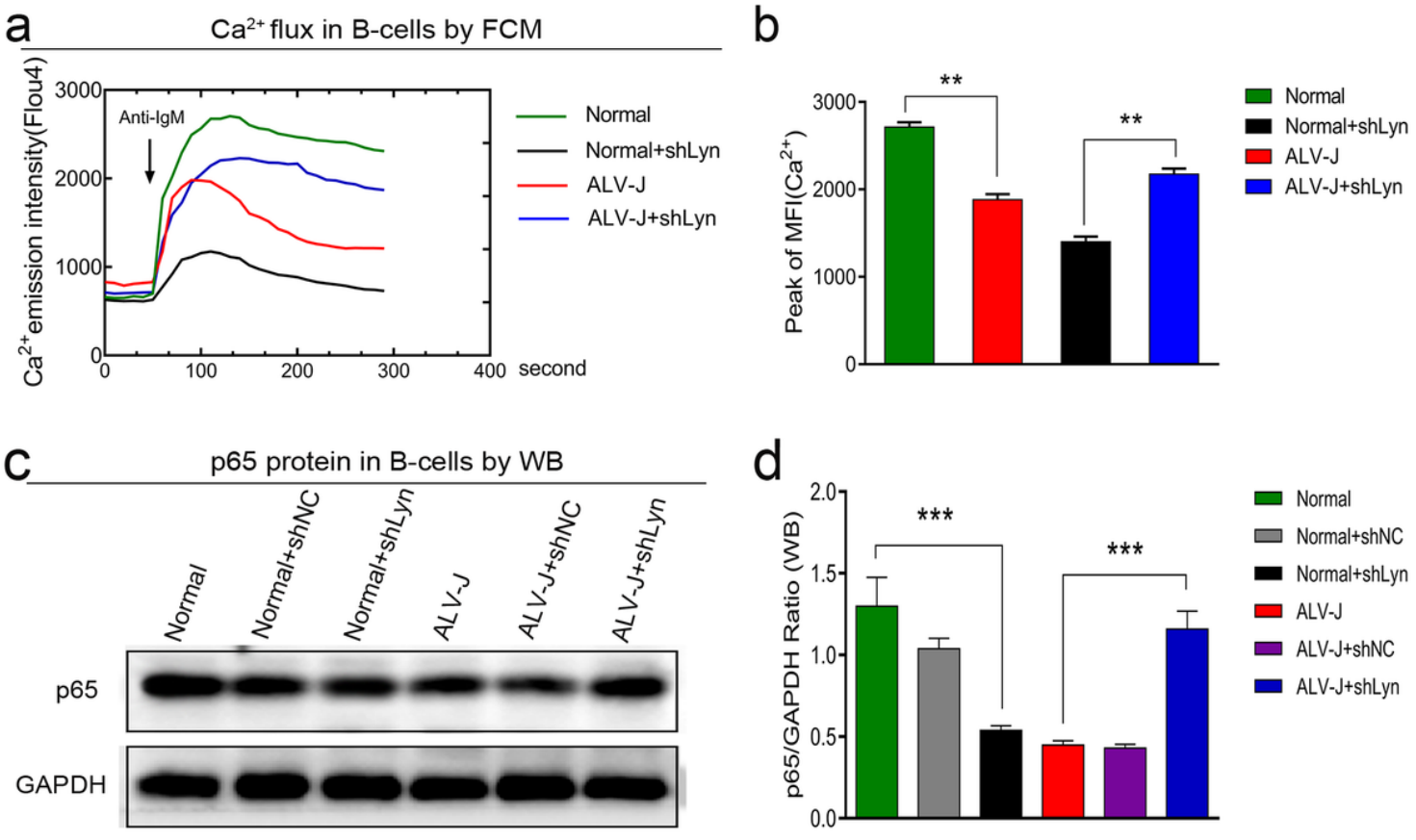

## Figure 3

Enhanced phosphorylation of Lyn and decreased phosphorylation of Syk in B-cell infected with ALV-J in vitro and vivo. a FCM analysis for the basis and activation-induced phosphorylation levels of Lyn and Syk at different phosphorylation sites in chicken B-cells. b Quantification of a. c Phosphorylation levels of Lyn and Syk in bursal cells by IHC. d, e, and f Quantification of c, phosphorylation levels of Lyn were significantly enhanced but phosphorylation levels of Syk were significantly decreased under ALV-J infection. ***p<0.001, ANOVA was performed. Data are expressed as the means ± SME.




## Figure 4

Phosphorylation levels of Syk were retrieved after Lyn interference in chicken B-cells infected with ALV-J. a Protein levels of Lyn in chicken B-cells by WB analysis. b Quantification of a, protein levels of Lyn in chicken B-cells were retrieved after Lyn interference in chicken B-cells infected with ALV-J. c Phosphorylation levels of Syk in chicken B-cells by FCM analysis. d Quantification of c, phosphorylation levels of Syk were significantly up-regulated after Lyn interference of under ALV-J infection. **p<0.01, ***p<0.001, ANOVA was performed. Data are expressed as the means ± SEM.




## Figure 5

Ca2+ flux and NF-κB p65 levels were retrieved after Lyn interference in chicken B-cells infected with ALV-J. a Flow cytometry analysis for the intracellular Ca2+fluxof chicken B-cell under different condition. b Quantification of a (peak of mean fluorescence intensity). c Expression levels of NF-κB p65 protein in B-cells under different condition by WB analysis. d Quantification of c, protein levels of p65 protein were significantly retrieved in B-cells after Lyn interference. **p<0.01, ***p<0.001, ANOVA was performed. Data are expressed as the means ± SEM.