Aspergillus fumigatus change Gasdermin-D-dependent pyrolysis of the lung through regulating TRL2-dependent Regulatory T cells differentiation

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Abstract

Aspergillus fumigatus, as opportunistic fungi, lead to infection in the lungs and even systemic infection. The *A. fumigatus* interacts with the body through a series of complex and dynamic processes. After the fungi have invaded the host, it has a series of adaptive mechanisms which it applies to evade being detected by a host immune system also ways to retaliate against the immune responses. Regulatory T cells (Tregs) in enhancing immune tolerance and immune escape by inhibiting the body's immune response are concerned. It could increase the sensitivity of bacterial infections. When *A. fumigatus* enters the host, it stimulates Toll-like receptor 2 (TLR2) and triggers the signal transduction pathway. However, how Tregs affects susceptibility to *A. fumigatus* and TLR2 changes Tregs in *A. fumigatus* infection is unclear. We examined whether *A. fumigatus* induces Tregs proliferation in vivo, molecular mechanisms underlying *A. fumigatus*-stimulated TLR2 activation and the contribution of Tregs activation to susceptibility to A. fumigatus. We observed that Tregs to $CD4^+$T cells ratio increased in samples from patients infected with *Aspergillus fumigatus*. After conducting tests in mice, it was revealed that when mice were infected with *A. fumigatus*, there was an enhancement in the Tregs to $CD4^+$T cells ratio and Foxp3 expression levels which happen in the lungs also up-regulate. Inhibited Tregs by using CD25 neutralizing antibodies, and we found that the number of fungal burdens in the lung was decreased when these CD25 neutralizing antibodies are used. In our research, *A. fumigatus* infection can indeed stimulate TLR2 expression to increase, and for the lungs, damages and fungal burden caused by $TLR2^{-/-}$mice were reduced. Then it also found that in $TLR2^{-/-}$-mice, pyrolysis-related proteins (GSDMD, IL-1α, and IL-1β) changed. It was concluded that for TLR2 knockout animals, the Treg cells quantities are significantly linked to vulnerability to be infected with *A. fumigatus*. The infection triggered the proliferation and differentiation of $CD4^+CD25^+Foxp3^+$ Tregs through the activation of TLR2 pathway. It is a potential mechanism to evade host defense in *A. fumigatus* infection of the lung. And this effect can regulate GSDMD-dependent pyrolysis, and may involve TRL2 signals partially.

1. Introduction

Aspergillus fumigatus is an important pathogenic microorganism for human infection[(Latge 2001)], with approximately 200,000 cases of invasive Aspergillus(IA) patients each year globally[(Kontoyiannis *et al.* 2010; Brown et al. 2012)]. As opportunistic fungi, it is one of the common conidia species in the air[(Sales-Campos et al. 2013; Latge and Chamilos 2019)]. And it could lead to *A. fumigatus* infection in the lungs and systemic infection(Kontoyiannis *et al.* 2010). Today, IA is among the deadliest disease in the world especially due to its extreme damage to the immune system(Kontoyiannis *et al.* 2010). The pathogen-associated molecular patterns(PAMPs) evidenced is majorly contributed by the dynamic and complex attributes of the *A. fumigatus* cells(Liu et al. 2017). When *A. fumigatus* infection develops further, the body's specific immune response was activated(Kontoyiannis *et al.* 2010). *A. fumigatus* specific structures could be recognized by specific pattern recognition receptors (PRRs) [(Liu et al. 2017)].TLRs is an example of these PRRS and it is the propagation of various signaling pathways, and trigger antimicrobial host responses[(Medzhitov 2001; Jie et al. 2009)]. After *A. fumigatus* infection and

exposed the PAMPs, which then consequently could trigger the activation of the TLR2-associated signaling pathway activation which may result in the innate immune response induction[(Balloy et al. 2005)].TLR2 can be activated by either *A. fumigatus* conidia or mycelium stimulation. And signal transduction induced by *A. fumigatus* cell extracts is strictly dependent on TLR2 signal transduction too[(Braedel et al. 2004)]. However, *A. fumigatus* has ability to bear the harsh conditions within the host and have ways to avoid degradation and create a favorable niche while abating being destroyed by the antibodies. Some of these adaptive mechanisms include germinating to avoid macrophages and impeding being acidified through phagolysosome or phagocytosis[(Slesiona et al. 2012)]. *A. fumigatus* hinders immune cells from perform their core functions and also prevents macrophages from undergoing phagocytosis by releasing a secondary metabolite[(Schlam *et al.* 2016)]. Previous studies have shown that monocytes treated with TLR5 silencing RNA have the ability to reduce the viability of conidia [(Rodland *et al.* 2011)]. And macrophages had a lowered secretion of pro-inflammatory cytokines in response to both Aspergillus conidia and hyphae stimulated production of IL-10 via TLR2-dependent mechanisms[(Netea *et al.* 2003)]. It can clearly be seen that TLRs play an important role in the escape mechanism of *Aspergillus*.

On the other hand, regulatory T cells (Tregs) role in promoting immune tolerance and immune escape by restraining the body's immune response is concerned. It could increase the sensitivity of bacterial infections[(George et al. 2020)]. In a mouse model used to understand infection caused by *A. fumigatus* it was revealed that Tregs increase is evident[(Wang *et al.* 2017)]. It showed the conventional T lymphocytes response in the field of amount and target specificity [(Montagnoli et al. 2006; Bacher et al. 2014; Stephen-Victor *et al.* 2017b)]. Treg cells can inhibit excessive tissue inflammation by inhibiting Th1 and Th17 responses during the first few days after infection by *A. fumigatus*(Montagnoli et al. 2006). However, this reaction may also make the fungus persistent. The conventional T cell response specific to *Aspergillus fumigatus* can be counteracted by the strong Tregs response, giving a clue to Tregs depletion strategies that may facilitate the host to improve antifungal immunity. Judging by the current analysis and research, in addition to innate immune cells like killer cells, dendritic cells and macrophages exhibiting TLRs, the same TLRs expression is seen on several adaptive immune cells like $CD4^+$ and $CD8^+$ T cells[(Kulkarni et al. 2011)],$CD4^+Foxp3^+$ Tregs[(Dai et al. 2009)], and B cells[(Buchta and Bishop 2014)]. The function of T cells is regulated by how they provoke the expression of TLRs[(George et al. 2017)]. The TLR2 on the cell membrane of Tregs is responsible for its proliferation, the proliferation of $CD4^+ Foxp3^+$ Treg cells can be induced by TLR2/MyD88 pathway in Dengue infection[(George et al. 2020)]. In a mouse test to analyze infection caused by *Candida* albicans, reducing the expression of TLR2 lowers the quantity of $CD4^+CD25^+$ Treg cells and decrease the fungal burden [(Netea *et al.* 2004)].

Thus, we hypothesized that *A. fumigatus* stimulates TLR2 signal activation to induce an increase of Treg cells, thereby mediating an inflammatory environment changes in the lung and promoting the persistence of fungi. To clarify the assumption, we analyze a small number of clinical biological samples of *A. fumigatus* infection. And created a mouse model of pulmonary infection of *A. fumigatus* in wild-type

mice and TLR2$^{-/-}$mice, respectively, and determined the mechanism of TLR2 in *A. fumigatus* infection by detecting the effect of inactivated *A. fumigatus* after stimulating CD4^{+} T cells.

2. Materials And Methods

2.1. Clinical samples collection

There were fourteen participants in this sample. Seven donors in perfect health provided the control samples to be use in this test. At the same time, seven adult patients with A. fumigatus infection were collected from the First Affiliated Hospital of Chongqing Medical University. Every participant in this test provided their consent before sample collections and the university committee on clinical research ethics ensured that all the required regulations and standards we upheld and enforced.

2.2. Human serum cytokine or chemokine measurements

After blood collection, the blood samples centrifuged at 3000 rpm for 15 min at 4 °C to obtain supernatant as for serum. These samples were directed to the Clinical Molecular Testing Center of the First Affiliated Hospital of Chongqing Medical University for cytokine/chemokine measurements.

2.3. Human PBMCs Isolation

EDTA tubes were utilized to store the collected blood samples and then subjected to a centrifuge for a period of 8 min, a constant speed of 2500 rpm while temperatures were kept constant at 4^{o}C. We used Ficoll-Paque PLUS reagent (GE Healthcare, Chicago, IL, USA) to separate PBMCs from blood.

2.4. Animals

The C57/BL6 mice (male, 6–8 weeks, 17–24 g) used in our experiment were acquired from Laboratory Animal Center of Chongqing Medical University. TLR2 knockout mice with C57/BL6 background were purchased from Jackson Laboratory. All experimental mice in this experiment maintained at Chongqing Medical University. Through the Institutional Animal Care and Use Committee of Chongqing Medical University, all experimental protocols were approved.

2.5. Strains of Fungi and Conditions for Cultivation

The strain of *A. fumigatus* used was Af293 with the required specifications for infections and cultivation were as previously described[(Mirkov *et al.* 2015)]. Briefly, conidia were matured on Sabouraud dextrose agar plates for seven days at 37 °C and 5% CO_2. To prepare a spore suspension, rinse with 10 mL sterile saline containing 0.1% Tween 20 and gently scrape the Aspergillus colonies on the Petri dish[(Dai *et al.* 2018)]. Then filter through eight layers of sterile gauze. After adjusting the fungal suspension to the desired concentration with a hemocytometer, the conidia suspension was stored at 4 °C.

2.6. Mouse Model with *Aspergillus fumigatus* infection and tissue samples collection

Mice were mildly anesthetized and then placed in a flat position, administered intratracheally at a concentration of 50 µl of 1×10^7 viable spores while maintaining an upright position to be used as the test model for infections caused by *A. fumigatus*[(Rivera et al. 2011)]. Within 1, 2 hours after injection, the mice recovered completely and had a healthy appearance. After the operation, the mice were kept at SPF laboratory and euthanized at 24 and 72 h. Blood was collected retro-orbitally. Lung tissue and spleen was removed of mice, and the colony-forming units (CFUs) per lung were evaluated for lung tissue homogenate.400µL PBS (divided into two) is injected into the trachea to collect bronchoalveolar lavage fluid (BALF). Spleen was taken out of mice, and gently ground the spleen with a mesh screen to obtain spleen cells for subsequent research.

2.7. Blockade of Treg cells in vivo

For this examination, we used mouse CD25/IL-2R neutralizing antibody (AF2438, R&D Systems, Minnesota, USA) to block Treg cells. One hour before *A. fumigatus* infection, each mouse in the inhibitor group was intraperitoneally injected with 20 µg of anti-CD25 antibody, and control mice were injected with antibody rat IgG1 [(Zhou *et al.* 2019)].

2.8. Histopathology

Samples of lung were fixed in 4% formaldehyde. Sectioning was also done after the samples were embedded in paraffin wax. For detection of fungus, Groccott's methenamine silver (GMS) was utilized to stain the lung samples. For the histological analysis procedure, samples of lung were stained with either hematoxylin and eosin (H&E). Analyzed through the use Coolscope digital light microscope (Nikon Co, Tokyo, Japan) and Lung injury was scored according to criteria for defined Mikawa[(Mikawa et al. 2003)] (Alveolar hyperemia, Hemorrhage, Interstitial or aggregation of interstitial or neutrophils, Thickening of alveolar septum or hyaline membrane formation). Pneumoniae pulmonary infection scores were approximated through the method by the scoring standard published by Cimolai[(Cimolai et al. 1992)] (The scoring standard is based on Infiltration degree of inflammatory cells around trachea and bronchiole 0–3; Quality of trachea and bronchiole infiltrate 0–3; Infiltration degree of inflammation in trachea and bronchiole cavity 0–2; Infiltration of inflammatory cells around blood vessels Degree 0–3; Inflammation of the lung parenchyma involves the range 0, 3, 5). The severity of the inflammation is directly proportional to the magnitude of the score.

2.9. In vivo quantification of viable conidia

The fungal burden in the lungs of mice was determined by the plate colony counting method. Separate mouse lungs aseptically, weigh their wet weight, add ice PBS, homogenize the tissue, and dilute the tissue proportionally. Each concentration gradient was added to the sandcastle plate medium, and each concentration gradient was inoculated with two dishes and cultivated at 37 °C for 72 hours. Count the colonies and multiply by the dilution factor.

2.10. Cytokine measurements

After collecting lung tissue of WT mice and TLR2$^{-/-}$ mice, they are stored under frozen temperatures of − 80 °C while waiting for analysis. Tumor necrosis factor (TNF)-α, IL-6, VEGF and CCL2 were measured in accordance with enzyme-linked immunosorbent assay kit (ELISA) as measured (4A Biotech, China).

2.11. Extraction of RNA, synthesis of cDNA and Real-time Quantitative PCR

In this experiment, TRIzol (TakaraBio, Tokyo, Japan) was used to extract total RNA from lung tissue and measure the RNA concentration. Before the start of the experiment, the experimental items need to be pretreated to prevent contamination of exogenous RNase. The specific experimental procedures follow the instructions of TBGreen®Premix Ex Taq™II (Tli RNaseH Plus) (TakaraBio, Tokyo, Japan). Added specific primers to the reaction system to perform qRT-PCR on mouse TLR2, Foxp3 and glyceraldehyde triphosphate dehydrogenase (GAPDH). The primer sequence were as follows:: TLR2 forward 5'-GATGAAGTCAGCTCACCGAT-3';reverse 5'-ACAGTTCCAAGATGTAACGC-3';Foxp3 forward 5'-CCTATGCCACCCTTATCCGATG-3';reverse 5'- CGAACATGCGAGTAAACCAA-3';GAPDH forward 5'-GGACACTGAGCAAGAGAGGC-3';reverse 5'-TTATGGGGGTCTGGGATGGAA-3'. Using a 25 μl systems, add TB Green Premix Ex TaqII (Tli RNASEH Plus) (2×), forward primer, reverse primer, DNA template and RNase free dH2O according to the instructions. Adopted two-step PCR reaction program, pre-distortion 95℃, 30 s; PCR reaction 40 cycles. The $2^{\Delta\Delta C(t)}$ approach is used for determining the expression of relative target gene.

2.12. Western blotting assay

Utilizing a homogenizer, homogenization of the lung tissue in 1 ml 50 mM Tris-HCl (pH 7.8) containing 15% glycerol, 150 mM NaCl, 0.1% Tween-20 and protease inhibitors was done and centrifuged. The level of protein concentrations was estimated through the use of a bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Shanghai, China). The supernatant (ie total protein) was separated sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotting onto a polyvinylidene difluoride membrane.The membrane was blocked with 5% (w/v) skimmed milk, and then incubated with TLR2 antibody, Foxp3 antibody, Gasdermin-D antibody, IL-1α antibody, IL-1β antibody or GADPH antibody at 4 °C for 14–17 h, and then used The horseradish peroxidase-conjugated secondary antibody (diluted 1:8000) was reacted at 37 °C for 1 hour. The membrane is exposed to enhanced chemiluminescence (ECL) reagents. Use ImageQuant TL software to detect protein expression.

2.13. CD4 $^{+}$ T lymphocytes isolation, proliferation and differentiation differentiation into Treg cells in vitro

After the heat-inactivation of the *A. fumigatus* conidia at rest under temperatures of 65 °C for a period of 60 minutes. Sabourad agar is applied to test the viability of these conidia. This is then followed by freezing these conidia while awaiting utilization under temperatures of − 80 °C. These experiments demand that the reagents used should have 1×10^{7} /mL concentration [(Raijmakers *et al.* 2017)]. Spleen samples should be collected from C57/BL6 mice according to the guidelines provided in the previous method[(Flaherty and Reynolds 2015)]. To isolate CD4 + T lymphocytes, the collected mouse primary splenocyte pellets were resuspended in a medium containing fluorescent antibodies against CD4, CD62L,

CD25, and CD44. In a 48-well plate, about 5×10^5 CD4 + T cells are inoculated in each well of the 48-well plate in 500 µl medium. The medium RPMI 1640 supplement includes50 ng/mL transforming growth factor- (TGF-) β1 (PeproTech), 5 µg/mL anti-mouse CD3 (eBioscience), 2 µg /mL anti-mouse CD28 (eBioscience),10 ng/mL cytokines IL-2 (PeproTech, Rocky Hill, NJ, USA),50 mM β-mercaptoethanol (Macklin, Shanghai, China) and 2 mML-glutamine (STEMCELL Technologies, Vancouver, Canada),with or without C29, incubate at 37 °C, 5% CO2 for 3 days, then perform flow cytometry detection, each group Repeat 5 times. $C_{16}H_{15}NO_4$(C29) (MCE, New Jersey, USA) was dissolved in DMSO as 50 mM stock solution(Grabowski et al. 2020).

2.14. Flow cytometry

The cultured T cells, isolated PBMCs and splenocytes to be tested were incubated in the dark with fluorescent antibodies to determine the percentage of $CD4^+ CD25^+ Foxp3^+$ Tregs in $CD4^+$ T cells. according to the manufacturer's instructions, simply put the collected cells are washed with PBS, centrifuged to pellet, and then stained with antibodies(anti-CD25-phycoerythrin-PE,anti-CD4-FITC,anti-Foxp3-APC) and a Fixation/Permeabilization kit (eBioscience)) in the dark for flow cytometry detection. At least 10^5 cells were collected and detected with FACS flow cytometer (Becton Dickinson), data was analyzed with FlowJo software V10.

2.15. Statistical analyses

Statistical analysis using SPSS 20.0 (IBM, Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). All experimental data were expressed as either mean ± standard deviation. Experimental data were assessed with Student's unpaired two-tailed t test, ONE-WAY or TWO-WAY Analysis of variance (ANOVA) attended by the Tukey Post Hoc test. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. *Aspergillus fumigatus* Infection cause susceptibility and lung damage in immunocompetent mice

We found that IL-1β($p < 0.0001$),IL-6($p < 0.001$) and IL-2R($p < 0.0001$) were massively raised among patients specimens infected with *A. fumigatus*, IL-10 was increased slightly after infection($p < 0.05$) (Fig. 1A).In order to better understand the lung damage caused by *A. fumigatus* infection, We established a mouse lung *Aspergillus fumigatus* infection model, and use GMS staining and the number of fungal colonies to confirm the lung infection of *A. fumigatus* in mice (Fig. 1B). We used H&E staining of C57BL/6 lung tissue to observe the inflammatory and pathological changes within A. fumigatus- infected lungs of mice. As shown in Fig. 1C, we found that H&E of lung tissue sections showed that infiltration of inflammatory cells in the bronchiole, peri vascular and vascular lumen. The degree of lung damage semi-quantitative injury index includes hemorrhage, alveolar hyperemia, Interstitial or neutrophil infiltration or aggregation, and inflammatory cell infiltration were severe in the mice undergoing *A. fumigatus* pneumonia compared to the control mice. Appreciably enhancive quantity of inflammatory cells and

increased lung histopathology Mikawa scores and Cimolai score[(Cimolai et al. 1992)] were observed *A. fumigatus* infection group ($p < 0.0001$). Obviously, *A. fumigatus* significantly increases the concentration of chemokines or cytokines inculding TNF-α, CCL2, IL-6 and VEGF in mouse lung tissue (Fig. 1D).

3.2. Increased of Treg cells ratios after *A. fumigatus* stimulated in the lung tissue of WT mice

We used a flow cytometry method to ascertain whether *A. fumigatus* infection gave rise to the proliferation of $CD4^+CD25^+Foxp3^+$ Treg cells in the PBMCs of human specifically, the number of Treg cells in patients infected with *A. fumigatus* also increased($p < 0.0001$) (Fig. 2A). As shown in Fig. 2B, to further understand the involvement of Tregs in pulmonary *A. fumigatus*, we studied the protein and mRNA levels of Foxp3 in the lungs post *A.fumigatus* challenged by Real-time Quantitative PCR and Western blotting. There was upregulated in expression of Foxp3 in the lungs of mice suffered *A. fumigatus* infection compared with controls. Subsequently we found that compared with noninfected mice, the Treg cells in the spleen of mice treated with *A. fumigatus* increased significantly within 72 hours after infection ($P < 0.0001$) (Fig. 2C).

3.3. The persistent presence of fungi in lung injury induced by *Aspergillus fumigatus* is related to Tregs

The above data suggested that the increased ratio of Treg cells was associated pulmonary Aspergillosis. To further clarify the role of Tregs in *Aspergillus fumigatus* infection, intraperitoneal injection of CD25 neutralizing antibodies 20 μg was used to inhibit Treg cells in WT mice in our research. Injection with CD25 neutralizing antibodies lower the quantity of Treg cells in spleen of mice compared with controls ($p < 0.0001$) (Fig. 2D). Additionally, we found that the number of fungal colonies in lung tissue was decreased in mice injected CD25 neutralizing antibodies compared with controls($P < 0.05$) (Fig. 2E). After inhibiting Treg cells, the damage of the we observed the lungs by morphology examination. As shown in Fig. 2F, higher amount at inflammatory cells and hemorrhage in the alveolus were discovered in mice treatment with *A. fumigatus*. However, overall, the inflammatory cells around the blood vessels are slightly lower, and mildly reduced lung histopathology Mikawa scores and Cimolai scores compared with CD25 neutralizing antibodies compared to IgG1-treated mice with *A. fumigatus* infection in the lung ($p < 0.05$).

3.4. TLR2 is increased in lung of mice treated with *Aspergillus fumigatus*

To investigate whether TLR2 affects the persistence in the pulmonary *A. fumigatus*, we measured the protein and mRNA levels of TLR2 in control and infected wild-type mice lung by western blot and RT-PCR. The relative TLR2 mRNA expression levels were upregulated in the infected mice lung tissue compared to the control group ($p < 0.01$) (Fig. 3A). The TLR2 protein levels were also increased in the infected mice lung compared to the control group, ($p < 0.001$) (Fig. 3A). These results confirmed that TLR2 expression higher after *A. fumigatus* infection in the lung of mice.

3.5. $TLR2^{-/-}$ mice are less susceptible to *Aspergillus fumigatus* infection

TLR2 is one of the cell membrane receptors involved by *A. fumigatus* [(Liu et al. 2017)]. To determine the influences of TLR2 while the *A. fumigatus* invade host, we infected $TLR2^{-/-}$ mice with 1×10^7 conidia

were inoculated and compared with wild-type mice, no deaths occurred in either of the two groups within 3 days (data not shown).Surprisingly, TLR2$^{-/-}$ mice were observed to have a slightly lower load of *A. fumigatus* in their lung compared with controls ($p < 0.05$) (Fig. 3B). As shown in Fig. 3C, histology of the lung showed that mainly infiltration of macrophages and monocytes in the lungs of TLR2$^{-/-}$ mice with *A. fumigatus*, alveolar congestion and hemorrhage. However, compared with TLR2$^{-/-}$ mice, WT mice suffered from interstitial congestion and hemorrhage more obvious after *A. fumigatus* infection, with severity was neutrophil infiltration. Assessed changes in lung tissue morphology, increased lung histopathology Mikawa scores and Cimolai scores were observed in the WT mice undergoing *A. fumigatus*. In order to study the role of TLR2 in *A. fumigatus* stimulating cytokines, as shown in Fig. 3D, the concentration of inflammatory cytokines and chemokines, including TNF-α, IL- 6,CCL2 and VEGF in lung were significantly treatment within mice after *A. fumigatus* infection, TNF-α and IL-6 were increased in lung from TLR2$^{-/-}$ mice compared with control wild-type mice to *Aspergillus fumigatus* infection ($p < 0.0001$).CCL2 and VEGF were mildly reduced compared with wild-type mice.

3.6. TLR2 plays a crucial role in induces the proliferation of CD4 $^{+}$ CD25 $^{+}$ Foxp3 $^{+}$ Tregs in lung injury induced by Aspergillus fumigatus

To investigate determine the molecular mechanisms of Treg cells differentiation and proliferation caused by TLR2 in *A. fumigatus* infection. We also tested Tregs in the spleen of TLR2$^{-/-}$ mice after infection with *Aspergillus fumigatus*. As shown in Fig. 4A, Treg cells ratio in the spleen of TLR2$^{-/-}$ mice were significantly lower than those in the WT mice, this is consistent with previous research($P < 0.0001$) (George et al. 2020). We also tested the expression of Foxp3 in the lungs of mice after infection with *A. fumigatus*. RT-PCR results showed that the expression of Foxp3 in the lungs of the control group TLR2$^{-/-}$ mice was reduced compared to wild-type mice ($P < 0.001$) (Fig. 4B). The expression of Foxp3 was decreased in lung of no infection TLR2$^{-/-}$mice compared with WT mice in control ($p < 0.05$).In addition, although the expression of Foxp3 was upregulation after infection with *A. fumigatus* in the lung of TLR2$^{-/-}$ mice and WT mice, TLR2$^{-/-}$ mice was also reduced compared to WT mice($P < 0.05$) (Fig. 4C).

3.8. Inhibitor of TLR2 reduces *Aspergillus fumigatus* -Induced CD4 $^{+}$ CD25 $^{+}$ Treg cells differentiation in CD4 + T lymphocytes

In order to confirm whether TLR2 in *A. fumigatus* infection can affect the differentiation of CD4^{+} T lymphocytes into Treg cells, we obtained primary CD4^{+} T lymphocytes from mouse spleens for subsequent in vitro cell culture experiments. Intervention by adding C29 (TLR2 inhibitor) to the medium. It was observed that flow cytometry analysis was performed after 72 hours of culture, and we found that the ratio of CD4 $^{+}$ T lymphocytes differentiated into Treg cells decreased after C29 treatment ($P < 0.001$) (Fig. 4D).

3.9*. Aspergillus fumigatus* promote Gasdermin-D-dependent pyrolysis

Gasdermin-D-dependent pyrolysis signal molecules play an important role in lung damage caused by infection[(Liu and Lieberman 2017)].We infected lung of TLR2$^{-/-}$ mice and WT mice with 1×10^7 conidia and evaluated Gasdermin-D,IL-1αand IL-βproteins by Western blot. As shown in Fig. 5, Gasdermin-D, IL-1α and IL-βproteins in lung tissue was induced after aspergillus fumigatus stimulation whether it is WT mice or TLR2- knockout mice. In addition, the expression of both IL1-β and GSDMD in TLR2$^{-/-}$mice decreased compared with WT mice($p < 0.01$), IL-1α also showed a downward trend.

4. Discussion

There are varieties of diseases caused by *A. fumigatus*, which range from malignant infections to hypersensitivity. Critical patients are more prone to *A. fumigatus* infection, which accompanied by severely immunocompromised and prolonged neutropenia mainly. Recent studies indicate that IA has a higher incidence and should be regarded as an emerging serious infectious disease in ICU patients. The lung was the most common site of infection (94%), and *A. fumigatus* has been identified as the commonest isolated species (92%) [(Taccone et al. 2015)]. IA due to *A. fumigatus* is associated with greater severity, high mortality and more frequently organ support need.

Currently, therapy for disease (such as cancer, autoimmune disease), which based immune escape mechanisms, become increasingly attractive in the biomedical field. On entry to the host, *A. fumigates* triggers complex and dynamic interactions with the host[(Stewart *et al.* 2020)]. Besides, after *Aspergillus fumigatus* invades the host, it can cause a series of mechanisms such as immune recognition, evading immune recognition and resisting host response. Although inflammation is mainly a defensive response to pathogens with harmful consequences, its downstream effects, such as the metabolic changes or influx of immune cells, may actually promote the growth of pathogens and the spread of tissues. The deflection of the immune response directed by microbes through specific signals may further reduce the antibacterial effect and enhance the benefits of pathogens[(Flieger *et al.* 2018)]. In the pathogen infection, Though the main role of the Tregs is to inhibit damage to tissues and also limit severe inflammation caused by the infection, the accumulation of Tregs also does the opposite, which weaken protecting immune response to pathogens and enhance pathogen persistence[(Stephen-Victor *et al.* 2017a)]. Tregs that specifically stimulate *A. fumigatus* have been explained in humans[(Bedke et al. 2014)]and mice[(Montagnoli et al. 2006)]. In our study, we identified that IL-1β, IL-6 and IL-2R were significantly elevated in clinical biological samples of *A. fumigatus* patients (Fig. 1A). In mice after *A. fumigatus* infection of lung, data showed that lung damaged and increased cytokines in mice with *A. fumigatus* infection too (Fig. 1B-D). However, the ratio of Tregs/CD4$^+$ T cells was heightened after *A. fumigatus* infection in patients (Fig. 2A). And we found that the expression of Foxp3 in lung and proportion of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in spleen were upregulated after *A. fumigatus* infection (Fig. 2B,2C). Tregs have been proven to the effect of inflammation protection. So, what is the significance of the increase in Tregs, which is synchronized with the increase of lung injury and inflammatory factors expression, after lung injury induced by *Aspergillus fumigatus* infection? Is it a response to the anti-inflammatory effects, or other possible values? To further understand the relationship between Tregs and

fungal persistence, we use CD25 neutralizing antibodies to inhibit Tregs (Fig. 2D) and found that the quantity of fungal burden in lung was lower in mice injected with CD25 neutralizing antibodies (Fig. 2E). Consistent with previous research, Study showed that $CD4^+CD25^+Foxp3^+$ Tregs are not generated in $B7^-2^-$ or $CD28^-$deficient mice, these mice capable of efficiently inhibiting the fungal growth[(Montagnoli et al. 2002)]. In *A. fumigatus* infection, fungal burden was higher in WT than in $CD4^+CD25^+Foxp3^+$ Tregs reduced mice[(Montagnoli et al. 2006)]. Immune dysfunction leads to worse outcomes pathogenic microorganism infection. Partial exhaustion of Tregs elevated IL-17A and IL-1β production, but decreased IL-10 levels and inducing decline bacterial load, attenuation of lung injury in secondary *P. aeruginosa* infection after sepsis[(Hu *et al.* 2018)]. The deleterious role of Tregs on the innate immune response was underscored in the improved resistance to *C. albicans* infection[(Netea *et al.* 2004)]. Our results show that the lung could be rescued partially, after after depletion of Tregs (Fig. 2F). These results may imply that the impacts of Tregs are adverse when pathogen invades the host, such as *A. fumigatus*, persistent.

Along with *A. fumigatus* infection developing, TLRs trigger antimicrobial host immune responses. TLR2, as an important pathogen pattern recognition receptor, plays a vital role in infection[(Raijmakers *et al.* 2017; Stephen-Victor *et al.* 2017b)]. It is an important receptor able to recognize the hypha and spores of *A. fumigatus*[(Netea *et al.* 2003)]. Our study found that *A. fumigatus* infection can indeed stimulate TLR2 expression to increase (Fig. 3A). We observed the survival rate of controls to *A. fumigatus* was similar to those of TLR2-deficient mice. These data illustrated the competence of the mice in the innate antifungal resistance level, as illustrated by reduced fungal growth in mice with primary disseminated candidiasis[(Bellocchio et al. 2004)]. Interestingly, 3 days after *A. fumigatus* infection, we noticed that the number of fungal amount and the degree of damage in the lung tissue of $TLR2^{-/-}$ mice were decreased compared to controls (Fig. 3B,3C). It implies that *A. fumigatus* could evade host defense through TLR2-mediated signals probably. And the expression of TNF-α and IL-6 were marginally impaired in $TLR2^{-/-}$ mice (Fig. 3D). It suggested that TLR4[(Taghavi *et al.* 2017)], C-type lectin receptors(CLRs)[(Werner *et al.* 2009; Gessner et al. 2012; Taylor et al. 2014)] and galectin family proteins involved in *A. fumigatus*-induced proinflammatory cytokine release too. TLRs may regulate the host's adaptive immune response by inhibiting or stimulating the function of Treg cells. Past research proves has proved that CD4 + Foxp3 + Treg cells may sense pathogens and regulate their differentiation via TLRs[(Zanin-Zhorov and Cohen 2013)]. Although there was evidence that $CD4^+ Foxp3^+$ Treg can express a series of TLR mRNA, but only a few of TLRs like TLR2, TLR5 and TLR8 were triggered to affect the proliferation, differentiation and/or inhibitory function of $CD4^+ Foxp3^+$ Treg[(Liu et al. 2006; Sutmuller *et al.* 2006)]. IL-10 initiates the growth of $CD4^+$ Tregs in a costimulation- and TLR-dependent fashion in fungi infection[(Mills 2004; O'Garra and Vieira 2004; Belkaid and Rouse 2005)]. Study showing that *C. albicans* results in immunosuppression via TLR2-derived signals that mediate increased IL-10 production and survival of Treg cells[(Netea *et al.* 2004)]. And current evidence showed that Dengue infection influenced the proliferation of Treg cells through TLR2/MyD88 pathway[(George et al. 2020)]. In our study, we found that TLR2 deficient mice have a significant decrease in Tregs of spleen (Fig. 4A) and the expression of Foxp3 of lung (Fig. 4B,4C). On the other hand, starting from naive cells, CD4 + T cells are able to undergo differentiation into various effector cell subsets with particular roles. Tregs display plasticity that allows for the functional

adaptation to various physiological and pathological environments during immune response process [(Wan et al. 2020)]. TLR signaling is involved in T cell population regulation [(Netea *et al.* 2004; Rau et al. 2015)]. After inhibiting TLR-2 pathway, the differentiation of Tregs from $CD4^+$ T cell promoted by *A. fumigatus* stimulation decreased (Fig. 4D). It suggested that the signal mediated by TLR2 is an important link that affects the development of Treg cells. The present our data prompted that *A. fumigatus* infection host induced the proliferation of $CD4^+CD25^+Foxp3^+$ Tregs through the activation of TLR2 pathway.

A. fumigatus produces plenty of spores, consequently triggering sprouting of several inflammasomes [(Said-Sadier et al. 2010)]. It can lead to the host's inflammasome activation, causing the activation of the pyroptosis pathway[(van de Veerdonk et al. 2015)]. Past studies have proved that the inflammasome sensors are capable of recognized the spores, DNA and polysaccharides produced by the fungi in vivo[(Karki et al. 2015; Huang et al. 2018)]. Such a situation would perhaps lead to activation of a cytosolic macromolecular signaling platform that mediates the release of the proinflammatory cytokines IL-1 and IL-18 and cleavage of the pore-forming protein gasdermin-D (GSDMD). Highly proinflammatory is a major feature of pyroptosis. Inflammasomes that rely on caspase-1 activation can promote the maturation of IL-1β ,and then released during cell death[(Miao et al. 2010)]. In previous studies, immunocompetent WT mice and mice lacking components of the inflammasome like NLRP3 or absent in melanoma 2(AIM2) do not succumb to infection with *A. fumigatus*[(Karki et al. 2015)]. In our research, we selected Aspergillus fumigatus to infect WT and $TLR2^{-/-}$ mice, which with immunocompetent. Then found that the expression of GSDMD, IL-1α and IL-1β increased in WT mice after lung infection of Aspergillus fumigatus (Fig. 5). The release of IL-1β in monocytes and the increased spread of pro-IL-1β is stimulated by hyphae while *A. fumigatus* triggers the release of NLRP3 inflammasomes[(Said-Sadier et al. 2010)]. Studies showed that the expression of NLRP3 was increased in lung tissue from patients with allergic bronchopulmonary aspergillosis (ABPA)[(Jeong *et al.* 2018)].For the inflammasome to induce the secretion of IL-1β the steps involved include proinflammatory stimuli triggering TLR to mediate signals, cytokines to be induced to produce proform and inflammasomes to be activated to ensure mature processing of cytokines[(Schroder and Tschopp 2010)]. To a certian extent IL-β mRNA was reduced in TLR2-deficient compared with wild-type macrophages during *C. difficile* infection[(Liu *et al.* 2018)]. H. pylori triggers the inflammasome in a TLR2 and NLRP3-dependent manner and activation of inflammasome benefits the *H. pylori* to ensure that infection persistent[(Koch et al. 2015)]. In $TLR2^{-/-}$ mice, the pyrolysis-related proteins (GSDMD, IL-1α, and IL-1β) upregulated, which showed the immunocompetent in the infection of *A. fumigatus*. But they were decreased after the infection of *A. fumigatus*, compared with WT mice (Fig. 5). Although the is no clarity on how intracellular receptors involved in the activation of inflammasomes and the physiological functions of the inflammasomes in response to Aspergillus fumigatus. Our results provided preliminary evidence to suggest that TRL2 play a role in GSDMD-dependent pyrolysis of lung after *A. fumigatus* infection partially.

5. Conclusion

Susceptibility to *A. fumigatus* is associated with quantity of Tregs in TLR2 knockout animals. The infection leads to the proliferation and differentiation of $CD4^+CD25^+Foxp3^+$ Tregs via the activation of TLR2 pathway. It is a potential mechanism to evade host defense in *A. fumigatus* infection of lung. And this effect can regulate GSDMD-dependent pyrolysis, and may involve TRL2 signals partially.

6. References

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Figures

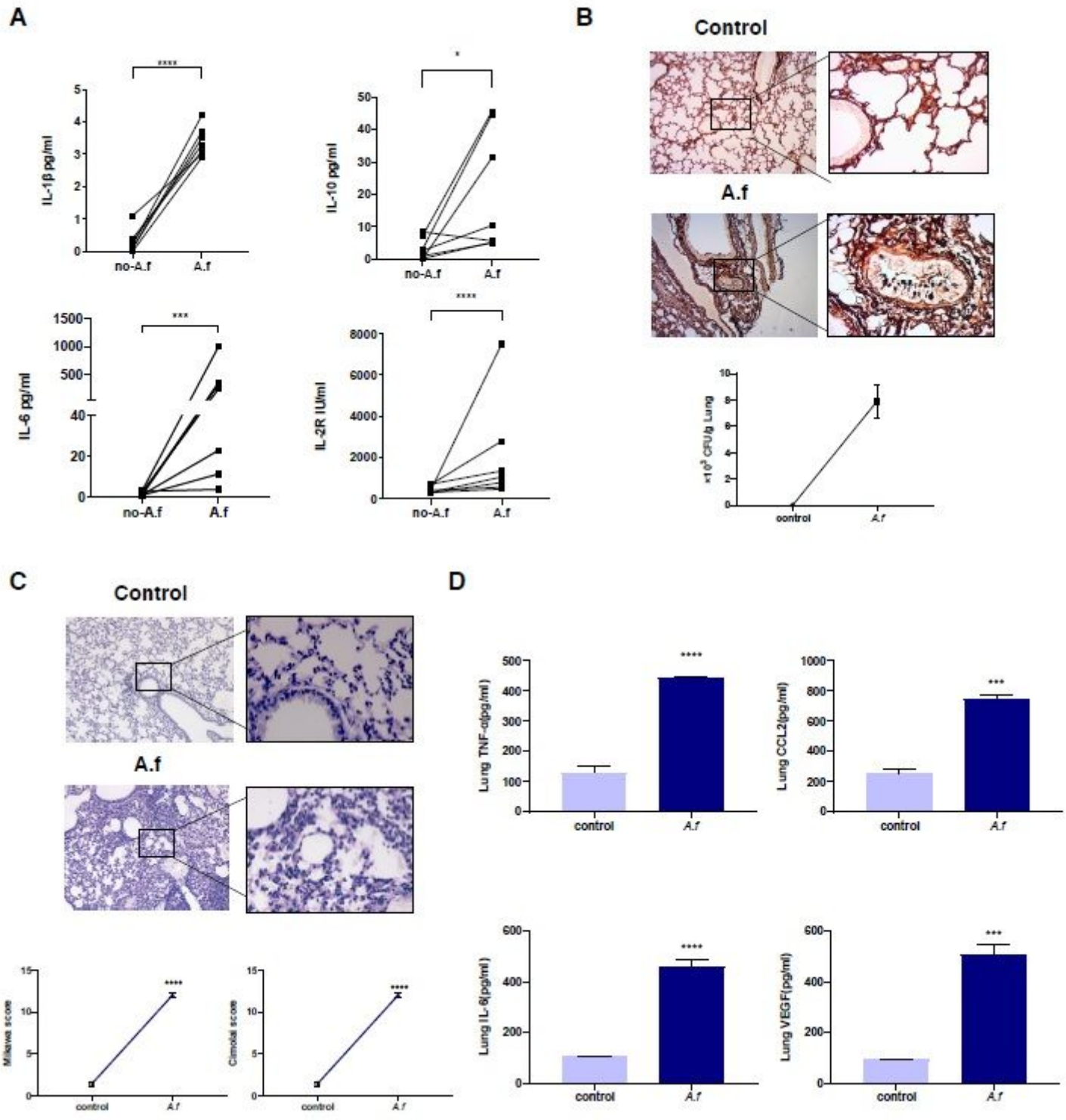

Figure 1

Aspergillus fumigatus infection cause pulmonary inflammatory response. (A) IL-1β,IL-6,IL-10 and IL-2R is elevated in serum of patients infected with Aspergillus fumigatus(n=7).(B) C57BL/6 mice were administered intratracheally at a concentration of 50 μl of 1× 107 viable spores and monitored for 3 days(n=5/group). Grocott's methenamine silver (GMS) and lung colony-forming units (CFUs)(n=5/group). (C) H&E staining of the lung tissues of A. fumigatus infected mice at 100× and 400× magnification,

compared with the noninfected control, alveolar hemorrhage and inflammatory cell in filtration were more after A. fumigatus challenge.(D) TNF-α, IL-6,CCL2, and VEGF expression levels in lung tissues of mice were detected by ELISA.A. fumigatus upregulated the production of proinflammatory cytokines/chemokines in lung tissues, including TNF-α, IL-6,CCL2, and VEGF(n=3/group). Experiments were done at least three times. ns: not significant; ∗p < 0.05, ∗∗p < 0.01, ∗∗∗ p < 0.001, and ∗∗∗∗ p < 0.0001 by Student's unpaired two-tailed t test. Error bars represent SEM.

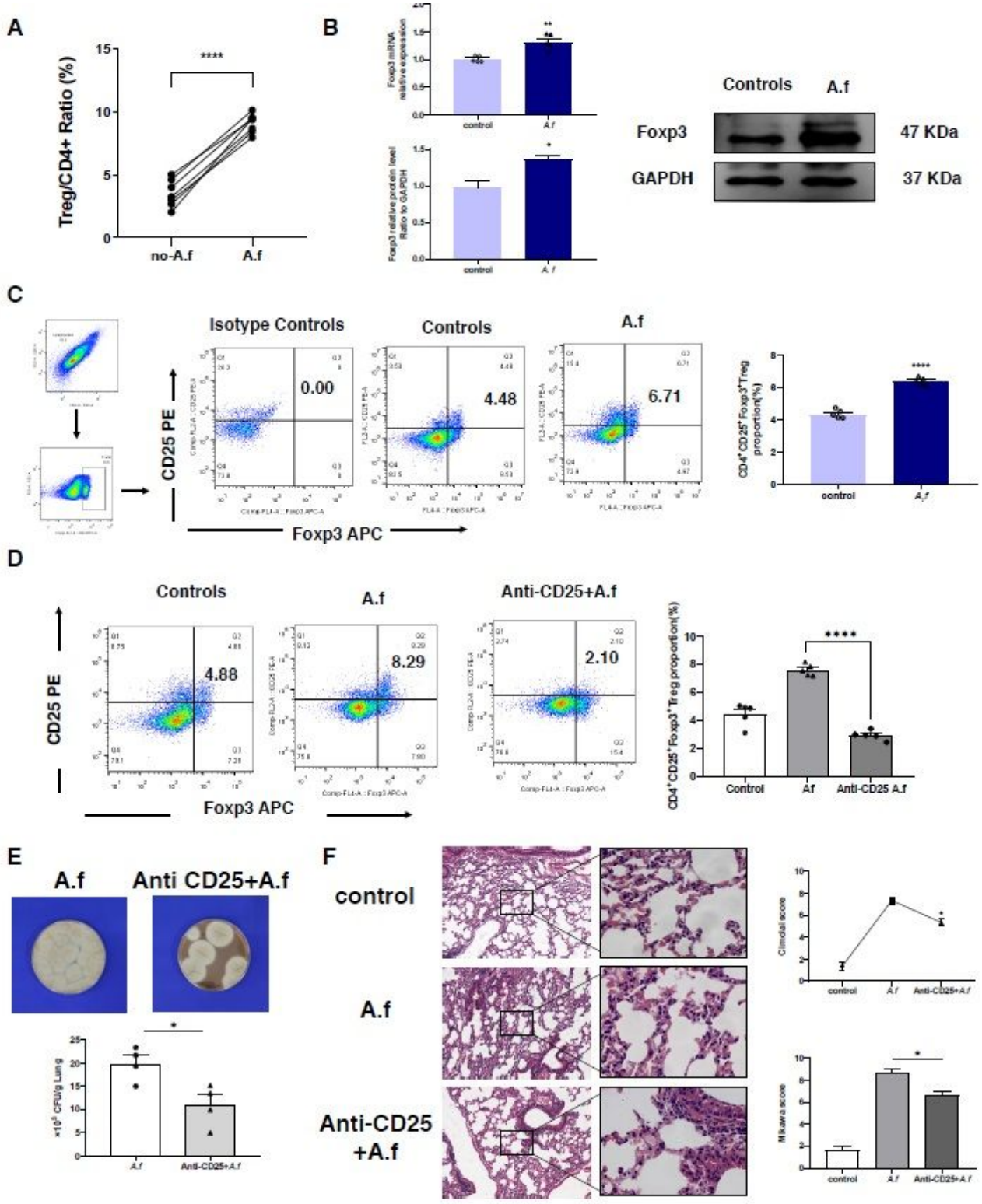

Figure 2

Increased number of Treg cells in mice infected with Aspergillus fumigatus. (A) Increased CD4+CD25+Foxp3+ Tregs proportion significantil in PBMCs of patients infected with Aspergillus fumigatus(n=7). (B) Foxp3 mRNA levels in the lungs were measured with qRT-PCR. Relative expression levels of the genes were expressed with the GAPDH housekeeping gene as an internal reference(n=5/group). The expression of Foxp3 protein levels in the lungs were measured with Western blotting.Relative expression levels of the protein were expressed with the GAPDH as an internal reference(n=3/group) (C) CD4+CD25+Foxp3+ Tregs in spleen were detected by flowcytometry and the proportions were analyzed by FlowJo10 as prior described in methods. Increased CD4+CD25+Foxp3+ Tregs proportion significantly in spleen of A. fumigatus infection mice(n=5/group).Decreased susceptibility of mice to A. fumigatus infection after Tregs depletion (D) After pretreatment with CD25 neutralizing antibodies, the number of Treg cells in the spleen of mice with A. fumigatus infection group was significantly reduced ,each group (n=5/group).(E) Fungal load (Colony-forming unit) after 1 days of infection(n=4/group). Experiments were done at least three times. Data are presented as mean ± standard deviation. *Statistically significant difference ($p < 0.05$) against noninfected control. (F)Lungs from each experimental group were processed for histological examination after H&E staining. Lung injury scores were evaluated by method described previously. *$p<0.05$ **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by Student's unpaired two-tailed t test and the one-way ANOVA followed by the Tukey Post Hoc test. Error bars represent SEM.

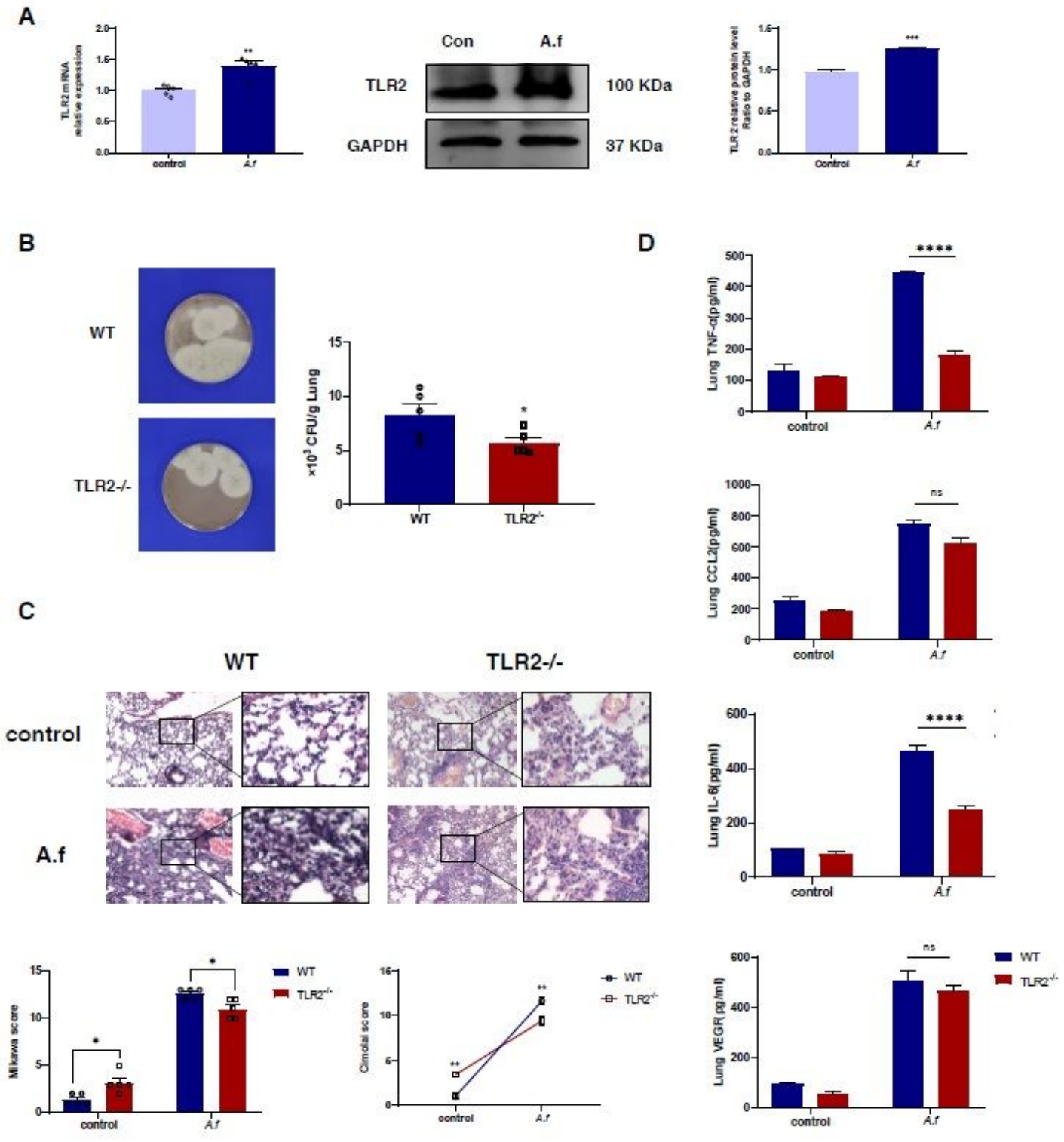

Figure 3

C57BL/6 mice and TLR2-/- mice were infected with A. fumigatus spores and monitored for 3 days. (A) The expression of TLR2 protein in lungs of mice were infected with A. fumigatus for 3 days was detected by western blot an analyzed by imager systemas described in methods, and the mRNA levels of TLR2 were determined by qRT-PCR(n=3/group).(B) Fungal load (Colony-forming unit) after 3 days of infection(n=5/group). (C)H&E staining of the lung tissues of A. fumigatus infected WT mice and TLR2-/-

mice at 100× and 400× magnification, mainly infiltration of macrophages and monocytes in the lungs of TLR2-/- mice with A. fumigatus, with alveolar congestion and hemorrhage. TLR2-/- mice suffered from interstitial congestion and hemorrhage less obviously after A. fumigatus infection compared whit WT mice. (n=5/group).(D)TNF-α, IL-6,CCL2, and VEGF expression levels in lung tissues of mice were detected by ELISA.TLR2-deficient downregulated the production of proinflammatory cytokines/chemokines in lung tissues treated by A. fumigatus, including TNF-α, IL-6,CCL2, and VEGF(n=3/group).Experiments were done at least three times. ns: not significant; $*p < 0.05$, $**p < 0.01$, $*** p < 0.001$, and $**** p < 0.0001$ by Student's unpaired two-tailed t test and the two-way ANOVA followed by the Tukey Post Hoc test. Error bars represent SEM.

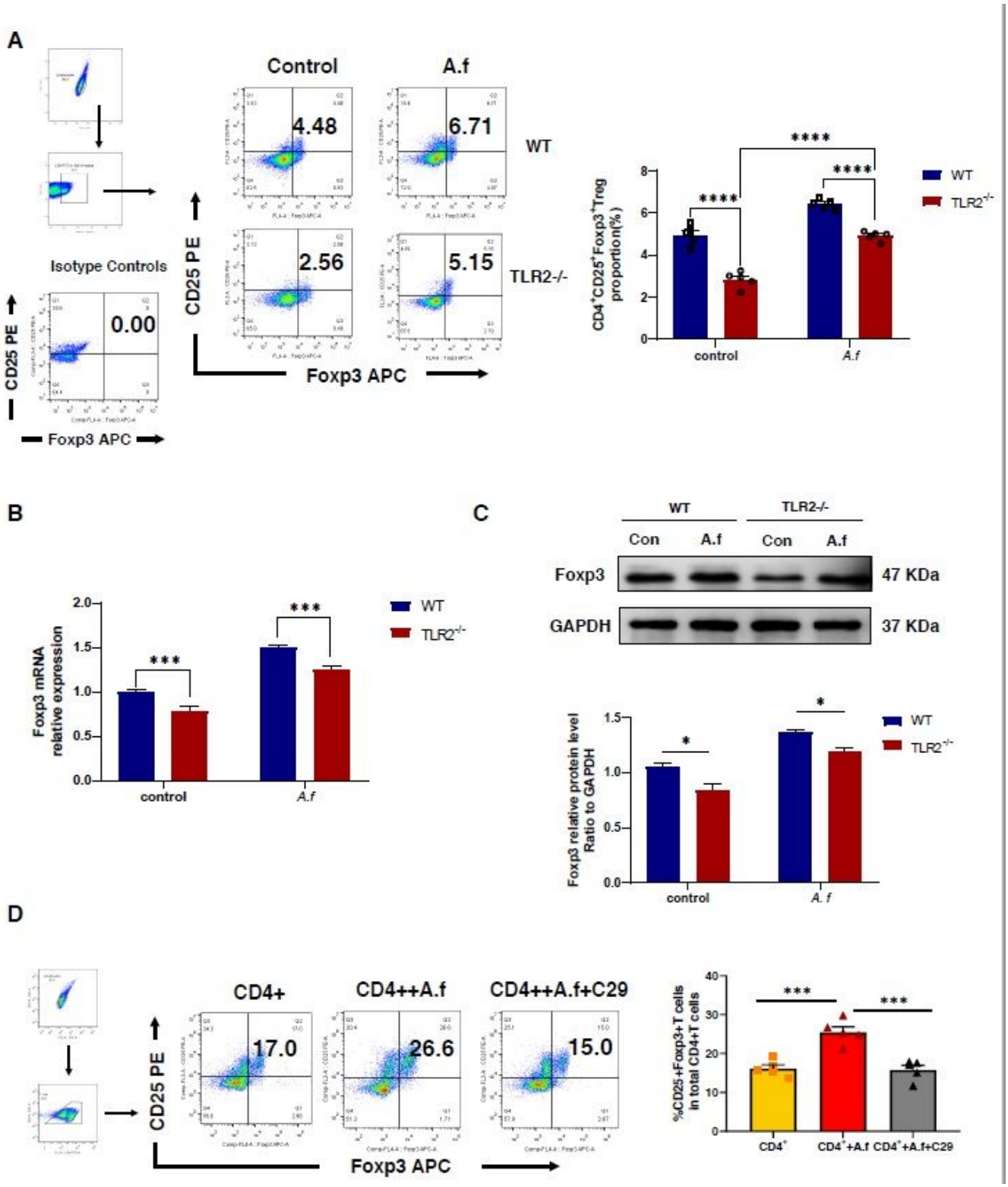

Figure 4

(A) CD4+CD25+Foxp3+ Tregs in spleen were detected by flowcytometry and the proportions were analyzed by FlowJo10 as prior described in methods. Reduced CD4+CD25+Foxp3+ Tregs proportion significantly in spleen of A. fumigatus infection TLR2-/- mice(n=5/group). (B)Foxp3 mRNA levels in the lungs were measured with qRT-PCR(n=3/group). (C) The expression of Foxp3 protein in lungs of C57BL/6 mice and TLR2-/- mice were infected with A. fumigatus for 3 days was detected by western blot an

analyzed by imager systemas described in methods(n=3/group). CD4+ T cells were sorted from the spleens of wild-type C57BL/6 mice and cultured. Tregs were detected by flow cytometry on day 3 and analysed by FlowJo10. (D) CD4+T lymphocytes differentiated less Treg cells after treatment with C29.In each group, n = 5; three replicate experiments were performed three tims. ns: not significant; ∗p < 0.05, ∗∗p < 0.01, ∗∗∗ p < 0.001, and ∗∗∗∗ p < 0.0001 by the one-way ANOVA and the two-way ANOVA followed by the Tukey Post Hoc test. Error bars represent SEM.

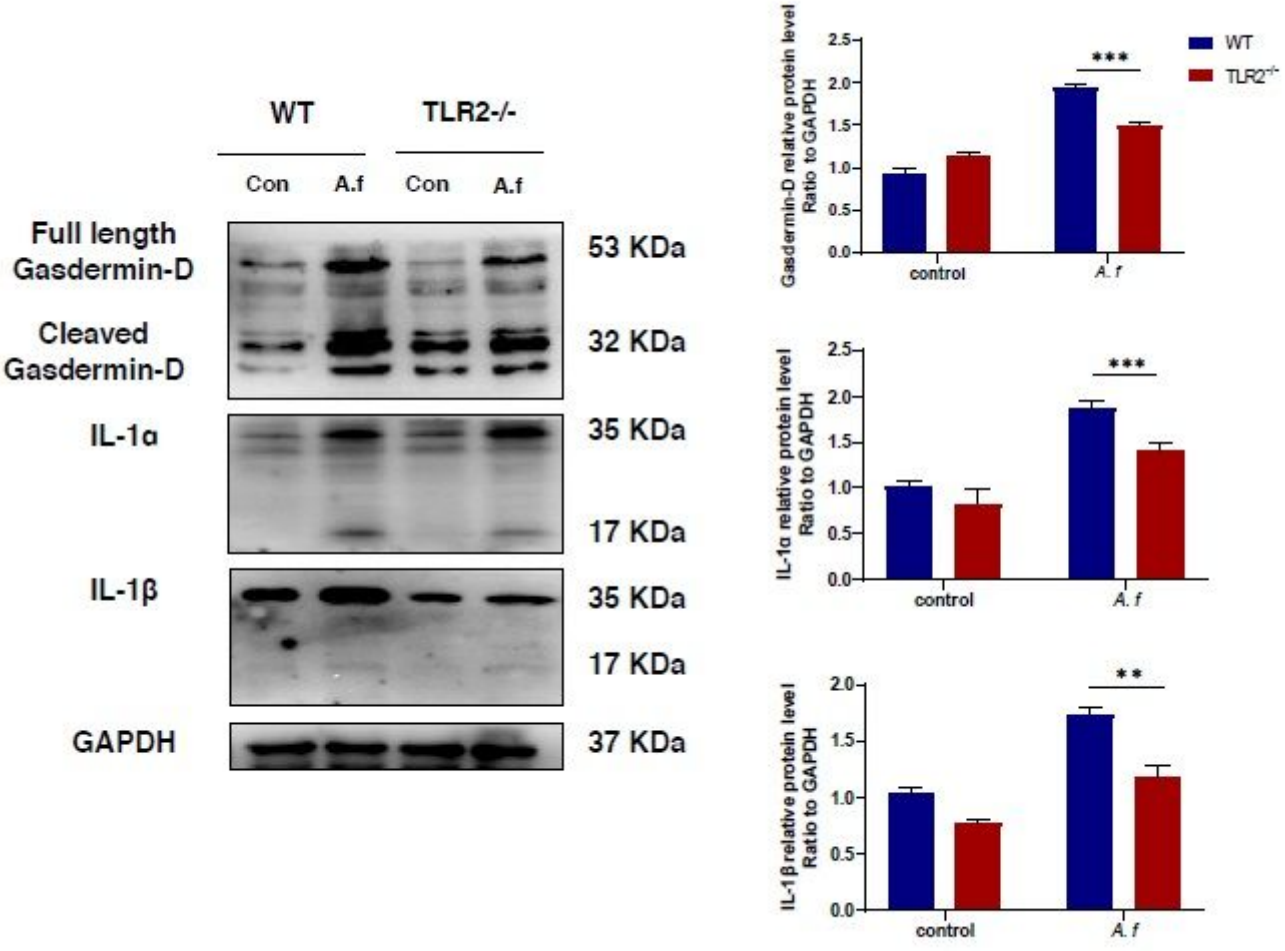

Figure 5

The expression of Gasdermin-D,IL-1α,and IL-1β protein in lungs of was detected by western blot an analyzed by imager systemas described in methods(n=3/group). ns: not significant; ∗p < 0.05, ∗∗p < 0.01, ∗∗∗ p < 0.001, and ∗∗∗∗ p < 0.0001 by two-way ANOVA followed by the Tukey Post Hoc test comparing the WT,WT+A,f.TLR2-/-, TLR2-/-+A.f groups. Error bars represent SEM.