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研究課題名	To study the molecular mechanism of COVID-19 protein ORF3a to stimulate the production of cytokine in macrophages	
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(Form 2)

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Joint Research Report (Project Completion)

Project Completion Report

Report

1. Confirm the effect of ORF3a protein of COVID-19 to stimulate the cytokine production of macrophages.

(1) Use ORF3a to elevate the cytokine level in macrophages cell line (Raw264.7). Use ELISA to detect the cytokine level in the cell culture supernatant and use FACS to detect the cytokine level in macrophages.

When the Raw264.7 were stimulated by ORF3a, the level of TNF-α, IFN-γ, IL-2, IL-6, IL-10 and IL-12 increased (P<0.001), while the IL-4 level had no change. Another protein of COVID-19, RBD had no the promotion effect (Figure 1). There was a dose-effect relationship between ORF3a and cytokine production of macrophage (Figure 2). The results showed that the ORF3a was one of the main proteins to stimulate macrophage to produce cytokine.

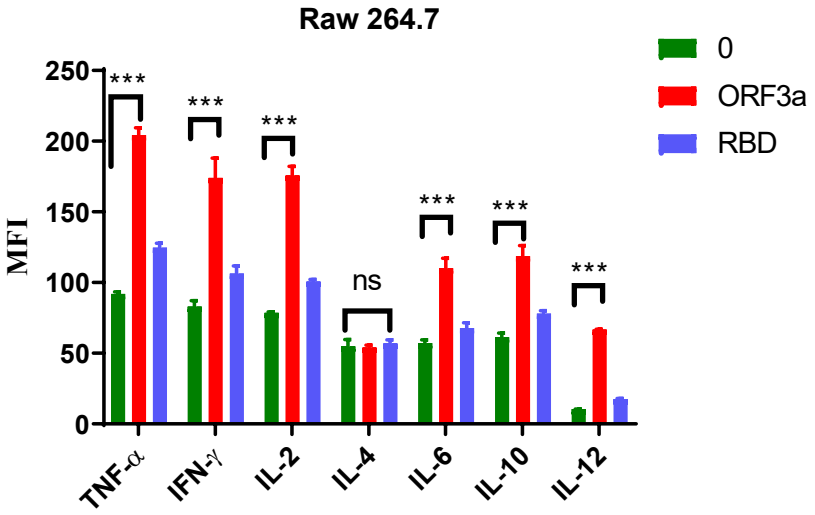

Figure 1 ORF3a protein promoted the production of cytokines in Raw264.7 cells

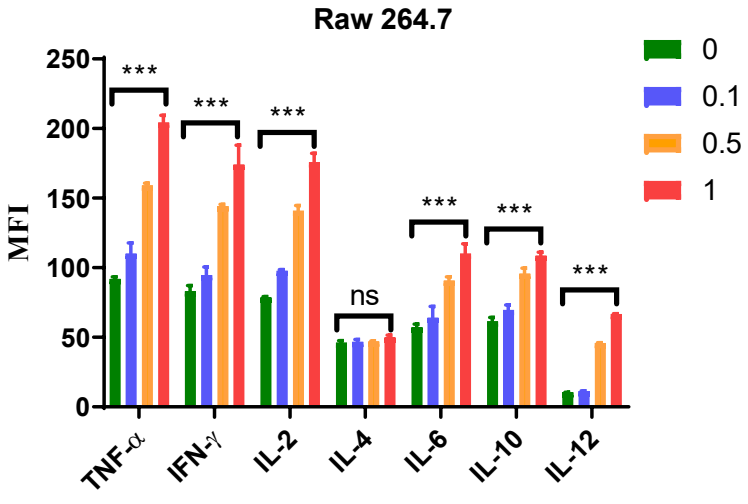

Figure 2 The dose relationship between ORF3a protein and the production of cytokines in Raw264.7 cells

(2) Use ORF3a to elevate the cytokine level in mice. Use ELISA to detect the cytokine level in serum and use FACS to detect the cytokine level in peripheral blood mononuclear cell (PBMC) and splenocytes.

ORF3a was used to stimulate the cytokine level in mice model. The results showed that the TNF-α, IFN-γ, IL-6 and IL-10 level of CD11b+ cells in PBMC were increased (P<0.001 or P<0.01) by ORF3a stimulation while the IL-4 level had no change (Figure 3). The level of TNF-α and IL-6 of CD11b+ cells in splenocytes also increased in ORF3a group (P<0.001), which IFN-γ and IL-4 had no change (Figure 4).

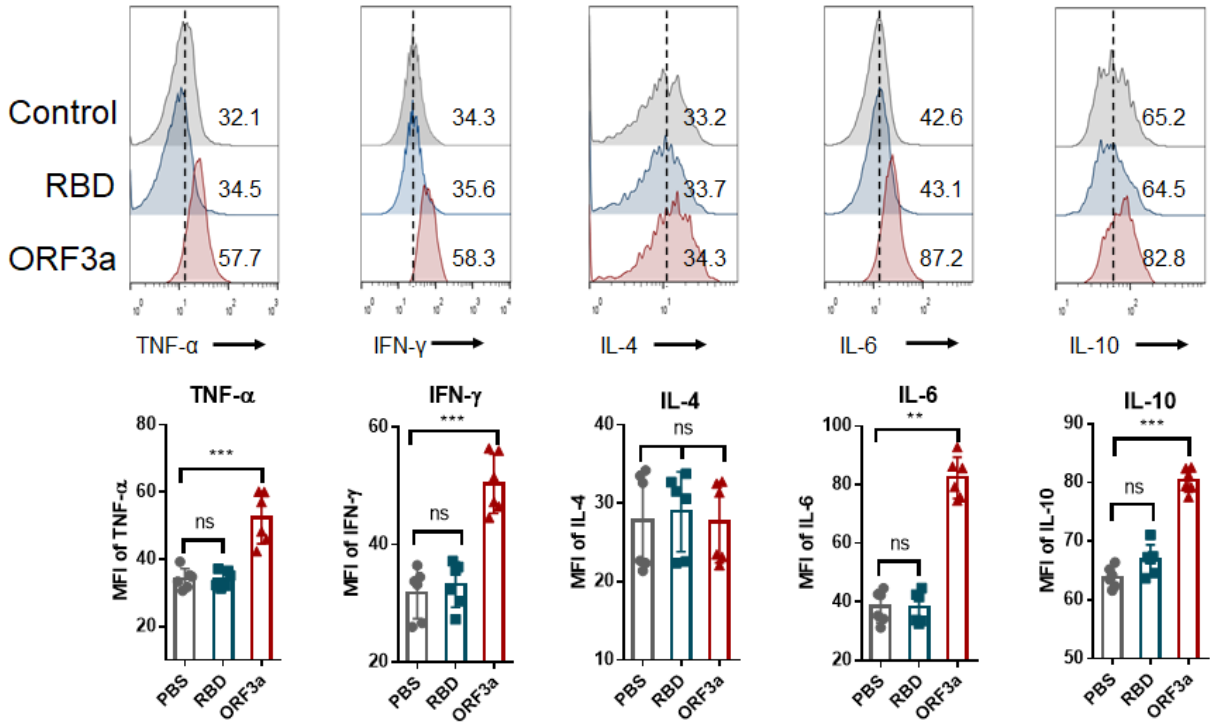

Figure 3 ORF3a protein up-regulates cytokines expression of $CD11b^{+}$ cells in PBMC

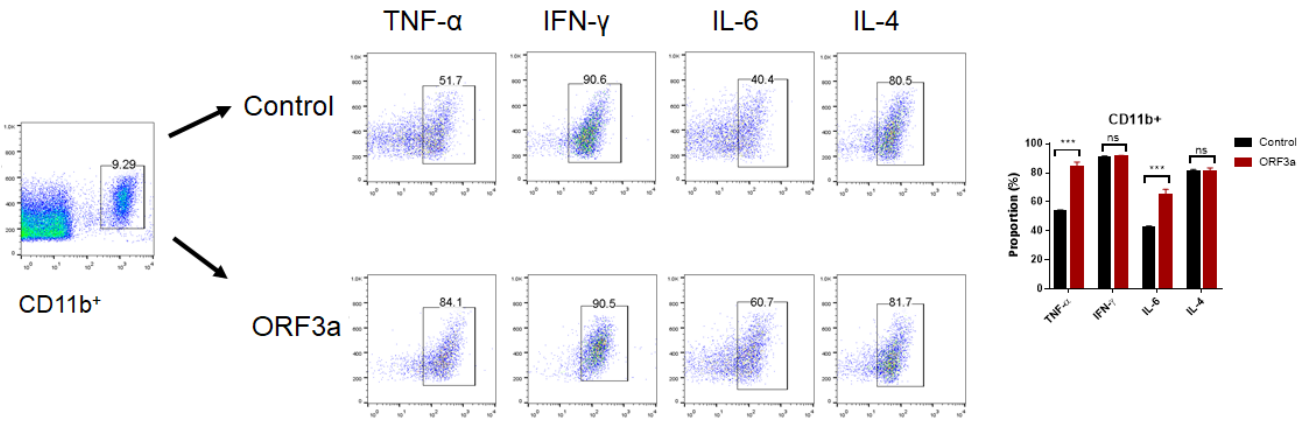

Figure 4 ORF3a protein up-regulates the expression of TNF-α and IL-6 in CD11b+ cells in splenocytes

2. Use proteomics to screen the signal pathway molecules during the process of ORF3a to stimulate macrophages to produce cytokine.

(1) Proteomics were used to screen the key molecules related to the cytokine production in macrophages.

Proteomics were used to analysis the protein profile change after the Raw264.7 was stimulated with ORF3a. The results showed that there were 73 proteins up-regulated and 42 proteins down-regulated. During which, the level of S100A4

increased in ORF3a group (Figure 5).

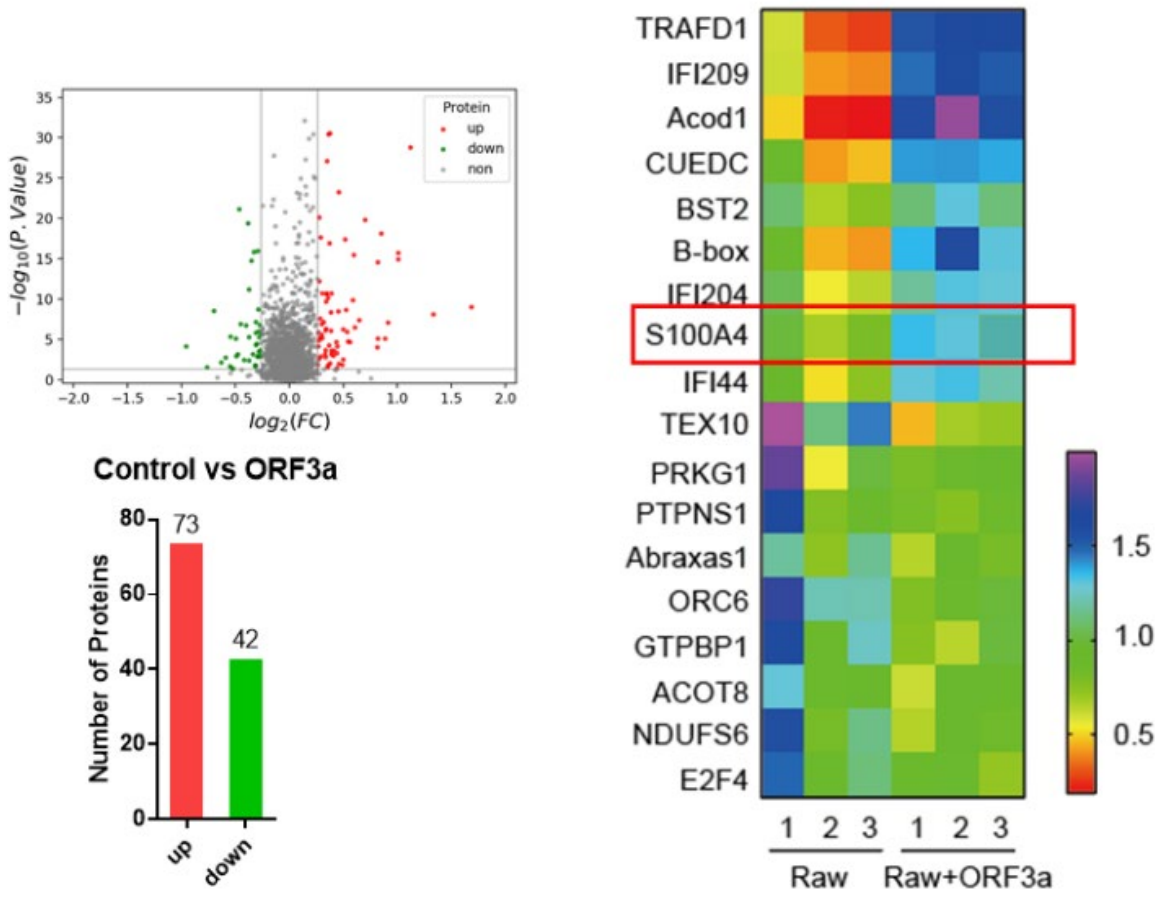

Figure 5 The proteomics results of Raw264.7 which were stimulated with ORF3a

(2) Western blot and qPCR were used to confirm the target protein of signal pathway molecules from proteomics results.

The express level of S100A4 in Raw264.7 increased after being stimulated by ORF3a, and the transcript level of S100A4 was also increased (Figure 6).

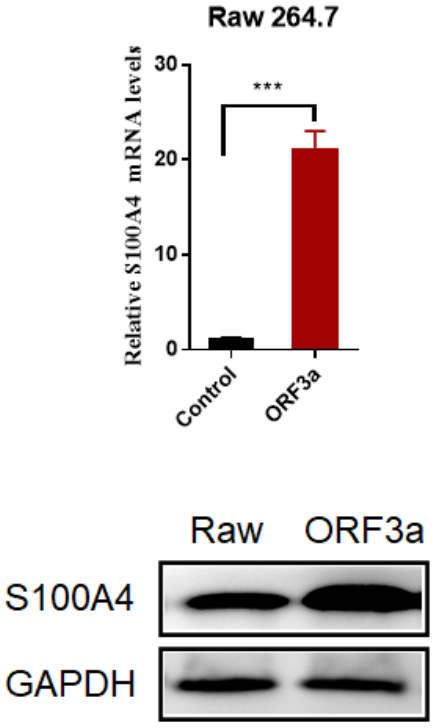

Figure 6 ORF3a up-regulates the expression and transcript level of S100A4 in Raw264.7

3. Construct the gene knockout macrophages cell line and gene knockout mice to study the molecular mechanism.

(1) Select one molecule with meaningful immunological function to act as candidate from proteomics results. CRISPR-Cas9 method is used to knock out target gene in Raw264.7 to construct the cell model. The expected results are that ORF3a effect to elevate cytokine level are blocked in gene knockout cells.

S100A4 was selected as the target molecule to study the molecular mechanism based on the results of proteomics, western blot and qPCR. The S100A4 knockout Raw264.7 was built up by using of CRISP-Cas9 method. Raw264.7 was transfected with ORF3a plasmid by 293T coating system, and the stable cell line was screened by puromycin (Figure 7a). It could endogenous express ORF3a which was confirmed by western blot (Figure 7b). The results showed that endogenous ORF3a induced Raw264.7 to proliferation, while it was blocked by S100A4 knockout (Figure 7c).

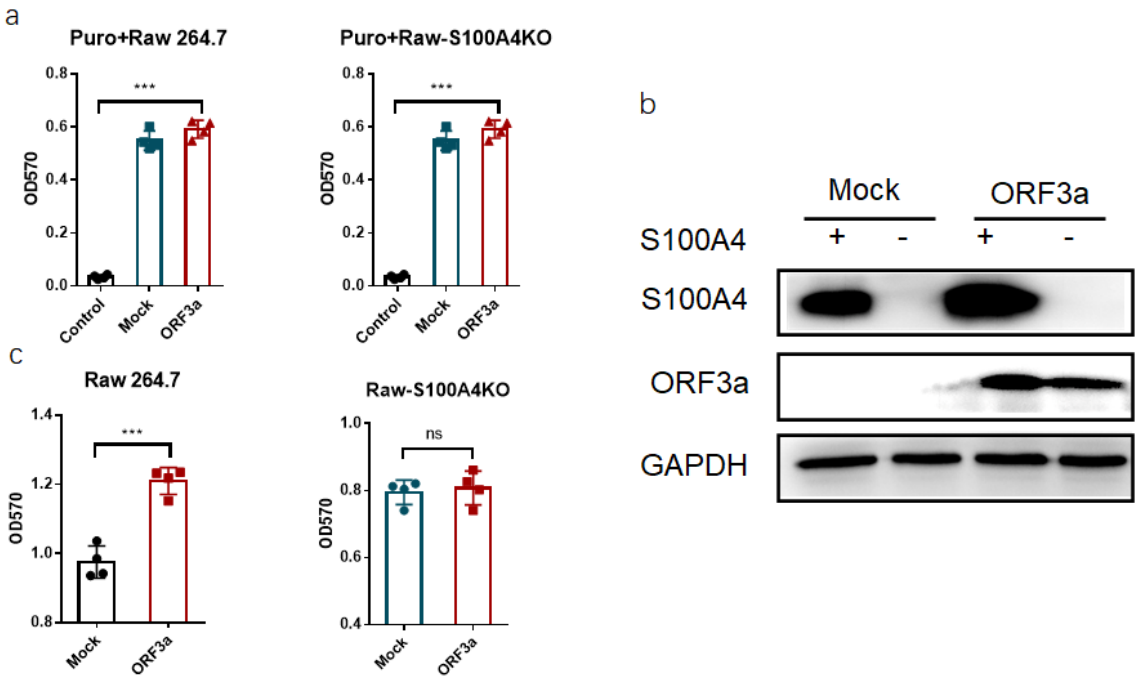

Figure 7 Endogenous ORF3a promotes Raw cell proliferation and S100A4 production

4. Construct the gene knockout macrophages cell line and gene knockout mice to study the molecular mechanism.

(1) Select S100A4 to act as candidate from proteomics results.

The results showed that endogenous ORF3a promoted the production of cytokines (TNF-α, IFN-γ, IL-2, IL-6 and IL-10) in Raw264.7, while they were blocked by S100A4 knockout (Figure 8a and 8b). The results were also confirmed by qPCR (Figure 8c).

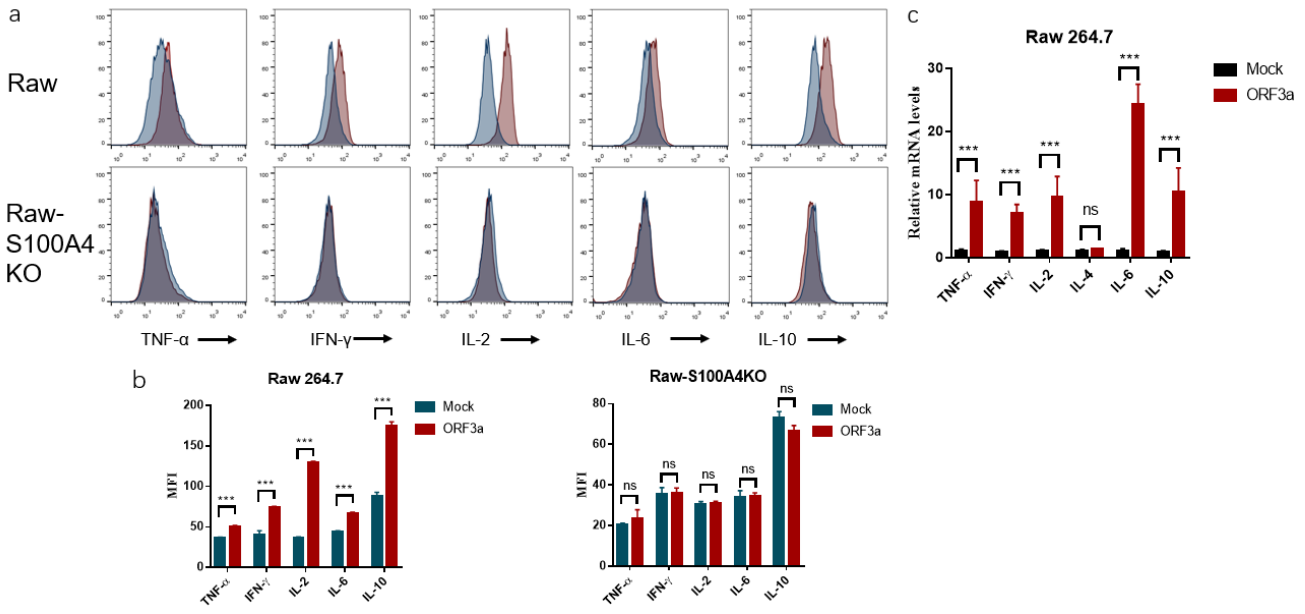

Figure 8 The endogenous ORF3a promotes the production of cytokines in Raw cells while no effect on Raw-S100A4KO cells.

(2) Gene knockout mice to study the effect of target gene in vivo. The expected results are that the effect of ORF3a to elevate cytokine level are blocked or reduced in gene knockout mice.

Macrophages S100A4-conditional knockout mice were constructed. ORF3a was administered to mice via intraperitoneal injection. The results showed that ORF3a increased the proportion of macrophages (CD11b+) in blood, while it was with no change in splenocytes. In macrophages S100A4 conditional knockout mice, the effect of ORF3a on macrophages had been blocked (Figure 9). The results indicated that S100A4 was a key signal pathway molecules of ORF3a on macrophages increasement.

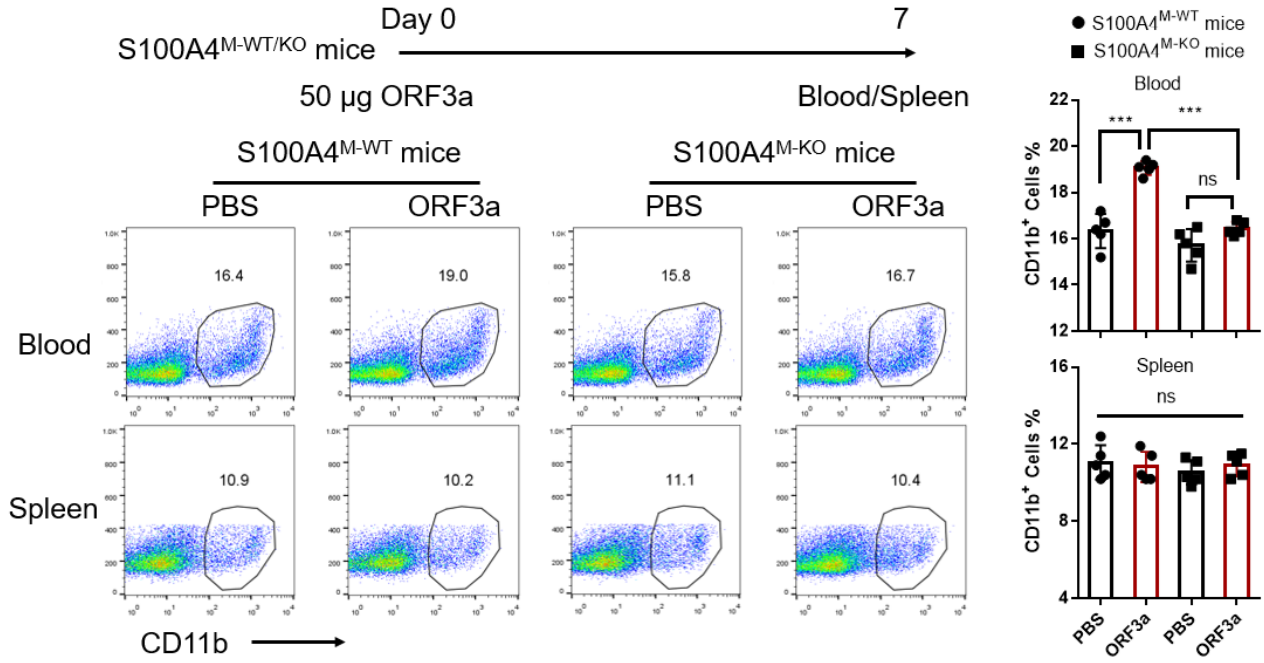

Figure 9 ORF3a upregulates the proportion of $CD11b^{+}$ cells in PBMCs of WT mice, while does not affect the proportion of S100A4 in macrophages conditional knockout mice.

ORF3a stimulated the production of TNF-α, IFN-γ, IL-6 and IL-10, while the effect were blocked in S100A4 macrophage conditional knockout mice (Figure 10). This result confirms the role of S100A4 in ORF3a-stimulated cytokine production in macrophages.

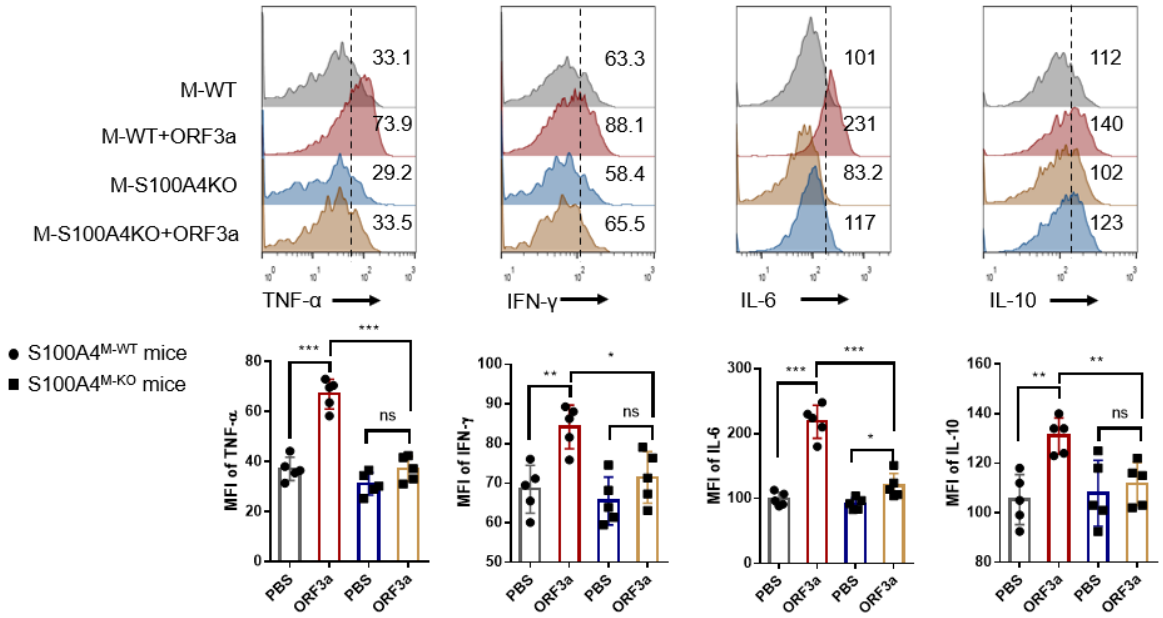

Figure 10 ORF3a does not affect cytokine expression of $CD11b^{+}$ cells in PBMCs of S100A4 in macrophage conditional knockout mice.

In the culture system of S100A4-knockout (S100A4KO) Raw cells, the expression levels of TNF-α, IFN-γ, IL-2 and IL-6 were basically unchanged or slightly increased after adding ORF3a or S100A4 alone (detected by flow cytometry). However, in the combined treatment group, the expression levels of these cytokines were significantly higher than those in the single treatment groups, indicating that exogenous S100A4 promotes ORF 3a-induced cytokine upregulation (Figure 11).

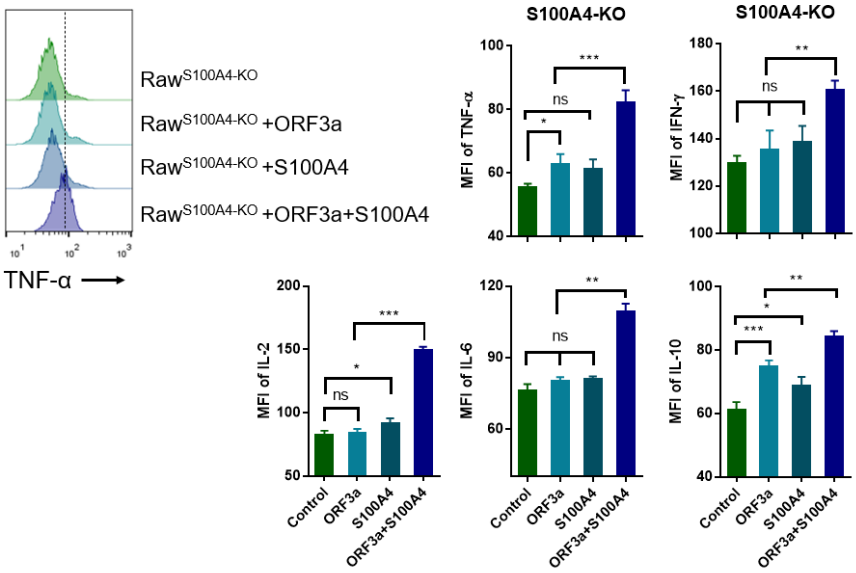

Figure 11 Exogenous S100A4 protein promotes ORF3a-induced macrophage production of cytokines

After ORF3a acted on Raw cells, the expression levels of TNF-α, IFN-γ, IL-2 and IL-6 increased significantly. However, this effect was abrogated by using S100A4 antibody, suggesting that S100A4 is a critical molecule for ORF3a-induced cytokine upregulation. This finding is consistent with the results of Figure 11 (Figure 12).

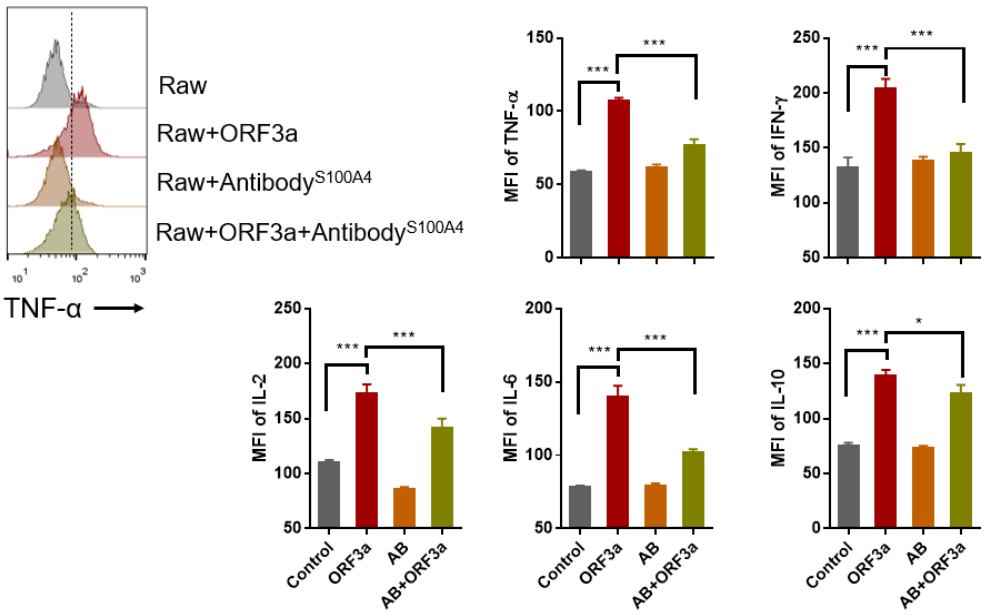

Figure 12 S100A4 antibody inhibits ORF3a-induced macrophage production of cytokines

This project suggests that the SARS-CoV-2 protein ORF3a is a major protein responsible for cytokine elevation, and S100A4 is involved in the process of ORF3a-induced cytokine elevation. Blocking this protein may provide new ideas and target proteins for the treatment of cytokine storms caused by SARS-CoV-2. In this project, an antagonistic protein of S100A4 has been prepared, and its therapeutic effect will be verified in in vivo experiments in the future. This conclusion also provides new insights for the treatment of cytokine storms caused by other viral infectious diseases, and even for the treatment of other types of cytokine storms.