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Immunomodulatory effect of tibetan medicine compound extracts against ORFV in vitro by metabolomics

Yueyuan Fan[1†], Jiao Wu[1†], Wei Huang[2†], Saiju Li[1], Qin Zeng[1], Zhuoga Gesang[3], Yuzhen Silang[3*], Chong Zhang[4*] and Guowen Fu[1*]

Abstract

Ovine contagious pustular dermatitis (ORF) is one of the main diseases of sheep and is a zoonotic disease caused by Ovine contagious pustular dermatitis virus (ORFV) infection, posing a significant constraint on sheep breeding industry and human health. The Tibetan medical formulation composed of *Polygonum leucoides*, *Polygonum xanthoxylum* and *Acanthophora rotunda* significantly regulated lymphocyte immune function following ORFV stimulation, although the mechanism remains unclear. In order to study the immunomodulatory effects and mechanism of three Tibetan medicinal extracts (*Polygonum leucoides*, *Polygonum xanthoxylum,* and *Acanthophora rotunda*) against ORFV in vitro, sheep peripheral blood lymphocytes were isolated in vitro and treated with different concentrations of Tibetan medicine compound extract solution after ORFV infection. The cytokine expression levels in lymphocytes were measured at 4 h, 8 h and 12 h. Additionally endogenous metabolites in lymphocytes at 0 h, 4 h, 8 h and 12 h were quantified by untargeted metabolomics method. The results showed that, the extracts could regulate the lymphocyte immune factors altered by ORFV, and regulate the lymphocyte immune function through cysteine and methionine metabolic pathways as well as the pyrimidine metabolic pathways, potentially alleviating the immune evasion induced by ORFV.

Keywords ORFV, Tibetan medicine compound, Untargeted metabolomics, Cytokines

Introduction

Ovine contagious pustular dermatitis (ORF), commonly known as "sheep mouth sore", is a contagious zoonotic disease characterized by more than skin cell proliferation. The disease's repeated infections can lead to secondary infection in young animals and, in immunosuppressed hosts, can be fatal, with lamb mortality reaching up to 10% during outbreak [1]. As the sheep industry globalizes and intensifies, and with the development of increasingly close international trade, ORF is emerging as a worldwide epidemic, inflicting substantial economic losses in livestock husbandry. ORF is caused by Ovine contagious pustular dermatitis virus (ORFV), a contact epitheliophilic DNA double-stranded virus belonging to the Parapoxvirus genus in Poxviridae family [2].

†Yueyuan Fan, Jiao Wu and Wei Huang these authors have contributed equally to this work and share the first authorship.

*Correspondence:
Yuzhen Silang
40089378@qq.com
Chong Zhang
leofighting@163.com
Guowen Fu
fuguowen1@126.com
[1] College of Veterinary Medicine, Yunnan Agricultural University, Kunming 650201, China
[2] College of Agronomy and Life Sciences, Kunming University, Kunming 650214, China
[3] Institute of Animal Science, Tibet Academy of Agricultural and Animal Husbandry Sciences, Lhasa 850000, China
[4] Kunming Customs Technology Center, Kunming 650228, China

Notably resilient, ORFV can survive for up to 17 years in dry condition and remains active on animal fur and contaminated materials for approximately one month [3]. Given its high infectivity and potential for harm, vaccination stands as the primary method for controlling ORF. However, the immune evasion mechanisms of ORFV can suppress the immune response [4], and the traditional vaccines used in clinical settings often have limitations, such as insufficient efficacy and brief antibody maintenance, rendering them ineffective for preventing ORFV. Therefore, there is an urgent need to develop innovative veterinary drugs to mitigate and control the spread of infectious ORF.

Taditional Chinese medicine (TCM) has emerged as a promising avenue for veterinary drug research and development. Recently, an increasing number of sheep infectious impetigo cases have been treated using TCM. A mixture of plantain seed, angelica dahuricae, berberis, scutellaria baicalensis, coptis chinensis, mint and poria cocos has demonstrated effective curative properties against viral infections and prevention of secondary infection [5]. Furthermore, in vitro antiviral test conducted with a compound solution of three Tibetan medicinal materials-*Polygonum leucoides*, *Polygonum xanthoxylum* and *Acanthophora rotunda*-have shown excellent inhibitory effects on infectious goat impetigo virus [6]. Medical research shows that TCM has anti-inflammatory, bacteriostatic, antiviral and regulating immune function. It has been found that the immune function of ginkgo biloba leaves was evaluated by phagocytosis of macrophages and secretion of a series of cytokines, which showed that ginkgo biloba leaves had excellent immune -boosting effect [7]. Research indicates that TLR2, a receptors for polysaccharides plays a key factor in the immunomodulatory effect of TCM polysaccharides, and its combination with TCM polysaccharides can regulate the synthesis of cytokines IL-6 and TNF-α through TLR2/NF-κB signaling pathway [8]. Lymphocyte mediated immune response is closely related to cytokines secreted by lymphocyte [9], such as Th1 cells mainly secrete cytokines like IL-2, IFN-γ and TNF-α to regulate cellular immune response [10], and Th2 cells mainly secrete cytokines such as IL-4, IL-6 and IL-10 to regulate humoral immune response [11].

Lymphocyte immune reactions are the main type response for early ORFV nfection, judging by the expression of cytokines in vivo immune efficacy. And the types and quantities of endogenous metabolites reflect the metabolic processes within lymphocyte, which demonstrates the changes for immune reaction. Metabolites by endogenous biology and external environmental factors work together could reveal the dynamic and small molecular changes response to the overall situation of the body, and the intuitive pathological process of physiological and biochemical reaction. Now, metabolomics has been widely used to clarify the therapeutic mechanism of drugs. As ORFV has a variety of proteins involved in the immune evasion mechanism, and the attenuated vaccines used abroad have shortcomings such as virulence regapitation and virus diffusion, there are no effective treatment drugs or effective vaccines for ORF in China, resulting in serious impacts of ORF on human and animal health. Consequently, the traditional prescription of *Polygonum leucoides*, *Polygonum xanthoxylum* and *Acanthophora rotunda* which have unique clinical effects were selected, and their effective ingredients were extracted to study the effects of compound drugs on ORFV-infected lymphocyte immune factors and endogenous metabolites by ELISA and untargeted metabolomics. The research establishes a foundation for the further investigation into the antiviral potential of *Polygonum leucoides*, *Polygonum xanthoxylum* and *Acanthophora rotunda* in antiviral research.

Materials and methods

Preparation of compound extracts of tibetan medicine

Firstly, 100 g of the prepared Tibetan medicine powder, *Polygonum leucoides*, *Polygonum xanthoxylum* and *Acanthophora rotunda* (the main components of Chinese herbal medicine were flavonoids compounds, such as quercetin, rutin, genistein and catechin) were accurately weighed with an electronic balance at the ratio of 1:1:1 in a conical flask, and hybrid by 75% ethanol at the ratio of 1:3. The powder was extracted for 30 min in an ultrasonic extraction instrument at 60℃, and repeated for 3 times. After filtration by gauze and suction and filtration by negative pressure suction and filtration pump, the obtained liquid and petroleum ether were fully mixed at a ratio of 1:1, and the petroleum ether extract was extracted after standing. Under the condition of 60℃ with 70r/min, the petroleum ether extract was concentrated by rotary evaporator, petroleum ether was removed, and the residual petroleum ether was dried in the air blowing oven at 60℃. The concentrated solution was frozen at -80℃ for 24 h and then dried to powder in a negative pressure freeze dryer, which was collected and stored in a refrigerator at 4℃.

Lymphocytotoxicity test of compound extracts of tibetan medicine in vitro

Sheep peripheral blood lymphocytes were isolated by Ficoll-Hypaque, cleaned, purified and cultured. The collected lymphocytes were stained with Trypan blue and counted, and a certain amount of lymphocytes were stained with Swiss-Giemsa staining. Cell morphology was observed under electron microscope, and the lymphocyte

purity was observed and calculated, and the cell activity and purity of the purified lymphocytes were 97.8% and 98.8%, respectively. Then the maximum safe concentration of DMSO and Tibetan medicine compound extract was determined, and the virus TCID was calculated by Reed-Muench method$_{50}$. Found that 6.25% DMSO, 7.6 μg/mL Tibetan medicine compound extract was the maximum safe concentration, ORFV virus $TCID_{50}$ on lymphocytes is $10^{-5.125}$/100μL. On the basis of the maximum safe concentration of compound extract of different concentrations of Tibetan medicine filtered by 0.22 μm membrane, three concentrations (1.9 μg/ml, 3.8 μg/ml and 7.6 μg/ml) were diluted by two-fold dilution method, and the isolated and purified lymphocytes were cultured in RPMI 1640 medium containing 10% FBS and 1% penicillomycin to 2×10^7. After centrifugation, ORFV virus was added into the lymphocytes culture medium with different concentrations (4 replicates in each group) of Tibetan medicine compound extract: ORFV + 1.9 μg/ml, ORFV + 3.8 μg/ml, ORFV + 7.6 μg/ml. Meanwhile, ORFV group (ORFV virus was added into the lymphocytes) and the control group (only lymphocytes) were set up with 4 replicates to compare with the experimental group. Then the cells were incubated for 2 h, and were incubated at 37℃ at 5% CO_2 in the cell incubator.

Determination of lymphocytes immune factor

After 0, 4, 8, 12 h, cell precipitation and culture supernatant were collected. And CD4, CD8, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, TNF-β, IFN-γ were determined and their contents were calculated according to the instruction of ELISA detection reagent (Shanghai Enzyme linked Technology Co., LTD, Shanghai, China). In short, added 10 μL serum sample and 40 μL diluent for each well, sealed the plate with a sealing plate film and incubated them at 37℃ for 30 min. After that, washed, then added 50 μL enzyme-labeled reagent to each well, then incubated and washed again. Mixed gently after added color developer and developed color at 37℃ for 15 min. Then add termination solution 50 μL to terminate the reaction. Finally, the absorbance (OD value) of each well was measured at 450 nm wavelength in microcoder (Thermo Fisher Technologies, Waltham, USA) [12].

Metabolomic analysis of lymphocytes

The lymphocytes were collected and cleaned with PBS preheated at 37℃ for 3 times, followed by centrifugation at 1000r/min for 10 min. The supernatant was discarded, and the cells were dried and precipitate, then quickly frozen at -80℃.

Sample was thawed on ice, then added 1 mL precooled extractant (80% methanol aqueous solution), and whirled for 2 min. Freezed the mixture for 3 min in liquid nitrogen after remove ice for 5 min, it will be whirled for 2 min, and circulated this at 3 times. Centrifuged the mixture again with 12,000 r/min at 4 ℃ for 10 min. Finally added 200 μL of supernatant into the inner liner of the corresponding injection bottle for on-board analysis.

The sample extracts were analyzed using an liquid chromatography-electrospray tandem mass spectrometry (LC–ESI–MS/MS) system (UPLC, ExionLC AD, https://sciex.com.cn/; MS, QTRAP® System, https://sciex.com/). The analytical conditions were as follows, UPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 mm × 100 mm); column temperature, 40 ℃; flow rate, 0.4 mL/min; injection volume, 2μL; solvent system, water (0.1% formic acid): acetonitrile (0.1% formic acid); gradient program, 95:5 V/V at 0 min, 10:90 V/V at 10.0 min, 10:90 V/V at 11.0 min, 95:5 V/V at 11.1 min, 95:5 V/V at 14.0 min [13].

LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP), QTRAP® LC–MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst 1.6.3 software (Sciex). The ESI source operation parameters were as follows: source temperature 500 ℃; ion spray voltage (IS) 5500 V (positive), -4500 V (negative); ion source gas I (GSI), gas II (GSII), curtain gas (CUR) were set at 55, 60, and 25.0 psi, respectively; the collision gas (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

Unsupervised PCA (principal component analysis) was performed by statistics function prcomp within R (www.r-project.org). The data was unit variance scaled before unsupervised PCA.

The HCA (hierarchical cluster analysis) results of samples and metabolites were presented as heatmaps with dendrograms was carried out by R package ComplexHeatmap. For HCA, normalized signal intensities of metabolites (unit variance scaling) are visualized as a color spectrum.

Significantly regulated metabolites between groups were determined by VIP > = 1 and absolute Log_2FC (fold change) > = 1. VIP values were extracted from OPLS-DA result, which also contain score plots and permutation plots, was generated using R package MetaboAnalystR. The data was log transform (log_2) and mean centering before OPLS-DA. In order to avoid overfitting, a permutation test (200 permutations) was performed. Identified metabolites were annotated using KEGG Compound

database, annotated metabolites were then mapped to KEGG Pathway database [14].

Results

Differential analysis of lymphocyte immune factor

Lymphocyte immune factor assay results were showed in Fig. 1. The contents of CD4, IFN-γ and IL-10 in 3.8 μg/mL + ORFV and 1.9 μg/mL + ORFV group were both up-regulated from 4 to 12 h. And the contents of IL-10 decreased in 4-8 h but increased in 8-12 h, well the contents of CD4 increased in 4-8 h but decreased in 8-12 h, and the contents of IFN-γ increased in 4-12 h, which was contrary to the results of ORFV group. The contents of TNF-β decreased in 4-8 h but increased in 8-12 h. And the contents of IL-2 decreased during 4-12 h, and the contents of other factors increased during 4-12 h. The contents of CD8, IL-4, IL-6, IL-12 and TNF-β in 3.8 μg/mL + ORFV and 1.9 μg/mL + ORFV group were up-regulated in different degrees within 4-12 h, and the contents of IL-2 in 1.9 μg/mL + ORFV group were significantly down-regulated within 4-8 h ($P < 0.01$).

Differential metabolites analysis of lymphocytes

Principal component analysis (PCA) was conducted on the samples (including the quality control samples) as shown in Fig. 2A. The results showed that the quality control sample clustered well, indicating that the test data was accurate and reliable. The separation of the control group, the ORFV virus group, 7.6 μg/mL + ORFV group, 3.8 μg/mL + ORFV group, and 1.9 μg/mL + ORFV group at 0 h was significantly separated with these at 4 h, 8 h and 12 h, indicating that the cell metabolism was different in the initial state of cells treated and after culture. Well, 3.8 μg/mL + ORFV group was significantly separated with others, showed that the expression of metabolites in lymphocytes was strongly disturbed after the treatment of 3.8 μg/mL Tibetan medicine compound extract with ORFV at 12 h.

The data were normalized and all samples were clustered in the heat map (Fig. 2B). The results showed that, compared with the expression of metabolites in lymphocytes of ORFV group at 0 h, 4 h, 8 h and 12 h, the contents of metabolites in lymphocytes of 7.6 μg/mL + ORFV group, 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group all changed significantly after ORFV infection. Particularly, at 12 h, the contents of amino acids and their metabolites, organic acids and their derivatives, nucleotides and their metabolites, carboxylic acids and derivatives, heterocyclic compounds, oxidized lipids, coenzymes, vitamins, bile acids and other cluster metabolites in 3.8 μg/mL + ORFV group were increased.

At 0 h, there were 65 different metabolites with 29 up metabolites and 36 down metabolites in 0-M1-O (1.9 μg/mL + ORFV group) and 0-CTRL (the control group) (Fig. 3A), 35 different metabolites with 18 up metabolites and 17 down metabolites in 0-M2-O (3.8 μg/mL + ORFV group) and 0-CTRL (Fig. 3B), 21 different metabolites with 14 up metabolites and 7 down metabolites in 0-M3-O (7.6 μg/mL + ORFV group) and 0-CTRL (Fig. 3C), 16 different metabolites with 13 up metabolites and 3 down metabolites in 0-O (ORFV group) and 0-CTRL (Fig. 3D). And there were 10 common metabolites in the three drug groups and control group, and 7 common metabolites in all the groups (Fig. 3E). Among them, the contents of N-Acetyl-L-Leucine, D-Glucose 6-Phosphate, Nα-Acetyl-L-Arginine, 2'-Deoxyadenosine-5'-Monophosphate, Dl-2-Aminooctanoic acid and Met-Glu in the three drug groups and ORFV group were higher than that in the control group except Heparin (except in 0-O vs 0-CTRL), and the contents of ADP-ribose, 4-Pyridoxic acid, Cortisol in the three drug groups were higher than that in the control group.

At 4 h, there were 47 different metabolites with 38 up metabolites and 9 down metabolites in 4-M1-O (1.9 μg/mL + ORFV group) and 4-CTRL (the control group) (Fig. 4A), 28 different metabolites with 23 up metabolites and 5 down metabolites in 4-M2-O (3.8 μg/mL + ORFV group) and 4-CTRL (Fig. 4B), 97 different metabolites with 94 up metabolites and 3 down metabolites in 4-M3-O (7.6 μg/mL + ORFV group) and 4-CTRL (Fig. 4C), 48 different metabolites with 38 up metabolites and 10 down metabolites in 4-O (ORFV group) and 4-CTRL (Fig. 4D). There were 19 common metabolites in the three drug groups and control group, and 16 common metabolites in all the groups (Fig. 4E). Among them, the contents of L-Isoleucine, Dulcitol, 5-Methylcytosine, 2-Aminoethanesulfinic acid, L-Cystathionine, Uridine 5-Monophosphate, N-Acetyl-L-Histidine, γ-L-Glutamate-Cysteine, N-Methyl-α-aminoisobutyric acid, Biotinamide, (2S, 3S)-3-methylphenylalanine, Carnitine C5:1, Cis-4-Hydroxy-D-Proline, Methylguanidine, 8-Azaguanine in three drug groups and the ORFV group were higher than that in the control group except Clupanodonyl carnitine, and the contents of 3-Hydroxypropanoic Acid, Guanidinoethyl sulfonate, N-Acetylaspartylglutamic acid in the three drug groups were higher than that in the control group.

At 8 h, there were 17 different metabolites with 8 up metabolites and 9 down metabolites in 8-M1-O (1.9 μg/mL + ORFV group) and 8-CTRL (the control group) (Fig. 5A), 26 different metabolites with 12 up metabolites and 14 down metabolites in 8-M2-O (3.8 μg/mL + ORFV group) and 8-CTRL (Fig. 5B), 31 different metabolites with 6 up metabolites and 25 down metabolites in 8-M3-O (7.6 μg/mL + ORFV group) and 8-CTRL (Fig. 5C), 18 different metabolites with 7 up metabolites

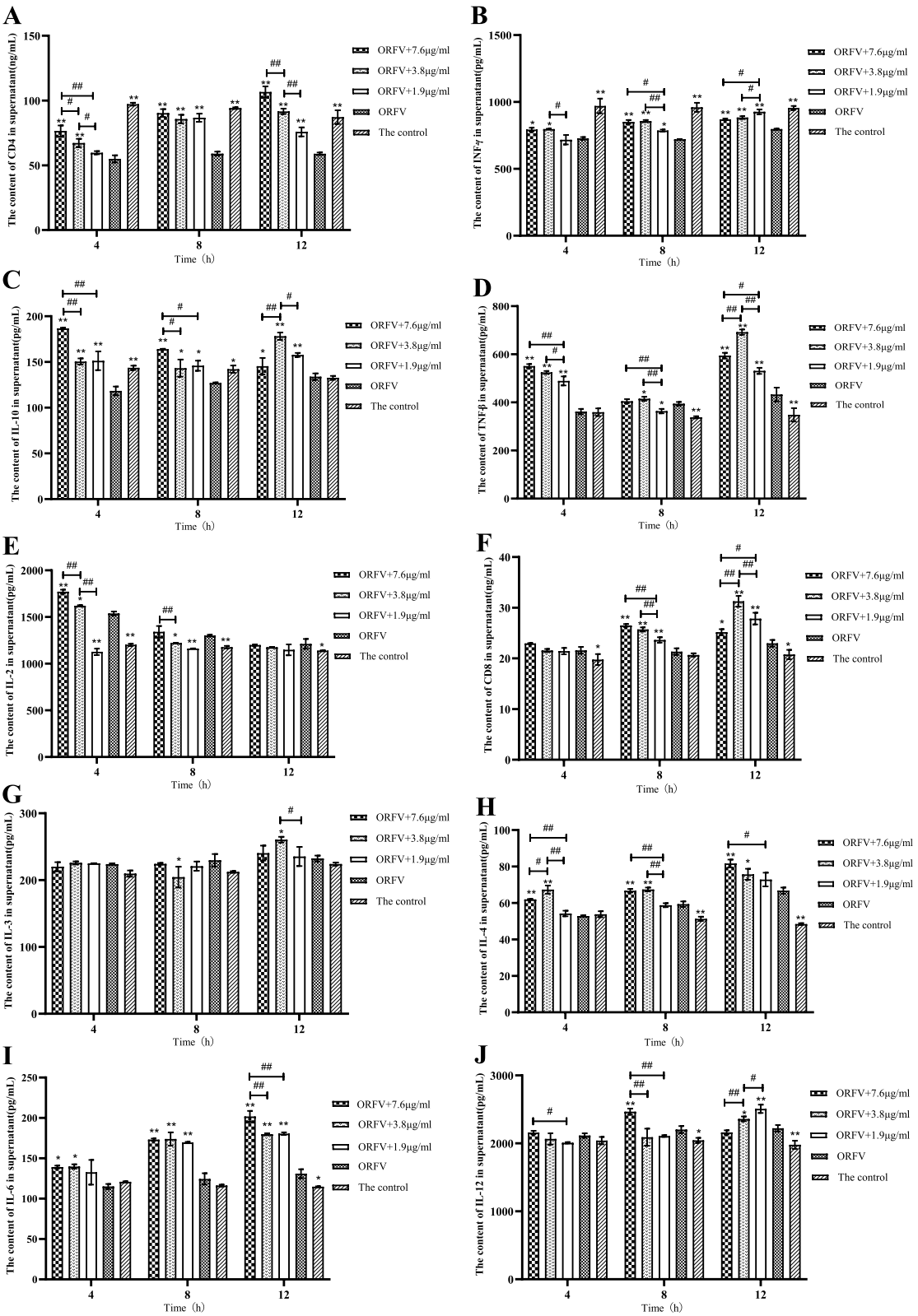


Fig. 1 Contents of lymphocyte immune factor CD4 (**A**), IFN-γ (**B**), IL-10 (**C**), TNF-β(D), IL-2 (E), CD8 (F), IL-3 (**G**), IL-4 (H), IL-6 (**I**), IL-12 (**J**) in the control group (only lymphocytes), the ORFV virus group (ORFV virus was added into the lymphocytes), Tibetan medicine compound extract group (Tibetan medicine compound and ORFV virus was added into the lymphocytes): ORFV + 1.9 μg/mL, ORFV + 3.8 μg/mL, ORFV + 7.6 μg/mL

and 11 down metabolites in 8-O (ORFV group) and 8-CTRL (Fig. 5D). There were 4 common metabolites in the three drug groups and control group, and 1 common metabolite in all the groups (Fig. 5E). Among them, the contents of Asp-phe and Guanine in the three drug groups were higher than that in the control group, the contents of 2-Hydroxy-6-Aminopurine in the three drug groups were lower than that in the control group, and the contents of Valyl-leucine in the 1.9 μg/mL + ORFV group and 7.6 μg/mL + ORFV group were lower than that in the control group. Moreover, the content of Valyl-leucine in ORFV group was higher than that in the control group.

At 12 h, there were 34 different metabolites with 7 up metabolites and 27 down metabolites in 12-M1-O (1.9 μg/mL + ORFV group) and 12-CTRL (the control group) (Fig. 6A), 50 different metabolites with 39 up metabolites and 11 down metabolites in 12-M2-O (3.8 μg/mL + ORFV group) and 12-CTRL (Fig. 6B), 17 different metabolites with 10 up metabolites and 7 down metabolites in 12-M3-O (7.6 μg/mL + ORFV group) and 12-CTRL (Fig. 6C), 45 different metabolites with 11 up metabolites and 34 down metabolites in 12-O (ORFV group) and 12-CTRL (Fig. 6D). And there were 2 common metabolites in the three drug groups and control group, and 1 common metabolite in all the groups (Fig. 6E). Among them, the contents of Dl-Threitol and Oxaloacetic acid in the three drug groups were higher than that in the control group, and the content of Oxaloacetic acid in the ORFV group was also higher than that in the control group.

Differential metabolites signaling pathway analysis of ORFV-infected lymphocytes and that treated with tibetan medicine compound extracts

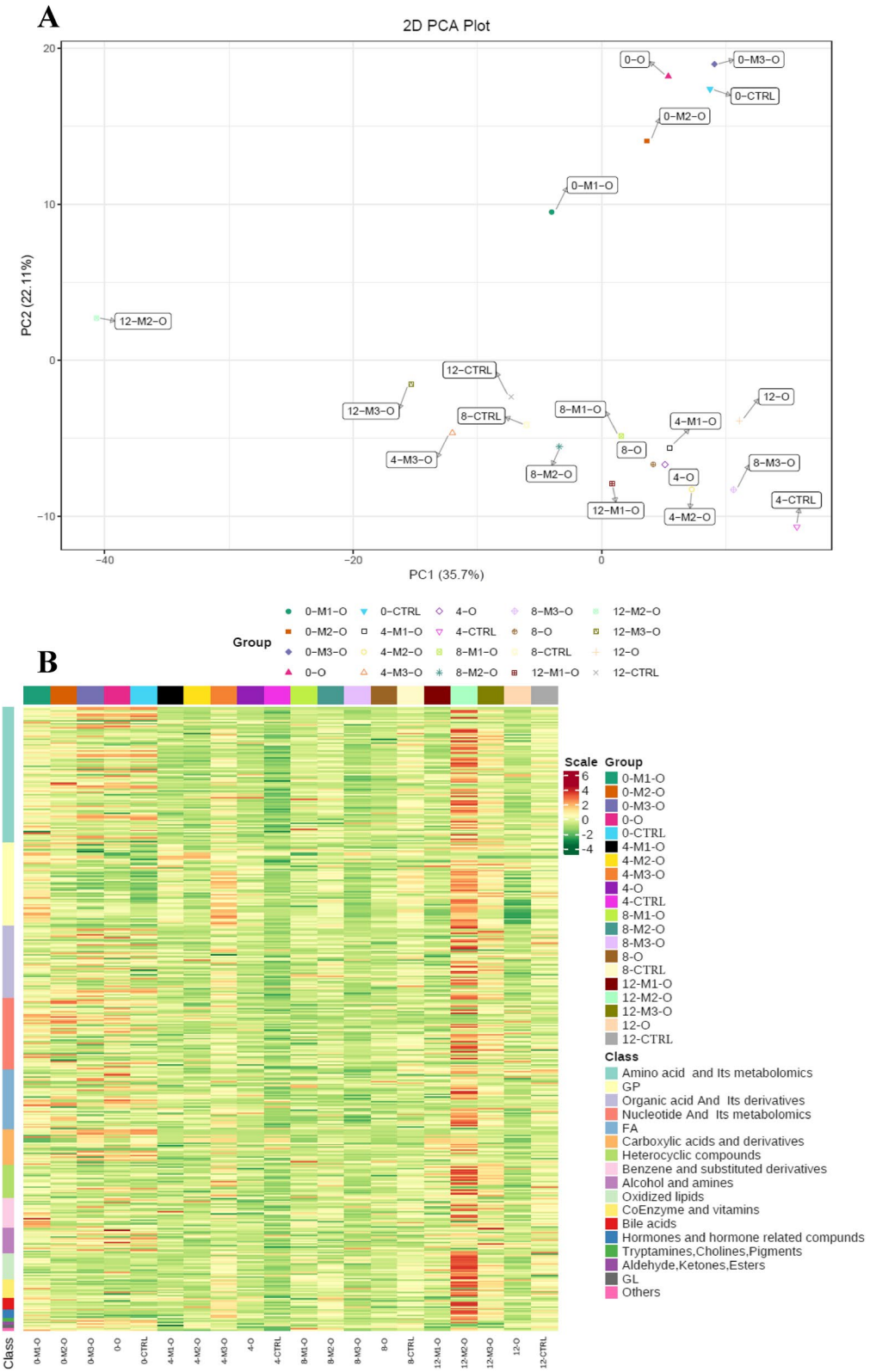
KEGG analysis of differential metabolites in all group at 0 h showed that (Fig. 7A), the metabolic pathways in 0-M1-O (1.9 μg/mL + ORFV group) and 0-CTRL (the control group) were galactose metabolism, starch and sucrose metabolism, carbohydrate digestion and absorption, ABC transporters, carbon metabolism, cysteine and methionine metabolism, glucagon signaling pathway, etc. 0-M2-O (3.8 μg/mL + ORFV group) and 0-CTRL showed starch and sucrose metabolism, carbohydrate digestion and absorption, glutathione metabolism, etc. And 0-M3-O (7.6 μg/mL + ORFV group) and 0-CTRL group had steroid hormone biosynthesis, prostate cancer, aldosterone-regulated sodium reabsorption, etc. And the metabolic pathways involved in 0-O (ORFV group) and 0-CTRL group were starch and sucrose metabolism pathways, insulin resistance, inositol phosphate metabolism, retinol metabolism, pertussis, insulin secretion, prolactin signaling pathway, AMPK signaling pathway, etc.

KEGG analysis of differential metabolites in all group at 4 h showed that (Fig. 7B), the metabolic pathways in 4-M1-O (1.9 μg/mL + ORFV group) and 4-CTRL (the control group) were cysteine and methionine metabolism, taurine and hypotaurine metabolism, mineral absorption, carbon metabolism, glycolysis/gluconeogenesis, biosynthesis of amino acids, etc. The metabolic pathways in 4-M2-O (3.8 μg/mL + ORFV group) and 4-CTRL were taurine and hypotaurine metabolism, ferroptosis, cysteine and methionine metabolism, etc. And the metabolic pathways in 4-M3-O (7.6 μg/mL + ORFV group) and 4-CTRL were glucagon signaling pathway, glyoxylate and dicarboxylate metabolism, central carbon metabolism in cancer, propanoate metabolism, biosynthesis of amino acids, carbon metabolism, etc. The metabolic pathways mainly involved in 4-O (ORFV group) and 4-CTRL include cysteine and methionine metabolic pathways, leucine, serine and threonine metabolic pathways, mineral absorption, pyrimidine metabolism, VB6 metabolic pathways, sucrose and starch metabolic pathways, etc.

KEGG analysis of differential metabolites in all group at 8 h showed that (Fig. 7C), the metabolic pathways in 8-M1-O (1.9 μg/mL + ORFV group) and 8-CTRL (the control group) were prostate cancer, steroid hormone biosynthesis, aldosterone-regulated sodium reabsorption, neuroactive ligand-receptor interaction, cortisol synthesis and secretion, adrenergic signaling in cardiomyocytes, etc. The metabolic pathways in 8-M2-O (3.8 μg/mL + ORFV group) and 8-CTRL were cysteine and methionine metabolism, ferroptosis, aldosterone-regulated sodium reabsorption, prostate cancer, steroid hormone biosynthesis, beta-Alanine metabolism, etc. And the metabolic pathways in 8-M3-O (7.6 μg/

(See figure on next page.)

Fig. 2 Sample quality control analysis. **A** PCA score chart of mass spectrum data of each group of samples and quality control samples. **B** Sample clustering analysis diagram. 0-CTRL, the control group at 0 h; 0-M1-O, the 1.9 μg/mL + ORFV group at 0 h; 0-M2-O, the 3.8 μg/mL + ORFV group at 0 h; 0-M3-O, the 7.6 μg/mL + ORFV group at 0 h; 0-O, the ORFV group at 0 h; 4-CTRL, the control group at 4 h; 4-M1-O, the 1.9 μg/mL + ORFV group at 4 h; 4-M2-O, the 3.8 μg/mL + ORFV group at 4 h; 4-M3-O, the 7.6 μg/mL + ORFV group at 4 h; 4-O, the ORFV group at 4 h; 8-CTRL, the control group at 8 h; 8-M1-O, the 1.9 μg/mL + ORFV group at 8 h; 8-M2-O, the 3.8 μg/mL + ORFV group at 8 h; 8-M3-O, the 7.6 μg/mL + ORFV group at 8 h; 8-O, the ORFV group at 8 h; 12-CTRL, the control group at 12 h; 12-M1-O, the 1.9 μg/mL + ORFV group at 12 h; 12-M2-O, the 3.8 μg/mL + ORFV group at 12 h; 12-M3-O, the 7.6 μg/mL + ORFV group at 12 h; 12-O, the ORFV group at 12 h



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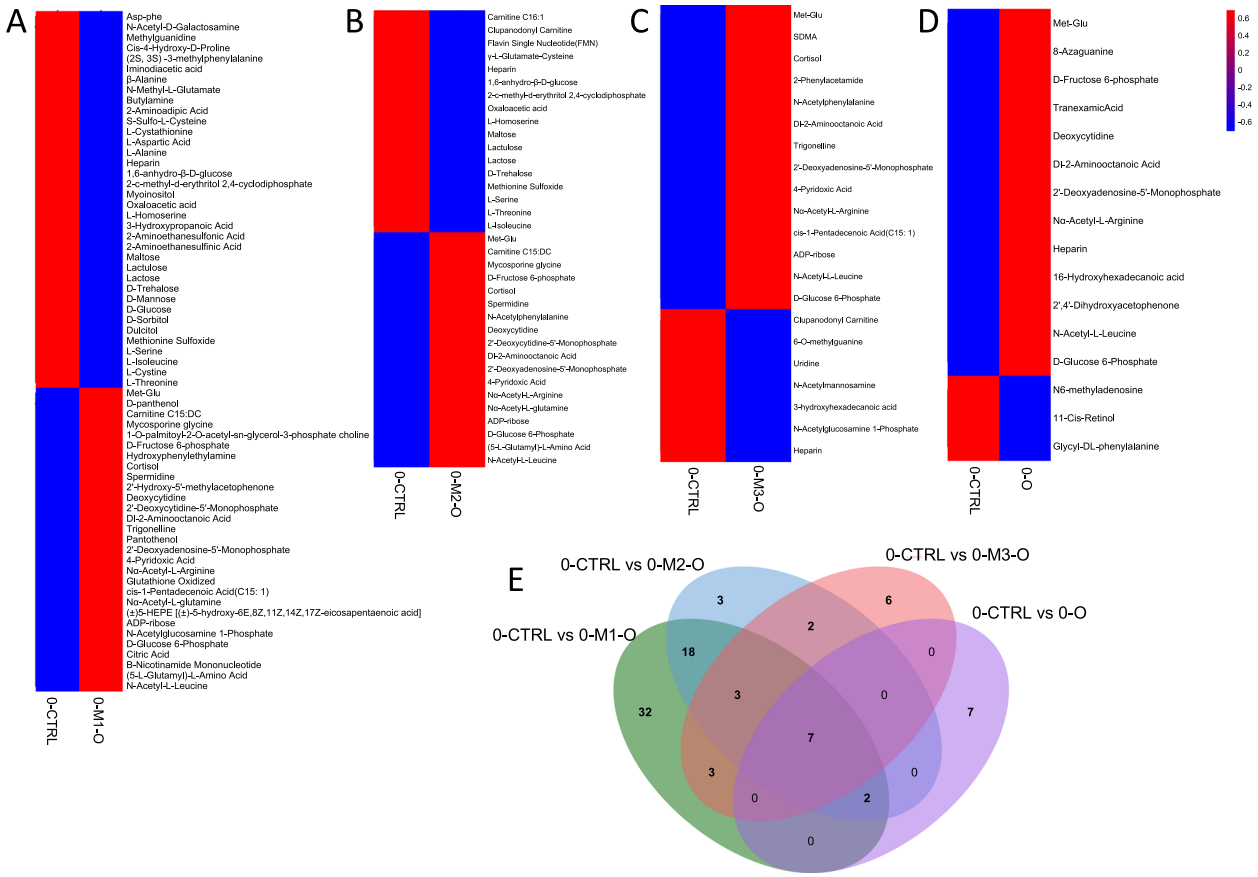

Fig. 3 Differential metabolites analysis at 0 h. **A** Differential metabolites in 0-M1-O (1.9 μg/mL + ORFV group) and 0-CTRL (the control group) at 0 h. **B** Differential metabolites in 0-M2-O (3.8 μg/mL + ORFV group) and 0-CTRL (the control group) at 0 h. **C** Differential metabolites in 0-M3-O (7.6 μg/mL + ORFV group) and 0-CTRL (the control group) at 0 h. **D** Differential metabolites in 0-O (ORFV group) and 0-CTRL (the control group) at 0 h. **E** Venn diagram of differential metabolites in all groups at 0 h

mL + ORFV group) and 8-CTRL were taste transduction, citrate cycle (TCA cycle), cysteine and methionine metabolism, pancreatic secretion, regulation of actin cytoskeleton, glucagon signaling pathway, glyoxylate and dicarboxylate metabolism, etc. The metabolic pathways involved in 8-O (ORFV group) and 8-CTRL were lysine degradation, beta-Alanine metabolism, cysteine and methionine metabolism, neuronal receptor interaction, arginine and proline metabolism, etc.

KEGG analysis of differential metabolites in all group at 12 h showed that (Fig. 7D), the metabolic pathways in 12-M1-O (1.9 μg/mL + ORFV group) and 12-CTRL (the control group) were neuroactive ligand-receptor interaction, metabolic pathways, alanine, aspartate and glutamate metabolism, biosynthesis of amino acids, renin secretion, cysteine and methionine metabolism, etc. The metabolic pathways in 12-M2-O (3.8 μg/mL + ORFV group) and 12-CTRL were galactose metabolism, glucagon signaling pathway, fructose and mannose metabolism, glycolysis/gluconeogenesis, citrate cycle (TCA cycle), etc. And the metabolic pathways in 12-M3-O (7.6 μg/mL + ORFV group) and 12-CTRL were C-type lectin receptor signaling pathway, lysosome, galactose metabolism, inositol phosphate metabolism, morphine addiction, phosphatidylinositol signaling system, etc. And the main metabolic pathways involved in 12-O (ORFV group) and 12-CTRL group include neuronal receptor interaction, starch and sucrose metabolism, galactose metabolism, bile secretion, etc.

Changes of differential metabolites in ORFV-infected lymphocytes and that treated with tibetan medicine compound extracts

The results showed that, at 0 h, compared with the control group, ORFV group down-regulated 3 metabolites and up-regulated 13 metabolites (Fig. 3D). While, these metabolites contents had not changed in 7.6 μg/mL + ORFV group, 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group (Fig. 3A, 3B, 3C). At 4 h, compared with the control group, the ORFV group down-regulated

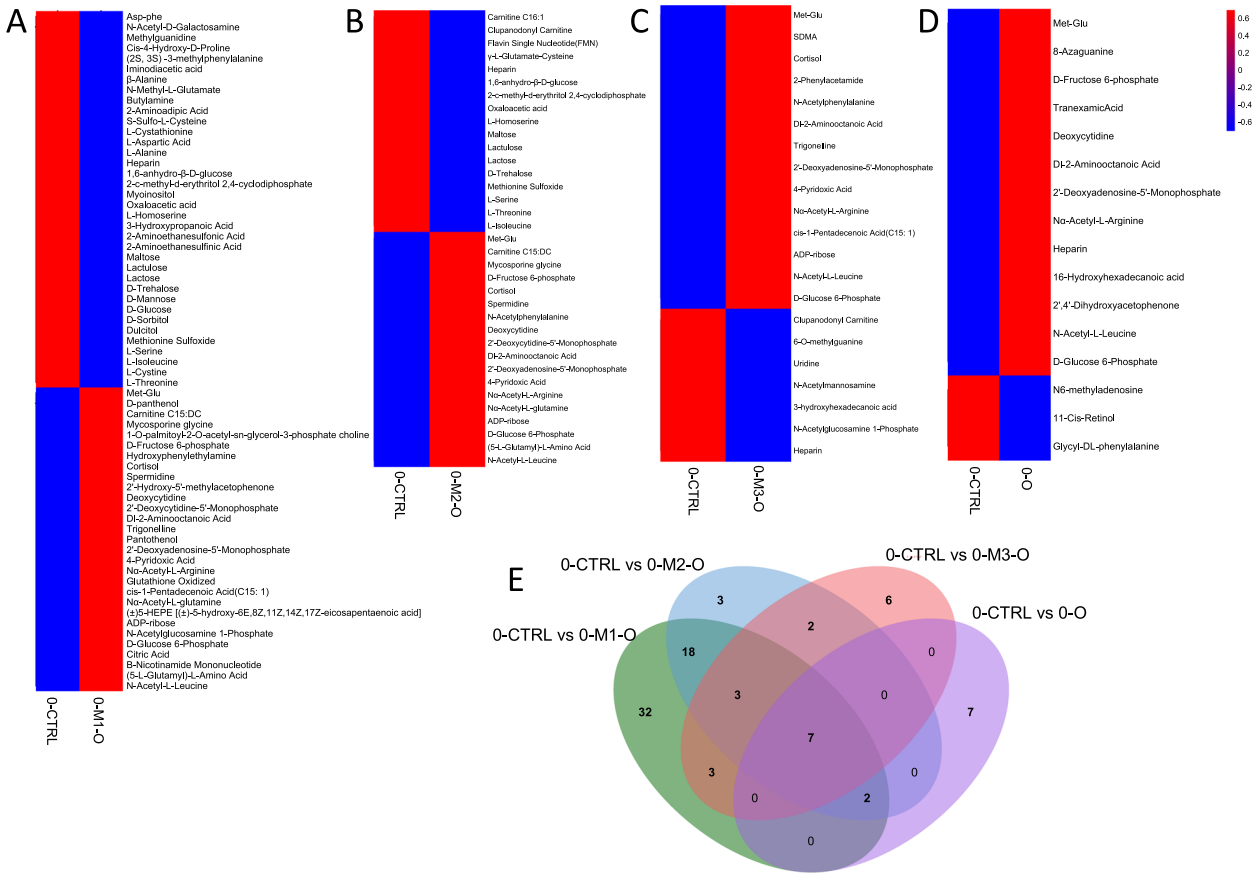

Fig. 4 Differential metabolites analysis at 4 h. **A** Differential metabolites in 4-M1-O (1.9 μg/mL + ORFV group) and 4-CTRL (the control group) at 4 h. **B** Differential metabolites in 4-M2-O (3.8 μg/mL + ORFV group) and 4-CTRL (the control group) at 4 h. **C** Differential metabolites in 4-M3-O (7.6 μg/mL + ORFV group) and 4-CTRL (the control group) at 4 h. **D** Differential metabolites in 4-O (ORFV group) and 4-CTRL (the control group) at 4 h. **E** Venn diagram of differential metabolites in all groups at 4 h

10 metabolites and up-regulated 38 metabolites (Fig. 4D), while the 7.6 μg/mL + ORFV group, 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group reversed 6, 12 and 15 different metabolites (Fig. 8A), respectively. Signaling pathways enriched by these metabolites showed that, 7.6 μg/mL + ORFV group had no key pathway, but the key pathways in 3.8 μg/mL + ORFV group were cysteine and methionine metabolic pathways, VB6 metabolic pathways and pyrimidine metabolic pathways, and the key pathways of the 1.9 μg/mL + ORFV group were cysteine and methionine metabolic pathway, VB6 metabolic pathway, starch and sucrose metabolic pathway and pyrimidine metabolic pathway. At 8 h, 11 metabolites were down-regulated and 7 were up-regulated in the ORFV group (Fig. 5D), and 6, 4 and 7 metabolites in the 7.6 μg/mL + ORFV, 3.8 μg/mL + ORFV and 1.9 μg/mL + ORFV groups were significantly down-regulated, respectively (Fig. 8B). And, signaling pathways enriched by these metabolites showed that, lysine hydrolysis and pyrimidine metabolism were the key pathways in the 7.6 μg/mL + ORFV group. Lysine hydrolysis was the key pathway in the 3.8 μg/mL + ORFV group. And the key pathways in the 1.9 μg/mL + ORFV group were β-alanine metabolic pathway, cysteine and methionine metabolic pathway, lysine hydrolysis and pyrimidine metabolic pathway. At 12 h, 34 metabolites were down-regulated and 11 were up-regulated in the ORFV group (Fig. 6D), while 17, 29 and 26 metabolites in the 7.6 μg/mL + ORFV group, 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group were significantly down-regulated, respectively(Fig. 8C). After pathway enrichment analysis, the key pathways in the 7.6 μg/mL + ORFV group were galactose metabolism, starch and sucrose metabolic pathways, taurine and subtaurine metabolic pathways, cysteine and methionine metabolic pathways, VB6 metabolic pathway, pyrimidine metabolic pathway. The key pathways in the 3.8 μg/mL + ORFV group were purine metabolic pathway, tricarboxylic acid cycle, galactose metabolism, starch and sucrose metabolic pathways, taurine and subtaurine metabolic pathways, cysteine and

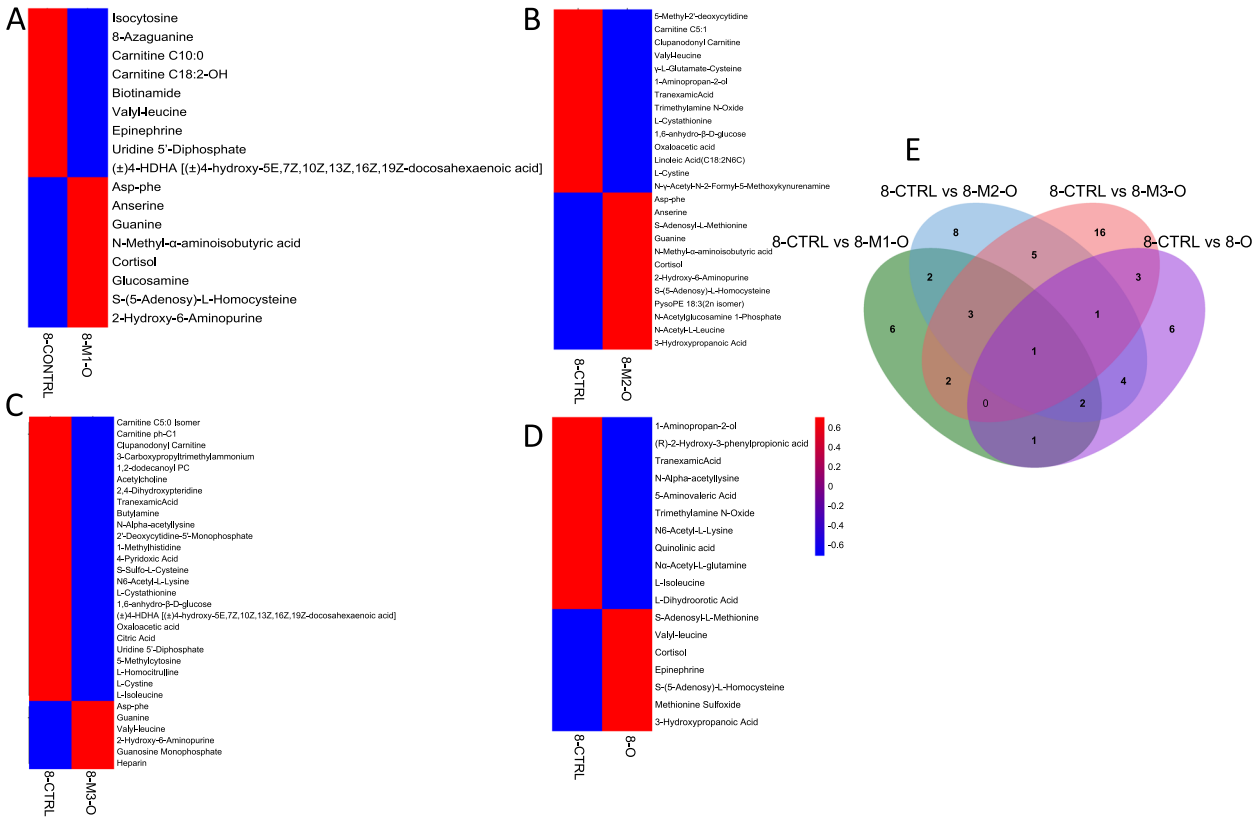

Fig. 5 Differential metabolites analysis at 8 h. **A** Differential metabolites in 8-M1-O (1.9 μg/mL + ORFV group) and 8-CTRL (the control group) at 8 h. **B** Differential metabolites in 8-M2-O (3.8 μg/mL + ORFV group) and 8-CTRL (the control group) at 8 h. **C** Differential metabolites in 8-M3-O (7.6 μg/mL + ORFV group) and 8-CTRL (the control group) at 8 h. **D** Differential metabolites in 8-O (ORFV group) and 8-CTRL (the control group) at 8 h. **E** Venn diagram of differential metabolites in all groups at 8 h

methionine metabolic pathways, VB6 metabolic pathway, pyrimidine metabolic pathway, tyrosine metabolic pathway, and bile secretion. And the key pathways in the 1.9 μg/mL + ORFV group were tricarboxylic acid cycle, galactose metabolism, starch and sucrose metabolic pathways, taurine and subtaurine metabolic pathways, cysteine and methionine metabolic pathways, VB6 metabolic pathway, pyrimidine metabolic pathway, tyrosine metabolic pathway, bile secretion and riboflavin metabolic pathway.

The expression of differentially significant metabolites in the ORFV virus group changed at 0 h, 4 h, 8 h and 12 h was compared with that after treatment, and the differentially significant metabolites in the callback were analyzed by pathway enrichment. The results showed that the 7.6 μg/mL + ORFV group, 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group recalled 6, 12 and 15 differentially significant metabolite expression changes caused by ORFV virus at 4 h, respectively (Table 1). The 1.9 μg/mL + ORFV group involved the most callback pathways. Cysteine and methionine metabolic pathways, VB6 metabolic pathways, starch and sucrose metabolic pathways and pyrimidine metabolic pathways, respectively. At 8 h, the expression of 6, 4 and 7 metabolites with significant difference caused by ORFV were recalled, respectively (Table 1). The 1.9 μg/mL + ORFV group had the most involved callback pathways, including β-alanine metabolism pathway, cysteine and methionine metabolism pathway, lysine hydrolysis and pyrimidine metabolism pathway. At 12 h, 17, 29 and 26 metabolites with significant difference caused by ORFV were called back, respectively (Table 1). There were 10 callback pathways involved in 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group. The pathways involved were purine metabolism pathway, tricarboxylic acid cycle, galactose metabolism pathway, starch and sucrose metabolism pathway, taurine and subtaurine metabolism pathway, cysteine and methionine metabolism pathway, VB6 metabolism pathway, pyrimidine metabolism pathway, tyrosine metabolism pathway, biliary secretion, riboflavin metabolism pathway. The purine metabolic pathway and riboflavin metabolic pathway were 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group respectively.

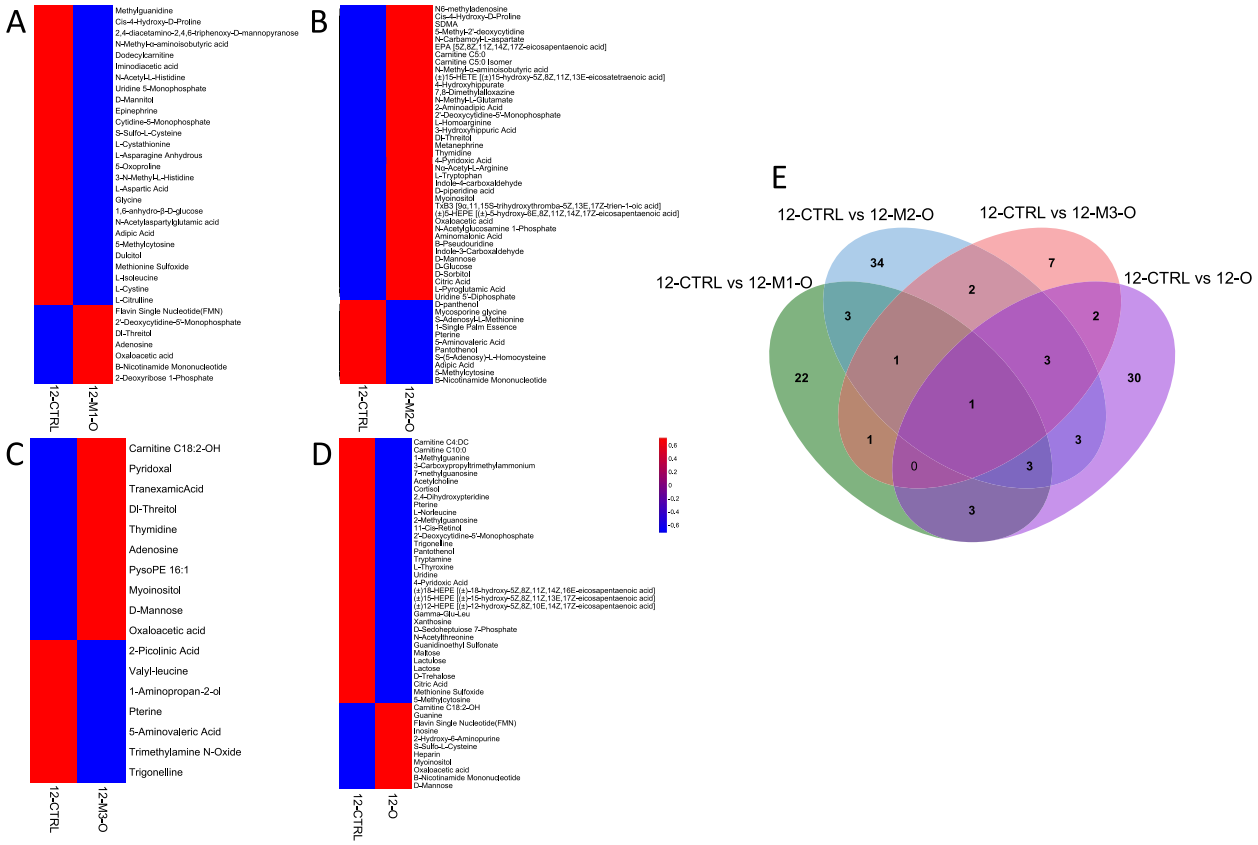

Fig. 6 Differential metabolites analysis at 12 h. **A** Differential metabolites in 12-M1-O (1.9 μg/mL + ORFV group) and 12-CTRL (the control group) at 12 h. **B** Differential metabolites in 12-M2-O (3.8 μg/mL + ORFV group) and 12-CTRL (the control group) at 12 h. **C** Differential metabolites in 12-M3-O (7.6 μg/mL + ORFV group) and 12-CTRL (the control group) at 12 h. **D** Differential metabolites in 12-O (ORFV group) and 12-CTRL (the control group) at 12 h. **E** Venn diagram of differential metabolites in all groups at 12 h

Discussion

Lymphocytes play an integral role in the body's immune response. Effector T cells are the important part of antigen-mediated cellular immunity and humoral immunity, which include helper T cells and cytotoxic T cells. Helper T cells can be divided into two subtypes: Th1 cells mediating cellular immunity and Th2 cells mediating humoral immunity. Th1 cells mainly secrete immune factors such as IL-2, IL-12, IL-18, IFN-γ and TNF-α, while Th2 cells mainly secrete immune factors such as IL-4, IL-6 and IL10 [15]. ORFV can bidirectional regulate the host immune system through a variety of immune regulatory mechanisms. On the one hand, ORFV's own membrane proteins F1L and B2L have strong immunogenicity, F1L can induce cellular immunity, and B2L can stimulate the body to produce humoral immunity and induce neutralizing antibody IgG [12]. On the other hand, ORFV encodes a series of immunoregulatory factors homologous to the host to interfere with the body's immune response to achieve immune evasion, such as IFN resistance factor (OVIFNR) and IL-10 homologue (VIL-10).

IFN-γ test results showed that, within 12 h after ORFV stimulation, the level of IFN-γ in peripheral blood lymphocytes of sheep cultured in vitro was significantly lower than that in the control group ($P < 0.01$), which indicated that ORFV could significantly inhibit the content of IFN-γ in peripheral blood lymphocytes of sheep. The reason is speculated to be that IFN resistance factor (OVIFNR) produced by ORFV plays a role in immune evasion. Studies have showed that ORFV ORF020 could produce viral dsRNA competitively binding protein kinase to form PKA-DSRNA and inactivate PKA, which leads to the down-regulation of host IFN mRNA expression and inhibits IFN secretion by host immune cells to produce antiviral response [16]. In addition, the results of this study showed that the extracts of three Tibetan medicine compounds, such as *Polygonum leucoides*, *Polygonum xanthoxylum* and *Acanthophora rotunda*, could effectively increase the content of IFN-γ in cell culture supernatant within 12 h. And at 12 h, 7.6 μg/mL + ORFV, 3.8 μg/mL + ORFV, 1.9 μg/mL + ORFV could significantly increase IFN-γ content ($P < 0.01$). The

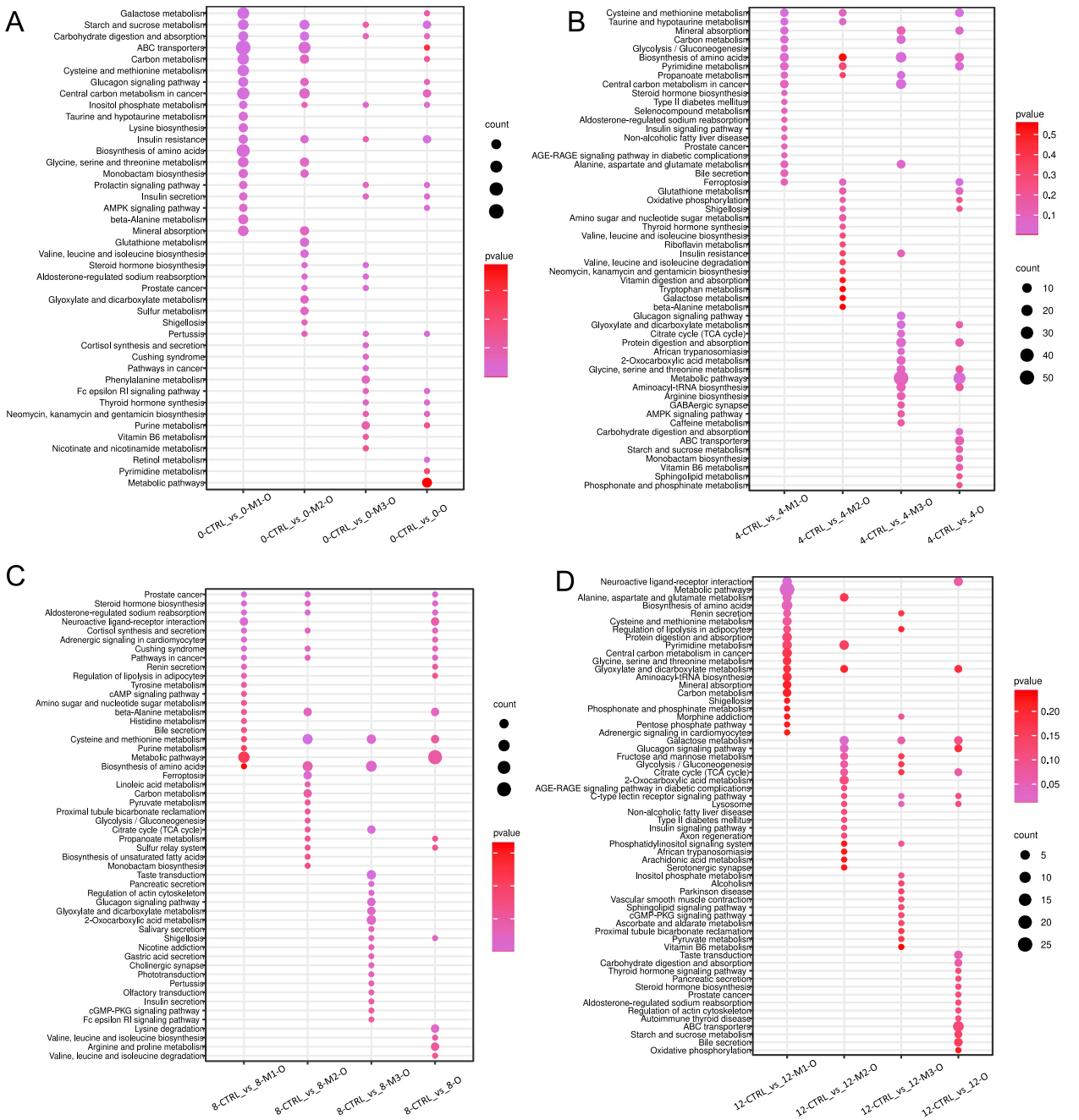

Fig. 7 KEGG analysis of differential metabolites. **A** KEGG analysis of differential metabolites in all group at 0 h. **B** KEGG analysis of differential metabolites in all group at 0 h. **C** KEGG analysis of differential metabolites in all group at 0 h. **D** KEGG analysis of differential metabolites in all group at 0 h. 0-CTRL, the control group at 0 h; 0-M1-O, the 1.9 μg/mL + ORFV group at 0 h; 0-M2-O, the 3.8 μg/mL + ORFV group at 0 h; 0-M3-O, the 7.6 μg/mL + ORFV group at 0 h; 0-O, the ORFV group at 0 h; 4-CTRL, the control group at 4 h; 4-M1-O, the 1.9 μg/mL + ORFV group at 4 h; 4-M2-O, the 3.8 μg/mL + ORFV group at 4 h; 4-M3-O, the 7.6 μg/mL + ORFV group at 4 h; 4-O, the ORFV group at 4 h; 8-CTRL, the control group at 8 h; 8-M1-O, the 1.9 μg/mL + ORFV group at 8 h; 8-M2-O, the 3.8 μg/mL + ORFV group at 8 h; 8-M3-O, the 7.6 μg/mL + ORFV group at 8 h; 8-O, the ORFV group at 8 h; 12-CTRL, the control group at 12 h; 12-M1-O, the 1.9 μg/mL + ORFV group at 12 h; 12-M2-O, the 3.8 μg/mL + ORFV group at 12 h; 12-M3-O, the 7.6 μg/mL + ORFV group at 12 h; 12-O, the ORFV group at 12 h

degree of up-regulation in 1.9 μg/mL + ORFV group was significantly different from 7.6 μg/mL + ORFV group and 3.8 μg/mL + ORFV group ($P < 0.05$). The results showed that the extracts of three Tibetan medicine compounds could regulate the immune suppression of IFN-γ caused by ORFV at a certain extent, and the 1.9 μg/mL additive amount showed the best effect at 12 h.

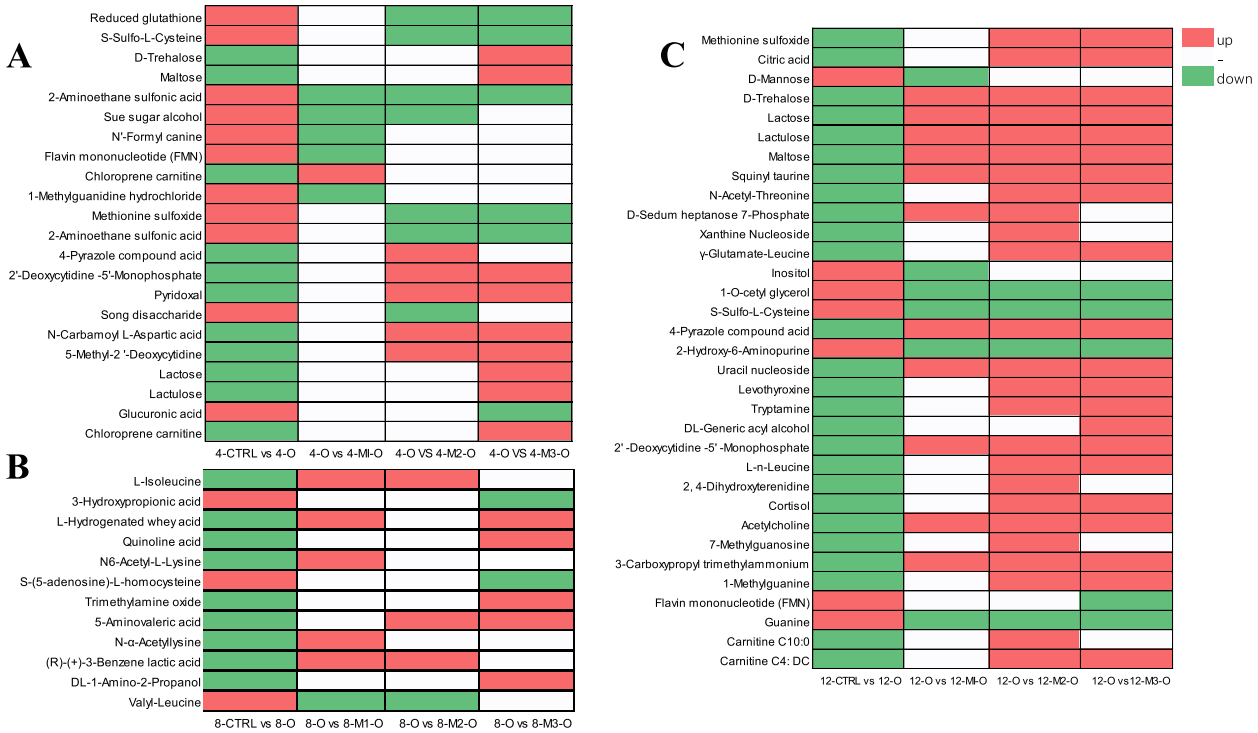

Fig. 8 Changes of Differential metabolites in ORFV-infected lymphocytes and that treated with Tibetan medicine compound extracts at 4 h (A), 8 h (A), 12 h (A). 4-CTRL, the control group at 4 h; 4-M1-O, the 1.9 μg/mL + ORFV group at 4 h; 4-M2-O, the 3.8 μg/mL + ORFV group at 4 h; 4-M3-O, the 7.6 μg/mL + ORFV group at 4 h; 4-O, the ORFV group at 4 h; 8-CTRL, the control group at 8 h; 8-M1-O, the 1.9 μg/mL + ORFV group at 8 h; 8-M2-O, the 3.8 μg/mL + ORFV group at 8 h; 8-M3-O, the 7.6 μg/mL + ORFV group at 8 h; 8-O, the ORFV group at 8 h; 12-CTRL, the control group at 12 h; 12-M1-O, the 1.9 μg/mL + ORFV group at 12 h; 12-M2-O, the 3.8 μg/mL + ORFV group at 12 h; 12-M3-O, the 7.6 μg/mL + ORFV group at 12 h; 12-O, the ORFV group at 12 h

ORFV ORF020 (VIL-10) is a key gene in early ORFV infection, which has a high homology with mammalian IL-10 in structure, and plays an important role in immunosuppression by inhibiting the synthesis and secretion of host cytokines, such as IL-8, IFN, IL-2, and IL-3. In this study, the content of IL-10 in the supernatant of peripheral blood lymphocytes stimulated by ORFV decreased significantly compared with the normal control group at 4 h and 8 h, maybe ORFV mainly induces Th1 cell immune response in the early stage followed by Th2 cell immune response [17], and IL-10 is an anti-inflammatory cytokine secreted by Th2 cells. At the same time, the content of IL-2 in the supernatant decreased with the increase of culture time. Compared with the normal control group, the content of IL-2 was significantly increased at 4 h and 8 h ($P < 0.01$), and significantly increased at 12 h. In addition, there was no significant difference in IL-3 content in normal control group within 12 h. Based on the changes of IFN-γ, IL-2, IL-3 and IL-10 in the supernatant of sheep peripheral blood lymphocytes stimulated by ORFV, it could be concluded that the synthesis of VIL-10 secreted by ORFV could inhibit the synthesis of IFN-γ, IL-2 and IL-3 in lymphocytes to achieve immunosuppression. The significant increase of IL-2 levels may be unrelated to the GM-CSF/IL-2 inhibitor of ORFV, for that the inhibitor is involved in the antigen-presenting cell pathway [18]. In addition, high levels of IL-2 lead to T cell overactivation and induce T cell death [19], and the results showed that 1.9 μg/mL solution could significantly reduce the content of IL-2 mediated by ORFV at 4 h and 8 h, indicating that the extracts of three Tibetan medicine compounds showed a regulatory effect on IL-2.

In addition, IL-6 is a multifunctional cytokine that plays a central role in many physiological inflammatory and immune processes, transmitting defense signals from the site of pathogen invasion or tissue injury to stimulate the acute phase response, immune response, hematopoiesis and various internal organs in preparation for host defense [20]. The results showed that the IL-6 content in the supernatant of peripheral blood lymphocytes stimulated by ORFV was significantly upregulated at 12 h, which indicated that the humoral immunity might be activated and related cytokines, such as IL-4 and IL-10, could be produced about 12 h after ORFV invasion. This result was consistent with the study results from Lin F Y

Table 1 Differential metabolites involved callback pathway at 4 h, 8 h and 12 h

	Metabolic pathway	Differential metabolites	7.6 μg / mL + ORFV	3.8 μg / mL + ORFV	1.9 μg / mL + ORFV
At 4 h	Cysteine and methionine metabolic pathways	Reduced glutathione	-	√	√
		S-Sulfo-L-cysteine			
	Starch and sucrose metabolic pathways	D-Trehalose	-	-	√
		Maltose			
	VB6 metabolic pathway	4-Pyrazole compound acid	-	√	√
		Pyridoxal			
	Pyrimidine metabolic pathway	2′-Deoxycytidine -5′-monophosphate	-	√	√
		N-carbamoyl L-aspartic acid			
At 8 h	β -alanine metabolic pathway	3-Hydroxyl acrylic acid	-	-	√
		Quinoline acid			
	Cysteine and methionine metabolic pathways	S-(5-adenosine) -L-homocysteine	-	-	√
	Lysine hydrolysis	N6-acetyl-L-lysine	√	√	√
		5-Aminovaleric acid			
	Pyrimidine metabolic pathway	L-Hydrogenated whey acid	√	-	√
At 12 h	Purine metabolic pathway	Xanthine nucleoside	-	√	-
		Guanine			
	Tricarboxylic acid cycle	Citric acid	-	√	√
	Galactose metabolism	D—mannose	√	√	√
		Lactose			
	Starch and sucrose metabolic pathways	Maltose	√	√	√
		D—trehalose			
	Taurine and subtaurine metabolic pathways	Squinyl taurine	√	√	√
	Cysteine and methionine metabolic pathways	S-sulfo-L-cysteine	√	√	√
	VB6 metabolic pathway	4-Pyrazole compound acid	√	√	√
	Pyrimidine metabolic pathway	Uracil nucleoside	√	√	√
		2′-Deoxycytidine -5′-monophosphate			
	Tyrosine metabolic pathway	Levothyroxine	-	√	√
	Bile secretion	Cortisol	-	√	√
		Levothyroxine			
	Riboflavin metabolic pathway	Flavin mononucleotide (FMN)	-	-	√

et al. [21], and they showed that serum IL-6 and TNF-α levels in ORFV-treated mice were significantly higher than those in the PBS blank control group before IAV infection ($P < 0.05$). Similarly, some studies suggested that ORFV could induce IL-6 production [22]. And, the results showed that 7.6 μg/mL + ORFV group, 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group could significantly up-regulate the contents of IL-6, IL-4, IL-12 and TNF-β in cell culture supernatant to varying degrees, and these factors play an important role in immune response. IL-4 also plays an important regulatory role in humoral and cellular immunity [23], and IL-12 plays an important role in the differentiation of T cell subsets [24]. T cells could produce TNF-β, IFN-γ, IL-10, IL-4 and IL-5 under the induction of IL-12. And TNF-β is an important mediator of inflammation and immune response [25]. So, the extracts of three Tibetan medicine compounds could regulate the lymphocyte immune response by increasing the contents of IL-6, IL-4, IL-12 and TNF-β in the supernatant of lymphocyte culture.

In this study, at 0 h, 4 h, 8 h and 12 h, the endogenous metabolites of lymphocytes in ORFV group and normal control group were detected, and 16, 48, 18 and 45 metabolites with significant differences were noted, respectively. The endogenous metabolites of lymphocytes in ORFV group were detected at 0 h and 4 h, 4 h and 8 h, 8 h and 12 h. There were 86, 23 and 27 metabolites with significant differences, respectively, so it was speculated that ORFV could regulate the endogenous metabolites of lymphocytes for 0-4 h and 8-12 h. It was found that the endogenous metabolites of lymphocytes in ORFV virus group and normal control group changed, involved cysteine and methionine metabolism pathway, and showed that, ORFV from start to stimulate lymphocyte

to develop 8 h, cysteine and methionine metabolism pathway as the main regulation pathways to regulate lymphocyte endogenous metabolites, and 12 h has been in a dynamic running state. At 0 h, 4 h and 12 h, the changes of endogenous metabolites of lymphocytes in ORFV group and normal control group were all involved in sucrose and starch metabolic pathways, suggested that the metabolites of D-glucose-6-phosphate and D-fructose 6-phosphate could be regulated through sucrose and starch metabolic pathways in 0-4 h and 8-12 h before ORFV stimulation. Metabolites of lymphocytes in ORFV group and normal control group at 0-4 h involved leucine, serine and threonine metabolic pathways, suggested that the metabolic pathways of leucine, serine and threonine was one of the main metabolic regulation pathways of lymphocytes cultured in vitro for 4 h under ORFV stimulation. The change of lymphatic endogenous metabolites in ORFV group and normal control group at 4 h and 8 h involved arginine and proline metabolism pathway, so it maybe one of the main metabolic regulation pathways, but related metabolites levels have no significant difference.

The extracts of three Tibetan medicine compounds could to some extent call back the metabolites up-regulated or down-regulated in lymphocytes after ORFV stimulation. At 4 h, the 7.6 μg/mL + ORFV group, 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group could regulate the expression levels of 6, 12 and 15 differentially significant metabolites caused by ORFV, respectively. The involved callback pathways were cysteine and methionine metabolic pathway, VB6 metabolic pathway, starch and sucrose metabolic pathway and pyrimidine metabolic pathway. At 8 h, the 7.6 μg/mL + ORFV group, 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group recalled 6, 4 and 7 differentially significant metabolites caused by ORFV, respectively, and these metabolites involved β-alanine metabolic pathway, cysteine metabolic pathway and methionine metabolic pathway, lysine hydrolysis and pyrimidine metabolic pathways. At 12 h, 17, 29 and 26 differentially significant metabolites were recalled in 7.6 μg/mL + ORFV group, 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group, respectively, and involved purine metabolic pathway, tricarboxylic acid cycle, galactose metabolic pathway, starch and sucrose metabolic pathway, taurine and subtaurine metabolic pathway, cysteine and methionine metabolic pathway, VB6 metabolic pathway, pyrimidine metabolic pathway, tyrosine metabolic pathway, bile secretion and riboflavin metabolic pathway. And cysteine and methionine metabolic pathways was the most important callback pathways, and the most significant biomarkers were reduced glutathione, S-(5-adenosine) -L-homocysteine, S-adenosine methionine, S-sulfo-L-cysteine, L-methionine, etc. These metabolites are directly or indirectly related to the immune function and antioxidant function of the body [26]. The production of glutathione, taurine and other metabolites increased due to methionine catabolism, while some studies showed that cysteine was a precursor of glutathione synthesis, and glutathione in immune cells regulates immune responses including Th cell action and antibody production [27]. Previous studies reported that methionine also regulates the immune system and reduces oxidative stress by producing glutathione[28, 29]. In addition, the pyrimidine metabolic pathway was also the main regulatory pathway of endogenous metabolites in lymphocytes after the effect of Tibetan drug combination on ORFV. Pyrimidine derivatives have been used as anticancer, antibacterial, antiviral, antigenic animal and antifungal drugs [30], and pyrimidine is the biological macromolecular amino acid, nucleic acid, lipid, carbohydrate synthesis of precursor, and actively dividing cells than static cells need higher levels of pyrimidine, therefore can be speculated that rock dragonhead red-knees herb, drought and other three kinds of Tibetan medicine compound extract solution on the one hand may by cysteine and methionine metabolism pathways to regulation of lymphocyte immune function [31, 32]. On the other hand, the pyrimidine metabolic pathway could stimulate lymphocyte proliferation and produce antiviral derivatives to form antiviral effects to regulate lymphocyte immune function. The molecular regulatory mechanism needs to be further verified by experiments.

It was observed that 7.6 μg/mL + ORFV group had the least amount of callback to the endogenous metabolites of lymphocytes stimulated by ORFV within 12 h. 3.8 μg/mL + ORFV group was the optimal concentration at 12 h, and it had the largest callback number of endogenous metabolites in lymphocytes after ORFV stimulation within 8-12 h. Meanwhile, the results of cytokine IL-3, IL-10, TNF-β and CD4 content at 12 h also showed that the 3.8 μg/mL + ORFV group had the best effect compared with the other two groups, and the IL-4 content at 8 h showed that the 3.8 μg/mL + ORFV group had the best effect compared with the other two groups. The 1.9 μg/mL + ORFV group had the largest number of callback pathways for endogenous metabolites of lymphocytes after ORFV stimulation within 8 h, and the same number of callback pathways were involved in the 3.8 μg/mL + ORFV group at 12 h. In addition, 3.8 μg/mL + ORFV group and 1.9 μg/mL + ORFV group showed similar trends in the detection results of immune factors, but were significantly different from 7.6 μg/mL + ORFV group. Therefore, the results preliminarily indicated that both 3.8 μg/mL and 1.9 μg/mL of compound extract of Tibetan medicine could be used as the optimal concentration of lymphocytes stimulated by ORFV.

Conclusion

Three kinds of compound extracts of Tibetan medicine such as *Polygonum leucoides*, *Polygonum xanthoxylum* and *Acanthophora rotunda* could regulate the changes of lymphocyte immune factors induced by ORFV, so it was speculated that the compound extracts of Tibetan medicine could alleviate the immune escape caused by ORFV to a certain extent. Cysteine and methionine metabolic pathways were not only the main pathway of ORFV-stimulated lymphocyte metabolite regulation, but also the main callback pathway of lymphocyte metabolite regulation after ORFV-stimulated treated with Tibetan medicine compound extracts. And pyrimidine metabolic pathway was the main metabolite callback pathway in a dynamic state. It was speculated that the extracts of three Tibetan medicine compounds may regulate the immune function of lymphocytes after ORFV-stimulated through cysteine and methionine metabolism pathway and pyrimidine metabolic pathway. Which indicated that Tibetan medicine could potentially mitigate the impact of ORFV on lymphocyte activity.

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Authors' contributions
Conceptualization, Yueyuan Fan and Guowen Fu; methodology, Jiao Wu; software, Saiju Li; validation, Jiao Wu and Saiju Li; formal analysis, Wei Huang; investigation, Qin Zeng; data curation, Zhuoga Gesang; writing—original draft preparation, Yueyuan Fan, Jiao Wu and Wei Huang; writing—review and editing, Yuzhen Silang, Chong Zhang and Guowen Fu; visualization, Wei Huang and Saiju Li; supervision, Chong Zhang; project administration, Yueyuan Fan and Saiju Li. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
All procedures conducted with the sheep were approved by the Yunnan Agricultural University Animal Care and Use Committee (approval ID: YNAU202203080). Animal use and care were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Research Council. The sheep provided by Yongren Jiajia Agricultural Animal Husbandry Technology Co., LTD, and we obtained informed consent from the owner to use the sheep in this study.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest.

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