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Linoleic acid stimulation results in TGF-β1 production and inhibition of PEDV infection *in vitro*

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ABSTRACT

Linoleic acid (LA) is recommended to improve pork quality. However, whether it affects the intestinal immune response in pigs is still unclear. Our *ex vivo* experiments demonstrated that LA stimulation resulted in increased frequencies of Tregs in PBMCs but not in Peyer's Patches (PPs). The results of RT-qPCR, flow cytometry, and ELISA indicated that LA increased the TGF-β1 expression level in DCs isolated from PEDV infected pigs. Furthermore, RT-qPCR and flow cytometry results demonstrated that TGF-β1 was associated with higher frequencies of Tregs both in PBMCs and PPs. Additional investigations showed that TGF-β1 inhibited PEDV infection *in vitro*. Besides, knocking-out TGF-β1 in IPEC-J2 cells resulted in higher viral load. Taken together, our results demonstrated that LA stimulation resulted in enhanced production of TGF-β1 by DC, which resulted in higher frequencies of Tregs production and inhibition of PEDV infection.

1. Introduction

Linoleic acid is an essential fatty acid (Cunha, 1991). Linoleic acid (LA; C18:2), also called linoleate, belongs to the omega-6 polyunsaturated fatty acids (*n*-6 PUFAs) (Radzikowska et al., 2019). As shown in human *in vitro* models, *n*-6 PUFAs are beneficial to the physical and immunological function of intestine. For example, n-6 PUFAs inhibit IL-1β production in the Caco-2 cell line and decrease levels of pro-inflammatory cytokines like IL-6 and IL-8 (Marion-Letellier et al., 2008). LA also induces the expression IL-10 in dendritic cells and inhibits the LPS-induced IL-1β secretion of neutrophils (Aggarwal et al., 2016; Loscher et al., 2005). In addition, PUFAs upregulate mRNA expression levels of FoxP3, cytotoxic T lymphocyte associated protein 4 (CTLA4) and tumor growth factor-beta (TGF-β), which are important for the immunosuppressive activity of regulatory T cells (Tregs) (Yessoufou et al., 2009). Tregs are identified based on the expression of Foxp3 (Travis and Sheppard, 2014). As a result of PUFAs inducing Tregs, production of TGF-β1 is increased, which in the end limits the tissue damage due to the infection (Sanjabi et al., 2017).

Nowadays, to improve pork quality and decrease lipid synthesis in growing pigs, LA or conjugated linoleic acid (CLA) are added as feed additives (Go et al., 2012). The increased CLA concentration may improve the performance of pork, especially on subcutaneous fat and lean tissue (Thiel-Cooper et al., 2001). Moreover, CLA supplementation dies not affect tissue lipid synthesis but increased levels of intramuscular lipid (Go et al., 2012). Increasing the amount of LA in the diet support the growth of the inner backfat lipid and thickness (Whittington et al., 1986). Besides, LA also plays an important role in disease prevention. Dietary LA is known to significantly influence oxidation of the jejunal mucosa in pigs (López Bote et al., 2001). Moreover, an increase in the concentration of LA in 10^{-6} mol/L induces relaxation and hyperpolarization of coronary vascular smooth muscle cells in pigs via influencing the Na^{+}/K^{+}-ATPase pump.

Porcine epidemic diarrhea virus (PEDV) is a member of the genus *Alphacoronavirus* in the family *Coronaviridae* (Lee, 2015). PEDV infection is characterized by serious diarrhea, vomiting, dehydration and even death in piglets less than 10-days old. This infection has been a major problem affecting the pig industry worldwide, which needs to be resolved (Yang et al., 2020). The main infection site of PEDV is the small intestine, especially the jejunum and ileum. Nowadays, many therapeutics are difficult to be applied by oral administration because they are easily to be degrades by enzymes in the digestive tract. LA, as a feed additive, can overcome this difficulty.

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Abbreviations

LA	Linoleic acid
PBMC	Peripheral blood mononuclear Cell
PP	Peyer's Patches
ELISA	Enzyme-linked Immunosorbent assay
FoxP3	Forkhead box P3
TNF-α	Tumor necrosis factor α
IPEC-J2	Intestinal porcine enterocyte cell line
TGF-β1	Transforming growth factor β1
DC	Dendritic cell
PEDV	Porcine epidemic diarrhea virus
CLA	Conjugated linoleic acid
BLIMP1	B-lymphocyte-induced maturation protein 1
PAX5	Paired box protein 5
IRF4	Interferon regulatory factor 4
BCL6	B-cell lymphoma 6
MHCII	Major Histocompatibility Complex II
GM-CSF	Granulocyte-macrophage colony-stimulating factor
PUFA	Polyunsaturated Fatty Acid
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
Treg	Regulatory T cell

PEDV infection leads to severe inflammatory response, and LA has the ability to inhibit inflammation (Chen et al., 2020; Marion-Letellier et al., 2008). Therefore, the aim of this study was to explore whether LA can be used as potential drug to inhibit PEDV infection. Firstly, we explored how LA supplementation affects the intestinal mucosal immune system. LA was demonstrated to induce production of Tregs and secretion of TGF-β1 in intestinal lymphocytes in this study. Furthermore, we showed that TGF-β1 was able to effectively inhibit porcine epidemic diarrhea virus (PEDV) infection *in vitro*. These findings further strengthen the significance of applying LA into the feed, and also imply a new strategy for preventing PEDV infection via feed additives.

2. Methods and materials

2.1. Viral strains and cell lines culture

The cell culture-adapted PEDV strain (GS-PEDV LJX) was generated and preserved in our lab. African green monkey kidney cell line (Vero-E6) and porcine jejunal cell line (IPEC-J2) were cultured in Dulbecco's minimum essential medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma) at 37 °C under 5% CO2.

2.2. Primary cell preparation and culture

For isolation of PBMCs, fresh porcine blood was collected. The heparin blood was diluted with PBS and gently layered over an equal volume of Percoll plus medium (GE Healthcare,17-5445-01) and density gradient centrifugation was performed. To this end, the tube was centrifuged for 30 min at 400 g without brake. The middle layer including PBMCs characterized as white and cloudy "blanket" was collected, washed with PBS for three times and counted for use.

For isolation of Peyer's patches lymphocytes (PPs), Peyer's patches were collected, then fatty portions, serosal surface, mucus and villi were discarded. The tissues were minced by ophthalmic scissors and homogenized by using a cell separator GentleMax (Miltenyi Biotec. No.031799). Then the cell suspension was diluted with PBS prior to being filtered through 100 μm, 70 μm, 40 μm filters to exclude debris, respectively. After filtering and centrifuging, the pellet was resuspended in 40% Percoll plus medium and then layered under 67.5% Percoll plus medium. Afterwards, the lymphocytes were collected from the middle layer after centrifuging at 1800 rpm for 30 min at room temperature without brake. In the process of isolating primary cells, all tissues and cells were treated with pre-cold PBS.

For some experiments, $CD3^+$ T cells and $CD14^+$ PBMCs were enriched by magnetic cell separation assay (Miltenyi) with specific antibodies according to the instructions of the manufacturer. The primary antibodies that were used were mouse anti-porcine CD3e-SPRD (Southern Biotech, 4510-13), mouse anti-porcine monoclonal antibody CD14-FITC (Southern Biotech, 4515-02), and the second antibodies were anti-mouse IgG coupled to microbeads (Miltenyi, 130-048-401) and anti-mouse FITC microbeads (Miltenyi, 130-048-701). To culture monocyte-derived dendritic cells (DCs), $CD14^+$ PBMCs were isolated via magnetic cell separation and then 5×10^6 cells were cultured in12-well plate with 10 ng/mL IL-4 (R&D, 654-P4-025) and 20 ng/mL GM-CSF (pig, R&D, 711-PG-010). Half of medium was replaced every 2 days. On the sixth day after stimulation, the $CD14^+$ PBMCs were differentiated into unmatured DCs with synapses, which were used in the experiments. All the primary cells were cultured in RPMI-1640 medium with 10% serum. When DCs were used in mixed culture with T cells, the ratio between DCs and T cells was 1:10.

2.3. RNA extraction and RT-qPCR

The total RNAs from cell or tissue samples were extracted using Trizol reagent (Takara, 9109), and reverse transcribed into cDNAs using random primers. RT-qPCR was performed to detected specific genes. The detailed information of the primers and probe targeting PEDV M gene were as follows: forward primer: 5′-GAT ACT TTG GCC TCT TGT GT-3′, reverse primer: 5′-CAC AAC CGA ATG CTA TTG ACG-3′, and Taqman probe: 5′-FAM-TTC AGC ATC CTT ATG GCT TGC ATC-TAMRA-3'. To detect the viral copies, the amplification enzyme was Probe qPCR SuperMix, from TransStart. For relative gene expression detection, the amplification enzyme was ChamQ SYBR qPCR Master Mix, from Vazyme. The primers are showed in Table 1.

2.4. Flow cytometry

The stimulated cells were collected and washed firstly with cold PBS. To stain for the cellular surface markers, cells were incubated with primary antibodies for 30 min at 4 °C, washed then resuspended in PBS and analyzed. For the intracellular markers, the cells were fixed and permeabilized prior to primary antibodies incubation. The primary antibodies are mouse-anti-porcine CD3e-SPRD (Southern Biotech, 4510-13), CD4-FITC (Southern Biotech, 4515-02), CD8-FITC (Southern Biotech, 4520-02), CD21-FITC (Southern Biotech, 4530-09), CD172-PE (Southern Biotech, 4525-09), CD1a (Abcam, ab24986), MHCII (Abcam, ab34000), CTLA4-PE (Ancell, 359-050), PI (BD51-66211E), Foxp3 (Bioss, bs-10211R). The primary antibodies incubation in intracellular staining is the same as surface marker staining. After washing with PBS, the secondary antibodies were incubated for 30 min at 4 °C, then washed in PBS and analyzed. The secondary antibodies, including FITC-labeled goat anti-mouse IgG (H+L) antibody and PE-labeled goat anti-rabbit. Lastly, 10^4 cells in gate were collected and analyzed by CytoFLEX S (Beckman). The data were analyzed by using FlowJo (FlowJo v10).

2.5. Enzyme linked immunosorbent assay (ELISA)

The concentrations of TGF-β1 (Genie, PRFI00153) was measured by ELISA Kits according to the manufacturer's instruction (Alhajj and Farhana, 2021).

2.6. Animal samples collection

All the samples from uninfected and infected pigs were from a

Table 1
Detailed sequences of primers for RT-qPCR analysis.

Names	Forward primers	Reversed primers
BLIMP1	ATGACACACAAATCCAGAGCCA	GGGAGTCCAATTTCAGGATTTC
PAX5	TGTTTGCCTGGGAGATCAGG	CCGTGGACACTATGCTGTGA
IRF4	CCGGCCTGTGAAAATGGTTG	GGACGTGGTCAGCTCTTTCA
BCL6	GTATCCAGTTCACCCGCCAT	AGGACCGTCTTATGGGCTCT
FoxP3	GTGGTGCAGTCTCTGGAACAAC	AGGTGGGCCTGCATAGCA
TNF-α	GTCTCAAACCTCAGATAAG	GTTGTCTTTCAGCTTCAC
IL-17	AAGTCCAGGATGCCCAAA	CGGTTCAAGATGTTCAAGTTG
TGF-β1	GGAACCTGTATTGCTCTC	AATCATTGCTGTATTTCTGG
IL-2	GTGAATATGATGATGAGACAGTAA	CAAGTCAGTGTTGAGTAGATG
IL-6	AATGCTCTTCACCTCTCC	TCACACTTCTCATACTTCTCA
IL-10	GTGAAGAGTGCCTTTAGC	TCTATGTAGTTGATGAAGATGTC
CTLA4	ACGGGACTCTACATCTGCAAGG	GGAGGAAGTCAGAATCTGGGCA
GAPDH	CATCCATGACAACTTCGGCA	GCATGGACTGTGGTCATGAGTC

previous study (Yang et al., 2020). All experimental procedures and animal care protocols were approved by the guidelines for Care and Use of Laboratory Animals of Lanzhou Veterinary Research Institute (LVRI), Chinese Academy of Agricultural Sciences, China (Yang et al., 2020).

2.7. Metabolites and relevant reagents

Linoleic acid (L1012) was purchased from Merck, which was of over 99% purity. The concentration of LA is 0.5 μg/mL.

2.8. Quantification and statistical analysis

All data and diagrams were analyzed and shown by GraphPad 7.0 and SPSS. The significant difference analysis was performed by one-way ANOVA ("***" means $p < 0.001$, "**" means $p < 0.01$, "*" means $p < 0.05$, $n \geq 3$). Data presented as Mean ± SEM.

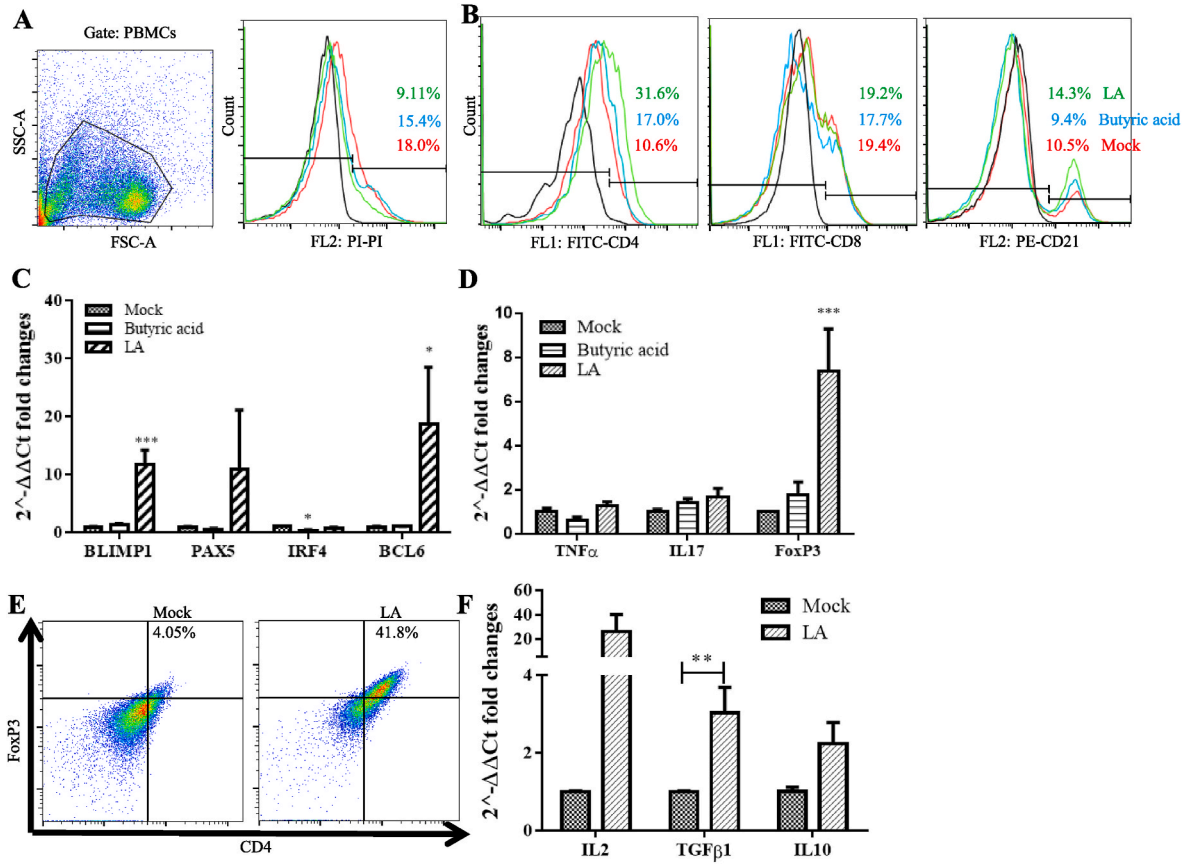

Fig. 1. Linoleic acid stimulation results in production of Tregs (A) Flow cytometry results demonstrating the percentage of PI^+ cells upon treatment with various acids of PBMCs. Different colored lines represent different groups. The black lines indicate unstained cells. The colored numbers represent the percentage of positive cells in each group. (B) Flow cytometry results of $CD4^+$, $CD8^+$, $CD21^+$ cell frequency after the treatment with various acids of PBMCs. (C) Expression of B cell differentiation related factors compared mock and LA treated PBMCs as detected by RT-qPCR. (D) T cell differentiation related molecules expression level in mock or different acid treated PBMCs were detected by RT-qPCR. (E) Frequencies of $CD4^+Foxp3^+$ T cells in mock or LA treated PBMCs were analyzed by flow cytometry. (F) Expression level of IL-2, TGF-β1 and IL-10 were detected in mock or LA treated PBMCs by RT-qPCR.

3. Results

3.1. Linoleic acid stimulation results in production of Tregs

To investigate whether LA affects specific immune cell subsets, we analyzed the effect of LA stimulation on PBMCs. The gating strategy is shown in Fig. 1A. To investigate whether LA affects cell viability compared to other organic acids like butyric acid, the effect of LA on cell viability was analyzed by flow cytometry. We found that the percentage of PI^+ cells was twice lower in LA treated PBMCs compared to the mock group (Fig. 1A). Next, we determined frequencies of CD21, CD4 and CD8 positive cells in PBMCs upon *in vitro* stimulation with LA. Flow cytometric analysis showed that LA stimulation resulted in an increase in $CD21^+$ immature B cells and $CD4^+$ T cells (Fig. 1B). To further explore how LA regulate the frequency of immature B cells, expression of intracellular molecules involved in B cell differentiation like BLIMP1, PAX5, IRF4, BCL6 were detected by RT-qPCR. No significant changes in the mRNA expression levels were observed except for BLIMP1 and BCL6 (Fig. 1C). However, BLIMP1 and BCL6, in addition to promoting B cell development, are also involved in production of Tregs, and limit differentiation of Th1, Th17 and follicular helper T cells. Therefore, mRNA expression levels of TNF-α, IL-17 and FoxP3, markers for Th1, Th17 and Tregs (Rudensky, 2011), in PBMCs or PPs that were stimulated with LA were analyzed. LA stimulation resulted in a significant increase in mRNA levels of FoxP3, suggesting that LA stimulation may affect the differentiation of Tregs (Fig. 1D). Base on the gating strategy shown in Fig. S1A, LA stimulation indeed resulted in an increase of the percentage $CD4^+$ $FoxP3^+$ T cells in PBMCs (Fig. 1E). In addition, LA stimulation resulted in expression of TGF-β1 (Fig. 1F). Similarly, LA inhibited the percentage of PI^+ cells in lymphocytes from Peyer's patches (PPs) (Fig. S1B). LA stimulation of lymphocytes isolated from PP's did not result in an increase in the percentage of $CD4^+$ T cells (Fig. S1C). Also mRNA expression levels of FoxP3 (Fig. S1D) and the percentage of $CD4^+FoxP3^+$ Treg cells were not affected (Fig. S1E).

3.2. Linoleic acid stimulates DCs secreting TGF-β1

To further explore how LA may affect differentiation of Tregs, $CD3^+$T cells were isolated from PBMCs, cultured and stimulated by LA. Results showed that stimulation with LA did not affect FoxP3 expression and frequency of $CD3^+CD4^+$ cells from $CD3^+$T cells (Fig. 2A and B). After literature reviewing, dendritic cells (DCs) could control Tregs fate and induce differentiation in the presence of TGF-β1 (Zou et al., 2010). In comparison to PPs, the percentage of $CD172a^+MHCII^+$ DCs was higher in the PBMCs in adult pigs (Fig. 2C). The higher percentage of DCs in PBMCs suggest that LA may stimulate DCs secreting specific cytokines to further induce the formation of Tregs. Culturing DCs from monocytes in the presence of LA (Fig. 2D), resulted in more viable cells compared to mock group (Fig. 2E). Base on the gating strategy shown in Fig. 2E, LA stimulation did not influence the percentage of $CD172^+CD1a^+$ DCs (Fig. 2F) but resulted in lower frequency of mature $MHCII^+CTLA4^+$ DCs (Fig. 2G). Moreover, LA stimulation inhibited the percentage of $MHCII^+$ or $CTLA4^+$ cells, respectively (Fig. 2H). Analysis of levels of cytokines including TGF-β1 in LA stimulated DCs showed a significant increase in TGF-β1 mRNA expression (Fig. 2I), as well as in TGF-β1 levels in the culture supernatant (Fig. 2J).

3.3. TGF-β1 induces Tregs differentiation in PPs

To explore whether TGF-β1 is directly involved in Tregs differentiation, TGF-β1 stimulation of $CD3^+$ T cells from PPs was performed. This stimulation resulted in an increase in mRNA levels of FoxP3, IL-10 and TGF-β1 (Fig. 3A). Meanwhile, also the percentage of $CD4^+$ $FoxP3^+$ T cells increased upon TGF-β1 stimulation (Fig. 3B). Besides, TGF-β1 stimulation resulted in increased percentage of $CTLA4^+$ T cells in PPs (Fig. 3C). In conclusion, LA stimulation of DCs results in the production of TGF-β1, and subsequently an increase in percentage.

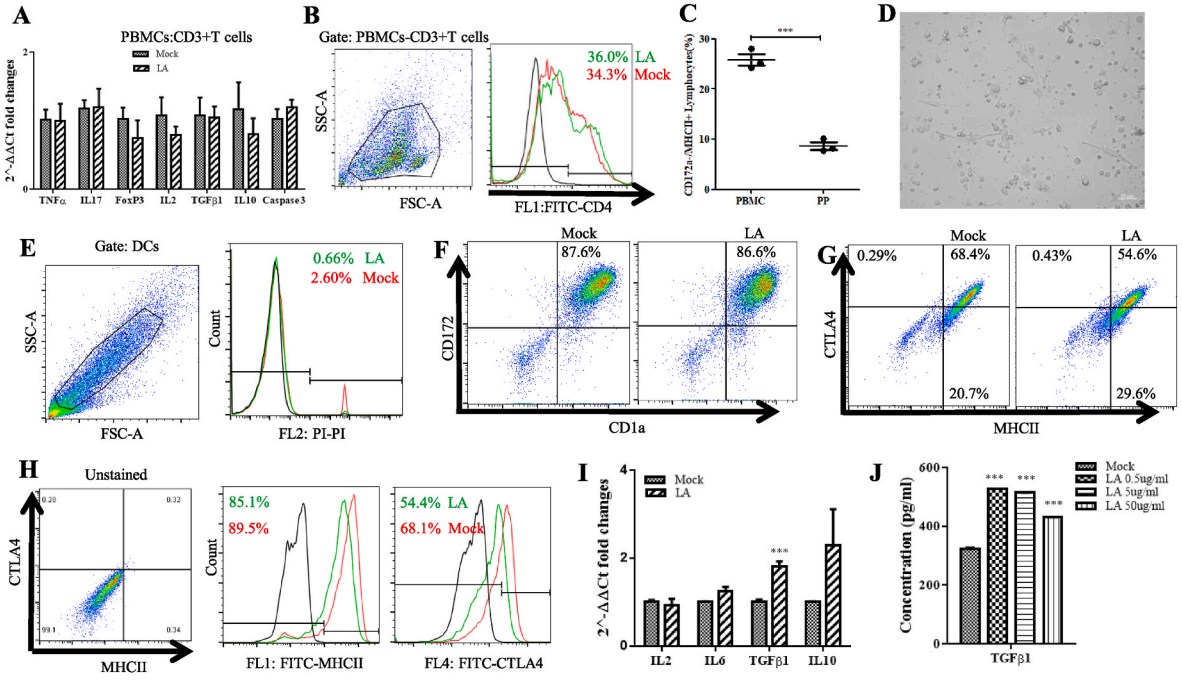

Fig. 2. Linoleic acid stimulates secretion of TGF-β1 by DC. (A) T cell differentiation related molecules in mock or LA treated $CD3^+$T cells were detected by RT-qPCR. (B) $CD3^+CD4^+$ T cells were also recorded by flow cytometry. (C) The frequencies of $CD172a^+MHCII^+$ cells between PBMCs and PPs were compared. (D) Morphology of DCs after 6 days of culture was shown. (E) Flow cytometry result in PI^+ cells was pictured after the treatment with LA of DCs. (F and G) $CD172^+CD1a^+$ and $CTLA4^+MHCII^+$ cells were tested in mock or LA treated DCs by flow cytometry. (H) The frequencies of $CTLA4^+$ or $MHCII^+$ cells were tested in mock or LA treated DCs by flow cytometry. (I) RT-qPCR was applied to test the key cytokines that secreted by DCS with LA stimulation. (J) ELISA was applied to test the concentration of TGF-β1 that LA induced in DCs.

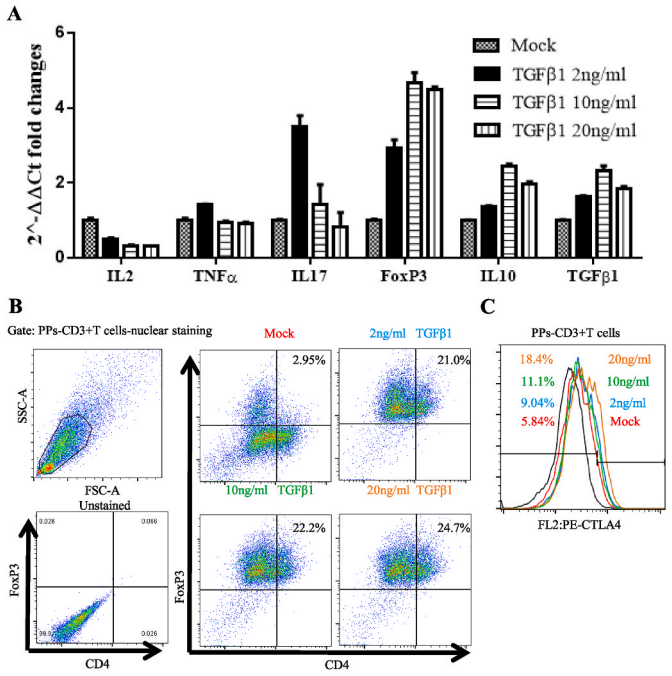

Fig. 3. TGF-β1 induces Tregs differentiation in PPs. (A) RT-qPCR was applied to analyze levels of cytokines that TGF-β1 induced in T cells (isolated from PPs). The frequency of $CD4^{+}Foxp3^{+}$ (B) and $CTLA4^{+}$ T cells (C) were measured in mock or TGF-β1 treated T cells (isolated from PPs) by flow cytometry and ELISA. The results presented are representative of triplicates.

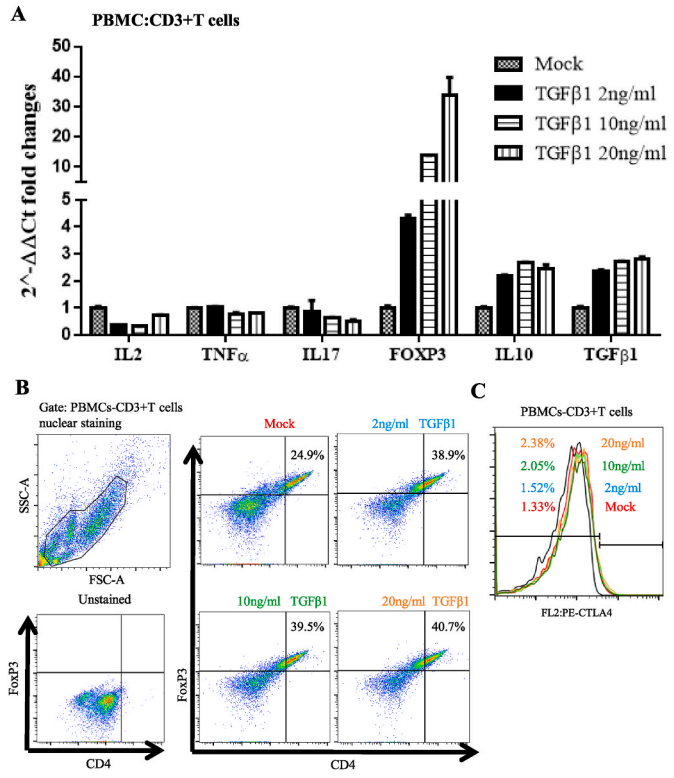

Fig. 4. TGF-β1 induces Tregs differentiation in PBMCs. (A) RT-qPCR was applied to test the key cytokines that TGF-β1 induced in T cells (isolated from PBMCs). The percentage of $CD4^{+}Foxp3^{+}$ (B), $CTLA4^{+}$ T cells and (C) were tested in mock or TGF-β1 treated T cells (isolated from PBMCs) by flow cytometry and ELISA. The results presented are representative of triplicates.

3.4. *TGF-β1 induces Tregs differentiation in PBMCs*

This induction of FoxP3, IL10 and TGF-β1 mRNA levels and Tregs frequency were observed both as well as in $CD3^{+}$ T cells from PBMCs (Fig. 4A and B). Besides, TGF-β1 stimulation increased the percentage of $CTLA4^{+}$ T cells in PBMCs (Fig. 4C).

3.5. *Linoleic acid induces TGF-β1 production*

To further investigate the role of LA in the induction of Tregs differentiation, a co-culture between DCs and T cells were established. As shown in Fig. 5A, characteristic factors for Treg like FoxP3, TGF-β1 and IL-10 were all raised in the co-culture system. Levels of cytokines IL-2 and IL-17, were also upregulated while the mRNA expression of IL-6 was significantly decreased (Fig. 5A). Moreover, percentage of $CD172^{+}CD1a^{+}$ DCs, surface expression of the activation markers MHCII and CTLA4 and the percentage of mature DCs ($MHCII^{+}CTLA4^{+}$) were inhibited after LA treatment (Fig. 5B and C). Percentages of Tregs (Fig. 5D), $CTLA4^{+}$ T cells (Fig. 5E) and protein levels of TGF-β1 (Fig. 5F) were increased as well.

3.6. *PEDV infection induces Tregs and TGF-β1 production*

To further explore whether LA may have the potential to inhibit PEDV infection, the samples from uninfected and PEDV infected pigs were collected (Yang et al., 2020). In samples from PEDV infected pigs, higher percentage of Tregs were observed compared to uninfected pigs, both in PBMCs and MLN lymphocytes (Fig. 6A and B). As shown in Fig. 6C, expression levels of IL-1β, TNF-α, FoxP3, CTLA4 and TGF-β1 were higher in lymphocytes from PEDV infected pigs. Furthermore, levels of TGF-β1 were increased both in serum and in intestinal content (Fig. 6D and E). Taken together, more Tregs and higher expression levels of Treg specific factor suggest that PEDV infection induces Tregs and TGF-β1 production.

3.7. *TGF-β1 is able to inhibit PEDV infection in vitro*

Next, we studied the effect of TGF-β1 on PEDV infection *in vitro* using Vero-E6 cells. Infection of TGF-β1 transfected cells resulted in lower viral copy numbers and a weaker band in a western blotting as shown in Fig. 7A, B and 7C. Overexpression of TGF-β1 in IPEC-J2 cells resulted in lower viral copies via qPCR detection (Fig. 7D). PEDV infection of TGF-β1 knockout IPEC-J2 cells resulted in a significantly higher number of viral copies compared to the parental cell line. When TGF-β1 was knocked out, viral load was increased both at 6h and 24 h post infection (Fig. 7E). Taken together, TGF-β1 is able to inhibit PEDV infection of an epithelial cell line *in vitro*.

4. Discussion

Although LA is added to the feed of pigs to improve fat synthesis and meat quality, the impact of LA on the mucosal immune system in pigs has not been explored. In this study, we demonstrated that LA stimulation result in TGF-β1 secretion by DC, which subsequently induces Treg differentiation. PUFA CLA is reported to inhibit the maturation of DC by lower expression of MHCII, CD80 and CD86 and to suppress the differentiation of Th1 and Th17 subsets and production of inflammatory cytokines (Draper et al., 2014). Increased levels of LA decreases the antigen presenting ability of human and murine DCs, as shown by lower expression levels of CD40, IL-6, IL-12 and less differentiation of Th1 and Th17 subsets (Huang et al., 2021b). In pigs, we also observed that stimulation with LA reduces the MHCII expression level on DCs, together with an increase in expression of CTLA4 of T cells and inhibit of IL-6 production. Moreover, LA stimulation induced Treg differentiation and TGF-β1 production, accompanied by high expression of CTLA4 and increased generation of immature DCs. These results all contribute to the

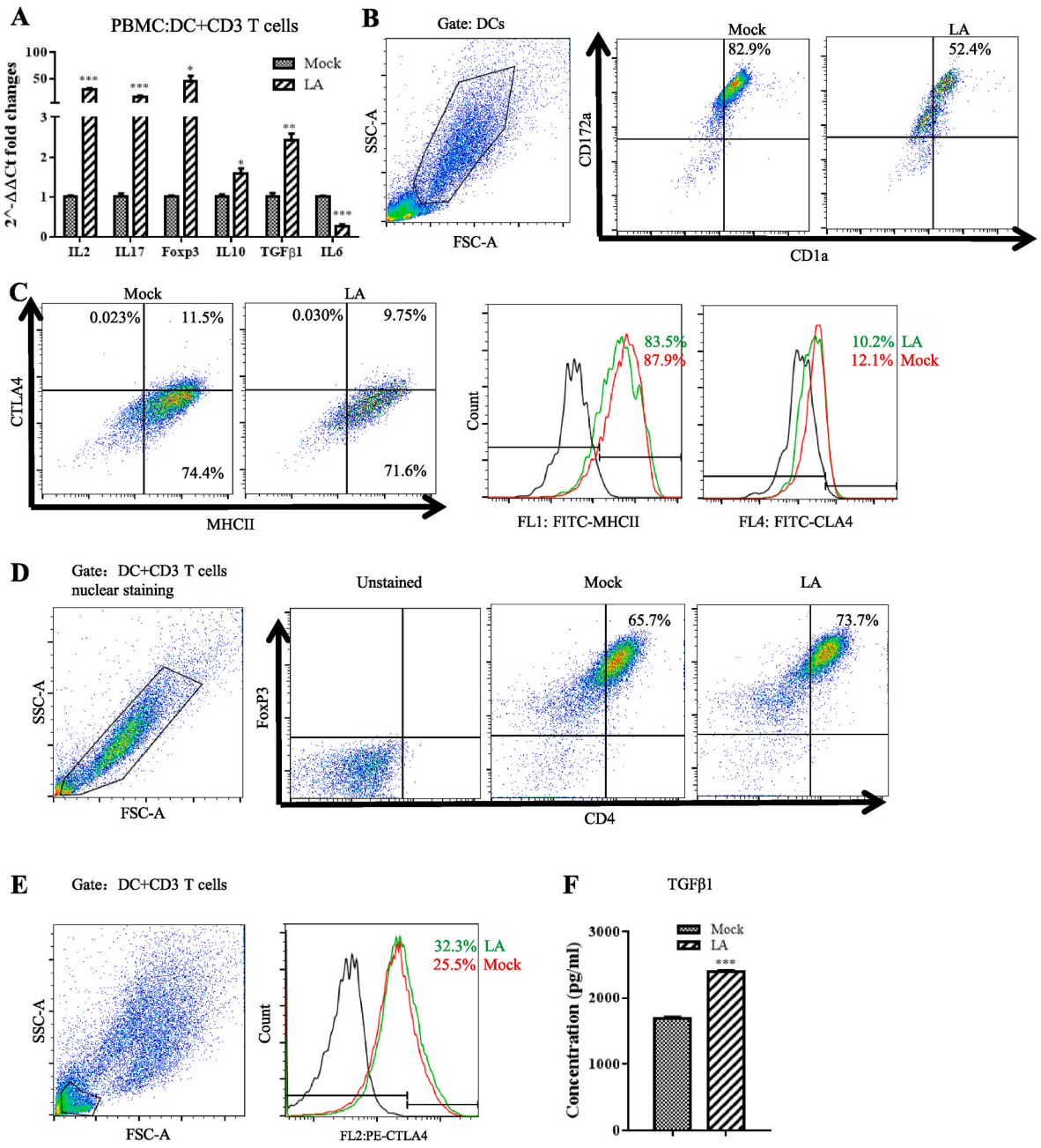

Fig. 5. Linoleic acid induces TGF-β1 production. (A) Co-culture experiments were performed with T cells and LA stimulated DCs for 72h. The key molecules including IL-2, IL-17, Foxp3, IL-10, TGF-β1, IL-6 expression level was detected by RT-qPCR. (B and C) $CD172^{+}CD1a^{+}$, $CTLA4^{+}MHCII^{+}$, $CTLA4^{+}$ and $MHCII^{+}$ DC cells were tested in mock or LA treated mixed system by flow cytometry. The percentage of $CD4^{+}Foxp3^{+}$(D), $CTLA4^{+}$ T cells (E) and TGF-β1 concentration (F) were tested in mock or LA treated mixed system (T cells isolated from PPs) by flow cytometry and ELISA.

induction of immunosuppression (Kondelkova et al., 2010). TGF-β1 is known to be involved in alleviating inflammation whether it is applied to the *in vitro* experiment or clinical treatment (Huang et al., 2021a; Melisi et al., 2019; Yang et al., 2015).These negative effects on inflammation may contribute to limit tissue injury caused by the infection. The anti-inflammatory therapy inhibits coronavirus complications and has potential to prevent coronavirus infection (Fratta Pasini et al., 2021).

PEDV infection induces strong cytokine storm and inflammatory response (Chen et al., 2020). Therefore, we speculate that this anti-inflammatory therapy may have affect PEDV infection. Then we demonstrate that anti-inflammatory cytokine TGF-β1 inhibit PEDV infection, which further indicate that LA is potential to inhibit enteric PEDV infection. Moreover, comparing to the other antiviral therapies, LA is difficult to be degraded by enzyme, therefore this feed compound could be potential and effective to prevent viral infection.

However, although beneficial effects of LA have been demonstrated in this study, the concentration of LA should be taken into account in further studies. Our data showed that high concentration of LA resulted in affect cell viability (Fig. S2A). The high dose LA that was used could also be toxic to the mice. Besides, A high dose of linoleic acid has been reported to be harmful to health, as it caused gastric disease and coronary heart disease in human (Farvid et al., 2014; Hirata et al., 2017; Moran et al., 2001). Therefore, the optimal dose should be taken into account in follow-up studies.

5. Conclusion

Taken together, we discovered that LA influenced the secretion of

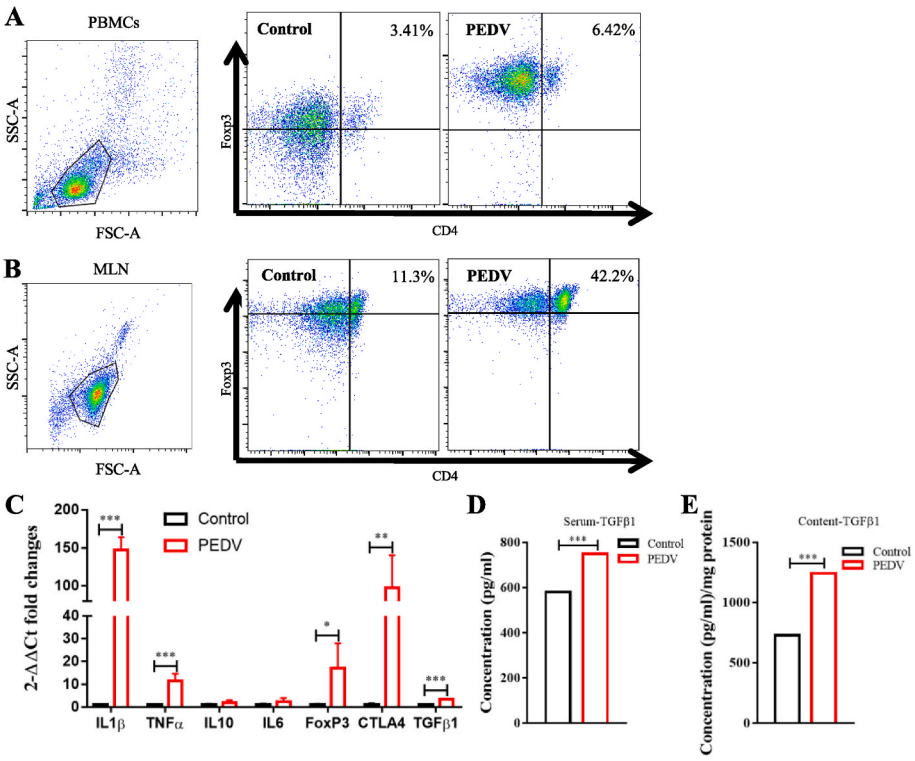

Fig. 6. PEDV infection induces differentiation of Tregs and production of TGF-β1. (A) Percentages of $CD4^{+}Foxp3^{+}$ cells in PBMCs were analyzed by flow cytometry in uninfected and PEDV infected pigs. (B) The percentage of $CD4^{+}Foxp3^{+}$ cells collected form MLNs between uninfected and infected pigs were detected by flow cytometry. (C) Relative molecules expression levels were detected by RT-qPCR. The concentration of TGFβ1 in serum (D) and intestinal content (E) was tested by ELISA kit according to protocol.

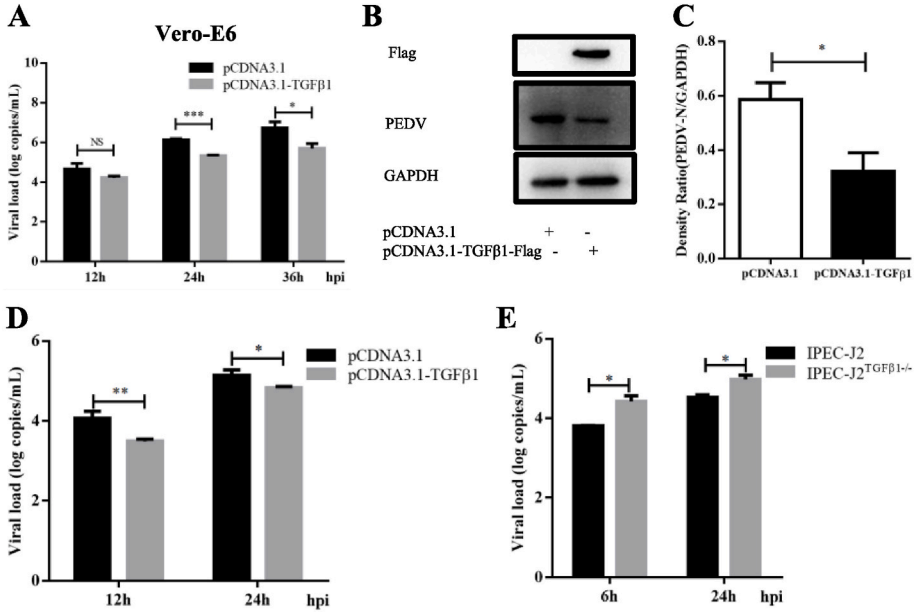

Fig. 7. TGF-β1 is able to inhibit PEDV infection *in vitro*. (A) Real-time RT-qPCR was applied to detected viral copies after transfection of TGF-β1 in Vero-E6 cells. (B) Western blotting was utilized to detect expression of viral protein expression after transfecting TGF-β1 plasmids into Vero-E6 cell. (C) Density ratio of western blotting results were analyzed by ImageJ. Results of three experiments are shown. (D) Real-time RT-qPCR was applied to detected viral copies after transfection of TGF-β1 plasmids. (E) Viral copies were compared between mock and TGF-β1-KO IPEC-J2 cell line by Real-time RT-qPCR assay.

TGF-β1 by DC, affected the differentiation of Tregs and further inhibited enteric PEDV infection. This suggests that LA may have potential for future use as clinical grade feed compound in the context of pro-inflammatory response and PEDV infection. The demonstrated effects of LA on the intestinal immune system of pigs suggests that administration of LA does not only improve pork quality, but may also regulate the intestinal immune system and has potential to prevent PEDV infection in pigs.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. The authors agree to make available all of the replicate data upon request.

CRediT authorship contribution statement

Shanshan Yang: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft,

Visualization, Project administration. **Caiying Wang:** Investigation. **Xin Huang:** Investigation. **Christine A. Jansen:** Data curation, Writing – review & editing, Supervision. **Huub F.J. Savelkoul:** Writing – review & editing, Supervision. **Guangliang Liu:** Conceptualization, Methodology, Validation, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no other competing.

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Appendix A. Supplementary data

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