Research Article

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Nicotinic acid receptor GPR109A promotes antiviral innate immune response through autophagy.

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ABSTRACT

Emerging evidence suggests that GPCRs play a pivotal role in virus infection. However, the mechanisms underlying GPCRs-mediated type I interferon responses to protect host from viral infection are not systematically explored. In order to screen key functioning GPCRs in interferon-induced expression process, we applied gene chips analysis, where the GPR109A, ranked as the uppermost regulated GPCR, functions as a powerful antiviral factor, implicating its potential role as an antivirus target. The expression of GPR109A was upregulated in mouse peritoneal macrophages (PEM) stimulated with IFNβ, IFNγ and virus, as determined by Q-PCR and Western blot analysis. Moreover, blocking STAT1 activation using inhibitor (Fludarabine) could decrease the expression of GPR109A expression induced by IFNβ and IFNγ stimulation. Above results suggested that GPR109A is an interferon-stimulated gene (ISG). Both VSV-virus infection and replication was remarkably enhanced in Gpr109a-/- macrophage compared to the WT macrophage. GPR109A deficiency reduced the survival of VSV-virus infection lethal mouse model with increased virus infection rate of peritoneal cells, and increased virus titers in various abdominal organs were observed. Mechanistically, increased viral infection in GPR109A deficient mouse was due to reduced IFN production by restraining the formation of autophagosomes. This is confirmed by an activation of autophagy in GPR109A deficient cells, where normal IFN production was restored. Finally, nicotinic acid induced the formation of autophagosomes in GPR109A dependent manner, and further increased the release of IFN, which contributed to the anti-VSV infection, implying the clinical potential of GPR109A in antiviral infection treatment.

Keywords : GPR109A, viral infection, ISGs, IFN-I, autophagy

Abbreviations

GPCRs: G protein-coupled receptors; ISGs: Interferon-stimulated genes; PAMPs: Pathogen-associated molecular patterns; pDCs: plasmacytoid dendritic cells; ROS: Reactive oxygen species; JAK/STAT: Janus kinase signal transducer and activator of transcription; ATG5: Autophagy protein 5; IFN-α 2b: Inerferon-alpha 2b.

INTRODUCTION

The innate immune system serves as the initial line of defense against viral infections. During an infection, the host recognizes pathogen-associated molecular patterns (PAMPs) via pattern-recognition receptors (PRRs), triggering the activation of the type I interferon (IFN-I) pathway and initiating an antiviral immune response [1]. IFN-I consists of multiple interferon alpha (IFNα) subtypes and one interferon beta (IFNβ) subtype [2]. Due to the intricate role of IFN-I to keep a balanced immunity, complex signaling pathways exist to regulate the respective expression. As a DNA or RNA virus has been sensed, PRRs soon induce the activation of a series of signaling molecules such as TBK1, MAVS, STING, TRIF, and transcription factors IRF3, IRF7, NF-κB in cells to induce the

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expression of IFN-I. These secreted IFN-I ultimately induce the expression of hundreds of interferon-stimulated genes (ISGs) by activating transcription factors represented by STAT1 and STAT2. These ISGs play an important role in regulation of antiviral immunity during different stages of viral infection [3]. Even though sufficient IFN-I expression would take an effect promptly during viral defense by quickly triggering apoptosis of virus-infected cells, excessive IFN-I may lead to tissue damage or immune disorders [4-6]. Therefore, IFN-I production during viral infection has to be controlled precisely in a spatial and temporal manner to initiate an appropriate immune response to defend against the invading virus without damaging the tissue.

G protein coupled receptors (GPCRs) are a receptor superfamily with seven transmembrane structures, form the largest family of receptors involved in cellular signaling , and can sense many extracellular stimuli, such as hormones, ions, neurotransmitters, amino acids, and even light [7,8]. Emerging evidence suggest that GPCRs are closely related to the anti-viral immunity, such as extracellular danger signal UDP that activates P2Y6 to promote the release of IFNβ to resist viral infection [9]. In addition, there are signal cross-talk between pattern recognition receptors TLR and GPCR downstream signaling pathways [10]. Moreover, many chemokine receptor GPCRs were found as a leading factor during viral infection [11]. GPR109A was identified as a niacin receptor [12], expressed in adipose tissue [13,14] and myeloid derived immune cells, such as neutrophils, macrophages and DCs [15-17]. As a classis GPCR, GPR109A couples to Gαi/o [18], which acts as an anti-inflammatory regulator. Niacin, as an agonist of GPR109A, effectively inhibits the occurrence of enteritis and intestinal cancer through activation of GPR109A [19]. However, the functions of niacin/GPR109A in host defense against viral infection remains unclear.

The viral defense could achieved through autophagy, an ongoing biophysiological process in the cell infected with pathogens, which were captured and delivered from autophagosomes to lysosomes for degradation [20]. In addition to maintaining homeostasis of protein turnover, autophagy is also a key part of the innate immune defense mechanism, such as xenophagy, which can selectively eliminate intracellular microorganisms. Previous study showed that plasmacytoid dendritic cells (pDCs) detect viruses through TLR7 in an autophagy process dependent [21]. Moreover, autophagy deficiency have been reported to accumulate cellular reactive oxygen species (ROS)[22]. In addition, autophagy lessen the number of virus-infected cells and limit virion spreading by delaying apoptosis [23]. The current body of research on GPCR as a regulator of antiviral activity through modulation of cellular autophagosomal biology remains limited. Here, we demonstrated an antiviral role of nicotinic/GPR109A axis in inducing type I IFN release through induction of autophagy, suggesting that GPR109A could serve as a potential clinical target for treating viral infections.

RESULTS

GPR109A is an interferon-stimulated gene

To screen valid candidate functional GPCRs involved in anti-viral immune responses, exogenous IFNα was administered to induce human PBMC-derived macrophages. The transcriptome profile of IFNα-induced human PBMC-derived macrophages by gene chips revealed that hundreds of genes were up-regulated, including many reported ISGs, and HCAR2 (GPR109A) ranked as a prominently featured 11th place within all the upregulated genes that had been sequenced (Fig. 1A). Furthermore, the results of differential screening analysis of GPCRs suggested that, GPR109A ranked as the first place in the up-regulated GPCRs profile with a nearly 40 folds change (Fig. 1B). In consistent with the result, GPR109A was up-regulated in IFNβ and IFNγ stimulated mouse peritoneal macrophages (PEM) (Fig. 1C-E). Furthermore, we also found that VSV, WSN33 and HSV-1 could stimulate the GPR109A expression (Fig. 1F and G). In consistent, the expression of GPR109A was downregulated by blocking type I IFN signaling using IFNAR antibody (Fig. 1H). Type I interferons could activate transcription factors of the signal transducer and activator of transcription (STAT) family, initiating synthesis of ISGs24-26. Fludarabine is a typical STAT1 activation inhibitor that effectively inhibits GPR109A expression induced by IFNβ and IFNγ stimulation (Fig. 1I and J), indicating that IFN induces GPR109A expression in a STAT1-dependent manner. Collectively, these data demonstrated that GPR109A serves as an interferon-stimulated gene, and is activated in an IFN/STAT1 signaling pathway-dependent manner.

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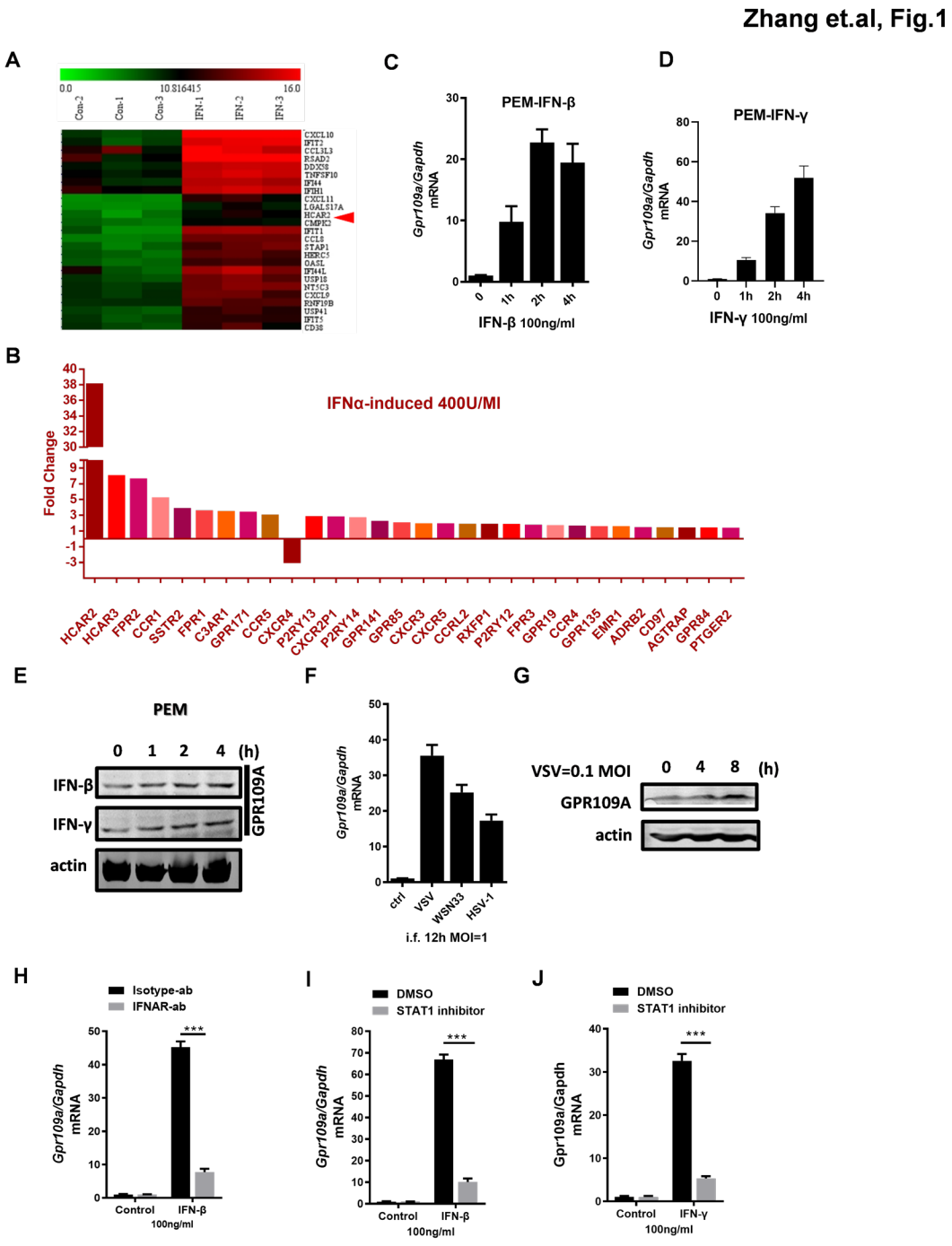
Figure 1. Gpr109a is an interferon-stimulated gene.



A-B, Human PBMC-derived macrophage was inoculated into a six-well plate at a cell density of 3 million per milliliter, and stimulated for 2.5 h by IFNα2b (400 U/mL), RNA was extracted and determined by gene chip. (A) Heap map showed significant differences in gene expression of control group (unstimulated) compared with IFNα2b - stimulated macrophages (n = 3). (B) Differentially expressed GPCRs are analyzed, and the top 25 GPCRs with the greatest differences are showed. (C-D) Mouse peritoneal macrophages were treated with IFNβ (100ng/mL) (C) and IFNγ (100ng/mL) (D) for the indicated time, Gpr109a mRNA expression was normalized to Gapdh in control untreated cells. (E) The expression of GPR109A detected by immunoblotting after stimulation by IFNβ and IFNγ. (F) Gpr109a mRNA was quantified in the presence of VSV□WSN33 and HSV-1 infection at an MOI = 1 for 12 h. (G) Peritoneal macrophages were infected with VSV (MOI, 0.1), the expression of GPR109A was analyzed by immunoblotting. (H) Relative mRNA levels of Gpr109a in the presence or absence of recombinant murine IFNβ and/or IFNAR antibody (IFNAR-ab) in peritoneal macrophages.(I) mRNA levels of Gpr109a in the presence or absence of IFNβ/IFNγ and/or STAT1 inhibitor Fludarabine (25 μM). Data are shown as mean ± SD, and the experiment was performed three times and a representative example is shown. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and determined by unpaired Student's t-test. NS, not significant.

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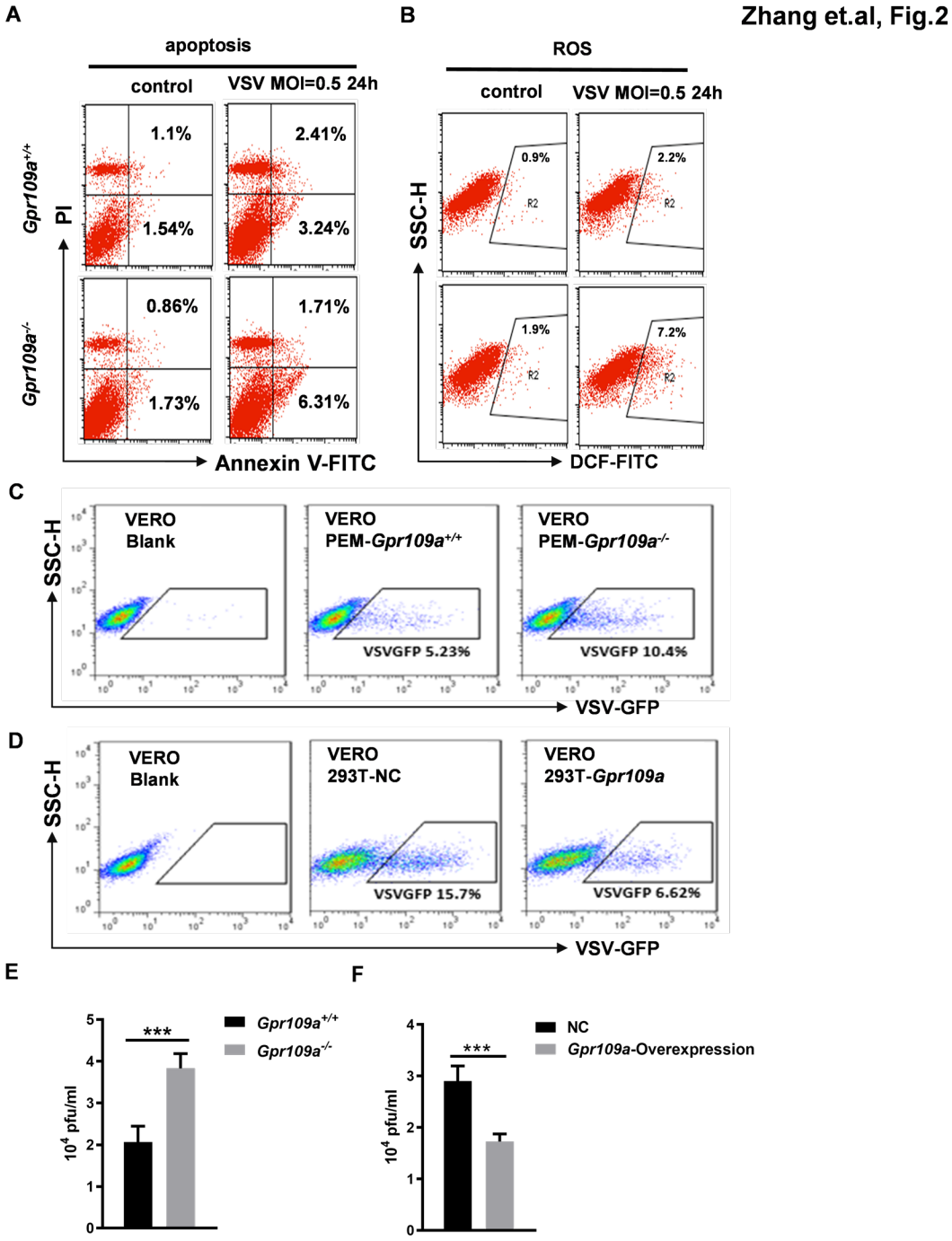
GPR109A deficiency promoted cell apoptosis following virus infection

The apoptotic cells were initially evaluated at 24 h post-infection, as viruses predominantly induce cell death through apoptosis. Increased number of apoptotic cells was observed from Gpr109a-/- PEMs compared with WT PEMs (Fig. 2A). Furthermore, GPR109A deficiency could increase the production of ROS in normal and VSV challenged PEMs (Fig. 2B). In VSV-GFP-challenged PEMs and 293T cells, we found that the virus infection was significantly increased in Gpr109a-/- PEMs (Fig. 2C), and that the overexpressed GPR109A reduced viral infection in 293T cells (Fig. 2D), which was confirmed by the elevated virus titers in Gpr109a-/- PEMs (Fig. 2E), and reduced virus titers in GPR109A overexpression 293T cells (Fig. 2F). Collectively, these data suggest that both cellular apoptosis and viral infection had been prominently promoted in the GPR109A deficient cells.

Figure 2. GPR109A deficiency induced cell apoptosis after virus infection.



(A) GPR109A+/+ and GPR109A-/- mouse peritoneal macrophages were infected with VSV (MOI, 0.5) for 24h, and the percentage of early apoptotic cells and late apoptosis were analyzed by flow cytometry. (B) GPR109A+/+ and GPR109A-/- mouse peritoneal macrophages were infected with VSV (MOI, 0.5) for 24h, ROS levels in cells was measured using DCFH-DA, and representative and quantitative flow cytometry values depicting percentages of DCF. (C) GPR109A+/+ and GPR109A-/- PEMs were infected with VSV-GFP viruses (MOI, 0.5), changed fresh

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medium after 4h infection and cultured for 24h. After 24 h, the viral progeny supernatant was used to infect VERO cells, and the infection rate of virus released in the PEMs supernatant was evaluated by flow cytometry.(D) 293t (overexpressing GPR109a and control) cells were infected with VSV-GFP (MOI, 0.5), changed fresh medium after 4h infection and cultured for 24 h.. After 24 h, the infection rate of virus released in the 293T supernatant was evaluated by flow cytometry. (E) GPR109A+/+ and GPR109A-/- PEMs were infected with VSV-GFP viruses (MOI, 0.5), changed fresh medium after 4h infection and cultured for 24h. After 24 h, the viral progeny supernatant was used to infect VERO cells, and titers of viral progeny released in PEM supernatants were evaluated by plaque assay. (F) 293t (overexpressing GPR109a and control) cells were infected with VSV-GFP (MOI, 0.5), changed fresh medium after 4h infection and cultured for 24 h. After 24 h, the viral progeny supernatant was used to infect VERO cells, and titers of viral progeny released in 293t supernatants were evaluated by plaque assay. Data are shown as mean ± SD, and the experiment was performed three times and a representative example is shown. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and determined by unpaired Student's t-test. NS, not significant.

GPR109A positively regulates host defense against viruses

In order to further validate the in vivo antiviral function of GPR109A, we conducted intraperitoneal injections in mice to assess virus infection and replication. After VSV infection, an elevated number of infected cells and VSV were observed in thoracic cavities of GPR109A deficient mice (Fig. 3A and Supplementary Fig. 1A). Congruently, higher VSV replication was found in peritoneal, spleen, and liver of GPR109A deficient mice compared to the WT mice (Fig. 3B-D). Likewise, higher VSV concentration was detected in the spleen (Fig. 3E). In corresponding to the evidence of increased viral infection in multiple organs, mesenteric abnormal congestion was more severe in GPR109A deficient mice than the WT mice after 24h viral infection (Supplementary Fig. 1B). Above results suggested that GPR109A plays a significant role in conferring resistance to viral infections in the mouse model. GPR109A deficiency markedly reduced the survival of viral infection mouse model (Fig. 3F), and the pathological analysis of lung using HE staining also revealed that GPR109A deficient mice exhibited more severe lung lesions compared to WT mice, accompanied by evident infiltration of inflammatory cells (Fig. 3G). Therefore, GPR109A deficiency promoted viral infection and deteriorated the virus-induced tissue damage and mortality.

Supplementary figure 1. GPR109A deficiency aggravated viral infection and inflammation.

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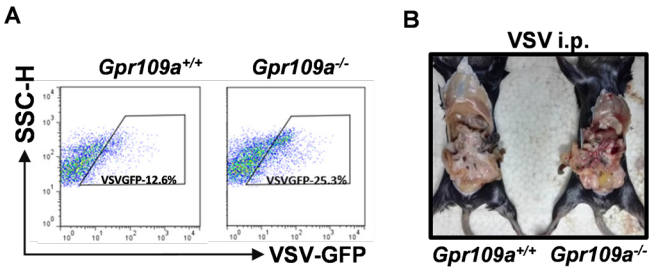

A and B, Gpr109a+/+ and Gpr109a-/- mice were infected with VSV-GFP (1×10^8 pfu/mouse) intraperitoneally for 24 hours. The percentage of VSV-GFP positive cell in peritoneal cells was analyzed by flow cytometry (A), and mesenteric bleeding in mice were shown (B).

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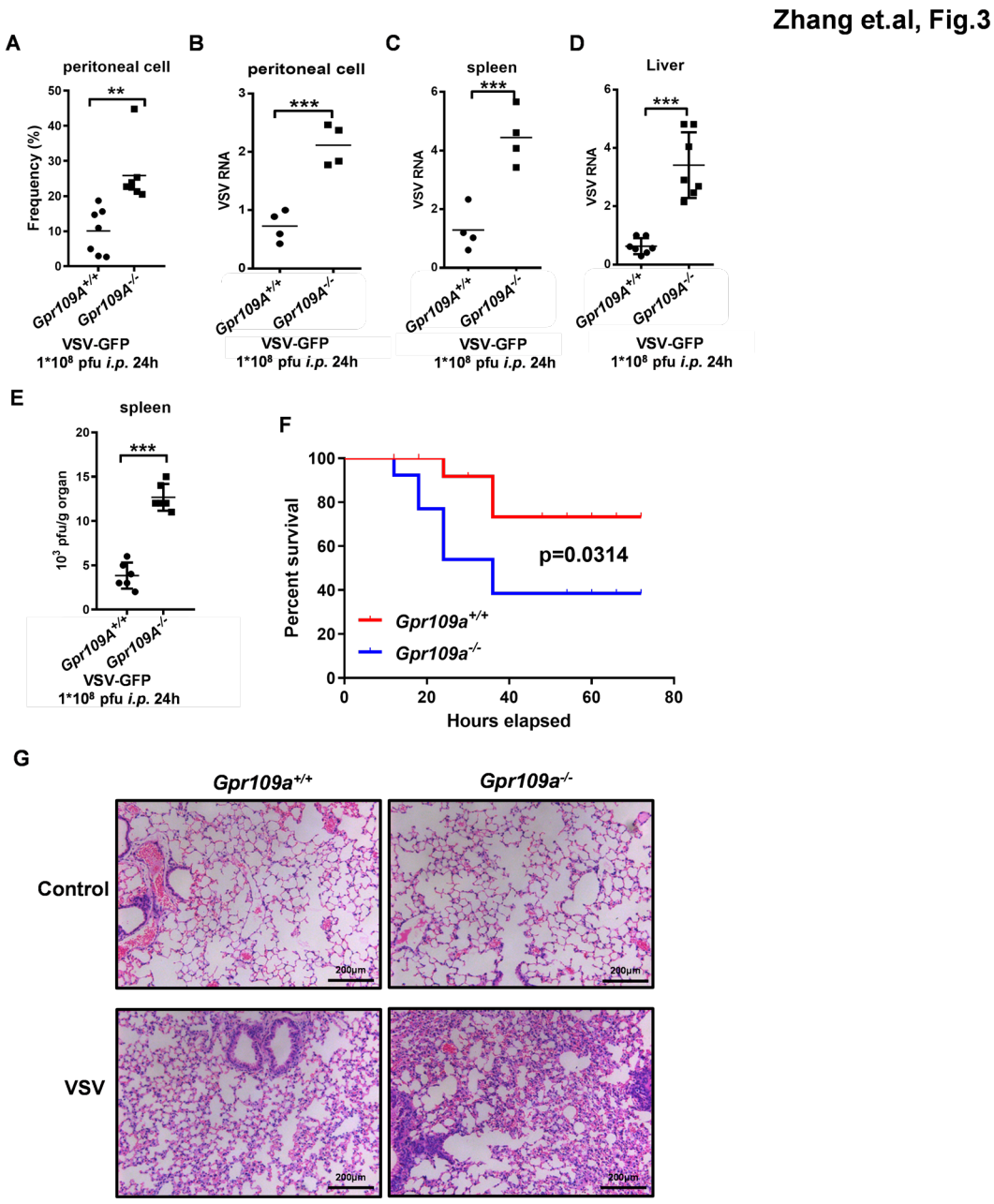
Figure 3. GPR109A positively regulates host defense against viruses.



A-G, Gpr109a+/+ and Gpr109a-/- mice were infected with 1×10^8 pfu/mouse VSV-GFP intraperitoneally for 24 h. (A) GFP expression frequency of peritoneal cells was detected by flow cytometry. B-D, qPCR analysis of VSV RNA replicates in the peritoneal cells (B), spleen (C), and liver (D) from Gpr109a+/+ and Gpr109a-/- mice infected with VSV intraperitoneally for 24 h. (E) Gpr109a+/+ and Gpr109a-/- mice were infected with 1×10^8 pfu VSV-GFP intraperitoneally for 24 h. Virus titers of spleen was measured by VERO cell plaque assay. (F) Gpr109a+/+ and Gpr109a-/- mice were infected with 0.2×108 pfu VSV /g (n=8), survival rate of Gpr109a+/+ and Gpr109a-/- mice was recorded (Log-rank (Mantel-Cox) test). (G) Hematoxylin and eosin staining of lung sections from mice in (E). Data are shown as mean ± SD, and the experiment was performed three times and a representative example is shown. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and determined by unpaired Student's t-test. NS, not significant.

GPR109A deficiency influences ISG-related cellular antiviral response

In order to further investigate the role of GPR109A in the modulation of inflammation induced by viral infection. The distribution of pro-inflammatory peritoneal cells were assessed by flow cytometry in VSV-challenged mouse model. GPR109A deficiency evidently increased the number of monocytes and neutrophils both in peritoneal (Fig. 4A and B) and spleen of mice following virus infection (Supplementary Fig. 2A and B). The expression of pro-inflammatory molecules, such as CCL2 (Fig. 4C), CXCL2 (Fig.

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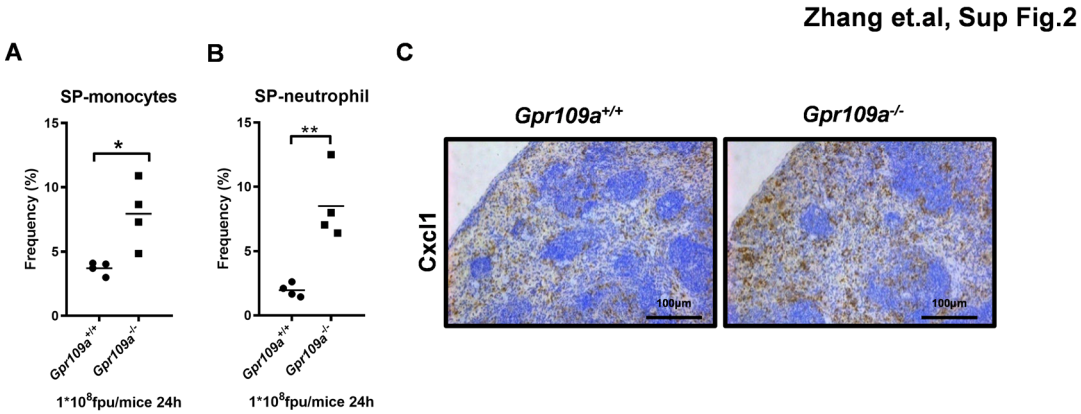
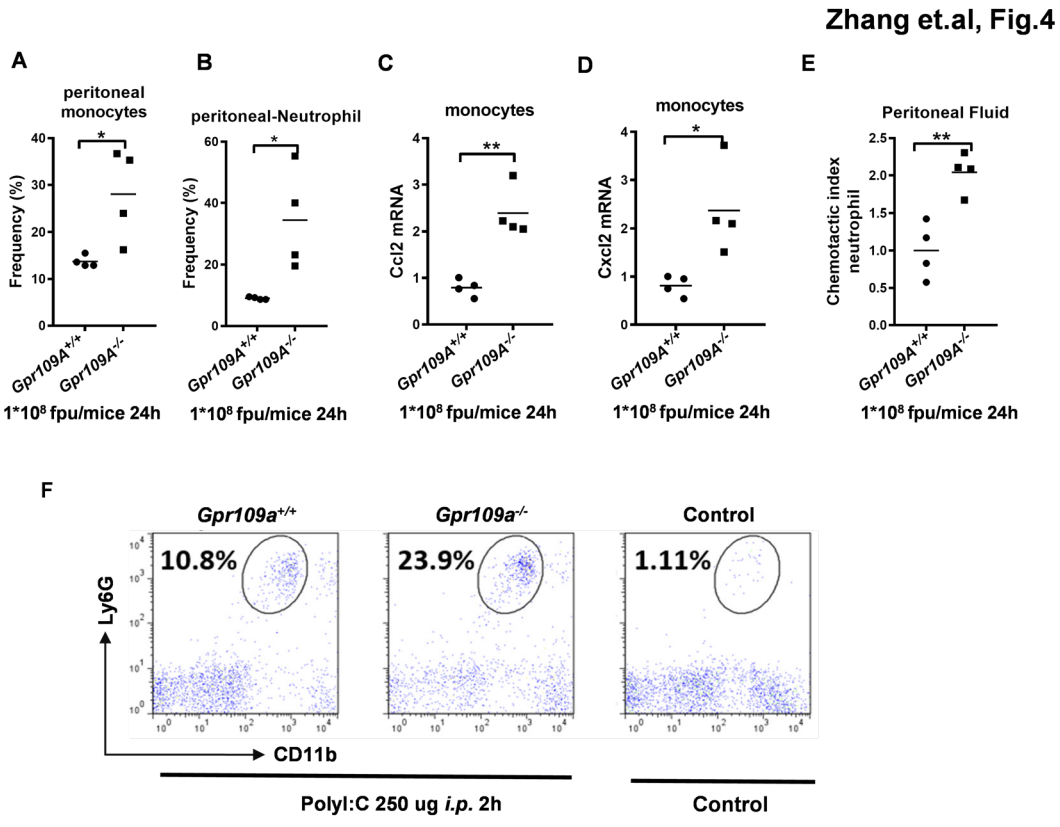
4D), and CXCL1 (Supplementary Fig. 2C), were increased in Gpr109a-/- monocytes after viral infection in vivo. We also found that the chemotactic index of neutrophils of the peritoneal fluid from the GPR109A deficient mice was markedly upregulated post-infection (Fig. 4E). A higher proportion of neutrophils was detected in GPR109A deficient mice than in WT mice, after the stimulation with 250 μg PolyI:C (Fig. 4F). The findings suggest that GPR109A plays a pivotal role not only in antiviral infection, but also in modulating the expression of chemokines and the recruitment of pro-inflammatory cells during viral infection, thereby attenuating the inflammatory response elicited by viral infection.

Supplementary figure 2. GPR109A deficiency aggravated viral infection in peritoneal monocytes and neutrophils, and increased CXCL1 expression of spleen.



A-C, Gpr109a+/+ and Gpr109a-/- mice were infected with VSV (1×10^8 pfu/mouse) intraperitoneally for 24 hours. The percentage of peritoneal monocytes (A) and neutrophiles (B) in spleen were analyzed by flow cytometry. (C) Representative images of immunohistochemistry staining of spleen for CXCL1 were shown.

Figure 4. GPR109A restricts infection by regulation of proinflammatory chemokines



A-E, Gpr109a+/+ and Gpr109a-/- mice were infected with 1×108 pfu/mouse VSV intraperitoneally for 24 h. (A) The frequency of peritoneal monocytes and peritoneal neutrophils (B) were analyzed by flow cytometry. C and D, qPCR analysis of Ccl2 (C) and Cxcl2 (D) expression in

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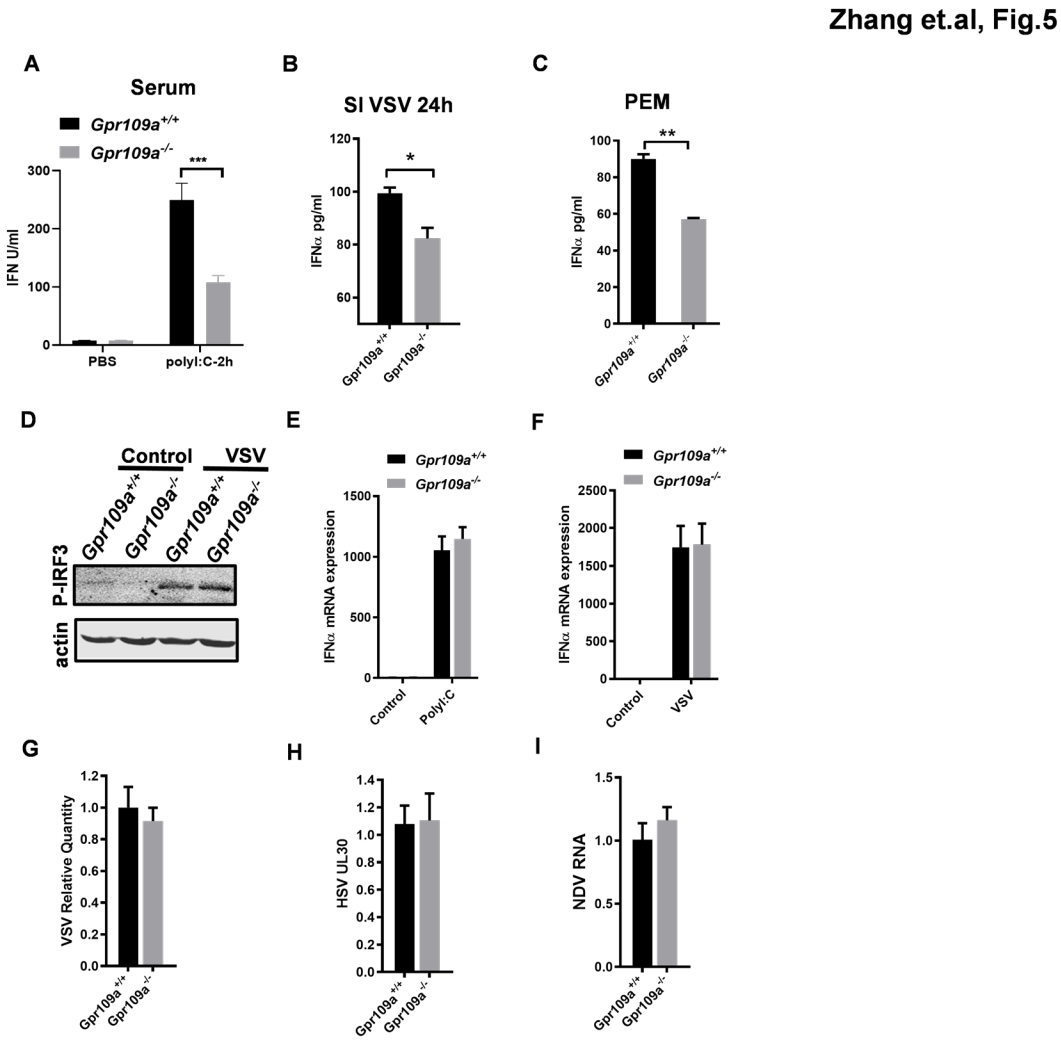
the peritoneal monocytes. (E) Chemotactic index of neutrophils in peritoneal fluid was detected after VSV infection for 24h. (F) Gpr109a+/+ and Gpr109a-/- mice were injected with 250 μg PolyI:C/mouse intraperitoneally for 2 h, percentage of neutrophils (CD11b+Ly6G+ cells) in the peritoneal cavity cells of the mice were detected by flow cytometry. Data are shown as mean ± SD, and the experiment was performed three times and a representative example is shown. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and determined by unpaired Student's t-test. NS, not significant.

GPR109A deficiency did not influence the early phase of the viral infection and the activation of IFN signaling

To further investigate the involvement of GPR109A in the modulation of interferon expression. Gpr109a+/+ and Gpr109a-/- mice were injected with PolyI:C (250 μg/mouse) intraperitoneally for 2h, and found that the concentration of IFN in serum Gpr109a-/- mice blood was lower than in wild type mice (Fig. 5A). Furthermore, GPR109A deficiency significantly reduced the viral infection induced IFNα expression in small intestine endothelial cells and PEM cells (Fig. 5B and 5C). Previous study demonstrated that the activation of IRF3 is always observed in IFN expression 27. However, there was no significant difference in phosphorylated IRF3 between Gpr109a-/- macrophage and WT macrophage after VSV infection (Fig. 5D). We also found that GPR109A deficiency did not influence the expression of IFNα in PEMs after short-term (6h) stimulation by VSV or polyI:C (Fig. 5E and F). In addition, GPR109A deficiency did not influence the virus replication after short-term challenge for PEMs (6 h) using VSV, HSV and NDV viruses (Fig. 5G-5I). Therefore, above data confirm that GPR109A does not affect the activation of antiviral signaling pathways.

Figure 5. GPR109A deficiency did not influence the early phase of the viral infection and the activation of IFN signaling.



(A) Gpr109a+/+ and Gpr109a-/- mice were injected with PolyI:C (250 μg /mouse) intraperitoneally for 2h. The concentration of IFN of serum was measured by enzyme-linked immunosorbent assay (ELISA) and IFN activity test (i.e. VSV bioactivity test). (B) Gpr109a+/+ and Gpr109a-/- mice were infected with VSV (1x108 pfu/mouse) intraperitoneally for 24 h. The concentration of IFNα of small intestine was measured by enzyme-linked immunosorbent assay (ELISA). (C) Peritoneal macrophages were harvested from Gpr109a+/+ and Gpr109a-/- mice, and infected with

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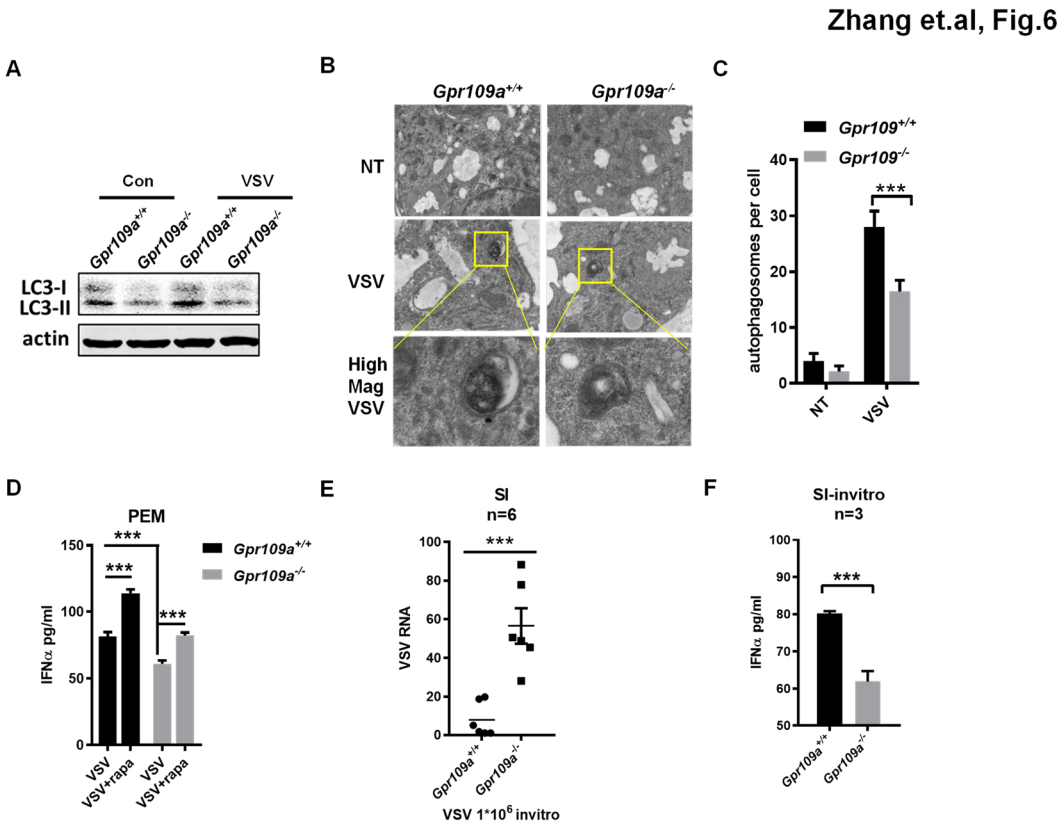
VSV (MOI, 0.1) for 12h, the concentration of IFN in cell cultures was measured by ELISA. (D) Gpr109a+/+ and Gpr109a-/- PEMs were infected with VSV (MOI, 1) for 6 h, the expression of phosphorylated IRF3 (p-IRF3) and β-actin were detected by Western blotting. (E) Gpr109a+/+ and Gpr109a-/- PEMs were transfected with Polyl:C (1 μg), after 6 h the expression of IFNα was analyzed by qPCR. (F) Gpr109a+/+ and Gpr109a-/- PEMs were stimulated by VSV (MOI, 1) for 6h, the expression of IFNα was analyzed by qPCR. G-I, Gpr109a+/+ and Gpr109a-/- PEMs infected with VSV (MOI, 0.5), HSV (MOI, 0.5) and NDV (MOI, 0.5) viruses for 8h (Short-term infection), VSV (G), HSV (H) and NDV (I) RNA replication were analyzed by qPCR. Data are shown as mean ± SD, and the experiment was performed three times and a representative example is shown. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and determined by unpaired Student's t-test. NS, not significant.

GPR109A deficiency inhibits autophagy-mediated IFN release

Emerging evidences suggested that ISGs and IFN-I can regulate antiviral immunity through autophagy 28. The LC3-I and LC3-II are the most widely adopted indicators to monitoring autophagy 29. We found that both expressions of LC3-I and LC3-II were significantly reduced in GPR109A deficient PEMs (Fig. 6A). In addition, autophagosomes within cells were quantified using transmission electron microscopy after viral infection. In consistent to our hypothesis, less autophagosomes in GPR109A deficient PEMs were assessed (Fig. 6B and C). Furthermore, we observed that GPR109A deficiency decreased IFNα release was rescued by rapamycin (autophagy agonist), indicating that GPR109A deficiency reduced IFNα release was dependent on autophagy (Fig. 6D). GPR109 is highly expressed in mouse and human small intestine tissue 30,31. Therefore, the virus replication and IFNα production of intestine tissue were measured after virus infection. Higher VSV replication and lower IFNα production were observed in the intestine tissue of viral infected GPR109A deficient mice (Fig. 6E and F). Together, above data demonstrate that GPR109A deficiency reduces IFNα release by inhibiting autophagy production and further promote viral infection.

Figure 6. GPR109A deficiency inhibits autophagy-mediated IFN release.



(A) Gpr109a+/+ and Gpr109a-/- PEMs were infected with VSV (MOI, 0.5) for 12h, the expression of LC3-I and LC3-II were detected by Western blotting. B and C, Gpr109a+/+ and Gpr109a-/- PEMs were infected with VSV (MOI, 0.5) for 12h, the autophagosomes were photographed (B) and quantitated (C) using transmission electron microscopy. (D) Gpr109a+/+ and Gpr109a-/- PEMs were treated with rapamycin (RAPA, 20μg/ml) for 2h, and following challenged with VSV (MOI, 0.5) for 12h,, the concentration of IFNα in the cell culture was measured by ELISA. E and F, Intestinal tissues (0.5 cm2) from Gpr109a+/+ and Gpr109a-/- Mouse were minced into small pieces, and infected with VSV (1×106pfu) for 8h, VSV RNA replication was analyzed by qPCR (E), the concentration of IFNα in the tissue culture supernant was measured by ELISA (F). Data are

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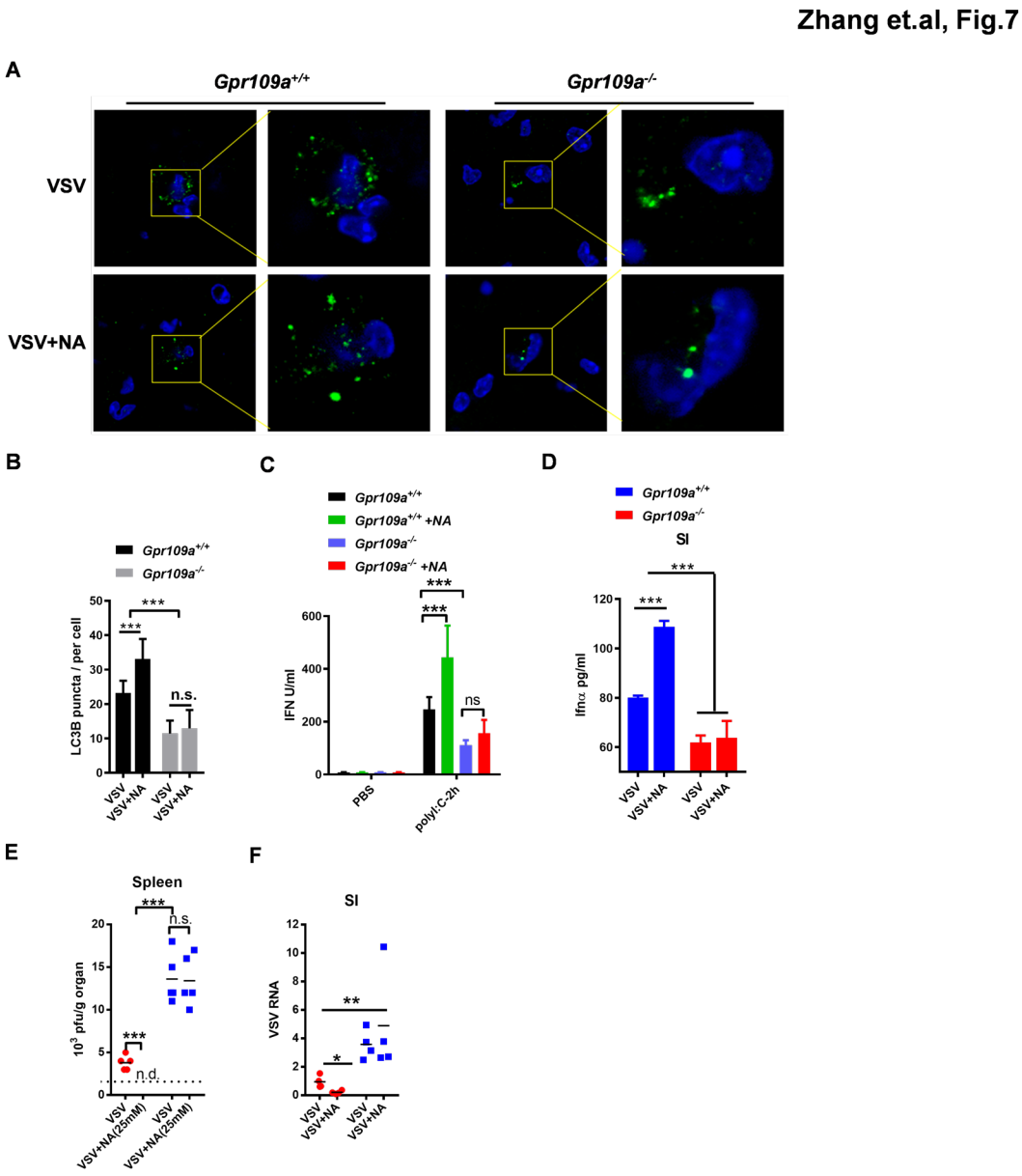
shown as mean ± SD, and the experiment was performed three times and a representative example is shown. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and determined by unpaired Student's t-test. NS, not significant.

Nicotinic acids promote the release of interferons and inhibit viral infection through GPR109A

We further challenged PEMs with VSV and found that GPR109A deficiency decreased the expression of LC3-II. However, the GPR109A agonist nicotinic acids (1mM) could promote the expression of LC3-II in a GPR109A dependent manner (Fig. 7A and B). In addition, nicotinic acids significantly promoted the release of IFN in the serum of WT mice but not in the serum of Gpr109a-/- mice after PolyI:C stimulation (Fig. 7C). Similar outcomes were also observed in intestine tissue after VSV infection (Fig. 7D). Moreover, nicotinic acids could reduce the virus titer of spleen and VSV replication of intestine tissue in WT mice, but not Gpr109a-/- mice (Fig. 7E and F). Therefore, above data suggest that nicotinic acids promote autophagy and further resist viral infection through GPR109A.

Figure 7. Nicotinic acid promote the release of interferons and inhibit viral infection through GPR109A.



A and B, Gpr109a+/+ and Gpr109a-/- PEMs were challenged with VSV (MOI, 0.5) and nicotinic acid (1 mM) for 12h. Representative images of immunofluorescence staining for LC3 (A), and the expression of LC3 per cells (B) were quantified. (C) The Gpr109a+/+ and Gpr109a-/- mice were fed a diet containing 25 mM nicotinic acid (NA) for 2 weeks, then treated with PolyI:C (i.p.250 μg/mouse) for 2h, the concentration of IFNα in the serum was measured by ELISA (n=5). (D) Intestinal tissues (0.5 cm2) from Gpr109a+/+ and Gpr109a-/- mouse were minced into small pieces, and treated with VSV (1×106pfu) and nicotinic acid (1 mM) for 8h, the concentration of IFNα in the supernatants from intestinal

tissue culture medium was measured by ELISA. (E) The Gpr109a+/+ and Gpr109a-/- mice were fed a diet containing 25 mM nicotinic acid (NA) for 2 weeks, following infected with VSV (1 × 108 pfu/mouse) for 24h. The spleen were grinded and centrifugated, virus titers in the grinding solution were measured by VERO cell plaque assay (E). (F) Intestinal tissues (0.5 cm2) from Gpr109a+/+ and Gpr109a-/- mouse were minced into small pieces, and treated with VSV (1×106pfu) and nicotinic acid (1 mM) for 8h, and virus RNA replication was analyzed by qPCR.Data are shown as mean ± SD, and the experiment was performed three times and a representative example is shown. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and determined by unpaired Student's t-test. NS, not significant.

DISCUSSION

ISGs, known as anti-viral and anti-bacterial genes, potently resist the virus life cycle during viral infection. However, the function of GPCRs as part of functional antiviral ISGs to resist viral life cycle has been rarely studied in the past. Here, we demonstrated that GPR109A belongs to ISGs, and that nicotinic acids/GPR109A axis promotes the expression of IFN to defense viral infection in an autophagy-dependent manner. GPR109A deficiency reduced the production of antiviral factors, thereby weakening the host's antiviral immunity. Being a bioactive ligand of GPR109A, nicotinic acids, could increase the release of IFN through a rescue of autophagosome formation in cells, thereby resisting viral infection in vivo and in vitro.

After interferon binds to the interferon receptor on the cell surface, a signaling cascade response is quickly initiated, particularly through the JAK/STAT signaling pathway (Janus kinase signal transducer and activator of transcription), where it transcriptionally regulates hundreds of downstream interferon-stimulated genes (ISGs) that exert direct antiviral effects32. Thus, the mechanism of type I interferon to kill viruses, is to regulate the antiviral genes. Moreover, different stage of virus life cycle can theoretically be manipulated as a target for ISGs intervention. However, most of the known ISGs are antiviral enzymes and membrane-localized proteins 32. The medicinal prospects of these known ISGs are not clear, because these ISGs originally play their own roles in cells, and targeting them will cause changes in the homeostasis of the cells themselves. Here, gene chips analysis revealed that GPR109A as the top ranked GPCRs in IFN-induced macrophage derived from PBMC. In addition, both interferon and virus can induce the expression of GPR109A, indicating that GPR109A is an ISG. Previous studies regarding the niacin/GPR109A signaling are primarily focusing on the attenuation of colonic inflammation and colonic cancer 18. However, the role of GPR109A in antiviral immunity remains unclear. Here, we showed that niacin inhibited the viral replication in vivo and in vitro by activating GPR109A, thereby significantly prolonging the survival of lethal infection mouse model. The high expression of GPR109A in immune cells and the intestines also presents novel therapeutic possibilities for localized enterovirus infections, such as seasonal outbreaks of norovirus, which are responsible for enteritis, diarrhea, and other related ailments. Oral niacin administration may potentially activate GPR109a. Additionally, the small intestine releases interferon (IFN) to combat viral effects.

Autophagy plays an important role in cell differentiation and development, and clearance of invading pathogens and starvation, and viral infection 33 and is also a part of host stress responses 20,34,35. Preliminary studies showed that autophagy protein 5 (ATG5) deficiency in plasmacytoid dendritic cells (pDCs) caused reduction in TLR7-dependent IFN expression in VSV-viral infection 21. Also, metabolism receptor GPCRs are found involved in regulation of biophysiological activities of autophagy in cells. Furthermore, emerging studies showed that some Gαi subunits also play an important role in the regulation of autophagy 36. Insulin inhibited anti-autophagic effect was abrogated in Gαi3 mice 37. And the impact of autophagy on viral replication remains uncertain and is contingent upon various factors, including the cell types being infected, the strains of the virus involved, and the specific conditions of infection 38. As a classic GPCR, GPR109A couples to Gαi, resulting in controlling inflammation in various and organs 18. Furthermore, GPR109A also is a member of the metabolic receptor family and belongs to carboxylic acid metabolism receptor. However, the role of GPR109A and its ligand in regulation of autophagy remains unknown. Herein, we confirmed that viral replication accelerated in GPR109A deficient PEMs, which was achieved through impaired formation of autophagosomes. Meanwhile, after inducing autophagy in GPR109A deficiency PEMs using the autophagy activator rapamycin, we observed a restoration of IFN content reduction caused by deletion of GPR109A and a recovery to normal cell levels. Furthermore, niacin significantly promoted the release of IFN in the serum of WT mice but not in the serum of Gpr109a-/- mice after infection. Moreover, niacin could promote autophagy and resist the viral replication in a GPR109A dependent manner, suggesting that GPR109A may be a potential target for antiviral therapy.

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MATERIALS AND METHODS

Reagent

Table

REAGENT	SOURCE	Catalog No.
Inerferon-alpha 2b(IFN-α 2b) human	ExCell Bio	CB055-0610
Mouse IFN-alpha protein	Sino Biological	50524-M08Y
Mouse IFN-beta/IFNB1 Protein	Sino Biological	50708-MCCH
GPR109A rabbit antibody	Santa Cruz Biotechnology	sc-377292
Fludarbine (STAT1 inhibitor)	Selleck	S1491
Nicotinic acid	SIGMA-ALDRICH	N4126
Agarose, low gelling temperature	SIGMA-ALDRICH	A9045
Annexin V:FITC apoptosis detection kit1	eBioscience	BMS500FI/300
Carboxy-H2DCFDA (General Oxidative Stress Indicator)	Thermo Fisher Scientific	C400
Anti-LC3A/B (D3U4C) XP® Rabbit mAb	Cell Signaling Technology	12741
Anti-p-IRF-3 (Ser396)	Cell Signaling Technology	4947
Anti-β-Actin (8H10D10) Mouse mAb	Cell Signaling Technology	3700
Anti-CD11b Mouse, PE FCAb	Thermo-eBioscience	12-0112-82
Anti-Ly-6G Mouse, FITCFCAb	Thermo-eBioscience	11-9668-82
Anti-Ly-6C Mouse, Alexa Fluor® 488 FCAb	Thermo-eBioscience	53-5932-80

Mice

Age-matched Gpr109a-/- mice on C57BL/6 background were a kind gift obtained from Professor Stephan Offermans (Max Plank institute, Bad Nauheim) and have been described39. All mice were bred in our own specified pathogen-free facilities and used at the age of 8-12 weeks. All animal experiments were performed with the approval of the Scientific Investigation Board of East China Normal University (M20150401).

Cell Culture

Gpr109a-/- peritoneal cells and their littermate WT controls were generated from age of 8-10 weeks mice 4 days after thioglycollate broth (Sigma) injection and maintained in complete DMEM medium. All cells were grown in 37°C incubator supplied with 5% CO2.

Virus Infection

For VSV in vivo infection, mice were infected i.p. with 108 pfu VSV (Indiana 1 serotype) for 24 h. For VSV in vitro intestinal infection, intestine tissue (0.5cm *0.5cm) were infected with 106 pfu VSV for 8 h. Male (50%) and female (50%) mice both were used in the mouse model. Mouse peritoneal macrophages were infected with MSN33 (influenza viruses; MOI = 1) in vitro for 12 h. Mouse peritoneal macrophages were infected with HSV-1 (herpes simplex virus type 1; MOI = 1) in vitro for 12 h.

Determination of virus titers

VSV titers were determined in a standard VSV plaque assay40. VSV load in spleen was determined on 24 h post infection using a previously described method40,41. Briefly, organs were weighed and pestle/tube-homogenized in 1 ml of PBS per gram, and VSV was titered in 10 fold serial dilutions on Vero cells by plaque assay. Results are expressed as plaque-forming units (pfu) per gram of tissue. For quantification of infectious VSV yields, cells were infected with VSV inoculum for 1 h, then removed uninfected virus by washing with PBS and cultured another 24 h, cell supernatants were collected and determined VSV yields by plaque assay on Vero cells.

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Cell isolation and flow cytometry

Splenocytes were isolated and stained for cell surface markers using fluorescence-conjugated antibodies that do not overlap as previously described42. FACSCalibur (BD Biosciences) was used for flow cytometry and data were analyzed with CellQuest software (BD Biosciences) and FlowJo software (Tree Star, Inc).

Niacin Treatment

For niacin treatment, mice between 6–8 weeks of age treated with 25 mM niacin that was administered in drinking water for 3 days before viral infection. Control groups of mice received water. The mice remained on niacin treatment during viral infection. Male (50%) and female (50%) mice both were used in the mouse model.

Quantitative Real-Time PCR

The total RNA from cells or tissues was extracted using TRIzol (TaKaRa), and the concentration of RNA was quantified using NanoDrop 2000 (Thermo Fisher Scientific). Subsequently, cDNA synthesis was performed using PrimeScript RT Master Mix (TaKaRa). Quantitative PCR analysis was conducted on the QuantStudio 3 Real Time PCR System (Applied Biosystems). The expression levels of each gene were normalized to GAPDH expression and presented as relative mRNA expression ($2^{-\Delta\Delta Ct}$) or fold change.

Immunofluorescence assay and Laser Confocal Imaging Analysis

WT or Gpr109a-/- peritoneal cells were seeded on 24-well glass slide. 12 h later, cells were incubated with VSV at the MOI of 0.5 for 1 h then removed the virus inoculum and replenished with complete medium for 12 h. Next, the cells were fixed with 4% paraformaldehyde containing 0.1% Triton X-100 in PBS for 20 min at room temperature. Then washed with PBS three times and blocked by 5% BSA for 1 h. The samples were stained with LC3B antibody (1:200 dilution) at 4°C overnight then incubated incubated for 1 h with Alexa Fluor 488 goat anti-rabbit antibody (1:2,000 dilution). After washing cells were satined with DAPI for 5 min. Confocal images were acquired using a Leica TCS SP5 confocal laser scanning microscope.

Western blot analysis

WT mouse peritoneal macrophages were treated with IFNβ (100ng/ml) and IFNγ (100ng/ml) for the indicated time. Gpr109a+/+ and Gpr109a-/- PEMs were infected with VSV (MOI, 0.5) for 12h. PEMs were harvested and lysed with radio immunoprecipitation assay (RIPA) buffer supplemented with complete Protease and Phosphatase inhibitor Cocktail. Cell lysates were separated by standard SDS-PAGE and analyzed by immunoblotting. The following antibodies were used: GPR109A, p-IRF3, LC3A/B, actin and appropriate fluorophoreconjugated secondary antibodies.

Apoptosis and ROS analysis

Cells were seeded on 24-well plates and treated with VSV at a MOI of 0.5 for 24 h. The apoptosis assays were conducted using the Annexin V: FITC apoptosis detection kit1. The measurement of ROS was performed by 2', 7'-dichlorofluorescein diacetate (DCFH-DA). Cells were incubated with DCFH-DA at 37 °C for 30 min. These assays were analysed by flow cytometry (FACSCalibur, BD).

Transmission Electron Microscopy

Peritoneal cells treated with virus were immediately fixed in 2.5% glutaraldehyde, washed with PBS (pH 7.4). The samples were treated with 1% osmium tetroxide in PBS and were sequentially dehydrated in graded alcohols and propylene oxide, followed by infiltration in Epon812 (SPI, 90529-77-4). Ultrathin sections of the tissue samples were made using a diamond knife (Daitome, Ultra 45°) and visualized with a transmission electron microscope (HITACHI).

Development of human macrophage from PBMCs

Collect fresh peripheral anticoagulant blood from healthy adults. Add 5ml of lymphocyte separation solution (Ficoll separation solution) into a 15ml centrifuge tube, thoroughly mix the heparin-containing anti-coagulant blood with an equal amount of RPMI1640 culture solution, and slowly add the blood along the tube wall to the upper layer of the lymphocyte separation solution using a pipette. Take care to avoid excessive force that may disrupt stratification and ensure clarity and distinctness in the separation layer. Then, above mix were centrifuged (2000rpm, 20 min) with a gradual acceleration and deceleration. The pipette was inserted into the mononuclear cell layer, and the mononuclear cells were aspirated and transferred to a new 15ml centrifuge tube. Subsequently, RPMI1640 culture medium was added in a volume five times that of the cells, followed by centrifugation at 1500rpm for 10 minutes to wash. After cell counting, the cell density was adjusted to 3×106/mL and inoculated onto a six-well plate. The plate was then placed in an incubator with suitable humidity at 37 °C and 5% CO2 for 3 h to allow attachment to the wall. Subsequently, preheated (37 °C) medium was utilized to wash away non-adherent cells. The cells were cultured in RPMI1640 medium supplemented with 2ml of 10% fetal bovine serum (FBS) and stimulated with human granulocyte-macrophage colony-stimulating factor (GM-CSF) at a final concentration of 1000 U/ml for 7 days. Human-PBMC derived macrophage was stimulated by IFNα2b (400 U/mL) for 2.5 h, and RNA was extracted and determined by gene chip.

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ETHICS STATEMENT

All animal experiments conformed to the regulations drafted by the Association for Assessment and Accreditation of Laboratory Animal Care in Shanghai and in direct accordance with the Ministry of Science and Technology of the People's Republic of China Animal Care guidelines. The protocol was approved by the East China Normal University Center for Animal Research (AR2013/08002). All surgeries were performed under anesthesia and all efforts were made to minimize suffering.

STATISTICAL ANALYSIS

Statistical significance between groups was determined by Student's t test. Differences were considered to be significant when $p < 0.05$.

Data Availability Statement

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplemental file.

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Author contributions

Experimental design, N.Z, Z.Z, J.Q, and B.D; manuscript preparation, N.Z, Z.Z, Q.W, J.Q and B.D; experiment execution, N.Z, Z.Z, J.Q, N.W, B.T; bioinformatics, X.L; data analysis, N.Z, Z.Z, Q.W, J.Q, and B.D.

Conflict of interest

We declare that we have no conflict of interest.

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