HHS Public Access

Author manuscript

Mucosal Immunol. Author manuscript; available in PMC 2023 July 10.

Published in final edited form as:

Mucosal Immunol. 2023 June ; 16(3): 302–311. doi:10.1016/j.mucimm.2023.03.004.

Novel neuroendocrine role of γ-aminobutyric acid and gastrin releasing peptide in the host response to influenza infection

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Abstract

Gastrin Releasing Peptide (GRP), an evolutionarily-conserved neuropeptide, significantly contributes to influenza-induced lethality and inflammation in rodent models. Since GRP is produced by pulmonary neuroendocrine cells (PNEC) in response to γ-aminobutyric acid (GABA), we hypothesized that influenza infection promotes GABA release from innervated PNECs that activate $GABA_B$ receptors on PNEC to secrete GRP. Oxidative stress was increased in lungs of influenza A/PR/8/34 (PR8)-infected mice, as well as serum glutamate decarboxylase 1 (GAD1), the enzyme that converts L-glutamic acid into GABA. Therapeutic administration of saclofen, a $GABA_B$ receptor antagonist, protected PR8-infected mice, reduced lung proinflammatory gene expression of CCR2, CD68, and TLR4, and decreased levels of GRP and HMGB1 in sera. Conversely, baclofen, a $GABA_B$ receptor agonist, significantly increased lethality and inflammatory responses. The GRP antagonist, NSC77427, as well as the $GABA_B$ antagonist, saclofen, blunted PR8-induced monocyte infiltration into the lung. Together, these data provide the first report of neuroregulatory control of influenza-induced disease.

Keywords

influenza; GABA; GRP; TLR4; ALI; HMGB1

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Author contributions: All authors contributed to the experimental design of this study. Specifically, KAS and WL carried out all mouse experiments. MES was responsible for the IHC staining. JCGB was responsible for histopathology and imaging. KAS, SNV, and FC oversaw the project. All co-authors participated in the writing of this manuscript.

Competing interests: The authors declare no competing interests.

INTRODUCTION

The crosstalk between the innate immune system and the peripheral nervous system plays an integral protective role in the host response by detecting and responding to both external threats such as pathogens as well as internal signals such as danger associated molecular patterns (DAMP)s and other inflammatory mediators[1,2]. The lungs are highly innervated with both autonomic and sensory neurons[3,4]. The sensory neurons interact with the immune system at mucosal surfaces such as in the lungs. Pulmonary neuroendocrine cells (PNEC) are rare epithelial cells that represent less than 1% of the lung epithelium[5]. PNECs function as chemosensors in the lungs by responding to changes in oxygen levels and chemical stimuli[6] and are a source of neuropeptides and neurotransmitters responsible for eliciting both physiological and aberrant immune responses[7].

Gastrin releasing peptide (GRP) is a 27 amino acid peptide first isolated from porcine intestine[8,9]. GRP is highly conserved evolutionarily: it shares 9 of 10 C-terminal amino acids with bombesin, an amphibian neuropeptide, and was first described for its ability to mediate gastric acid secretion in the mammalian gut[10,11]. GRP is translated as an inactive “pre-proform” that, upon cleavage and amidation of the C-terminal methionine, becomes a mature, active, secreted peptide[10]. GRP interacts primarily with a 7-transmembrane, G-protein coupled, high affinity receptor, GRPR/BB2, to stimulate many signaling pathways including cAMP, MAPK, PI3K, and AKT, directly or indirectly through the transactivation of other ligand/receptor systems[10,12]. GRP stimulates fetal lung growth/maturation, but has also been associated with pulmonary inflammatory diseases and malignancies including bronchopulmonary dysplasia, chronic obstructive pulmonary disease, emphysema, fibrosis, small cell lung cancer, and non-small lung cancer[10,13,14].

Influenza is a highly contagious respiratory illness. The WHO estimate of influenza-associated deaths globally is ~300,000–650,000[15], and The Centers for Disease Control and Prevention has estimated ~8–13 million influenza-associated illnesses with 5,000–14,000 deaths in the U.S.A. between October 2021 and June 2022[16]. With new antigenic variants emerging annually, the ability to predict the influenza strains to be incorporated into vaccines can be difficult and has resulted in reduced efficacy due to an unanticipated strain predominance[17,18]. The role of the neuroimmune response to influenza has not been closely studied. We previously reported that GRP plays a contributory role in influenza-mediated disease[19]. Herein, we have further investigated the mechanism(s) by which GRP is induced in response to influenza infection and contributes to significant inflammatory disease. Influenza induces oxidative stress as well as production of γ-aminobutyric acid (GABA), which acts on $GABA_B$ receptors on PNEC to release GRP[20,21]. GRP, in turn, mediates monocytic infiltration into the lung. Therapeutic administration of $GABA_B$ receptor agonist (baclofen) or antagonist (saclofen) enhance and depress the inflammatory response, respectively, with a corresponding effect on survival and the proinflammatory response to infection. This study represents the first direct evidence for neuroendocrine cross-talk in the host inflammatory response to virus infection.

RESULTS

Influenza infection induces oxidative stress and GABA in mice

Influenza induces oxidative stress through production of a variety of products[22–25]. To confirm this, wild type (WT) C57BL/6J mice were infected intranasally (i.n.) with an ~LD_{90} of a mouse-adapted influenza strain, PR8[26]. Mice were euthanized on day 6 post-infection (p.i.) and the sera collected for measurement of markers of oxidative stress. Malondialdehyde (MDA), a lipid peroxidation marker, and 8-hydroxy 2-deoxyguanasine (8-OHdG), one of the predominant forms of free radical-induced oxidative stress, were measured[27,28]. Levels of both MDA and 8-OHdG were significantly increased in the lungs of PR8-infected mice (Figure 1A), consistent with previous studies[29–32].

The lung has been characterized as an endocrine organ that produces immunoregulatory peptides such as GRP[9,13]. Rare cells of the bronchiolar columnar epithelium, PNEC[33], cluster in response to noxious stimuli to form “neuroepithelial bodies” (NEBs)[34–36]. These specialized cells secrete GRP in response to environmental agents that lead to oxidative stress, including hypoxia, hyperoxia, ozone, smoking, and others[13,34]. Many such oxidative stressors have been associated with influenza infection[23,24]. The PNECs/NEBs are the only innervated epithelial cells in the lung[37]. When PNEC/NEBs are activated by the neurotransmitter γ-aminobutyric acid (GABA), they secrete GRP[20,21]. We previously reported that GRP is detectable in lung homogenates of influenza-infected mice and in the sera of influenza-infected cotton rats[19]. Therapeutic treatment of mice with a well-characterized small molecule GRP inhibitor, NSC77427[38–40], a monoclonal anti-GRP antibody (2A11)[41], and a GRPR/BB2 small molecule receptor antagonist (BW2258U89)[14] resulted in significant survival after infection as well as decreased numbers of GRP-producing PNECs[19].

Multiple groups have reported the effects of oxidative stress on GABA with regard to traumatic brain injury and related diseases[42–44], however, its role in influenza infection has not been studied. Since the PNECs are the only cell type in the lung known to be in close juxtaposition to neurons[45], we hypothesized that influenza-induced oxidative stress would stimulate neurons to produce GABA by producing glutamate decarboxylase-1 (GAD1). GAD1 is a pyridoxal 5’-phosphate-dependent enzyme that catalyzes the conversion of the L-glutamate to the neurotransmitter GABA[46]. Protein levels of both GAD1 and GABA were significantly upregulated in the sera of mice following influenza PR8 infection (Figure 1B).

Effect of antagonizing or stimulating the $GABA_B$ receptor during influenza infection in mice

There are three classes of GABA receptors, $GABA_A$, $GABA_B$, and $GABA_C$[47]. $GABA_A$ and $GABA_C$ receptors are ionotropic. $GABA_B$ is a metabotropic G-protein coupled receptor that is often found pre-synaptically in the nervous system where it inhibits the release of other transmitters[47]. Therefore, we hypothesized that blocking or stimulating the $GABA_B$ receptor on PNEC/NEBs would, in turn, modulate secretion of GRP and its subsequent role in influenza-induced disease. To assess the role of the $GABA_B$ receptor during influenza infection, WT C57BL/6J mice were infected intranasally (i.n.) with an ~LD_{90} of PR8. On

days 2 through 4 post-infection (p.i.), mice were treated once daily with either vehicle (saline) or with saclofen, a competitive antagonist for the $GABA_B$ receptor[48]. Mice treated with saclofen were significantly protected from influenza PR8-induced lethality (~67% survival; $p = 0.0022$) compared to infected mice treated with vehicle (Figure 2A). Saclofen treatment of influenza PR8-infected mice also showed ameliorated lung pathology compared to vehicle-treated, PR8-infected mice at day 6 p.i. (Figure 2B). Therapeutic treatment of influenza PR8-infected mice with saclofen also led to reduced levels of inflammatory cytokines: 6 days p.i, lungs of mice treated therapeutically with saclofen produced significantly lower mRNA levels of proinflammatory genes *Tnfa*, *Il1b*, *Ifnb*, and *Ccl5* compared to mice infected with PR8 and treated with vehicle (Figure 2C). This reduction in gene induction was also paralleled at the protein level for TNF-α, IL-1β, IFNβ, CCL5, and CCL2 (Supplemental Figure 1). Additionally, the saclofen-treated PR8-infected mice produced significantly lower protein levels of GRP and the danger-associated molecular pattern (DAMP), High Mobility Group Box 1 (HMGB1), in their sera than measured in PR8-infected, vehicle-treated control mice (Figure 2D). We have previously reported that influenza-induced HMGB1 elicits TLR4-mediated influenza-induced disease[49].

While antagonizing the $GABA_B$ receptor resulted in increased protection from influenza disease, we tested whether stimulating $GABA_B$ receptor after infection would worsen influenza-induced disease. Baclofen is a well characterized $GABA_B$ receptor agonist that is used clinically for muscle spasticity[50]. To test this hypothesis, WT C57BL/6J mice were infected with a low dose of influenza PR8 (~LD_{10})[51] and subsequently administered either vehicle or baclofen once daily from days 2 to 4 p.i. Survival was significantly greater (~79%) in vehicle-treated, PR8-infected mice compared to baclofen-treated, PR8-infected mice ($p < 0.0001$) with ~94% of the baclofen-treated mice succumbing to infection (Figure 3A). Baclofen treatment alone did not affect survival of mice (data not shown). Baclofen treatment of PR8-infected mice also led to a significant increase in lung pathology (Figure 3B). Post-infection stimulation of the $GABA_B$ receptor with baclofen also significantly increased the inflammatory response to low dose PR8 infection: at day 6 p.i., baclofen-treated mice produced significantly higher levels of *Tnfa*, *Il1b*, *Ifnb*, and *Ccl5* mRNA and protein levels (Figure 3C; Supplemental Figure 2), as well as increased protein levels of GRP and HMGB1 in their sera (Figure 3D), compared to vehicle-treated, infected mice, consistent with their increased sensitivity to low dose influenza PR8 infection.

Effect of GABA on GRP-mediated infiltration of lung macrophages during influenza infection in mice

Previous studies suggested that acute lung injury (ALI) induced by oxidative stress leads to enhanced GRP expression and this, in turn, acts as a chemotactic factor for neutrophils and monocytes via stimulation of the high affinity GRP receptor, GRPR/BB2[13,52,53]. It has been widely reported that in response to influenza, the infiltrating cells are largely monocytic[54–57]. Since influenza infection induced oxidative stress (Figure 1), as well as increased the number of PNEC/NEBs producing GRP[19], we examined the effect of the GRP small molecule antagonist, NSC77427[19,39], on the infiltration of monocytic cells into the lungs of mice in response to PR8 infection. Expression of CD68 (a pan-monocyte marker), CCR2 (the infiltrating monocyte chemokine receptor for CCL2), and TLR4 (the primary

target of HMGB1[58]), were first measured in the lungs of mice infected with influenza PR8 and treated therapeutically with vehicle or with NSC77427, the small molecule GRP antagonist that we previously reported to be protective during PR8 infection[19]. Mice infected with influenza PR8 and treated with vehicle exhibited significantly increased mRNA levels of all three monocytic markers, while therapeutic treatment of PR8-infected mice with NSC77427 on days 2–6 p.i. significantly decreased expression of all three genes (Figure 4). Immunohistochemical staining of lung sections from these same mice was carried out for CD68 and TLR4. Blinded quantification of CD68- and TLR4-positive cells in multiple sections per slide confirmed the mRNA data that influenza infection induced a significant infiltration of monocytes, and that treatment of influenza-infected mice with NSC77427 significantly decreased the number of infiltrating $CD68^+$, $TLR4^+$ monocytic cells (Table I). Collectively, this data indicates that blocking GRP during influenza infection results in a reduction of $TLR4^+$ monocytes infiltrating into the lungs.

The data presented in Figures 2D and 3D indicate that treating influenza PR8-infected mice with either saclofen or baclofen resulted in the decreased or increased production of GRP, respectively. We hypothesized that if less GRP were produced, or its ability to interact with its receptor is blocked, then fewer TLR4+ inflammatory monocytes would be recruited to the lungs. Conversely, if more GRP were produced, then more TLR4+ inflammatory cells would be recruited, resulting in more severe disease. To test whether antagonizing (saclofen) or stimulating (baclofen) the $GABA_B$ receptor also affects the recruitment of TLR4+ inflammatory monocytic cells, we examined induction of *Cd68* and *Tlr4* mRNA in the lung samples of influenza PR8-infected mice that were treated with vehicle only, saclofen, or baclofen as shown in Figures 2C and 3C. Lungs from mice infected with influenza PR8 and treated with saclofen expressed significantly less *Cd68* and *Tlr4* mRNA compared to untreated PR8-infected mice (Figure 5A), while mice that were infected and treated with baclofen showed a significant increase in both *Cd68* and *Tlr4* mRNA compared to infection alone (Figure 5B). Immunohistochemical staining for the monocytic marker, CD68, in saclofen-treated, PR8-infected mice confirmed a marked reduction in the number of $CD68^+$ cells compared to vehicle-treated PR8-infected mice (Figure 6). Panels A and B from mock-infected mice show typical lung architecture with one normal CD68+ macrophage visible in an alveolar space as shown by an arrow in panel B. Mice infected with influenza PR8 and treated with vehicle, as shown in panels B and C, show severe acute pneumonia with multiple small veins surrounded by CD68+ monocytes and macrophages crossing through the vascular wall as indicated with arrows. Importantly, mice infected with influenza PR8 and treated with saclofen showed very few CD68+ macrophages as pointed out by arrows in panel F. Taken together, our data support the conclusion that influenza infection leads to the release of GABA that, in turn, elicits production of GRP by PNEC/NEBs. GRP then recruits CD68+ monocytic cells to the lung that mediate the TLR4-dependent inflammation associated with influenza infection.

DISCUSSION

The lungs and respiratory tract act as a key innate mechanical barrier against respiratory pathogens. The lung, which has been classified as an endocrine organ, is highly innervated throughout the airway by neurons that are able to respond and work in conjunction with the

immune response to a variety of chemical and microbial insults[59]. While much work has been done on a neuro-immune response during allergic inflammation[59], the neuro-immune response during the innate immune response to viral infections is less understood with the preponderance of studies focused on mechanisms by which viral infections elicit airway hyperreactivity and sympathetic reflex responses (*i.e.*, cough and sneezing)[60] and not on the initial response to infection. Our previous study focused on the role of GRP during influenza infection[19]. We were the first to show that influenza infection triggered GRP production in mice and cotton rats, a unique rodent species that is susceptible to non-adapted human respiratory virus strains[61], and showed that inhibiting either GRP or its receptor greatly increased survival in mice, reduced the inflammatory response, and improved lung histology[19].

PNEC/NEBs are the only lung epithelial cell that is innervated[37]. GABA is a potent neurotransmitter and neuromodulator that has been assumed to be produced by neurons that act on PNEC/NEB to produce GRP[20,21]. However, a study by Barrios et al.[62] provided convincing evidence that PNECs are the major source of GABA in the lungs in primate and human models of Th2-mediated allergic inflammation induced by exposure to ovalbumin and LPS; however, their study does not exclude the possibility that in a more Th1-driven model of inflammation, such as that induced by influenza infection, neurons and/or PNECs are a source of GABA. Future studies will be required to determine the cellular source of GABA in response to influenza infection. Regardless of the cellular source of the GABA, stimulation of PNEC/NEBs in an autocrine/paracrine manner would result in enhanced GRP production as we have shown.

In this study, we focused on the production of GABA in response to influenza infection and how its modulation influences the outcome of infection, specifically on the neuroendocrine response and resulting monocytes coming into the tissue after infection. While GABA is known as an inhibitory neurotransmitter in the brain[47], it can also be involved in migration and cellular proliferation in neuronal and non-neuronal cells[63–65]. We specifically focused on modulation of the metabotropic $GABA_B$ receptor on PNEC/NEBs due to our previous study showing release of GRP during influenza infection. $GABA_B$ receptor-specific agonists, such as baclofen, have been shown to decrease the airway responses by modulating acetylcholine release to bronchoconstricting agents[66,67]. However, baclofen has also been shown to induce more severe respiratory responses in asthma studies following administration of methacholine[68]. These studies were again focused on Th2 models, thus, the role of modulating $GABA_B$ during a severe Th1 model such as influenza had not been reported. In our current study, inhibiting the $GABA_B$ receptor with a specific inhibitor improved the response to a lethal influenza infection, while administration of a $GABA_B$ receptor-specific agonist worsened the response to an otherwise low dose infection and resulted in increased inflammatory mediator production and more severe lung pathology.

In conclusion, the interplay of GABA and the host innate immune response plays a pivotal role in the response to influenza-induce ALI. In Figure 7, we provide a hypothetical model that encompasses our previous and new data. We posit that influenza-infected epithelial cells lead to inflammatory and oxidative stress responses resulting in cell death and the release of HMGB1. Our data presented herein shows that influenza-induced release of

GABA acts on the PNEC/NEBs through the $GABA_B$ receptor to produce GRP that, in turn, acts as a chemotactic factor to recruit $TLR4^+$ monocytic cells into the lung that respond to HMGB1-mediated TLR4/MD-2 signaling to release inflammatory mediators of lung damage. Blocking GRP with a small molecule inhibitor or monoclonal antibody or blocking the GRPR/BB2 reduced lung damage and lethality[19]. Blocking $GABA_B$ signaling also blocked GRP production and release, and the recruitment of TLR4+ monocytic cells that amplify TLR4-dependent, HMGB1-mediated signaling and lung damage. Conversely, stimulating $GABA_B$ signaling leads to further damage and sustained inflammation. To our knowledge, our findings represent the first report of a novel neuroendocrine pathway leading to the induction of inflammation and lung pathology during influenza-induced ALI.

Methods

Reagents:

GRP Enzyme ImmunoAssay (EIA), that is specific for the mature form of GRP (amide 1–27), was purchased from Phoenix Pharmaceuticals, Inc. (Catalog # EK-027–07; Burlingame, CA). GABA ELISA kit was purchased from Aviva Systems Biology (Catalog # OKEH02564; San Diego, CA). HMGB1 ELISA kit was purchased from IBL International (Catalog # ST51011; Toronto, Ontario, Canada). The 8-hydroxy 2 deoxyguanosine (8-OHdG) ELISA kit was purchased from Abcam (Catalog # ab201734; Branford, CT). GAD ELISA was purchased from Lifeome Biolabs (Catalog # EL009159MO-96; Oceanside, CA). The ELISA kit for IFN-β was purchased from PBL Assay Science (Catalog # 42400; Piscataway, NJ). The ELISA kits for TNF-α (Catalog # MTA00B), IL-1β (Catalog # MLB00C), CCL5/RANTES (Catalog # MMR00), and CCL2/MCP-1 (Catalog # MJE008) were purchased from R&D Systems (Minneapolis, MN). The GRP inhibitor, NSC77427, was made by the Small Molecule Library Reagent Program (National Cancer Institute, Division of Cancer Treatment & Diagnosis/Developmental Therapeutics Program, NIH). Saclofen and baclofen were purchased from Tocris Bioscience (Minneapolis, MN). The TLR4 antibody was purchased from Invitrogen (Catalog # 14–991782; Waltham, MA) and the CD68 antibody purchased from Biolegend (San Diego, CA).

Mice:

Six-week old, WT C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal experiments were conducted with institutional IACUC approvals from University of Maryland, Baltimore.

Viruses:

Mouse-adapted H1N1 influenza A/PR/8/34 virus (“PR8”) (ATCC, Manassas, VA) was grown in the allantoic fluid of 10-day old embryonated chicken eggs as described[69] and was kindly provided by Dr. Donna Farber (Columbia University).

Virus challenge and treatment:

For survival experiments, WT C57BL/6J mice were infected with mouse-adapted influenza virus, strain A/PR/8/34 (PR8; ~7500 $TCID_{50}$i.n., 25 ml/nares), a dose of PR8 that kills ~90% of infected mice[26,49] for saclofen experiments. For baclofen experiments, WT

C57BL/6J mice were infected with mouse-adapted influenza virus, strain A/PR/8/34 (PR8; ~1500 $TCID_{50}$ i.n., 25 μl/nares), a dose of PR8 that kills ~10% of infected mice[51]. Two days after PR8 infection, mice received either vehicle (indicated in the figure legend), saclofen (20 μg; 100 ml i.p.), or baclofen 100 μg; 100 ml i.p.) daily for 3 consecutive days (day 2 until day 4). For tissue analysis, mice were treated as above and the sera and lungs harvested on day 6 p.i. for histology, gene expression, and protein levels.

Histology and staining:

Lungs were inflated and perfused and fixed with 4% PFA. Fixed sections (5 μm) of paraffin-embedded lungs were stained with hematoxylin and eosin (H&E). Four inflammatory parameters were scored independently from 0 to 4 for each section: peribronchiolitis (inflammatory cells, primarily lymphocytes, surrounding a bronchiole), perivasculitis (inflammatory cells, primarily lymphocytes, surrounding a blood vessel), alveolitis (inflammatory cells within alveolar spaces), and interstitial pneumonitis (increased thickness of alveolar walls associated with inflammatory cells). Slides were randomized, read blindly, and scored for each parameter. Data is shown as a cumulation of the four parameters measured.

Immunohistochemistry:

IHC staining for CD68 for imaging in Figure 6 was carried out as follows: Paraformaldehyde-fixed, paraffin-embedded lung sections were treated for 10 min with Triton X-100 (0.3% in PBS), then normal serum blocking. Slides were incubated with anti-CD68 antibody diluted 1:100 overnight at 4°C, followed by PBS washes and incubation for 2 h at 4° C with 1:200 dilution of biotinylated secondary antibody, then ABC-alkaline phosphatase complex (Vector #AK-5000), and ImmPACT Vector Red Substrate kit for alkaline phosphatase (AP; Vector #SK-5105). Slides were counterstained with hematoxylin. IHC staining for CD68 and TLR4 was carried out as follows for quantitation shown in Table I: Paraformaldehyde-fixed, paraffin-embedded lung sections were treated for 10 min with Triton X-100 (0.3% in PBS), then normal serum blocking. Diluted primary antibodies were added to serial sections overnight at 4°C, followed by PBS washes and incubation for 2 h at 4° C with 1:250 dilution of biotinylated secondary antibodies. After blocking with 3% H_2O_2 in methanol, and slides were incubated with ready to use avidin-biotin complex (peroxidase ready; Vectastain), all slides were then washed and incubated with diaminobebzidine (DAB) solution then counterstained with 2.5% aqueous methyl green. All slides were finally blinded for semi-quantitative analysis by a board-certified pathologist. Results are expressed as the mean numbers of cells positive for CD68 or TLR4 per mm^2 lung tissue section (Table I). Statistical analysis was carried out using a one-tailed Student's *t* test.

Quantitative real-time PCR (qRT-PCR):

Total RNA isolation and qRT-PCR were performed as previously described[70,71]. Levels of mRNA for specific genes were normalized to the level of the housekeeping gene, HPRT, in the same samples and are expressed as "fold-increase" over the relative gene expression measured in mock-infected lungs.

Protein levels in serum and lung homogenates:

Protein levels in sera for GABA, GRP, HMGB1, 8-OHdG, MDA, and GAD were measured by ELISA according to the manufacturers' protocols in the serum of mice infected with influenza as described above. Protein levels in lung tissue homogenates for TNF-α, IL-1β, IFN-β, CCL5/RANTES, and CCL2/MCP-1 were measured by ELISA according to the manufacturers' protocols.

Statistics:

Statistical differences between two groups were determined using an unpaired, one-tailed Student's *t* test with significance set at $p < 0.05$. For comparisons between ≥3 groups, analysis was done by one-way ANOVA followed by a Tukey's multiple comparison post-hoc test with significance determined at $p < 0.05$. For survival studies, a Log-Rank (Mantel-Cox) test was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

This work was supported by the following grants: NIH AI159507 (KAS) and NIH AI123371 (SV). We thank Professor Margaret McCarthy (UMB) for thoughtful discussions.

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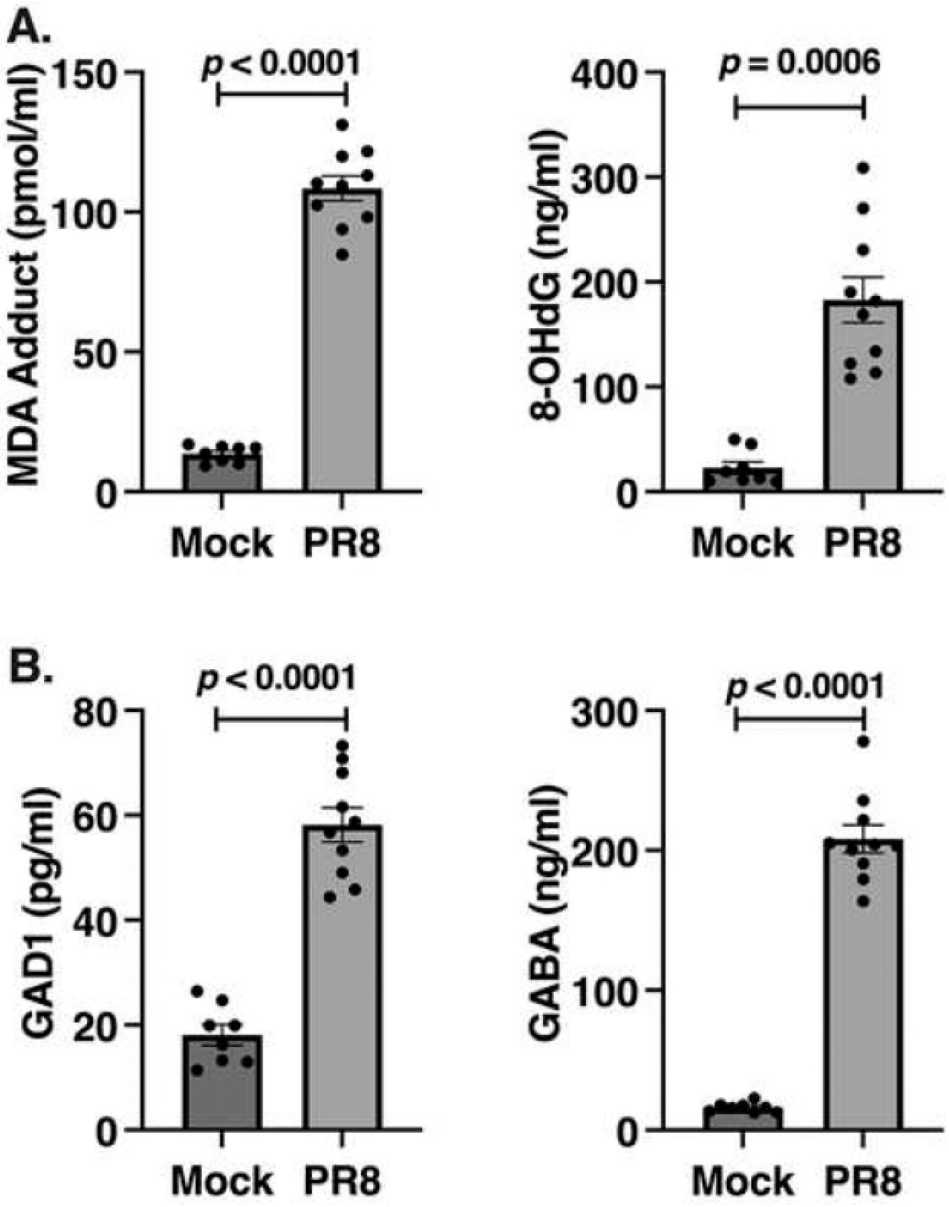

Figure 1. Oxidative stress induced by influenza PR8 infection is accompanied by the release of GAD1 and GABA.

WT C57BL/6J mice were infected with and LD_{90} dose of mouse-adapted influenza strain PR8. On day 6 post-infection, protein levels were measured by ELISA in sera for oxidative stress markers, MDA and 8-OHdG (A) and GAD1 and GABA (B). Data shown are combined results of 2 separate experiments (N = 4–5 mice/group/experiment).

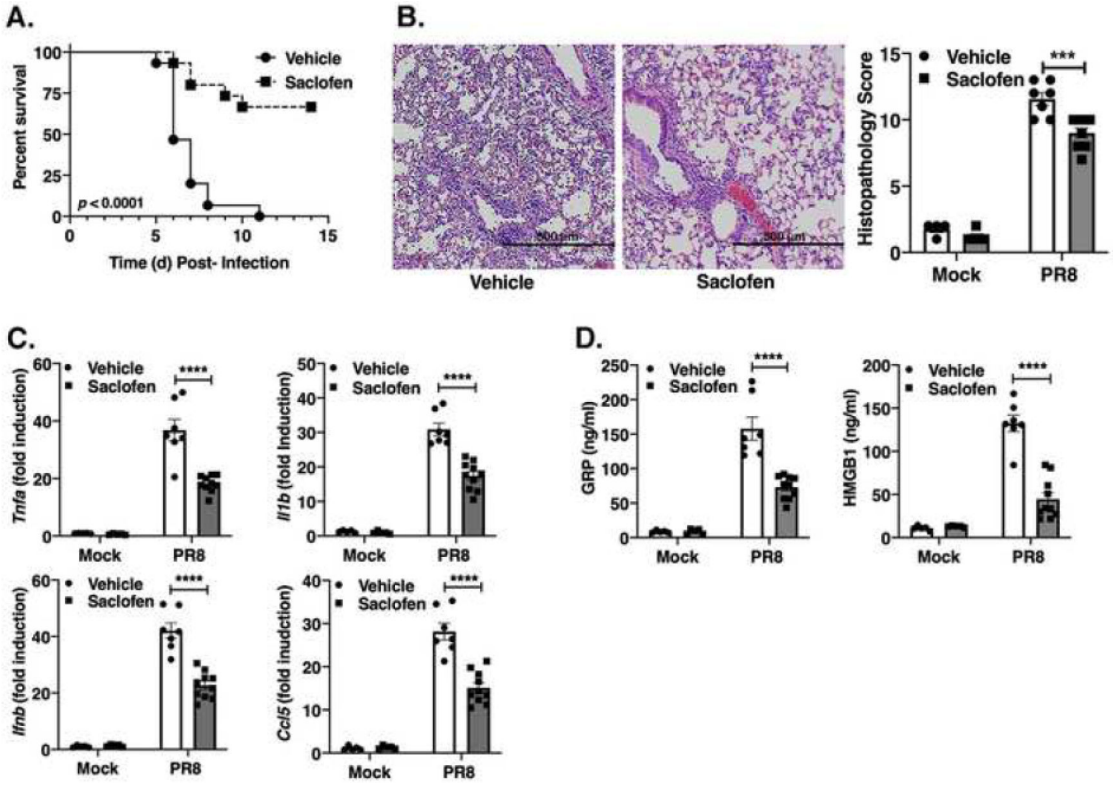

Figure 2. Effect of antagonizing the $GABA_B$ receptor during influenza PR8 infection.

(A) Mice were infected with and LD_{90} of mouse-adapted influenza strain PR8. Mice received vehicle (saline) or $GABA_B$ receptor antagonist (saclofen) daily from day 2 to day 4 p.i. Survival was monitored for 14 days. Data shown are combined results of 3 separate experiments (5 mice/treatment group/experiment). (B,C,D) Mice were infected and treated as described in (A). Lungs were harvested on day 6 p.i. for histopathology (B), lung gene expression by qRT-PCR (C), and serum levels of GRP and HMGB1 (D). Data represent the combined results of 2 separate experiments N = 4–5 mice/treatment group/mock; N = 7–10 mice/treatment group. *** $p = 0.0002$; **** $p < 0.0001$.

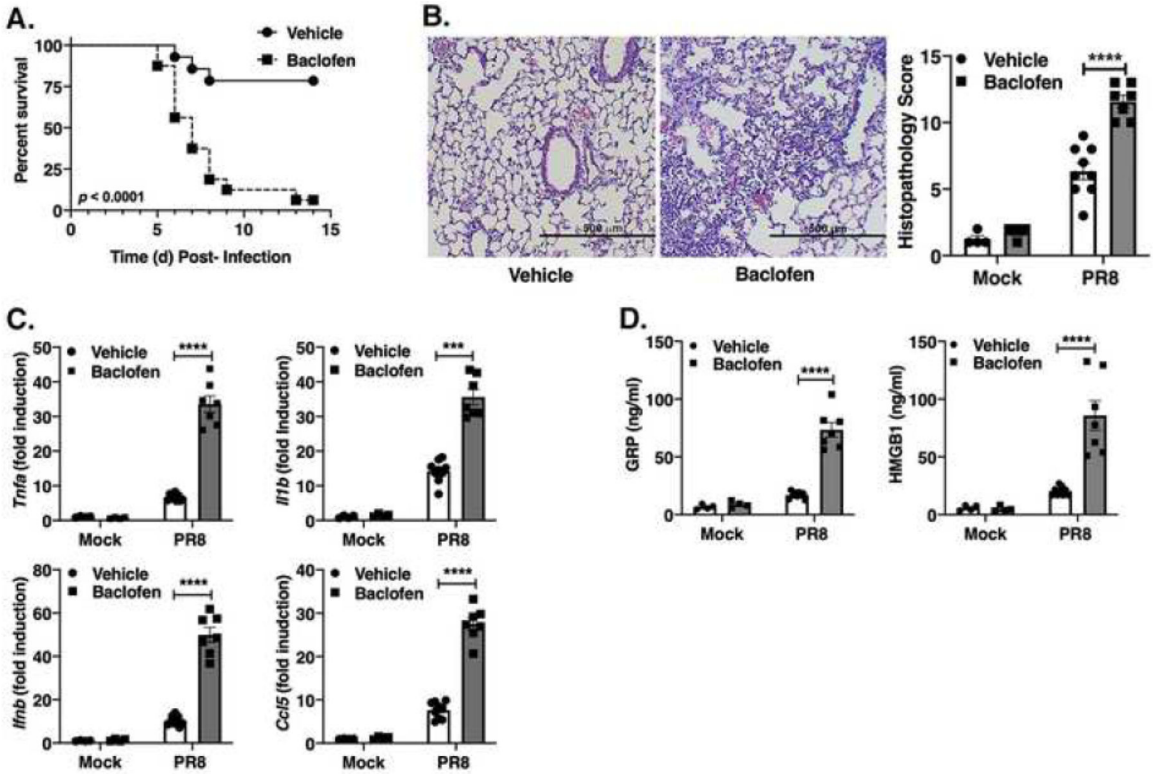

Figure 3. Effect of stimulating $GABA_B$ receptor during influenza PR8 infection.

(A) Mice were infected with an LD_{10} of mouse-adapted influenza strain PR8. Mice received vehicle (saline) or $GABA_B$ agonist (baclofen) daily from day 2 to day 4 p.i. Survival was monitored for 14 days. Data shown are combined results of 2 separate experiments N = 4–5 mice/treatment group/mock; N = 7–10 mice/treatment group/influenza). (B,C,D) WT C57BL/6J mice were infected with mouse-adapted influenza strain PR8. Mice received vehicle (saline) or $GABA_B$ agonist (baclofen) daily from day 2 to day 4 p.i. Lungs were harvested on day 6 p.i. for histopathology (B), lung gene expression by qRT-PCR (C), and levels of GRP and HMGB1 (D). Data represent the combined results of 2 separate experiments N = 4–5 mice/treatment group/mock; N = 7–10 mice/treatment group. *** $p = 0.0002$; **** $p < 0.0001$.

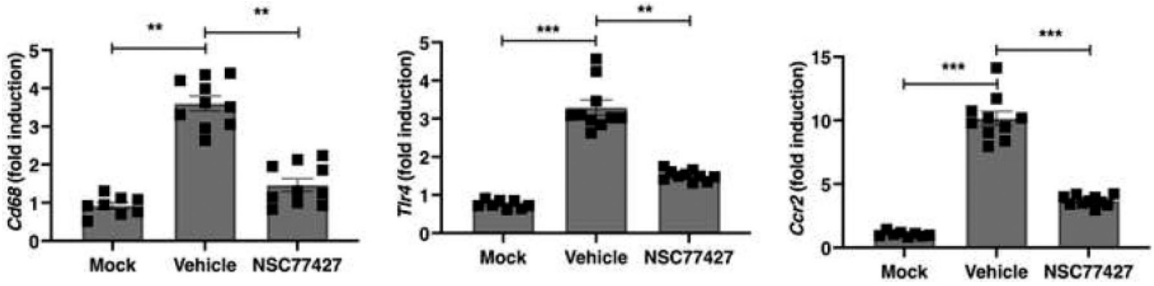

Figure 4. GRP mediates infiltration of CD68+, TLR4+ CCR2+ monocytic cells.

WT C57BL/6J mice were infected with and LD_{90} of mouse-adapted influenza strain PR8 (LD_{90}; ~7500 $TCID_{50}$) and treated with vehicle (0.0096% DMSO in saline; i.v.) or NSC77427 (20 mM; i.v.) daily from days 2 to 6 p.i. Lungs were harvested on day 7 post-infection for mRNA gene expression by qRT-PCR. Data represent the combined results of 2 separate experiments. N = 4–5 mice/treatment group/mock; N = 5 mice/treatment group. *$p < 0.01$; **$p < 0.007$.

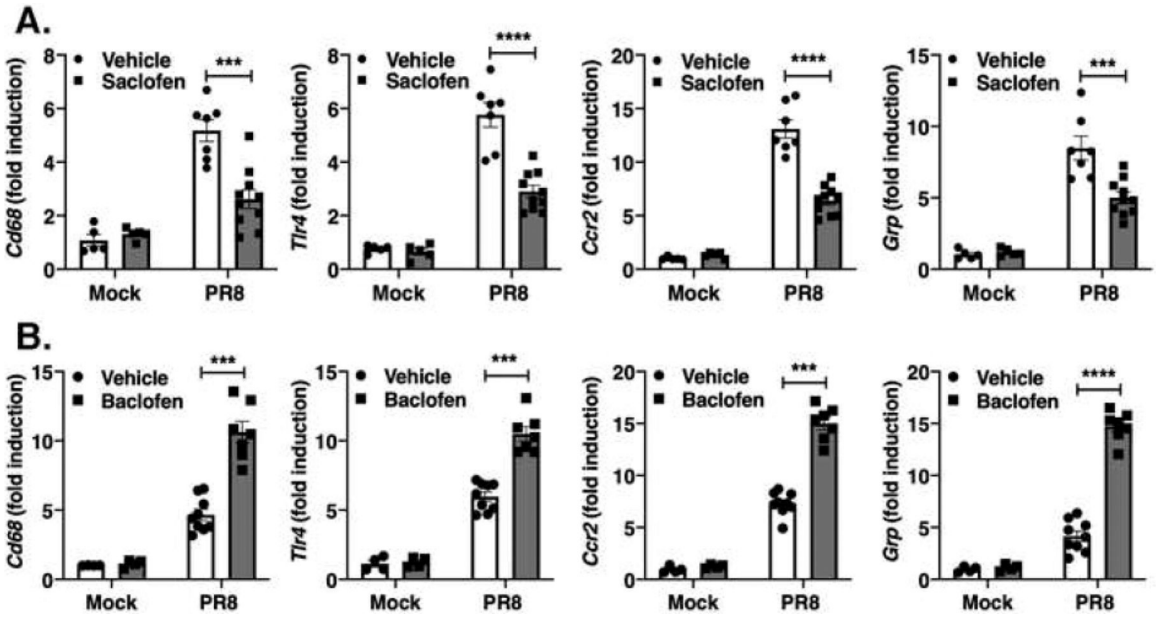

Figure 5. Antagonizing or stimulating $GABA_B$ receptor during influenza PR8 infection alters gene expression of markers of monocytic infiltration and GRP.

(A) Mice were infected with an LD_{90} of mouse-adapted influenza strain PR8. Mice received vehicle (saline) or $GABA_B$ receptor antagonist (saclofen) daily from day 2 to day 4 post-infection. Lungs were harvested on day 6 p.i. and gene expression analyzed by qRT-PCR. Data represent the combined results of 2 separate experiments. N = 4–5 mice/treatment group/mock; N = 7–10 mice/treatment group. (B) Mice were infected with an LD_{10} of mouse-adapted influenza strain PR8. Mice received vehicle (saline) or $GABA_B$ agonist (baclofen) daily from day 2 to day 4 p.i. Lungs were harvested on day 6 p.i. and gene expression was analyzed by qRT-PCR. Data represent the combined results of 2 separate experiments N = 4–5 mice/treatment group/mock; N = 7–10 mice/treatment group. *** $p = 0.0002$; **** $p < 0.0001$.

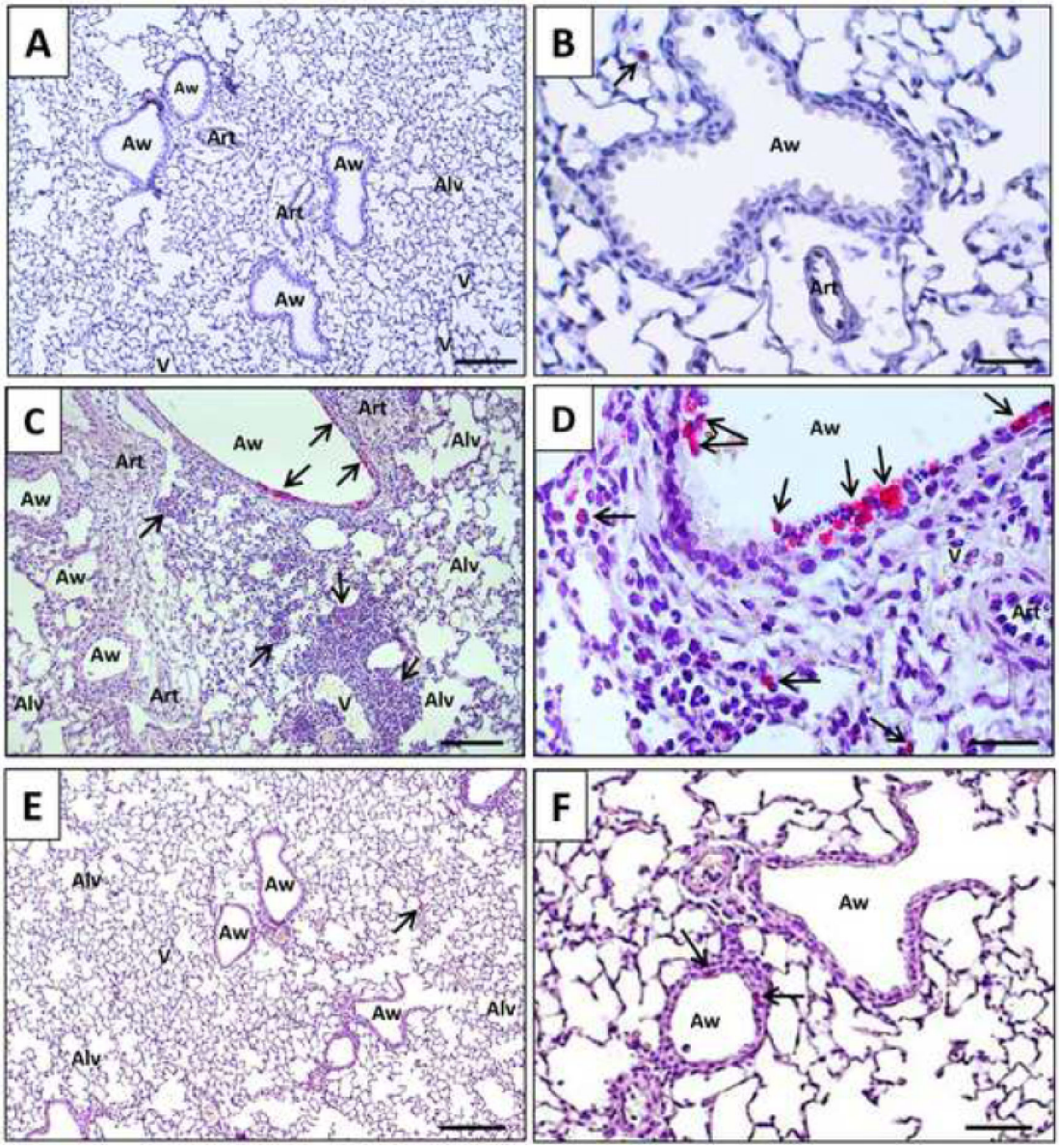

Figure 6. IHC staining for CD68 in mouse lungs infected with influenza.

Lung sections from Figure 2 were stained in mock-infected (panels A and B), PR8 + vehicle treatment (panels C and D), and PR8 + saclofen treatment (panels E and F). Representative images shown are results of 2 separate experiments N = 4–5 mice/treatment group/mock; N = 7–10 mice/treatment group. Scale bars for panels A, C, and E at 100X are 200 μm and at 400X are 50 μm for panels B, D, and F. Arrows point to CD68+ cells. Abbreviations: Alv, alveoli; Art, artery; Aw, airway; V, vein.

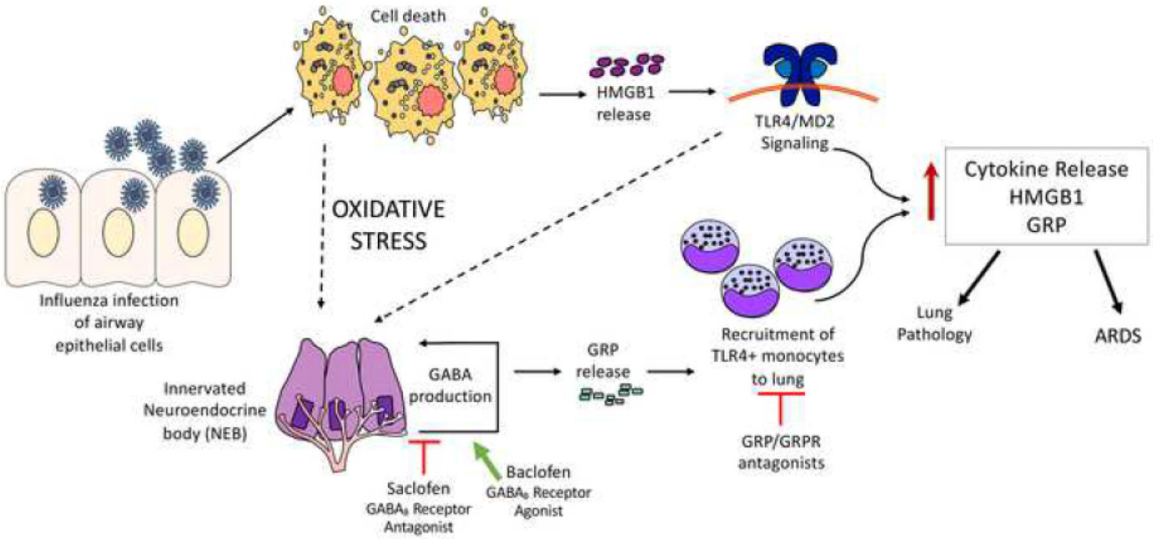

Figure 7. Hypothetical model for involvement of GABA, GRP, and TLR4 during influenza infection.

Influenza-infected epithelial cells lead to inflammatory and stress responses resulting in HMGB1 release and the production of secretion of GABA by neurons and NEBs. HMGB1 signals through TLR4/MD2, leading to further oxidative stress, cytokine release, tissue damage, and lethality. GABA production by neurons and/or PNECs/NEBs triggers GRP production, which recruits TLR4+ monocytes into the lung that are the targets of HMGB1-mediated TLR4/MD-2 signaling, leading to proinflammatory mediator production and subsequent lung damage. Blocking GRP reduces lung damage and lethality as previously reported. Blocking $GABA_B$ signaling blocks GRP production and release, thereby preventing further recruitment of TLR4+ monocytes that amplify signaling and damage. Stimulating $GABA_B$ signaling leads to further damage and sustained inflammation.

Table I.

Blocking GRP decreases infiltrating TLR4+ monocytes

	CD68+ cells/20X field	SEM	TLR4+ cells/20X field	SEM
PR8+Vehicle	2.6	0.11	3.1	0.23
PR8+NSC77427	1.5	0.34	1.19	0.31
Statistics	$p = 0.04$		$p = 0.0002$	