Nutritional Gel Supplementation Reduces Weight Loss and Mortality in Mice Infected with Influenza A/PR/8/34 Virus

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ABSTRACT

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Influenza virus A (IAV) places a high-burden on public health. Studies of IAV commonly use mice to model the response of the immune system *in vivo*. Complications of IAV studies include anorexia and dehydration with subsequent weight loss resulting in early removal of mice from a study based on euthanasia criteria. To reduce the number of mice prematurely removed from an experiment, we assessed various support strategies in mice infected with IAV: 1) standard of care (SOC), 2) nutritional gel (NG), 3) subcutaneous fluids, 4) oral gavage - nutritional formula (OG ICU), and 5) oral gavage - PBS (OG PBS). We hypothesized that when compared to the SOC, supplementation with NG would lead to decreased weight loss and mortality in mice infected with IAV without impacting the initial immune response. For assessment of NG, both male and female C57Bl6/J mice were infected with mouse-adapted IAV, A/PR/8/34, at low, medium or high doses. SQ fluids, OG ICU and OG PBS were assessed in male mice infected with the middle influenza dose. Euthanasia criteria removed mice at 30% weight loss. Mice supplemented with NG and subcutaneous fluids lost significantly less weight

and the NG group had a significant reduction in mortality. Supplementation with OG ICU and PBS had no benefit. Supplementation with NG did not alter the pulmonary recruitment of immune cells as measured with cell counts and flow cytometry of cells recovered in bronchoalveolar lavage fluid. In summary, the results of this study show mice infected with mouse-adapted IAV that are supplemented with NG have reduced weight loss and mortality. These results suggest that NG should be considered as a support strategy for mice infected with IAV.

DEDICATION

This work is dedicated to my family who have supported me and are who I have inherited my love of science and medicine from.

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TABLE OF CONTENTS

ABSTRACT---III
DEDICATION---V
ACKNOWLEDGMENTS---VI
INTRODUCTION---1
MATERIALS AND METHODS---4
Animal and Housing---4
Influenza Virus---5
Nutritional Gel (NG) Supplementation---5
Subcutaneous (SQ) Fluid Administration---6
Oral Gavage of Nutritional Formula or PBS---6
Measurements---7
Treatment and Recovery Times---8
Study Design: Evaluation of Immune Response---8
Statistical Analysis---10
RESULTS---13
Nutritional Gel Supplementation---13
Subcutaneous Fluid Administration---15
Oral Gavage of Nutritional Formulation or PBS---16
Evaluation of Time of Treatments---17
Evaluation of Immune Response---17
DISCUSSION---18
FIGURES AND FIGURE LEGENDS---24
TABLES---33
REFERENCES---37

INTRODUCTION

Influenza A virus (IAV) infections place a major strain on public health world-wide. The World Health Organization estimates that influenza viral infections, alone, cause severe illness in 3-5 million people and death in up to 650,000 people world-wide each year. [1-3] Due to the burden that influenza has on public health, there is continued need to develop a better understanding of the interactions of this virus with the mammalian immune system. This need has led to research studies of the virus that utilize a variety of species to examine the host response to IAV to develop new concepts and novel therapeutics. Of the species used to study influenza, the most commonly used are mice. [4,5] In order to study the immune response to influenza, well-designed studies are required that take into consideration specific complications inherently associated with in vivo studies of influenza infection. Clinical signs of influenza in mice include anorexia, dehydration, respiratory distress, hypothermia, hunched posture, unkempt hair coat and ocular discharge. [4,6-8] Anorexia and dehydration are a complication of influenza infection that leads to excessive weight loss in mice. This weight loss is routinely used as a euthanasia end point criteria for mice infected with IAV, and leads to early removal of mice from study, which negatively impacts sample size for many studies. [6]

Weight loss in mice infected with influenza virus is associated with systemic inflammation leading to anorexia. Pro-inflammatory cytokines have been shown to inhibit normal feeding behavior in mice with IAV and other infections, specifically through increased expression of tumor necrosis factor-alpha (TNFα), interleukin-1β (IL-1β), and interleukin-6 (IL-6). [8-15] For example, following infection with IAV, the amount of TNFα synthesized begins to rise around 2-3 days post infection (dpi) and peaks at 7 dpi. [10] Mice begin to lose weight around 4-5 dpi with

peak weight loss around 9 dpi, which correlates with the increases in TNFα and other pro-inflammatory mediators. [9,10,16]

Under the guidance of Institutional Animal Care and Use Committees (IACUCs), a 20 to 30% loss in body weight is one of the endpoint criteria used in studies of IAV. Due to concerns for animal welfare, it difficult for an IACUC to justify a weight loss of over 30%. Based on the three R's, replacement, reduction, and refinement alternatives, an important responsibility of scientists is to refine techniques that utilize animal models of disease to minimize discomfort. [17] Additionally, finding ways to support mice and reduce the loss of animals in IAV studies will lead to a reduction in the number of animals required to adequately power a study. Therefore, an important question is whether there is supportive care that reduces weight loss and therefore mortality in mice infected with IAV?

The goal of the work performed for this study was to evaluate treatment strategies that result in reduced weight loss and increased survival in mice infected with IAV, without affecting the innate immune response. To accomplish this goal we evaluated a number of support strategies in mice for up to 14 days after infection with IAV and compared these to the SOC, moist chow and hydrogel. The support strategies evaluated included 1) nutritional gel (NG) - Clear H_2O DietGel Recovery, 2) subcutaneous fluids (SQ fluids), 3) oral gavage of a nutritional formulation (OG-ICU; Abbott Promote ICU formula), and 4) oral gavage of PBS (OG-PBS). These support strategies were chosen as a way to compare and contrast both nutritional and hydration support in IAV infected mice.

There is inherent stress induced by handling mice, and the impact of stresses of influenza infection combined with handling for oral gavage or subcutaneous fluid administration was suspected to overcome any benefit of the treatments.[18-21] Therefore, due to the physical (soft consistency) and nutritional properties along with minimal handling required to provide the supplement, we hypothesized that nutritional gel (DietGel Recovery) would be the support strategy that showed the most benefit to mice infected with influenza virus by minimizing weight loss and increasing the number of mice who reached endpoint compared to the other support strategies. The overall goal of this work is to improve the standard of care for influenza infected mice, which could ultimately reduce the number of animals necessary for influenza research.

MATERIALS AND METHODS

Animal and Housing

Studies were performed with male and female C57BL/6J mice (Jackson Labs, Sacramento, CA) aged 8 weeks at time of arrival, and 9 weeks at initiation of studies. All mice were housed in a specific pathogen-free animal facility throughout the experiment. Animals were co-housed (2-5 mice per cage) in standard plastic cages with corn-cob bedding with nestlet material provided for enrichment in an American Association for the Accreditation of Laboratory Animal Care (AAALAC) accredited facility. Following infection with IAV mice were housed under biosafety level-2 (BSL-2) conditions. The room temperature was held at a range of 68 to 79°F with the goal of 72°F and an acceptable temperature variation of no more than a 4°F over a 24-h period. The acceptable room humidity range was 30 to 70%. In addition to the various supplements described below, mice were given free access to food and acidified (pH 2.5-2.8) water after infection and were maintained on a 14/10 hour light/dark cycle. Daily health checks were performed by the University of Washington husbandry staff who are overseen by lab animal veterinarians. All procedures were approved by the University of Washington's Institutional Animal Care and Use Committee (IACUC).

Influenza Virus.

Mouse-adapted Influenza A/Puerto Rico/8/34 (H1N1) virus was grown in the allantoic fluid of research grade specific pathogen-free (SPF) embryonic chicken eggs (Charles River Avian Vaccine Services, Norwich, CT) and a hemagglutination assay was performed to determine the viral titer.[22] As sex differences are documented in mice infected with influenza[23,24], a lethal dose 50 (LD50) was performed in both male and female mice to determine the appropriate plaque

forming units (PFU) to use for infections for each sex. Mice were anesthetized with isoflurane gas anesthetic and infected with influenza via oropharyngeal aspiration[25,26] for LD50 studies as previously described. Using the Reed and Muench calculation, the LD50 for each sex was determined by assessing the number of mice who reached endpoint criteria infected at serial dilutions of influenza A/PR/8/34 virus. [22,27] Mice were monitored and removed when they reached end-point criteria with care taken to avoid death without euthanasia. The LD50s were determined to be 20 PFU for female mice, and 50 PFU for male mice.

Study Design: Evaluation of Support Strategies for Mice Infected with IAV

Nutritional Gel (NG) Supplementation.

On the day of infection, mice were anesthetized with isoflurane gas anesthetic and infected via oropharyngeal aspiration of virus with one of three doses: 1) low dose- 0.2 LD50, 2) middle dose - 0.5 LD50 or 3) high dose- 1.25 LD50 IAV (**Table 1**). In addition to regular access to food and water, on the day of infection mice were given either standard care (SOC) for mice with influenza or NG supplementation. SOC consisted of chow pellets moistened with water (moistened irradiated chow; LabDiet 5053, Rodent Diet 20, St. Louis, MO) and hydration gel (HydroGel; Clear H_2O, Portland, ME) provided in two separate paper cups. NG groups received moistened chow and nutritional gel (DietGel Recovery; Clear H_2O, Portland, ME) provided in two separate paper cups. Moistened chow was made using one pellet of chow per mouse in the cage in order to allow for a visual assessment of how much supplemented feed was consumed each day. NG was given at 5 grams per mouse (10.7 kcal/ mouse) in cage and was measured using a scale each day. Table 2 provides additional nutritional details of feeds and gels provided in this study. The weights were monitored daily for the mice. At 14 days post infection mice

were euthanized. Mice that reached end point criteria were removed from study early (see Monitoring and End Point Criteria).

Subcutaneous (SQ) Fluid Administration.

Male mice were infected with the middle dose (0.5 LD50, 25 PFU) of IAV. Mice in both groups were provided with the SOC for influenza infected mice, while only one group was supplemented with SQ fluids (SOC only: n=18, SQ fluids: n=8). Weights were monitored daily for the mice. Once any mouse in a cage lost 5% of initial weight, 0.9% Sodium Chloride (Monoject™ 0.9% Sodium Chloride Flush Syringe) was administered subcutaneously two times daily at 50 ml/kg, for a total of 100ml/kg/day. The site of SQ fluid administration was never in the same location for a given day. SQ fluids were discontinued once a mouse regained weight to 15% of the initial weight. At 14 days post infection mice were euthanized. Mice that reached end point were removed from study early (see Monitoring and End Point Criteria).

Oral Gavage of Nutritional Formula or PBS.

Male mice were infected with the middle dose (0.5 LD50) of influenza. Mice in all groups were provided with the standard of care for influenza infected mice, with two groups supplemented with oral gavage of either 1) protein rich nutritional formula (OG ICU) or 2) PBS (OG PBS) (SOC only: n=18, OG ICU: n=4, OG PBS: n=3). The nutritional formula used was Abbott Promote Intensive Care Unit Formula (Abbott Laboratories, Chicago, IL). Table 2 provides additional nutritional information for Abbott Promote formula. Once any mouse in a cage lost 5% of their initial weight, either nutritional formula or PBS was administered via oral gavage two times daily at a volume of 10 ml/kg.[28] Mice were gavaged using a 22 gauge, 1.5 inch straight stainless steel gavage needle with a 1.25mm ball diameter (Cadence Science Inc,

Cranston, RI). Oral gavage was discontinued once a mouse regained weight to 15% of the initial weight. At 14 days post infection mice were euthanized. Mice that reached end point were removed from study early (see Monitoring and End Point Criteria).

Measurements

Body Weight.

Body weights were measured in grams, at minimum once daily. Weights were taken on the same scale at approximately the same time each day. Initial weight was defined as the weight on the day of influenza infection. Once mice lost 20% of their initial weight, weight was measured two times daily to ensure mice did not lose greater than 30% loss of their initial weight. When mice received twice daily weight measurements, the weight used for statistical analysis was the lowest weight during periods of a downward trend in weight, and the highest weight in periods of an upward trend in weight.

Monitoring and Endpoint Criteria.

Mice, similar to humans, have stereotypical responses to acute infection with influenza virus that includes anorexia leading to significant weight loss. It has been shown that mice can reach greater than 30% weight loss due to influenza infection before they begin to recover.[4-6] In this study mice were monitored daily post influenza infection, and euthanized when they reach a body condition score <2, or $\geq 30\%$ weight loss, or displayed severely labored breathing. Additionally, mice were removed from study if they showed a grouping of other signs of severe illness including eye crusting, severe dehydration, lethargy and decreased movement with loss of resistance to handling and abnormal posture such as hunching and piloerection. No mice in this

study displayed the additional euthanasia criteria and mice reaching end point criteria were solely euthanized due to a weight loss of ≥ 30%.

Treatment and Recovery Times.

The time of administration was recorded for each treatment. For both SOC and NG groups, the time was recorded as the time from beginning to prepare the feed to the time it was placed into the cage. The time of subcutaneous fluid administration was measured beginning at the time the saline was collected into the syringe and to the time the mouse was placed back into the cage. The time of oral gavage treatments were measured for both OG ICU and OG PBS groups. This was measured as the time from when the substance was collected into syringe, and completed when the mouse recovered from the oral gavage treatment and placed back into the cage. Time of recovery from oral gavage was measured as the time the mouse was placed back on the wire top after restraint to the time the mouse walked 3 consecutive steps.

Study Design: Evaluation of Immune Response Differences with Nutritional Gel

Female C57Bl/6J mice were instilled with either PBS (n=6) or 0.5 LD50 influenza in order to determine if NG alters the immune response in mice. The mice infected with influenza were maintained on either SOC (n=10) or NG (n=10). At 7 days post infection mice were euthanized. Bronchoalveolar lavage (BAL) fluid was then collected and the cells were counted, stained and flow cytometry was performed to determine the immune cell populations specifically looking at alveolar macrophages, interstitial macrophages, neutrophils, eosinophils, T-cells and B-cells.

Bronchoalveolar Lavage (BAL).

Seven days post infection mice were euthanized via cardiac exsanguination while under isoflurane anesthesia. BAL fluid was collected using 5 mM EDTA (Invitrogen, 0.5M EDTA, Ref AM9260G) diluted in sterile PBS as previously described. [29] To collect the BAL fluid in all mice, the trachea was intubated and 5mM EDTA was perfused within the lungs and then withdrawn. This step was performed three times per mouse with the same volume of EDTA+PBS (1.) 800 ul, then 2.) 700 ul, and finally 3.) 700ul) used in each mouse for BAL fluid collection.

Flow Cytometry.

Control (PBS) and IAV infected mice were euthanized 7 dpi and BAL fluid was collected. The BAL fluid was stored on ice during use. The resultant cells were counted using a Nexcelom cellometer (Nexcelom Bioscience, Lawrence, MA). The dead cells were excluded, using Cellometer AO/PI stain (Nexcelom Bioscience, Lawrence, MA).

Red blood cells were lysed from the sample using Red Blood Cell Lysis Solution (eBioscience, San Diego, CA). The remaining cells were then stained with PE Rat anti-mouse Siglec F (BD biosciences; Pharmingen, San Diego, CA), APC/Cy7 anti-mouse CD45 (Biolegend, San Diego, CA), PB anti-mouse CD11c (eBioscience, San Diego, CA), PE/Cy7 anti-mouse/ human CD11b (Biolegend, San Diego, CA), APC anti-mouse Ly6G (Biolegend, San Diego, CA), PerCP/Cy 5.5 anti-mouse CD3e (TONBO biosciences, San Diego, CA). Cells were incubated with Fc Block (BioLegend, San Diego, CA). Compensation was acquired using compensation beads, UltraComp eBeads (Invitrogen, Waltham, MA).

Cell populations were determined using a sequential gating strategy using FlowJo software program (FlowJo, LLC, Ashland, OR) (**Figure 8**). Populations of cells were determined as follows: 1) resident/ alveolar macrophages: $CD45^+$Siglec $F^+CD11b^+CD11c^+$, 2) interstitial macrophages: $CD45^+$Siglec $F^-CD11b^+CD11c^+$, 3) neutrophils: $CD45^+Ly6G^+CD11b^+$, 4) eosinophils: $CD45^+$Siglec F^+CD11c^-, 5) T-cells: $CD45^+CD3e^+$, and 6) B- cells: $CD45^+B220^+$. The percentage of cells within each inflammatory cell gate was multiplied by the total number of live cells (after AO/PI exclusion) to obtain an absolute live-cell count for each of the populations of immune cells identified with flow cytometry.

Statistical Analysis.

Sex as a Biological Variable

As both male and female mice are used in research of influenza virus, this study was performed in both male and female mice to ensure any results or benefit seen from NG was consistent between sexes. Mice received a low, middle, or high dose of influenza based on their gender specific LD50. Whereas the total PFUs used to treat male and female mice differed, the clinical course of disease was similar when the same gender specific LD50 dose was compared. To ensure that data from male and female mice could be grouped together, the data was combined and a normal distribution was confirmed using the % weight loss over time data and maximum % weight loss data. A normal distribution was confirmed through the D'Agostino and Pearson test, as well as assessment of the skewness and kurtosis of the data. If skewness was greater than -1 and less than 1, and kurtosis is greater than -2 and less than 2 then data was considered to have an approximately normal distribution. A normal distribution was confirmed

in the treatments and at all doses: 1.) low dose (SOC: skewness=-0.3210, kurtosis=-0.9077, 95% CI= 19.61-23.73; NG: skewness=0.3689, kurtosis=-0.3219, 95% CI= 16.16 -20.18), and 2.) middle dose (SOC: skewness=-0.5632, kurtosis=0.1403, 95% CI= 26.09-28.36; NG: skewness=-0.9744, kurtosis=-0.2461, 95% CI= 21.11 -24.79). The variance between male and female mice was also assessed using an F-test for variance, and no variance was seen. Therefore, both the male and female doses were combined and used for statistical analysis of each dose of influenza.

Power Calculations

Power calculations for group size were made using data obtained from the preliminary studies for this project using both male and female mice at 0.5 LD50 influenza doses. The power calculations were performed for both male and female mice separately. Power analysis suggested groups of n = 10-12 are recommended to reject the null hypothesis with 95% probability using the statistical tests.

Considerations for removal of mice from a study

Individual variations in response to viral infections are known to occur as shown by multiple studies, which means individual mice will respond differently to IAV infection. As we are assessing the benefits of supplementations to help support mice through infections leading to severe weight loss, only data from mice who lost at least 10% of total body weight in this study were evaluated, under the assumption that those who lost at maximum less than 10% of body weight did not have as severe of a response to influenza virus and would not benefit from supplementation, and ultimately resulting in skewed data.

Analysis of Data

All statistical analyses were performed using Prism 8.1.1 (GraphPad Software, La Jolla, CA). Unpaired, two-tailed, t-tests were used to determine significance of the maximum % weight loss between support strategy groups as well as inflammatory response data. Significance in weight loss during the progression of disease from 0-14 dpi was determined using multiple t-tests corrected for multiple comparisons using the Holm-Sidak method with few assumptions (alpha=0.05). Kaplan-Meier survival graphs and the log-ranked (Mantel-Cox) test were used to assess differences in mortality in each support strategy group. p-values less than 0.05 were considered statistically significant. Unpaired, two-tailed, t-tests were used to determine significance between total cell numbers and populations of inflammatory cells of BAL fluid from flow cytometry.

RESULTS

For all three doses of IAV studied, mice began to lose weight around 5 days post infection (dpi) and reached their maximum weight loss between 9-12 dpi. In both the middle and high doses, mice were removed from study due to reaching 30% weight loss at approximately 9-12 dpi. With the exception of one mouse in the SQ fluid group who was found dead, all mortalities were associated with mice who were euthanized due to reaching end point criteria of 30% weight loss. Mice were removed due to reaching end point in both the middle and high IAV doses that make statistical analysis of the % weight loss over time difficult due to survivor bias. Therefore, the maximum weight loss for each mouse on study was calculated so that mice that had reached the end point criteria could be included in the analysis of the different support strategies.

Nutritional Gel Supplementation

Low Dose Influenza (0.2 LD50):

Nutrient gel supplementation reduces weight loss in low dose influenza infected mice.

Mice infected with the low dose of IAV (0.2 LD50) provided the SOC reached a maximum % weight loss of 21.67% ± 4.53% (SEM = 0.99), while those provided NG reached 18.17% ± 4.30% (SEM = 0.96). There was a significant effect seen with the addition of NG, p=0.0153 with a 95% confidence interval of -6.29 to -0.710, implicating that the addition of NG helped mice recover from IAV infection sooner than mice receiving SOC (**Fig. 1B**). In analyzing the % weight loss over the time of infection, there was no significant difference seen between groups (**Fig. 1A**). There was no mortality associated with mice given the low influenza dose in both the SOC and NG supplemented groups (**Fig. 1C**).

Middle Dose Influenza (0.5 LD50):

Nutrient gel supplementation decreases weight loss and mortality in middle dose influenza infected mice. Mice infected with the middle dose of IAV (0.5 LD50) provided the SOC reached a maximum % weight loss of 27.2% ± 3.04% (SEM = 0.56), while those in NG reached 23.0% ± 4.66 % (SEM = 0.90), showing there was a significant reduction in maximum % weight loss with the addition of NG, p=0.0001 with a 95% confidence interval of -6.35 to -2.21 (**Fig. 2B**). There was also a significant difference seen in the % weight loss over the time of infection at 9 and 10 dpi demonstrating that supplementation with NG results in a more rapid recovery post influenza infection (**Fig. 2A**). There was no mortality associated with reaching end-point criteria of 30% loss of initial weight in mice supplemented with NG, while there was a 17% mortality in mice given the SOC with 5 mice removed early due to weight loss. Mortality was plotted on a Kaplan-Meijer curve and statistical significance was shown through a log-rank (Mantel-Cox) test (p=0.028, Chi squared=4.834, df=1) (**Fig. 2C**).

High Dose (1.25 LD50):

Nutritional gel supplementation provides no benefit to mice infected with high dose influenza. Only female mice were assessed at the high dose of influenza. Female mice infected with the high dose of influenza (1.25 LD50) provided the SOC reached a max % weight loss of 29.9% +/- 1.63% (SEM = 0.45), while those in NG reached 29.8% +/- 2.44% (SEM = 0.70). Looking at the maximum % weight loss between the groups, there was no significant effect seen with the addition of NG, p=0.929 with a 95% confidence interval of -1.774 to 1.626 (**Fig. 3B**). There was no significant benefit seen over the time of the disease (**Fig. 3A**). There was mortality associated with both SOC and NG. Six mice reached euthanasia criteria resulting in early removal and a mortality of 46.2% of mice who were provided SOC, while 5 mice receiving NG

reached euthanasia criteria resulting in a mortality of 41.7%, with no significant difference between groups (p=0.754) (**Fig. 3C**).

Subcutaneous Fluid Administration

Middle Dose Influenza (0.5 LD50):

SQ fluid administration decreases weight loss in middle dose influenza infected mice. This study was performed only in male mice given the middle dose of influenza. Mice infected with the middle dose of IAV (0.5 LD50) provided the SOC reached a max % weight loss of 27.3% ± 2.5% (SEM = 0.60), while those given SQ fluids had a max loss of 22.4% ± 6.2% (SEM = 2.2), showing there was a significant reduction in maximum % weight loss with SQ fluid administration, p=0.007, df t=2.926, df=24, 95% confidence interval -8.399 to -1.451 (**Fig. 4B**). There was also a significant difference seen between the groups regarding the % weight loss over the time of infection at 6, 8, and 9 dpi (**Fig. 4A**). There was no significant difference in mortality associated with reaching end-point criteria of 30% loss of initial weight as there was 17% mortality associated in the SOC group, and a 13% mortality associated with the SQ fluid group. Mortality was plotted on a Kaplan-Meijer curve and no statistical significance was found using a log-rank (Mantel-Cox) test (p= 0.750, Chi squared= 0.1019, df=1) (**Fig. 4C**). One mouse in the SQ fluid group was found dead in the cage on 12 dpi. On gross necropsy signs of gastroenteritis were noted with no overt gastrointestinal ulceration(s) seen grossly or based on histopathology.

The benefit from SQ fluid administration was directly compared to NG supplementation, by analyzing the maximum % weight loss. This comparison found no significant difference between SQ fluids and NG; p=0.557. There was only a significant difference seen at 6 dpi when analyzing % weight loss over progression of disease (**Fig. 5**).

Oral Gavage of Nutritional Formulation or PBS

Middle Dose Influenza (0.5 LD50):

Oral gavage of nutritional formula or PBS provides no benefit in middle dose IAV infected mice. This study was performed only in male mice provided the middle dose of influenza. There was no significant benefit in minimizing weight loss in mice provided OG ICU or OG PBS compared to SOC. Mice provided the SOC reached a max % weight loss of 27.3% +/- 2.5% (SEM = 0.60), while those given OG ICU had a max loss of 28.0% +/- 3.3% (SEM = 1.92). Using an unpaired, two-tailed, t-test comparing the max % weight loss between the groups, there was no significant effect seen with OG ICU supplementation, p=0.691, confidence interval -2.771 to 4.093 (**Fig. 6B**). OG PBS had a maximum % weight loss of 24.6% +/-1.6 (SEM = 0.92). p=0.086, 95% confidence interval -5.976 to 0.4312 (**Fig. 6B**). There was no significant difference in % weight loss over the time of the disease in either oral gavage treatment (p>0.05 at each dpi) (**Fig. 6A**).

There was increased mortality seen in the mice provided oral gavage of human ICU formula. Mortality was plotted on a Kaplan-Meijer curve and no statistical differences were observed using a log-rank (Mantel-Cox) test (p= 0.1696, Chi squared= 3.549, df=2) (**Fig. 6C**) One mouse was removed from study early due to the presence of formula noted in nares bilaterally during gavage, signifying a likely aspiration event. The mouse was immediately euthanized and lungs were submitted for histology, with no evidence of aspiration noted in lungs. The lungs were submitted for bacterial culture and returned with no bacterial growth.

It was noted that mice did not respond well to oral gavage, especially as their disease progressed, therefore the time to recovery from oral gavage was measured in both OG ICU and OG PBS groups of mice. The recovery from oral gavage was measured in mice (n=2 for each

group) and there was an increase in the recovery time seen as the IAV infection worsened clinically, especially in mice being given OG ICU (**Fig. 7**).

Evaluation of Time of Treatments

Another consideration is the amount of time it will take research personnel to perform these tasks, as a faster support strategy has the potential to reduce stress on the mice. Therefore, the total time to provide treatments, including preparing and administration, was timed. Overall there was no significant difference in the time to provide SOC and NG, while it took considerably more time to prepare and give SQ fluids and both oral gavage treatments (**Table 3**).

Evaluation of Immune Response Differences with Nutritional Gel Supplementation

Nutritional gel supplementation does not alter immune responses to middle dose IAV infection 7 dpi. Nutritional gel was found to be a beneficial support strategy resulting in decreased weight loss and mortality in mice post IAV infection at the low and middle doses. Therefore, further studies were performed to determine if NG altered the immune response. The immune cells recovered in BAL fluid 7 dpi was measured in female mice given the middle-dose of IAV and provided either SOC or NG. Cell counts were performed to get the total number of cells recovered in BAL fluid. Flow cytometry with specific markers for immune cells was performed to identify resident/alveolar macrophages, interstitial macrophages, neutrophils, eosinophils, T-cells, and B- cells. There was no significant difference observed in the total cell numbers of cells when mice in the SOC and NG groups were compared 7 dpi (**Table 4**). Additionally, flow cytometry showed that there was no significant difference ($p>0.05$) in the total number of alveolar macrophages (AM), interstitial macrophages (IM), neutrophils, eosinophils, B cells or T cells when mice in the SOC and NG groups were compared (**Fig. 9**).

DISCUSSION

This study was initiated to evaluate and identify support strategies that decrease weight loss and mortality in mice infected with IAV. A common standard of care (SOC) practice for sick mice at the University of Washington, including those infected with IAV, is the placement of moistened pellets and hydration gel (Clear H_20 HydroGel) on the cage floor to allow easy access to feed and hydration. This raised the question as to whether supplementing both hydration and/or nutrition with the support strategies utilized in this study would improve outcomes. Initially this study was performed in all treatment groups (SOC, NG, SQ fluids, OG ICU, OG PBS), until it became evident that the stress involved with those treatments likely outweighed their benefit, and the study shifted to focus on NG supplementation.

In this study, we found that NG supplementation had a positive effect over the course of infection, specifically with recovery in IAV infected mice. When compared to Clear H_2O HydroGel, the nutritional gel (NG), DietGel Recovery, provides hydration, calories, and electrolytes indicating that the replacement of the HydroGel with DietGel may improve outcomes in mice infected with IAV (**Table 2**). In this study, NG showed the most benefit to mice infected with influenza compared to other support strategies evaluated, and continued investigation was performed to further assess this treatment. Laboratories studying influenza virus implement a variety of doses depending on the research being performed. Due to these variances, we chose to assess the effect of NG on mice given low, middle and high doses of influenza to try and determine if this strategy would be useful for a range of influenza research models **(Table 1).** In order to appropriately determine if supplementation reduces weight loss, for this study, the doses of influenza used were selected due to their ability to cause notable weight loss after infection and with the potential for mortality at the middle and higher doses.

At the low and middle doses of influenza, there was a benefit to the supplement of NG seen by significant differences in weight and mortality compared to the SOC, however, at the high dose there was no benefit seen. At the high dose of influenza, the period of anorexia, as observed through the visual estimates each day, lasted for longer periods of time than in the other influenza doses (data not shown), which negatively impacted weights of the mice. It is probable that at a high enough infectious dose, the illness is too great and the inflammatory response too strong resulting in continued anorexia for longer periods of time. Even with no added benefit to mice at higher doses of influenza, a benefit was seen at the low and middle doses of influenza. Further study would be required to determine the exact reason for the benefit acquired by influenza infected mice, however, there are multiple factors that may have played a role.

From observation of the feed intake of mice, the nutritional gel was readily eaten the first time offered on the day of infection. Thus, no acclimation time was required. It was noted that mice began to consume the NG more quickly following the period of anorexia from influenza infection than mice in the SOC group. It is suspected that the taste of the nutritional gel, which contains corn syrup, was a key player in the rate at which mice ate NG, as there was an evident preference for NG over the flavorless hydration gel seen between treatment groups. It has been shown that C57Bl/6J mice favor the taste of certain sugars, including maltose which is a main ingredient in corn syrup, which could have contributed to the high consumption rate of NG.[30, 31] There is a large variety of nutritional gels available on the market, and it is possible others containing mouse preferred sugars could also provide a benefit in mice during infection studies.[30,32-35]

The goal of this study is to provide evidence toward a support strategy that research laboratories studying influenza can apply to decrease the number of mice used in research. In

order to be able to comfortably recommend NG supplementation, we wanted to ensure there were no alterations to the immune response that may have influenza recovery from IAV. 7 dpi was chosen as the appropriate time to evaluate as it would include components of both the innate and adaptive immune response at this time period.[9,10] No further time points were assessed under the assumption that the initial inflammatory response would be a good representation of the course of the immune response to influenza. The inflammatory response to influenza infection in mice provided NG was comparable to mice provided the SOC, and no differences were noted. Therefore, the implementation of NG to influenza infected mice did not alter the results of immunologic data.

This study began by evaluating all support strategies concurrently (SOC, NG, SQ fluids, OG ICU, OG PBS) in male mice infected with the middle dose of influenza, until inherent problems associated with both the oral gavage and subcutaneous fluid groups were revealed. Once it became apparent that mice in the oral gavage and subcutaneous groups were being negatively impacted by the treatment process, it was deemed that the harm of repeating the studies did not outweigh the benefit of increasing power and these studies were not continued and the focus of the study shifted to evaluation of NG supplementation.

Wang et al. found that nutritional supplementation using oral gavage with nutritional supplement (Abbott Promote ICU nutritional formulation) protected against mortality from influenza infection, suggesting that the provision of a nutritional supplement via oral gavage could be a support strategy utilized for IAV infected mice.[36] However, in our study the use of oral gavage of either nutritional formula or PBS to IAV infected mice is not a recommended support strategy as these strategies showed no significant benefit to the weight loss due to viral infection and caused distress to the mice as well as mortality. Mice are restrained tightly for oral

gavage to take place, which impedes breathing in already respiratory challenged mice, and it was evident during the procedure that mice did not tolerate the oral gavage well with prolonged recovery times and marked respiratory distress post-gavage. It was interesting to note, that the time of the treatment and recovery was increased in mice in the OG ICU group compared to OG PBS. It is possible the thicker consistency of the nutritional formula was the cause of this through increased time to draw up the liquid into a syringe (**Table 3**). And this thicker consistency also could have led to increased restraint time required to gavage the ICU solution and therefore leading to the increased recovery time seen in the mice (**Fig. 7**). There are intrinsic complications associated with oral gavage in mice including the potential for trauma, stress, aspiration of nutritional formula that could affect the pulmonary immune response and thereby confounding immunologic data. For all of these reasons, the use of oral gavage in influenza infected mice is not endorsed by this study.

Unlike the study by Wang et al,[36] there was no benefit seen in using oral gavage of human ICU formula in the mice infected with influenza. Our sample size was small which could affect significance, however, the continued use of mice for these treatments seemed contraindicative to our overall goal of study: to improve welfare and minimize the number of mice needed for influenza experiments. As there was benefit seen in subcutaneous fluids and nutritional gel, the experiment then focused on these strategies.

Work by Sanders et al. evaluated whether the support strategy of intraperitoneal fluid therapy improved weight loss in C57BL/6 and BALB/c mice infected with IAV. This study showed that infection of BALB/c and C57BL/6 mice with IAV caused significant weight loss that was not improved by intraperitoneal administration of either normal saline or compound sodium lactate (20 ml/kg).[37] This raised the question as to whether hydration strategies with slower absorption,

such as subcutaneous administration of fluids, may provide benefits? In our study, SQ fluid administration showed a significant benefit in altering weight loss in mice infected with influenza compared to the SOC. Mice began treatment with SQ fluids once any mouse in the cage reached 5% weight loss. This occurred between 5-6 dpi in all groups of mice. It is interesting to note that in the graph showing % weight loss over time (**Figure 4A**), the lines diverge at approximately the time subcutaneous fluid administration was initiated, and there was also a significant difference in the % weight loss at 6 dpi between the SQ fluid group and both SOC and NG groups. The weight measurements were always taken at minimum 12 hours post subcutaneous fluid administration, when fluids should be absorbed. However, this divergence does bring up the question as to whether fluids were retained in the subcutaneous space and therefore deceptively increasing the weight of the mice.

While SQ fluids did show a benefit by minimizing weight loss (Fig. 4A and B), twice daily handling for fluid administration is stressful for mice. One mouse in this group was found dead in the cage with evidence of enteritis on gross necropsy, which could be indicative of a stress response. It is unknown, but possible that increased stress from subcutaneous fluid administration played a role in the death of this mouse. While this treatment did provide a significant difference in the weights of influenza infected mice, there was no significant difference in the max % weight loss of mice administered SQ fluids when compared to nutritional gel supplementation (**Fig. 5B**). The only significance seen comparing these treatments was at 6 dpi, which is 1) an early time point in the disease process and 2) the time when all mice are receiving SQ fluids (**Fig. 5A**). It is possible the cause of this difference is due to weight of fluids, and has no relevance to the disease itself. While there was a significant benefit was seen with the provision of SQ fluids, this strategy requires regular handling/ stress on

mice and takes longer to give, therefore NG was a stronger candidate for recommendation for influenza infected mice. With the same reasoning as with the oral gavage treatments, it was deemed unnecessary to continue using mice for studying the response to SQ fluid administration.

Overall, NG supplementation showed the most benefit and its use is recommended for use in mice being utilized in influenza research. This support strategy minimized weight loss and mortality, and is fast and easy to give, readily consumed, involves no handling of mice and does not alter immune response to the influenza disease process at 7 dpi. The implementation of this support strategy to mice on influenza studies is a way to provide support to the mice and minimize premature loss of animals due to reaching weight related end point criteria.

FIGURES

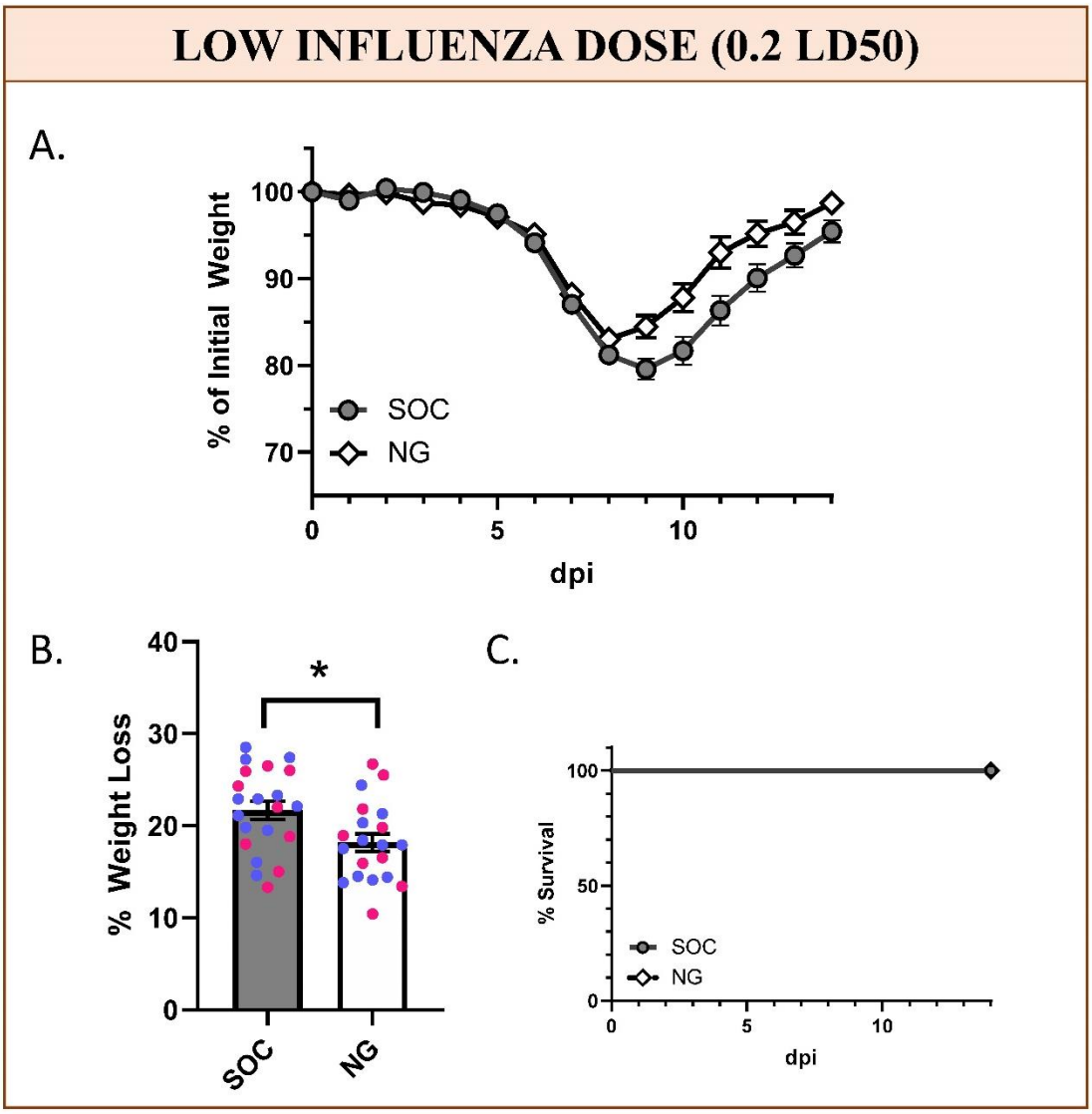

Figure 1. Nutritional support using NG in mice infected with low influenza dose. (A) % of initial body weight following exposure to IAV. Values are the mean ± SEM. Statistical analysis was performed using multiple t-tests corrected for multiple comparisons using the Holm-Sidak method ($p< 0.05$, $n = 15$), and no significance was seen over progression of disease course. (B) Maximum percent weight loss for individual mice on the study. Statistics were performed using an unpaired, two-tailed, t-test with significantly reduced max % weight loss in NG group compared to SOC ($p=0.0153$). Male mice are represented by blue circles and females by red circles. (C) Kaplan- Meijer survivor graph. No mortality was seen in either SOC or NG groups at the lower influenza dose. SOC n=21; DG n=20.

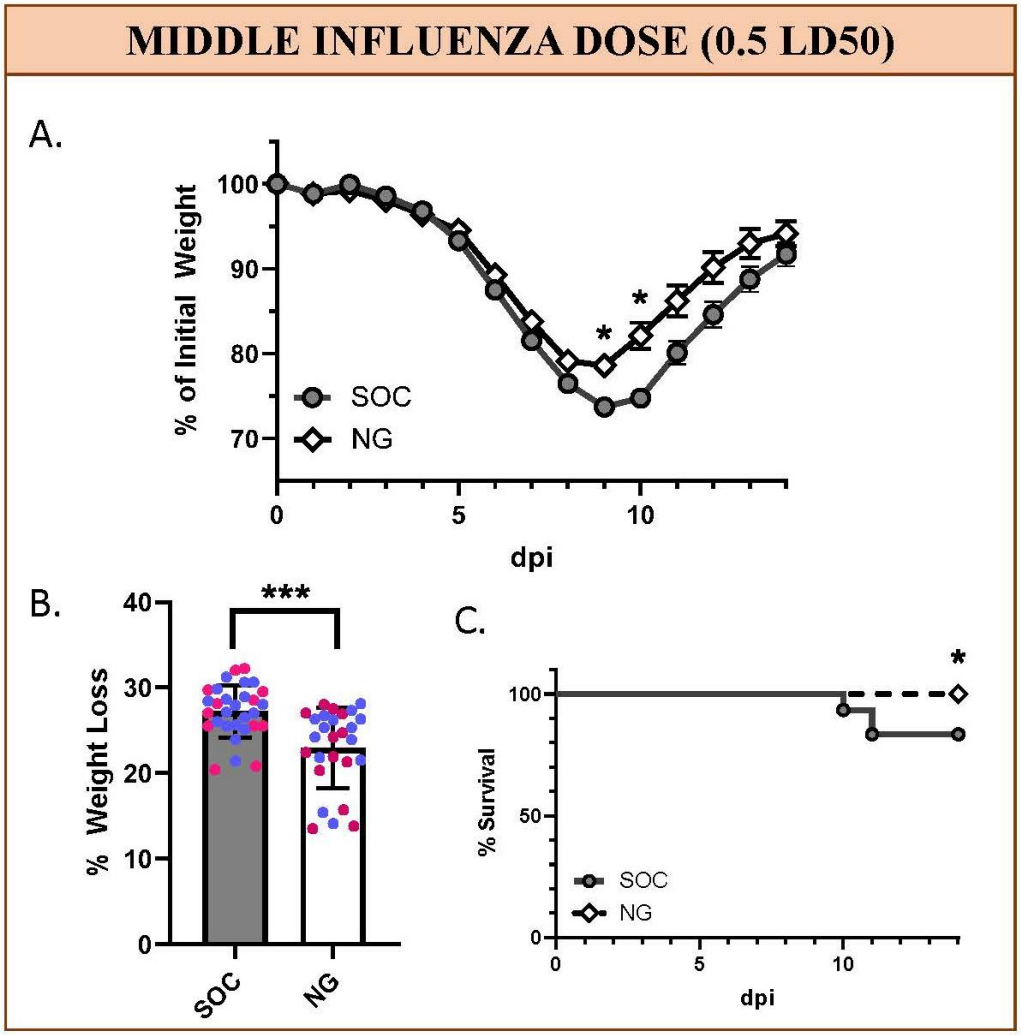

Figure 2. Nutritional support using NG in mice infected with middle influenza dose. (A) % of initial body weight following exposure to IAV. Values are the mean ± SEM. Statistical analysis was performed using multiple t-tests corrected for multiple comparisons using the Holm-Sidak method ($p< 0.05$, $n = 15$), and significance was seen at 9 (adjusted $p= 0.004$) and 10 dpi (adjusted $p= 0.003$). (B) Maximum percent weight loss for individual mice on the study. Statistics were performed using an unpaired, two-tailed, t-test with significantly reduced max % weight loss in NG group compared to SOC ($p=0.0001$). Male mice are represented by blue circles and females by red circles. (C) Kaplan- Meijer survival graph. A significant reduction in mortality was seen in mice supplemented with NG using the Log-rank (Mantel-Cox) test ($p=0.028$). SOC n=30; DG n=27.

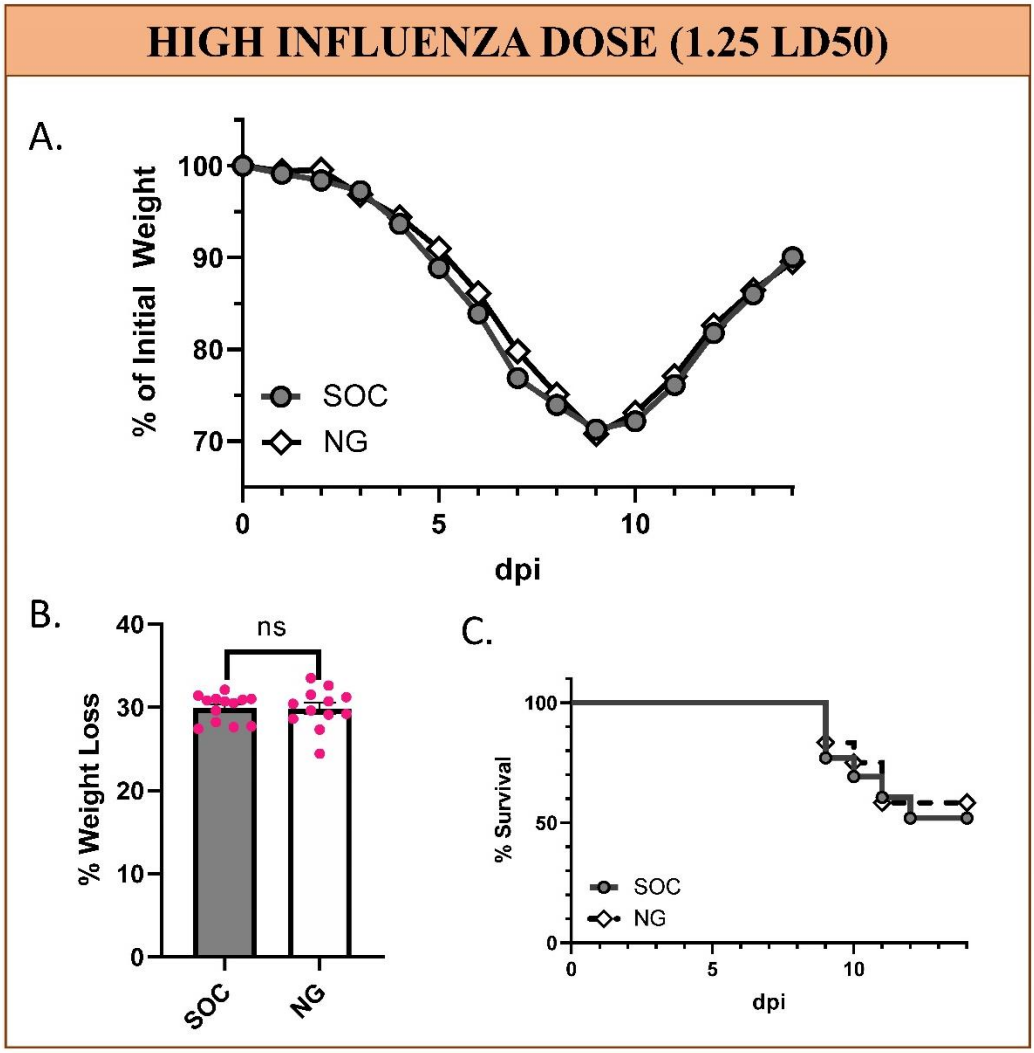

Figure 3. Nutritional support using NG in mice infected with high influenza dose. (A) % of initial body weight following exposure to IAV. Statistical analysis was performed using multiple t-tests corrected for multiple comparisons using the Holm-Sidak method ($p< 0.05$, $n = 15$), and no significance was seen over progression of disease process. (B) Maximum percent weight loss for individual mice on the study. Statistics were performed using an unpaired, two-tailed, t-test with no significantly reduced max % weight loss in NG group compared to SOC ($p=0.929$). (C) Kaplan- Meijer survival graph. No significant reduction in mortality was seen in mice supplemented with NG using the Log-rank (Mantel-Cox) test ($p=0.754$). SOC n=13; DG n=12.

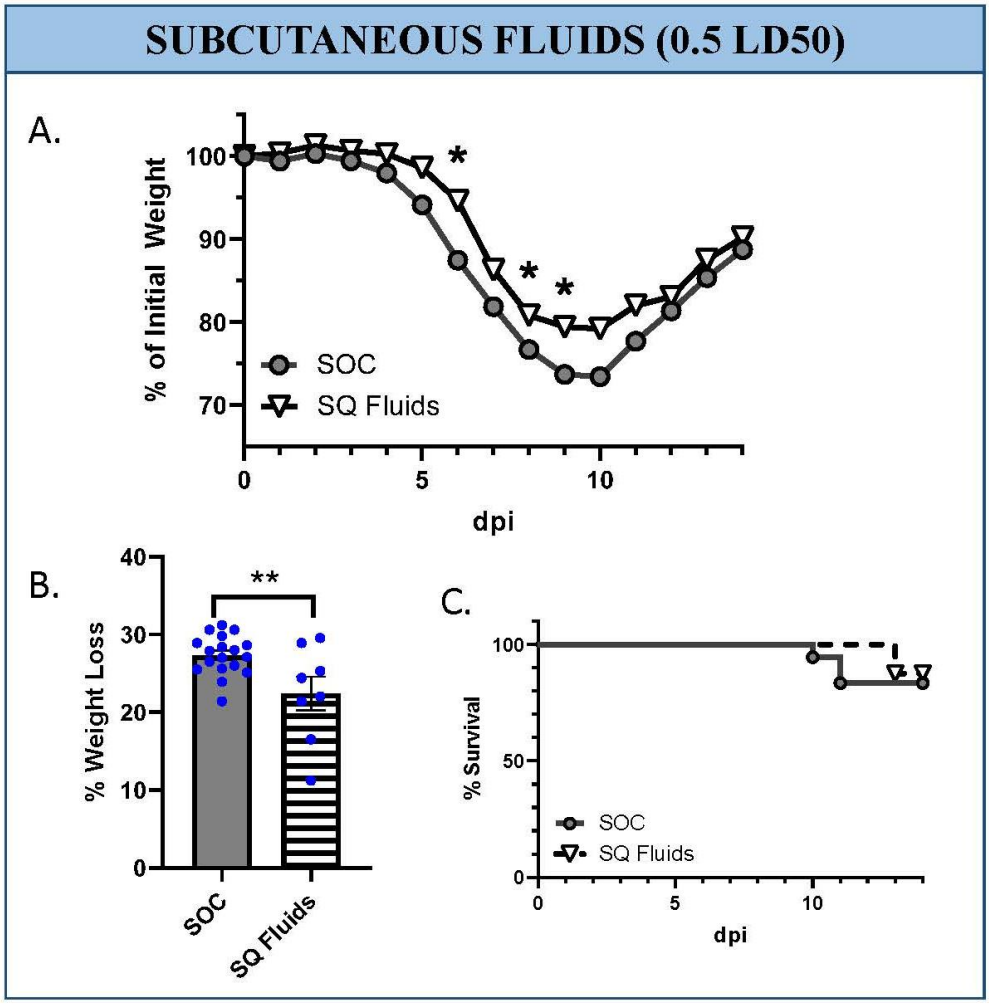

Figure 4. Subcutaneous fluid administration in mice infected with middle influenza dose. (A) % of initial body weight following exposure to IAV. Statistical analysis was performed using multiple t-tests corrected for multiple comparisons using the Holm-Sidak method ($p< 0.05$, $n = 15$), and significance was seen at 6 (adjusted $p=0.003$), 8 (adjusted $p=0.030$), and 9 dpi (adjusted $p=0.005$). (B) Maximum percent weight loss for individual mice on the study. Statistics were performed using an unpaired, two-tailed, t-test with significantly reduced max % weight loss in mice given SQ fluids compared to SOC ($p=0.007$). (C) Kaplan- Meijer survival graph. No significant reduction in mortality was seen in mice administered SQ fluids using the Log-rank (Mantel-Cox) test ($p=0.750$). SOC n=18; SQ fluids n=8.

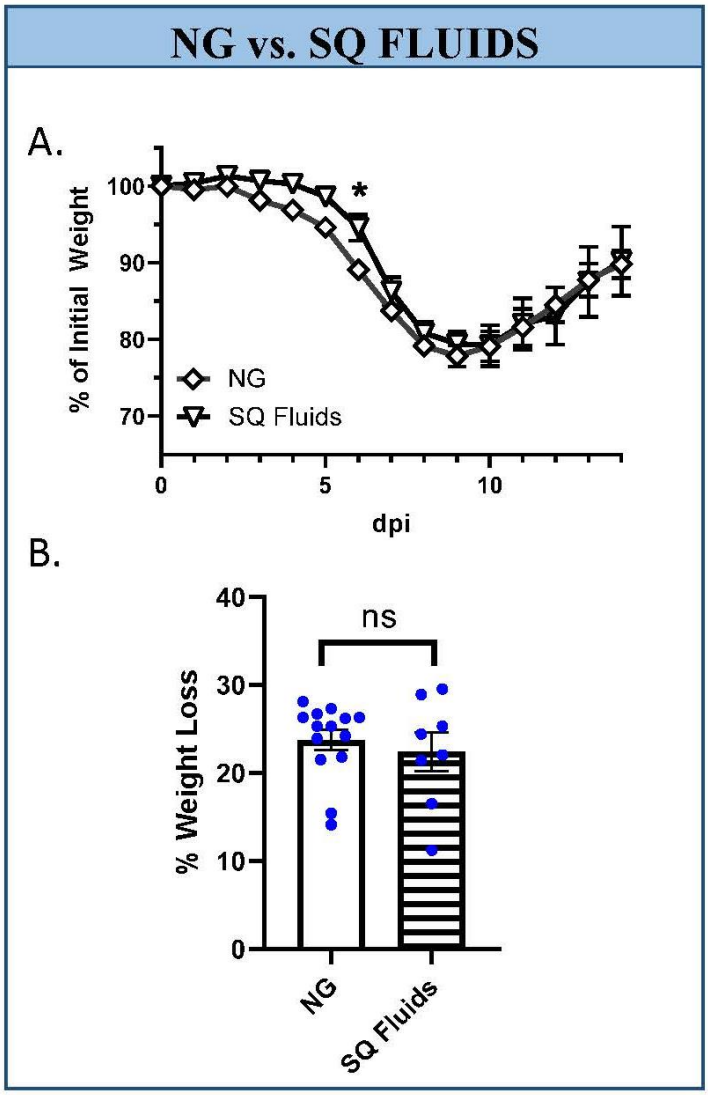

Figure 5. NG supplementation compared to subcutaneous fluid administration in mice infected with middle influenza dose. (A) % of initial body weight following exposure to IAV. Statistical analysis was performed using multiple t-tests corrected for multiple comparisons using the Holm-Sidak method ($p< 0.05$, $n = 15$), and significance was seen on 6 dpi ($p=0.039$) between NG and SQ fluids. (B) Maximum percent weight loss for individual mice on the study. Statistics were performed using an unpaired, two-tailed, t-test with no significantly reduced maximum % weight loss in mice seen comparing NG with SQ fluids ($p= 0.557$). NG n=14; SQ fluids n=8.

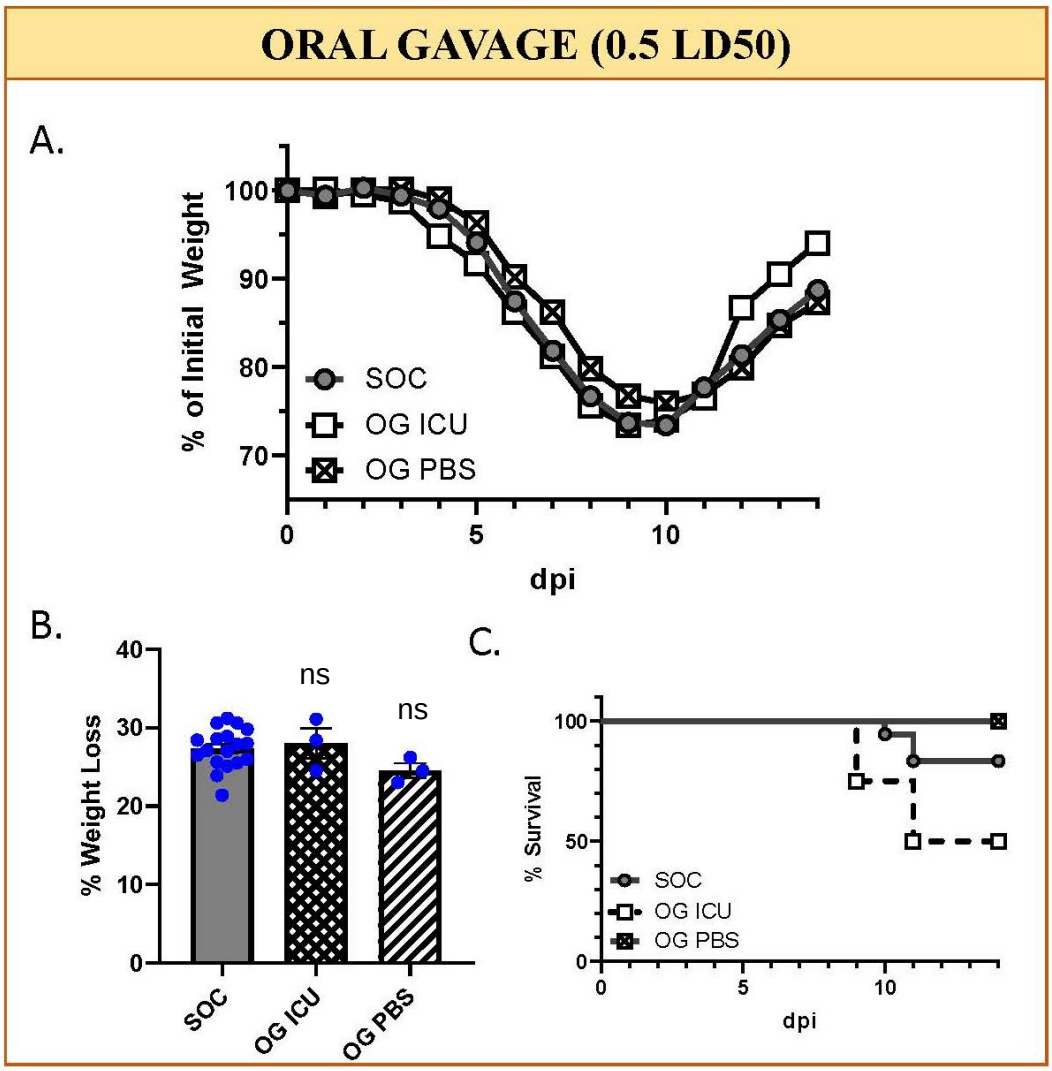

Figure 6. Oral gavage strategies in mice infected with middle influenza dose, including OG ICU and OG PBS. (A) % of initial body weight following exposure to IAV. Statistical analysis was performed using multiple t-tests corrected for multiple comparisons using the Holm-Sidak method ($p< 0.05$, $n = 15$), and no significance was seen through progression of disease. (B) Maximum percent weight loss for individual mice on the study. Statistics were performed using an unpaired, two-tailed, t-test with no significant benefit seen with support with either oral gavage supplement (OG ICU $p=0.691$; OG PBS $p=0.086$). (C) Kaplan-Meijer survival graph. No significant reduction in mortality was seen in either oral gavage support strategy using the Log-rank (Mantel-Cox) test ($p=0.750$). SOC n=18; OG ICU fluids n=4, OG PBS n=3.

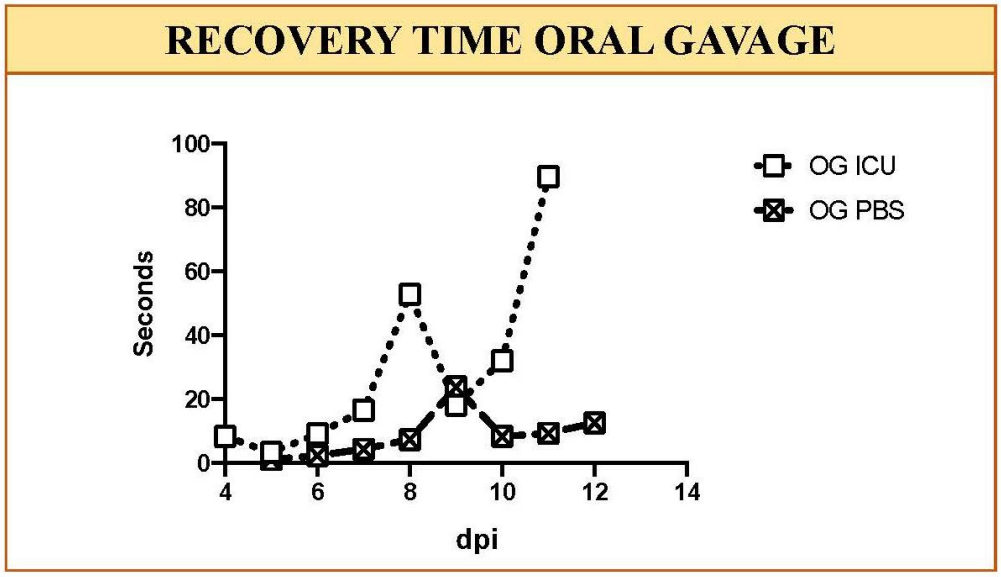

Figure 7. Time to recovery of oral gavage treatments. Time of recovery measured as when mouse placed back on cage top after restraint until time mouse walks 3 steps. There was an increase in the time (seconds) it took mice to recover from restraint for oral gavage as disease severity progressed. Mice receiving oral gavage ICU formula appeared to take longer to recover than those given PBS. OG ICU n=2, OG PBS n=2.

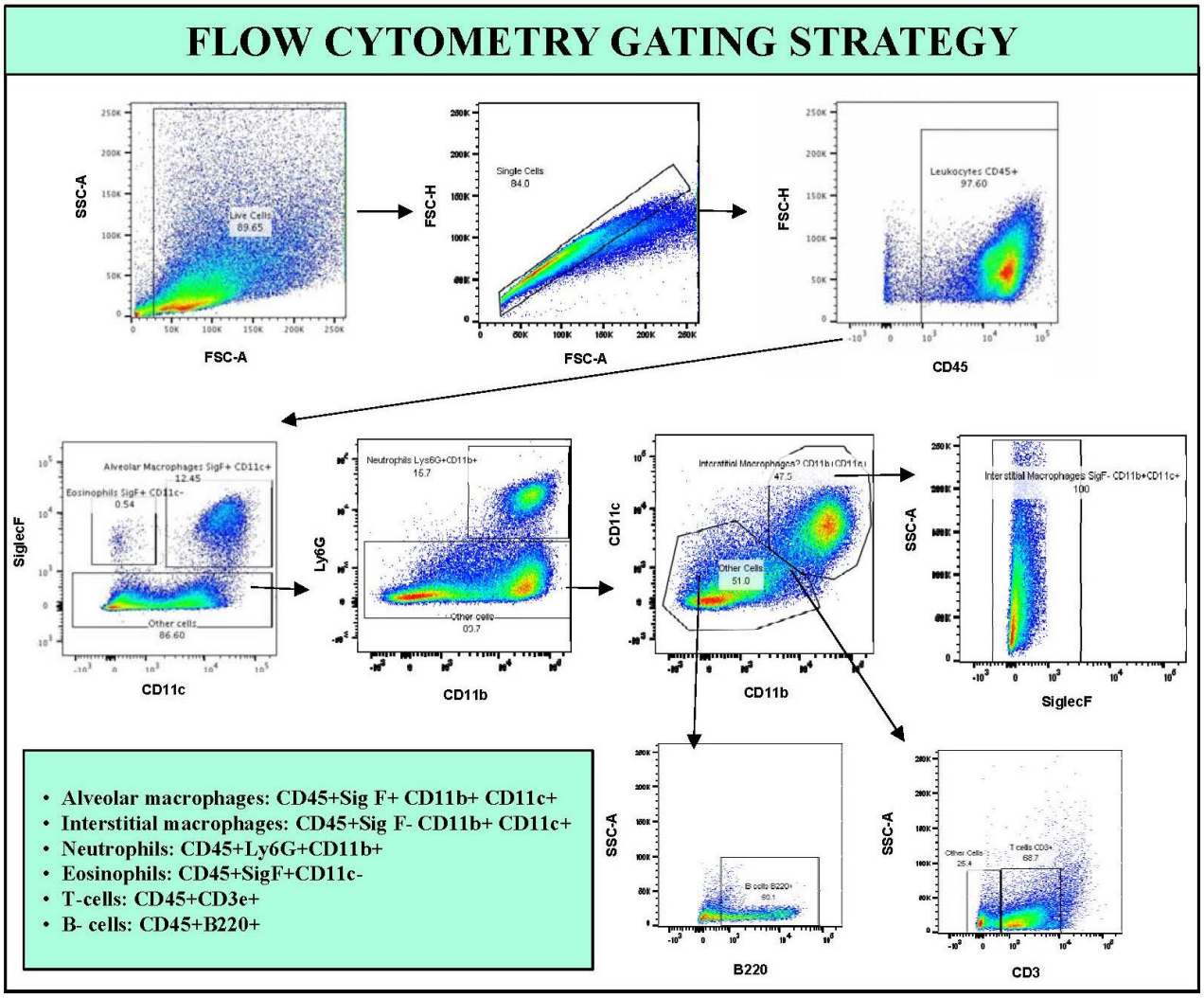

Figure 8 . Flow cytometry gating strategy used for identification of individual cell populations for analysis of inflammatory response between groups of mice. PBS n=6, SOC n=10, NG n=10

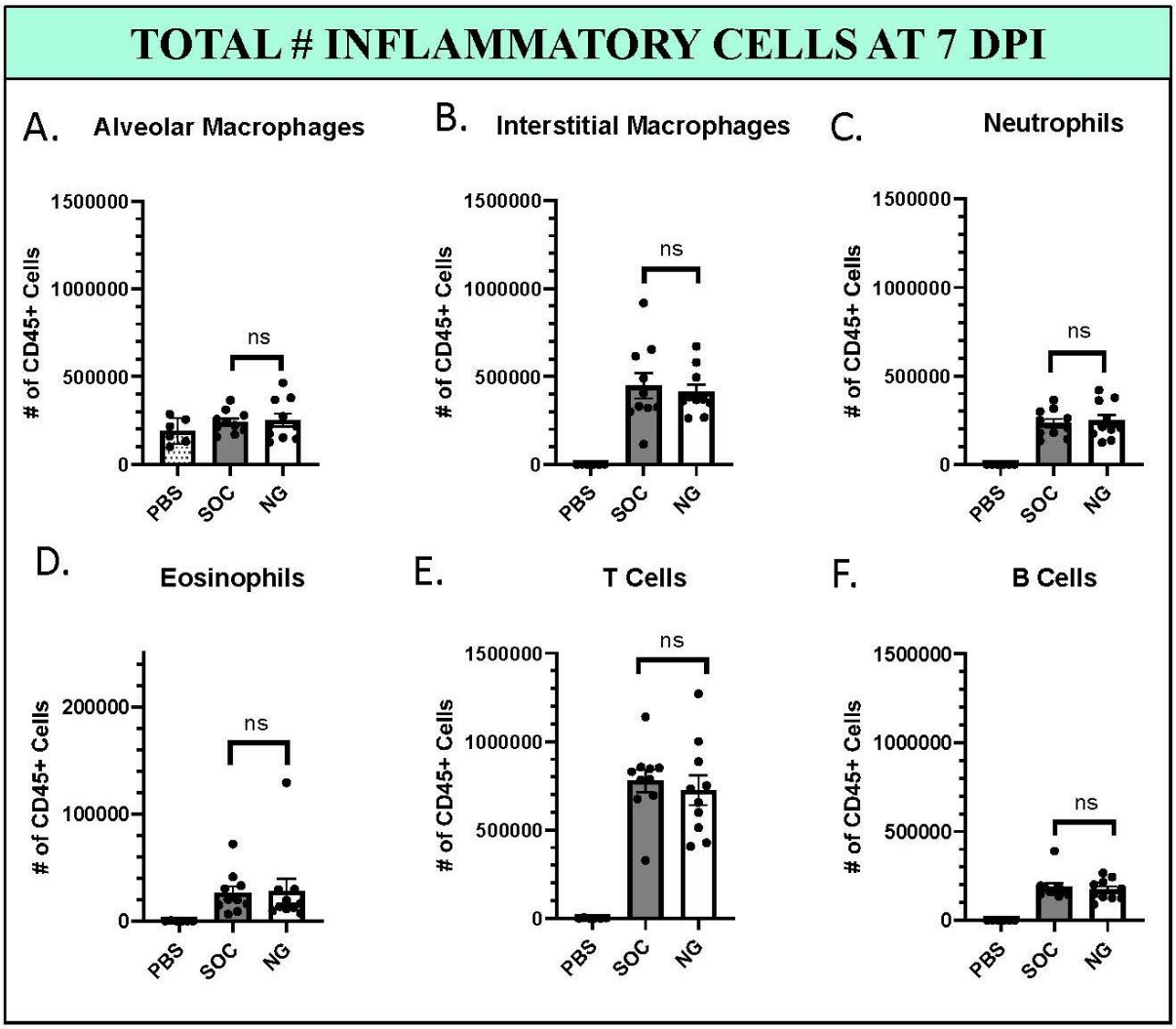

Figure 9. Total number of CD45+ staining inflammatory cells from BAL fluid collected 7 dpi from female mice infected with PBS or middle dose IAV given SOC or NG. Populations of inflammatory cells identified through flow cytometry. Statistics performed comparing only SOC and NG. A) Total # of alveolar macrophages, B) total # of interstitial macrophages, C) total # of neutrophils, D) total # of eosinophils, E) total # of T cells, F) total # of B cells. No significant difference seen between NG and SOC for any cell population assessed (Table 4). PBS n=6, SOC n=10, NG n=10.

TABLES

	Female Mice		Male Mice	
Dose	SOC	NG	SOC	NG
Low – 0.25 LD50	4 PFU n = 9	4 PFU n= 9	10 PFU n =12	10 PFU n = 11
Middle– 0.5 LD50	10 PFU n = 12	10 PFU n = 13	25 PFU n =18	25 PFU n = 14
High – 1.25 LD50	25 PFU n = 14	25 PFU n = 13		

Table 1. Doses of IAV for Studies of Nutritional Gel Supplementation.

	LabDiet 5053 (per gram)	HydroGel (per gram)	DietGel Recovery (per gram)	Promote ICU Formula (per ml)
Calories (kcal)	*4.07*	*0.063*	*2.4*	*1*
Calories from fat	*0.13*	*0*	*0*	-----
Protein (g)	*0.2*	*0*	*0.006*	*0.062*
Carbohydrates (g)	*0.62*	*0.015*	*0.126*	*0.130*
Sugars (g)	*0.3884*	*0*	*0.23*	-----
Dietary Fiber (g)	*0.047*	*0.014*	*0.008*	-----
Fat –(g)	*0.05*	-----	*0.019*	*0.026*
Saturated Fat(g)	-----	-----	*0.002*	-----
Moisture (%)	*10*	-----	*70-75*	-----
Calcium (mg)	*0.008*	*0.036*	*0.06*	*1.2*
Chloride (mg)	*0.0051*	-----	*1.052*	*1.266*
Potassium (mg)	*0.011*	*0.30*	*1.013*	*1.99*
Sodium (mg)	*0.003*	*0.38*	*0.518*	*1.01*
Phosphorous (mg)	*0.0063*	*0.23*	*0*	*1.20*

Table 2. Nutritional information for nutritional support strategies used in the study. SOC consists of moistened LabDiet 5053 and HydroGel. Per LabDiet, mice will eat approximately 5 grams/ day/ mouse. NG consists of moistened LabDiet 5053 and DietGel Recovery. Mice received 5 gram/ mouse/ day of DietGel Recovery when on study. OG ICU mice are provided with Promote ICU Formula mice on study received 10ml/kg which was approximately 1 ml total per mouse per day.

Treatment	Time of treatment/ mouse (seconds)
SOC	*4.84 ± 1.04*
NG	*5.40 ± 1.44*
SQ Fluids	*43.2 ± 12.2*
OG ICU	*68.7 ± 32.5*
OG PBS	*51.4 ± 13.3*

Table 3. Measurements of the times to perform each treatment per mouse in the cage. Average time + standard deviation to perform support strategies per mouse in a cage.

Cell Type	Total Cell #		p-value
	SOC	NG	
AMs	$2.40 \times 10^5 \pm 6.47 \times 10^4$	$2.51 \times 10^5 \pm 1.16 \times 10^5$	0.789
IMs	$4.48 \times 10^5 \pm 2.29 \times 10^5$	$4.12 \times 10^5 \pm 1.32 \times 10^5$	0.665
Neutrophils	$2.33 \times 10^5 \pm 7.78 \times 10^4$	$2.48 \times 10^5 \pm 1.04 \times 10^5$	0.732
Eosinophils	$2.63 \times 10^4 \pm 1.94 \times 10^4$	$2.79 \times 10^4 \pm 3.64 \times 10^4$	0.904
T cells	$7.78 \times 10^5 \pm 2.02 \times 10^5$	$7.25 \times 10^5 \pm 2.71 \times 10^5$	0.616
B cells	$1.86 \times 10^5 \pm 7.38 \times 10^4$	$1.73 \times 10^5 \pm 5.81 \times 10^4$	0.650

Table 4. Mean total cell numbers + standard deviation of inflammatory cells identified via flow cytometry comparing total number of cells retrieved from BAL fluid from SOC (n=10) compared to mice supplemented with NG (n=10). Results of unpaired t-tests comparing the total cell numbers between the groups are listed under p-value. There was no significant difference seen between any of the inflammatory cell types ($p>0.05$).

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