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Original Research

Evaluation of Nutritional Gel Supplementation in C57BL/6J Mice Infected with Mouse-Adapted Influenza A/PR/8/34 Virus

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Mice are a common animal model for the study of influenza virus A (IAV). IAV infection causes weight loss due to anorexia and dehydration, which can result in early removal of mice from a study when they reach a humane endpoint. To reduce the number of mice prematurely removed from an experiment, we assessed nutritional gel (NG) supplementation as a support strategy for mice infected with mouse-adapted Influenza A/Puerto Rico/8/34 (A/PR/8/34; H1N1) virus. We hypothesized that, compared with the standard of care (SOC), supplementation with NG would reduce weight loss and increase survival in mice infected with IAV without impacting the initial immune response to infection. To assess the effects of NG, male and female C57BL/6J mice were infected with IAV at low, intermediate, or high doses. When compared with SOC, mice given NG showed a significant decrease in the maximal percent weight loss at all viral doses in males and at the intermediate dose for females. Mice supplemented with NG had no deaths for either sex at the intermediate dose and a significant increase in survival in males at the high viral dose. Supplementation with NG did not alter the viral titer or the pulmonary recruitment of immune cells as measured by cell counts and flow cytometry of cells recovered in bronchoalveolar lavage (BAL) fluid in either sex. However, mice given NG had a significant reduction in IL6 and TNFα in BAL fluid and no significant differences in CCL2, IL4, IL10, CXCL1, CXCL2, and VEGF. The results of this study show that as compared with infected SOC mice, infected mice supplemented with NG have reduced weight loss and increased survival, with males showing a greater benefit. These results suggest that NG should be considered as a support strategy and indicate that sex is an important biologic variable in mice infected with IAV.

Abbreviations: IAV, Influenza A Virus; SOC, Standard of Care; NG, Nutritional Gel; EuD50, Euthanasia Dose 50; LD50, Lethal-Dose 50; PFU, Plaque Forming Unit; dpi, days post infection; IL6, Interleukin 6; SEM, Standard Error of Mean; ns, no significance

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Influenza A virus (IAV) infections place a major strain on public health services world-wide. The World Health Organization estimates that influenza viral infections cause severe illness in 3 to 5 million people and death estimated at 145,000 to 650,000 people each year.[22,27,35,40,48] Due to the significant burden that influenza places on public health, the need to learn more about the interactions of this virus with the mammalian immune system continues. This need has led to studies that use a variety of species to examine the host response to IAV. Mice are the most common species used to study influenza.[10,47] Clinical signs of influenza in mice include anorexia, dehydration, respiratory distress, hypothermia, hunched posture, unkempt hair coat and ocular discharge.[10,43,49,51] Anorexia and dehydration are common signs of influenza infection that lead to excessive weight loss in mice.

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Weight loss in mice infected with influenza virus is associated with anorexia induced by systemic inflammation. Proinflammatory cytokines have been shown to inhibit normal feeding behavior in mice with IAV, specifically through increased expression of tumor necrosis factor-α (TNFα), interleukin-1β (IL1β) and interleukin-6 (IL6).[8,12,16,19,24,38,51] For example, after infection with IAV, the levels of both TNFα and IL6 begin to rise around 2 to 3 d post infection (dpi) and peak at 7 dpi.[12,24] Mice begin to lose weight at approximately 4 dpi, with peak weight loss around 9 dpi, which correlates with the levels of TNFα, IL6 and other proinflammatory mediators.[12,24,31] Weight loss is routinely used as a euthanasia endpoint criterion for mice infected with IAV. The early removal of mice from a study can have a negative impact on sample size and may compromise the accuracy of a study by introducing survivor bias.[43]

Under the guidance of Institutional Animal Care and Use Committees (IACUCs), a 20 to 30% loss in body weight is a common endpoint criterion in studies of IAV.[43] However, an IACUC may be reluctant to justify a weight loss of over 30%. Based on the 3 Rs, replacement, reduction, and refinement, scientists should refine techniques used in animal models of disease to minimize discomfort.[20] In addition, finding ways to

provide physiologic support for mice in order to 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, whether supportive care reduces weight loss and consequently mortality in mice infected with IAV is an important question.

The objective of the work performed here was to identify a support strategy that resulted in reduced weight loss and increased survival in mice infected with IAV, without altering the nature or degree of their immune response. The overall goal of this investigation is to improve the standard of care for influenza infected mice. If an appropriate strategy is identified, the number of mice needed for IAV studies may be reduced and providing additional support for infected mice will refine the model. To accomplish this goal, the effects of nutritional gel (NG) supplementation were evaluated in mice infected with IAV and compared with the SOC, defined as moist chow and hydrogel. Due to its physical (soft consistency) and nutritional properties, along with the minimal handling required to provide the supplement, we hypothesized that NG (DietGel Recovery) would minimize weight loss and increase the number of mice reaching the end of study as compared with the current SOC.

In this paper we show that NG supplementation benefited influenza infected mice by reducing weight loss and the number of mice euthanized based on the endpoint criteria of 30% weight loss. These effects were seen at all doses in male mice, and at the low and intermediate doses in female mice. Evaluation of the immune response to influenza infection using the 0.5 EuD50 dose revealed no significant differences in the number of immune cells in mice given SOC or NG. Measurements of cytokines in BAL fluid showed lower recovery of IL6 in female mice and TNFα in male mice supplemented with NG. Thus, to summarize, both sexes should be included in study design, and mice infected with mouse-adapted IAV and supplemented with NG demonstrate less weight loss and increased survival than do SOC mice. Our data suggest that NG should be considered as a support strategy for mice infected with IAV.

Materials and Methods

Animals and husbandry. Studies were performed with male and female C57BL/6J mice (Jackson Labs, Sacramento, CA) aged 9 wk at initiation of studies. Initial weights of male mice were 26.5g ± 1.7g and female mice were 19.9g ± 1.5g. Mice were cohoused with 2 to 5 mice per cage in standard plastic cages with corn-cob bedding and autoclaved acidified reverse osmosis purified water (pH 2.4 to 2.8) in bottles. Cotton square material was provided for enrichment. The facility is accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

After inoculation with IAV, mice were housed under animal biosafety level-2 (ABSL-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 supplements described below, mice had free access to food and water and were maintained on a 14/10 h 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).

University of Washington rodent health surveillance is performed via exhaust air dust (EAD) PCR of Allentown individually vented cage (IVC) racks every 16 wk.[34] Testing is performed at Charles River Laboratories. Excluded pathogens include: mouse hepatitis virus (MHV), mouse parvovirus (MPV), minute virus of mice (MVM), reovirus 3 (Reo-3), pneumonia virus of mice (PVM), epizootic diarrhea of infant mice (EDIM), Theiler murine encephalomyelitis virus (TMEV), lymphocytic choriomeningitis virus (LCMV), ectromelia (mouse pox), Sendai virus, *Mycoplasma pulmonis*, pinworms and fur mites.

Influenza virus. Mouse-adapted Influenza A/Puerto Rico/8/34 (A/PR/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.[17] Because sex differences have been documented in mice infected with influenza,[2,32] a euthanasia dose 50 (EuD50) was identified in both male and female mice to determine the appropriate infectious dose in plaque forming units (PFU) that would be used for each sex. The EuD50 was determined using the same methods described for the Reed and Muench calculation of lethal dose 50 (LD50).[17,41] The EuD50s for the A/PR/8/34; H1N1 stock solutions were determined to be approximately 20 PFU for female and 50 PFU for male C57BL/6J mice inoculated via oropharyngeal aspiration as previously described.[14,15,18,33,36] For the infection studies, female and male mice were anesthetized with isoflurane gas anesthetic and inoculated with IAV via oropharyngeal aspiration at 3 doses: 1) low dose- 0.2 EuD50, 2) intermediate dose - 0.5 EuD50 or 3) high dose- 1.25 EuD50 IAV. The dose in PFU and the number of animals are provided in Table 1.

Evaluation of nutritional gel (NG) supplementation. Male and female mice were inoculated with a low, intermediate, or high dose of IAV (Table 1). In addition to regular access to food and water, beginning on the day of infection all cages were given chow pellets moistened with water and supplemented with either hydration gel (SOC, control) or nutritional gel (NG) (Table 2) that was placed on the cage floor and replaced daily during study. All cages received moistened chow (one pellet of chow per mouse, irradiated LabDiet 5053, Rodent Diet 20, St Louis, MO). Mice placed in the SOC group received moistened chow and hydration gel (HydroGel; Clear H_2O, Portland, ME). NG groups received moistened chow and nutritional gel (DietGel Recovery; Clear H_2O, Portland, ME). NG was provided at 5 grams per mouse in a cage (10.7 kcal/ mouse). Visual estimates of moistened chow, hydration gel and NG consumption were made daily. Nutritional details of chow, hydration gel and NG are provided in Table 3. Body weights were recorded daily. At 14 dpi mice were euthanized. Mice that reached endpoint criteria prior to day 14 were euthanized at that time (see monitoring and endpoint criteria).

Monitoring and endpoint criteria. Mice can lose greater than 30% weight with influenza infection before they begin to recover.[10,43,47] In our study mice were monitored daily after influenza infection, and euthanized when they reached or exceeded 30% body weight loss, displayed severely labored breathing or became moribund. In addition, mice were removed from study if they showed a grouping of other signs of severe illness, including eye crusting, severe dehydration, lethargy, loss of resistance to handling, and abnormal posture such as hunching and piloerection. Mice that reached endpoint criteria and were euthanized were considered not to have survived.

Body weight measurements. Body weights were measured once daily in the morning. Initial body weight was defined as the weight on the day of influenza infection (0 dpi). Once mice had lost 20% of their initial weight, body weight was measured twice daily. If body weight was measured twice a day, the weight used for statistical analysis was the lowest weight

Table 1. Doses of IAV used for NG supplementation studies.

	Female mice		Male mice	
Dose	SOC	NG	SOC	NG
Low – 0.25 EuD50	4 PFU $n = 9$	4 PFU $n = 9$	10 PFU $n = 13$	10 PFU $n = 14$
Intermediate– 0.5 EuD50	10 PFU $n = 12$	10 PFU $n = 13$	25 PFU $n = 18$	25 PFU $n = 14$
High – 1.25 EuD50	25 PFU $n = 13$	25 PFU $n = 12$	62 PFU $n = 10$	62 PFU $n = 10$

Table 2. In addition to regular access to food and water, mice were provided with moistened supplemental feed and hydration or nutritional gel on the cage floor.

Treatment	Additional ad lib feed/supplement provided
SOC	Moistened chow + hydration gel
NG	Moistened chow + nutritional gel

during periods of a downward trend in weight, and the highest weight in periods of an upward trend in weight. Because removal of mice meeting euthanasia criteria may bias comparisons of weights between groups, we also calculated the maximal weight loss for each mouse in the study.[37,50] The maximum percent weight loss was measured using the lowest measured weight for each mouse on study.

Evaluation of Immune Response Differences with Nutritional Gel. Male and female C57BL/6J mice were inoculated with either PBS ($n = 6$/sex) or the intermediate influenza dose of IAV (0.5 EuD50) and were maintained on either SOC ($n = 10$/sex) or NG ($n = 10$/sex). Mice were euthanized on day 7 after inoculation based on reports in the literature.[12,24] Bronchoalveolar lavage (BAL) fluid was collected and total cell counts were determined, followed by flow cytometry to characterize the immune cell populations (specifically, alveolar macrophages, interstitial macrophages, neutrophils, eosinophils, T-cells and B-cells). The BAL fluid supernatant was collected and frozen at -80 °C until it was used to evaluate proinflammatory chemokine and cytokine levels using a multiplex assay.

Bronchoalveolar lavage (BAL) fluid collection. Mice were euthanized 7 dpi via cardiac exsanguination under inhaled isoflurane anesthesia. BAL fluid was collected using 5 mM EDTA (Invitrogen, 0.5M EDTA, Ref AM9260G) diluted in sterile PBS as previously described.[14] To collect the BAL fluid, the trachea was intubated and PBS containing 5mM EDTA was perfused into the lungs and then withdrawn. This step was performed 3 times per mouse using sequential volumes of 800 uL, 700 uL, and 700 uL. BAL fluid was stored on ice after collection until further processing.

Flow cytometry. BAL cells were collected by centrifugation for 10 min at 350g, 4 °C, and cell-free BAL fluid was stored at -80 °C. Total live cells were counted using a Nexcelom cellometer 2000 with AO/PI staining (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 incubated with Fc Block (BioLegend, San Diego, CA) and then stained with PE Rat antimouse Siglec F (BD biosciences; Pharmingen, San Diego, CA), APC/Cy7 antimouse CD45 (Biolegend, San Diego, CA), PB antimouse CD11c (eBioscience, San Diego, CA), PE/Cy7 antimouse/ human CD11b (Biolegend, San Diego, CA), APC antimouse Ly6G (Biolegend, San Diego, CA), FITC antihuman/ mouse B220 (eBioscience, San Diego, CA), or PerCP/Cy 5.5 antimouse CD3e (TONBO biosciences, San Diego, CA). Compensation was acquired using compensation beads, UltraComp eBeads (Invitrogen, Waltham, MA). Flow data were collected using a BD FACS Canto RUO maintained at the University of Washington cell analysis facility flow and imaging cytometry core lab.

Cell populations were determined using a sequential gating strategy using FlowJo 10.6.1 software program (FlowJo, LLC, Ashland, OR). Populations of cells were determined as follows: 1) resident/ alveolar macrophages: $CD45^+SiglecF^+CD11c^+$, 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 B- cells: $CD45^+B220^+$.

Measurement of Total Protein and Inflammatory Mediators in BAL Fluid. To address the degree of lung inflammation and injury in mice, we measured total protein and select cytokines, chemokines and growth factors in BAL fluid. Total protein concentration of BAL fluid supernatant was measured using the Pierce BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA). Chemokine and cytokine analysis was performed using a Milliplex ZMAP Mouse Cytokine/ Chemokine magnetic bead panel (EMD Millipore Corporation, Billeric, MA) with magnets specific for IL6, CCL2, TNFα, IL4, IL10, CXCL1, CXCL2, and vascular endothelial growth factor (VEGF).

Viral plaque assay for pulmonary IAV quantification. Male and female mice were infected with the intermediate dose of IAV via oropharyngeal instillation and maintained on either SOC ($n = 5$/sex) or NG ($n = 5$/sex). On 5 dpi, mice were euthanized and left lungs excised, placed into Omni bead ruptor in 2 mL tubes containing 2.8mm ceramic beads, and snap frozen in liquid nitrogen. Lungs were stored at -80oC until plaque assay was performed. For the plaque assay, one million Madin-Darby canine kidney (MDCK) cells grown in Eagles Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum and penicillin/streptomycin were seeded per well in 6 well plates and grown overnight. Lungs were kept frozen until 1 mL cold PBS was added immediately before homogenization with an Omni 24-bead ruptor. A 25 s burst of homogenization was done, tubes removed from the homogenizer and placed in wet ice for 1 min to cool tubes, and a 25 s homogenization performed. Lung homogenates were stored on ice until serial dilutions performed. Dilutions (10E-1 to 10E-3) were performed in duplicate in infection medium (PBS with 0.2 mg/mL bovine serum albumin and XuM Ca Mg). MDCK monolayers where washed with PBS and 1 mL infection medium added to the wells. 40 uL of each dilution were added to each of 2 wells and the plates incubated at 37 °C. After 1 h, unattached virus was removed by PBS washes and 2.5 mL of EMEM containing TPCK trypsin and agar added to each well. After 20 min at room temperature, the plates were inverted and incubated at 37 °C for 48 to 72 h Plaques were visualized and counted using a chick anti-PR8 antibody and a rabbit antichick HRP conjugated antibody and visualized using TrueBlue peroxidase substrate.

Statistical Analysis. *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 EuD50 influenza doses. The power calculations were performed for both male and female mice separately. Power analysis suggested groups of $n = 10$ to 12 to reject the null hypothesis with 95% probability.

Table 3. Nutritional information for supplemental support used in the study.

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

Considerations for removal of mice from a study. Individual variations in response to viral infections are known to occur. In addition, instances of inadequate inoculation of virus (for example, due to partial oral ingestion of instilled virus) are possible in murine influenza studies and result in no clinical signs of disease. As the goal of this study is to develop a better standard of care for mice who become clinically sick due to influenza infection, data from mice who gained weight or lost less than or equal to 5% of initial body weight consistent with a failed viral inoculation were not used for data analysis. In addition, one mouse in the high IAV dose group was removed using the Grubbs test for outliers (described in Results).[5,25] Removal of these mice from the analysis was based on the assumption that they did not experience the expected severe response to influenza infection or received a partial inoculation of influenza and would not be appropriate to include in data analysis. Including data from these mice could ultimately lead to skewed weight data and perhaps misrepresent the benefit of the support strategies analyzed in this study.

Analysis of Data. All statistical analyses were performed using Prism 8.1.1 (GraphPad Software, La Jolla, CA). Unpaired, 2-tailed, Student *t* tests were used to determine significance of the maximum % weight loss between groups. Weight curves over the time course of infection were evaluated using 2-way ANOVA with repeated measures, testing for differences in percent weight loss during the progression of disease from 0 to 14 dpi in mice that survived to the end of study. The repeated measure factor that was primarily assessed was the interaction between the treatment groups and the time (dpi). Post hoc multiple comparisons (Sidak) of mean weight by day were also performed on this data set. Kaplan–Meier survival graphs and the log-ranked (Mantel–Cox) test were used to assess differences in mortality among groups. A one-way ANOVA with Dunnett multiple comparisons test was used to assess total cell numbers in BAL fluid, populations of inflammatory cells of BAL fluid identified via flow cytometry, total protein concentration in supernatant of BAL fluid, and chemokine/ cytokine concentrations in BAL fluid supernatant. If PFU values for viral titers or the amount of cytokines in a biological sample were not detectable in PBS treated mice, these animals were excluded; statistical analysis was then performed to compare the SOC group to the NG group using an unpaired, 2 tailed Student *t* test. P values ≤ 0.05 were considered statistically significant and all values are described as the mean ± the standard error of the mean (SEM).

Results

Nutritional Gel Supplementation. Consistent with NIH policies, these studies were performed using both male and female mice to determine whether any conclusions relevant to NG applied to both sexes. The EuD50 studies revealed significant differences in the host response of male and female mice to the A/PR/8/34; H1N1 virus. The EuD50 for females was approximately 20 PFU compared with 50 PFU for male C57BL/6 mice. To account for greater susceptibility of female mice to A/PR/8/34; H1N1 virus, we designed studies in which the doses of the virus for males and females were calculated as a percent of the EuD50. Three doses were used: 1) low dose- 0.2 EuD50, 2) intermediate dose - 0.5 EuD50 or 3) high dose- 1.25 EuD50 IAV (Table 1).

Low dose influenza. *Females.* NG reduced the time to peak weight loss as compared with mice provided SOC, indicating that the addition of NG helped mice recover from IAV infection sooner than mice receiving SOC (Figure 1 A). Weight curves over the course of infection were evaluated by 2-way ANOVA, with significant interaction of treatment over time and with NG showing less weight loss over time than did mice given SOC (Table 4). No significant differences in mean weights per day were detected in multiple comparisons posttests. The maximal % weight loss was calculated for each animal on study; no significant decrease was found between mice that received NG and those that received SOC, with a maximal % weight loss of 21% ± 5% for SOC mice and 19% ± 5% for NG mice (P = 0.36; Figure 1 C). No deaths occurred in either NG or SOC mice given the low influenza dose (Figure 1 E). One mouse was removed from data analysis for both the SOC and NG groups due to an inadequate disease response (see considerations for removal of mice from a study in Materials and Methods).

Males. Mice supplemented with NG reached their peak weight loss sooner than those given SOC, indicating that the addition of NG helped mice recover from IAV infection earlier than mice receiving SOC (Figure 1 B). A significant difference was seen in NG mice, whose weight loss was minimized over the total course of infection as compared with SOC mice (Table 4), but no significant differences were detected in multiple comparisons posttest of mean weight by day. Male mice in the SOC group reached a maximal % weight loss of 22% ± 4%, while male NG mice reached 18% ± 3%, resulting in significantly lower maximal % weight loss in mice that received NG (P = 0.013; Figure 1 D). No deaths occurred in male mice given

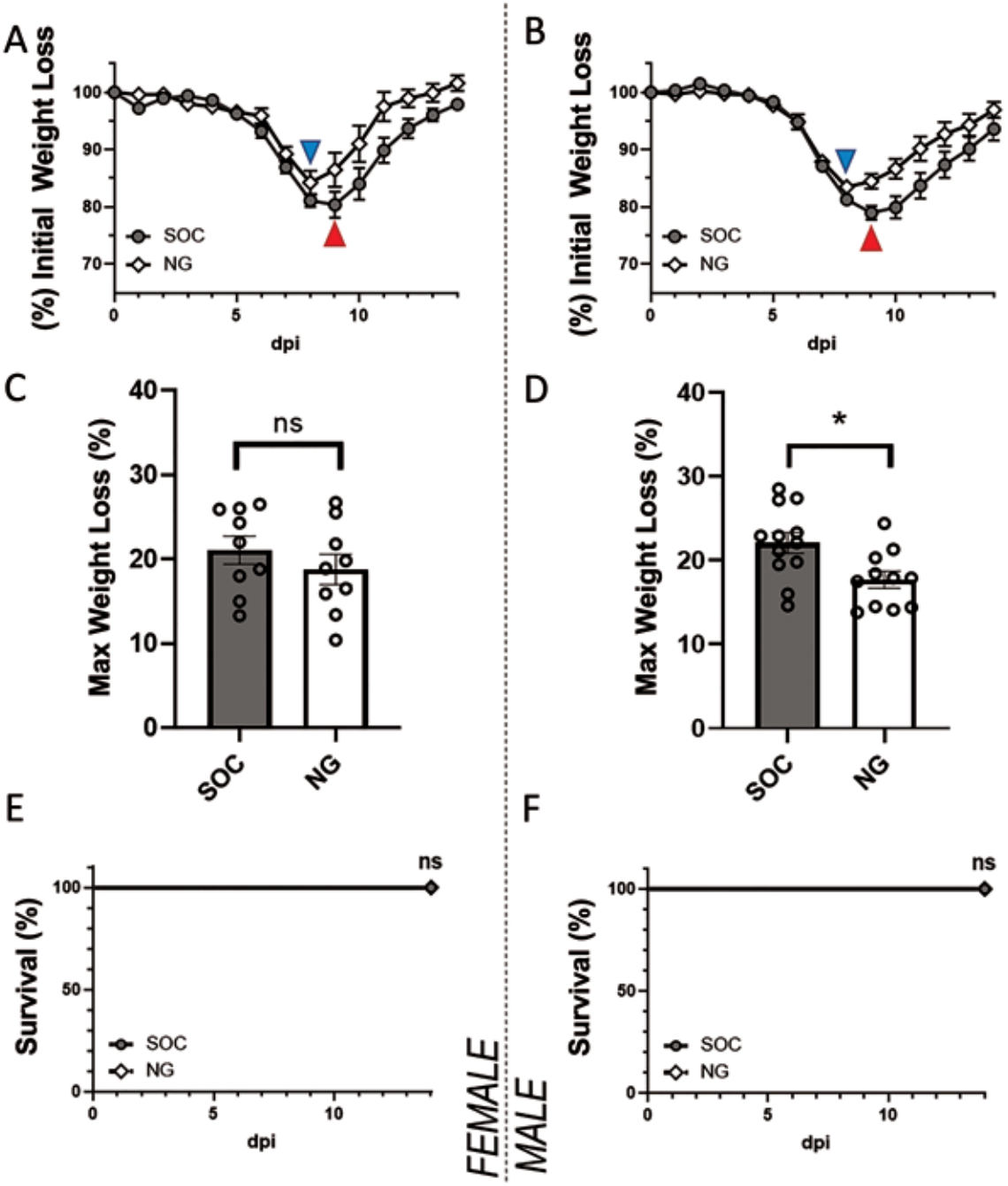

Figure 1. NG supplementation in female (A, C, E) and male (B, D, F) mice infected with low influenza dose. (A, B) % of initial body weight following exposure to IAV over 14 dpi. Arrows represent the day SOC (red arrow) and NG (blue arrow) supplemented mice reached their peak weight loss. (C, D) Maximum percent weight loss for individual female (C) and male (D) mice on the study. Analysis was performed using an unpaired, 2-tailed, *t* tests. (E, F) Kaplan–Meijer survivor graph. Statistics performed using log-ranked (Mantel–Cox) test. Values are the mean ± SEM * indicates a significant finding (P £ 0.05). Females (SOC n = 9; NG n = 9); Males (SOC n = 13; NG n = 14).

the low influenza dose, in either the SOC or NG groups (Figure 1 F). Two mice in both the SOC and NG groups were removed from data analysis due to an inadequate disease response (see considerations for removal of mice from a study in Materials and Methods).

In summary, these results show that NG supplementation decreases weight loss over course of IAV infection in both male and female mice and reduces the maximal weight loss in low dose influenza infected male mice. No deaths occurred in either male or female mice given the low dose of influenza virus.

Intermediate dose influenza. *Females.* Mice supplemented with NG reached their peak weight loss earlier than did those given SOC (Figure 2 A). Mice given SOC reached a maximal % weight loss of 27% ± 4%, while NG mice reached 22% ± 5%, resulting in a significant reduction in maximal % weight loss in mice supplemented with NG (P = 0.01; Figure 2 C). NG supplementation had no significant effect on mouse survival. However, no deaths were associated with reaching endpoint criteria of 30% loss of initial weight in NG mice (P = 0.13), whereas SOC mice had 17% mortality, with 2 mice removed early due to

Table 4. Results of 2-way ANOVA with repeated measures comparing differences in % weight loss over course of infection in mice who survived to 14 dpi in the low (female: SOC $n = 9$, NG $n = 9$; male: SOC $n = 13$, NG $n = 14$), intermediate (female: SOC $n = 10$, NG $n = 13$; male: SOC $n = 15$, NG $n = 14$), and high (female SOC $n = 6$, NG $n = 7$; male: SOC $n = 2$, NG $n = 6$) IAV doses. $P \leq 0.05$ is considered a significant value.

	P-value	
Source of variation	Female	Male
Low	0.0008	<0.0001
Time × treatment		
Intermediate	0.0006	0.0011
Time × treatment		
High	0.9966	<0.0001
Time × treatment		

reaching weight loss endpoint criteria (Figure 2 E). To evaluate if NG was beneficial to mice that survived to the end of study, we excluded from data analysis any mice that were removed from study due to reaching an endpoint or death and evaluated the % loss of initial weight over the course of infection in a 2-way ANOVA. We found a significant interaction between weight loss and time, with less weight loss over the time of infection in NG mice as compared with SOC mice (Table 4). No significant differences were detected in multiple comparisons posttest of mean weight by day. Three SOC mice and 2 NG mice removed from data analysis for inadequate disease response (see considerations for removal of mice from a study in Materials and Methods).

Males. The time to peak weight loss was reduced in NG mice as compared with SOC mice, suggesting that the addition of NG helped mice recover from IAV infection (Figure 2 B). Mice given SOC reached a maximal % weight loss of 27% ± 2%, while NG mice reached 24% ± 4%, resulting in a significant reduction in maximal % weight loss in mice supplemented with NG ($P = 0.006$; Figure 2 D). However, NG provided no significant benefit in terms of mouse survival, although no deaths were associated with reaching endpoint criteria of 30% loss of initial weight in NG mice ($P = 0.12$). In contrast, the SOC group had 17% mortality, with 3 mice removed early due to reaching weight loss endpoint criteria (Figure 2 F). Evaluation of response to treatment in surviving mice revealed a significant interaction between weight loss and the time of infection in mice given NG compared with SOC (Table 4), but no significant differences detected in multiple comparisons posttest of mean weight by day. One mouse in the SOC group was removed from data analysis for inadequate disease response (see considerations for removal of mice from a study in Materials and Methods).

In summary, these results show that NG supplementation promoted recovery in male and female mice infected with the intermediate dose of IAV. In addition, NG supplementation minimized the maximal % weight loss and was not associated with deaths in male and female mice. When evaluating only mice who survived to endpoint, NG also minimized weight loss over course of IAV infection.

High dose influenza. ***Females.*** Weight curves were similar in SOC and NG mice, with both groups reaching their peak weight loss on the same day (Figure 3 A). Both SOC and NG female mice infected with the high dose of IAV reached a maximal % weight loss of 30% ± 2%, with no significant reduction in maximal % weight loss with NG supplementation ($P = 0.75$; Figure 3 C). NG mice also showed no significant increase in survival as compared with SOC mice ($P = 0.33$; Figure 3 E). Nine SOC mice reached euthanasia criteria, resulting in a mortality of 70%, while 6 NG mice died, a mortality rate of 50%. The surviving mice showed no significant interaction between treatment and time, indicating no benefit of NG supplementation over the course of IAV infection (Table 4). One mouse in the NG group was removed due to inadequate disease response[5,25] (see considerations for removal of mice from a study in Materials and Methods).

Males. Both SOC and NG mice reached their peak weight loss at 9 dpi, however, by 14 dpi, NG mice had recovered to 85% of their initial weight, while SOC mice had only recovered to 74% of their initial weight, indicating that the addition of NG helped mice recover from IAV infection at a faster rate (Figure 3 B). SOC mice infected with the high dose of IAV reached a maximal % weight loss of 31% ± 2%, while NG mice reached 27% ± 3%, resulting in a significant reduction in maximal % weight loss with NG supplementation ($P = 0.003$; Figure 3 D). NG mice also showed a significant increase in survival ($P = 0.01$; Figure 3 F). Eight SOC mice reached euthanasia criteria, resulting in a mortality of 80% in that group, whereas 3 NG mice died, resulting in 30% mortality. Surviving mice showed a significant interaction between time and treatment, with a benefit from NG treatment over the course of disease for surviving mice (Table 4). No significant differences were detected in multiple comparisons posttest of mean weight by day. Only 2 mice survived until the end of study in the SOC group, which must be considered when evaluating the statistical analysis of this data set.

In summary, at the high IAV dose, NG increased the rate of recovery in male but not female mice. In addition, the maximal % weight loss was lower in male mice supplemented NG, but not females. NG supplementation also led to fewer deaths in both sexes but had a greater affect in males. In evaluating only those mice who survived to the end of study, NG supplementation minimized weight loss over course of IAV infection in male, but not female, mice.

Assessment of the Pulmonary Host Response to IAV. Nutritional gel was found to be a beneficial support strategy, resulting in decreased weight loss and mortality in mice post IAV infection, at all doses studied. Therefore, subsequent studies were performed to determine if NG supplementation had altered the immune response. To address the potential impact of NG supplementation on the pulmonary host response, we measured viral titers at 5 dpi in whole lung homogenates using a viral plaque assay. Pulmonary inflammation and injury was measured in mice at 7 dpi using measurements of total protein, changes in the number and subset of immune cells, and measurements of inflammatory mediators in BAL fluid. These studies were performed in male and female mice receiving an intermediate dose of A/PR/8/34; H1N1 virus and comparing SOC with NG.

Evaluation of IAV Titer in Whole Lung Homogenates. No significant difference in viral replication was measured at 5 dpi, using viral plaque assay, in either treatment group in both female ($P = 0.34$) or male ($P = 0.67$; Figure 4).

Pulmonary Inflammation and Injury in Mice Supplemented with Nutritional Gel. The immune cells recovered from BAL fluid at 7 dpi were measured in both male and female mice given the intermediate dose of IAV and either SOC or NG. An inflammatory response to influenza infection was confirmed based on significant differences between PBS (sham infected) and influenza infected groups in total cell numbers and inflammatory cell populations recovered from BAL fluid, as well as total protein and the amount of select chemokines and cytokines in BAL fluid supernatant.

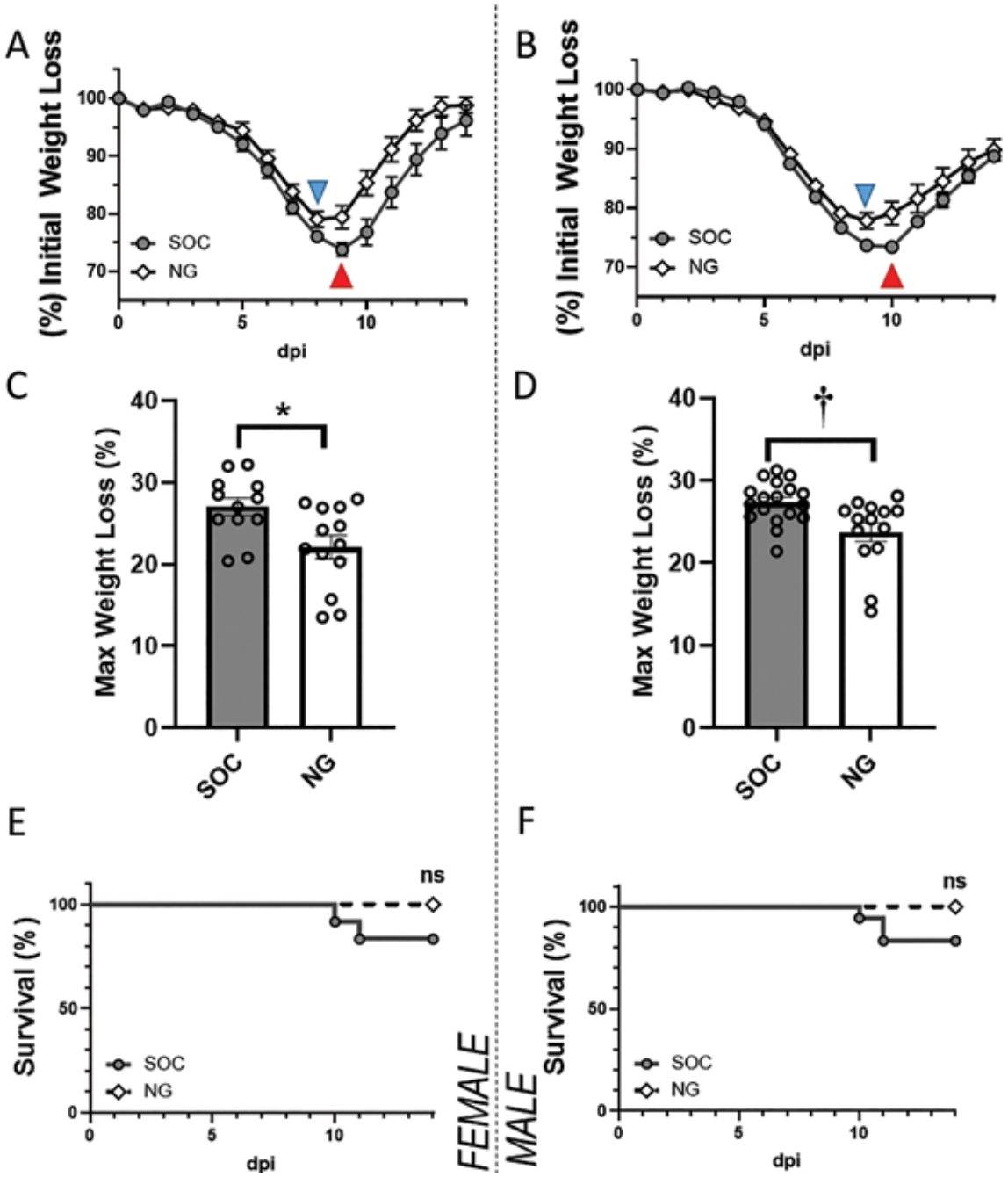

Figure 2. NG supplementation in female (A, C, E) and male (B, D, F) mice infected with intermediate influenza dose. (A, B) % of initial body weight following exposure to IAV over 14 dpi. Arrows represent the day SOC (red arrow) and NG (blue arrow) supplemented mice reached their peak weight loss. (C, D) Maximum percent weight loss for individual female (C) and male (D) mice on the study. Analysis was performed using an unpaired, 2-tailed, *t* tests. (E, F) Kaplan–Meijer survivor graph. Statistics performed using log-ranked (Mantel–Cox) test. Values are the mean ± SEM. For each data set significance is shown by *, $P \leq 0.05$ and †, $P \leq 0.01$. Females (SOC $n = 12$; NG $n = 13$); Males (SOC $n = 18$; NG $n = 14$).

Comparison of SOC and NG groups showed no significant differences in BAL total cell counts or in BAL total protein levels (Figure 5). No significant differences ($P > 0.05$) were detected between SOC and NG mice in the total number of alveolar macrophages (AM), interstitial macrophages (IM), neutrophils, T-cells, B-cells, or eosinophils identified using established flow cytometric markers (Figure 6 A through F and 7 A through F) . Flow cytometry was performed using a sequential gating strategy to identify specific cell populations from BAL fluid (Figure 6 G). IL6 expression was significantly lower in female NG mice ($P = 0.03$), as was TNFα in male NG mice ($P = 0.04$), as compared with SOC mice (Figure 8). No significant differences were detected between SOC and NG groups in the amount of CXCL1, CXCL2, CCL2, IL4, IL10 or VEGF measured with a mouse immunology multiplex panel (Figure 8).

Discussion

The goal of this study was to evaluate and identify a support strategy for mice infected with IAV that was intended to reduce weight loss and minimize the number of mice removed early from a study because they had reached endpoint criteria

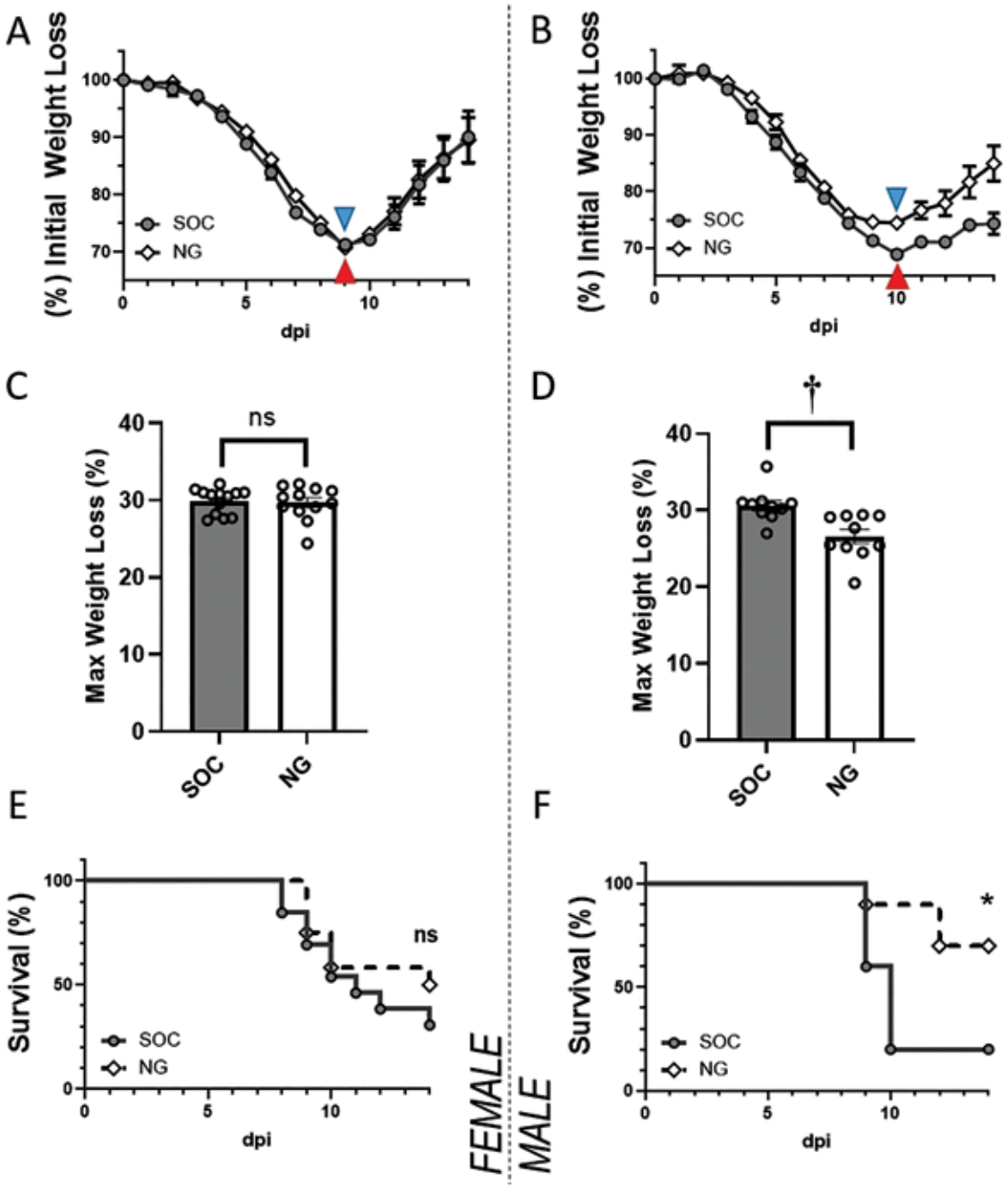

Figure 3. NG supplementation in female (A, C, E) and male (B, D, F) mice infected with high influenza dose. (A, B) % of initial body weight following exposure to IAV over 14 dpi. Arrows represent the day SOC (red arrow) and NG (blue arrow) supplemented mice reached their peak weight loss. (C, D) Maximum percent weight loss for individual female (C) and male (D) mice on the study. Analysis was performed using an unpaired, 2-tailed, *t* tests. (E, F) Kaplan–Meijer survivor graph. Statistics performed using log-ranked (Mantel–Cox) test. Values are the mean ± SEM. For each data set significance is shown by *, $P \leq 0.05$ and †, $P \leq 0.01$. Females (SOC $n = 13$; NG $n = 12$); Males (SOC $n = 10$; NG $n = 10$).

based on body weight. Our standard of care (SOC) practice for sick mice, including those infected with IAV, is the placement of moistened food pellets and hydration gel on the cage floor to give the mice easy access to feed and hydration. However, due to the anorexia induced by IAV, mice generally do not consume the moistened food pellets or hydration gel over the course of disease. This raised the question of whether supplementing infected mice with nutrition gel would improve outcomes. In our study, NG supplementation was found to reduce weight loss and thus reduce early weight-based removal of mice from studies. Furthermore, providing NG for nutritional support does not require animal handling, and so imposes no additional handling stress in mice infected with IAV.

A recent study showed that nutritional supplement with glucose decreased mortality in mice infected with IAV, providing evidence that nutrition contributes to recovery from influenza.[53] In that study, the investigators used oral gavage to provide nutritional supplementation; this procedure can be stressful in a mouse with a compromised respiratory system.[11,28] Therefore, we used the nutritional gel DietGel Recovery, which provides

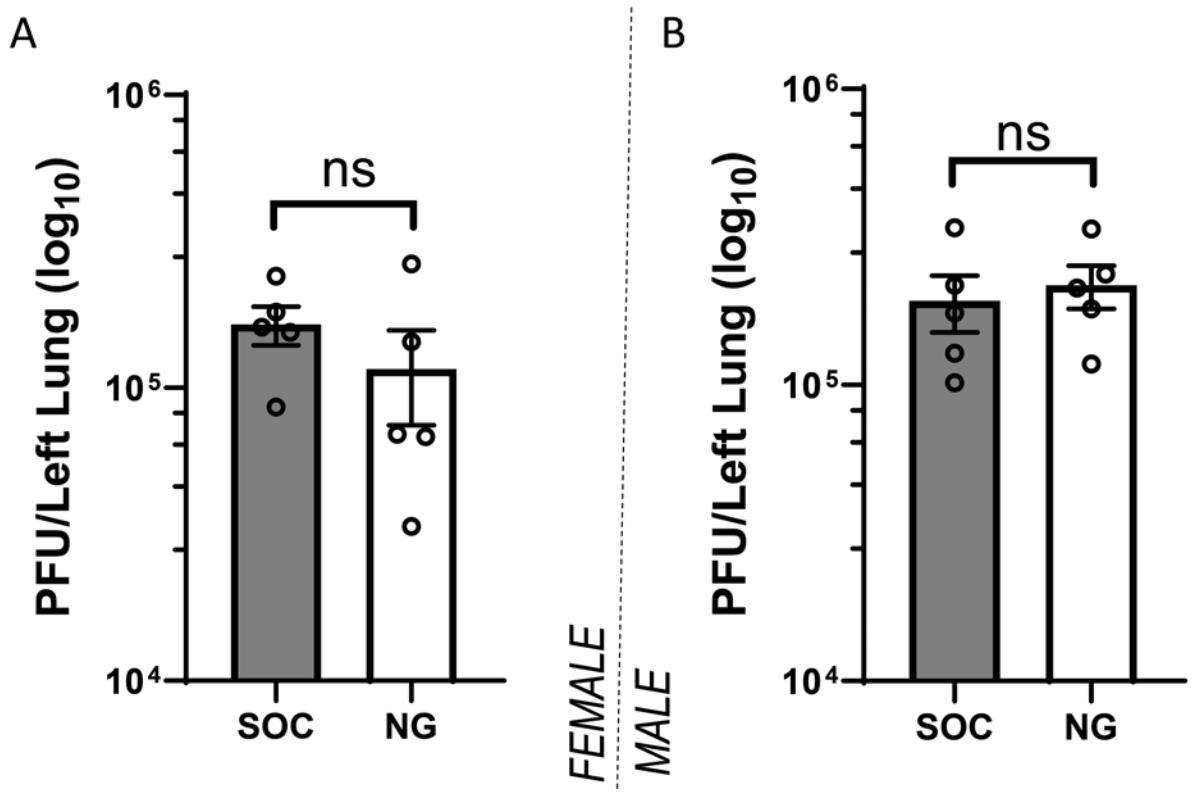

Figure 4. Viral titers were performed at 5 dpi in A) female and B) male mice infected with the intermediate dose of IAV. Values are the mean ± SEM. Analysis performed by unpaired *t* test. SOC $n = 5$/sex; NG $n = 5$/sex.

both nutrition and hydration based on calories, electrolytes, sugars and water content. Overall, NG supplementation was beneficial for mice at all IAV doses because it reduced the percent body weight loss, leading to quicker recovery and decreasing the numbers of mice requiring euthanasia prior to the end of study. These effects were seen for both the high and intermediate doses, and were more predominant in male mice. NG supplementation also lead to earlier recovery and minimized weight loss at time points normally associated with the most severe clinical signs of IAV. While this quicker recovery was seen in mice supplemented with NG, with the exception of the males provided the high dose, by day 14 both groups had similar weights as compared with their initial body weight. In addition, recovery time by treatment interactions in mice that survived to end of study were significant for all but the high-dose treatment group of females.

The reduced weight loss in mice supplemented with NG led to fewer mice reaching weight-based euthanasia criteria. While the finding was not significant for the intermediate dose, none of the mice supplemented with NG were removed from the study due to weight loss, while 17% of both female and male SOC mice were removed from study early due to the reaching weight loss endpoint criteria. Male NG mice given a high dose of influenza showed a significant increase in survival as compared with SOC male mice. Weight loss is a common cause of early removal of mice from influenza studies, and prevention of these deaths benefits both the mice and the overall study. By minimizing weight loss, the number of mice required to adequately power a study is decreased. In addition, survival bias, also known as incidence-prevalence bias, is minimized. Survival bias is a form of selection bias that can lead to distorted or misleading results or conclusions. It is caused by the loss of data from animals that did not survive to the end of a study, therefore focusing data analysis and interpretation only on those who survived.[50] By minimizing or removing survival bias, internal validity of a study is increased, resulting in more valid interpretations because all mice, including those more severely affected by the disease process, reach the end of a study and can be included in data analysis.[37,56] Because NG reduces the number of mice euthanized due to weight loss and minimizes selection bias, replacing hydration gel with NG is recommended for mice infected with IAV.

A concern with adding NG to a study is that it might result in the loss of a disease phenotype in studies where weight loss is used as an objective clinical parameter to monitor disease progression. Conversely, humans with IAV infections, especially those admitted to hospitals, are provided nutritional support. Therefore, the use of NG supplementation may provide a more translational study design that more closely approximates what happens to humans. Both points should be taken into consideration when developing the experimental design for a study.

In a research model using C57BL/6J mice infected with influenza A/PR/8/34, NG supplementation refines the model by providing additional physiologic support to mice during the course of their infection. Although a limitation of this study is that a blinded, clinical scoring system was performed to evaluate behaviors and clinical signs of disease other than weight loss, improvement was observed in NG mice compared with SOC, including in appetite recovery. As compared with hydration gel (HG), DietGel Recovery (NG) provides calories in the form of sugar, as well as electrolytes and hydration. During this study, daily estimates of gel consumption were made (data not shown). These observations showed that NG was readily eaten and that acclimation time was not required. In contrast, mice showed relatively less interest in HG. The consumption of NG fell as infection progressed, with the period of no to minimal consumption of NG lasting approximately one day in the

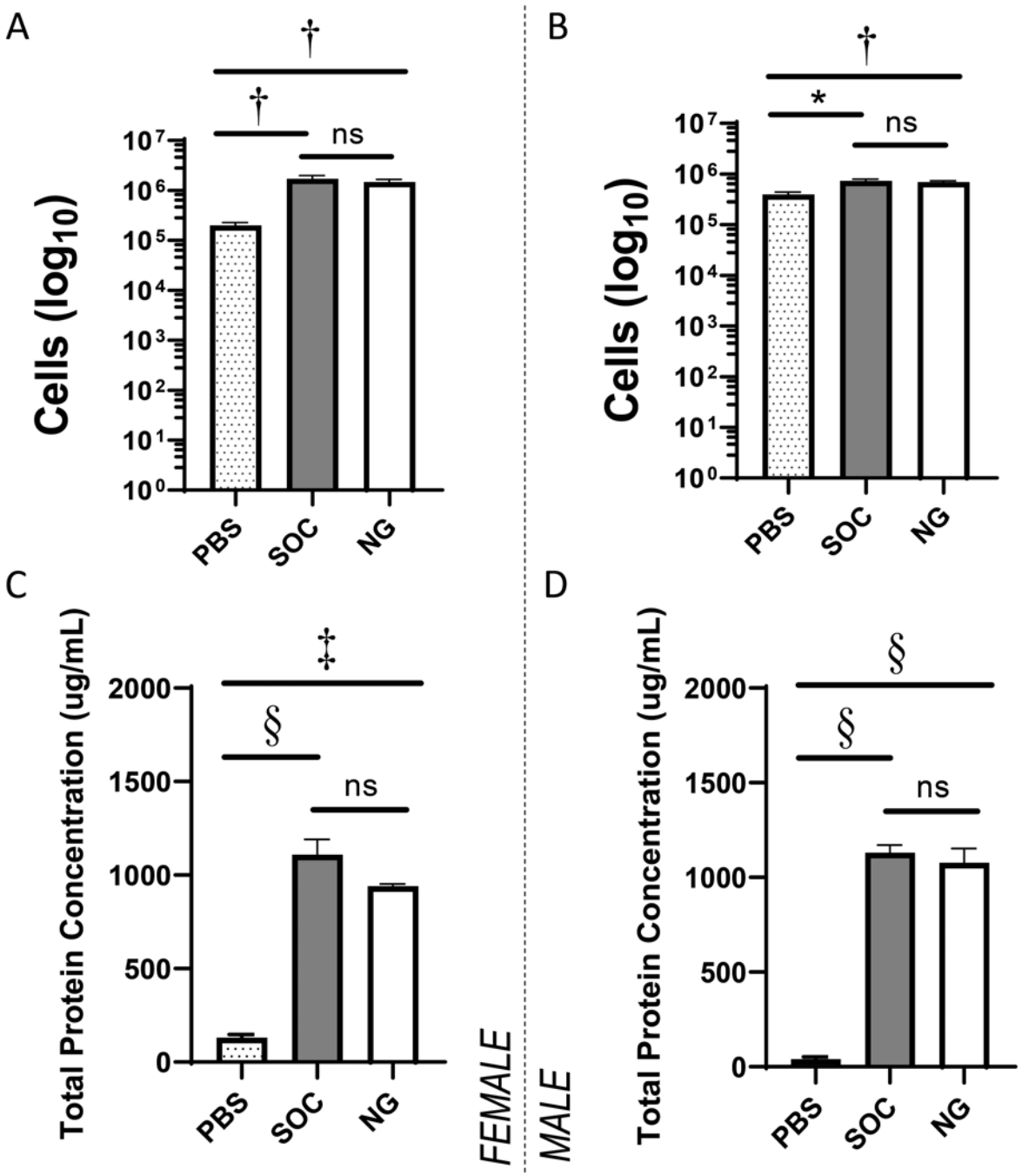

Figure 5. Total number of cells and total protein concentration in BAL fluid collected 7 dpi from female (A, C) and male (B, D) mice infected with PBS or the intermediate dose IAV given SOC or NG. (A, B) Total number of cells in BAL fluid in mice. (C, D) Total protein concentration levels from BAL fluid supernatant. Values are the mean ± SEM. Statistics were performed using a one-way ANOVA with Dunnet multiple comparisons test. For each data set significance is shown by *, $P \leq 0.05$, †, $P \leq 0.01$, ‡, $P \leq 0.001$, and §, $P \leq 0.0001$. For females and males, PBS $n = 6$/sex; SOC $n = 10$/sex; NG $n = 10$/sex.

low and intermediate IAV doses. In contrast, the period of no to minimal consumption of HG occurred over multiple days (2 to 5 d) in mice given SOC in the low and intermediate IAV doses. Inbred mouse strains have taste preferences for sugars, and C57BL/6J mice are sweet sensitive, favoring the taste of sugars, including maltose which is a main ingredient in corn syrup.[3,4,26,44] This taste preference likely contributed to the high consumption rate of the NG used in our study, as it contains high fructose corn syrup. A large variety of nutritional gels are available on the market, and others may contain maltose or other sugars that mice prefer; these could provide a greater benefit to mice during infection studies. However, while nutritional gels high in sugars directed at strain taste preferences could be a benefit, some studies implicate high sugar diets in altering responses to some disease processes, including increasing mortality in bacterial pneumonia[53] and increasing tumor growth and development.[23,53] In fact, some work has shown that anorexia was beneficial, whereas nutritional supplementation with glucose reduced survival in mice infected with *Listeria monocytogenes*.[53] Consequently, support strategies will most likely vary based on the disease process being studied. In addition, the benefits of NG supplementation may vary based on the strain

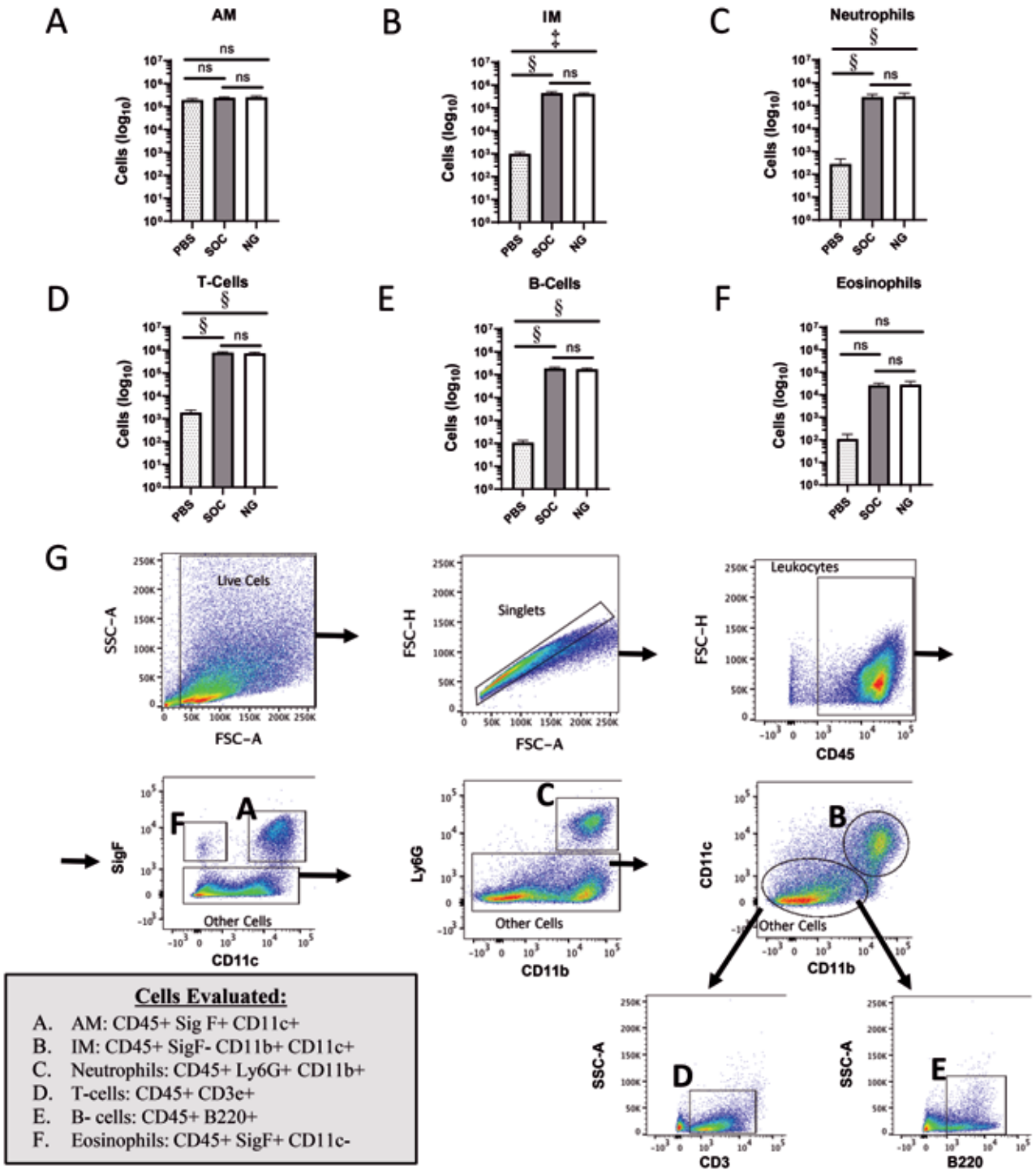

Figure 6. Total number of CD45+ staining inflammatory cells from BAL fluid collected 7 dpi from female mice infected with PBS or the intermediate dose IAV given SOC or NG. Populations of inflammatory cells identified through flow cytometry. An inflammatory response secondary to IAV infection was confirmed due to the increased levels of inflammatory cells noted between PBS and influenza infected mice. Total no. of A) alveolar macrophages (AM), B) interstitial macrophages (IM), C) neutrophils, D) T-cells, E) B-cells, F) eosinophils. Values are the mean ± SEM. G) Flow cytometry gating strategy used for identification of individual cell populations. Statistics were performed using a one-way ANOVA with Dunnet multiple comparisons test. For each data set significance is shown by *, $P \leq 0.05$, †, $P \leq 0.01$, ‡, $P \leq 0.001$, and §, $P \leq 0.0001$. PBS $n = 6$; SOC $n = 10$; NG $n = 10$.

of influenza virus and the strain of mice being used. Therefore, although our study showed a benefit of NG in C57BL/6J mice infected with influenza A/PR/8/34, the response we saw may not occur under other conditions.

The NG we used was chosen because it provided both nutritional and hydrational support. We found a significant benefit of NG in mice infected with IAV, which raised the question of the benefit of the hydration support in IAV infected mice. Hydration provided by NG may contribute to the improved outcomes we observed with this support strategy. One study showed that mice infected with IAV were protected against death when given nutritional supplementation that included glucose, which

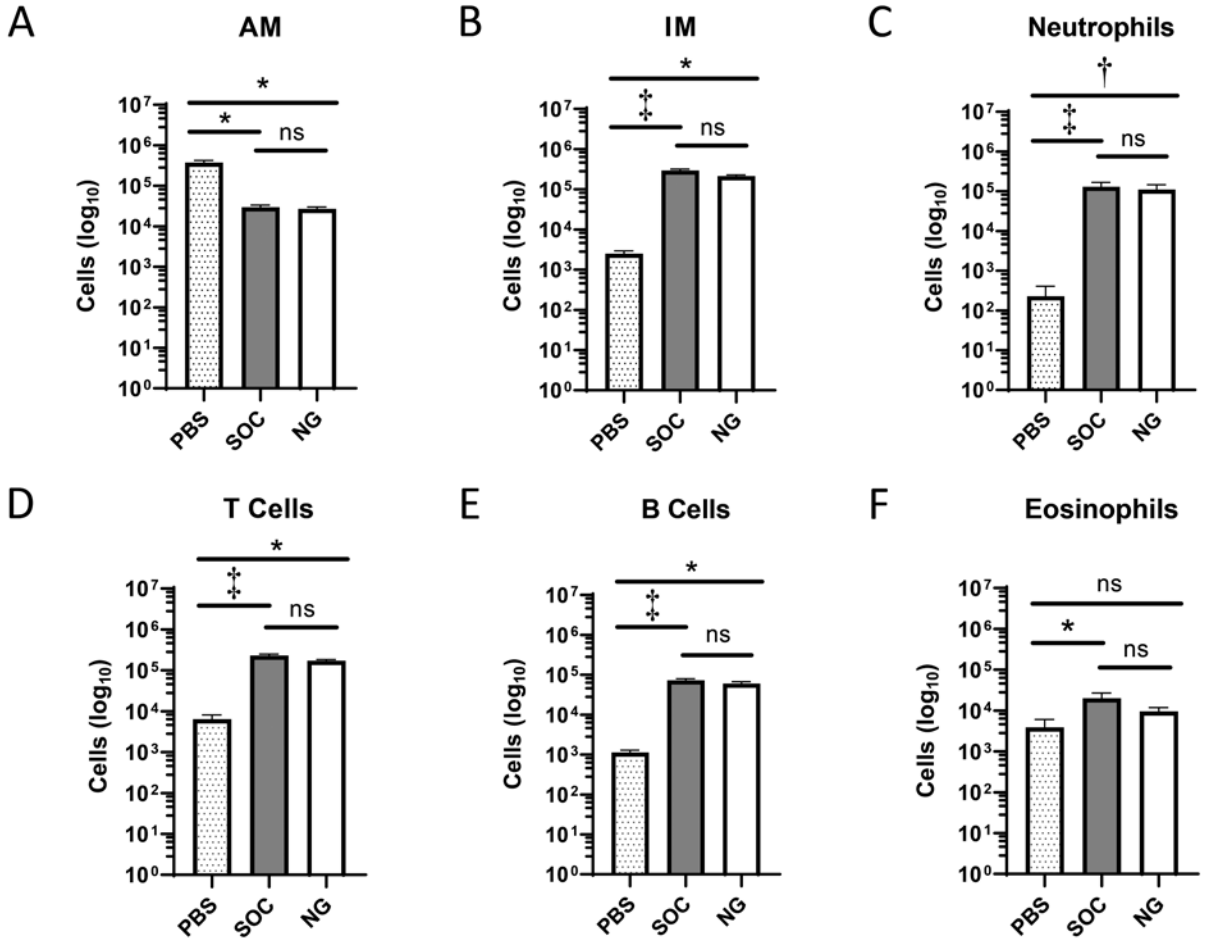

Figure 7. Total number of CD45+ staining inflammatory cells from BAL fluid collected 7 dpi from male mice infected with PBS or the intermediate dose IAV given SOC or NG. Populations of inflammatory cells identified through flow cytometry. Total no. of A) AM, B) IM, C) neutrophils, D) T-cells, E) B-cells, F) eosinophils. Values are the mean ± SEM. Statistics were performed using a one-way ANOVA with Dunnet multiple comparisons test. For each data set significance is shown by *, $P \leq 0.05$, †, $P \leq 0.01$, and ‡, $P \leq 0.001$. PBS $n = 6$; SOC $n = 10$; NG $n = 10$.

suggests a metabolic need for glucose in viral infections.[53] Because NG contains sugar, the benefit of NG supplementation was possibly related to its metabolic effect, in addition to its hydrational support. Future studies could examine the contribution of both hydration and glucose that in the benefits we saw in IAV infections in this study.

To determine that the benefits seen from NG supplementation were not due to alteration of viral replication in the lungs, viral titer was measured at 5 dpi in both male and female mice infected at the intermediate IAV dose. No significant differences were detected in virus titer, indicating that the benefits of NG supplementation were not due to lower viral levels in the lungs. To determine if NG altered lung inflammation and/or injury, we measured immune cells, total protein, and inflammatory mediators recovered in BAL fluid from mice 7 dpi with the intermediate dose of IAV. No differences were found in the recovery of immune cell populations in BAL fluid or in lung injury as estimated by BAL total protein concentration. The amount of IL6, CCL2, TNFα, IL4, IL10, CXCL1, CXCL2, and VEGF recovered in BAL fluid was measured. The only significant findings were a modest decrease in the amount of IL6 in female mice, and in the amount of TNFα in male NG mice. IL6 has both pro- and antiinflammatory properties and is created and released in response to lung epithelial cell damage during influenza infection.[12,16,24,52,55] TNFα is a cytokine, mainly produced by macrophages, that contributes to multiple critical cell functions (including cell proliferation, survival, differentiation and apoptosis) and coordinates the cytokine cascade in various inflammatory diseases.[39] IL6 and TNFα have also been shown to contribute to mediating symptoms, including anorexia, during IAV infection.[9,12,21,24,42,46] We cannot rule out the possibility that NG supplementation reduced IL6 and TNFα production in response to IAV infection, leading to decreased anorexia and improved outcomes.[1,45,53,54,57] It is interesting to note that TNFα was initially named cachectin by the investigators who were trying to delineate the basic mechanisms of cachexia in chronic infections.[6,7] Defining the roles of IL6 and TNFα in mediating this response merits further work, but is beyond the scope of this study. However, due to the lower recovery of IL6 and TNFα in BAL fluids collected at 7 dpi in NG mice, it is recommended that all mice in a study be given NG if it is being used.

Sex differences in mice in response to PR8 and other strains of influenza have been well documented, and the results of this study reiterated the importance of including both male and female mice in IAV studies.[2,13,29,30] Clear differences between sexes in response to IAV infections were evident in the EuD50 studies performed prior to this study. Female C57BL/6J mice were more sensitive to disease than male mice, with increased weight loss and higher mortality rates at lower doses than male mice. Because of these differences, our study was designed using doses of influenza given at the same fraction of the sex specific EuD50 to provide comparable results between

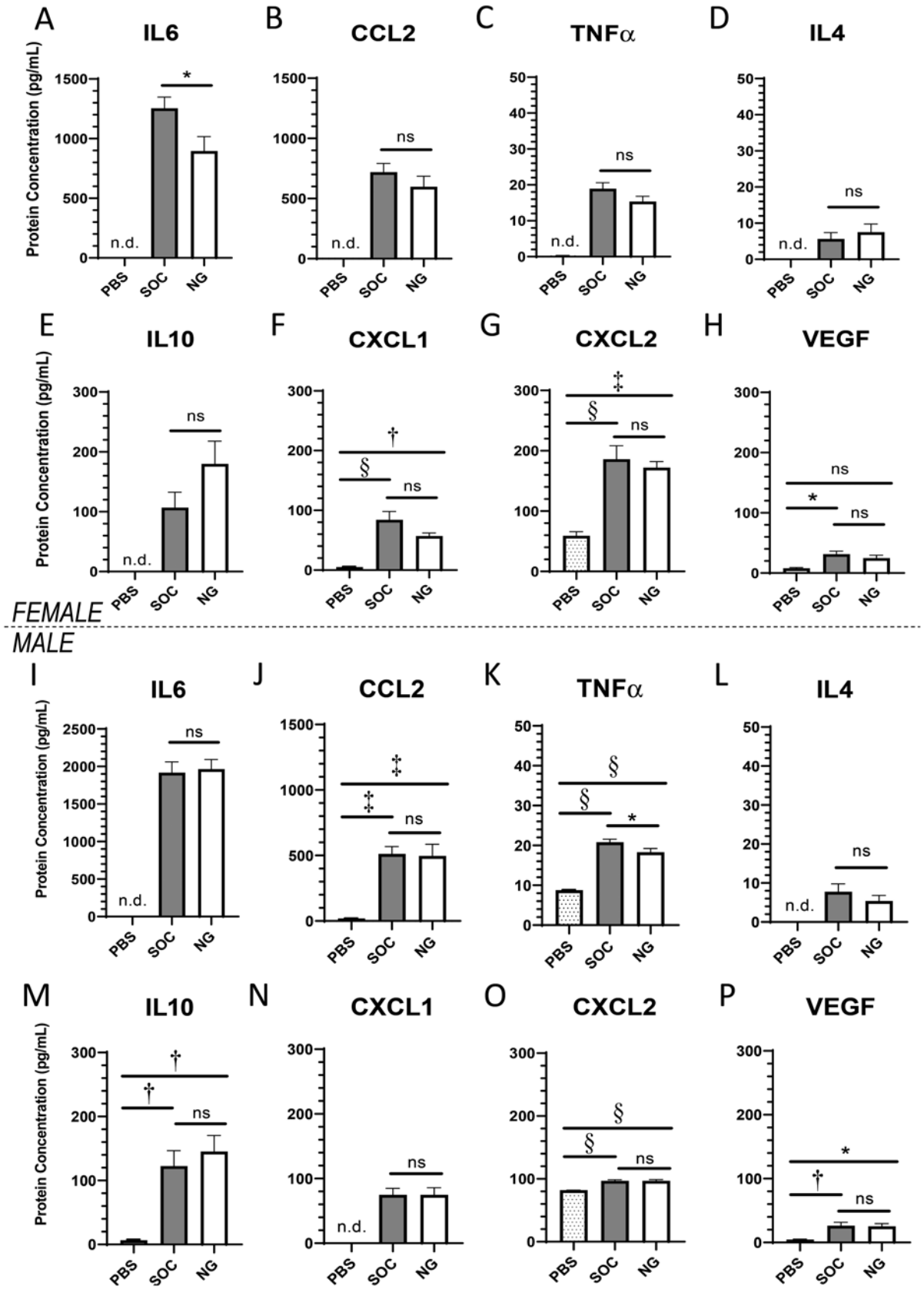

Figure 8. Concentration of chemokines/ cytokines from BAL fluid collected 7 dpi from female (Athrough H) and male (I through P) mice infected with the intermediate dose IAV given SOC or NG. Protein concentration of: A and I) IL6, B and J) CCL2, Cand K) TNFa, Dand L) IL4, E/M) IL10, Fand N) CXCL1, G and O) CXCL2, and Hand P) VEGF. Values are mean ± SEM. Statistics were performed using a one-way ANOVA with Dunnet multiple comparisons test. When cytokines/chemokines were nondetectable in mice given PBS, statistical analysis was performed using an unpaired *t* test. Note that y-axis values are different between graphs. For each data set significance is shown by *, $P \leq 0.05$, †, $P \leq 0.01$, ‡, $P \leq 0.001$, and §, $P \leq 0.0001$. No significance is indicated by 'ns' ($P > 0.05$). PBS $n = 6$/sex; SOC $n = 10$/sex; NG $n = 10$/sex.

sexes. Male and female mice presented a similar clinical picture at the low and intermediate IAV doses, but a deviation between the sexes was clearly seen at the highest dose in that clinical outcomes such as % survival were more severe for males at the high IAV dose. These differences between male and female mice at the high dose emphasizes the importance of considering sex as a separate variable for the purpose of data analysis.

In our study, we found differences in response to IAV in male and female mice, especially at the higher IAV dose independent of NG supplementation. At the high dose of influenza, the female mice in the SOC and NG groups showed minimal to no difference in weight loss. In contrast, the male mice showed a greater benefit from NG supplementation when compared to SOC, with less weight loss and a greater proportion of survivors. At the high dose of influenza, the period of anorexia, as observed by daily visual estimates, lasted for a longer period of time than with the other influenza doses (data not shown), which led to greater weight loss in the mice. These periods of anorexia were likely affected by cytokine responses, as both male and female mice in the NG groups showed lower levels of anorexia-inducing cytokines. At the high infectious dose, the illness likely is more severe, and the inflammatory response stronger, resulting in continued anorexia for longer periods of time than the female mice could recover from. While sex differences are apparent in the response to NG supplementation, differences in response to IAV are a known phenomenon between sexes, and the differences noted between sexes in our study are unlikely due to the NG supplementation. Overall, when given NG, both sexes seemed to show faster recovery of weights, which occurred in females at the intermediate dose and the males at all doses, indicating the benefit of NG supplementation for mice infected with A/PR/8/34 influenza. The differences observed between male and female mice in this study also merit additional research.

Based on the results of this study, we recommend NG supplementation for C57BL/6J mice used in IAV research. NG supplementation minimizes weight loss, reduces the need to remove mice from a study due to euthanasia criteria, and may lead to more robust studies by minimizing survival bias. NG supplementation did not affect immune cell subsets and resulted in minimal change to cytokines, chemokines, and growth factors recovered in BAL fluid. NG supplementation is also easy to implement, minimizes handling of mice, is readily consumed, and is cost effective (approximately $0.57/ day/ cage of 5 mice). In addition, while NG supplementation can benefit both sexes with IAV infections, these data suggest biologic differences in host response to IAV infection, emphasizing the importance of 1) including both sexes within study designs and 2) keeping all mice/sexes in a study on the same supplemental feed/diet. In conclusion, the implementation of NG supplementation in mice infected with IAV is an effective way to refine a study by providing nutritional and hydration support. It also reduces the number of mice required for a study by decreasing premature loss of animals due to reaching weight related endpoint criteria, which will ultimately result in more robust studies.

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