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Original Research

Antibody Production Remains Intact Despite Loss of Bone Marrow B cells in Murine Norovirus Infected *Stat1*$^{-/-}$ Mice

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Murine norovirus (MNV), which can be used as a model system to study human noroviruses, can infect macrophages/monocytes, neutrophils, dendritic, intestinal epithelial, T and B cells, and is highly prevalent in laboratory mice. We previously showed that MNV infection significantly reduces bone marrow B cell populations in a *Stat1*-dependent manner. We show here that while MNV-infected *Stat1*$^{-/-}$ mice have significant losses of bone marrow B cells, splenic B cells capable of mounting an antibody response to novel antigens retain the ability to expand. We also investigated whether increased granulopoiesis after MNV infection was causing B cell loss. We found that administration of anti-G-CSF antibody inhibits the pronounced bone marrow granulopoiesis induced by MNV infection of *Stat1*$^{-/-}$ mice, but this inhibition did not rescue bone marrow B cell losses. Therefore, MNV-infected *Stat1*$^{-/-}$ mice can still mount a robust humoral immune response despite decreased bone marrow B cells. This suggests that further investigation will be needed to identify other indirect factors or mechanisms that are responsible for the bone marrow B cell losses seen after MNV infection. In addition, this work contributes to our understanding of the potential physiologic effects of *Stat1*-related disruptions in research mouse colonies that may be endemically infected with MNV.

Abbreviations: MNV, murine norovirus; PI, postinfection; TD, T cell dependent; TI, T cell independent

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Noroviruses are the most common cause of diarrheal illness across the globe, estimated to cause over 200,000 deaths annually in developing countries, and are the principal cause of foodborne disease in the United States.[13,22] Historically, the study of human noroviruses was limited due to lack of both an in vitro culture system and an animal model. However, the discovery of the first murine norovirus (MNV) in 2003[21] has resulted in a substantial gain in our understanding about the pathogenesis and cellular tropisms of norovirus infection. Although murine models of norovirus infection only recapitulate human clinical symptoms in immune-compromised or neonatal animals,[20,25,29] murine models are nonetheless valuable tools to help identify pathogenic mechanisms, components of genetic susceptibility, and potential targets for therapies and vaccines.

Initially described to infect macrophages and dendritic cells, MNV have more recently been shown to also infect neutrophils, inflammatory monocytes, T cells, and B cells, and the infection of these cell types may modulate immune responses.[10,19,28,39,42] The susceptibility of myeloid cells, T cells, and B cells to infection depends on the expression of the CD300lf receptor, which is necessary for viral entry.[10,12,27,39] We previously reported that MNV-4 infection causes a significant loss of developing B cells in the bone marrow that can be prevented by signal transducer and activator of transcription 1 (STAT1), an important antiviral transcription factor.[17] We found that inhibition of B cell development by MNV-4 infection in the absence of STAT1 was likely due to a combination of direct and indirect mechanisms, rather than solely from direct infection and cell death of maturing B cells.[17] We also reported that along with these B cell losses, MNV-infected *Stat1*$^{-/-}$ mice had a profound increase in the number of granulocytes in the bone marrow and significant elevations of serum granulocyte colony stimulating factor (G-CSF).[17,34] In the current study, we sought to determine whether the bone marrow B cell losses seen after MNV infection in *Stat1*$^{-/-}$ mice resulted in an impaired humoral immune response, and whether the increased granulopoiesis in the bone marrow influences the bone marrow B cell losses seen after MNV infection.

Given that B cells are the major antibody producing cells and can also function as antigen presenting cells, we speculated that MNV infection could affect both adaptive and innate immunity. We hypothesized that MNV infection of *Stat1*$^{-/-}$ mice would result in altered antibody production in response to novel antigenic stimulation due to depletion of B cell populations and/or alteration of B cell function. In addition, because lymphopoiesis and granulopoiesis occur in the same developmental niche in the bone marrow and exhibit a reciprocal relationship,[37] we hypothesized that prevention of granulopoiesis in the bone marrow would rescue the B cell losses observed during MNV infection. Herein, we report that despite MNV's ability to infect B cells and diminish bone marrow B cell populations, MNV-infected *Stat1*$^{-/-}$ mice can nonetheless mount a robust antibody response to novel antigens that is comparable to the antibody response

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of uninfected *Stat1*$^{-/-}$ mice. Furthermore, we demonstrate that administration of anti-G-CSF antibody inhibits MNV-induced granulopoiesis in the bone marrow of *Stat1*$^{-/-}$ mice but does not rescue the MNV-induced loss of bone marrow B cells.

Materials and Methods

Virus. MNV-4 was propagated in RAW 264.7 cells and quantified by plaque-assay as previously described[18] with the modifications of omitting HEPES and using 1% penicillin/streptomycin instead of ciprofloxacin.

Mice. *Stat1*-deficient (*Stat1*$^{-/-}$) mice on a 129 background (129S6/SvEv-*Stat1*^{tm1Rds}) and wild-type (WT) 129 mice (129S6/SvEvTac) were purchased from Taconic Biosciences (Germantown, NY). Mice were fed standard irradiated rodent chow ad libitum (Purina Lab Diet 5053, Brentwood, MO), housed in autoclaved, individually ventilated cages (Allentown, Allentown, NJ) with corncob bedding (The Andersons, Maumee, OH), and provided acidified, reverse-osmosis purified, autoclaved water in bottles. Mice were housed in groups of 3-5 mice per cage and room temperature was set at 72°F (22.2°C). All manipulations were performed in a vertical flow animal transfer station (Ani-Gard II, The Baker Company, Sanford, ME) disinfected with chlorine dioxide (dilution 1:18:1; Clidox S, Pharmacal Research Laboratories, Naugatuck, CT).

Mice were maintained SPF via a rodent health monitoring program and were certified by the vendor to be free of specific rodent pathogens including ectoparasites, endoparasites, *Pneumocystis murina*, *Helicobacter* spp., known enteric and respiratory bacterial pathogens, and antibodies to murine norovirus, mouse hepatitis virus, Sendai virus, pneumonia virus of mice, reovirus 3, Theiler murine encephalomyelitis virus, ectromelia virus, polyoma virus, lymphocytic choriomeningitis virus, mouse adenovirus, minute virus of mice, mouse parvovirus, mouse rotavirus, mouse cytomegalovirus, mouse thymic virus, Hantaan virus, K virus, *Encephalitozoon cuniculi*, cilia-associated respiratory bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme*. The University of Washington's animal facilities are AAALAC accredited and all animal studies were approved by the University of Washington's IACUC.

Experimental infections. Mice were acclimated for at least 1 wk prior to study initiation. Female, 5- to 9-wk-old mice were used for experimental infections with MNV. Mice were inoculated with 200 µL of clarified supernatants of RAW 264.7 cells by oral gavage with MNV-4 (passage 7) at approximately 1×10^6 PFU per mouse. Clarified supernatant from uninfected RAW 264.7 cell lysates were used for control inoculations. Mice were group housed by infection status for the duration of the experiment.

KLH and NP-Ficoll Immunization. Female, 5- to 9-wk-old *Stat1*$^{-/-}$ and 129 wild-type mice were inoculated with MNV-4 or uninfected RAW 264.7 cell lysate by oral gavage. At 7 d after infection, mice were either given NP-Ficoll (NPF) (50 µg per mouse, Biosearch Technologies, Beverly, MA) diluted in sterile phosphate buffered saline and administered by intraperitoneal injection, or Keyhole Limpet Hemocyanin (KLH) (100 µg per mouse, EMD Millipore, St. Louis, MO) suspended in TiterMax Gold Adjuvant (Sigma-Aldrich, St. Louis, MO) and administered by subcutaneous injection. Mice were euthanized via CO_2 asphyxiation at 5 wk after infection (4 wk after immunization). Two independent experiments were performed, with each experiment consisting of 4 to 5 mice per group. Total group sizes for 129 wild-type mice: KLH+MNV (n =10), KLH+lysate (n=10), NPF+MNV (n=10), NPF+lysate (n=10); total group sizes for *Stat1*$^{-/-}$ mice: KLH+MNV (n =10), KLH+lysate (n=10), NPF+MNV (n=9), NPF+lysate (n=10).

ELISA. Whole blood was collected at necropsy into serum separator microtainers (BD Biosciences, San Jose, CA) and spun down at 12,000 x g. Sera were frozen at –80 °C until used. KLH- or NP-Ficoll-specific levels of IgM, IgG, IgG1, IgG2a, and IgG3 antibodies in the serum were measured using AffiniPure goat anti-mouse secondary reporter antibody (Jackson ImmunoResearch, West Grove, PA) on either KLH- or NP-BSA-coated Maxisorp ELISA plates (Thermo Scientific, Wilmington, DE). Serum samples were serially diluted with phosphate buffered saline (PBS) (1:66, 200, 600, 1800, 5400, 16200, 48600, 145800) and optical density (OD) was recorded with a Multiskan Spectrum spectrophotometer (Thermo Scientific, Wilmington, DE) at 10 min after ELISA substrate addition. Serum samples were run in duplicate and averaged.

G-CSF and Anti-G-CSF treatment. Female, 5- to 9-wk-old, *Stat1*$^{-/-}$ mice were given daily intraperitoneal injections of either carrier-free recombinant mouse G-CSF protein (0.5 µg per mouse, R and D Systems, Minneapolis, MN), anti-G-CSF antibody (10 µg per mouse, monoclonal rat IgG1, clone #67604, R and D Systems, Minneapolis, MN), or control IgG isotype antibody (10 µg per mouse, monoclonal rat IgG1, clone #43414, R and D Systems, Minneapolis, MN) diluted in PBS. At the same time the intraperitoneal injections began, mice were inoculated with MNV-4 or uninfected control lysate by oral gavage. Mice were euthanized via CO_2 asphyxiation after either 7 or 14 d of antibody treatment. Two independent experiments were run for the 7 d time point with 4 to 5 mice per group. Total group sizes for the 7 d time point: isotype+lysate (n=9), isotype+MNV (n=10), anti-G-CSF+MNV (n=10), G-CSF+lysate (n=10). A single experiment with 6 mice per group was run for the 14 d time point omitting the G-CSF+lysate group. Total group sizes for the 14 d time point: isotype+lysate (n=6), isotype+MNV (n=6), anti-G-CSF+MNV (n=6).

Flow cytometry. Spleen cells and bone marrow cells harvested from the femurs and tibias were evaluated by flow cytometry using a BD FACSCanto II (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR). Red blood cells were lysed with ammonium-chloride-potassium lysing buffer. Bone marrow and splenic cells (1 to 2×10^6 cells) were blocked with anti-CD16/CD32 (2.4G2) antibody (Tonbo Biosciences, San Diego, CA) and stained with antibodies specific for the following cell surface markers: B220/CD45R (RA3-6B2), CD23 (B3B4), CD93 (AA4.1), CD21/CD35 (CR2/CR1) (BioLegend, San Diego, CA); CD43 (S7), Gr1-Ly6G/Ly6C (RB68C5), TCRβ (H57-597), Ly-6G (1A8), Ly-6C (AL-21), CD11b (M1/70) (BD Biosciences, San Diego, CA); IgM (Jackson ImmunoResearch, West Grove, PA). Live macrophage, granulocyte and lymphocyte populations were gated based on FSC-A and SSC-A and then evaluated by cell surface markers. In the bone marrow, developing B lymphocytes were classified by phenotypic fraction as previously described[14] and grouped: pro-B/pre-B cells (Fraction A-C', B220$^+$CD43$^+$), pre-B/immature B cells (Fraction D-E, B220^{lo}CD43$^-$), and long-lived mature B cells (Fraction F, B220^{hi}CD43$^-$). Granulocytes and macrophages were classified by cell surface markers as GR-1$^+$CD11b$^+$ and GR-1$^-$CD11b$^+$, respectively. Alternatively, granulocytes and macrophages were classified by Ly6C$^+$Ly6G$^+$ and Ly6C$^+$Ly6G$^-$, respectively. In the spleen, mature and transitional B cells were classified as B220$^+$CD93$^-$ and B220$^+$CD93$^+$, respectively. Mature B cells in the spleen were further classified as follicular (IgM^{mid}CD21^{mid}CD23$^+$), marginal zone (IgM^{hi}CD21^{hi}CD23$^-$), or marginal zone precursor (IgM^{hi}CD21^{hi}CD23$^+$).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA). Data

are expressed as the mean values ± SD An unpaired Student *t* test was used to compare 2 groups, and a one-way ANOVA with Sidak multiple comparisons test was used for greater than 2 groups. Statistical significance was defined as a *P* value of less than 0.05.

Results

MNV infection, B cell loss and antibody responses to novel antigens. We previously reported that MNV infection resulted in a decrease in the percentage and total number of both immature and mature B cells in the bone marrow of *Stat1*$^{-/-}$ mice as compared with uninfected *Stat1*$^{-/-}$ mice.[17] Because antibodies are exclusively produced by B cells during the humoral immune response, we examined whether MNV-induced B cell losses influenced the production of serum antibodies against novel antigens in vivo. Previous data showed that B cell losses could be detected as early as 7 d after infection across all stages of developing B cells in the bone marrow.[17] Thus, WT and *Stat1*$^{-/-}$ mice were immunized with either the T-dependent (TD) antigen KLH or the T-independent (TI) antigen NPF at 7 d after MNV-4 infection to coordinate the immunization with the beginning of when B cell losses. Four weeks after immunization (5 wk after infection), bone marrow was evaluated for B cell (Hardy Fractions A-F), granulocyte, and macrophage populations, and serum was evaluated for antibody isotypes and subclasses (IgM, IgG, IgG1, IgG2a, and IgG3) for either the KLH-TD or NPF-TI antigen.

As expected, MNV-infected *Stat1*$^{-/-}$ mice had a significant decrease in the total number of developing pro-B/pre-B cells (B220^{+}CD43^{+}, Fraction A-C'), pre-B/immature B cells (B220^{lo}CD43^{-}, Fraction D-E), and long-lived mature B cells (B220^{hi}CD43^{-}, Fraction F) in the bone marrow as compared with uninfected *Stat1*$^{-/-}$ mice (Figure 1A). In contrast, the bone marrow B cell populations of MNV-infected WT mice did not change significantly as compared with uninfected WT mice (Figure 1B). However, at 4 wk after immunization, all MNV-infected *Stat1*$^{-/-}$ mice produced a serum antibody response that was close to or in some cases, even exceeded those of uninfected *Stat1*$^{-/-}$ mice (Figure 2), despite a significant loss of developing and mature B cells in the bone marrow. To compare serum antibody levels of MNV-infected mice with uninfected mice, statistical analysis (*t* test) was performed at select antibody dilutions that were in the linear range of the dilution curve. In NPF-TI-immunized mice, total IgG and IgG2a were significantly higher than in MNV-infected *Stat1*$^{-/-}$ mice as compared with uninfected *Stat1*$^{-/-}$ mice (Figure 2). In *Stat1*$^{-/-}$ mice immunized with either the KLH-TD or NPF-TI antigen, significantly lower mean OD values of serum IgM were found in MNV-infected *Stat1*$^{-/-}$ mice as compared with uninfected *Stat1*$^{-/-}$ mice (Figure 2).

Because serum antibody levels were largely unchanged, and in some cases even higher in MNV-infected *Stat1*$^{-/-}$ mice despite significantly fewer developing and mature B cells in the bone marrow, we evaluated splenic B cell populations. In the spleen, follicular B cells are important for antibody production to T-dependent antigens, while marginal zone B cells are involved with antibody production to T-independent antigens.[1,7,8,24,33] MNV-infected *Stat1*$^{-/-}$ mice immunized with either the KLH-TD or NPF-TI antigen had significantly higher total splenic cellularity as compared with uninfected *Stat1*$^{-/-}$ mice (Figure 3). The higher splenic cellularity was largely due to significant increases in TCRβ^{+} (T cells), B220^{+}CD93^{-} (mature B cells), Gr1^{+}CD11b^{+} (granulocytes), and Gr1^{-}CD11b^{+} (macrophages) cell counts (Figure 3). B220^{+}CD93^{+} (transitional B cells) cell counts did not differ significantly (Figure 4). Mature B cells were further characterized as IgM^{hi}CD21^{hi}CD23^{+} (marginal zone precursor), IgM^{hi}CD21^{hi}CD23^{-} (marginal zone), and IgM^{mid}CD21^{mid}CD23^{+} (follicular). After MNV-infection, marginal zone B cells in *Stat1*$^{-/-}$ mice immunized with either KLH or NPF did not significantly differ from those of similarly immunized uninfected *Stat1*$^{-/-}$ mice (Figure 4). However, marginal zone precursor and follicular B cells were significantly higher in MNV-infected *Stat1*$^{-/-}$ mice as compared with

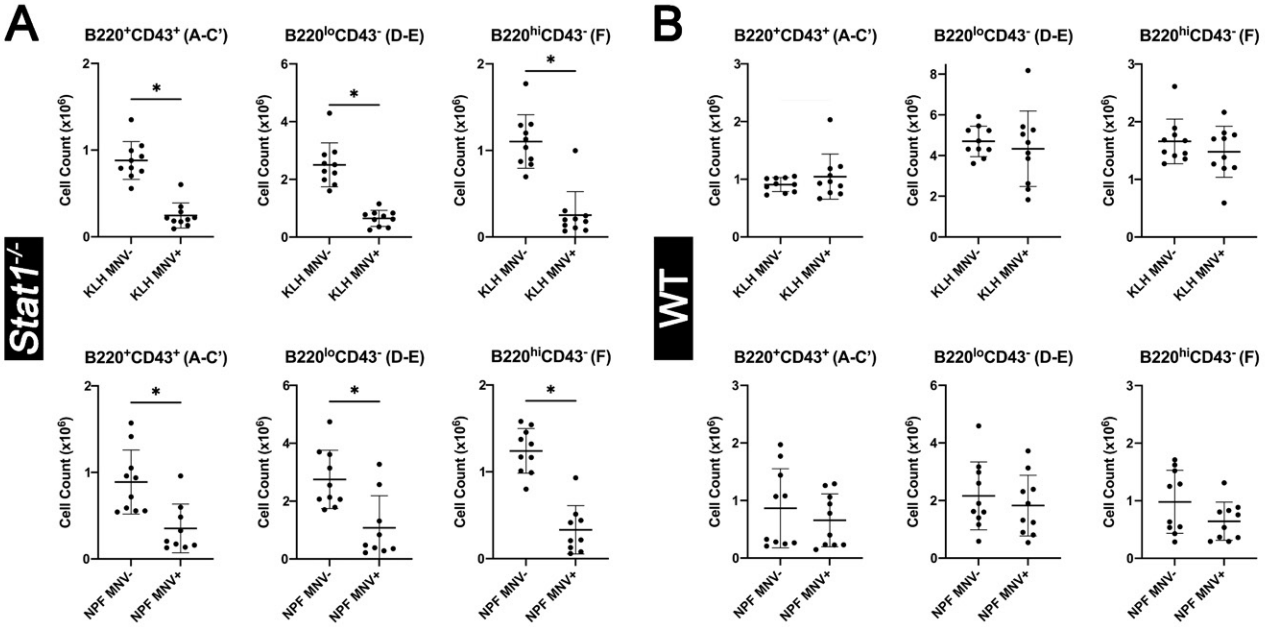

Figure 1. MNV-4 infection decreases developing bone marrow B cells in a STAT1-dependent manner. *Stat1*$^{-/-}$ mice (A) and wild-type (WT) 129 mice (B) were infected with MNV-4 or mock-inoculated, immunized 1 wk post infection (PI) with KLH or NPF, and the bone marrow B cells evaluated by flow cytometry at approximately 5 wk PI. Developing B cells were separated into pro-B/pre-B (Fraction A-C'), pre-B/immature B (Fraction D-E), and long-lived mature B (Fraction F) cells based on B220 and CD43 surface antigen staining. Data shown is combined from 2 independent experiments, each experiment with 4-5 mice per group. Bars represent mean ± SD, * = $P < 0.05$.

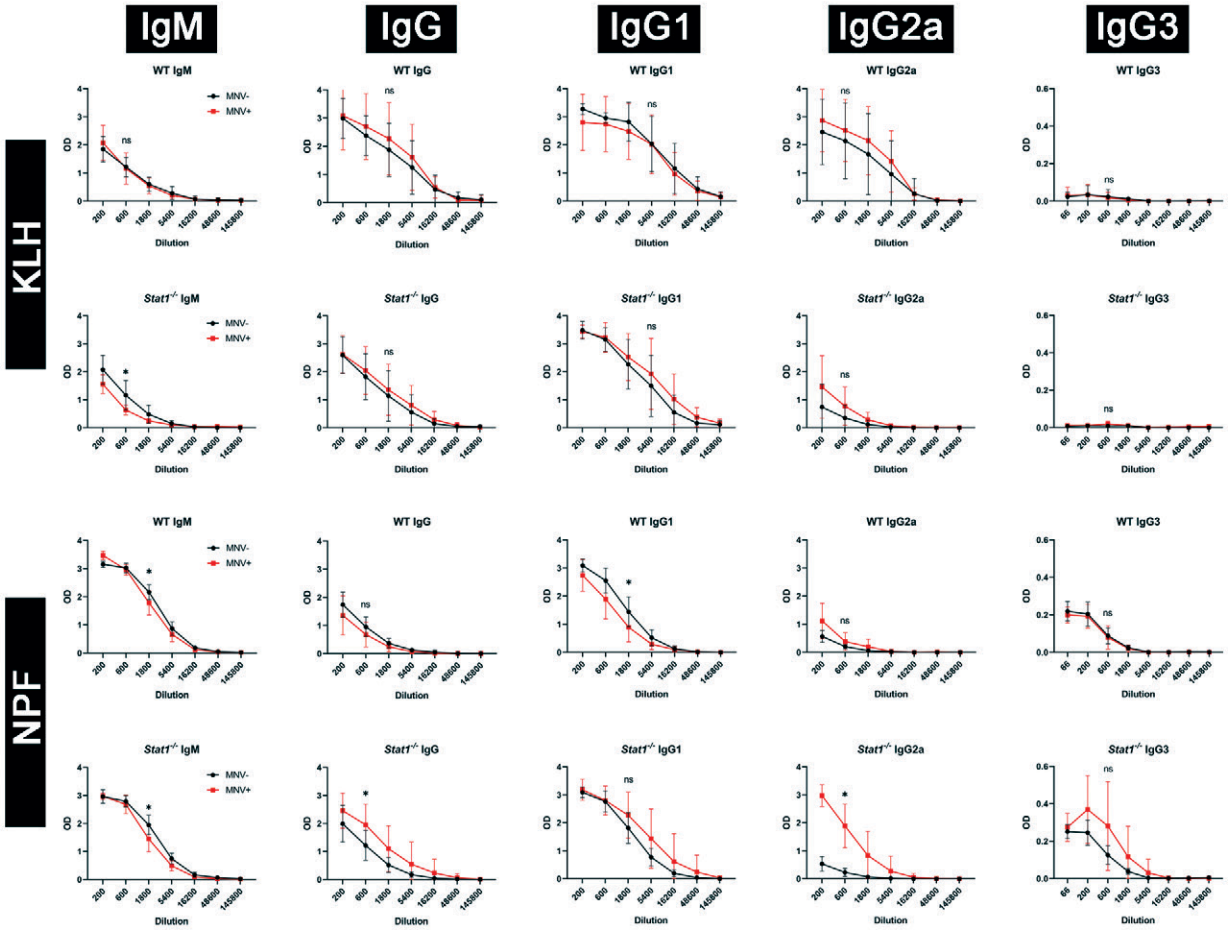

Figure 2. MNV-infected WT and *Stat1*$^{-/-}$ mice mount a robust humoral immune response to novel antigens. Wild-type (WT) 129 mice and *Stat1*$^{-/-}$ mice were infected with MNV-4 or mock-inoculated, and immunized 1 wk post infection (PI) with KLH or NPF. Serum antibodies were evaluated via ELISA at 5 wk PI. Dilution curves were generated and evaluated to ensure ELISA assays were not saturated. A dilution along a linear portion of each curve was chosen for statistical analysis. Data shown is combined from 2 independent experiments, each experiment with 4-5 mice per group. Red curves represent MNV-infected mice and black curves represent uninfected mice. Points along each curve represent mean optical density (OD) value ± SD, * = $P < 0.05$, ns = not significant.

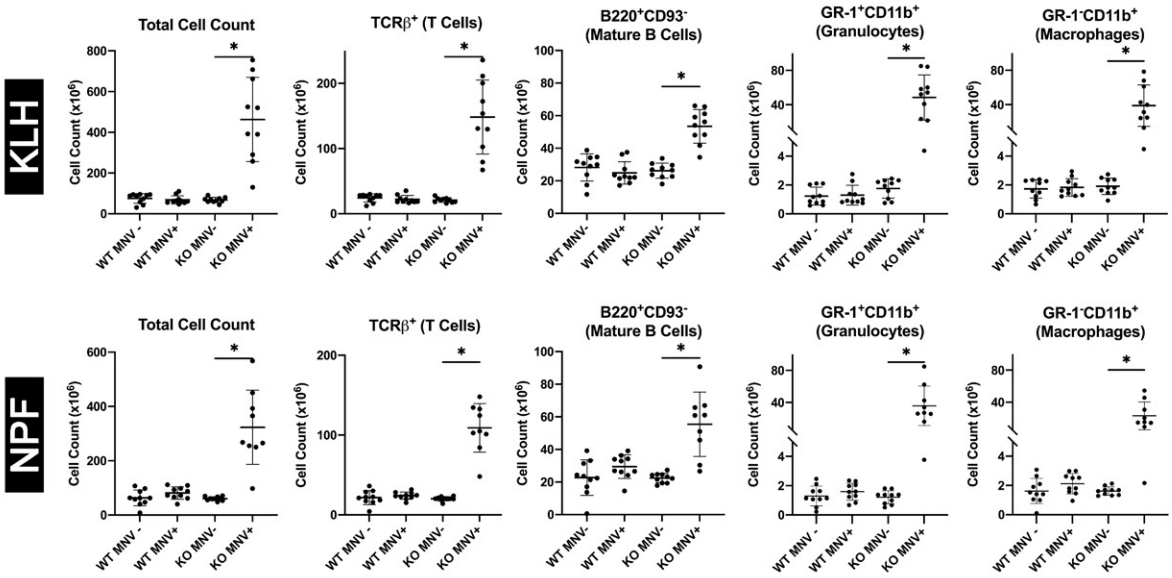

Figure 3. MNV-4 infection increases total splenic cellularity, T cells, mature B cells, granulocytes, and macrophages in a STAT1-dependent manner. Wild-type (WT) 129 mice and *Stat1*$^{-/-}$ (KO) mice were infected with MNV-4 or mock-inoculated, immunized 1 wk post infection (PI) with KLH or NPF, and splenic cell populations were evaluated by flow cytometry at approximately 5 wk PI. Cells were categorized based on cell surface antigen staining: T cells (TCRβ$^+$), mature B cells (B220$^+$CD93$^-$), granulocytes (GR-1$^+$CD11b$^+$), and macrophages (GR-1$^-$CD11b$^+$). Data shown is combined from 2 independent experiments, each experiment with 4-5 mice per group. Bars represent mean ± SD, * = $P < 0.05$.

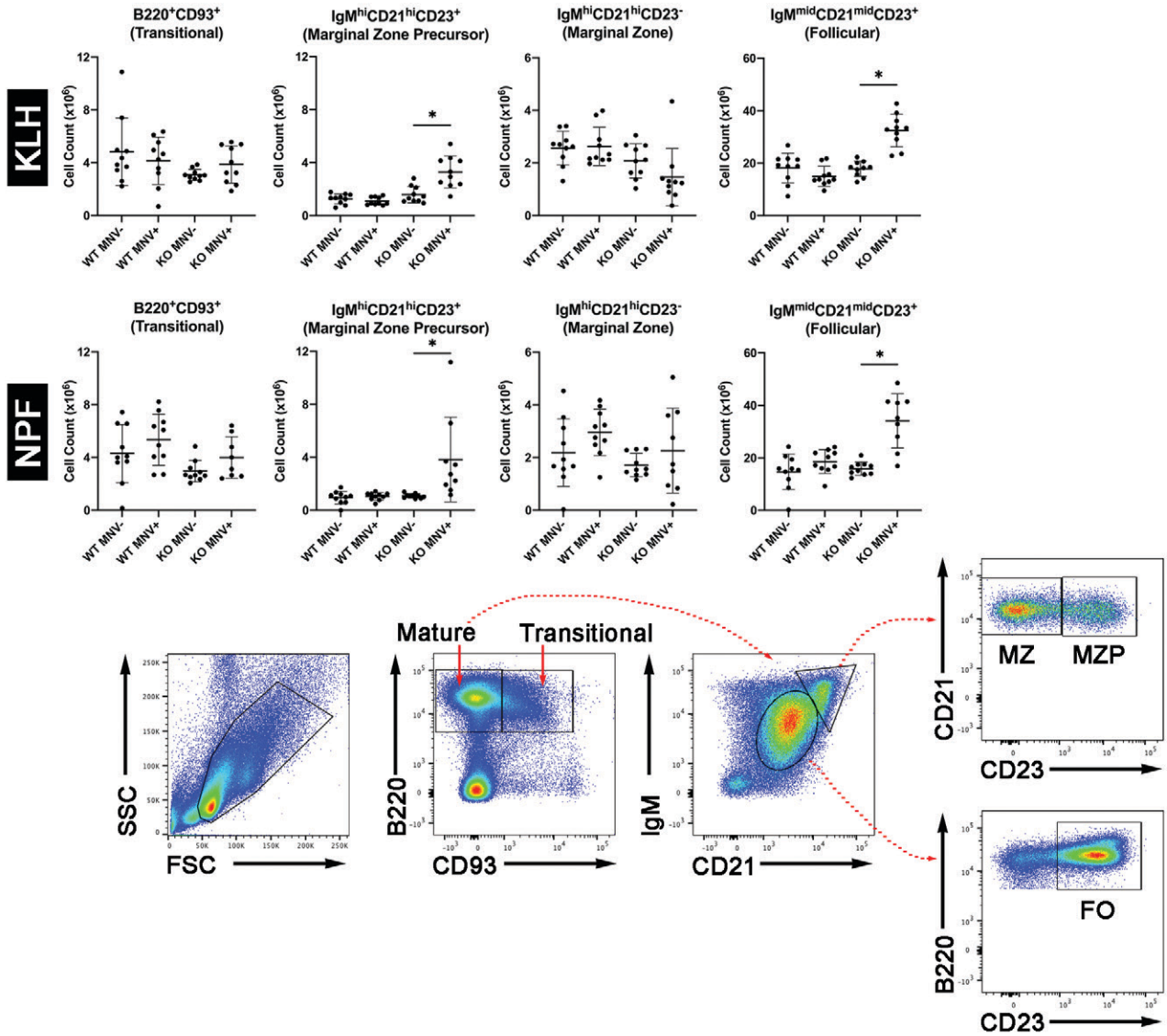

Figure 4. MNV-4 infection increases splenic mature marginal zone precursor and follicular B cell populations in a STAT1-dependent manner. Wild-type (WT) 129 mice and *Stat1*$^{-/-}$ (KO) mice were infected with MNV-4 or mock-inoculated, immunized 1 wk post infection (PI) with KLH or NPF, and splenic cell populations were evaluated by flow cytometry at approximately 5 wk PI. Mature (B220^{+}CD93^{-}) and transitional (B220^{+}CD93^{+}) B cells were separated by cell surface antigen staining. Mature (B220^{+}CD93^{-}) B cells were further separated into marginal zone precursors (MZP, IgM^{hi}CD21^{hi}CD23^{+}), marginal zone (MZ, IgM^{hi}CD21^{hi}CD23^{-}), and follicular (FO, IgM^{mid}CD21^{mid}CD23^{+}) B cells. Data shown is combined from 2 independent experiments, each experiment with 4-5 mice per group. Bars represent mean ± SD, * = $P < 0.05$.

uninfected *Stat1*$^{-/-}$ mice immunized with either the KLH-TD or the NPF-TI antigen (Figure 4). These results indicate that the loss of developing and mature bone marrow B cells in MNV-infected *Stat1*$^{-/-}$ mice does not result in concurrent or proportional losses of all B cell populations in the spleen, but rather that some of these splenic B cell populations are significantly increased.

Effects of prevention of granulocyte proliferation on B cell losses. In previous studies, we showed that concurrent with the B cell losses seen in the bone morrow after MNV-infection in *Stat1*$^{-/-}$ mice, granulocytes and macrophages were significantly higher in the bone marrow.[17] We also observed that serum levels of granulocyte colony-stimulating factor (G-CSF) were 5.7 fold higher at 7 d PI and 3.9 fold higher at 21 d PI in MNV-4-infected *Stat1*$^{-/-}$ mice.[34] Because of the reciprocal relationship between granulopoiesis and B lymphopoiesis in the bone marrow,[37] and because G-CSF is a major regulator of neutrophil maturation[31] and can suppress B lymphopoiesis in the bone marrow,[6,40] we hypothesized that the loss of bone marrow B cells observed in our studies may be due to increased granulopoiesis and G-CSF induced by MNV-4 infection in *Stat1*$^{-/-}$ mice. To test this, we administered daily injections of mouse recombinant G-CSF to uninfected *Stat1*$^{-/-}$ mice to mimic infection with MNV, or we administered anti-G-CSF antibody to MNV-infected *Stat1*$^{-/-}$ mice to try to prevent bone marrow B cell loss due to infection.

As expected, after 7 d of treatment with G-CSF, uninfected *Stat1*$^{-/-}$ mice had significantly more bone marrow granulocyte and macrophage cells than did uninfected *Stat1*$^{-/-}$ mice treated with IgG isotype control antibody (Figure 5). Uninfected *Stat1*$^{-/-}$ mice receiving G-CSF also had significantly fewer pre-B/immature B cells (Fraction D-E) and long-lived mature B cells (Fraction F) as compared with uninfected IgG isotype treated controls (Figure 5). Pro-B/pre-B (Fraction A-C') cell counts were

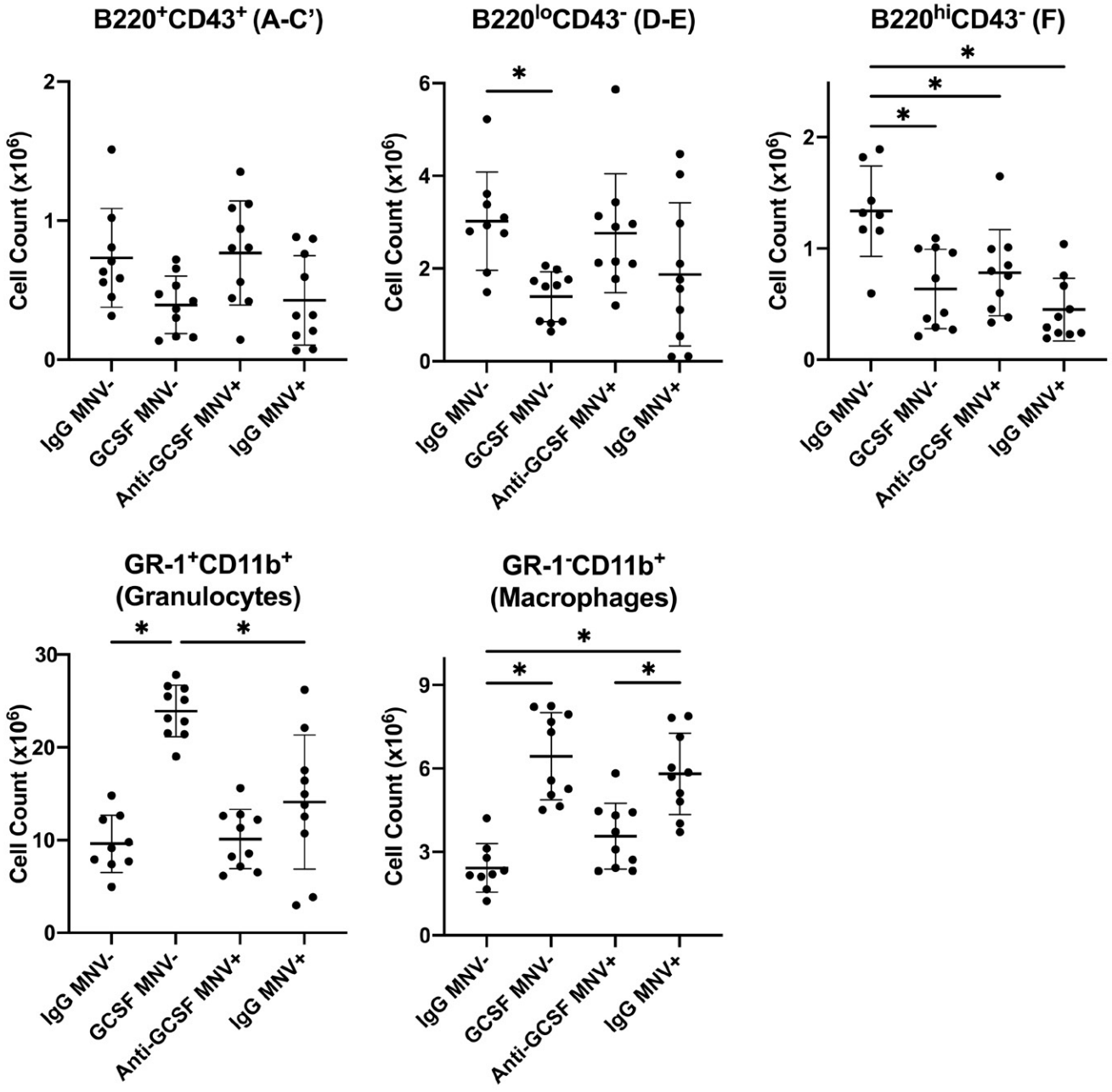

Figure 5. Exogenous G-CSF recapitulates MNV infection in *Stat1*$^{-/-}$ mice. *Stat1*$^{-/-}$ mice were infected with MNV-4 or mock-inoculated and administered daily intraperitoneal injections of recombinant G-CSF, anti-G-CSF antibody, or IgG isotype control antibody for 7 d. Bone marrow cells were evaluated by flow cytometry at 7 d post infection (PI) based on surface antigen staining: pro-B/pre-B cells (Fraction A-C'), pre-B/immature B cells (Fraction D-E), long-lived mature B cells (Fraction F), granulocytes (GR-1$^+$CD11b$^+$), and macrophages (GR-1$^-$CD11b$^+$). Data shown is combined from 2 independent experiments, each experiment with 4-5 mice per group. Bars represent mean ± SD, * = $P < 0.05$.

not significantly lower than those of uninfected isotype treated controls. These results indicate that G-CSF treatment can cause granulopoiesis and a concurrent loss of B cell populations in the bone marrow, similar to that observed after MNV-infection in *Stat1*$^{-/-}$ mice.

To determine whether G-CSF contributes to the bone marrow B cell losses seen after MNV-infection in *Stat1*$^{-/-}$ mice, anti-G-CSF antibody was administered to MNV-infected *Stat1*$^{-/-}$ mice. Seven daily anti-G-CSF treatments, resulted in no statistically significant effects in mean cell counts of any Hardy fractions of B cells (Fractions A-C', D-E, and F) in MNV-infected *Stat1*$^{-/-}$ mice as compared with MNV-infected *Stat1*$^{-/-}$ mice treated with IgG isotype control antibody (Figure 5). Anti-G-CSF treatment of MNV-infected *Stat1*$^{-/-}$ mice had significantly lower mean macrophage counts than did MNV-infected IgG isotype treated controls; granulocyte counts were not significantly different between the 2 groups (Figure 5).

We expected in this experiment that bone marrow B cells would be significantly lower at 7 d PI in the positive control MNV-infected *Stat1*$^{-/-}$ mice (IgG MNV+ mice) as compared with the negative control uninfected *Stat1*$^{-/-}$ mice (IgG MNV- mice), as shown in our previous work.[17] However, in the current study, we found that the difference in pro-B/pre-B cells (Fraction A-C') and pre-B/immature B cells (Fraction D-E) did not reach statistical significance. Therefore, given that 7 d PI was the *earliest* time point at which we observed a statistical

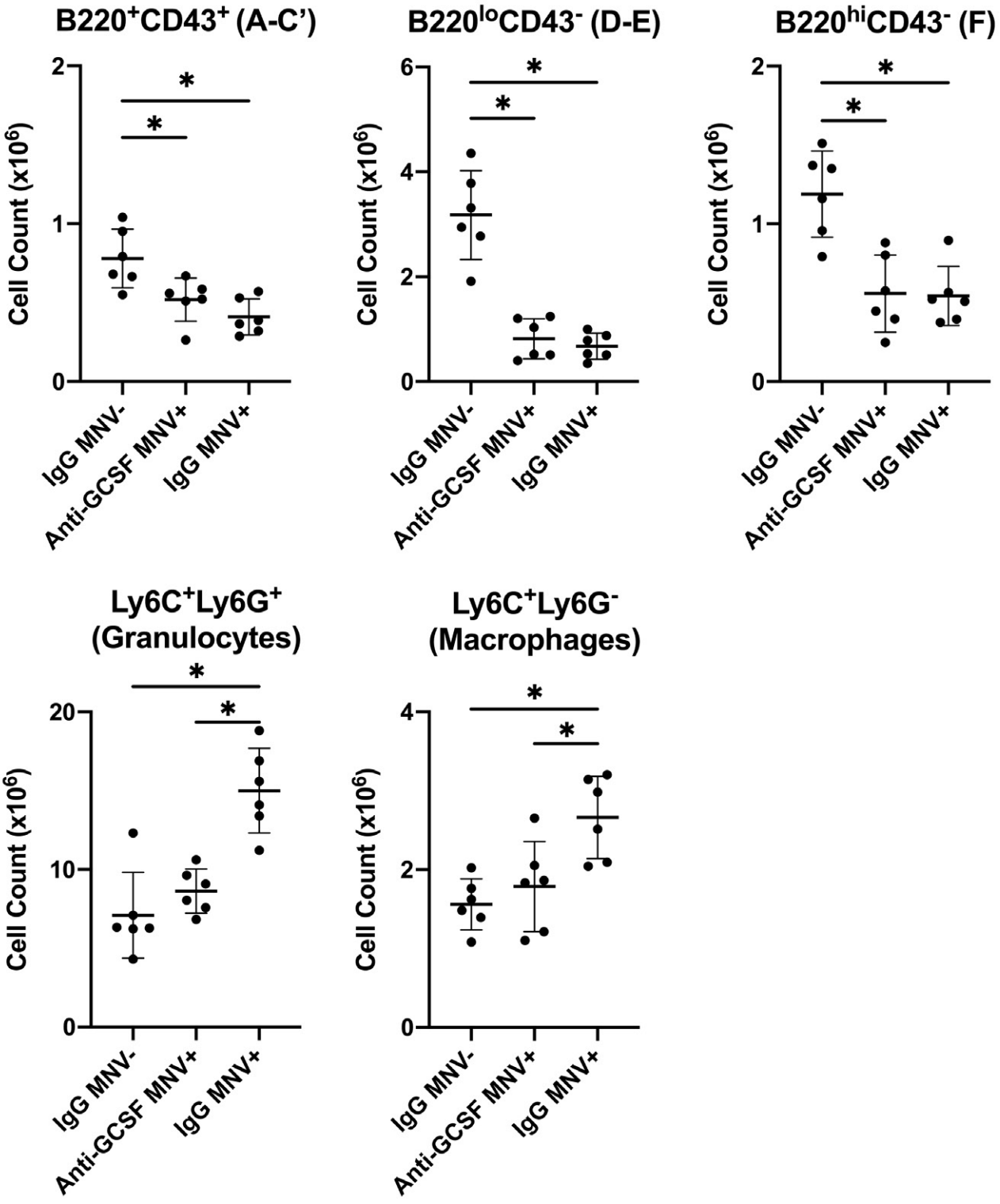

Figure 6. Prevention of granulopoiesis during MNV infection does not prevent bone marrow B cell losses in *Stat1*$^{-/-}$ mice. *Stat1*$^{-/-}$ mice were infected with MNV-4 or mock-inoculated and administered daily intraperitoneal injections anti-G-CSF antibody or IgG isotype control antibody for 14 d. Bone marrow cells were evaluated by flow cytometry at 14 d post infection (PI) based on surface antigen staining: pro-B/pre-B cells (Fraction A-C'), pre-B/immature B cells (Fraction D-E), long-lived mature B cells (Fraction F), granulocytes ($Ly6C^+Ly6G^+$), and macrophages ($Ly6C^+Ly6G^-$). $n = 6$ mice per group. Bars represent mean ± SD, * = $P < 0.05$.

difference in all bone marrow B cell populations in our previous study,[17] we performed a second anti-G-CSF treatment with the MNV infection period extended to 14 d, thus allowing more time for statistically significant losses of all bone marrow B cell populations in MNV-infected *Stat1*$^{-/-}$ mice to occur. At 14 d PI, all B cell populations were significantly lower in MNV-infected *Stat1*$^{-/-}$ mice (IgG MNV+) as compared with uninfected controls (IgG MNV-) (Figure 6). Treatment of MNV-infected *Stat1*$^{-/-}$ mice with anti-G-CSF antibody inhibited granulopoiesis in the bone marrow and also prevented increases in macrophage cell counts as compared with those of MNV-infected IgG isotype controls (Figure 6). However, despite the inhibition of granulopoiesis,

MNV-infected mice treated with 14 d of anti-G-CSF still showed decreased bone marrow B cell counts that were consistent with those of MNV-infected IgG isotype treated controls and were significantly lower than in uninfected controls. Taken together, these data suggest that granulopoiesis induced by G-CSF is not responsible for the chronic impairment of B cell development observed in the bone marrow of MNV-infected *Stat1*$^{-/-}$ mice.

Discussion

This study evaluated whether *Stat1*$^{-/-}$ mice would have an altered humoral immune response to novel antigens during MNV infection, since MNV can infect B cells and result in a significant depletion of both mature and immature B cells in the bone marrow.[17,19] We examined the production of serum IgM, total IgG, and 3 IgG subclasses in *Stat1*$^{-/-}$ mice treated with either a T-dependent or T-independent antigen in the presence or absence of MNV infection and assessed whether the comprehensive antibody profile of the humoral immune response could be impacted by either direct infection of B cells or by impaired B cell development due to MNV infection. We found that MNV-infected *Stat1*$^{-/-}$ mice, despite a significant depletion of bone marrow B cells, can mount a robust serum antibody response to the KLH-TD or NPF-TI antigen comparable to those of uninfected mice. Given that marginal zone B cells are the primary cells responsible for the antibody response to TI antigens, and that follicular B cells are the primary cells responsible for the antibody response to TD antigens,[1,7,8,24,33] we evaluated the splenic B cell populations of immunized mice. We found that although MNV-infected *Stat1*$^{-/-}$ mice have significantly lower bone marrow B cell populations, their splenic B cells numbers are not lower than those of uninfected *Stat1*$^{-/-}$ mice. Splenic B cell populations in MNV-infected *Stat1*$^{-/-}$ mice were either equivalent to or in some cases significantly higher than those of uninfected *Stat1*$^{-/-}$ mice (for example, marginal zone precursor and follicular B cells). Therefore, these results suggest that MNV-infected *Stat1*$^{-/-}$ mice can mount a robust humoral immune response to TD or TI antigens due to adequate or elevated B cell populations in the spleen.

Our data showed that IgG2a antibody levels were higher in MNV-infected *Stat1*$^{-/-}$ mice than in uninfected *Stat1*$^{-/-}$ mice when immunized with the NPF-TI antigen. Another study has also reported that mice given poly (I:C), a synthetic double-stranded RNA that mimics viral nucleic acids, had a greater IgG2c (expressed instead of IgG2a in certain inbred mouse strains) antibody response after immunization with NPF than did mice given NPF alone.[36] Although all 4 IgG subclasses (IgG1-4) can be produced after immunization with TI antigens, the proportions of each subclass will vary as a result of the prevailing cytokine environment.[4] After viral infection, IgG2a is the predominant antibody produced, and its production is upregulated by the Th1 cytokine interferon-γ.[5,9] Recently, we reported that MNV-infected *Stat1*$^{-/-}$ mice have elevated serum interferon-γ (a 13.9 fold increase at day 7 PI);[34] this may account for the increased production of IgG2a antibody in response to immunization with a TI antigen in our current study. Although the biologic significance of this increase in IgG2a antibody production in MNV-infected *Stat1*$^{-/-}$ mice is not known, IgG2a has been associated with stronger FcγR-mediated activity than other antibody isotypes.[4,26] Specifically, IgG2a is the most efficient antibody isotype for viral clearance and for directing antibody-dependent cellular cytotoxicity.[30] This antibody isotype also provides greater protection from neurologic disease induced by lactate dehydrogenase-elevating virus, lymphocytic choriomeningitis virus, or yellow fever virus.[2,23,32] Overall, our results suggest that while MNV infection in *Stat1*$^{-/-}$ mice decreases the number of bone marrow B cells, this decrease did not result in decreased antibody production, but rather infection may have enhanced the T-independent antibody response to a novel antigen.

This study also evaluated the role of G-CSF and bone marrow granulopoiesis in the loss of bone marrow B cells in *Stat1*$^{-/-}$ mice after MNV infection. G-CSF is an important regulator of the mobilization of granulocytes, hematopoietic stem cells, and other myeloid cells from the bone marrow.[3,11,31] Levels of G-CSF increase in the blood and lungs of mice in response to influenza and parainfluenza infections; this increase is critical for host survival to these infectious agents by mobilizing and recruiting activated granulocytes to promote viral clearance.[15,16] Given that MNV-infected *Stat1*$^{-/-}$ mice have significant elevations in serum G-CSF and greatly increased granulocyte populations in the bone marrow, spleen, and liver,[17,34,35] we hypothesized that increased granulopoiesis and G-CSF could contribute to the significant depletion of bone marrow B cells after MNV infection. This idea is supported by a reciprocal relationship between B lymphopoiesis and granulopoiesis, as they both occupy a common developmental niche in the bone marrow.[37] In addition, G-CSF has been shown to suppress B lymphopoiesis in the bone marrow by reducing CXCL12 and IL-7, both of which are essential cytokines that are produced by stromal cells and are required for B cell development.[6,40] Furthermore, G-CSF can deplete specific populations of bone marrow macrophages essential for maintaining the hematopoietic stem cell niches that give rise to developing B cells.[41] In the current study, we found that administration of exogenous G-CSF for 7 d to uninfected *Stat1*$^{-/-}$ mice significantly reduced B lymphopoiesis and concurrently increased granulopoiesis in the bone marrow, mimicking the bone marrow changes observed after MNV-infection of *Stat1*$^{-/-}$ mice and thus suggesting a similar mechanism. To further define the role of G-CSF during MNV infection, we administered anti-G-CSF to MNV-infected *Stat1*$^{-/-}$ mice, which after 14 d effectively reduced granulopoiesis and bone marrow granulocyte numbers to levels equivalent to those of uninfected mice. However, prevention of G-CSF-induced granulopoiesis alone was not sufficient to rescue bone marrow B cell losses after MNV-infection, as the bone marrow B cell numbers in these mice and those in MNV-infected *Stat1*$^{-/-}$ control mice (without anti-G-CSF) were similarly decreased. Although these results suggest that G-CSF and granulopoiesis alone do not contribute to how MNV infection causes decreased bone marrow B cells in *Stat1*$^{-/-}$ mice, G-CSF may still be involved in a multifactorial mechanism, such as altering the expression of a cofactor like IL-7. We previously showed that MNV infection blunted IL-7 gene expression in MNV-infected *Stat1*$^{-/-}$ bone marrow derived macrophages.[17] We also showed that while daily administration of IL-7 to uninfected *Stat1*$^{-/-}$ mice greatly increased bone marrow B cell numbers as compared with baseline levels in untreated-uninfected mice, daily administration of IL-7 to MNV-infected *Stat1*$^{-/-}$ mice only partially rescued the bone marrow B cell losses.[17] B cell numbers in these mice did not rise to the levels seen in uninfected IL-7 treated *Stat1*$^{-/-}$ mice.[17] Therefore, given that myeloid and lymphoid cells occupy the same developmental niche in the bone marrow, we suspect that the IL-7 treated MNV-infected *Stat1*$^{-/-}$ mice in our previous study may have been limited in their capacity to expand their bone marrow B cells to levels equivalent to those of uninfected IL-7 treated *Stat1*$^{-/-}$ mice due to the concurrent expansion of bone marrow granulocytes caused by infection. Further investigation will be required to determine if a combination of anti-G-CSF treatment

in addition to IL-7 treatment (and/or other potential cofactors such as CXCL12) would wholly return B cells levels to those of similarly treated, uninfected control mice.

Our study focused on G-CSF mediated granulopoiesis and its influence on bone marrow B cells in MNV-infected *Stat1*$^{-/-}$ mice, yet why infection induces granulopoiesis and increased monocytes in these mice remains uncertain. A recent report indicates that recruitment of monocytes and neutrophils to sites of infection with MNV promotes viral persistence, because these cells express the CD300lf receptor necessary for viral entry and are thus susceptible to infection.[39] That study also reported that the cytokine IL-1α mediated this recruitment, but detection of IL-1α in the serum of *Stat1*$^{-/-}$ mice at day 2 after infection was variable and dependent on differences in the viral capsid.[39] In our previous studies with MNV-4 in *Stat1*$^{-/-}$ mice, we could not detect increases in serum IL-1α at days 3, 7, or 21 after infection.[34] However, future studies may determine whether IL-1α increases locally in infected tissues, or if another mechanism is responsible for the increased granulocytes seen in the tissues of MNV-4 infected *Stat1*$^{-/-}$ mice in our studies.

Together, the studies reported here corroborate and expand upon our previous novel finding that MNV infection in the absence of *Stat1* can suppress the number of B cells in the bone marrow and induce granulopoiesis. We determined that this significant loss of both developing and mature bone marrow B cells does not impair the humoral immune response to novel antigens, possibly due to sufficient levels of B cells in peripheral sites such as the spleen. This finding is significant because it suggests that although MNV has a tropism for B cells, the functional role of antibody production is not impaired after norovirus infection even in the face of an immune-compromising condition like the absence of *Stat1* expression. We also provide evidence that the significant granulopoiesis observed during MNV infection in the absence of *Stat1* can be effectively reduced with administration of anti-G-CSF antibodies. However, this reduction in granulopoiesis does not rescue the B cell losses seen in the bone marrow. Thus, our studies add to the knowledge of norovirus pathogenesis and the potential physiologic effects on mice with *Stat1*-related disruptions in research mouse colonies that may be endemically infected with MNV.

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