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Murine norovirus inhibits B cell development in the bone marrow of STAT1-deficient mice

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ABSTRACT

Noroviruses are a leading cause of gastroenteritis in humans and it was recently revealed that noroviruses can infect B cells. We demonstrate that murine norovirus (MNV) infection can significantly impair B cell development in the bone marrow in a signal transducer and activator of transcription 1 (STAT1) dependent, but interferon signaling independent manner. We also show that MNV replication is more pronounced in the absence of STAT1 in *ex vivo* cultured B cells. Interestingly, using bone marrow transplantation studies, we found that impaired B cell development requires *Stat1*$^{-/-}$ hematopoietic cells and *Stat1*$^{-/-}$ stromal cells, and that the presence of wild-type hematopoietic or stromal cells was sufficient to restore normal development of *Stat1*$^{-/-}$ B cells. These results suggest that B cells normally restrain norovirus replication in a cell autonomous manner, and that wild-type STAT1 is required to protect B cell development during infection.

1. Introduction

Noroviruses are a leading cause of acute gastroenteritis worldwide and of foodborne illnesses in the United States (Ahmed et al., 2014; Belliot et al., 2014). They are non-enveloped RNA viruses that generally cause self-limiting diarrhea and vomiting in both children and adults. Each year in the United States, noroviruses account for 19–21 million cases of acute gastroenteritis and approximately 400,000 emergency room visits, mostly involving young children (Hall et al., 2013). Though noroviruses have large impacts on human health, the study of norovirus pathogenesis was previously hampered by the lack of a small animal model of infection and the inability to grow the virus in cell culture, until 2003 when a novel norovirus, murine norovirus-1 (MNV-1), was isolated from laboratory mice and could be grown in cell culture (Karst et al., 2003; Wobus et al., 2004). MNV-1 was found to have a tropism for macrophages and dendritic cells, and could be propagated in the RAW 264.7 mouse macrophage cell line (Wobus et al., 2004). Many additional MNV strains have been subsequently identified, and all could similarly be propagated in RAW 264.7 cells, suggesting that macrophages could be the primary reservoir (Basic et al., 2014; Hsu et al., 2006; Shortland et al., 2014). These discoveries were significant advancements for the study of noroviruses as they have contributed to the understanding of norovirus infection and immunity, as well as to the search for finding novel vaccines and treatments.

Recently, Jones et al. reported that noroviruses can infect B cells by demonstrating that a human norovirus could infect the human B cell line BJAB, while in mice, intestinal Peyer's patch CD19^{+} B cells had detectable levels of viral genome after MNV-infection (Jones et al., 2014). MNV was also shown to infect and propagate in the cultured B cells lines WEHI-231 and M12 (Jones et al., 2014). Interestingly, MNV-induced cytopathic effects (cell death) in cultured B cell lines was variable – MNV infection induced cell death in the immature B cell line WEHI-231 but not in the mature B cell line M12 (Jones et al., 2014). A subsequent study found that MNV infection of CD19^{+} Peyer's patch cells resulted in upregulation of MHC class I and co-stimulatory molecules in vivo (Zhu et al., 2016), implying that MNV might modulate B cell immune responses. Because B cells function to make antibodies in response to infection or vaccination and also act as antigen presenting cells, these data suggested that MNV infection could have a direct impact on adaptive as well as innate immunity.

Since the bone marrow is the primary site of B cell development and maturation, and because MNV-induced cytopathic effect in vitro was variable in immature vs. mature cultured B cell lines, we hypothesized that MNV infection in laboratory mice could alter developing B cell populations in the bone marrow. In this report, we describe for the first time that MNV infection causes a significant loss of developing B cells in the bone marrow which can be prevented by signal transducer and activator of transcription 1 (STAT1), an important anti-viral transcription factor. We also report that this inhibition of B cell development by MNV infection in the absence of STAT1 is likely due to a combination of

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direct and indirect mechanisms, rather than solely by direct infection and cell death of maturing B cells. Since B cells are the primary antibody producing cells and can also serve as antigen presenting cells, the studies reported herein are intriguing as they contribute to the knowledge about norovirus infection and pathogenesis.

2. Materials and methods

2.1. Viruses

MNV-4 was propagated in RAW 264.7 cells and plaque-assayed as previously described (Hsu et al., 2005) with the modifications of omitting HEPES and using 1% penicillin/streptomycin instead of ciprofloxacin. Also, a novel MNV strain (MNV-UW) was isolated from the mesenteric lymph node of a sentinel mouse from our institution as previously described (Hsu et al., 2006). In contrast to MNV-4, this MNV-UW strain did not cause a cytopathic effect in RAW 264.7 cells. Therefore, viral propagation was confirmed by passaging the virus three times in RAW 264.7 cells and verifying the presence of viral genome by quantitative RT-PCR.

2.2. Mice

Stat1-deficient (*Stat1*$^{-/-}$) mice on a 129 background (129S6/SvEv-*Stat1*^{tm1Rds}), wild-type 129 mice (129S6/SvEvTac), CD45.1 mice (B6.SJL-*Ptprc*^{a}/BoyAiTac), and *Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ mice (B10;B6-*Rag2*^{tm1Fwa}*Il2rg*^{tm1Wjl}) were purchased from Taconic Biosciences, Inc. (Germantown, NY). *Stat1*$^{-/-}$ mice on a C57BL/6 background (B6.129S (Cg)-*Stat1*^{tm1Dlv}/J) were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in house. *IfnαβγR*$^{-/-}$ mice on a 129/SvEv background (AG129) (van den Broek et al., 1995) were a kind gift from Dr. Michael Gale (University of Washington, Seattle, WA). Mice were fed standard irradiated rodent chow ad libitum (Purina Lab Diet 5053, Brentwood, MO), housed in autoclaved, individually ventilated cages (Thoren, Hazleton, PA) with corncob bedding (The Andersons, Maumee, OH), and provided acidified, reverse-osmosis purified, autoclaved water in bottles. All manipulations were performed in a vertical flow animal transfer station (AniGard II, The Baker Company, Sanford, ME) disinfected with chlorine dioxide (dilution 1:18:1; Clidox S, Pharmacal Research Laboratories, Naugatuck, CT). Mice were maintained specific pathogen free 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.

2.3. Experimental infections

Mice were acclimated for at least 1 week prior to study initiation. Female, 6- to 8-week-old mice were used for experimental infections with MNV except for one experiment where we evaluated sex-dependent effects of MNV infection in male *Stat1*$^{-/-}$ mice. Mice were inoculated with MNV-4 (passage 7) at ~1×10^6 PFU in 200 μL of clarified supernatants of RAW 264.7 cells per mouse by oral gavage. For infection with the MNV-UW strain, since this strain did not cause a cytopathic effect and thus could not be plaque titrated, mice were infected with a similar volume (200 μL) of clarified supernatant from infected RAW 264.7 cells. Clarified supernatants of uninfected RAW 264.7 cell lysates were used for control inoculations. Mice were grouped by infection status and group sizes were 3–5 mice per group unless otherwise indicated. Mice were humanely euthanized via CO_2 asphyxiation and tissues were evaluated 3 weeks post infection (PI) unless otherwise indicated.

2.4. Bone marrow chimera

Male 10-week-old *Stat1*$^{-/-}$ mice, or female 6-week-old recombinase activating gene 2 (*Rag2*) and interleukin 2 receptor, gamma chain (*Il2rg*) deficient mice (*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$) were irradiated (900 rads) and administered ~1×10^7 total donor bone marrow cells via retro-orbital injection under isoflurane anesthesia. Donor bone marrow was from either *Stat1*$^{-/-}$ mice (CD45.2^{+}), or from a 1:1 mixture of *Stat1*$^{-/-}$ and wild-type (CD45.1^{+}) bone marrow cells. Mice were maintained on water containing enrofloxacin for 3 weeks post irradiation. After 10–11 weeks to allow reconstitution of bone marrow cells, mice were inoculated with MNV-4 or with uninfected control lysate, and the bone marrow evaluated at 3 weeks PI.

2.5. Antibiotic depletion

Depletion of the gut microbiota with antibiotics was performed similarly as previously described (Gounder et al., 2016). Briefly, mice were oral gavaged with 100 μL of a compounded antibiotic cocktail (ampicillin 100 mg/mL, neomycin sulfate 100 mg/mL, metronidazole benzoate 100 mg/mL, vancomycin HCl 50 mg/mL in Oro-Sweet Syrup vehicle and peanut butter flavoring) daily for 5 days, and then maintained on antibiotic water (2 mL compounded antibiotic cocktail in 8 oz. of water, changed twice a week) for the remainder of the experiment. Microbial depletion was confirmed by aerobic and anaerobic culture of fecal samples 5 days after initiation of the antibiotic administration (i.e., on the last day of oral gavage with antibiotics) and again just prior to necropsy at the end of study. Mice were inoculated with MNV-4 or uninfected control lysate 7 days after initiation of antibiotics and evaluated at 3 weeks PI.

2.6. IL-7 treatment

Female, 7- to 8-week-old, *Stat1*$^{-/-}$ mice were administered daily intraperitoneal injections of carrier-free recombinant mouse IL-7 protein diluted in phosphate buffered saline (1 μg per mouse, R&D Systems, Minneapolis, MN) or mock injected with phosphate-buffered saline. At the same time as when IL-7 administration began, mice were inoculated with MNV-4 or uninfected control lysate by oral gavage, and then evaluated at 7 days PI.

2.7. Flow cytometry

Splenic cells and bone marrow cells harvested from the femurs and tibias of mice 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 (1×10^6 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), CD19 (ID3), CD45.1 (A20), CD45.2 (104) (Tonbo Biosciences); IgD (11–26 c.2a) (BioLegend, San Diego, CA); CD43 (S7), Gr1-Ly6G/Ly6C (RB6-8C5), TCRβ (H57-597) (BD Biosciences); IgM (Jackson ImmunoResearch, West Grove, PA). Caspase staining was performed using the CellEvent Caspase-3/7 Green Flow Cytometry Assay Kit (Life Technologies) and live/dead staining using Ghost Dye Red 780 (Tonbo Biosciences). Live cells or lymphocyte populations were gated based on FSC-A and SSC-A, then evaluated by cell surface markers. Developing B lymphocytes were classified by phenotypic fraction as previously

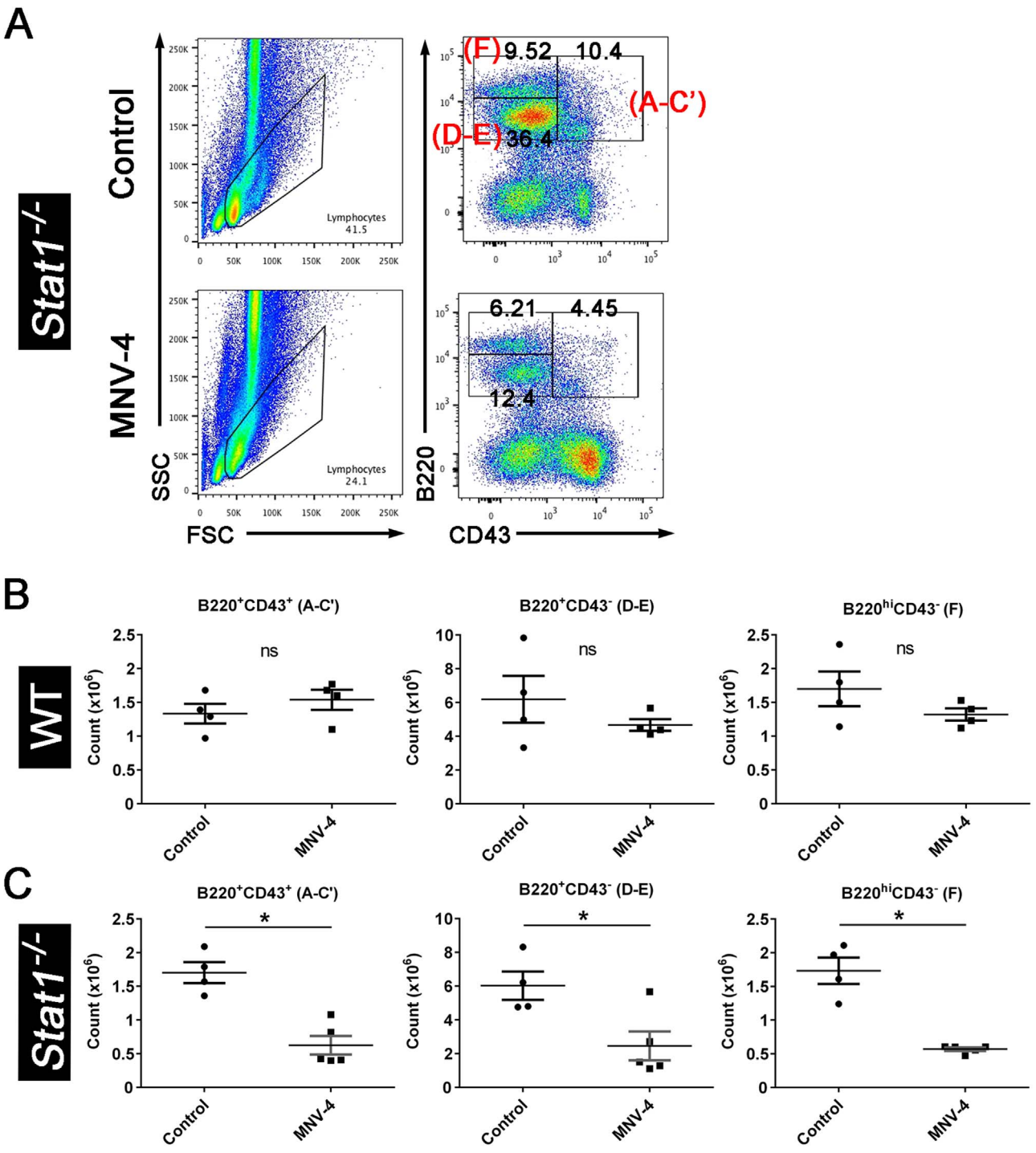

Fig. 1. MNV-4 infection decreases developing bone marrow B cells in a STAT1-dependent manner. Wild-type (WT) 129 mice (B) and *Stat1*$^{-/-}$ mice (A and C) were infected with MNV-4 and the bone marrow B cells evaluated by flow cytometry at approximately 3 weeks post infection. Developing B cells were separated into pro-B/pre-B (Fractions A-C'), pre-B/immature B cells (Fraction D-E), and long-lived mature B cells (Fraction F) based on B220 and CD43 surface antigen staining. Representative of 1 of 2 independent experiments, n = 3–10 mice per group. Bars represent mean ± SEM, * = $P < 0.05$, ns = not significant.

described (Hardy et al., 2007) and grouped: developing pro-B/pre-B cells (Fractions A-C', B220^{+}CD43^{+}), pre-B/immature B cells (Fraction D-E, B220^{+}CD43^{-}), and long-lived mature B cells (Fraction F, B220^{hi}, CD43^{-}). Alternatively, B cells were classified by B220^{+}IgM^{-} (Fractions A-D), B220^{+}IgM^{+} (Fraction E), and B220^{hi}IgM^{+} (Fraction F).

2.8. Cell cultures

Bone marrow-derived macrophages (BMDM) were isolated, differentiated and cultured as previously described (Hsu et al., 2014). BMDM were plated in 6 well plates at 1 × 10^{6} cells per well and incubated with MNV-4 at a multiplicity of infection (MOI) of 0.2 or with uninfected lysate control. After 24 h, cells were washed twice with PBS, and RNA isolated for quantitative RT-PCR. B cells were isolated from the bone marrow of uninfected mice (n = 3) by positive selection using CD45R (B220) MicroBeads and from the spleen by negative selection using the B Cell Isolation Kit (Miltenyi Biotec, San Diego, CA). Isolated B cells from each mouse were mock infected or incubated with MNV-4

for 1 h at a MOI of 0.5, washed with B cell culture media (RPMI, 10% FBS, 1% GlutaMAX, 0.01 M HEPES, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% sodium pyruvate, β-mercaptoethanol, and 10 ng/mL recombinant IL-7) and plated in 96 well plates at 1×10^6 cells in 200 μL per well. Purity of B cells after separation was evaluated by B220 staining and flow cytometry and was 97–99% for the spleen, and 82–89% for the bone marrow.

2.9. RT-PCR

RNA extraction from tissues and feces, RT-PCR for MNV, and primers have been previously described (Hsu et al., 2015, 2006). A real-time quantitative RT-PCR (qRT-PCR) assay for MNV was developed using primers previously described (Hsu et al., 2006). The PCR product was cloned into a plasmid using the pGEM-T Easy Vector System II, isolated with the PureYield Plasmid Miniprep System, linearized with PSTI, and purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) according to manufacturer's recommendation. Plasmid copy number was calculated based on nucleic acid concentration measured using a NanoDrop Lite (Thermo Scientific, Wilmington DE). cDNA was created from RNA samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and real-time qRT-PCR performed using the GoTaq qPCR Master Mix (Promega). Primers for interleukin-7 (IL-7) gene expression were obtained from PrimerBank (ID 6680433a1).

2.10. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). An unpaired Student's t-test was used to compare 2 groups, and a one-way ANOVA with Sidak multiple comparison test was used for greater than 2 groups. Statistical significance was defined as a P value of less than 0.05.

3. Results

3.1. STAT1 prevents MNV-4 induced loss of bone marrow B cells independent of interferon signaling

Since MNV has been shown to infect B cells in vivo and cytopathic infection appears to be dependent on the maturity of B cells in vitro (Jones et al., 2014), we determined whether MNV infection influences developing B cells in the bone marrow in vivo. We infected WT and *Stat1*$^{-/-}$ mice with MNV-4, a strain that causes persistent infections in mice (Hsu et al., 2006), and evaluated B cell development at approximately 3 weeks post MNV-4 infection by flow cytometry. We chose to assess *Stat1*$^{-/-}$ mice because it had previously been shown that STAT1 provided some protective immunity to MNV (Karst et al., 2003; Mumphrey et al., 2007). Developing B cells within the bone marrow can be categorized into pre-pro-B, pro-B, pre-B, immature B, and mature B cells based upon expression of cell surface markers such as B220, CD43, and IgM (Hardy et al., 2007). More specifically, B cell development can be roughly divided into "Hardy Fractions" by letter designations "A" through "F", with "A" representing the earliest stage (pre-pro B cell) while "F" refers to the mature B cell (Hardy et al., 2007).

Effects of MNV on B cell development in the bone marrow differed depending on the presence or absence of STAT1. In *Stat1*$^{-/-}$ mice, MNV-4 infection resulted in a significant depletion in the percentage and total number of developing pro-B/pre-B (Fractions A-C'), pre-B/immature B cells (Fraction D-E), and long-lived mature B cells (Fraction F) in the bone marrow (Fig. 1A and C). However, B cell development was not altered in WT mice after MNV-4 infection (Fig. 1B). Concurrent with this decrease in B cell subsets, MNV-4 infected *Stat1*$^{-/-}$ mice also had a corresponding increase in the percentage and total number of Gr1$^+$CD11b$^+$ (granulocytes) and Gr1$^-$CD11b$^+$ (macrophages) cells in the bone marrow (Fig. 2B) while WT mice had no significant changes in these cell populations after infection (Fig. 2A). Ter119$^+$ erythroid precursor cells were significantly decreased after MNV-4 infection in *Stat1*$^{-/-}$ mice ($1.88 \times 10^6 \pm 1.1 \times 10^5$ cells, mean ± SEM) compared to uninfected mice ($3.24 \times 10^6 \pm 1.9 \times 10^5$ cells). MNV-4 infection did not affect total bone marrow cellularity in either WT or *Stat1*$^{-/-}$ mice. Bone marrow cell counts were not significantly different between uninfected *Stat1*$^{-/-}$ ($3.84 \times 10^7 \pm 2.6 \times 10^6$ cells, mean ± SEM) and MNV-infected *Stat1*$^{-/-}$ mice ($4.53 \times 10^7 \pm 5.9 \times 10^6$ cells), or between uninfected WT ($3.05 \times 10^7 \pm 3.1 \times 10^6$ cells) and MNV-infected WT mice ($2.85 \times 10^7 \pm 1.3 \times 10^6$ cells). MNV-4 infection of total bone marrow was confirmed by RT-PCR in infected WT and *Stat1*$^{-/-}$ mice, and was absent in uninfected mice. MNV-4 genome copy number in total bone marrow was not significantly different between infected WT vs. *Stat1*$^{-/-}$ mice (Supplementary Figure 1A), indicating that the lack of B cell changes observed in WT mice was not the result of clearance of the virus.

To determine the timing of B cell depletion caused by MNV-4 infection in *Stat1*$^{-/-}$ mice, bone marrow was evaluated by flow cytometry at 3, 5, and 7 days PI. MNV infection resulted in significantly decreased mature B cells (Fraction F) at 3 days PI, while differences in pro-B/pre-B (Fractions A-D) and immature B cells (Fraction E) was observed beginning at day 7 PI (Fig. 3). These results indicate that alterations of *Stat1*$^{-/-}$ B cell populations in the bone marrow occurs as early as 3 and 7 days after MNV-4 infection. Additionally, MNV-4 was detected by RT-PCR in the total bone marrow of infected mice at 3 and 7 days PI (day 5 PI not performed) confirming that virus was present in the bone marrow at the same time when B cell depletion was observed. MNV-4 genome copy number in total bone marrow cells significantly increased from day 3 PI to day 7 PI, and then significantly decreased from day 7 to day 21 PI (Supplementary Figure 1B).

Since STAT1 is activated by type 1 and type 2 interferons when bound to their cognate receptors, we next evaluated whether lack of interferon signaling would likewise result in alterations in bone marrow B cells after MNV infection. Mice deficient in the type 1 and type 2 interferon receptors (*IfnαβγR*$^{-/-}$) were infected with MNV-4, and bone marrow B cells were evaluated by flow cytometry. At approximately 3 weeks PI, although virus could be detected by qRT-PCR in the bone marrow (Supplementary Figure 1A), no differences in B cell populations were seen between MNV-infected and uninfected mice (Supplementary Figure 2).

Collectively, these results suggest that MNV infection causes depletion of developing B cells with concurrent increases in granulocytes and macrophages in the bone marrow in a STAT1-dependent, but IFNαβγ-independent manner.

3.2. Enteric bacteria are not required for MNV-4 to inhibit B cell development

It has been previously reported that enteric bacteria contribute to MNV infection in vivo by enhancing infection or by facilitating viral persistence (Baldridge et al., 2015; Jones et al., 2014). Therefore, we evaluated whether enteric bacteria contributed to the B cell depletion observed in the bone marrow after MNV-4 infection. *Stat1*$^{-/-}$ mice were treated with a mix of four antibiotics, inoculated with MNV-4 or lysate control, and bone marrow B cells were evaluated by flow cytometry at 21 days PI. Depletion of enteric bacteria was confirmed by aerobic and anaerobic culture of the feces that exhibited no growth. MNV-4 infection in antibiotic treated mice caused a significant decrease in the developing bone marrow B cell subsets pro-B/pre-B (Fractions A-C') and pre-B/immature B cells (Fraction D-E), but not in mature B cells (Fraction F), when compared to uninfected control mice treated with antibiotics (Fig. 4). qRT-PCR confirmed that MNV-4 could be detected at high levels in the bone marrow of infected *Stat1*$^{-/-}$ mice treated with antibiotics (Supplementary Figure 1A). These results indicate that MNV-4 can inhibit B cell development in some cell subsets in the bone marrow of infected mice independent of enteric bacteria.

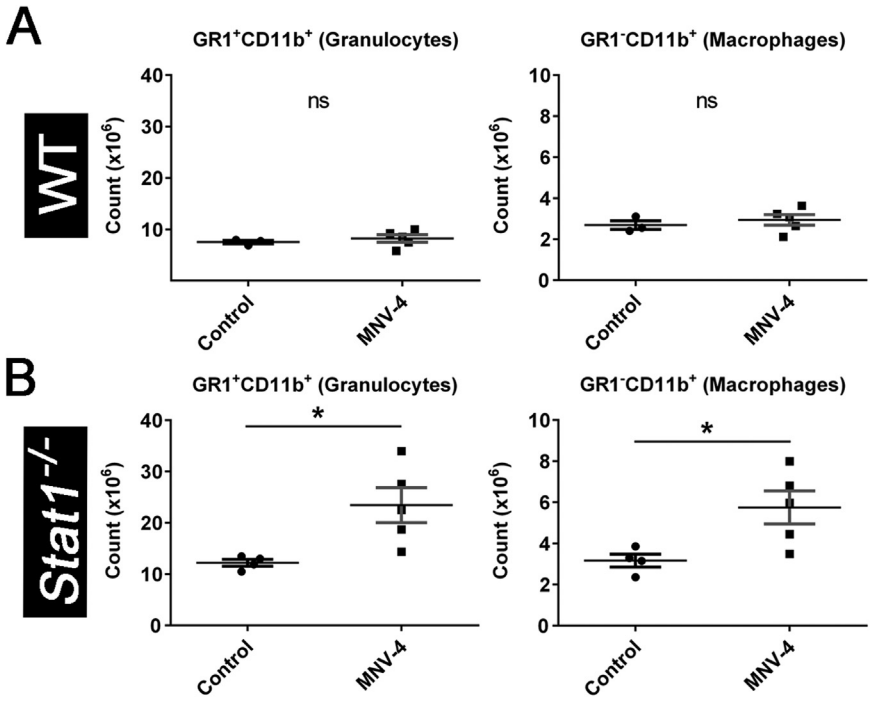

Fig. 2. MNV-4 infection increases granulocytes and macrophages in the bone marrow of *Stat1*$^{-/-}$ mice but not in wild-type mice. Wild-type (WT) 129 mice (A) and *Stat1*$^{-/-}$ mice (B) were infected with MNV-4 and the bone marrow cells evaluated by flow cytometry at approximately 3 weeks post infection by GR1 and CD11b surface antigen staining. n = 3–5 mice per group. Bars represent mean ± SEM, * = $P < 0.05$, ns = not significant.

3.3. MNV induces B cell loss independent of viral strain, mouse background strain, and mouse sex

To determine whether another strain of MNV could likewise inhibit B cell development in the bone marrow, we utilized a novel MNV strain (MNV-UW) we isolated from the mesenteric lymph node of a sentinel mouse housed in another facility at our institution. Whereas MNV-4 causes 100% cytopathic effect by 48 h post-inoculation in the RAW 264.7 mouse macrophage cell line, MNV-UW did not cause a cytopathic effect even after 72 h post-inoculation. Since it has been reported that MNV may replicate in RAW 264.7 cells without causing a cytopathic effect (Shortland et al., 2014), we instead used quantitative RT-PCR to verify if viral replication was occurring and to measure viral concentration. The MNV-UW isolate was passaged 3 times in RAW 264.7 cells to dilute out non-replicating virus, and the clarified cell lysate was evaluated by quantitative RT-PCR for MNV genome copies. The P3 supernatant contained 2.88×10^9 viral genome copies/mL. As expected, no plaques were visible by plaque assay. By comparison, the MNV-4 inoculum used throughout these experiments had a copy number of 4.02×10^{10} viral genome copies/mL and a plaque titer of 5.2×10^6 plaques/mL. These results indicate that after 3 passages, the MNV-UW isolate could infect and replicate to a high genome copy number, and that the MNV-UW isolate is distinct from the MNV-4 isolate based on the differential induction of a cytopathic effect. Similar to *Stat1*$^{-/-}$ mice infected with MNV-4, mice infected with MNV-UW also showed significant depletion of developing pro-B/pre-B (Fractions A-C'), pre-B/immature B cells (Fraction D-E), and long-lived mature B cells (Fraction F) in the bone marrow, as well as a corresponding increase in Gr1$^+$CD11b$^+$ (granulocytes) and Gr1$^-$CD11b$^+$ (macrophages) cells (Fig. 5) when examined at 21 days PI. qRT-PCR confirmed that MNV-UW could be detected at high levels in the bone marrow of infected *Stat1*$^{-/-}$ mice at 21 days PI (Supplementary Figure 1A).

To determine whether the sex or background strain of the mouse significantly contributed to MNV's ability to alter B cell development in the bone marrow, male *Stat1*$^{-/-}$ mice on a 129 background and female *Stat1*$^{-/-}$ mice on a C57BL/6 background were infected with MNV-4 and bone marrow B cells evaluated by flow cytometry at 21 days PI. Up to this point, our studies were limited to only female *Stat1*$^{-/-}$ mice on a 129 background. Similar to our results in female *Stat1*$^{-/-}$ mice on a 129 background, MNV-4 infection induced losses in developing B cells in the bone marrow of male *Stat1*$^{-/-}$ mice (129 background) (Supplementary Figure 3A), as well as female *Stat1*$^{-/-}$ mice on a C57BL/6 background (Supplementary Figure 3B).

Collectively, these results indicate that MNV can induce B cell losses in the bone marrow of *Stat1*$^{-/-}$ mice independent of viral strain, mouse background strain, and mouse sex.

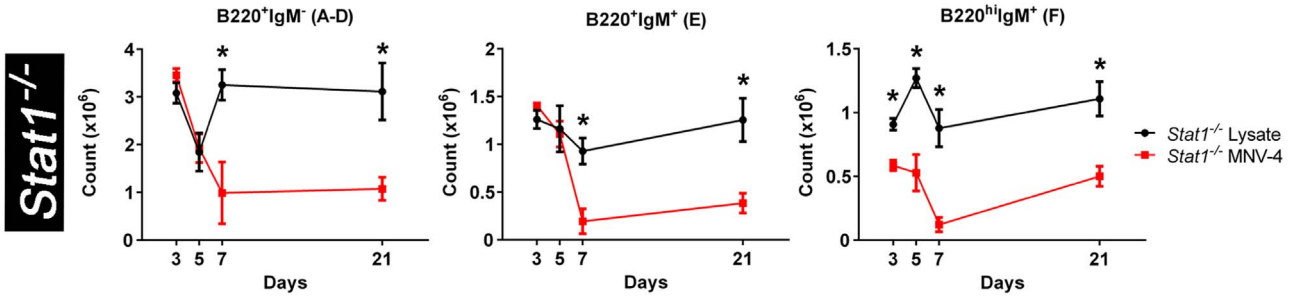

Fig. 3. MNV-4 infection results in decreased pro-B/pre-B and immature B cells at day 7 PI, and decreased mature B cells at day 3 PI, in the bone marrow of *Stat1*$^{-/-}$ mice. *Stat1*$^{-/-}$ mice were infected with MNV-4 and the bone marrow B cells evaluated by flow cytometry at 3, 5, 7 and 21 days post infection. Developing B cells were separated into pro-B/pre-B (Fractions A-D), immature B cells (Fraction E), and long-lived mature B cells (Fraction F) based on B220 and IgM surface antigen staining. Days 3, 5 and 7: n = 3–5 mice per group at each time point; day 21: n = 5–10 mice per group. Bars represent mean ± SEM, * = $P < 0.05$.

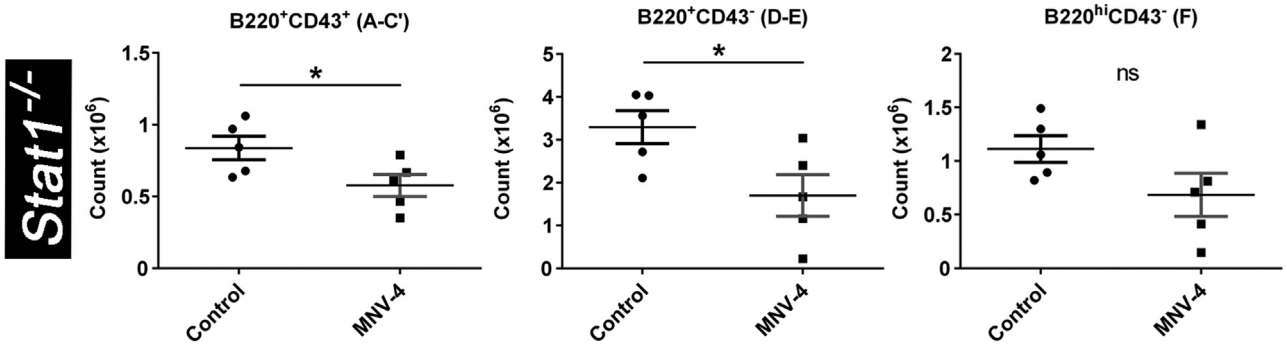

Fig. 4. Enteric bacteria are not required for MNV-4 to decrease developing bone marrow B cells in *Stat1*$^{-/-}$ mice. *Stat1*$^{-/-}$ mice were depleted of enteric bacterial by antibiotic treatment, infected with MNV-4, and the bone marrow B cells evaluated by flow cytometry at 3 weeks post infection. Developing B cells were separated into pro-B/pre-B (Fractions A-C'), pre-B/immature B cells (Fraction D-E), and long-lived mature B cells (Fraction F) based on B220 and CD43 surface antigen staining. $n = 5$ mice per group, bars represent mean ± SEM, * = $P < 0.05$, ns = not significant.

3.4. MNV infection is enhanced in STAT1-deficient B cells in vitro but does not induce cytopathic effect

We hypothesized that loss of developing B cells in MNV-infected *Stat1*$^{-/-}$ mice was due to either direct infection of *Stat1*$^{-/-}$ B cell progenitors by MNV resulting in cell death of bone marrow B cells (cell autonomous), or alternatively, due to an indirect mechanism such as through the formation of a cytotoxic bone marrow environment or through the loss of a necessary B cell growth factor (non-cell autonomous). To test whether direct infection by MNV in STAT1-deficient B cells results in a cytopathic effect, we isolated B cells by B220 positive selection from the bone marrow of three naïve *Stat1*$^{-/-}$ mice and placed the purified cells in culture. Cells from each mouse were incubated with MNV-4 at an MOI of 0.5 or uninfected control inoculum for 1 h, after which the inoculum was removed and replaced with culture media. Cells were then evaluated by trypan blue exclusion assay or flow cytometry to assess cell death at 2, 24 and 48 h PI. There was no difference in the percentage of dead cells between MNV-4 infected and uninfected cells by trypan blue exclusion (data not shown) or by flow cytometry at any time point (Fig. 6A). Similar results were observed with cultured splenic B cells isolated from naïve *Stat1*$^{-/-}$ mice by negative selection (Fig. 6B) despite a steady increase of MNV-4 viral RNA measured by quantitative RT-PCR (Fig. 6C). Interestingly, cultured splenic B cells from WT mice showed a less pronounced increase of MNV-4 viral RNA levels over time compared to cultured splenic B cells from *Stat1*$^{-/-}$ mice (Fig. 6C). These data indicate that while MNV-4 can infect and replicate in cultured B cells (more pronounced in *Stat1*$^{-/-}$ cells), infection did not result in increased cell death.

3.5. MNV does not induce caspase activation in bone marrow B cells of infected Stat1$^{-/-}$ mice

We also evaluated whether caspase activity, a marker of apoptosis, was increased in bone marrow B cells after MNV-4 infection in *Stat1*$^{-/-}$

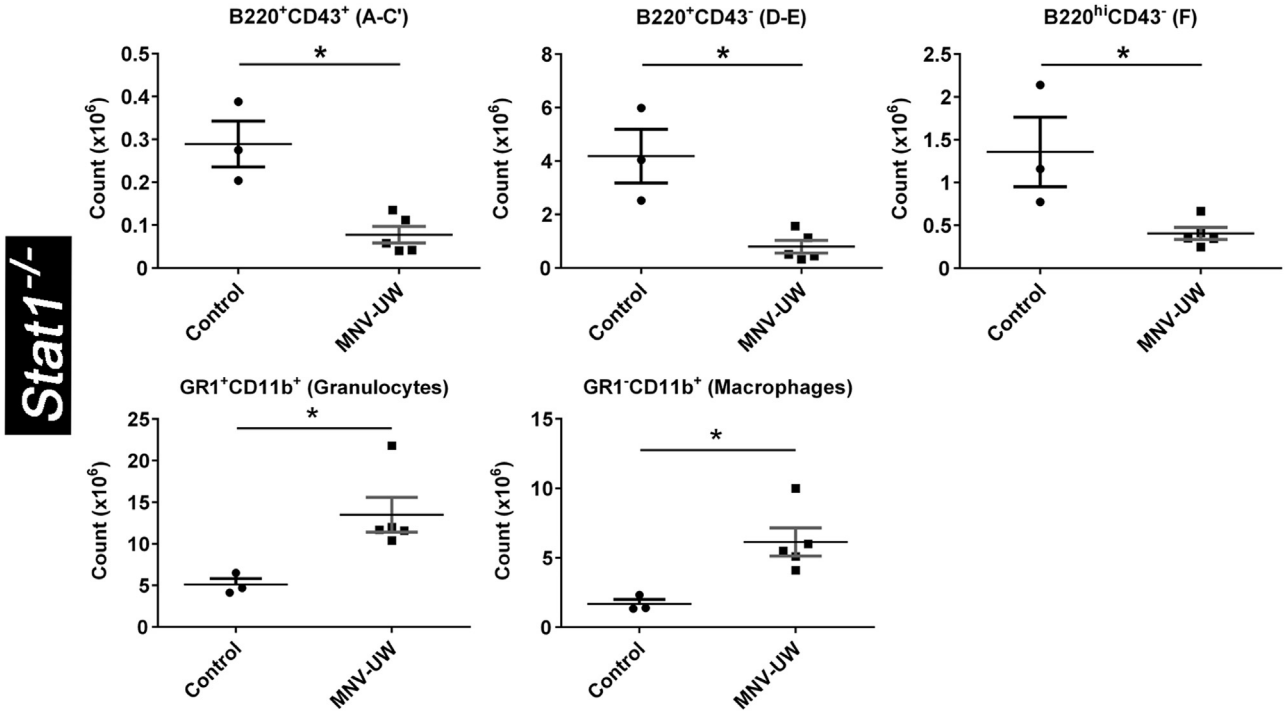

Fig. 5. Infection with a novel MNV strain (MNV-UW) decreases bone marrow B cells and increases granulocytes and macrophages in *Stat1*$^{-/-}$ mice. *Stat1*$^{-/-}$ mice were infected with a novel MNV-UW strain isolated from the mesenteric lymph node of a sentinel mouse and bone marrow cells were evaluated by flow cytometry at 3 weeks post infection. Developing B cells were separated into pro-B/pre-B (Fractions A-C'), pre-B/immature B cells (Fraction D-E), and long-lived mature B cells (Fraction F) based on B220 and CD43 surface antigen staining, while granulocytes and macrophages were characterized by GR1 and CD11b surface antigen staining. $n = 3$–5 mice per group, bars represent mean ± SEM, * = $P < 0.05$.

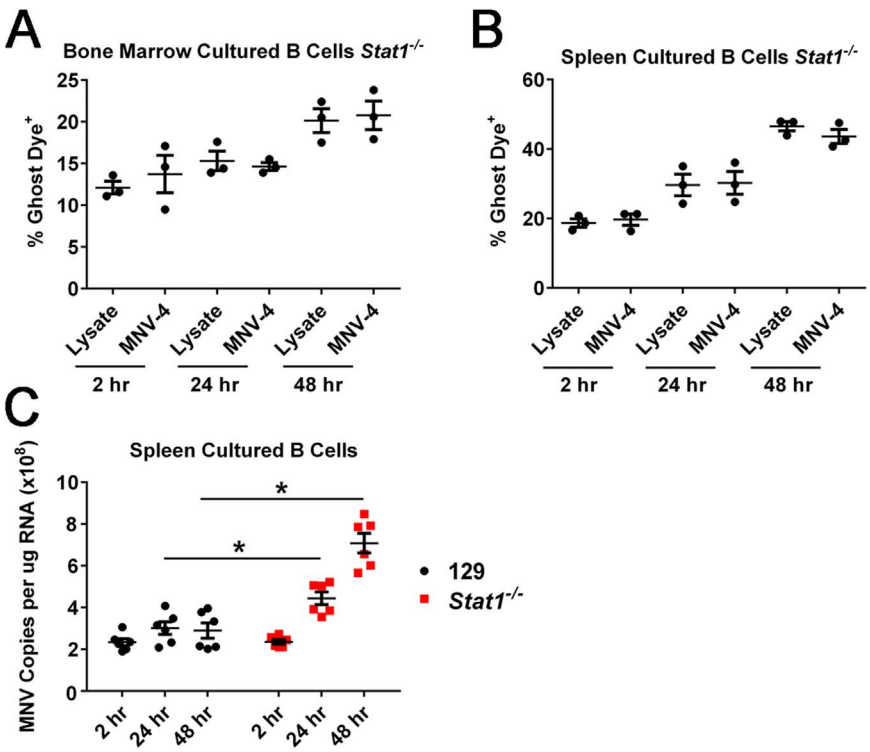

Fig. 6. MNV-4 replicates in cultured B cells isolated from the spleens of *Stat1*$^{-/-}$ mice but does not induce cell death. B cells were isolated from the bone marrow (A) or spleen (B) of $n = 3$ uninfected *Stat1*$^{-/-}$ mice and infected in vitro with MNV-4 at an MOI of 0.5 or mock infected with lysate control. Cells were evaluated at 2, 24 and 48 h for cell death by flow cytometry and Ghost Dye staining. (C) MNV genome copy number evaluated by quantitative RT-PCR in B cells isolated from the spleens of uninfected *Stat1*$^{-/-}$ and wild-type 129 mice and then infected in vitro at an MOI of 0.5. Fig. C is pooled data from 2 independent experiments. Bars represent mean ± SEM, * = $P < 0.05$.

mice. It has been previously reported that after direct infection, MNV activated caspase and induced apoptosis of RAW 264.7 cells (Bok et al., 2009; Furman et al., 2009; McFadden et al., 2011). Mice were evaluated 5 days after MNV-4 infection to target a time point where bone marrow cells are known to be infected (detected by RT-PCR at day 3 PI) but prior to significant losses in all B cell subsets (day 7 PI, see Fig. 3). Activated caspase 3/7 staining did not differ between uninfected and MNV-4 infected mice when evaluated in total B220$^+$, pro-B/pre-B (B220+IgM$^-$, Fractions A-D), immature (B220$^+$IgM$^+$, Fraction E), or mature (B220^{hi}IgM$^+$, Fraction F) bone marrow B cells (Supplementary Figure 4). These results reveal that apoptosis via caspase activation could not be detected in bone marrow B cells 5 days PI.

3.6. Loss of STAT1 in bone marrow derived hematopoietic cells and bone marrow stromal cells contribute to decreased B cell development after MNV infection, while wild-type cells can prevent B cell losses

To further evaluate whether direct or indirect effects of MNV-4 infection were responsible for B cell losses, we generated bone marrow chimeras and then evaluated bone marrow B cells after infection. Bone marrow from *Stat1*$^{-/-}$ mice alone, or a 1:1 mixture of WT (CD45.1$^+$) and *Stat1*$^{-/-}$ (CD45.2$^+$) bone marrow, was transferred to irradiated *Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ recipients which are T-, B- and NK-cell deficient, but have normal STAT1-sufficient stroma cells. Recipient mice were allowed to reconstitute for 10 weeks and then infected with MNV-4. Bone marrow B cells were evaluated by flow cytometry at ~3 weeks PI. We expected that if MNV-4 was directly infecting and killing *Stat1*$^{-/-}$ developing B cells, then only *Stat1*$^{-/-}$ (CD45.2$^+$) developing B cells would be depleted in both groups of mice, while WT B cells with intact STAT1 would be unaltered. Alternatively, if an indirect mechanism derived from the administered *Stat1*$^{-/-}$ bone marrow cells was causing B cell loss, then both WT and *Stat1*$^{-/-}$ developing B cells would be decreased in MNV-4 infected mice administered the 1:1 mix of WT:*Stat1*$^{-/-}$ bone marrow compared to uninfected controls because both cell types in the 1:1 mix are exposed to the same *Stat1*$^{-/-}$ bone marrow cells. Mean reconstitution rates of B220$^+$ cells in the bone marrow of uninfected mice administered the 1:1 bone marrow mix of WT:*Stat1*$^{-/-}$ cells were 66.6% WT:17.4% *Stat1*$^{-/-}$ cells, indicating that *Stat1*$^{-/-}$ bone marrow cells cannot efficiently compete with wild-type bone marrow cells. Interestingly, neither of the bone marrow chimeras showed B cell depletion after MNV-4 infection compared to uninfected controls (Supplementary Figure 5A-B). Since *Stat1*$^{-/-}$ B cells were unaltered after infection even when administered alone to *Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ recipient mice (Supplementary Figure 5A), these results suggest that stromal cells deficient in STAT1 contribute to causing the B cells loss seen in MNV infected *Stat1*$^{-/-}$ mice, and that WT stroma present in the *Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ host is sufficient to rescue B cell development.

To further evaluate whether stromal cells deficient in STAT1 contribute to MNV induced B cell depletion in the bone marrow, we generated an additional bone marrow chimera in which a 1:1 mixture of WT and *Stat1*$^{-/-}$ bone marrow cells was transferred to irradiated *Stat1*$^{-/-}$ recipients. Surprisingly, at 3 weeks PI, neither CD45.1$^+$ WT B cells, nor CD45.2$^+$ *Stat1*$^{-/-}$ B cells were decreased in the bone marrow (Supplementary Figure 6). Mean reconstitution rates of B220$^+$ cells in the bone marrow of uninfected mice were 49.3% WT:33.3% *Stat1*$^{-/-}$ cells. These results indicate that co-administration of WT bone marrow is sufficient to prevent the loss of *Stat1*$^{-/-}$ bone marrow B cells seen in MNV infected *Stat1*$^{-/-}$ mice.

Collectively, these data indicate that MNV does not induce loss of *Stat1*$^{-/-}$ bone marrow B cell progenitors solely by direct infection and cell death. Rather, these data suggest that an indirect mechanism may play a role in which loss of STAT1 in both bone marrow derived cells and in stromal cells are required to induce the B cells losses seen in the bone marrow. Additionally, these data suggest that STAT1-sufficient WT cells can prevent *Stat1*$^{-/-}$ B cell losses in MNV infected *Stat1*$^{-/-}$ mice, such as by co-administration of WT bone marrow cells or by the presence of WT stromal cells.

3.7. IL-7 treatment partially rescues B cell development in the bone marrow of MNV-infected Stat1$^{-/-}$ mice

The cytokine interleukin-7 (IL-7) is an essential factor for B cell development in the bone marrow and can also provide a survival signal for B cells (Fry and Mackall, 2002; Nagasawa, 2006). We hypothesized

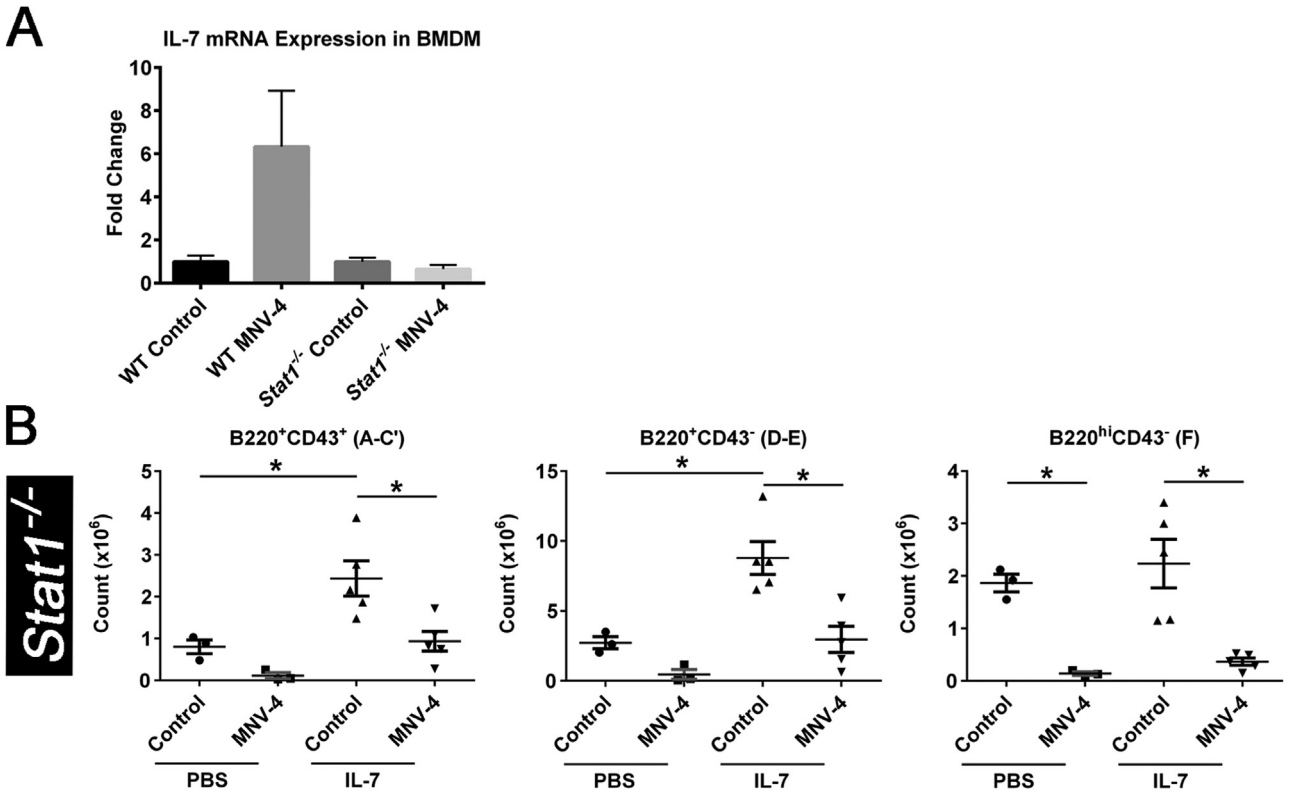

Fig. 7. MNV-4 infection decreases developing bone marrow B cells in *Stat1*$^{-/-}$ mice treated with IL-7. Bone marrow-derived macrophages were isolated and differentiated from uninfected wild-type and *Stat1*$^{-/-}$ mice, infected in vitro with MNV-4 or mock infected, and then evaluated for mRNA expression of IL-7 by quantitative RT-PCR (A). *Stat1*$^{-/-}$ mice were treated with recombinant mouse IL-7 or PBS daily and infected with MNV-4 or mock infected on the first day of treatment (B). Bone marrow B cells were evaluated by flow cytometry at 7 days post infection. Developing B cells were separated into pro-B/pre-B (Fractions A-C'), pre-B/immature B cells (Fraction D-E), and long-lived mature B cells (Fraction F) based on B220 and CD43 surface antigen staining. n = 3–5 mice per group. A one-way ANOVA with Sidak multiple comparison test was used to evaluate Control PBS vs. MNV-4 PBS, Control PBS vs. Control IL-7, Control IL-7 vs. MNV-4 IL-7, and MNV-4 PBS vs. MNV-4 IL-7. Bars represent mean ± SEM, * = $P < 0.05$.

that reduced IL-7 expression in the bone marrow after infection could account for an indirect mechanism by which MNV caused decreased B cell progenitors. To test this, we first evaluated IL-7 mRNA expression in bone marrow-derived macrophages (BMDM) after in vitro infection since MNV has a tropism to infect macrophages. In WT BMDM, MNV-4 infection increased IL-7 gene expression 6.3-fold compared to uninfected WT BMDM (Fig. 7A). However, in *Stat1*$^{-/-}$ BMDM, MNV-4 infection decreased IL-7 gene expression approximately 1.5 fold (Fig. 7A). These results suggest that IL-7 production in response to MNV-4 infection could be blunted in *Stat1*$^{-/-}$ mice and thus potentially affect B cell progenitor numbers. To test this in vivo, *Stat1*$^{-/-}$ mice were administered recombinant IL-7 for 7 days beginning on the day of MNV-4 infection to determine whether exogenous treatment would rescue the loss of B cells. Treatment with IL alone (i.e., uninfected mice) increased the number of B220^{+}CD43^{+} (A-C') and B220^{+}CD43^{-} (D-E) B cells approximately 3-fold, indicating that treatment with IL-7 was at a sufficient dose to stimulate B cell lymphopoiesis (Fig. 7B, PBS Control vs. IL-7 Control). IL-7 also stimulated an approximately 2- to 8-fold increase in the number of *Stat1*$^{-/-}$ B cells in the MNV-4 infected group (Fig. 7B, PBS MNV-4 vs. IL-7 MNV-4) partially rescuing B cell development. However, B cell levels were still significantly lower in the MNV-4 IL-7 treated group relative to the uninfected IL-7 treated group (Fig. 7B, IL-7 Control vs. IL-7 MNV-4), indicating that IL-7 could not completely rescue B cell development back to uninfected IL-7 baseline levels. These results indicate that a reduction in IL-7 following MNV infection in *Stat1*$^{-/-}$ mice may play a partial role in the B cell losses seen after infection, but that other factors such as the loss of adequate innate immunity contributes to impaired B cell development and the loss of B cells in *Stat1*$^{-/-}$ mice.

4. Discussion

In this study, we evaluated the consequence of MNV infection on the developing B cell populations in the bone marrow, since it has recently been reported that human and murine noroviruses can differentially infect B cells depending on the stage of development (Jones et al., 2014). Specifically, Jones *et al.* reported that MNV infection caused cell death in vitro in the immature B cell line WEHI-231 but not in the mature B cell line M12. Since the bone marrow is the primary site of B cell development and contains immature and mature stages of B cells, we evaluated whether MNV infection in vivo would likewise cause a differential effect on B cell death depending on the cell stage of maturation of bone marrow B cells. We utilized MNV-4, a strain that causes persistent and systemic infections in mice (Hsu et al., 2006), to evaluate changes in bone marrow B cells after infection. Herein, we report the novel finding that MNV-4 infection caused alterations in developing bone marrow B cells in vivo, however this effect did not differ between immature vs. mature B cells, as was seen in vitro. We show that MNV-4 infection resulted in a significant loss of pro-B/pre-B, immature B, and mature B cells within the bone marrow in *Stat1*$^{-/-}$ mice, and that this loss of B cells can be prevented by STAT1 in wild-type mice.

STAT1 is a transcription factor induced by type I (α and β) and type II (γ) interferons and is important in the immune response against viral infections (Kallal and Biron, 2013; Karst et al., 2003; Schneider et al., 2014). *Stat1*$^{-/-}$ mice have been shown to be more susceptible to MNV infection resulting in increased clinical and histopathologic disease as compared to wild-type mice (Kahan et al., 2011; Karst et al., 2003; Mumphrey et al., 2007). Similarly, mice deficient in the receptors for interferon α,β,γ or α,β alone also develop more severe disease following MNV infection compared to wild-type mice (Karst et al., 2003;

Mumphrey et al., 2007; Rocha-Pereira et al., 2013; Thackray et al., 2012). These data reveal the importance of the interferon/STAT1 signaling pathway in preventing MNV associated disease. However, in contrast to the B cell losses seen in our studies with *Stat1*$^{-/-}$ mice, we did not observe any changes to the bone marrow B cell populations of MNV-4 infected *IfnαβγR*$^{-/-}$ mice, suggesting that STAT1 is essential for normal B cell development and survival following MNV infection in a manner independent of interferon signaling. In a previous report on severe acute respiratory syndrome coronavirus infection, a similar difference in response to infection was reported between *Stat1*$^{-/-}$ mice and interferon-receptor deficient mice: viral infection caused severe lung disease and mortality in *Stat1*$^{-/-}$ mice, but only transient disease with recovery in *IfnαβR*$^{-/-}$, *IfnγR*$^{-/-}$, and wild-type mice (Frieman et al., 2010). These data suggest that STAT1 may have specific modulatory functions independent of interferon signaling, such as in cell growth and proliferation (Frieman et al., 2010; Levy and Darnell, 2002; Ramana et al., 2000).

MNV causes a cytopathic effect in RAW 264.7 mouse macrophage cells by activating caspases and inducing apoptosis after direct infection (Bok et al., 2009; Furman et al., 2009; McFadden et al., 2011; Wobus et al., 2004). A cytopathic effect is also seen in bone marrow-derived macrophages and bone marrow-derived dendritic cells derived from naïve *Stat1*$^{-/-}$ mice upon MNV infection in vitro (Wobus et al., 2004). MNV can also cause a cytopathic effect in the immature B cell line WEHI-231 (Jones et al., 2014). However, we did not observe increased cytopathic effect in primary *Stat1*$^{-/-}$ B cells, despite viral replication in these cells. One possible explanation for the lack of cell death observed is that perhaps our MOI of 0.5 was too low to cause significant MNV-induced cell death above the baseline level of cell death that was occurring in uninfected cells. By comparison, M12 and WEHI-231 cells were infected at an MOI of 5 (Jones et al., 2014). Alternatively, our data could suggest that after MNV-4 infection, an indirect mechanism is also required to cause decreased bone marrow B cell populations in *Stat1*$^{-/-}$ mice rather than simply direct infection and cell death since: 1) there was no detectable increase in caspase 3/7 activation suggestive of apoptosis in B cells isolated from the bone marrow of MNV-4 infected *Stat1*$^{-/-}$ mice, 2) there was no difference in the amount of cell death between uninfected and MNV-infected bone marrow and splenic B cells purified from naïve *Stat1*$^{-/-}$ mice and infected in vitro, and 3) bone marrow transplant experiments revealed that there was no depletion of CD45.2^{+} *Stat1*$^{-/-}$ B cells after MNV-4 infection compared to uninfected controls in the presence of STAT1-sufficient stromal or WT bone marrow cells. Infection and replication without causing cell death has been reported for other viruses and may serve as a survival mechanism to propagate without killing the host (Hay and Kannourakis, 2002; Kaminskyy and Zhivotovsky, 2010). Collectively, our data suggest that a combination of direct infection and an indirect mechanism is causing the loss of developing B cells in the bone marrow of *Stat1*$^{-/-}$ mice after MNV infection. In particular, our bone marrow chimera experiments suggest that B cell loss is dependent on having STAT1-deficiency in hematopoietic cells and in bone marrow stromal cells, and that a cellular component of STAT1-sufficient cells in WT bone marrow after reconstitution can protect against MNV induced B cell loss. Multiple cell types have been reported to be involved in the immune response against MNV including macrophages, dendritic cells, B cells, CD4^{+} and CD8^{+} T cells (Baldridge et al., 2016; Chachu et al., 2008a, 2008b; Nice et al., 2016). Therefore, further investigation will be required to determine which component(s) of the WT bone marrow help prevent the depletion of B cells in *Stat1*$^{-/-}$ mice.

Interleukin 7 (IL-7) is an essential cytokine required for B cell development and is produced by stromal cells in the bone marrow (Nagasawa, 2006). We observed that IL-7 gene expression was increased in WT BMDM but not in *Stat1*$^{-/-}$ BMDM upon MNV-4 infection. Therefore, we postulated that MNV-4 infection 'normally' induces IL-7 production that is necessary to maintain homeostatic levels of B cell progenitors in the bone marrow in WT mice, but that this increase does not occur to sufficient levels in infected *Stat1*$^{-/-}$ mice. In *Stat1*$^{-/-}$ mice, whereas MNV-4 infection decreased B cell subsets as expected in PBS treated mice, daily treatment with IL-7 partially rescued *Stat1*$^{-/-}$ B cell development in infected mice by causing expansion of B cells to similar levels as uninfected PBS treated mice. However, even with this expansion, MNV-4 infected *Stat1*$^{-/-}$ mice treatment with IL-7 still showed a significant decrease in B cell subsets when compared to uninfected IL-7 treated mice. These results suggest that reduced IL-7 may be involved, at least in part, in the mechanism by which MNV decreases bone marrow B cells in *Stat1*$^{-/-}$ mice.

Type I interferons such as interferon α and β have been shown to suppress B cell development by causing apoptosis independent of STAT1 (Gongora et al., 2000; Lin et al., 1998; Wang et al., 1995). However, it is unlikely that MNV is causing decreased B cells in the bone marrow by this mechanism since MNV infection in *Stat1*$^{-/-}$ mice does not induce a robust IFN-β response (Mumphrey et al., 2007) and we did not observe any changes in bone marrow B cell populations in MNV-4 infected *IfnαβγR*$^{-/-}$ mice. Although wild-type mice have been reported to produce type I interferons after MNV infection (Mumphrey et al., 2007; Wobus et al., 2004), in our studies, we did not observe any changes in bone marrow B cells after MNV infection in wild-type mice. Finally, we did not detect any caspase activation to suggest that apoptosis was occurring in bone marrow B cells of MNV infected *Stat1*$^{-/-}$ mice.

An increase in neutrophilic inflammation in the spleen and livers of *Stat1*$^{-/-}$ mice after MNV infection has been previously reported (Shortland et al., 2014). We also observed that in MNV-infected *Stat1*$^{-/-}$ mice, the number of granulocytes significantly increased in the bone marrow as the B cell populations decreased. Gr1^{+}CD11b^{+} granulocytes increased from approximately 38–60% of the total bone marrow population, while B220^{+} B cells decreased from 42% to 16% after MNV-4 infection. During inflammation, there is a reciprocal relationship between granulopoiesis and B lymphopoiesis as both occupy a common developmental niche within the bone marrow (Ueda et al., 2005, 2004). The proinflammatory cytokine TNFα reduces the expression of the chemokine CXCL12, a crucial factor produced by stromal cells for the homing of hematopoietic progenitor cells and for B cell development in the bone marrow (Cain et al., 2009; Nagasawa, 2007). This TNFα/CXCL12 mediated response results in B cell depletion in the bone marrow secondary to B cell mobilization to the periphery, and is potentiated by IL-1β which stimulates granulocyte expansion in the bone marrow (Cain et al., 2009; Ueda et al., 2004). Alternatively, it has been reported that administration of granulocyte colony-stimulating factor (G-CSF), a factor that promotes neutrophil proliferation, can suppress B lymphopoiesis in the bone marrow in mice by increasing B cell apoptosis mediated by decreased gene expression of CXCL12 and IL-7, independent of TNFα (Day et al., 2015; Winkler et al., 2013). Administration of G-CSF has also been shown to deplete specific populations of bone marrow macrophages essential for maintaining the hematopoietic stem cell niches (Winkler et al., 2010), including those that give rise to developing B cells. Further investigation will be required to determine whether MNV, which has a tropism to infect macrophages, can similarly disrupt *Stat1*$^{-/-}$ bone marrow stromal macrophages essential for maintaining hematopoietic stem cell niches, or whether the B cell phenotype reported in our studies is a consequence of the increased cytokines and growth factors of bone marrow granulopoiesis.

Chemotherapeutics used for the treatment of cancer such as doxorubicin can cause bone marrow suppression. In wild-type and *Stat1*$^{-/-}$ mice administered doxorubicin to induce bone marrow toxicity, repopulation of the bone marrow B lymphocytes was delayed in mice lacking STAT1 compared to those with functional STAT1 (Datta et al., 2014). Similarly, in our studies, STAT1 deficiency contributed to the diminished recovery of B cells in the bone marrow after MNV infection compared to WT mice. Our bone marrow chimera experiments further support the notion that cells lacking STAT1 are at a proliferative disadvantage compared to their WT counterparts with functional STAT1

since reconstitution of STAT1-deficient cells was lower compared to WT cells even when equally co-administered to either *Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ or *Stat1*$^{-/-}$ uninfected recipient mice. In people, complete or partial STAT1 deficiency has been described to result in an increased susceptibility to various infectious agents (Averbuch et al., 2011; Boisson-Dupuis et al., 2012; Dupuis et al., 2003). Therefore, it would be interesting to determine whether norovirus infection can cause alterations in B cell populations in the bone marrow of infected humans, especially in those who have altered STAT1 expression as a result of genetic polymorphisms. Additionally, because many viruses have evolved to inhibit STAT1 signaling (Andrejeva et al., 2002; Fleming, 2016; Lin et al., 2005; Liu et al., 2005), our results suggest that co-infection with noroviruses could result in significantly impaired B cell development and/or humoral immunity.

In the studies reported herein, we corroborate the findings of others that MNV can infect and replicate in B cells. We show for the first time that MNV decreases bone marrow B cells after infection in mice lacking STAT1 and that this decrease can be prevented by STAT1. We provide evidence that although MNV does directly infect and propagate in primary B cells, the decrease in B lymphopoiesis appears to require both direct infection and an indirect mechanism. Our studies reveal that perturbations of IL-7 expression in the bone marrow may play a role in the B cell losses seen, and that loss of STAT1 in both bone marrow cells and bone marrow stromal cells together may be essential components contributing to the B cell phenotype observed after MNV infection. We also show that WT bone marrow or stromal cells are able to prevent the B cell losses seen in *Stat1*$^{-/-}$ mice after MNV infection. It remains to be determined which cells from the bone marrow are responsible for the protection of *Stat1*$^{-/-}$ bone marrow B cells, as well as whether the granulocytic response is involved in the loss of *Stat1*$^{-/-}$ bone marrow B cells, in *Stat1*$^{-/-}$ mice infected with MNV.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2017.12.013.

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