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Original Research

Effects of Murine Norovirus on Atherosclerosis in *Ldlr*$^{-/-}$ Mice Depends on the Timing of Infection

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We previously reported that murine norovirus (MNV), a virus prevalent in United States research institutions, increased atherosclerotic lesion size in *Ldlr*$^{-/-}$ mice when the mice were infected 8 wk after feeding an atherogenic diet. To determine whether the timing of MNV infection relative to atherosclerosis development altered the disease phenotype and to examine potential mechanisms by which MNV influences the disease process, we fed *Ldlr*$^{-/-}$ mice an atherogenic diet for 16 wk. Three days after initiating the atherogenic diet, half of the mice received MNV4 and the other half vehicle only (clarified cell-culture lysate; controls). Both groups of mice developed large aortic sinus lesions (control compared with MNV4: $133 \pm 8 \times 10^3$ μm^2 compared with $140 \pm 7 \times 10^3$ μm^2) that were not significantly different in size. Because the timing of MNV infection relative to atherosclerosis development and hypercholesterolemia differed between our previous and the current studies, we examined whether hypercholesterolemia altered MNV4-induced changes in bone-marrow–derived macrophages. MNV4 infection increased the potential of macrophages to take up and store cholesterol by increasing CD36 expression while suppressing the ABCA1 transporter. Thus, the effects of MNV4 infection on atherosclerotic lesion size appear to be dependent on the timing of the infection: MNV4 infection promotes only established lesions. This effect may be due to MNV4's ability to increase cholesterol uptake and decrease efflux by regulating CD36 and ABCA1 protein expression.

Abbreviations: ABCA1, ATP-binding cassette A1; BMDM, bone-marrow–derived macrophage; iNOS, inducible nitric oxide synthase; MNV, murine norovirus.

Chronic viral infection, such as occurs with HIV and hepatitis C virus, has been associated with an increased risk for atherosclerosis,[2,19,46,48] although the mechanisms by which this occurs are not clearly defined. Our laboratory has been studying the effects of murine norovirus (MNV), which chronically infects immunocompetent mice, on murine models of inflammatory diseases, including atherosclerosis. MNV is a single-stranded RNA intestinal virus that belongs to the family *Caliciviridae* and has shown tropism toward antigen-presenting cells such as dendritic cells and macrophages.[54] Whereas human norovirus is a major cause of nonbacterial acute gastroenteritis,[52] MNV does not cause clinical disease in immunocompetent mice.[55] However, the high prevalence of MNV in biomedical research facilities throughout the world,[42,55] combined with its tropism for antigen-presenting cells, has prompted concern regarding potential effects on disease phenotypes in murine models of human diseases. Therefore, we previously examined 2 diseases, obesity and atherosclerosis, where macrophages have critical roles.[41,42] We found that MNV infection did not influence glucose metabolism and weight gain,[41] but it significantly increased the size and macrophage content of aortic sinus lesions in *Ldlr*$^{-/-}$ mice fed an atherogenic diet.[42] These findings suggest that MNV might be a potential tool to determine how viral infection alters the risk of atherosclerosis.

Many factors influence the progression of atherosclerosis. Accordingly, we examined whether the timing of MNV infection relative to the stage of atherosclerosis progression influenced disease phenotype and evaluated potential mechanisms by which MNV could affect the disease process. To this end, we modeled the infection of macrophages by using in vitro cultures of bone-marrow–derived macrophages (BMDM).

Materials and Methods

Animals and diet. Male B6.129S7-*Ldlr*^{tm1Her}/J (*Ldlr*$^{-/-}$; age, 4 wk) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and acclimated for 1 wk in an SPF facility[42] with a 12:12-h light:dark cycle in a temperature-controlled room (20 to 23 °C). This mouse strain lacks the LDL receptor and thus is susceptible to developing atherosclerosis secondary to an elevated serum cholesterol level induced by the consumption of a high-fat diet. Mice were housed in groups of 5 in autoclaved ventilated microisolator cages, provided autoclaved and acidified water, and given an irradiated regular rodent chow (catalog no. 5053, PicoLab Rodent Diet 20, LabDiet, St Louis, MO) ad libitum during the 1-wk acclimation period. Mice were tested and determined to be free of MNV by the supplier. Our group has established standard operating protocols for our mouse housing facility to

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restrict MNV infection to intended groups only.[41] All experimental protocols were reviewed and approved by the IACUC at the University of Washington.

After the acclimation period, all mice were fed an atherogenic (high fat, high cholesterol) diet (catalog no. TD88137, Harlan Laboratories, Dublin, VA) ad libitum for 16 wk to induce atherosclerosis. At 3 d after diet initiation, mice were infected with MNV4 (1×10^6 pfu, unknown passage) or vehicle (clarified RAW264.7 cell lysate) by oral gavage, as previously reported.[42] Infection status was determined by fecal RT-PCR analysis 2 wk after MNV4 infection and at the end of the study.[24] All MNV4-infected mice shed virus in the feces 2 wk after infection and remained positive for virus at the end of the study period (16 wk), whereas all mice treated with vehicle remained MNV-free throughout the study.

Atherosclerotic lesion analyses. At the termination of the study, mice were euthanized by CO_2 asphyxiation, followed by cardiocentesis to obtain blood. After perfusion with PBS, hearts and aortas were dissected and fixed in formalin for morphologic analyses. Aortic sinus sections were stained with Movat pentachrome stain, and lesion area was determined as previously described.[42] The extent of macrophage infiltration in the lesion area was determined after Mac2 staining.[42]

Analysis of the Ly6C^{hi} monocyte population in spleen. Single-cell suspensions were generated from the spleen at necropsy,[8] and cellularity was determined by using a hemocytometer. Cell subsets were analyzed as previously described.[50] Cells were blocked with antiCD16/CD32 (BD Biosciences, San Jose, CA) and then stained with antigen-specific antibodies for linage markers (Lin: NK1.1, CD90, CD45R, Ly6G), CD11b, CD11c, F4/80, MHC class II, and Ly6C (all obtained from BD Biosciences). Data were collected by flow cytometry (model LSRII, BD Biosciences) and analyzed by using FlowJo software (Tree Star, Ashland, OR). Single cells were gated by using forward-scatter parameters A and W, and leukocytes were gated for size via forward-scatter A and side-scatter A parameters. Cells were identified on the basis of surface staining as neutrophils (Lin$^+$CD11b$^+$F480$^-$class II$^-$), dendritic cells (CD-11c^{hi}), macrophages (Lin$^-$CD11c$^-$class II$^+$ and CD11b$^+$ or F4/80$^+$ or both), or monocytes (Lin$^-$CD11b$^+$class II$^-$F4/80$^-$). Monocytes were further characterized according to Ly6C staining.

BMDM. Bone marrow cells were isolated from *Ldlr*$^{-/-}$ mice (age, 10 to 12 wk) and differentiated to macrophages (7 to 10 d of culture) by using a previously reported method.[32] Differentiated macrophages were plated at 1 to 1.4×10^6 cells per well in 6-well plates and infected for 24 h with MNV4 (multiplicity of infection, 0.2) with various amounts of oxLDL. For qRT–PCR analysis, RNA was extracted by using the RNeasy kit (Qiagen, Venlo, Limburg, The Netherlands), and for western blot analysis, protein was extracted with the MPER protein extraction kit (Thermo Scientific, Waltham, MA). For flow cytometric analysis, BMDM were dissociated by cold shock in PBS (Ca^{++}- and Mg^{++}-free).

qRT-PCR. Total RNA (0.5 to 1 µg) was converted to cDNA by using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen) and used for qRT-PCR with specific primers for inducible nitric oxide synthase (iNOS),[35] IFNβ,[53] IL10,[1] CD36,[43] ATP-binding cassette A1 (ABCA1),[43] and IL6 (forward, 5′ AGA GTT GTG CAA TGG CAA TTC TGA 3′; reverse, 5′ TGG TAC TCC AGA AGA CCA GAG GAA 3′). The housekeeping gene HPRT[14] was used to normalize the expression levels, and data are presented relative to a single replicate of the control (untreated) sample.

Western blot analysis. Protein concentrations were determined by using BCA protein assay reagent (Thermo Scientific). Samples (15 to 20 µg total protein) were separated by denaturing gel electrophoresis (4% to 15% gradient gel; BioRad, Hercules, CA) and transferred onto polyvinylidene difluoride membranes (BioRad) with 1× CAPS buffer (10 mM CAPS in 10% methanol, pH 11). The membrane was incubated with ABCA1 antibody (polyclonal; Novus Biologic, Littleton, CO) in 5% bovine serum albumin in TBST (1× Tris-buffered saline containing 0.1% Tween 20) overnight, washed with TBST, and incubated with antirabbit–horseradish peroxidase secondary antibody (Thermo Scientific). Signal was detected by using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific). For a loading control, the housekeeping protein GAPDH was detected (antiGAPDH, catalog no. G9545, Sigma, St Louis, MO) on the same blot.

Flow cytometric analysis. The expression of CD36 in BMDM was detected by using flow cytometry. Cells were stained with antiCD36 (dilution, 1:50; clone number 72-1, allophycocyanin conjugate, eBiosciences, San Diego, CA) and analyzed by using flow cytometry (FACSCanto II, BD Biosciences) and FlowJo software (version 10). Specificity of the antiCD36 antibody was confirmed by using an isotype-matched control antibody (rat IgG2a K isotype, eBiosciences).

Statistics. All data was analyzed by using Prism statistical software (GraphPad, La Jolla, CA). A 2-tailed *t* test was performed for comparison between the 2 treatment groups, and statistical significance was defined as a P value of less than 0.05.

Results

Effects of MNV4 infection during early-stage atherosclerosis on Ly6C^{hi} monocytes and lesion size in *Ldlr*$^{-/-}$ mice. We previously determined that hyperlipidemic *Ldlr*$^{-/-}$ mice infected with MNV4 during lesion development (8 wk after initiation of an atherogenic diet) had significantly larger lesions in the aortic sinus than did uninfected controls.[42] To determine whether MNV4 infection alters atherosclerosis development and progression when mice are infected before they develop lesions due to sustained hypercholesterolemia, we infected *Ldlr*$^{-/-}$ mice with MNV4 3 d after the atherogenic diet was initiated.

Development of atherosclerotic lesions is associated with increased numbers of circulating Ly6C^{hi} monocytes,[50] therefore, we characterized splenic monocytes and other cell populations and analyzed aortic sinus lesions at the end of the study period. The splenic cellularity (Figure 1 A) and absolute cell numbers of Ly-6C^{hi} monocytes (Figure 1 B), MHC class II-positive macrophages (Figure 1 C), dendritic cells (Figure 1 D), and neutrophils (Figure 1 E) were significantly ($P < 0.05$) increased at the study endpoint. However, the cell percentages of these populations (Figure 1 F through I) did not differ between treatment groups, indicating that MNV infection was associated with a general increase in cell numbers and not with a specific increase in any particular cell type in the context of atherosclerosis development. In agreement with the lack of specific difference in monocyte populations in response to MNV4 infection, the size of atherosclerotic lesions in the aortic sinus at the end of the study did not differ between MNV4-infected mice compared with uninfected controls (Figure 2 A); the percentage of the lesion area showing macrophage infiltration was also similar between the 2 groups (Figure 2 B). This result is in contrast to our previous report,[42] where MNV4 infection significantly increased lesion size. However, in the previous study,

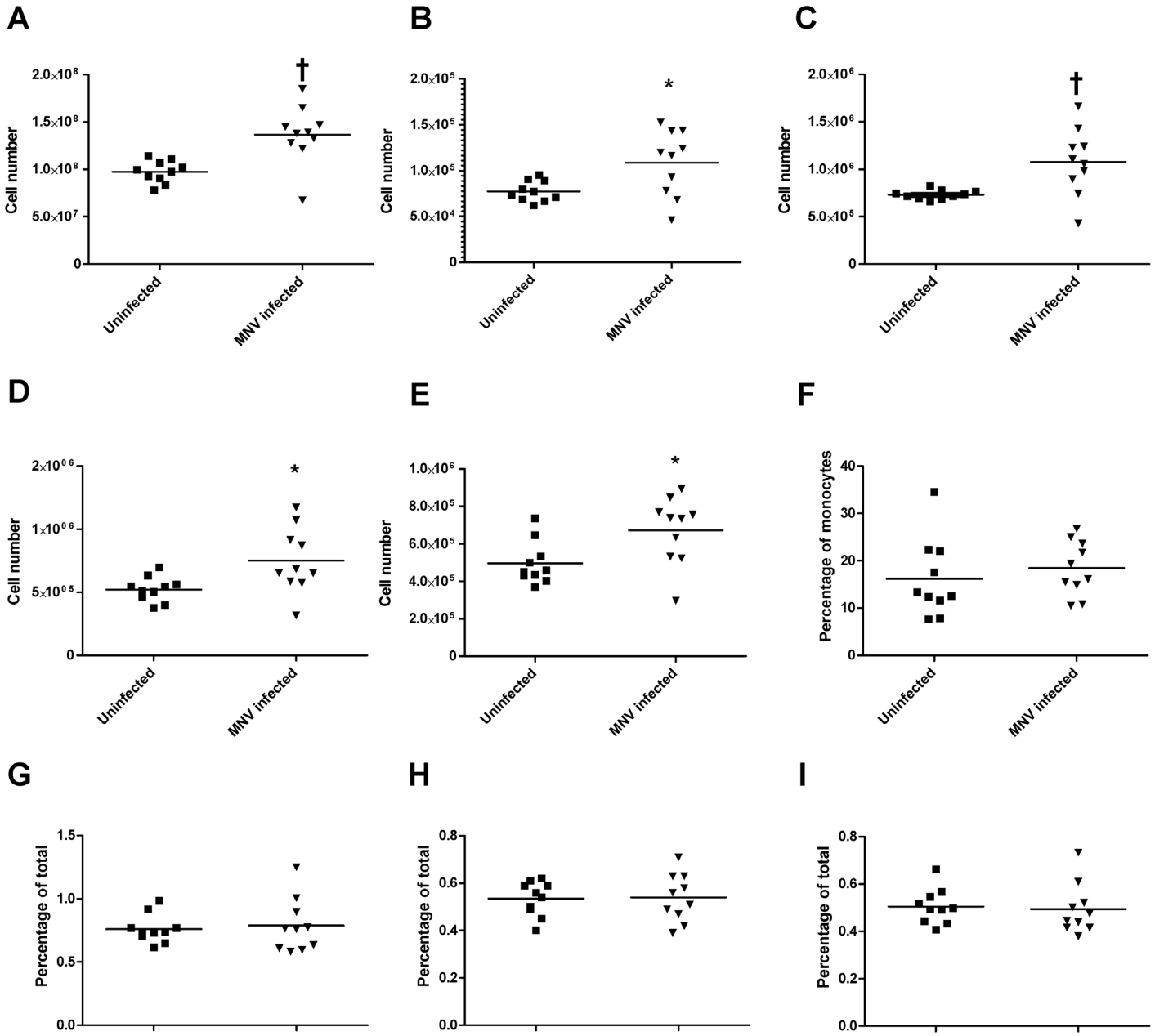

Figure 1. MNV infection does not alter the frequency of Ly6C^{hi} monocytes. Spleens of *Ldlr*$^{-/-}$ mice were processed at the end of the study to determine whether MNV influences composition of immune cell subtypes by staining for antigen-specific antibodies for linage markers (Lin: NK1.1, CD90, CD45R, Ly6G), CD11b, CD11c, F4/80, MHC class II, and Ly6C. Total cell numbers of (A) the spleen, (B) Ly6C^{hi} monocytes (Lin^{-}CD11b^{+}F4/80^{-}class II^{-} CD11c^{-}Ly6C^{hi}), (C) macrophages (Lin^{-}CD11c^{-}class II^{+} and CD11b^{+} or F4/80^{+} or both), (D) dendritic cells (CD11c^{hi}), and (E) neutrophils (Lin^{+}CD11b^{+}class II^{-}F4/80^{-}) are shown. (F) The percentage of Ly6C^{hi} monocytes (of total monocytes) as well as percentages of total splenocytes of (G) macrophages, (H) dendritic cells, and (I) neutrophils are shown. Bars represent means; values significantly (*, $P < 0.05$; †, $P < 0.01$) different from those of uninfected animals are indicated.

MNV4 infection occurred 8 wk after initiation of the atherogenic diet and thus, the mice would have sustained hyperlipidemia and developed lesions[42] by the time of infection. Another infectious agent, *Chlamydia pneumoniae*, also alters lesion size only in hyperlipidemic mice and not in mice prior to the induction of hyperlipidemia.[6] To explain potential differences between our previous study and the results we present here, we next examined the effect of hyperlipidemia on the responses of macrophages to MNV4 infection in vitro by using BMDM.

Effect of MNV4 infection on cytokine expression in BMDM. Modified LDL, such as oxLDL, are a risk factor for atherosclerosis because they are more readily taken up by macrophages than are native LDL.[49] We therefore treated BMDM with various amounts of oxLDL with or without MNV4 infection and assessed the expression of inflammation-associated genes. MNV4 infection increased expression levels of the inflammatory cytokines IL6 and IFNβ and of a classic macrophage activation marker, iNOS, in all treatment groups (comparison between no infection and MNV

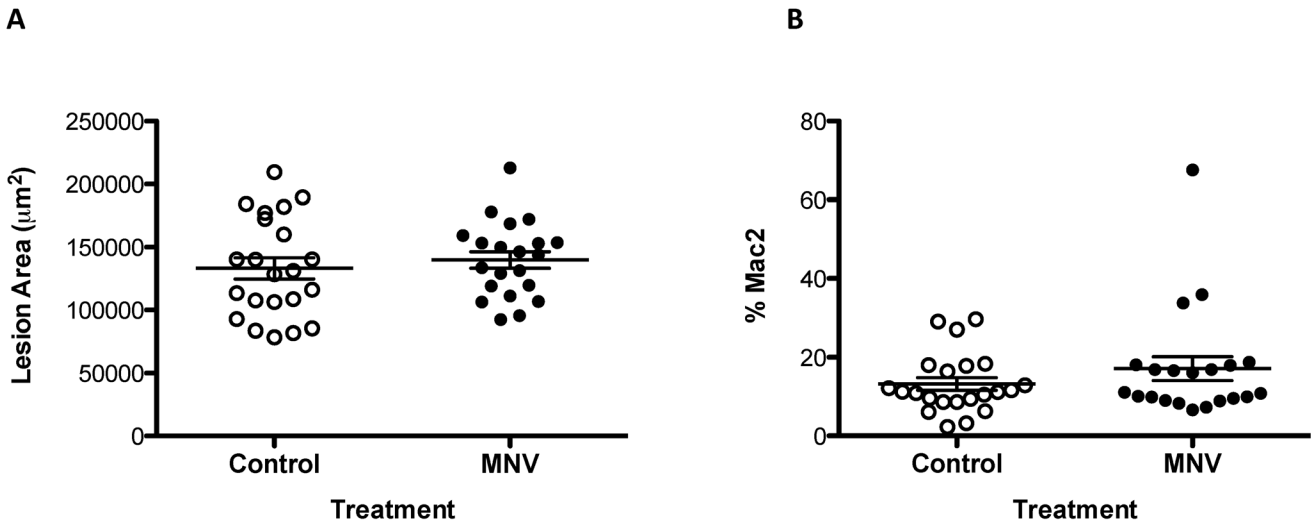

Figure 2. MNV infection at early stages of atherosclerosis development does not alter lesion size. (A) The size of the aortic sinus lesion was determined from Movat-pentachrome–stained serial sections of hearts of *Ldlr*$^{-/-}$ mice fed an atherogenic diet and treated with either MNV or vehicle only (clarified RAW264.7 cell lysate). (B) Macrophage infiltration was assessed by using antiMac2 antibody and is expressed as percentage of lesion area. Bars represent means ± SEM.

infection at different levels of oxLDL, Figure 3). In addition, the expression of the antiinflammatory cytokine IL10 was increased with MNV4 infection except at high levels of oxLDL (25 μg/mL; Figure 3).

Effects of MNV4 infection on CD36 and ABCA1 expression. Cellular cholesterol levels are regulated by both uptake and efflux mechanisms, and an increase in uptake or decrease in efflux in macrophages could contribute to the development of atherosclerosis. We therefore determined whether MNV4 directly influences these processes by evaluating the expression of the scavenger receptor CD36 and efflux protein ABCA1. CD36 preferentially binds oxLDL and facilitates its uptake in macrophages,[59] and its expression (RNA and protein) is induced as monocytes differentiate into macrophages in culture.[25] We first determined whether MNV4 infection and oxLDL influence CD36 expression in BMDM by using qRT–PCR analysis and flow cytometry (Figure 4). In agreement with previous reports,[47] oxLDL treatment increased CD36 RNA expression; specifically, CD36 RNA expression was significantly different among uninfected samples with varying amounts of oxLDL ($P < 0.0001$, one-way ANOVA). In addition, all samples treated with oxLDL differed from the control sample according to post hoc testing using Bonferroni correction for multiple comparisons ($P < 0.01$ for control compared with 5 μg/mL oxLDL; $P < 0.001$ for control compared with 10 or 25 μg/mL oxLDL). However, MNV4 infection did not further influence CD36 mRNA expression levels (no infection compared with MNV4 infection at all levels of oxLDL treatment). In contrast, cell-surface CD36 levels were significantly increased in MNV4-infected BMDM regardless of oxLDL treatment ($P <0.05$ for untreated groups ; $P \leq 0.0001$ for oxLDL-treated groups, Figure 4 B), suggesting that MNV4 infection potentially increases oxLDL uptake in macrophages.

Increased cellular cholesterol levels trigger cholesterol efflux through the ABCA1 pathway.[31] We therefore examined both the RNA and protein levels of *Abca1* in response to oxLDL and MNV4 infection in BMDM. RNA expression levels of *Abca1* did not change in response to either oxLDL treatment or MNV4 infection (Figure 5 A). However, ABCA1 protein levels were increased with oxLDL treatment (control compared with oxLDL, $P < 0.01$ for post hoc test after one-way ANOVA) and, notably, reduced with MNV4 infection in the presence of oxLDL (MNV4 + oxLDL compared with oxLDL, $P < 0.01$ for post hoc test after one-way ANOVA, Figure 5 B) indicating that MNV4 infection may decrease ABCA1-medated cholesterol efflux.

Discussion

Since the first report of MNV4 in a research mouse colony,[28] its high prevalence has been reported in mouse colonies in the United States, Canada, and Europe.[21,23,29,38,44,57] In addition, many investigators have reported diverse strains of the virus and its varied influence on animal models.[4,9,12,22,28,32,33,41,42] We have studied the effects of MNV4, the strain first reported by Hsu and colleagues,[23] on various murine models of human disease.[32,33,41,42] MNV4 infection persists for an extended period of time, and infected mice shed the virus in feces for as long as 16 wk after infection.[41,42] However, the effects of MNV4 on disease phenotype vary depending on the disease and model. For example, MNV4 infection increased the size of atherosclerotic lesions but did not alter obesity or diabetes phenotypes in *Ldlr*$^{-/-}$ mice.[41,42] MNV4 accelerated the development of inflammatory bowel disease in *Mdr1a*$^{-/-}$ mice[33] but did not alter colitis-associated cancer in *Smad3*$^{-/-}$ mice.[32] Therefore, it is difficult to predict whether MNV4 would be an intercurrent variable for studies using murine models.

Because of the tropism of MNV for macrophages, our laboratory examined the potential effects of MNV infection on atherosclerosis models.[54] We previously reported that MNV4 infection during atherosclerosis development increases aortic sinus lesion area in *Ldlr*$^{-/-}$ mice.[42] However, in research settings, MNV infection in mice can occur at different times. If breeding colonies are infected or newly purchased young mice are moved into a facility that does not exclude MNV, *Ldlr*$^{-/-}$ mice might be infected before significant atherosclerosis develops. Therefore, in these current studies, we examined whether MNV4 infection at an early stage of disease, that is, before hyperlipidemia is induced, alters disease

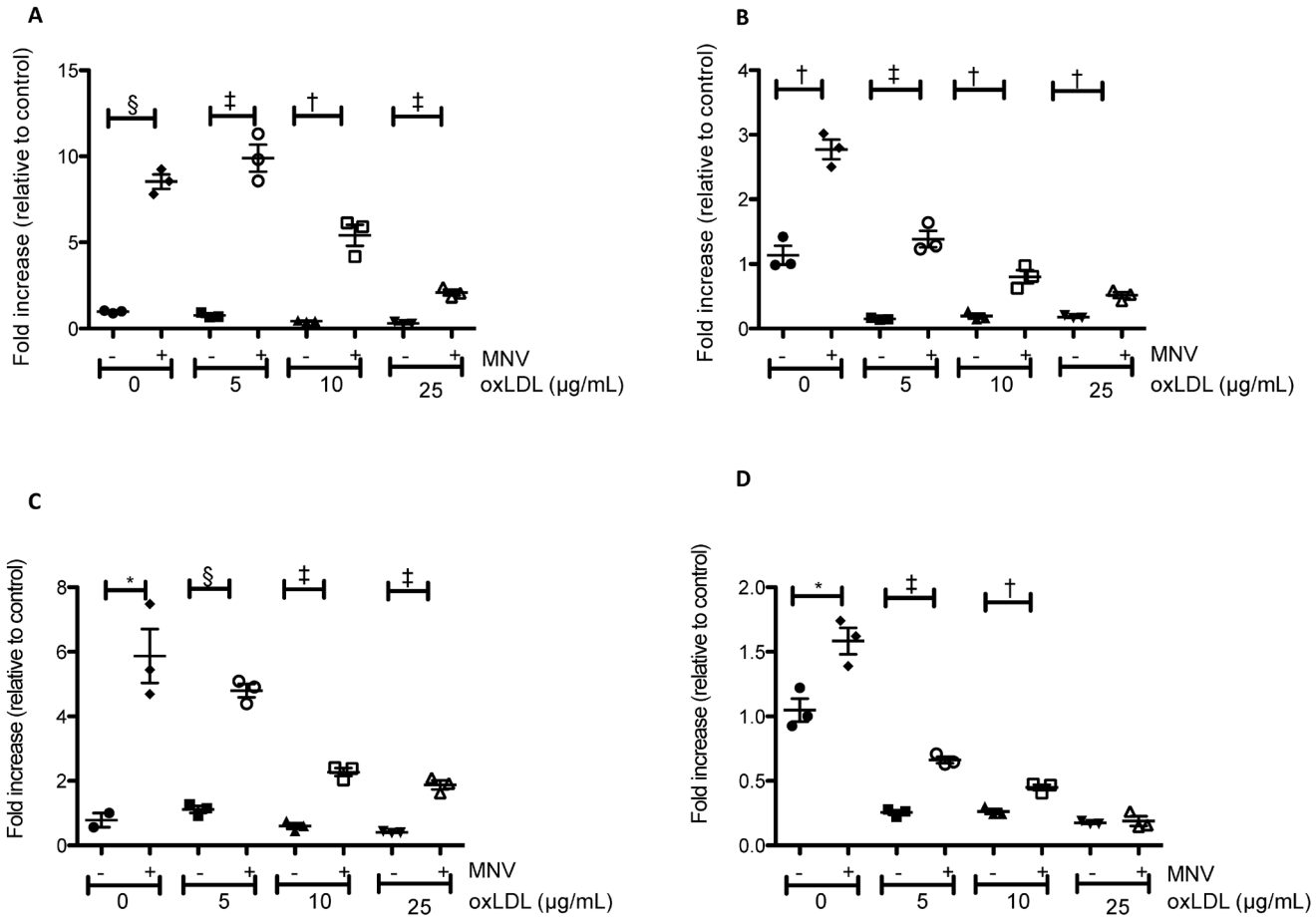

Figure 3. MNV infection alters cytokine mRNA expression in BMDM in the presence and absence of oxLDL. (A) iNOS, (B) IFNβ, (C) IL6, and (D) IL10 expression were determined by qRT–PCR analysis. Samples were normalized to HPRT and plotted relative to a single replicate of the control sample (no oxLDL and no MNV). Bars represent means ± SEM; expression levels were compared between uninfected and infected samples of the same concentration of oxLDL by using the Student *t* test, and values that differ significantly (*, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; §, $P < 0.0001$) are indicated.

outcome. We found that MNV infection during early atherosclerosis did not significantly alter lesion size or macrophage infiltration into the lesion area (Figure 2), suggesting that the timing of MNV4 infection may modulate its influence on atherosclerosis progression in this model.

Ldlr$^{-/-}$ mice were developed as a model to study familial hypercholesterolemia in humans due to defects in the LDLR pathway[26] and were instrumental in understanding lipid uptake and metabolism. However, unlike humans with LDLR defects, *Ldlr*$^{-/-}$ mice do not develop severe hypercholesterolemia unless fed an atherogenic, high-cholesterol containing diet.[26] This discrepancy is mainly due to the differential composition of apolipoprotein B associated with VLDL in humans compared with mice. In humans, VLDL is secreted with apoB100, a ligand for LDLR, whereas in mice, much VLDL is associated with apoB48, which is recognized by a remnant receptor[10,26] rather than by LDLR. Therefore, LDLR defects in humans have more severe consequences in endogenous lipoprotein clearance and circulating cholesterol levels than do mice with LDLR defects, because these mice can clear LDL through remnant receptors in addition to LDLR. Consequently, a diet containing high cholesterol is needed to induce severe hypercholesterolemia and atherosclerosis in *Ldlr*$^{-/-}$ mice.[27]

Because of this characteristic of *Ldlr*$^{-/-}$ mice and because another infectious agent, *Chlamydia pneumoniae*, was found to increase lesion size only when the animals were hyperlipidemic,[6] we postulated that the differential response to infection in our current and previous studies might be due to the timing of MNV4 infection in relation to disease stage or severity of hypercholesterolemia. To address this question, we examined whether MNV4 infection alters factors known to be associated with atherosclerosis in a manner dependent on oxLDL in vitro. MNV4 infection was associated with increased cytokine expression (IL6, IL1β, IFNβ) at all levels of macrophage exposure to oxLDL. Interestingly, oxLDL treatment decreased MNV4-induced cytokine expression in a dose-dependent manner, suggesting that MNV4's effect on macrophages might differ depending on the severity of hyperlipidemia (Figure 3). In addition, MNV4 infection increased the expression of a marker of classically activated macrophages, iNOS, and oxLDL exposure suppressed iNOS expression in a dose-dependent manner. We also noted that expression of the antiinflammatory cytokine IL10 paralleled changes in proinflammatory cytokines, suggesting that IL10 expression likely mirrors proinflammatory cytokine signals in an attempt to control inflammation. These data suggest that the effects of MNV4 on macrophages are modulated by the presence of oxLDL. Our observation

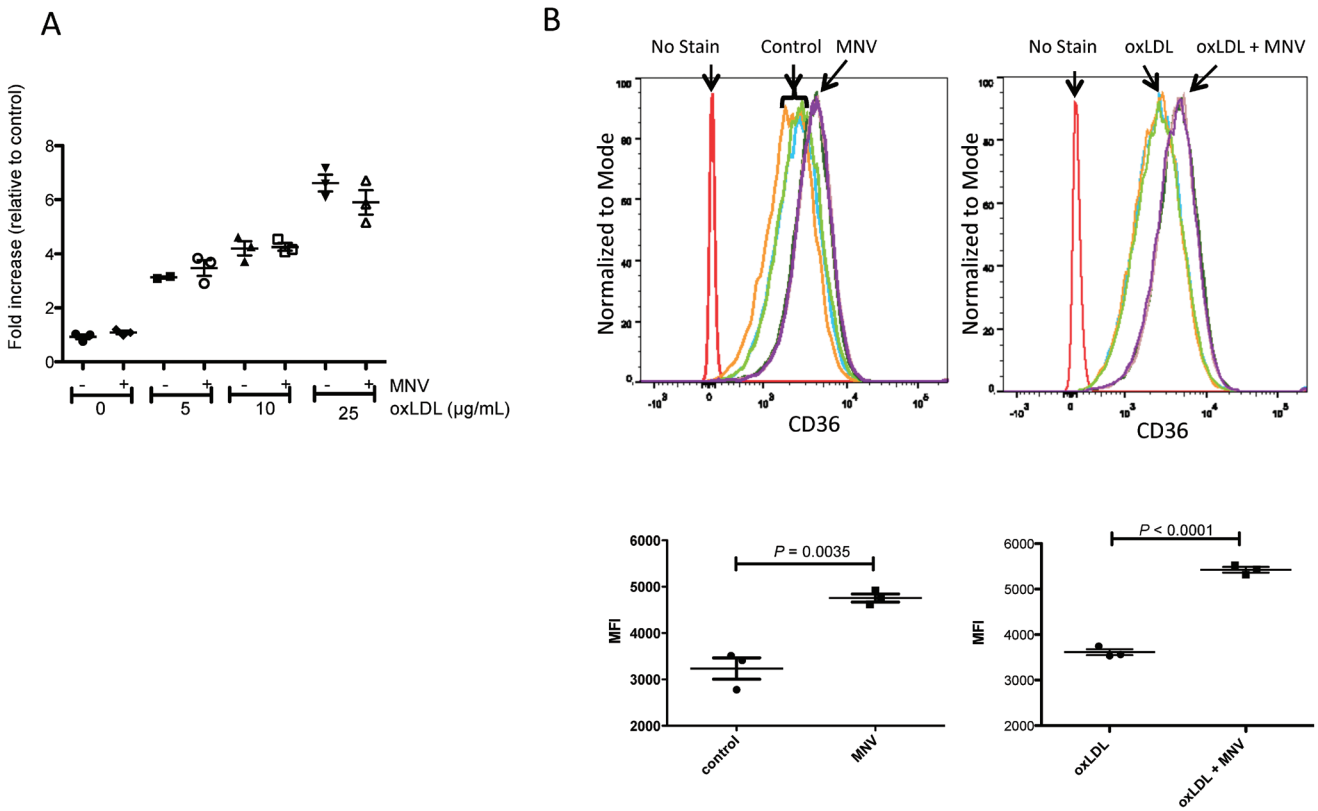

Figure 4. CD36 expression is influenced by oxLDL treatment and MNV infection. (A) CD36 mRNA expression in BMDM in response to different levels of oxLDL and the presence or absence of MNV was determined by qRT-PCR analysis. Expression levels were normalized to HPRT and expressed relative to a single replicate of the control sample (no oxLDL, no MNV) (B) Cell-surface expression of CD36 protein was determined in response to MNV in the presence or absence of oxLDL from triplicate samples per treatment by using flow cytometry. The fluorescent intensity (bar, mean ± SEM) of triplicate samples is shown.

agrees with previous reports that oxLDL inhibits inflammatory cytokine (IL1β, IL6, and TNFα) and iNOS expression induced by lipopolysaccharide or IFNγ.[17,20,40] How the oxLDL-associated suppression of immune responses in vitro relates to an increased risk of developing atherosclerosis in vivo is unclear. However, a decrease in normal inflammatory responses may allow macrophages to develop a chronic, smoldering inflammation, thus favoring progression to atherosclerosis.[20] Therefore, increased oxLDL at the time of MNV4 infection may promote a chronic inflammatory state, resulting in the progression of lesions rather than in acute inflammation that can be controlled quickly.

Another potential reason for why MNV4's influence on atherosclerosis differs between our current study and previous report is that the immune response to MNV4 infection may vary due to the age of the mice at the time of MNV 4 infection. In our previous experiment, we infected *Ldlr*$^{-/-}$ mice at 14 wk of age whereas in the current study, they were infected at approximately 6 wk of age. Because rodents' immune system is considered mature by 1 mo of age and because they can effectively establish immunologic memory in the first 6 mo of age,[30] we believe that the quality of the immune response to MNV (after what is a first-time exposure to MNV in our colony) is unlikely to differ according to the timing of infection during this time frame. However, this difference in infection timing introduced another variable, obesity. In our previous study, mice were fed a high-fat diet for 8 wk before the MNV4 infection and, thus, mice were obese prior to infection; in contrast, in the current study, infection occurred before significant obesity developed. Because obesity can influence macrophage response to infectious agents,[3,58] obesity in the context of the atherosclerosis-promoting high-cholesterol diet may influence the influx of macrophages into lesion areas. Finally, another possible explanation for the differences between our current and previous studies could be related to differences in the biologic potential between the 2 viral isolates. RNA viruses such as MNV have a high mutation rate owing to the lack of a 3′-to-5′ exonuclease proofreading activity in their RNA-dependent RNA polymerase,[13,56] allowing mutations to occur during viral propagation. Although the same viral stock was used to propagate MNV in Raw cells, viral propagation occurred at different times for the previous study compared with the study reported here, potentially resulting in the use of slightly different viral isolates between the 2 studies. Minor changes (even single amino-acid substitutions) in the MNV sequence can influence its biologic effects.[5,7,39,51]

The uptake and efflux of cholesterol regulates intracellular cholesterol content, which is a key factor in macrophage foam-cell formation.[34] We therefore examined whether MNV4 infection alters one or both of these processes by analyzing the RNA and protein levels of CD36 (oxLDL uptake) and ABCA1 (cholesterol efflux) in *Ldlr*$^{-/-}$ BMDM. CD36 is a large glycoprotein that belongs to a scavenger receptor class B family and binds various ligands, including oxLDL.[11] Studies to elucidate the role of CD36 in atherosclerosis in *Apoe*$^{-/-}$ *Cd36*$^{-/-}$ mice compared with *Apoe*$^{-/-}$ mice reported conflict-

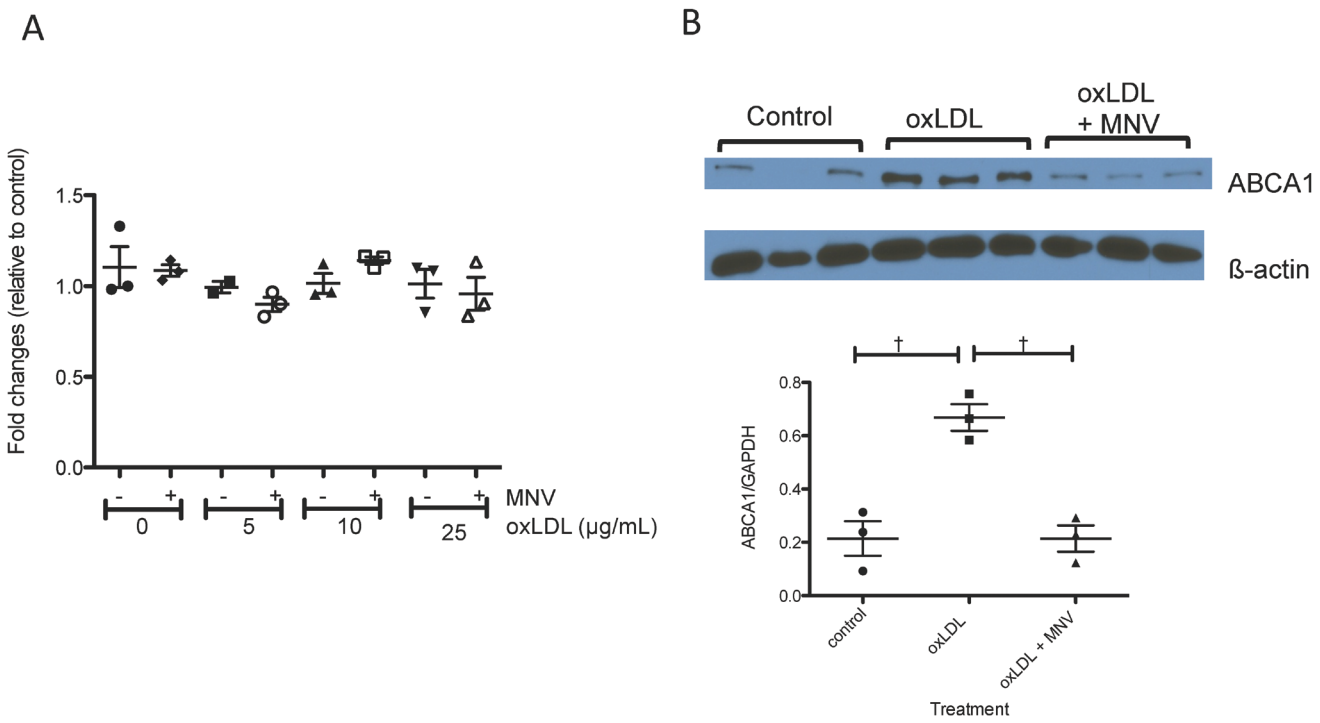

Figure 5. In the presence of oxLDL, MNV infection suppresses protein but not mRNA levels of ABCA1. (A) *Abca1* mRNA levels in BMDM were determined by qRT-PCR assay and normalized to HPRT. Normalized values are expressed relative to a single replicate of the control sample (no oxLDL, no MNV). (B) ABCA1 protein levels (bar, mean ± SEM) were determined by western blot analysis followed by densitometry and normalized to GAPDH. Values that differ significantly (†, $P < 0.01$) are indicated.

ing data: one study showed dramatic decreases in lesion size,[15] whereas the other showed a slight or moderate increase in lesion size depending on the analysis site.[36] However, in both studies, peritoneal macrophages showed impaired uptake of oxLDL and cholesterol in vitro[15] and in vivo,[36] demonstrating the involvement of CD36 in oxLDL uptake. In agreement with a previous study,[11] we found that *Ldlr*$^{-/-}$ BMDM expressed elevated levels of CD36 in response to oxLDL treatment (Figure 4). Although mRNA levels of CD36 did not change after MNV4 infection, CD36 protein expression was increased in MNV4-infected BMDM regardless of oxLDL treatment (Figure 4 B), suggesting that MNV4 infection increases the oxLDL uptake capacity of macrophages.

Intracellular cholesterol levels also are regulated by efflux pathways, which involve ATP-binding cassette proteins. We found that *Ldlr*$^{-/-}$ BMDM treated with oxLDL showed significantly elevated levels of ABCA1, whereas ABCA1 expression was suppressed to control levels in BMDM infected with MNV4 despite concomitant treatment with oxLDL (Figure 5B). These data suggest that MNV4 infection induces macrophages to take up more oxLDL and not release it; consequently existing hypercholesterolemia and MNV4 infection would result in increased cholesterol levels in macrophages. This scenario may explain why MNV4 infection prior to induction of severe hypercholesterolemia (current study) did not alter lesion size, whereas MNV4 infection in animals with severe hypercholesterolemia increases lesion size. Virus-induced alterations in cholesterol transport mechanisms have been reported. For example, HIV is associated with an increased risk of developing atherosclerosis in part because of inhibition of cholesterol efflux via ABCA1.[16,37] Monocytes and macrophages infected with HIV had a decreased levels of ABCA1 protein due to its increased degradation,[37] and this accelerated degradation of ABCA1 is mediated by an HIV accessory protein, Nef, which secures sufficient cholesterol to improve the infectivity of HIV.[37] Although MNV1 infection of macrophages are dependent on cholesterol,[18,45] whether increased intracellular cholesterol improves its infectivity is unknown. Additional studies need to determine whether MNV alters cholesterol homeostasis to benefit its infectivity or ability to replicate.

In summary, we show that MNV4 infection influences cellular cholesterol levels in macrophages by regulating both uptake (CD36) and efflux (ABCA1) pathways in BMDM. Whether this modulation translates to a detectable in vivo effect is likely dependent on many factors, including the stage of atherosclerosis and the potency of the viral isolate. Our findings suggest that careful consideration should be given when performing atherosclerosis studies in MNV-infected mouse colonies, given that predicting the influence of MNV on the disease phenotype is difficult in murine models.

Acknowledgments

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