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Involvement of microglial CD40 in murine retrovirus-induced peripheral neuropathy

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Abstract

B6 mice infected with LP-BM5 develop severe immunodeficiency (termed MAIDS) and peripheral neuropathy. To determine whether microglial CD40 is involved in LP-BM5-induced peripheral neuropathy, B6-CD40 knockout (KO) mice and B6-CD40 KO mice adoptively transferred either total leukocytes or B cells were examined for behavioral sensitivity, tissue viral loads, cytokine responses, and the development of MAIDS. All three CD40 KO groups developed MAIDS, the severity of which was correlated with peripheral cytokine responses. CD40 KO mice displayed significantly reduced mechanical hypersensitivity post-infection compared to wild-type mice regardless of cell transfer. These findings support microglial CD40 involvement in LP-BM5-induced peripheral neuropathy.

Keywords

Murine AIDS; LP-BM5; CD40; peripheral neuropathy; cytokine

1. Introduction

Human immunodeficiency virus (HIV)-associated peripheral neuropathy is the most common neurological complication associated with HIV infection, and distal symmetrical polyneuropathy (DSP) is the most common form of HIV-associated peripheral neuropathy. It is estimated that about one out of three HIV/acquired immunodeficiency syndrome (AIDS) patients experience DSP (Pettersen et al., 2006; So et al., 1988). Despite the extensive study of HIV infection, HIV-associated neuropathy is an understudied area, in part due to the lack of an appropriate animal model. Currently, there are no US FDA-approved pharmacological agents specifically designed for the treatment of HIV-associated neuropathy (Verma et al., 2005).

LP-BM5 is a retroviral mixture that contains mainly a pathogenic yet replication-defective virus (BM5def) and a non-pathogenic helper virus (ecotropic (mouse tropic) virus (BM5eco)) (Chattopadhyay et al., 1989). LP-BM5 infection in susceptible C57BL/6 (B6) mice induces a HIV-like immunodeficiency syndrome, termed murine acquired immunodeficiency syndrome (MAIDS). Following LP-BM5 infection, B6 mice experience

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splenomegaly, lymphadenopathy, polyclonal hypergammaglobulinemia, profoundly decreased T and B cell responses to mitogens, increased susceptibility to opportunistic pathogens, and the development of terminal B cell lymphomas (Jolicoeur, 1991). The mechanisms of viral pathogenesis in MAIDS have been extensively studied. Significantly, it has been found that both CD4⁺ T lymphocytes and B lymphocytes and the CD154 (CD40L)-CD40 interaction between these two cell populations are necessary for MAIDS induction and progression (Green et al., 1996; Green et al., 2001; Green et al., 1998; Simard et al., 1997). Specifically, the CD4⁺ T - B cell interaction during the early phase following infection is thought to be critical in the development of MAIDS, as blocking the CD40L-CD40 interaction beginning at 3–4 weeks post-infection (p.i.) lead to a significant reduction in MAIDS severity in about 60% of infected mice (Green et al., 1998). Recently, we demonstrated that B6 mice infected with LP-BM5 displayed both behavioral and pathological signs of peripheral neuropathy along with tissue-specific cytokine responses. Further, these changes could be reduced or reversed by the anti-retroviral agent zidovudine (AZT) (Cao et al., 2012).

In the current study, to determine whether spinal cord microglial CD40 signaling is involved in LP-BM5- induced peripheral neuropathy, B6-CD40 knockout (KO) mice were adoptively transferred either total leukocytes or B cells to establish LP-BM5-induced immune deficiency, and LP-BM5-induced sensory hypersensitivity, a behavioral sign of peripheral neuropathy, was then examined. Our data support a contributing role of microglial CD40 in the development of sensory hypersensitivity following LP-BM5 infection and suggest that the lumbar spinal cord viral load and the subsequent cytokine responses are involved in the microglial CD40-mediated peripheral neuropathy following LP-BM5 infection.

2. Materials and Methods

2.1. Mice

B6 mice and B6-CD40 KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and male adult mice (8–9 wks old) were used in all experiments. All mice were allowed to habituate to the institutional animal facility for at least one week before experimental use. All mice were group-housed (four per cage) with food and water *ad libitum* and maintained on a 12-h light/dark cycle. The Institutional Animal Care and Use Committee (IACUC) at UNE approved all experimental procedures used in this study.

2.2. LP-BM5 virus preparation and inoculation

LP-BM5 viral isolates were prepared as previously described (Green et al., 2001; Klinken et al., 1988). The titer of the viral stock was determined by a standard retroviral XC plaque assay for BM5eco virus (Rowe et al., 1970). Each mouse was infected with approximately 5×10^4 ecotropic plaque forming unit (PFU) via intraperitoneal (i.p.) injection. This dose of LP-BM5 has been shown to induce typical MAIDS (Li and Green, 2006) and signs of peripheral neuropathy (Cao et al., 2012).

2.3. Overall experimental procedure

A total of six experimental groups were used with 8 mice per group: 1) wild type (WT) mice without cell transfer or infection, 2) WT mice without cell transfer but with infection, 3) CD40 KO mice without cell transfer or infection, 4) CD40 KO mice without cell transfer but with infection, 5) CD40 KO mice with total leukocyte transfer and infection, and 6) CD40 KO mice with B cell transfer and infection. The cell transfers for the mice in groups (5) and (6) were performed weekly starting from one week before LP-BM5 infection (Day of infection = Day 0) to 5 weeks p.i.. Mice that did not receive weekly cell transfers were not manipulated in any way. To assess mechanical sensitivity, all groups of mice were baseline

tested before infection and weekly tested up to 12 weeks p.i.. During the weeks when both a cell transfer and behavioral testing were performed, the cell transfer was conducted the day before the behavioral testing. At 12 weeks p.i., all mice were euthanized via CO₂ inhalation. Blood from each mouse was collected via cardiac puncture and then sera were obtained following centrifugation and stored at -20°C until analysis. Following blood collection, the spleen, the hind paw skin, the lumbar spinal cord, and the lumbar (L4-L6) DRG from each animal were collected and stored at -80°C until further analysis of viral loads and cytokine responses was performed.

It should be noted that in this study, the adoptive transfer of leukocytes, and not WT → CD40 KO bone marrow chimeras, was used to generate mice that express CD40⁺ cells (primarily B cells) in the periphery (thus sufficient to induce MAIDS) but not in the CNS. In theory, in a WT → CD40 bone marrow chimera model, the recipient microglia, due to their lack of sensitivity to irradiation, would remain in the recipient mice after radiation and transplantation, whereas the transplanted bone marrow cells would repopulate the peripheral leukocyte population that was depleted by the irradiation. However, generation of new microglia from transplanted bone marrow cells (CD40⁺) is possible (Hickey et al., 1992), and it may become significant during our chronic study (up to 12 weeks p.i.). To avoid possible interference from newly generated CD40⁺ microglia from WT bone marrow cells, we chose an adoptive transfer regimen for this study. The leukocytes transferred in our studies are obtained from the spleen and lymph nodes and are mostly terminally developed cells that are much less likely to transform into CNS microglia.

It is also known that the early involvement of the CD4⁺ T - B cell CD154/CD40 interaction is critical in the development of MAIDS and that beyond 4 weeks p.i this interaction. is not essential in the progression of MAIDS (Green et al., 1998). Thus, we chose to transfer leukocytes weekly from week -1 to week 5 p.i. to ensure the development of MAIDS in adoptively transferred mice.

2.4. Leukocyte isolation and adoptive transfer

Splenocytes and lymph node (LN) (axillary and inguinal LNs) cells were aseptically harvested as previously described (Cao and DeLeo, 2008) from age-matched WT male B6 mice. All cells were pooled together. One portion of the cells were washed, resuspended in sterile phosphate buffered saline (PBS) at 50×10⁶ cells/ml, and kept on ice until being used as the “total leukocytes” in an adoptive transfer experiment. The rest of the cells were resuspended in sterile PBS containing 2% fetal bovine serum (FBS) (PAA, New Bedford, MA, USA) and 1mM EDTA at 1×10⁸ cells/ml for B cell isolation. B cell isolation was performed with the EasySep® magnet using the EasySep® negative selection mouse B cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada) following the manufacturer’s protocol. Collected B cells were resuspended in sterile PBS at 50×10⁶ cells/ml and kept on ice until being used as the “B cells” in an adoptive transfer experiment. 10×10⁶ of either total leukocytes or B cells in 200 µl PBS were intravenously injected into the tail vein (i.v.) of each recipient mouse. Cell transfers were performed weekly starting from one week before LP-BM5 infection to 5 weeks p.i..

After each cell isolation, the B cell content (identified as CD19⁺ cells) and their CD40⁺ expression were examined via flow cytometry as previously described (Cao and DeLeo, 2008) using APC-anti-mouse CD19 (Clone ID3) and Biotin-anti-mouse CD40 (clone 1C10)/Streptavidin-PE (1:50). All antibodies and the secondary reagent for flow cytometric analysis were obtained from eBiosciences (San Diego, CA). The B cell content (CD19⁺ cells) was 36.46% ± 2.23 and 88.79% ± 1.01 within the total leukocyte population and the B cell population, respectively. The content of CD40⁺ cells within CD19⁺ cells was 96.92% ±

0.55 and $97.89\% \pm 0.53$ in the total leukocyte population and the B cell population, respectively.

2.5. Behavioral sensitivity

Both mechanical and heat sensitivities of the hind paws of the mice were measured under nonrestrained conditions as previously described (Cao et al., 2012). Mechanical sensitivity was assessed via von Frey filaments (Stoelting, Wood Dale, IL) following the up-down paradigm (detailed in (Chaplan et al., 1994)). The 50% threshold force needed for paw withdrawal was calculated and used to represent mechanical sensitivity. Thermal sensitivity was determined via the Hargreaves test using the Ugo Basile thermal plantar analgesia instrument for mice (Ugo Basile Srl, Comerio VA, Italy). The paw withdrawal latency upon heat stimulation was recorded and used to represent animal's heat sensitivity. Due to the lack of differences between the left and the right hind paws (Cao et al., 2012), the average response from the left and the right hind paws was calculated and presented here. The person performing the behavioral tests was blinded to the experimental groups.

2.6. Measurement of MAIDS development

Spleen weight and serum immunoglobulin levels were measured to monitor the development of MAIDS as described previously (Li and Green, 2006). Serum IgM and IgG2a were measured via an established Cytokine enzyme linked immunosorbent assay (ELISA) protocol (Li and Green, 2006). The spleen of each animal was collected and weighed before storing at -80°C for further analysis.

2.7. RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

Hind paw skin, lumbar spinal cord, lumbar DRG, and about half of the spleen (the other half was used for cytokine ELISA (see below)) were subjected to total RNA extraction and subsequent qRT-PCR. Total RNA isolation and cDNA synthesis were performed as previously described (Cao et al., 2012). Tissue viral loads were determined by evaluating the expression of both BM5def (pathogenic component of LP-BM5) and BM5eco (helper virus of LP-BM5) *gag* RNAs via qRT-PCR (using β -actin expression as the control) as previously described (Cook et al., 2003; Giese et al., 1994). mRNA levels of selected cytokines (IL-1 β , IL-6, TNF α , IFN γ , and IL-12p40) in the lumbar spinal cord and DRG were determined via qRT-PCR as previously described (Cao et al., 2012).

2.8. Cytokine enzyme linked immunosorbent assay (ELISA)

Splenic tissue (about half of the spleen) was homogenized in a tissue lysis buffer and processed prior to ELISA as previously described (Cao et al., 2009). Serum and splenic levels of IL-1 β , IL-6, TNF α , IFN γ , IL-12/IL-23 p40, and IL-12 p70 were determined with DuoSet ELISA kits (R&D Systems, Minneapolis, MN) following the manufacturers' protocols. All cytokine levels in splenic tissue were normalized based on the protein concentration of the sample as determined by the BCA protein assay (Pierce-Thermo Scientific, Rockford, IL).

2.9. Statistical analyses

All statistical analyses were performed with SigmaStat 3.5 software (Systat Software, Inc.). Appropriate analyses of variance (ANOVA) (one-way, two way or two way repeated measure ANOVA) with group and/or time as factors were performed, followed by Student-Newman-Keuls (SNK) *post hoc* analysis. All data are presented as mean $\pm$ SEM, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Establishment of LP-BM50 induced immunodeficiency in CD40 KO mice after adoptive transfer

As described in the Materials and Methods, B6-CD40 KO mice were adoptively transferred either total leukocytes or B cells (for both, 10×10^6 /mouse via intravenous injection) from week -1 to week 5 p.i. (the day of infection = day 0). Mice were randomly assigned to non-infection and infection (5×10^4 ecotropic PFU/mouse via intraperitoneal injection) groups. All animals were sacrificed at week 12 p.i., and the development of immunodeficiency was then examined. As expected, LP-BM5 infection induced significant increases in spleen weight and serum levels of IgM and IgG2a in WT mice (Figure 1, one-way ANOVA, $p < 0.001$ for all). In CD40 KO mice, LP-BM5 induced significant increases in spleen weight only in the groups receiving cell transfer, and these increases were significantly less than that in WT mice (Figure 1A). While LP-BM5 induced significant elevations of both IgM and IgG2a in all infected CD40 KO mice, this elevation trended higher in the groups of CD40 KO mice receiving cell transfer compared to the “no cell transfer” CD40 KO group (particularly with IgG2a, Figure 1B and C). These data indicate that both of our cell transfer regimens were sufficient for LP-BM5 to induce immunodeficiency in CD40 KO mice.

3.2. Reduced sensory hypersensitivity in CD40 KO mice post-LP-BM5 infection

Mice within all treatment groups were tested weekly from before infection to 12 weeks p.i. for their hind paw sensory sensitivity to mechanical and heat stimulation via the up-down test (using von Frey filaments) and the Hargreaves test, respectively. As expected, WT mice developed mechanical hypersensitivity starting from 4–6 weeks p.i. Alternatively, CD40 KO mice regardless of cell transfer displayed transient mechanical hypersensitivity (between week 7–9) p.i., and the increases in mechanical sensitivity in these mice were marginal and significantly less than those in WT mice (Figure 2, two-way RM ANOVA, $p_{\text{time}} < 0.001$, $p_{\text{group}} < 0.001$, and $p_{\text{time} \times \text{group}} < 0.001$). Further, changes in heat sensitivity showed trends similar to those found for mechanical sensitivity (two-way RM ANOVA, $p_{\text{time}} < 0.001$, $p_{\text{group}} = 0.004$, and $p_{\text{time} \times \text{group}} = 0.461$; $p < 0.05$ when the “WT_No transfer_LP-BM5” group was compared to both non-infected groups and the “CD40 KO_No transfer_LP-BM5” group, and $0.05 < p < 0.10$ when the “WT_No transfer_LP-BM5” group was compared to the “CD40 KO_Total leukocytes_LPBM5” and “CD40 KO_B cells_LP-BM5” groups). However, due to the limited numbers of animals, no significant group differences were detected for any of the individual time points in *post hoc* tests (data not shown). Since CD40 KO mice transferred either total leukocytes or B cells lack CD40⁺ microglia, these data suggest that microglial CD40, but not CD40 expressed by peripheral leukocytes, contributes to the development of LP-BM5-induced sensory hypersensitivity.

3.3. LP-BM5 infection-induced changes in viral RNA levels in CD40 KO mice receiving adoptive transfer

To determine the relationship between viral infection and the development of sensory hypersensitivity, viral loads in all collected tissues (spleen, lumbar spinal cord, lumbar dorsal root ganglia (DRG), and hind paw skin) were determined by assessing the expression level of both BM5def and BM5eco *gag* RNA via quantitative real-time RT-PCR (qRT-PCR) as described in the Materials and Methods. Interestingly, despite the differences in LP-BM5-induced immunodeficiency and sensory hypersensitivity among the treatment groups, increased viral *gag* RNA levels (both BM5def and BM5eco) were detected in all the peripheral tissues (spleen, hind paw skin, and lumbar DRG) in all the groups of infected mice, and no significant differences were found among the infected groups (Figure 3A–F, one way ANOVA, $p < 0.001$ for all). Alternatively, although LP-BM5 also induced a significant elevation of viral RNA in the spinal cords of CD40 KO mice, the levels of

BM5def *gag* RNA were lower in all groups of infected CD40 KO mice than those of infected WT mice and were not affected by either type of cell transfer (Figure 3G and H, one way ANOVA, $p < 0.001$ for both). These data suggest that the viral load (particularly the pathogenic component of the virus) within the spinal cord is positively correlated with the development of behavioral signs of peripheral neuropathy following LP-BM5 infection.

3.4. LP-BM5 infection-induced changes in cytokine levels in CD40 KO mice receiving adoptive transfer

We previously showed that LP-BM5 induced tissue specific cytokine responses. To determine whether specific cytokine(s) are associated with microglial CD40-mediated, LP-BM5-induced sensory hypersensitivity, the cytokines IL-1 β , IL-6, TNF α , IFN γ , and IL-12 were measured in the serum and spleen via ELISA and in the lumbar spinal cord and lumbar DRG via qRT-PCR. Since changes in paw skin cytokines were transient and prior to week 12 p.i. (Cao et al., 2012), cytokine levels in the paw skin were not measured in this study. Only IL-12/IL-23 p40 was detectable in the serum, while in the spleen, only IL-1 β , IFN γ , and IL-12/IL-23 p40 were detectable. LP-BM5 induced significant increases of all these cytokines in WT mice, while the increases in them in CD40 KO mice varied depending on the individual cytokine and the type of cell transfer (Figure 4A–D, one way ANOVA, $p < 0.05$ for all). The general order of the infected groups in terms of the magnitude of cytokine increase was CD40 KO mice without cell transfer $\approx$ CD40 KO mice with total leukocyte transfer $\approx$ CD40 KO mice with B cell transfer $\approx$ WT without cell transfer. Of the cytokines, IFN γ appeared to be induced the least by LP-BM5 in CD40 KO mice, while a similar level of induction of splenic IL-1 β and IL-12/IL-23 p40 was observed in the total leukocyte- and B cell-transferred CD40 KO groups compared to the WT mice. These cytokine changes were consistent with the severity of immunodeficiency as measured by the spleen weights and serum immunoglobulin levels in the different treatment groups (see Figure 1).

In the lumbar DRG, IL-1 β displayed trends similar to those described above for the serum and splenic cytokines (Figure 5A, one way ANOVA, $p < 0.05$), whereas LP-BM5 did not induce significant increases of all the other cytokines measured in WT mice. In the lumbar spinal cord of WT mice, LP-BM5 infection induced significant increases of most of the spinal cord cytokines measured, but unlike in the peripheral tissues, LP-BM5-induced cytokine changes were not enhanced by either type of cell transfer in CD40 KO mice (Figure 5B–E, one way ANOVA, $p < 0.05$ for B, D and E and $p = 0.090$ for C). Interestingly, the LP-BM5-induced increase in lumbar spinal cord IFN γ was higher in CD40 KO mice without cell transfer than in the cell transferred CD40 KO groups (Figure 5D, one way ANOVA, $p < 0.05$). Compared to its effects in WT mice, LP-BM5 did not induce an increase in lumbar spinal cord IL-1 β in CD40 KO mice, regardless of cell transfer (Figure 5B, one way ANOVA, $p < 0.05$). Similar changes as those with IL-1 β were observed with lumbar spinal cord IL-6, although they did not reach statistical significance (Figure 5C, one way ANOVA, $p = 0.090$). Together with the behavioral data (Figure 2) and the reduced viral load in the lumbar spinal cord (Figure 3), these data suggest an association between lumbar spinal cord infection and the subsequent cytokine responses and the development of LP-BM5 induced peripheral neuropathy.

4. Discussion

In the current study, we examined the involvement of CD40 in the development of LP-BM5-induced sensory hypersensitivity, a behavioral sign of peripheral neuropathy that we have previously demonstrated to be associated with neuropathological changes (early, transient reductions in the number of hind paw intraepidermal nerve fibers) post-infection (Cao et al., 2012). Data obtained from experiments in which leukocytes were adoptively transferred to CD40 KO mice suggest the involvement of CD40 expressed in the CNS, primarily by

microglia, but not by peripheral leukocytes, in the development of LP-BM5-induced peripheral neuropathy. The transfer of either peripheral total leukocytes or B cells was able to at least partially restore LP-BM5-induced MAIDS and the associated cytokines responses in peripheral tissues (spleen, serum, and DRG) but had little to no effect on the viral load of the pathogenic component (BM5def) and selected LP-BM5-induced cytokine changes (particularly IL-1 β , IL-6, and IFN γ) within the lumbar spinal cord. It has been demonstrated that inflammatory responses mediated by central nervous system glial cell activation (including both astrocytes and microglia) are critical in the development of peripheral nerve injury-induced behavioral hypersensitivity (DeLeo et al., 2004; Milligan and Watkins, 2009; Ren and Dubner, 2010). Although there have only been a limited number of reports regarding the actions of microglia in HIV peripheral neuropathy, studies on HIV-associated optic neuropathy showed upregulation of proinflammatory cytokines in the microglia of patients with HIV/AIDS (Lin et al., 1997; Saadati et al., 1999). Microglia are known reservoirs for HIV in the CNS and are suggested to contribute to the persistent CNS LP-BM5 infection (Alexaki et al., 2008; Sei et al., 1998). Thus, we propose that the lack of central CD40, primarily microglial CD40, leads to reduced infection in the lumbar spinal cord and reductions in the associated spinal cord cytokine responses, likely due to the resistance of CD40⁻ microglia to LP-BM5 infection, which limits viral dissemination in the spinal cord. This reduction in central activation in turn decreases peripheral sensory hypersensitivity in part via reduced central sensitization. However, the ability of LP-BM5 to infect microglia (including both CD40⁺ and CD40⁻ microglia) requires further investigation. This investigation would provide further knowledge regarding the role of microglia, particularly microglial CD40, in HIV-associated peripheral neuropathy. It is known that microglia in HIV-1 encephalitic brain tissue expressed increased levels of CD40 (D'Aversa et al., 2002), and that this increased CD40 can synergize with the HIV Tat protein to induce microglial TNF α production, leading to further HIV-1 infection-associated neurotoxic effects (Sui et al., 2007).

On the other hand, since in this study cell transfer was only performed weekly from one week before infection to 5 weeks p.i. and it is not known whether the development of peripheral neuropathy is dependent on the development of MAIDS, it is possible that unlike what has been found for the development of MAIDS, peripheral CD40 is needed for longer than the first several weeks in order to induce behavioral hypersensitivity following LP-BM5 infection, i.e., a longer period of cell transfer could result in behavioral hypersensitivity in CD40 KO mice. Future studies can be conducted to test this possibility. In addition, it appears that non-microglial CD40-mediated mechanisms could contribute to the development of certain level of LP-BM5-induced mechanical hypersensitivity, as all CD40 KO groups displayed slight, transient behavioral hypersensitivity following infection.

In our previous study, we demonstrated the association between tissue viral loads, cytokine responses, and the development of peripheral neuropathy through the use of the anti-retroviral agent, AZT. Our current data further suggest a specific association between the lumbar spinal cord viral load (particularly the spinal cord viral load of the BM5 def component), spinal cord cytokine responses, and the development of peripheral neuropathy following LP-BM5 infection. These data suggest that the development of behavioral hypersensitivity following LP-BM5 infection is primarily a centrally mediated event that may be most effectively treated or prevented by a centrally targeted approach. Whether this association exists in HIV-associated peripheral neuropathy requires further investigation. Consistent with our findings, persistent gliosis in the spinal cord, but not pathological changes in the affected peripheral nerve, has been shown to accompany epineural HIV gp120-induced behavioral hypersensitivity (Herzberg and Sagen, 2001).

Altogether, our study suggests an involvement of microglial CD40 in the development of LP-BM5- induced peripheral neuropathy. Further investigation of the associated centrally mediated mechanisms could help to elucidate the underlying mechanisms of, and develop effective treatment for, HIV-associated peripheral neuropathy.

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Highlights

- A murine model of retroviral infection-induced peripheral neuropathy was used.
- Microglial CD40 plays a crucial role in the development of peripheral neuropathy.
- Data indicate a role for lumbar spinal cord infection and cytokine responses.

Figure 1. Development of LP-BM5-induced immunodeficiency in CD40 KO mice receiving adoptive transfer

As described in the Materials and Methods, adult B6 or B6-CD40 KO mice were randomly assigned to each treatment group, and selected groups were infected with LP-BM5. The MAIDS-associated features splenomegaly (A), hyper serum IgM (B), and hyper serum IgG2a (C) were examined upon sacrifice (mean $\pm$ SEM, $n = 8$ for all). One-way ANOVA was performed for each data set within each graph, followed by SNK *post hoc* analysis. ** indicates $p < 0.05$ between the indicated group and all other groups. * indicates $p < 0.05$ between the indicated group and both of the non-infected groups. # indicates $p < 0.05$ between the indicated group and both of the non-infected groups and "CD40 KO, no cell

transferred, LP-BM5 infected” group. “-“ = no LP-BM5 infection and “+” = with LP-BM5 infection. In B and C, the “WT, no cell transferred, no infection” group = 100%.

Figure 2. Reduced mechanical hypersensitivity in CD40 KO mice post-LP-BM5 infection

As described in the Materials and Methods, adult B6 or B6-CD40 KO mice were randomly assigned to each treatment group, and selected groups were infected with LP-BM5. Hind paw mechanical sensitivity was tested weekly from before infection to 12 weeks p.i. via the up-down method. Due to the lack of differences between the left and the right hind paws, the average response from the left and the right hind paws was calculated and presented here (mean $\pm$ SEM, $n = 8$). Two-way RM ANOVA was performed with “time” and “group” as factors, followed by SNK *post hoc* analysis. # indicates $p < 0.05$ between the indicated infected group and the corresponding non-infected group at the indicated time. *_w indicates $p < 0.05$ between the indicated infected WT group and the corresponding “wk 0” group; *_n indicates $p < 0.05$ between the indicated infected “CD40 KO, no cell transferred” group and the corresponding “wk 0” group; and *_b indicates $p < 0.05$ between the indicated infected “CD40 KO, B cell-transferred” group and the corresponding “wk 0” group.

Figure 3. LP-BM5 infection-induced increases in viral RNA levels in CD40 KO mice receiving adoptive transfer

As described in the Materials and Methods, adult B6 or B6-CD40 KO mice were randomly assigned to each treatment group, and selected groups were infected with LP-BM5. LP-BM5 viral RNA (A, C, E and G for BM5Def; B, D, F, and H for BM5eco) were determined in the spleen (A and B), the hind paw skin (C and D), the lumbar DRG (E and F), and the lumbar spinal cord (G and H) upon sacrifice via qRT-PCR using β -actin for normalization (mean $\pm$ SEM, $n = 8$ for all). One-way ANOVA was performed for each data set within each graph, followed by SNK *post hoc* analysis. In all tissues, both non-infected groups showed expression levels too low to be displayed in the graph. ** indicates $p < 0.05$ between the

indicated group and all other groups. * indicates $p < 0.05$ between the indicated group and both of the non-infected groups. “-“ = no LP-BM5 infection and “+” = with LP-BM5 infection.

Figure 4. LP-BM5 infection-induced changes in serum and splenic cytokine levels in CD40 KO mice receiving adoptive transfer

As described in the Materials and Methods, adult B6 or B6-CD40 KO mice were randomly assigned to each treatment group, and selected groups were infected with LP-BM5. Selected cytokines were measured in the serum and spleen upon sacrifice via ELISA. Data for serum IL-12/IL-23 p40 (A), splenic IL-1 beta (B), splenic IFN-gamma (C), and splenic IL-12/IL-23 p40 (D) are shown here (mean $\pm$ SEM, $n = 8$ for all). One-way ANOVA was performed for each data set within each graph, followed by SNK *post hoc* analysis. ** indicates $p < 0.05$ between the indicated group and all other groups. # indicates $p < 0.05$ between the indicated group and both of the non-infected groups and “CD40 KO, no cell transferred, LP-BM5 infected” group. Additional statistical results are marked in the relevant graphs. “-” = no LP-BM5 infection and “+” = with LP-BM5 infection.

Figure 5. LP-BM5 infection-induced changes in cytokine levels in DRG and the lumbar spinal cord in CD40 KO mice receiving adoptive transfer

As described in the Materials and Methods, adult B6 or B6-CD40 KO mice were randomly assigned to each treatment group, and selected groups were infected with LP-BM5. Selected cytokines were measured in the lumbar DRG and lumbar spinal cord upon sacrifice via qRT-PCR. Data for lumbar DRG IL-1 beta (A), lumbar spinal cord (LSC) IL-1 beta (B), LSC IL-6 (C), LSC IFN-gamma (D), and LSC IL-12 p40 (E) are shown here (mean $\pm$ SEM, $n = 8$ for all). One-way ANOVA was performed for each data set within each graph, followed by SNK *post hoc* analysis. * indicates $p < 0.05$ between the indicated group and all other groups except the group indicated with @. Additional statistical results are marked in the relevant graphs. “-” = no LP-BM5 infection and “+” = with LP-BM5 infection.