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MURINE IMMUNODEFICIENCY VIRUS-INDUCED PERIPHERAL NEUROPATHY AND THE ASSOCIATED CYTOKINE RESPONSES

Ling Cao,* M. Brady Butler,* Leonard Tan,* Kyle S. Drapeau,* and Woon Yuen Koh†

Distal symmetrical neuropathy is the most common form of HIV infection-associated peripheral neuropathy and is often associated with pain. C57BL/6 (B6) mice infected with LP-BM5, a murine retroviral isolate, develop a severe immunodeficiency syndrome similar to that in humans infected with HIV-1, hence the term murine AIDS. We investigated the induction of peripheral neuropathy after LP-BM5 infection in B6 mice. Infected B6 mice, like HIV-infected humans, exhibited behavioral (increased sensitivity to mechanical and heat stimuli) and pathological (transient loss of intraepidermal nerve fibers) signs of peripheral neuropathy. The levels of viral gag RNA were significantly increased in all tissues tested, including spleen, paw skin, lumbar dorsal root ganglia, and lumbar spinal cord, postinfection (p.i.). Correlated with the development of peripheral neuropathy, the tissue levels of several cytokines, including IFN-γ, IL-1β, IL-6, and IL-12, were significantly elevated p.i. These increases had cytokine-specific and tissue-specific profiles and kinetics. Further, treatment with the antiretroviral agent zidovudine either significantly reduced or completely reversed the aforementioned behavioral, pathologic, and cytokine changes p.i. These data suggest that LP-BM5 infection is a potential mouse model of HIV-associated distal symmetrical polyneuropathy that can be used for investigating the roles of various cytokines in infection-induced neuropathic pain. Further investigation of this model could give a better understanding of, and lead to more effective treatments for, HIV infection-associated painful peripheral neuropathy. The Journal of Immunology, 2012, 189: 3724–3733.

Human immunodeficiency virus infection is now considered a chronic disease owing to more effective viral suppressive treatments that significantly prolong survival postinfection (p.i.). As such, greater attention is now focused on the treatment of numerous HIV infection-related complications, including neurological disorders, opportunistic infections, and tumors.

HIV-associated peripheral neuropathies are the most common neurological disorders associated with HIV infection. The most common forms of HIV-associated peripheral neuropathies that have been characterized include antiretroviral agent-induced toxic neuropathy and infection-induced distal symmetrical polyneuropathy (DSP) (1, 2). HIV DSP occurs in about one third of HIV/AIDS patients (3, 4). Patients with DSP present with symptoms typical of neuropathic pain, which is pain caused by a lesion or disease of the somatosensory system (5), including both spontaneous and evoked pain. In general, the symptoms of DSP are more pronounced in the distal lower extremities and are symmetrically distributed, with the upper extremities being affected later in the course of DSP (1, 2). Neuropathologic changes have been correlated with the clinical course and described to progress in a “dying back” pattern, as usually the distal regions of nerve fibers are first affected, with the changes eventually progressing proximally (2, 6). The major pathologic changes associated with HIV DSP include reduced intraepidermal nerve fiber (IENF) density, damage to primary sensory fibers and neurons, and macrophage infiltration into peripheral nerves and the dorsal root ganglia (DRG). However, the underlying mechanism of the pathogenesis of HIV DSP is, in large measure, unknown owing, at least in part, to the lack of appropriate animal models. As such, HIV DSP is often underdiagnosed and/or undertreated, and no FDA-approved pharmacologic agents specifically designed for the treatment of HIV DSP are available.

Because humans are the only natural hosts of HIV-1, only a limited number of approaches are available for the study of HIV DSP. LP-BM5, first isolated by Latarjet and Duplan in 1962 (7), is a retroviral mixture that contains mainly a pathogenic yet replication-defective virus (BM5def) and a nonpathogenic helper virus (ectropic [mouse tropic] retrovirus [BM5eco]) (8). BM5eco is necessary for the replication and cell-to-cell spreading of BM5def. Despite certain differences between LP-BM5 and HIV-1, decades of research have established that LP-BM5 infection in susceptible C57BL/6 (B6) mice induces an HIV-like immunodeficiency syndrome, hence the term murine AIDS (MAIDS). The characteristic responses following LP-BM5 infection in B6 mice (9) include splenomegaly, lymphadenopathy, polyclonal hypergammaglobulinemia, profoundly decreased T and B cell responses (particularly CD4+ T lymphocyte responses to mitogens), increased susceptibility to opportunistic pathogens, and the development of terminal B cell lymphomas. This model also mimics HIV-1 infection in humans, in that LP-BM5 induces encephalopathy and cognitive deficits characterized by impairments in spatial learning and memory in B6 mice that appear to be associated with the progression of LP-BM5–induced MAIDS (10, 11).
and neuronal degeneration (12). Further, various cytokines, including IFN-γ, TNF-α, IL-1β, IL-6, and IL-12, are known to be critical in the development of LP-BM5–induced immunodeficiency and CNS deficits (13–17). Treatment with the antiretroviral agent zidovudine (AZT) significantly inhibits LP-BM5–induced immunodeficiency and CNS deficits (10, 18, 19). The MAIDS system may at present represent the best small-animal model for retrovirus-induced immunodeficiency and provides a valuable tool in studying certain aspects of HIV-1 infection and antiretroviral drug development (20).

In the current study, we, for the first time, to our knowledge, explored the potential of using LP-BM5 infection as a rodent model of HIV DSP. We found that B6 mice infected with LP-BM5 displayed both behavioral and pathologic signs of peripheral neuropathy, and tissue-specific cytokine responses were observed. Most of these LP-BM5–induced changes, including those in mechanical sensitivities, density of IENFs, and the expression of selected cytokines, could be reversed by AZT treatment. We believe that this model will help to further elucidate the underlying mechanisms of HIV DSP and could be used to delineate and test candidate therapeutic drugs for HIV DSP in the future.

Materials and Methods

Mice

Male adult B6 mice were purchased from the National Cancer Institute (Frederick, MD) and were allowed to habituate to the institutional animal facility for at least 1 wk before experimental use (8–9 wk old). All mice were group housed (four per cage) with food and water ad libitum and maintained on a 12-h light/dark cycle. For selected experiments, the antiretroviral drug AZT (Sigma-Aldrich, St Louis, MO) was added to the drinking water (at 0.2 mg/ml) from the day of infection to week 12 p.i. This dose was chosen based on previous studies regarding MAIDS-induced neurological deficits (10). AZT-containing water was replaced weekly during regular cage changes. All water bottles that contained AZT were covered with foil to avoid direct light exposure. The mice treated with AZT water did not show any signs of dehydration, atypical growth, or abnormal behavior. In addition, it is known that AZT alone does not cause peripheral neuropathy in patients or neurotoxicity in vitro (21–23). The Institutional Animal Care and Use Committee at University of New England (Biddedford, ME) approved all experimental procedures used in this study.

LP-BM5 virus preparation and inoculation

The initial complete LP-BM5 isolate was provided by Dr. William Green in the Geisel School of Medicine at Dartmouth College (Hanover, NH). The LP-BM5 viral isolates used in our experiments were prepared in either Dr. Green’s laboratory or our laboratory, as previously described (24, 25). The titer of the viral stock was determined by a standard retroviral XC plaque assay for BM5eco virus (26). Mice were infected (i.p.) with ~5 × 10^3 ecotropic PFU per mouse, a dose of LP-BM5 that has been shown to induce typical MAIDS (27).

Mechanical sensitivity

Mechanical sensitivities of the hind paws of the mice were assessed under nonrestrained conditions, as previously described (28). During the test, each animal was subjected to stimulation from a series of von Frey filaments ranging from 0.008 g to 2 g (Stoelting, Wood Dale, IL) following the up-down paradigm (detailed in Ref. 29). The 50% threshold force needed for paw withdrawal was calculated and used to represent mechanical sensitivity. For each experiment, testing of both hind paws was performed before (baseline) and weekly p.i. for up to 12 wk. The person performing the behavioral tests was blinded to the experimental groups. On the basis of our previous experience, a minimum of four animals per group are needed for the mechanical sensitivity test, to obtain statistical significance. Thus, a total of 20 animals were used in each group in the experiment that tested the effects of LP-BM5 (Fig. 1B).

For both of these sensitivity tests, the hind paw was chosen because the symptoms of HIV DSP usually start from the lower limbs in humans (1, 2). Separate animals were used in each of the sensory tests to avoid interference between the tests.

Measurement of the development of MAIDS disease

Spleen weight and serum Ig levels were measured to monitor the development of MAIDS disease, as described previously (27). Mice were euthanized through CO2 inhalation. Blood from each mouse was collected via cardiac puncture. Sera were obtained following centrifugation and stored at −80°C until analysis. Serum IgM and IgG2a were measured via an established ELISA protocol (27). The spleen of each animal was harvested following blood collection, and the total weight of each spleen was measured before storing the tissue at −80°C for further analysis. On the basis of experiences in Dr. William Green’s laboratory, a minimum of four animals per group are needed for statistical analyses of these MAIDS disease–related parameters to reach significance. We collected all available samples from our studies to determine the changes in these parameters. Thus, there are 16–19 mice per group in Fig. 1C–E and 7 mice per group in Fig. 6B–D.

RNA isolation and real-time quantitative RT-PCR for viral load

Spleens, lumbar (L4-L6) DRG, and lumbar spinal cord tissue were collected upon sacrificing each animal and stored at −80°C until viral load determination. The viral load in the tissue was ascertained by the evaluation of expression of both BM5def and BM5eco gag RNAs via quantitative RT-PCR (qRT-PCR), as previously described (30, 31). Briefly, total RNA was isolated from the selected tissue via the RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA). cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) from 1 µg total RNA, and qRT-PCR was performed by amplifying the cDNA (0.5 µl) with the SYBR Green Supermix (Bio-Rad) on an iCycler with iCycler iQ software (Bio-Rad). Mouse β-actin RNA expression was measured in parallel. As described earlier (30), the relative expression levels of the BM5def gag and BM5eco gag genes were calculated via the ΔΔC(T) method (32), using the 18S rRNA gene as the control. On the basis of our preliminary experiment, the expression of β-actin did not change with LP-BM5 infection or AZT treatment. Further, it was determined that a minimum of four animals per group from each type of tissue were needed for the statistical analyses of tissue levels of viral RNA to reach significance. We collected all available samples from our studies to determine the changes in viral RNA levels. In Fig. 3, the numbers of samples used to analyze changes in viral load in splenic, hind paw skin, lumbar DRG, and lumbar spinal cord tissue were n = 16–18, n = 4–8, n = 8, and n = 7–8, respectively. Seven animals per group were used to test the effects of AZT on viral loads (Fig. 6E–H).

IENF density

At the indicated times (see Results), selected groups of mice were euthanized by inhalation of CO2, and hind paw plantar foot pads were harvested for the evaluation of IENF density according to a previously published method (33). Briefly, foot pads tissues were fixed in 4% formaldehyde for 24 h, cryoprotected with 30% sucrose, and then sectioned into 15-µm sections. At least three sections were collected for analysis from each foot pad. Fluorescent immunohistochemistry for specific markers was conducted following our published procedure (33). Sample sections were stained with either a rabbit Ab against the pan-axonal marker protein gene product (PGP) 9.5 (1:500; AbD Serotec, Raleigh, NC), a rabbit Ab against calcitonin gene–related peptide (CGRP) (1:5000; Sigma-Aldrich), or a mouse Ab against growth-associated protein (GAP) 43 (1:1000; a gift from Dr. Frank Rice at the Albany Medical College, Albany, NY), followed by a fluorescence-labeled secondary Ab (goat anti-rabbit-Cy3 for PGP9.5 and CGRP [1:800 for both]; Jackson ImmunoResearch Laborato
cies, West Grove, PA) and goat anti-mouse-FTTC for GAP43 (1:250; Invitrogen/Life Technology, Grand Island, NY). The numbers of positively stained IENFs were counted in each section in a blinded manner, and the density of IENFs per millimeter of skin was determined based on the total numbers of positively stained IENFs and the length of the corresponding skin tissue. The final IENF density for each foot pad tissue was obtained by calculating the average IENF density of the sections obtained from a foot pad. According to our preliminary experiment, a minimum of four animals per group were needed for the statistical analysis of IENF density to reach significance. Thus, four to seven animals per group were used in the initial experiment for testing the effects of LP-BM5 (Fig. 2C, 2D), and four animals per group were used to test the effects of AZT treatment (Fig. 7A, 7B).

Cytokine qRT-PCR

The mRNA levels of selected cytokines in the lumbar spinal cord and DRG were determined via qRT-PCR. cDNA samples obtained for the determination of viral loads (described above) were also used for cytokine qRT-PCR. qRT-PCR was performed with 0.5 μl cDNA per sample and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an iCycler with iCycler iQ software (Bio-Rad). Primers for mouse cytokine IL-1β, TNF-α, IL-6, and IL-12p40, and β-actin, were purchased from OriGene (Rockville, MD), and the PCR protocols suggested by the manufacturer were used. For each of these cytokines, OriGene supplies the mixture of both the forward and reverse primers. Accession numbers for manufacturer were used. For each cytokine, OriGene supplies the PCR protocols suggested by the manufacturer. The following primers and reverse primers for the cytokines were used: (sequences were obtained from Dr. Paul Massa, SUNY Upstate Medical University, Syracuse, NY). A published PCR protocol (34) was used for IFN-γ PCR. The relative expression levels of all the cytokines measured were calculated using β-actin expression as the control via the ΔΔCt method. According to our preliminary experiment, a minimum of 6 animals per group were needed for the statistical analysis of the levels of cytokine RNA to reach significance. Thus, the number of samples used during the initial experiment for testing the effects of LP-BM5 were n = 7–8 for lumbar spinal cord and n = 8 for lumbar DRG (Fig.4), and 7 animals per group were used to test the effects of AZT treatment (Fig. 6–K).

Cytokine multiplex assay

Hind paw skin tissue was quickly collected after sacrificing each animal and processed as described previously (33) for the determination of cytokine levels via the FlowCytomix Multiplex assay. Briefly, skin from the left and right hind paws of each animal was pooled and lysed in lysis buffer (PBS supplemented with 1 mM Na3EDTA, 1% BSA [Sigma-Aldrich], and protease inhibitor mixture [Sigma-Aldrich]). The supernatants were collected after centrifugation (10,000 × g at 4°C for 15 min) and stored at −80°C until analysis. The FlowCytomix Multiplex assay was performed with the mouse FlowCytomix Basic Kit and mouse FlowCytomix Simplex sets for IL-1β, IL-6, TNF-α, IL-12 p70, and IFN-γ (eBioscience, San Diego, CA), following the manufacturers’ protocols, with an Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI). All cytokine levels were normalized based on the protein concentration of each sample, which was determined by the BCA Protein Assay Kit (Pierce-Thermo Scientific, Rockford, IL). The following formula was used: normalized level of a cytokine = cytokine concentration obtained from the multiplex assay (pg/ml)/protein concentration obtained from the BCA assay (mg/ml). On the basis of our preliminary experiment, a minimum of six animals per group were needed for the statistical analysis of protein levels of cytokines. Eight animals per group were used in the experiment for testing the effects of LP-BM5 (Fig. 5).

Statistical analyses

All statistical analyses were performed with raw data, using SigmaStat 3.5 software (Systat Software). When different animals were measured at different time points, appropriate ANOVAs (one-way or two-way ANOVA; details in the figure legends), with treatment and/or time as factors, were performed, followed by Student–Newman–Keuls post hoc analysis (all graphs except Figs. 1A, 1B, 6A). When data regarding the left and right side of the same animal were obtained, a paired t test was used to determine the effects of “side” prior to the evaluation of the effects of various treatments (Figs. 2C, 2D, 7A, 7B). When the same animals were repeatedly measured for their responses on both the left and the right sides at different time points, two-way repeated-measures ANOVA (RM ANOVA) (use side and time as factors) was performed to determine the effects of “side” over “time” prior to evaluation of the effects of various treatments over time via two-way RM ANOVA (Fig. 1A, 1B). In Fig. 6A, because no differences in mechanical sensitivities were observed between the left and right hind paws in Fig. 1A, average responses from the left and right paws were calculated and analyzed via two-way RM ANOVA to determine the effects of “treatment” over.” All data are presented as mean ± SEM, and p < 0.05 was considered statistically significant.

Results

LP-BM5 infection-induced hind paw sensory hypersensitivity

To determine whether LP-BM5 infection would induce behavioral signs of peripheral neuropathy in adult B6 mice chronically infected with LP-BM5, we examined the hind paw sensitivity to both mechanical and heat stimulation via the up-down test (using von Frey filaments) and the Hargreaves test, respectively. To avoid interference between the two behavioral tests, separate groups of animals were used for the two tests. All animals were baseline tested before infection and weekly p.i. for 12 wk. Both the left and the right hind paws were tested at each time point. No significant differences were found between the left and right hind paws in either of the sensory tests, regardless of the infection (for mechanical sensitivity: p = 0.771; for heat sensitivity: p = 0.591), whereas infected mice displayed mechanical and heat hypersensitivity, compared with noninfected mice, starting at week 6 and lasting to week 12 p.i. (for both sensory sensitivity tests: p < 0.001) (Fig. 1). No significant hind limb paralysis or weakness was observed in any infected mice. We did notice an adaptation in the animal’s response to heat stimulation over time. This adaptation can be seen more clearly in the noninfected group (Fig. 1B).

The development of splenomegaly and hypergammaglobulinemia, hallmark manifestations of MAIDS, was examined in separate mice in parallel. Fig. 1C–E summarizes the time course of the development of MAIDS. This disease can be readily detected as early as week 2 p.i. by any one of the parameters used; that is, at any selected time point p.i., significant differences were evident between the infected and noninfected groups in all parameters examined (p < 0.001) (Fig. 1C–E). In addition, the development of MAIDS was confirmed in the mice tested for behavioral sensitivities at week 12 p.i. (data not shown). Thus, LP-BM5–induced behavioral hypersensitivity manifests after LP-BM5–induced immunodeficiency is well developed.

LP-BM5 infection-induced loss of peripheral nerve fibers

To evaluate the pathological changes after LP-BM5 infection, hind paw foot pad tissue from both infected and noninfected B6 mice was collected at various times p.i., and the IENF density was examined via immunohistochemistry. The density of the hind paw IENFs was determined with an Ab against a pan-axonal marker, PGP9.5, as previously described (32, 33) (Fig. 2A, 2C). Although no side differences were found in any of the groups (p = 0.534), we did observe significant age-related effects in noninfected controls (p < 0.05). The number of PGP9.5+ IENFs was significantly reduced at 2 wk p.i. (p < 0.001) and then gradually recovered to levels similar to that of noninfected age-matched controls (Fig. 2A, 2C). The LP-BM5–induced reduction of PGP9.5+ IENFs at week 2 p.i. did not appear to be due to a loss of PGP9.5 expression because a similar reduction was observed when nerve fibers were identified by an Ab against GAP43 (data not shown), a neuronal membrane protein involved in axonal growth, regeneration, and remodeling that has been shown to be expressed by the majority of skin IENFs (35, 36). Further, it is known that skin IENFs consist of mainly sensory nerve fibers, including both peptidergic (CGRP+) and nonpeptidergic (CGRP–)
fibers. Thus, we examined the density of CGRP+ IENFs (Fig. 2B, 2D). Similarly, no side difference was found in any of the groups (p = 0.058). LP-BM5–induced reductions in CGRP+ fibers were detected at weeks 2 (p < 0.001) and 4 p.i. (p < 0.05), although these reductions were not accompanied by significant age-related changes (Fig. 2B, 2D).

FIGURE 1. LP-BM5–induced behavioral hypersensitivity along with MAIDS-associated features in B6 mice. Adult B6 mice were randomly assigned to LP-BM5 infection and no-infection groups. Separate animals were used for the assessment of the hind paw mechanical sensitivity (A) (n = 12), hind paw heat sensitivity (B) (n = 20), and MAIDS-associated features [splenomegaly (C), hyper serum IgM (D), and hyper serum IgG2a (E); n = 16–19 for (C)–(E)] in time course studies. In (A) and (B), no differences between the left and right sides were detected in both behavioral tests; thus two-way RM ANOVA was performed for each data set, with “infection” and “time” as factors (regardless of “side”). *p < 0.05 between the infected group and the noninfected group at the indicated time (regardless of “side”), #p < 0.05 between the indicated infected group and the corresponding “no infection wk 0” group (regardless of “side”), and ##p < 0.05 between the indicated noninfected group and the corresponding “no infection wk 0” group (regardless of “side”). In (C)–(E), two-way ANOVA was performed for each data set, with “infection” and “time” as factors. *p < 0.05 between the infected group and the noninfected group at the indicated time, #p < 0.05 between the indicated infected group and the “no infection wk 0” group. In (D) and (E), the “no infection wk 0” group = 100%.

FIGURE 2. LP-BM5–induced reduction of hind paw skin IENFs in B6 mice. Adult B6 mice were randomly assigned to LP-BM5 infection and no-infection groups. The density of total (PGP9.5) and CGRP+ IENFs in the hind paw skin were examined via immunohistochemistry and presented as the number of each type of nerve fiber per 1-mm length of skin tissue. Representative PGP9.5 (A) (×20) and CGRP (B) (×20) images are shown (scale bar, 50 μm). All images were taken at 2 wk p.i. and from animals’ left paws. Arrows indicate examples of positively stained nerve fibers. Corresponding quantitative data are shown in (C) (PGP 9.5) and (D) (CGRP) [n = 4–7 for both (C) and (D)]. Because no differences between the left and right sides were detected in either (C) or (D), two-way ANOVA was performed for each data set, with “infection” and “time” as factors (regardless of “side”). *p < 0.05 between the infected group and noninfected group at the indicated time (regardless of “side”), #p < 0.05 between the indicated group and the “no infection wk 0” group (regardless of “side”).
Viral loads in various tissues after LP-BM5 infection

To determine the viral loads in peripheral neuropathy-related tissues, BM5def and BM5eco gag (one of the viral genes that encode the group-specific viral core proteins) RNA expression levels were measured in spinal cord, lumbar DRG, and the hind paw skin of infected B6 mice via qRT-PCR. Splenic mRNA levels were also measured for comparison purposes. In the spleen, lumbar spinal cord, and lumbar DRG, viral RNA levels, especially BM5def gag levels, showed significant increases 2 wk p.i. and reached maximal levels 4 wk p.i. \((p < 0.001)\) (Fig. 3A, 3B, 3E–H). Alternatively, in the paw skin, viral RNA levels started to increase 4 wk p.i. and reached maximal levels at 6 wk p.i. \((p < 0.05)\) (Fig. 3C, 3D). Further, no significant differences in viral RNA were detected between the different segments of the spinal cord (cervical, thoracic, and lumbar), between the left and right hind paws, or between left and right lumbar DRG (data not shown). At any given time p.i., the levels of viral RNA were as follows: spleen > paw skin > DRG > spinal cord. In all, these data indicate that local viral infection is established early during the development of peripheral neuropathy.

Changes of cytokine levels in various tissues after LP-BM5 infection

Previous studies have indicated that several cytokines, IL-1\(\beta\), IL-6, TNF-\(\alpha\), IFN-\(\gamma\), and IL-12, were critical for the development of LP-BM5–induced immunodeficiency and/or CNS deficits (13–17). Thus, to assess the involvement of these cytokines in the development of peripheral neuropathy, their levels were determined over a time course in lumbar spinal cord and lumbar DRG by qRT-PCR and in the hind paw skin by FlowCytomix Multiplex assay. Because of the lack of side differences and the limited amount of tissue that could be collected from each animal, the left and right DRG and the left and right paw skin were pooled before processing for cytokine measurement. In the lumbar spinal cord, the only infection-induced changes detected were IL-12p40 mRNA levels \((p < 0.05)\), which trended upward over time p.i. (Fig. 4A). Of interest, the mRNA levels of IL-6 and IFN-\(\gamma\) significantly increased with time in both infected and noninfected mice (for both cytokines, \(p < 0.001\); data not shown), whereas no significant changes in the mRNA levels of IL-1\(\beta\) and TNF-\(\alpha\) were observed (data not shown). In the DRG, the mRNA levels of both IL-1\(\beta\) and IFN-\(\gamma\) started to increase as early as 2 wk p.i. and remained elevated until 12 wk p.i. \((p < 0.001)\) (Fig. 4B, 4C). However, no significant changes in the mRNA levels of IL-6, TNF-\(\alpha\), and IL-12p40 p.i. were detected (data not shown). In the hind paw skin, TNF-\(\alpha\) was not detectable at the protein level in most animals. No changes in the levels of IL-12p70 were detected. All other cytokines measured via FlowCytomix Multiplex assay showed transient increases p.i. Specifically, LP-BM5 induced a significant elevation of IL-6 at week 2 p.i. \((p < 0.01)\) that was followed by a significant elevation of IFN-\(\gamma\) at week 4 p.i. \((p < 0.01)\). Meanwhile, IL-1\(\beta\) levels appeared to be elevated for a longer time period (from week 4 to week 10 p.i.; \(p < 0.01)\) (Fig. 5). These data indicate that tissue-specific cytokine responses are associated with...
LP-BM5 infection and provide temporal associations between the changes of selected cytokines and the development of peripheral neuropathy.

Effects of AZT treatment on LP-BM5–induced responses

It has been shown that the antiretroviral agent AZT can significantly reverse LP-BM5–induced immunodeficiency and CNS deficits (10, 18, 37). To evaluate the effects of AZT on LP-BM5–induced peripheral neuropathy, AZT was administered via drinking water (0.2 mg/ml) from day 0 (day of infection) to week 12 p.i. Mechanical sensitivity was determined prior to infection and weekly up to 12 wk p.i. AZT did not significantly affect the mechanical sensitivities of uninfected animals but significantly prevented LP-BM5–induced mechanical hypersensitivities (*p < 0.05 between the infected group and the noninfected group at the indicated time, *p < 0.05 between the indicated group and the “no infection wk 0” group, #0.05 < p < 0.10 between the infected group and the noninfected group at the indicated time, ^0.05 < p < 0.10 between the indicated group and the “no infection wk 0” group).

The features of MAIDS (as described in Figs. 1, 6B–D), the viral loads in various tissues (Fig. 6E–H), as well as the expression of selected cytokines in the lumbar spinal cord and DRG (Fig. 6I–K), were determined at 12 wk p.i. upon completion of behavioral testing. AZT treatment was able to significantly reduce LP-BM5–induced increases in spleen weight, serum IgM and IgG2a, and the viral load in all the tissues examined (for all parameters, *p < 0.001) (Fig. 6B–H). The same AZT treatment also completely reversed the LP-BM5–induced elevation of selected cytokines in the lumbar spinal cord and DRG (for all parameters, *p < 0.05) (Fig. 6I–K). In addition, AZT did not induce significant changes in all the parameters measured in uninfected animals (Fig. 6A–K).

In a separate experiment, hind paw skin tissue was collected from AZT–LP-BM5–treated mice 2 wk p.i., and the densities of PGP9.5+ and CGRP+ IENFs were determined as described in Fig. 2. Similar to the previous results, no side differences were found in the numbers of both PGP9.5+ and CGRP+ IENFs (p = 0.089) (Fig. 7A, 7B). AZT completely reversed the LP-BM5–induced reduction of the density of both PGP9.5+ (p < 0.01) and CGRP+ (p < 0.05) IENFs, whereas AZT alone did not induce significant changes in IENF density (Fig. 7). In all, these data indicate that AZT can prevent LP-BM5–induced peripheral neuropathy.
Discussion

Although HIV-associated peripheral neuropathy is the most common neurological disorder linked to HIV infection, it is still poorly understood. One notable reason is the lack of an appropriate model that can be used to investigate the underlying mechanisms of HIV-associated neuropathy. Many studies have been carried out in vitro by the coculturing of DRG cells with either the intact virus or various viral components. Ultimately, the findings that result from such studies require further validation in an in vivo system. In vivo, feline immunodeficiency virus and SIV have been used as model systems to study HIV-associated peripheral neuropathy (38–41). Although the SIV model is considered one of the most appropriate models for HIV infection in humans, its use is significantly hampered by cost and the lack of species-specific reagents.

FIGURE 6. Effects of AZT on LP-BM5–induced responses in B6 mice. Adult B6 mice were randomly assigned to four treatment groups: no treatment, AZT, LP-BM5 infection, and LP-BM5 + AZT. AZT was administered via the drinking water (0.2 mg/ml) for 12 wk, beginning at the day of infection. Hind paw mechanical sensitivity (A) (n = 7) was tested weekly from week 0 before infection to week 12 p.i. (the average responses from the left and the right paws are shown). Two-way RM ANOVA was performed, with “infection” and “time” as factors. At each indicated time, *p < 0.05 between the indicated group and all other groups, †p < 0.05 between the indicated group and the corresponding “no infection wk 0” group, ‡p < 0.05 between the indicated group and both the “no treatment” and “AZT” groups at the indicated time, §p < 0.05 between the indicated group and both the “no treatment” and “AZT” groups at the indicated time, ¶p < 0.05 between the indicated group and both the “no treatment” and “LP-BM5” groups at the indicated time. Upon sacrifice at 12 wk p.i., the development of MAIDS-associated features (B–D), changes in viral gag expression (E–H); similar results were obtained from BM5def gag and BM5eco gag measurement; thus only the BM5def gag data are shown here), and cytokine responses (I–K) were also determined. In (B)–(K), all data are presented as mean ± SEM (n = 7). One-way ANOVA was performed for each data set, with “treatment” as the main factor. *p < 0.05 between the indicated group and all other groups. Note that the result of the statistical analysis for DRG IFN-γ is described in (K). In (C) and (D), the “no treatment” group = 100%. ND, Not detectable.
Rodent models of HIV DSP have been generated by either injecting HIV gp120 (either peripherally into the sciatic nerve or intrathecally) (42–45) or by the expression of HIV gp120 in the nervous system of mice (32). However, these rodent models are established without the natural progression of a systemic viral infection and strongly rely on the assumption that HIV gp120 is the major viral protein involved in HIV DSP. In our current study, we tested LP-BM5 infection as a potential model system for the study of HIV DSP. We demonstrated that besides developing an immunodeficiency syndrome, B6 mice infected with LP-BM5 also displayed both behavioral (sensory hypersensitivity) and pathological (decrease in IEFN density) changes similar to those found in HIV DSP. Many of these cytokines, such as IL-1β, IFN-γ, and TNF-α, are known to be associated with the development of HIV DSP (46–50). Our results indicate that these cytokines may also be involved in the development of LP-BM5–induced peripheral neuropathy. We believe that despite certain differences between LP-BM5 and HIV, this rodent LP-BM5 infection system, which is a small-animal model in which the development of peripheral neuropathy is associated with natural infection-induced immunodeficiency, will complement the current in vitro and in vivo model systems and can be used to investigate certain types of HIV-associated peripheral neuropathy, including HIV DSP. Because it is difficult, if not impossible, to obtain human samples from various tissues (including DRG and spinal cord) from patients at various disease stages (including prior treatment versus ongoing treatment), this model can be particularly useful in the investigation of cytokine network responses, the interactions between the peripheral nervous system and the CNS, the dynamic changes of the sensory pathway, and drug responses associated with HIV DSP. In fact, the LP-BM5 model was found to be the “most suitable for the rapid and cost effective initial screening of drugs, drug combinations” in a comprehensive review on commonly used in vivo HIV/AIDS models (20).

Changes in several cytokines, particularly proinflammatory cytokines, have been observed in patients with HIV-associated peripheral neuropathy (49, 50), and it has been suggested that proinflammatory cytokines are involved in the pathogenesis of HIV-associated peripheral neuropathy. The upregulation of selected cytokines has also been observed in animal models of HIV DSP (51). However, the specific roles of these cytokines in the development of HIV-associated peripheral neuropathy are still unclear and are often simply summarized as promoting neuroinflammation. In the current study, we observed tissue-specific changes in selected cytokines that occurred along with the development of LP-BM5–induced peripheral neuropathy (i.e., a gradual increase in IL-12p40 in the lumbar spinal cord, the persistent elevation of IL-1β and IFN-γ in lumbar DRG, and the transient increase of IL-6, IFN-γ, and IL-1β in the paw skin). Data suggest an association between infection-induced cytokine responses and the development of peripheral neuropathy. In particular, further investigation is necessary to delineate the role of specific cytokines in LP-BM5–induced behavioral and pathological changes in particular tissues and organs. Moreover, other relevant cytokines/chemokines can also be investigated in the future. In addition, colocalization studies using immunohistochemical techniques will help identify the source(s) of each cytokine involved, and targeted treatment strategies could be developed accordingly in the future. Currently, although changes in certain cytokines have been identified in humans with sensory neuropathy (49, 50), the tissue-specific cytokine expression data from human patients are very limited and often affected by antiviral treatment. We believe that our investigation with the LP-BM5 model will help determine the role of individual cytokines and cytokine networks in the development of HIV-associated peripheral neuropathy.

Paradoxically, patients with HIV-associated peripheral neuropathy often present with enhanced neuroinflammation/immune activation and systemic immunodeficiency simultaneously. A clear association exists between the diagnosis of AIDS/low CD4 count and the development of HIV-associated peripheral neuropathy (3, 52, 53). Whether the development of peripheral neuropathy depends directly on viral-induced immunodeficiency has not been investigated in detail. Our results indicate that the development of behavioral hypersensitivity is closely associated with the development of MAIDS-related features, whereas the reduction of IEFN density is transient and does not worsen with increased MAIDS severity. However, both of these peripheral neuropathy-related changes are dependent on LP-BM5 infection, as AZT treatment effectively reduced the viral loads in all tissues examined and reversed these changes simultaneously. These data suggest that peripheral neuropathy-related features may be differentially dependent on the development of immunodeficiency. This may aid in understanding the differences observed in various types of HIV-associated peripheral neuropathy (2). Further, in patients with HIV-associated peripheral neuropathy, IEFN density was found to be inversely correlated with neuropathic pain, and this correlation seemed to be stable over time (i.e., the fiber density did not recover) (6), which is somewhat different from what we have observed in LP-BM5–infected mice. However, careful examination of the underlying mechanisms that lead to the recovery in IEFN density in LP-BM5–infected mice may prove valuable in the development of novel treatments for HIV-associated peripheral neuropathy.

In this current study, we focused on the hind paw because the development of HIV-associated peripheral neuropathy often starts.
in the lower limits in humans. However, we cannot exclude pathological changes in the front paws of infected animals. To further assess this animal model, we have begun to examine the behavioral and histological changes in the front paws of LP-BM5–infected animals.

In summary, we believe that the LP-BM5 infection model can aid the investigation of HIV infection-associated peripheral neuropathy, particularly HIV DSP, and can be used to evaluate various drugs for alleviating this chronic neurological condition.

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Disclosures

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