Cerebral Expression of Interleukin-12 Induces Neurological Disease via Differential Pathways and Recruits Antigen-Specific T Cells in Virus-Infected Mice

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Transgenic expression of interleukin-12 (IL-12) in astrocytes causes a spontaneous inflammatory central nervous system disorder in aged mice. Here we show that spontaneous disorder developed only when both mature lymphocytes and interferon (IFN)- γ were present. Infection with noncytolytic Borna disease virus (BDV) did not affect wild-type mice but accelerated disease of IL-12 transgenic mice. Infection of transgenic mice lacking lymphocytes did not result in neurological symptoms. In contrast, BDV infection of transgenic mice lacking IFN- γ induced neurological disease with delayed onset of symptoms that resembled those in infected transgenic mice with a functional IFN- γ gene. In BDV-infected transgenic mice devoid of IFN- γ no cerebellar calcification was observed, and multiplication of BDV was not inhibited. To determine the antigen specificity of lymphocytes in brains of diseased animals, the IL-12 transgene was introduced into an H-2^k genetic background. Infection of IL-12 transgenic H-2^k mice resulted in extensive lymphocytic infiltration into the cerebellum but not into other brain regions that also contained viral antigen but expressed the transgene at lower levels. Tetramer analysis revealed that most CD8 T cells in the cerebellum of such mice were BDV-specific. Our results thus demonstrate that IFN- γ secreting lymphocytes are responsible for disease of IL-12 transgenic mice. They further suggest that expression of IL-12 in the central nervous system may lead to localized recruitment of T cells that recognize antigens expressed in the brain. (*Am J Pathol* 2004, 165:949–958)

Interleukin 12 (IL-12) is a key proinflammatory cytokine in the regulation of immune responses. It is secreted mainly by macrophages, dendritic cells, and granulocytes after stimulation and exerts its effects mainly on T and B lymphocytes and natural killer (NK) cells. In CD4 T cells IL-12

induces the differentiation from a Th0 to a Th1 phenotype^{1,2} that is characterized by up-regulation of numerous proinflammatory cytokines, including several chemokines and interferon (IFN)- γ .^{3–5} IFN- γ is the only member of the type II family of IFNs.⁶ On secretion it activates various host defense pathways.^{7–13} These include up-regulation of MHC class I and II molecules, expression of cytokines, chemokines such as CXCL10, and inflammation-related molecules such as the inducible NO-synthetase (NOS-2). This causes further recruitment of immune cells and additional production of IL-12. Thus, the IL-12/IFN- γ system is regulated by a positive feedback mechanism that is critical for the initiation and aggravation of inflammatory immune responses.

We have shown previously that transgenic expression of IL-12 in the cerebellum of mice (termed GF-IL12 mice) causes a spontaneous neurological disorder at 4 to 6 months of age.¹⁴ This disease is characterized by leukocyte infiltration, tissue destruction, calcification, hypomyelination, and up-regulation of proinflammatory cytokines (tumor necrosis factor- α , IL-1 α , IL-1 β , and IFN- γ) at the sites of transgene expression. Furthermore, stimulation of presymptomatic GF-IL12 mice with complete Freund's adjuvant lacking neural antigens as well as neonatal infection of newborn GF-IL12 mice with Borna disease virus (BDV) significantly accelerates the onset of the disease.^{15,16}

BDV is a neurotropic nonsegmented negative-stranded RNA virus that naturally infects horses and sheep causing an often lethal nonpurulent meningoencephalitis.¹⁷ BDV also replicates well in the central nervous system (CNS) of most mouse strains but induction of disease is limited to few susceptible strains. In susceptible mice the disease is characterized by a cellular immune response that depends on both CD4 and CD8 T cells.^{18–20} In mice bearing the H-2^k haplotype such as MRL and B10.BR mice a specific T cell epitope (TELEISSI) of the BDV-nucleoprotein (BDV-N) has been

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identified that is recognized by most infiltrating cytotoxic CD8 T cells.²¹ However, different mouse strains of this haplotype show considerable differences in the susceptibility to Borna disease (BD). Thus, neonatally BDV-infected MRL mice develop BD within several weeks²² whereas B10.BR mice stay healthy unless their immune system is stimulated further by peripheral expression of viral antigens. This demonstrates that B10.BR mice like MRL mice possess virus-specific T cells although they are otherwise disease resistant.²³ Resistant strains show only few lymphocyte infiltrates despite high BDV antigen load in the CNS.

In the present work, we show 1) that IFN- γ secreted by infiltrating lymphocytes is critical for the spontaneous disease and the observed cerebellar calcifications in GF-IL12 mice; 2) that lymphocytes but not IFN- γ are necessary for disease induction in BDV-infected GF-IL12 mice; 3) that IFN- γ mediates the reduced multiplication of BDV in the cerebellum of GF-IL12 mice; and (4) that cerebral expression of IL-12 may specifically trigger the localized recruitment of CD8 T cells that recognize cerebrally expressed antigens, thereby inducing an immune-mediated disorder in otherwise disease-resistant animals.

Materials and Methods

Animals

The generation and characterization of GF-IL12 mice expressing both subunits of the IL-12 heterodimer under transcriptional control of the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter have been described previously.¹⁴ GF-IL12 mice on a C57BL/6 background were crossed with C57BL/6 and B10.BR mice providing litters on two different genetic backgrounds. In addition, GF-IL12 mice were crossbred with mice bearing a disruption of the IFN- γ gene²⁴ leading to offspring with no functional IFN- γ genes (GF-IL12xIFN $\gamma^{-/-}$). The GF-IL12 mice were also crossbred with mice lacking a functional recombination activating gene (RAG2)²⁵ providing GF-IL12 mice with a homozygous deletion of RAG2 (GF-IL12xRAG2 $^{-/-}$). In all breedings only one parent was heterozygous for the IL-12 transgene providing offspring consisting of mice bearing one copy of the IL-12 transgene and wild-type littermates. IFN $\gamma^{-/-}$ and RAG2 $^{-/-}$ mice were on a C57BL/6 genetic background. Breeding was performed in the animal facility of the University of Freiburg.

Virus Stocks and Infection of Mice

A rat-adapted strain of BDV was adapted to the mouse by four consecutive passages through brains of newborn BALB/c mice, and two further passages through brains of adult MRL mice and a single passage through brains of adult rats. The seed virus derived from strain RW98, previously described as He/80.²² Ten μ l of brain homogenate containing virus was injected intracerebrally within

72 hours after birth. Animals were examined daily for disease symptoms such as ataxia and hunched posture.

RNA Isolation

Mice were anesthetized and brains were removed. One hemisphere was dissected into forebrain and cerebellum, snap-frozen in liquid nitrogen, and stored at -80°C until RNA extraction was performed. Total RNA was extracted with Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. RNA was dissolved in TE (10 mmol/L Tris, pH 7.4, 1 mmol/L ethylenediaminetetraacetic acid) and stored at -80°C .

RNase Protection Assays

RNase protection assays were performed as described previously.²⁶ Five μ g of total RNA were used for each sample and hybridized with the following probe sets: mCK5 (RiboQuant Multi-Probe template sets; BD Biosciences, Franklin Lakes, NJ) and AP9-containing probes for CD3, IL-10, tumor necrosis factor- α , NOS-1, NOS-2, NOS-3, IL-1 α , IFN- γ , IL-12 p40-subunit, GFAP, BDV-N (BDV nucleoprotein), and the RPL32-4A gene²⁷ that served as an internal loading control.¹⁵ Biomax films (Eastman-Kodak, Rochester, NY) were exposed for various periods of time and scanned using a ScanJet 4C (HP, Hewlett-Packard, Palo Alto, CA). NIH Image software, version 1.62, was used to quantify the autoradiographs.

Histology and Immunohistochemistry

The remaining brain hemispheres were either embedded in Tissue Tek (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and shock-frozen in liquid nitrogen-chilled isopentane or fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Paraffin sections (4 μ m thick) were processed according to standard procedures and used for routine staining and immunohistochemistry for GFAP (DAKO, Hamburg, Germany) and BDV-N (kind gift from I. Lipkin, Columbia University, New York, NY). Alizarin Red stain was used for the detection of calcification. Cryostat sections (12 μ m thick) were air-dried and fixed in cold (-20°C) acetone-methanol (1:1) for 45 seconds. Endogenous peroxidase was blocked for 18 minutes with 0.3% H_2O_2 in PBS. Subsequently, sections were incubated with normal serum (ABC kit; Vector Laboratories, Burlingame, CA) for 30 minutes. Primary rat monoclonal antibodies against infiltrating lymphocytes (CD4, CD8, CD45 receptor), macrophages [MAC3 (membrane attack complex 3), BD-Pharmingen, San Diego, CA], MHC class II (I-A^b MHC II alloantigen biotinylated; BD-Pharmingen, San Diego, CA) and NOS-2 (BD-Transduction Laboratories, Franklin Lakes, NJ) were incubated overnight at 4°C . Bound primary antibody was detected using either a mouse-adsorbed, biotinylated anti-rat antibody for the cell surface molecules (Southern Biotechnology Associates, Birmingham, AL), or the biotinylated secondary antibody from the ABC kit followed by horseradish peroxi-

Table 1. Frequency and Onset of Disease in Uninfected and BDV-Infected Wild-Type, RAG2^{-/-}, IFN γ ^{-/-}, GF-IL12, GF-IL12xRAG2^{-/-}, and GF-IL12xIFN γ ^{-/-} Mice

	Strain	Frequency of disease	Age at onset of disease (mean), days	Observation period, days
Uninfected	GF-IL12	56/60 (93 %)	92–175 (131.9)	90–270
	GF-IL12xRAG2 ^{-/-}	0/30		150–730
	GF-IL12xIFN γ ^{-/-}	0/30		150–730
BDV-infected	Wild type	0/6	31–50 (37.5)	64–140
	RAG2 ^{-/-}	0/8		102–134
	IFN γ ^{-/-}	0/7		84–91
	GF-IL12	10/10 (100%)		45–84
	GF-IL12xRAG2 ^{-/-}	0/12		102–134
	GF-IL12xIFN γ ^{-/-}	11/11 (100%)		84–91

z-dase-coupled avidin biotin complex (ABC kit) and 3–3'-diaminobenzidine (Merck, Darmstadt, Germany) as a substrate. Sections were counterstained with Mayer's hematoxylin for 15 seconds, dehydrated in graded ethanol and xylene, and mounted.

Flow Cytometry Analysis of Brain Lymphocytes

Lymphocytes were isolated from the brains of severely diseased BDV-infected GF-IL12 mice on a C57BL/6 and a C57BL/6xB10.BR genetic background as described previously.^{28,29} Lymphocytes were resuspended in phosphate-buffered saline containing 2% fetal calf serum and 0.1% NaN₃. The cell suspensions were divided into three samples and incubated either with fluorescein isothiocyanate-labeled anti-CD8 and phycoerythrin-labeled anti-CD4 antibodies, with an APC-labeled anti-CD8 antibody alone or with APC-labeled anti-CD8 antibody and an H-2K^k tetramer complexed with the immunodominant BDV peptide epitope TELEISSI (H-2K^k/TELEISSI). Tetramer staining with or without anti-CD8 antibodies was performed at room temperature at 1:100 dilution for 30 minutes. The specificity of the tetramer was demonstrated by using brain mononuclear cells derived from the inflamed CNS of lymphocytic choriomeningitis virus-infected mice at the peak of neurological disease as negative control (Engelhardt KR, Staeheli P, and Hausmann J, submitted). The MHC I tetramer was kindly provided by the National Institute of Allergy and Infectious Diseases tetramer facility, Atlanta, GA. After washing, analysis of cells was performed on a FACSsort flow cytometer (Becton Dickinson, Heidelberg, Germany).

Statistical Evaluation

Statistical significance was tested with the Mann-Whitney U-test for unpaired groups.

Results

No Spontaneous Neurological Disease in GF-IL12 Mice Lacking B and T Cells or IFN- γ

Spontaneous neurological disease in GF-IL12 mice is accompanied by mononuclear cell infiltration into the

cerebellum.¹⁴ Whether and how these immune cells might contribute to disease remained elusive. To determine whether mature B and T lymphocytes are indeed required for the development of disease, we introduced the GF-IL12 transgene into mice with a homozygous deletion of the RAG2 gene. Unlike GF-IL12 mice that usually develop spontaneous neurological disorder at ~4 to 5 months of age, none of the 30 GF-IL12xRAG2^{-/-} mice that we observed in our facility for 150 to 730 days developed symptoms of neurological disease (Table 1). Histological analysis of brains of such mice revealed no overt pathological alterations (data not shown). Unlike in brains of diseased GF-IL12 mice, leukocyte infiltrates, tissue destruction, hypomyelination, or calcification were absent in GF-IL12xRAG2^{-/-} mice. Furthermore, expression of cytokines and cell surface molecules was normal (data not shown). Thus, IL-12-induced neurological disorder is mediated by infiltrating lymphocytes.

Because IL-12 can enhance IFN- γ synthesis by T cells, we next determined whether transgenic IL-12 expression would induce neurological disease in mice with a disrupted IFN- γ gene. None of the 30 GF-IL12xIFN γ ^{-/-} mice developed spontaneous neurological disorder during the entire observation period of 150 to 730 days (Table 1). Brain tissue of GF-IL12xIFN γ ^{-/-} mice showed no increased infiltration by mononuclear cells, and hypomyelination or calcifications were not observed. Expression of proinflammatory cytokines and chemokines such as tumor necrosis factor- α , NOS-2, CXCL10, CCL5, and XCL1 was not altered as compared to wild-type mice (data not shown). Thus, induction of disease and recruitment of lymphocytes into the cerebellum by transgenically expressed IL-12 involves at least one step that depends on IFN- γ .

Accelerated Disease in Virus-Infected GF-IL12 Mice Requires Lymphocytes but Not IFN- γ

If newborn GF-IL12 mice are infected with a noncytolytic, neurotropic virus that does not cause disease by itself, the onset of neurological disease is greatly accelerated.¹⁵ To determine whether virus infection would trigger neurological disease in GF-IL12 mice in the absence of lymphocytes, we challenged newborn GF-IL12xRAG2^{-/-} mice with BDV. Like wild-type and RAG2^{-/-} control mice,

Figure 1. Development of neurological disorder in BDV-infected, IL-12 transgenic C57BL/6 mice with and without defective RAG2 or IFN- γ genes. Newborn mice were infected intracerebrally with BDV as described in Materials and Methods. The animals were examined daily for neurological symptoms. Group sizes varied between 10 and 12 animals. The onset of disease in infected GF-IL12xIFN γ ^{-/-} mice was significantly delayed as compared to infected GF-IL12 mice (*, $P < 0.0005$; Mann-Whitney) while all infected GF-IL12xRAG2^{-/-} mice stayed healthy.

none of the 12 BDV-infected GF-IL12xRAG2^{-/-} mice developed neurological symptoms during the entire observation period of 102 to 134 days (Table 1). RNase protection assays revealed no alteration of cytokine and chemokine gene expression in the brains of these mice (data not shown). Multiplication of BDV in brains of GF-IL12xRAG2^{-/-} was comparable to wild-type and RAG2^{-/-} mice. Furthermore, no histological signs of tissue destruction, hypomyelination, or calcification were detected in brains of infected GF-IL12xRAG2^{-/-} mice (Figure 2, E and F).

We next evaluated whether viral infection of the brain would induce disease in GF-IL12 mice if no IFN- γ is present. Table 1 shows that all 11 GF-IL12xIFN γ ^{-/-} mice that we infected with BDV as newborns developed neurological disease. Symptoms were similar to those observed in infected GF-IL12 mice with an intact IFN- γ gene. However, disease onset was significantly delayed ($P < 0.0005$, Mann-Whitney test) in BDV-infected GF-IL12xIFN γ ^{-/-} mice (Figure 1). Whereas transgenic mice with an intact IFN- γ gene started to show signs of disease between days 31 and 50 after infection, BDV-infected GF-IL12xIFN γ ^{-/-} mice developed disease between days 56 and 64. Thus, simultaneous presence of IL-12 and virus in the CNS seemed to generate a sufficiently strong signal for induction of neurological disease, even if IFN- γ was absent.

Altered Pathology in the Cerebellum of Virus-Infected GF-IL12 Mice Lacking IFN- γ

Key pathological features in the cerebellum of GF-IL12 mice with neurological disease after BDV infection include severe infiltration by mononuclear cells, reactive astrocytosis, loss of granule cells, and prominent calcium deposits (Figure 2A). Furthermore, widespread demyelination and spongiosis of the cerebellar white matter is observed (Figure 2B). Similarly, virus-infected GF-IL12xIFN γ ^{-/-} mice with neurological disease also showed extensive mononuclear infiltrates in the menin-

ges and perivascular cuffs as well as numerous lymphocytes within the cerebellar parenchyma (Figure 2C). Immunohistochemistry showed that the infiltrates consisted mainly of CD4 and CD8 T cells, macrophages, and low numbers of infiltrating B cells (data not shown). As in diseased GF-IL12 mice that possess an intact IFN- γ gene, we observed pronounced reactive astrocytosis and signs of tissue destruction such as a severe loss of granule cells and demyelination in BDV-infected GF-IL12xIFN γ ^{-/-} mice (Figure 2D). However, these pathological alterations were less pronounced in IFN- γ -deficient mice. The most striking difference between these two groups of mice was that no calcifications were detected in BDV-infected GF-IL12xIFN γ ^{-/-} mice (Figure 2C). Thus, IFN- γ appears to be essential for the formation of the characteristic calcium deposits in the CNS of mice transgenically expressing IL-12. Other CNS regions of BDV-infected GF-IL12 and GF-IL12xIFN γ ^{-/-} mice displayed no overt pathological alterations.

Cytokine Gene Expression in the Cerebellum of Infected GF-IL12 Mice Lacking IFN- γ

RNase protection assays were performed to characterize alterations in cytokine gene expression in the cerebellum of diseased mice. In BDV-infected GF-IL12 mice with or without functional IFN- γ genes we observed enhanced expression of the T-cell marker CD3 and of GFAP, indicating inflammation and astrocytosis. Up-regulation of several cytokine and chemokine genes including tumor necrosis factor- α , IL-1 α , XCL1, CXCL10, CCL2, CCL3, CCL4, and CCL5 in the cerebellum of symptomatic BDV-infected GF-IL12xIFN γ ^{-/-} mice was similar to that seen in BDV-infected GF-IL12 mice (data not shown).¹⁵ Importantly, there was approximately a fourfold up-regulation of the IFN- γ -inducible NOS-2 gene in diseased GF-IL12 mice, while there was no altered expression of this gene in the cerebellum of GF-IL12xIFN γ ^{-/-} mice (Figure 3A).

IFN- γ Mediates the Reduced Multiplication of BDV in the Cerebellum of GF-IL12 Mice

Our previous analysis showed that transgenic expression of IL-12 in the cerebellum has a negative effect on BDV multiplication in this brain area.¹⁵ This was also true for our present experiments with GF-IL12 mice on a C57BL/6 genetic background (Figure 3B). We found that viral BDV-N gene transcript levels were almost twofold lower in the cerebellum of transgenic mice as compared to nontransgenic littermates ($P < 0.02$, Mann-Whitney test). In other areas of the brain, expression of the viral N gene was not influenced by the transgene (data not shown). In the absence of IFN- γ , however, IL-12 had no antiviral effect (Figure 3B). Thus, BDV inhibition at the site of prominent IL-12 transgene expression was dependent on IFN- γ .

Figure 2. Histological alterations in the cerebellum of BDV-infected GF-IL12 mice with and without functional IFN- γ or RAG2 genes. Newborn mice were infected intracerebrally with BDV as described in Materials and Methods. The animals were examined daily for neurological symptoms. Infected GF-IL12 and GF-IL12xIFN $\gamma^{-/-}$ mice were symptomatic at the time of sacrifice while GF-IL12xRAG2 $^{-/-}$ mice were healthy. **A** and **B**: Cerebellum of diseased GF-IL12 mice. Note the meningeal, parenchymal, and perivascular (**arrows**) mononuclear infiltrates, granule cell loss, calcification (**arrowheads**) and extensive demyelination. **C** and **D**: Cerebellum of diseased GF-IL12xIFN $\gamma^{-/-}$ mice. Note the similar but less pronounced alterations and the absence of calcifications. **E** and **F**: Cerebellum of healthy GF-IL12xRAG2 $^{-/-}$ mice. No overt histopathological alterations were found as compared to wild-type mice. gcl, granule cell layer; me, meninges; ml, molecular layer; wm, white matter. H&E stain: **A**, **C**, **E**; Klüver-Barrera myelin stain: **B**, **D**, **F**. Original magnifications, $\times 20$.

Localized Recruitment of Antigen-Specific T Cells into Brain Areas with IL-12 Expression

The antigen specificity of T cells in the cerebellum of diseased GF-IL12 mice is unknown. The lack of obvious

candidate antigens complicates experimental approaches in animals that develop disease spontaneously. We reasoned that this type of analysis might be feasible in BDV-infected H-2^k mice. The brain-infiltrating CD8 T cells of these mice mainly recognize a single, well-char-

Figure 3. Expression of NOS-2 (A) and BDV-N (B) in the cerebellum of BDV-infected IL-12 transgenic and nontransgenic mice with or without a functional IFN $\gamma^{-/-}$ gene. Newborn mice were infected intracerebrally with BDV as described in Materials and Methods. Transgenic mice showed neurological symptoms. Healthy nontransgenic control mice were sacrificed at similar ages. Cerebellar RNA was prepared from individual mice ($n = 4$) and subjected to RNase protection assay as described in Materials and Methods. Autoradiographs were scanned and optical densities of the bands were measured. Graphs show mean values, standard deviations, and the statistical significance (Mann-Whitney).

acterized viral epitope derived from the BDV nucleoprotein (TELEISSI).²¹ We therefore crossbred our transgenic GF-IL12 mice with B10.BR (H-2^k) mice to produce F1 offspring of the desired genetic makeup. We first analyzed whether this mixed genetic background might affect spontaneous and BDV-induced disease development in transgenic mice. Uninfected transgenic animals of this type developed spontaneous disease at approximately day 145, which is well within the range observed in GF-IL12 mice that have a pure C57BL/6 background (compare Table 1). After infection with BDV, transgenic mice of mixed genetic background started to develop neurological symptoms at an age of 33 days. By day 77, 33 of the 34 infected transgenic animals had developed disease. The average onset of disease was 51.8 days compared to 37.5 days in GF-IL12 mice of a pure C57BL/6 genetic background. The onset of disease in transgenic mice of mixed genetic background was generally abrupt with severe symptoms within the first 2 days. Therefore, most of these mice had to be killed within 2 days for ethical reasons. This contrasted to the slowly progressive course of disease in GF-IL12 mice with a pure C57BL/6 genetic background. Histological and immunohistochemical examination of the cerebellum revealed similar pathological features except for the ab-

Figure 4. Histological alterations in the cerebellum (A) and hippocampus (B) and flow cytometric analysis of brain lymphocytes (C–G) from BDV-infected IL-12 transgenic mice with pure C57BL/6 and mixed C57BL/6x6B10.BR genetic background. Newborn mice were infected intracerebrally with BDV as described in Materials and Methods. The animals were examined daily for neurological symptoms. Mice were symptomatic at the time of sacrifice. **A:** There were mononuclear infiltrates in the meninges (arrows) as well as perivascular cuffing (arrowheads) and granule cell loss in the cerebellum of diseased GF-IL12 mice. **B:** There were no leukocyte infiltrates or signs of neurodegeneration in the hippocampus. **C–F:** CNS mononuclear cells isolated from brains of diseased BDV-infected GF-IL12 mice on a pure C57BL/6 (C, D) or mixed C57BL/6x6B10.BR background (E, F) were stained for flow cytometric analysis with anti-CD8-APC alone (C, E) or double stained with anti-CD8-APC and H-2K^b/TELEISSI-PE tetramer (D, F). The percentages of CD8 T cells co-staining with the MHC I tetramer in the CD8 T-cell population are indicated by the numbers in the corresponding quadrant. Note that the majority of CD8 T cells were specific for TELEISSI, which is the immunodominant BDV epitope in mice with an H-2^k haplotype. **G:** CNS mononuclear cells isolated from the brain of a diseased BDV-infected C57BL/6x6B10.BR animal were stained for flow cytometric analysis with anti-CD4-phycoerythrin and anti-CD8-fluorescein isothiocyanate. Numbers in the respective quadrants refer to the percentage of stained cells from all cells in the lymphocyte/monocyte gate. Note that CD4 and CD8 T cells were present at comparable frequencies. gcl, granule cell layer; ml, molecular layer; wm, white matter; ca, Cornu ammonis; dg, dentate gyrus. H&E stain. Original magnifications, $\times 10$.

sence of calcification in BDV-infected transgenic mice of mixed genetic background. The absence of calcification in these mice is most likely a consequence of the short duration between onset of symptoms and death.

Surprisingly, inflammation in virus-infected transgenic mice of mixed genetic background was still restricted to the cerebellum (Figure 4A). Virtually no immune cells were observed in the neocortex and in the hippocampus (Figure 4B) where BDV antigen was present even more abundantly than in the cerebellum (data not shown). This observation suggested that the brain lymphocytes of these animals were not specific for BDV. Alternatively, virus-specific T cells were present but they did not accumulate at the sites of highest antigen load but rather migrated to the site of IL-12 expression. Flow cytometric analysis of lymphocytes extracted from the brains of diseased animals was performed to distinguish between these possibilities. The brain immune cell infiltrates of diseased GF-IL12 mice of mixed genetic background contained comparable numbers of CD4 cells and CD8

cells (Figure 4C). The vast majority of the CD8 T cells in these lymphocyte preparations specifically bound to TELEISSI peptide-loaded tetrameric MHC I complexes (Figure 4D). Values in different animals ranged from 54.4 to 79.5% (average, 70%). Such TELEISSI-specific CD8 T cells could not be detected in cervical lymph nodes and spleens of BDV-infected mice at the peak of neurological disease (Engelhardt KR, Staeheli P, and Hausmann J, submitted), which reflects the strict neurotropism of BDV. These results strongly favor the view that activated lymphocytes do not enter virus-infected brain areas in a random manner. They rather seem to accumulate preferentially at the sites of transgenic IL-12 expression.

Discussion

Interleukin-12 is a key mediator of innate and adaptive cellular immune responses causing activation of NK cells and lymphocytes and initiation of a Th1-type immune response via induction of IFN- γ .^{3,30} We previously showed that transgenic expression of IL-12 in the cerebellum of mice is an adequate trigger for a Th1-type immune response in the areas of transgene expression.^{14,15} The present study was aimed to dissect the impact of infiltrating lymphocytes and IFN- γ as mediators of disease in GF-IL12 mice.

Physiologically activated T lymphocytes enter the parenchyma of the central nervous system and perform an immune surveillance.³¹ Under normal conditions, these lymphocytes will be eliminated from the central nervous system by apoptosis unless they recognize an antigen or are otherwise stimulated.^{32,33} Our observations in GF-IL12 mice have demonstrated that the presence of IL-12 in the CNS not only is sufficient for the retention of lymphocytes in the brain but also stimulates these cells to exert a localized Th1-like immune response that is accompanied by demyelination, calcification, and reactive astrogliosis.¹⁴ To elucidate the relevance of infiltrating lymphocytes and NK cells we crossbred GF-IL12 mice with mice deficient of the RAG2 gene. These mice lack functionally active T and B lymphocytes while NK cell function is normal.²⁵ In contrast to IL-12 transgenic mice with normal lymphocyte-function GF-IL12xRAG2^{-/-} developed neither signs of a neurological disease nor overt histological alterations such as inflammatory lesions or calcifications or up-regulation of cytokines and cell surface markers. These observations suggest that in the course of the spontaneous disease of GF-IL12 mice lymphocytes are the predominant source of IFN- γ in the CNS. Similar observations have been reported from lymphocytic choriomeningitis virus-infected mice stimulated with lipopolysaccharide in which infiltrating CD8 T lymphocytes were the predominant source of IFN- γ , whereas NK cells contributed only negligibly to IFN- γ production.³⁴

Because IFN- γ holds a central position in the mediation of IL-12-induced Th1-type immune responses^{4,35-37} we crossbred GF-IL12 mice with mice lacking functional IFN- γ genes. Like GF-IL12xRAG2^{-/-} mice, GF-IL12xIFN γ ^{-/-} mice did not develop clinical symptoms

during their normal life span. Likewise, histology and RNA analysis showed no overt pathological changes or induction of cytokines and chemokines in the hindbrain of these mice. This demonstrates that IL-12-triggered IFN- γ production is an essential component in the development of spontaneous disease. Induction of proinflammatory molecules such as adhesion molecules and activators of immune responses was greatly reduced in GF-IL12 mice lacking IFN- γ . This might minimize lymphocyte retention and activation. These findings clearly demonstrate that in mice with transgenic expression of IL-12 in the CNS, activation and retention of lymphocytes is crucial for the spontaneous development of disease and that IFN- γ is a pivotal mediator in the pathogenesis of the disorder.

The onset of the spontaneous disease in GF-IL12 mice can be significantly accelerated by stimulation with either complete Freund's adjuvant and pertussis toxin¹⁶ or infection with an otherwise benign virus such as BDV.¹⁵ Both, uninfected as well as BDV-infected GF-IL12xRAG2^{-/-} mice did not develop signs of a neurological disorder demonstrating the requirement of lymphocytes for both the spontaneous and the BDV-accelerated disease in GF-IL12 mice. In contrast, neonatal BDV infection of GF-IL12xIFN γ ^{-/-} mice caused neurological symptoms comparable to those found in BDV-infected GF-IL12 mice. The combination of IL-12 expression and infection with BDV seems to exceed a threshold leading to disease that is not reached in uninfected GF-IL12 mice lacking IFN- γ . We have shown before that BDV induces production of various chemokines in the CNS of infected animals.^{38,39} In combination with transgenically expressed IL-12 these IFN- γ -independent mechanisms are obviously a sufficient stimulus for infiltrating lymphocytes to initiate an immune reaction resulting in disease and tissue destruction.

However, there were distinct differences in the disease of BDV-infected GF-IL12xIFN γ ^{-/-} mice as compared to the disease in uninfected and infected GF-IL12 mice. First, the onset of the disorder was significantly delayed in BDV-infected GF-IL12xIFN γ ^{-/-} mice as compared to infected GF-IL12 mice. Although up-regulation of various proinflammatory cytokine and chemokine genes was less pronounced in infected GF-IL12xIFN γ ^{-/-} mice as compared to infected GF-IL12 mice, RNA levels of the IFN- γ -inducible NO-synthetase (NOS-2) were not altered in infected GF-IL12xIFN γ ^{-/-} mice. Second, histological alterations in the cerebellum of GF-IL12xIFN γ ^{-/-} mice resembled the cerebellar lesions seen in GF-IL12 mice in many ways. Strikingly, calcification was absent in GF-IL12xIFN γ ^{-/-} mice. Interestingly, reports from four independent lines of transgenic mice expressing IFN- γ in the CNS described either spontaneous or EAE-induced demyelination and clinical symptoms (ataxia) similar to those found in GF-IL12 mice, however, calcifications were observed in none of these mice.⁴⁰⁻⁴³ These observations suggest that IFN- γ expression in the transgenic models was not high enough or that IFN- γ expression in the CNS alone is not sufficient for the development of calcification and that IL-12 and IFN- γ together mediate the formation of calcification in the cerebellum of GF-IL12 mice. Interestingly, mice with cerebellar IFN- α expression

showed calcifications too.⁴⁴ These were even more pronounced in IFN- α transgenic mice with deletion of the STAT1 gene,⁴⁵ which is part of the signal transduction complex of both IFN- α and IFN- γ . Although the mechanisms leading to calcification in IFN- α transgenic mice have not been resolved, it has been shown that IFN- α and IL-12 share some overlapping functions.^{46,47} Therefore, the calcification seen in GF-IL12 mice might be because of the same underlying mechanism as in IFN- α transgenic mice.

Independent of their genetic background (C57BL/6 and C57BL/6xSJL¹⁵) BDV-infected GF-IL12 mice showed a significantly reduced virus multiplication in the cerebellum as compared to wild-type mice. In contrast no reduced virus multiplication was observed in the cerebellum of BDV-infected GF-IL12xIFN $\gamma^{-/-}$ mice. This clearly demonstrates that IFN- γ mediates the observed reduction of virus multiplication in GF-IL12 mice¹⁵ and highlights the significance of our observations in BDV-infected cerebellar and hippocampal slice cultures in which IFN- γ treatment significantly inhibits virus multiplication.⁴⁸ Our results are in line with reports that demonstrated that IFN- γ mediates suppression of different viruses such as murine cytomegalovirus, vesicular stomatitis virus, polio virus, influenza virus, alphavirus, and Theiler's murine encephalomyelitis virus.^{7-9,49-51} In the case of murine cytomegalovirus Tanaka and Noda⁴⁹ demonstrated further that IFN- γ exerts this effect through induction of NOS-2, which is crucial for virus elimination. However, induction of NOS-2 was not detectable in BDV-infected cerebellar slice cultures treated with IFN- γ .⁴⁸ This indicates that at least in slice cultures NOS-2 is not required for the inhibition of BDV proliferation.

The antigen specificity of infiltrating lymphocytes in the spontaneous and BDV-accelerated disease of GF-IL12 mice still remains unresolved. Because of the large number of potential antigens in uninfected GF-IL12 mice it is more feasible to determine potential antigens in the presence of a specific well-defined pathogen such as BDV. Because CD8 T cells of mouse strains with an H-2^k haplotype recognize a specific epitope of the BDV-nucleoprotein (TELEISSI),²¹ we crossbred GF-IL12 mice on a C57BL/6 (H-2^b) background with B10.BR (H-2^k) mice. Uninfected GF-IL12 mice on this mixed background developed a spontaneous disease that was indistinguishable from the spontaneous disease in GF-IL12 mice on a pure C57BL/6 background. In contrast, the disease of BDV-infected GF-IL12 mice on the mixed C57BL/6xB10.BR background showed differences from the disease in BDV-infected GF-IL12 mice on a C57BL/6 background. The observed differences suggest a dual pathology in BDV-infected GF-IL12 mice on the mixed background. Lymphocyte infiltrates with a predominance of CD4 T cells over CD8 T cells as well as reduced virus multiplication in the cerebellum and only minute changes in the forebrain resembled the IL-12-mediated spontaneous disease in GF-IL12 mice on other genetic backgrounds (C57BL/6 and C57BL/6xSJL¹⁵). However, BDV-infected GF-IL12 mice on the mixed background showed a fierce onset of disease symptoms characteristic for BD in disease-sensitive mouse strains²² whereas BDV-in-

fecting GF-IL12 mice on a pure C57BL/6 and a mixed C57BL/6xSJL background present with a slowly progressive course of disease.¹⁵ Most important, analysis of brain lymphocytes revealed a substantial percentage of CD8 T cells recognizing the TELEISSI fragment of BDV-N. In contrast to peripheral immunization of BDV-infected B10.BR mice, in which inflammation is observed throughout the brain,²³ cerebellar IL-12 production merely induced a localized specific immune response in areas of transgene expression. Intriguingly, in the latter mice lymphocytic infiltration did not spread to other CNS areas. The containment of the immune response to the cerebellum highlights that lymphocyte entry into the CNS not only depends on the presence of a particular antigen but even more so on the presence of certain cytokines in the brain that provide a danger signal⁵² required for an immune response. BDV-induced chemokine expression and IL-12 together seem to provide such a danger signal that fuels the invasion of BDV-specific lymphocytes into the cerebellum. Another reason for the absence of leukocyte infiltrates in the cerebrum might be the short duration of the disease causing death within few days in most mice that might prevent the inflammation from dissemination.

Taken together, we have shown that both infiltrating lymphocytes and IFN- γ are essential for the development of the spontaneous neurological disease observed in mice with transgenic expression of IL-12 in the CNS. Stimulated infiltrating lymphocytes secrete IFN- γ as the key mediator causing leukocytes and resident cells in the CNS to secrete additional proinflammatory cytokines and chemokines. These in turn attract additional immune cells to the brain parenchyma thereby promoting a devastating inflammatory process in the areas of transgene expression.¹⁴ In addition, we have shown that BDV causes disease in GF-IL12xIFN $\gamma^{-/-}$ mice by mechanisms that are IFN- γ independent. IFN- γ is also essential for the reduced virus multiplication in BDV-infected GF-IL12 mice. Our finding that cerebral expression of IL-12 may render mice susceptible to BD is of particular importance because cerebral expression of IL-12 has been observed in pathological conditions such as endotoxemia.^{53,54} This might be a paradigm for the development of CNS immune disorders that are directed against self or foreign antigens such as viral antigens expressed in the brain after bacterial infections. This notion is supported by several studies that demonstrated that viral infections are a potent trigger for the human demyelinating disease multiple sclerosis^{55,56} and that cerebral IL-12 expression is up-regulated in patients suffering from multiple sclerosis attacks.^{57,58}

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