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RESEARCH ARTICLE

TLR3 agonism re-establishes CNS immune competence during α4-integrin deficiency

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Abstract

Objective: Natalizumab blocks α4-integrin-mediated leukocyte migration into the central nervous system (CNS). It diminishes disease activity in multiple sclerosis (MS), but carries a high risk of progressive multifocal encephalopathy (PML), an opportunistic infection with JV virus that may be prompted by diminished CNS immune surveillance. The initial host response to viral infections entails the synthesis of type I interferons (IFN) upon engagement of TLR3 receptors. We hypothesized that TLR3 agonism reestablishes CNS immune competence in the setting of α4-integrin deficiency. **Method**: We generated the conditional knock out mouse strain Mx1.Cre^{+}α4-integrin$^{fl/fl}$, in which the α4-integrin gene is ablated upon treatment with the TLR3 agonist poly I:C. Adoptive transfer of purified lymphocytes from poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ donors into naive recipients recapitulates immunosuppression under natalizumab. Active experimental autoimmune encephalomyelitis (EAE) in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice treated with poly I:C represents immune-reconstitution. **Results**: Adoptive transfer of T cells from poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice causes minimal EAE. The in vitro migratory capability of CD45^{+} splenocytes from these mice is reduced. In contrast, actively-induced EAE after poly I:C treatment results in full disease susceptibility of Mx1.Cre^{+} α4-integrin$^{fl/fl}$ mice, and the number and composition of CNS leukocytes is similar to controls. Extravasation of Evans Blue indicates a compromised blood-brain barrier. Poly I:C treatment results in a 2-fold increase in IFNβ transcription in the spinal cord. **Interpretation**: Our data suggest that TLR3 agonism in the setting of relative α4-integrin deficiency can reestablish CNS immune surveillance in an experimental model. This pathway may present a feasible treatment strategy to treat and prevent PML under natalizumab therapy and should be considered for further experimental evaluation in a controlled setting.

Introduction

Antagonism of alpha(α4)-integrin (CD49d) substantially reduces leukocyte trafficking into the central nervous system (CNS), and the accumulation of clinical disease activity in multiple sclerosis (MS)[1] and its model experimental autoimmune encephalomyelitis (EAE).[2,3] Natalizumab, a humanized monoclonal anti-α4-integrin IgG_4 diminishes the capability of leukocytes to migrate from the blood into the CNS,[4–7] and is approved for patients with relapsing forms of MS.

Natalizumab therapy has been associated with an increased risk of multifocal leukoencephalopathy (PML), a rare opportunistic infection of the CNS caused by a

human polyomavirus JC.[8] PML is most prevalent in the setting of severe and prolonged immunosuppression, in which the host defense against pathogens is diminished. However, PML has also been reported in individuals who had limited exposures to pharmacological agents associated with an increased risk. There is no effective anti-viral agent for JCV. In patients with Acquired Immune Deficiency Syndrome (AIDS) who developed PML, the incidence and survival only improved after immune-reconstitution with highly active antiretroviral therapy (HAART).[9,10] In PML under natalizumab, removal of natalizumab with plasma exchange with the goal to allow the reentry of leukocytes into the CNS may have beneficial effects, and is widely utilized in this scenario.[11]

An effective treatment and prevention strategy for PML under natalizumab may be to reestablish immune surveillance in the CNS in an antigen-independent manner. Anti-viral innate immunity in the CNS and other organs is mediated by different sensors that detect viral-pathogen-associated molecular patterns (PAMPs).[12] Viral double-stranded RNA (dsRNA) is recognized as a PAMP by Toll-like receptor 3 (TLR3),[13] and it mediates anti-viral defense by diverse host cells. Engagement of TLR3 results in the activation of interferon (IFN) regulatory factors (IRF), and subsequently in high levels of type I IFN production. Type I IFN, which in humans include IFNα, IFNβ, and IFNκ, IFNω, and IFNν possess anti-viral effects through the activation of genes that inhibit protein synthesis and viral replication.[14] The downstream effect of TLR3 agonism in viral diseases is the activation and expansion of T cells that are reactive to a wide variety of viral pathogens. Amongst type I IFN, IFNα has the most potent anti-viral properties,[15–17] and IFNα preparations are approved by the Food and Drug Administration for the treatment of hepatitis B and hepatitis C. While controlled trials have never been conducted, data from in vitro assays,[18] animal model experiments,[19] and treatment of HIV-positive PML patients[20,21] suggest that type I IFNs are capable of antagonizing polyoma virus, including JCV.

We hypothesized that TLR3 agonism re-establishes CNS immune competence in the setting of α4-integrin deficiency.

To address this hypothesis, we generated the Mx1.Cre$^+$α4-integrin$^{fl/fl}$ mouse strain. This experimental approach was chosen because anti-α4-integrin mAb therapy has very different clinical and immunological effects in the EAE model than natalizumab does in MS patients. Theien and colleagues showed that anti-α4-integrin treatment at the peak of acute EAE or during remission worsened clinical disease and increased the infiltration of CD4$^+$ T cells into the CNS.[3] In Mx1.Cre$^+$α4-integrin$^{fl/fl}$ mice, the Cre recombinase is under the control of the *Mx1* promoter which can be induced to high levels by administration of the TLR3 agonist polyinosinic-polycytidylic acid (poly I:C). Poly I:C engagement of TLR3 results in the expression of type I IFN, which subsequently bind IFN type I receptors in adjacent cells. Consequently, downstream transcription factors initiate transcription of antiviral genes, including *Mx1*. In Mx1.Cre$^+$α4-integrin$^{fl/fl}$ mice, Cre recombinase targets loxP sites flanking the *Itga4* (α4-integrin) gene, causing its deletion. This system allows the conditional deletion of α4-integrin on all IFN-receptor expressing cells, which includes leukocytes. Adoptive transfer of purified lymphocytes from poly I:C-treated Mx1.Cre$^+$α4-integrin$^{fl/fl}$ donors into naive recipients recapitulates immunosuppression under natalizumab (Fig. 1). Active experimental autoimmune encephalomyelitis (EAE) in Mx1.Cre$^+$α4-integrin$^{fl/fl}$ mice treated with poly I:C represents immune-reconstitution (Fig. 1).

We assessed the effect of poly I:C on α4-integrin deletion on leukocytes in vivo and in vitro. To test the role of TLR3 agonism on the re-establishment of CNS immune competence, we induced EAE by active immunization of poly I:C treated Mx1.Cre$^+$α4-integrin$^{fl/fl}$, and by immunophenotyping of leukocytes subsets in secondary lymphoid tissues and the CNS. Integrity of the blood-brain barrier was tested with Evans Blue, and CNS cytokine expression was determined by quantitative polymerase chain reaction (qPCR).

Materials and Methods

Mice

C57BL/6J mice and *Mx1.Cre* $^+$ (B6.Cg-Tg(Mx1-cre)1Cgn/J) were purchased from The Jackson Laboratory, Bar Harbor, ME, USA.[22] *α4* $^{fl/fl}$ mice were generated, described, and obtained from Dr. Thalia Papayannopoulou, University of Washington.[23] Briefly, a targeting vector was constructed including the promoter and the first two exons of α4 integrin gene, a PGK-neo-p(A) cassette flanked by *lox*P elements, with an additional *lox*P inserted distal to the second exon. AK7 cells were electroporated with linearized vector and floxed clones resulted from homologous recombination. *α4*^{floxed} clones were identified with specific primers. Clones with normal XY karyotype were injected into C57BL/6 blastocysts and transferred into pseudo pregnant females. Resulting male chimeras were then bred to C57BL/6 females. Offspring were genotyped and animals heterozygous for the floxed *α4* allele were crossed to generate floxed homozygotes.

α4$^{fl/fl}$ females were bred to *Mx1.Cre*$^+$ males. Progeny were genotyped for the *cre* transgene by PCR utilizing Mx1.*cre* primers (5′ –CGGTTATTCAACTTG CACCA- 3′

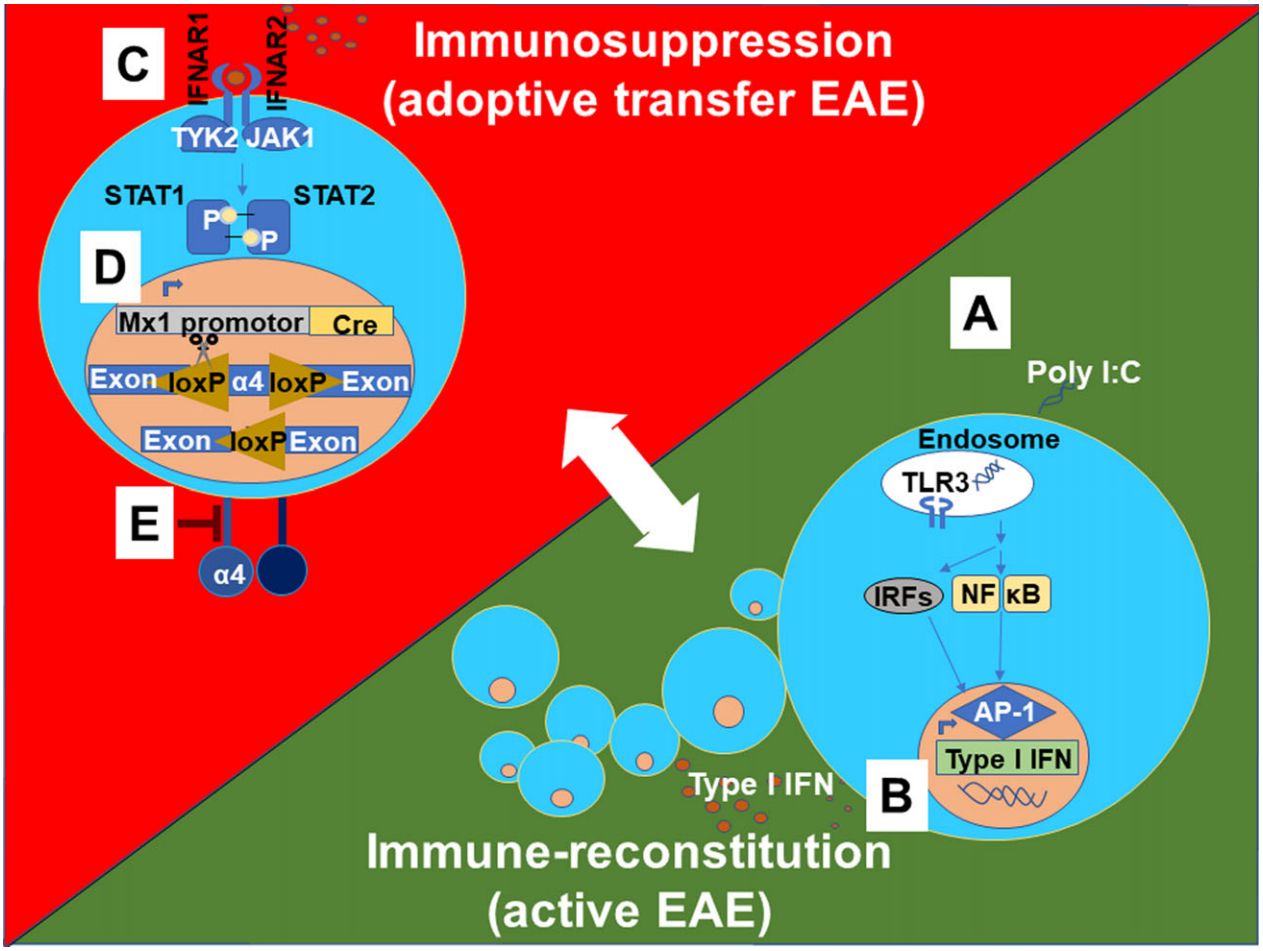


Figure 1. Generating a model to assess the effects of Toll-like receptor 3 (TLR3) agonism on CNS immune re-constitution in the setting of relative α4-integrin deficiency. We hypothesized that agonism of TLR3 with polyinosinic-polycytidylic acid (poly I:C) would fully reestablish EAE disease activity in mice that lack α4-integrin. To address this hypothesis, we generated the Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mouse strain as described in the Methods section. In these mice, the Cre recombinase is under the control of the *Mx1* promoter which can be induced to high levels by administration of poly I:C. (A) Poly I:C engagement of TLR3 results in the endosomal compartment (B) leads to the activation of interferon (IFN) regulatory factors (IRF) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) in the cytosol and the transcription factor activator protein-1 (AP-1), and eventually in the transcription and expression of type I interferons, which (C) subsequently bind IFN type I receptors in adjacent IFN Type I receptor-expressing cells. All tissues and organs and all hematopoietic cells express IFN type I receptors.[81] Activation of tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1) ensues, and (D) downstream transcription factors, including signal transducer and activator of transcription 1 (STAT1) translocate to the cell nucleus, and start transcription of antiviral genes, including *Mx1*. In Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, Cre recombinase targets loxP sites flanking the *Itga4* (α4-integrin) gene, causing its deletion. (E) This model allows the conditional deletion of α4-integrin on all IFN type I receptor-expressing cells, which includes leukocytes. Adoptive transfer experiments, in vitro migration assays, and some leukocyte immunophenotyping (all Fig. 2) were performed in the absence of poly I:C, and therefore only represent the immunosuppressive aspects of this model.

and 5′ –GTGAGTTTCGTTTCTGAGCTC C- 3′). *Mx1.Cre*$^{+}$ *α4*$^{fl/+}$ mice were intercrossed, and *Mx1.Cre*$^{+}$ progeny were genotyped for the *α4* allele (5′-GTCCACTGTTGGGCAAGTCC-3′ and 5′-AAACTTGTCT CCTCTGCCGTC3′). Eight to twelve weeks old, both female and male mice were used for all experiments. *Mx1.Cre*$^{+}$ *α4*$^{fl/fl}$ mice received three intra peritoneal injections of 300 μg poly(I)-poly(C) (poly I:C; Sigma Chemical Company, St. Louis, Mo.) given at 2 days intervals in order to activate the Cre recombinase. This was followed by a "wash-out" period of 21 days in which mice were then analyzed or immunized for EAE.

All mice described in this work were crossed and maintained at the UT Southwestern Medical Center Animal Resource Center in a pathogen-free animal facility. All animal procedures were performed in accordance with protocols approved by the UT Southwestern Institutional Animal Care and Use Committee (IACUC).

Active induction of EAE

Mice were anesthetized with 200 mg/kg tribromomethanol (1.5% Avertin) injected i.p. Active EAE was induced by s.c. injections into the flanks with 200 μg of mouse myelin oligodendrocyte glycoprotein$_{35–55}$ (MOG_{p35-55}) (MEVGWYRSPFSRVVHLYRNGK; CS Bio Menlo Park, CA, USA) emulsified in complete Freund's adjuvant (CFA) (DIFCO Laboratories, Detroit, MI, USA) containing 400 μg of heat inactivated *Mycobacterium tuberculosis* (Difco, Detroit, MI, USA). Mice also received i.p. injections of 200 ng pertussis toxin on days 0 and 2 (List Biological Laboratories Inc., Campbell, CA, USA). Clinical signs of EAE were assessed daily and reported following the classical criteria: 0 = no clinical disease, 1 = limp tail, 2 = partial hind leg paralysis, 3 = complete hind leg and uni-lateral paralysis, 4 = complete hind leg and partial front leg paralysis, 5 = moribund.[24] At least three independent experiments were conducted with a minimum of five mice per group.

Adoptive transfer EAE

For passive induction of EAE by adoptive transfer of myelin-specific T cell, single cell suspensions were prepared from splenocytes isolated from actively immunized mice. Cells were activated for 72 h with MOG_{35-55} and IL-12 in vitro.[25] After incubation, 5 million cells were injected i.p. into C57BL/6 recipients. Clinical signs of EAE were assessed daily and reported following the classical criteria: 0 = no clinical disease, 1 = limp tail, 2 = partial hind leg paralysis, 3 = complete hind leg and unilateral paralysis, 4 = complete hind leg and partial front leg paralysis, 5 = moribund.

Isolation of lymph node cells and splenocytes

Lymph node cells and splenocytes were isolated by pressing through a 70 μmol/L nylon mesh cell strainer. Cells were treated with RBC lysis buffer (Sigma-Aldrich, St. Louis, MO, USA), washed twice with cold PBS, and resuspended in culture medium consisted of Roswell Park Memorial Institute medium 1640 (Invitrogen) supplemented with L-glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), non-essential amino acids (0.1 mmol/L), penicillin (100 U/mL), streptomycin (0.1 mg/mL), 2-mercaptoethanol (1 μL of stock), and 10% fetal bovine serum (FBS) or PBS for counting with hemocytometer.

Enzymatic CNS digestions

As previously described,[26] brains and spinal cords were first finely minced using a sterile scalpel, washed with cold PBS, then processed based on the specific enzymes used. The commercially available Neural Tissue Dissociation Kit (P) (Kit) was used following the manufacturer's protocol (Neural Tissue Dissociation Kit (P), Miltenyi Biotec, San Diego, CA, USA). Following enzymatic dissociation, brains and spinal cords were washed with cold PBS, and then subjected to one wash with 37% Percoll PLUS™ to remove remaining myelin. The myelin-free single cell suspensions were counted using a hemocytometer.

Immunophenotyping

$1x10^6$ cells from spleens, lymph nodes, bone marrow, and CNS were resuspended in FACS buffer (5% FBS in PBS) and Fc receptors were blocked with anti-CD16/32 (Tonbo Biosciences) for 10 min at 4°C. For blood analysis, 60 μL of blood were also treated with 1 μg anti-CD16/32 but incubated and stained at room temperature. Cells were then stained for surface markers with fluorochrome-conjugated mAbs: Integrin α4-FITC from Santa Cruz Biotechnology, Inc.; CD3e-Pacific Blue, CD19-Alexa Fluor 700, CD11c-PE, CD11b-APC, GR-1-APC-Cy7 all from BD Biosciences (San Jose, CA, USA); CD45-PE-Cy7 from eBioscience (San Diego, CA, USA); CD4-PE-Texas Red, CD8-Pacific Orange both from Invitrogen (Grand Island, NY, USA); CD11a-PE and CD49e-PE from BioLegend (San Diego, CA, USA); biotinylated PDCA-1 from Miltenyi (Auburn, CA, USA) which was revealed with SA-Q Dot 655 from Invitrogen (Grand Island, NY, USA). Cells were then washed, resuspended in staining buffer, and fixed in 0.5% paraformaldehyde.

Fluorescence minus one (FMO) controls were prepared by adding all antibodies but one, for each parameter to discriminate positive staining from non-specific background. Up to 500,000 events were acquired on a BD FACS LSR II at UT Southwestern Flow Core or FACS LSRFortessa SORP at The Moody Foundation Flow Cytometry Facility, Children's Medical Center Research Institute at UT Southwestern. Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

In vitro *migration assay*

We performed an in vitro migration assay by Boyden Chamber as described before.[27] This assay quantifies the usage of α4-integrin by leukocytes to migrate across a fibronectin barrier.[28] Briefly, we utilized a Boyden chamber containing a polycarbonate membrane filter (Transwell® Permeable Supports, Corning Inc., Corning NY) pre-coated on its upper surface with FN. A total of $5x10^5$ splenocytes from mice actively immunized for EAE and sacrificed at day 10, suspended in media, were added to the upper chamber. Chambers were then incubated at

37°C for 6 h. Following incubation, the content of the lower chamber was collected, and the number of cells was counted with a hemocytometer and the phenotype of the cells determined by flow cytometry.

Evaluation of BBB permeability

Mice were injected intravenously (i.v.) with 200 μL of 3% (weight/volume) Evans Blue dye and perfused with 4% paraformaldehyde after 3 h. Brains and spinal cords were fixed in 4% paraformaldehyde and photographed with a dissecting microscope. For quantification of Evans Blue dye, tissues were dried at 56°C overnight, then incubated with 8 mL/g *N, N*-dimethylformamide at 56°C for 48 h. Evans Blue dye is soluble in *N, N*-dimethylformamide, therefore we prepared exponential dilutions for a standard curve and measured absorbance with spectrophotometer at 650 nm.[29,30]

Histology

Brains were perfused and isolated as described above and fixed in 10% formalin. Brains were then coronally sectioned, embedded in Tissue-tek O.C.T. Compound, and snap frozen in liquid nitrogen. 6 μm thick section were cut utilizing a freezing microtome and mounted on Fisher Brand Superfrost Plus glass slides. Samples were stained with hemotoxylin and eosin (H&E (Fisher Scientific, Pittsburgh, PA)), and prepared for light microscopy examination.

Proliferation assay

15 days post immunization, single cell suspensions were generated by isolating the LNs of the immunized mice. Utilizing the CellTrace™ CFSE (5(6)-carboxyfluorescein N-hydroxysuccinimidyl ester) Cell Proliferation kit (Life Technologies, Carlsbad, CA), $CD4^+$ T cell proliferation against antigens was determined. Briefly, isolated 20×10^6 LN cells were incubated for 5 minutes at room temperature with 1 μmol/L CFSE. After incubation, cells were washed with RPMI media twice, then incubated in a 96-well-round bottom plate at 1×10^6 cells per well with specified antigen for 96 h. Post incubation, cells were washed with staining FACS buffer two times, then the Fc receptors were blocked with anti-CD16/32 (BD Biosciences, Franklin Lakes, NJ,) for 15 min at 4°C before staining with mAbs for 30 min at 4°C. Cells were stained utilizing the following monoclonal antibodies: CD3e-Pacific Blue, CD45-PE-Cy7, and CD4-PE-Texas Red. Cells were analyzed with a LSRII flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA).

RNA isolation and quantitative real time PCR

TRI Reagent® Solution was utilized for RNA extraction of freshly isolated tissues of mice sacrificed on day 15 post immunization. Mice were overdosed with 400 mg/kg tribromomethanol. Brain and spinal cord tissues were placed in 10–20 volumes of TRIzol Reagent and immediately flash frozen. RNA was extracted by TRIzol chloroform extraction and RNeasy plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was stored at -80C^0 until use. RNA concentration was measured with Epoch Microplate Spectrophotometer (BioTek, Winooski, VT). cDNA was made with iScript™ cDNA synthesis kit (Bio-Rad). qPCR was performed using iTaq™ Universal SYBR® Green Supermix green (Bio-Rad, Hercules, CA), and Step One Plus (Applied Biosystems, Foster City, CA). Each reaction was performed in triplicate, using 100 ng cDNA per reaction and normalized to endogenous control R18s. The following verified KiCqStart SYBR® Green primers (Sigma-Aldrich) were used: IL-1α, IL-1β, IL-6, IL-12a, IL-17a, FoxP3, Csf2 (GM-CSF), IFNβ, and IFNγ. Fold change in expression relative to the control group was determined using the ΔΔCt algorithm method. The ΔCt was normalized to the housekeeping gene R18s.

Statistical analysis

All experiments were repeated at least twice, and at least five mice were utilized per treatment group. For parametric tests, data were checked for normality by using the Kolmogorov–Smirnov test. The means of samples were compared using an unpaired Student's t-test. Mean clinical scores significance between groups was analyzed by Mann-Whitney *U* test. The criterion for significance (alpha) has been set at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Data are given as mean ± standard error. All analyses were performed with Prism 6 for Windows (GraphPad Software, La Jolla, CA, USA).

Results

In the absence of poly I:C, Mx1.Cre$^+$ α4-integrin$^{fl/fl}$ mice behave clinically like control animals

To ablate α4-integrin, *Mx1.Cre*$^+$ *α4*$^{fl/fl}$ mice received three intra peritoneal injections of 300 μg poly(I)-poly(C) (poly I:C; Sigma Chemical Company) given at 2 days intervals in order to activate the Cre recombinase (Fig. 2A). This was followed by a "wash-out" period of 3 weeks in which

mice were then analyzed or immunized for EAE. To ascertain that *Mx1.Cre*$^{+}$ *α4-integrin*$^{fl/fl}$ *mice* do not possess a clinical phenotype distinct from C57BL/6 control mice, active EAE was induced in the absence of poly I:C (Fig. 2B). EAE disease incidence, onset, clinical severity were similar between Mx1.Cre^{+} α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice.

The frequency α4-integrin (CD49d)-positive leukocytes is reduced in primary and secondary lymphoid organs of poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice

The frequency of α4-integrin (CD49d)-positive leukocyte subsets was assessed by multi-parameter flow cytometry in poly I:C-treated Mx1.Cre^{+} α4-integrin$^{fl/fl}$ mice, and in poly I:C-treated C57BL/6 control mice on day 15 post active EAE induction. In the lymph nodes (Fig. 2C), spleen (Fig. 2D), and bone marrow (Fig. 2E) of poly I:C-treated Mx1.Cre^{+} α4-integrin$^{fl/fl}$ mice, the frequency of α4-integrin (CD49d)-expressing CD3^{+} T cells, CD8^{+} T cells, CD11c^{+} monocyte-derived dendritic cells (DC), and CD22b^{+}Ly6G^{+} myeloid-derived granulocytes was significantly diminished. In spleen (Fig. 2D), and bone marrow (Fig. 2E), the frequency of α4-integrin expressing CD4^{+} T cells, CD19^{+} B cells, and CD22b^{+}Ly6G^{-} macrophages was also significantly reduced. Similar trends for the latter cell subsets were seen in lymph nodes but do not reach statistical significance. (Fig. 2C).

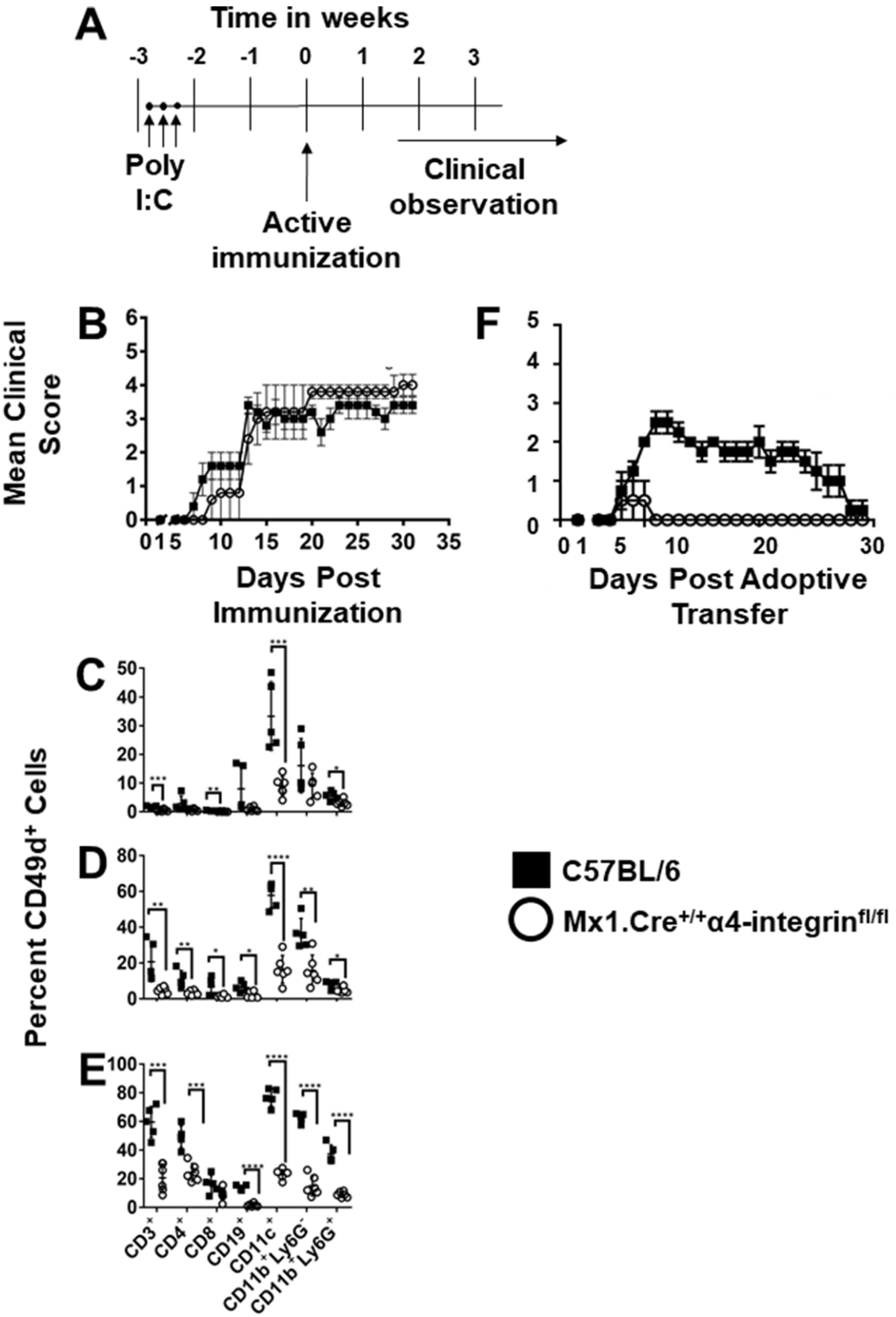
Adoptive transfer of lymph node cells from poly I:C-treated Mx1.Cre^{+}α4- integrin$^{fl/fl}$ donor mice results in ameliorated EAE disease activity

Next, we tested the in vivo role of genetic α4-integrin ablation by passively transferring activated cells from myelin oligodendrocyte glycoprotein peptide (MOG_p) 35-55-immunized poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, or poly I:C-treated C57BL/6 mice into naïve C57BL/6 recipient mice. In the adoptive transfer model, the recipient mice are not exposed to the effects of poly I:C. Transfer of cells from both strains resulted in the onset of clinic disease at day 7 (Fig. 2F). However, transfer of poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ donor T cells was associated with a disease incidence of only 75%, and in a significantly ameliorated and shortened disease course in recipient mice. In this experimental paradigm, recipient mice fully recovered by day 15 post transfer. In contrast, adoptive transfer of poly I:C treated C57BL/6 donor T cells resulted in 100% disease incidence, and a significantly more severe course (Fig. 2F).

Lymph node cells and splenocytes from systemically poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ show altered migratory behavior in vitro

To test the effect of α4-integrin deletion after poly I:C treatment on migratory competence of cells, we performed an in vitro migration assay on lymph node cells and splenocytes by Boyden Chamber as described before.[27] The composition of lymph node cells from poly I:C treated C57BL/6 control mice (Fig. 3A) and poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice (Fig. 3B) entered into the upper chamber, and the composition of lymph node cells from poly I:C treated C57BL/6 control mice (Fig. 3C) and poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice (Fig. 3D) that migrated into the lower chamber were assessed by multi-parameter flow cytometry. There were subtle differences in the composition of lymph node cells between the mouse strains at the outset of the migration experiments (Fig. 3A and B). After

Figure 2. In vivo and in vitro Characterization of Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice with or without exposure of the Toll-like receptor 3 (TLR3) agonist polyinosinic-polycytidylic acid (poly I:C). (A) To ablate α4-integrin, *Mx1.Cre*$^{+}$ *α4* $^{fl/fl}$ mice received three intra peritoneal injections of 300 μg poly(I)-poly(C) (poly I:C; Sigma Chemical Company) given at 2 days intervals in order to activate the Cre recombinase. This was followed by a "wash-out" period of three weeks in which mice were then analyzed or immunized for EAE. (B) EAE disease incidence, onset, clinical severity are similar between Mx1.Cre^{+} α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice not exposed to poly I:C. Then, the frequency of α4-integrin (CD49d)-expressing leukocyte subsets was assessed by multi-parameter flow cytometry in poly I:C-treated Mx1.Cre^{+} α4-integrin$^{fl/fl}$ mice, and in poly I:C-treated C57BL/6 control mice on day 15 post active EAE induction. (C) In the lymph nodes, (D) spleen, and (E) bone marrow of poly I:C-treated Mx1.Cre^{+} α4-integrin$^{fl/fl}$ mice, the frequency of α4-integrin expressing CD3^{+} T cells, CD8^{+} T cells, CD11c^{+} monocyte-derived dendritic cells (DC), and CD22b^{+}Ly6G^{+} myeloid-derived granulocytes is significantly diminished. (D) In spleen, and (E) bone marrow, the frequency of α4-integrin expressing CD4^{+} T cells, CD19^{+} B cells, and CD22b^{+}Ly6G^{-} macrophages is also significantly reduced. (C) Similar trends for the latter cell subsets are seen in lymph nodes but do not reach statistical significance. Next, we tested the in vivo role of genetic α4-integrin ablation by passively transferring activated cells from myelin oligodendrocyte glycoprotein peptide (MOG_p) 35-55-immunized poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, or poly I:C-treated C57BL/6 mice into naïve C57BL/6 recipient mice. In the adoptive transfer model, the recipient mice are not exposed to the effects of poly I:C. (F) Transfer of cells from poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ donor T cells was associated with decreased disease and severity in recipient mice.



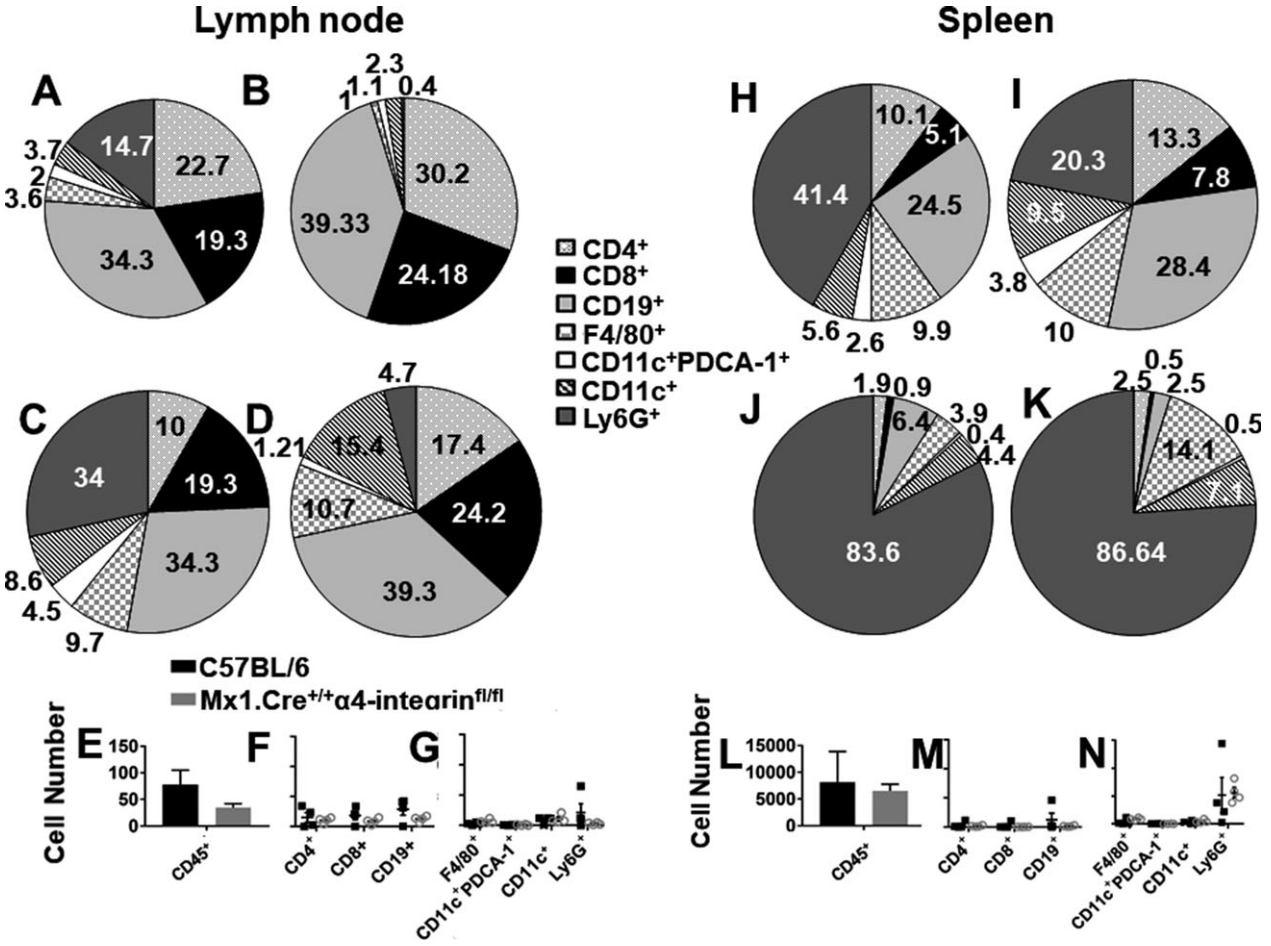


Figure 3. Lymph node cells and splenocytes from systemically poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ show altered migratory behavior in vitro. To test the effect of α4-integrin deletion after poly I:C treatment on migratory competence of cells, we performed an in vitro migration assay on lymph node cells and splenocytes by Boyden Chamber as described before.[27] Briefly, a total of 5×10^5 splenocytes from mice actively immunized for EAE and sacrificed at day 10, suspended in media, were added to the upper chamber. Chambers were then incubated at 37°C for 6 h. Following incubation, the content of the lower chamber was collected, and the number of cells was counted with a hemocytometer and the phenotype of the cells determined by flow cytometry. (A) The composition of lymph node cells from poly I:C treated C57BL/6 control mice and (B) poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice entered into the upper chamber, and (C and D) the composition of lymph node cells of both mouse strains that migrated into the lower chamber were assessed by multi-parameter flow cytometry. (A and B) There were subtle differences in the composition of lymph node cells between the mouse strains at the outset of the migration experiments. After 6 h of migration, the composition in the lower chamber reflected (C) an enhanced migratory capability of conventional CD11c^{+} dendritic cells (DC) in poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, and (D) a reduced migratory capability of Ly6G^{+} neutrophils compared to poly I:C treated C57BL/6 control mice. (E) Overall, there was a non-significant reduction in the migration of CD45^{+} lymph node cells of poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, which was driven by diminished migration of (F) CD4^{+} T cells, CD8^{+} T cells, CD19^{+} B cells, and (G) Ly6G^{+} neutrophils. (H) The composition of splenocytes from poly I:C treated C57BL/6 control mice and (I) poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice in the upper chamber showed a decreased percentage of Ly6G^{+} neutrophils in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, which was compensated by an expansion of all other leukocytes subsets. (J and K) The composition of splenocytes in the lower chamber was similar between mouse strains. (L) In splenocytes, there was also a non-significant reduction in the migration of CD45^{+} lymph node cells of poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, which was driven by diminished migration of (M) CD19^{+} B cells, and (N) Ly6G^{+} neutrophils.

6 hours of migration, the composition in the lower chamber reflected an enhanced migratory capability of conventional CD11c^{+} dendritic cells (DC) in poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, and a reduced migratory capability of Ly6G^{+} neutrophils compared to poly I:C treated C57BL/6 control mice (Fig. 3C and D). Overall, there was a non-significant reduction in the migration of CD45^{+} lymph node cells of poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice (Fig. 3E), which was driven by diminished migration of CD4^{+} T cells, CD8^{+} T cells, CD19^{+} B cells (Fig. 3F), and Ly6G^{+} neutrophils (Fig. 3G).

The composition of splenocytes from poly I:C treated C57BL/6 control mice (Fig. 3H) and poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice (Fig. 3I) in the upper chamber showed a decreased percentage of Ly6G^{+} neutrophils in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, which was compensated by an expansion of all other leukocytes subsets (Fig. 3I). The composition of splenocytes in the lower chamber was similar between mouse strains, suggesting a differential us of integrins (Fig. 3J and K). In splenocytes, there was also a non-significant reduction in the migration of CD45^{+} lymph node cells of poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice (Fig. 3L), which was driven by diminished migration of CD19^{+} B cells (Fig. 3M), and Ly6G^{+} neutrophils (Fig. 3N).

In vivo TLR3 agonism through systemic poly I:C treatment reestablishes EAE disease susceptibility and CNS immune competence in the setting of relative α4-integrin deficiency

After establishing that the frequency α4-integrin (CD49d)-positive leukocytes is significantly reduced in primary and secondary lymphoid organs of poly I:C-treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, and that the capacity of lymphocytes from these mice to induce passively transferred EAE, and to migrate across biological membranes in vitro is substantially diminished, we wanted to demonstrate that in vivo TLR3 agonism through poly I:C treatment reverses the effects of relative α4-integrin deficiency on EAE disease activity. Full EAE susceptibility requires the entry of leukocytes into the brain and spinal cord, and consequently cannot occur in the setting of compromised CNS immune competence.

When active EAE was induced in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice that were treated with poly I:C, EAE disease incidence, susceptibility, and severity were similar in both groups (Fig. 4A). There was a trend toward more severe clinical EAE disease in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice treated with poly I:C (Fig. 4A). Poly I:C treatment was clinically characterized by an ascending paralysis. An atypical ataxic syndrome that has been described in atypical EAE was not observed in any of the experimental animals.

In vivo TLR3 agonism through systemic poly I:C treatment leads to cellular immune reconstitution in the setting of relative α4-integrin deficiency

Next, the percentage of leukocytes in lymph nodes (Fig. 4B), spleen (Fig. 4C), brain (Fig. 4D), and spinal cord (Fig. 4E) was assessed in mice that were actively immunized for EAE and treated with poly I:C on disease day 15. In all compartments, there were no differences in composition of leukocytes between the two strains, indicating a full cellular immune re-constitution.

In vivo TLR3 agonism through systemic poly I:C treatment is associated with activated and functional antigen-specific lymphocytes in the setting of relative α4-integrin deficiency

To investigate possible causes of immune reconstitution Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice treated with poly I:C, we investigated antigen recall and activation status of CD4^{+} T cells from lymph nodes obtained at day 10 after active induction of EAE from Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice treated with poly I:C. There was no difference between the capacity of Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice that were treated with poly I:C to mount recall responses to MOG_{p35-55} (Fig. 4F). There was a trend toward strong MOG_{p35-55} CD4^{+} T cell proliferation from Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice treated with poly I:C (Fig. 4F). The number of activated CD4^{+}CD25^{+} T cells was increased in the brain of Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice treated with poly I:C, and similar between both mouse strains in the spinal cord (Fig. 4G). In the brain and spinal cord, we also observed a significant expansion of CD19^{+}SSC^{hi} B cells in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice treated with poly I:C (Fig. 4H). These cells were not further characterized, but may be plasmablasts. Poly I:C structural analogues are known to promote robust mucosal and systemic IgG antibody synthesis.[31]

TLR3 agonism through poly I:C treatment compromises the blood-brain barrier (BBB) in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice

Our data clinical and cellular data in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice that were treated with poly I:C indicated that leukocytes are capable of obtaining access to the CNS in the relative absence of α4-integrin when TLR3 is engaged by poly I:C: To test the effect of in vivo poly I:C treatment on blood-brain barrier BBB integrity, we performed an Evans Blue Dye (EBD) permeability assay. EBD has a high affinity for serum albumin. In the setting of BBB compromise, the serum-dye complex can penetrate the CNS parenchyma, and it can be visualized and quantified by spectrophotometry. There was no difference in the amount of EBD detected in the CNS of Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice treated in vivo with poly I:C (Fig. 5A). We also did not observe a difference in the absolute number of inflammatory infiltrates in the spinal cords between

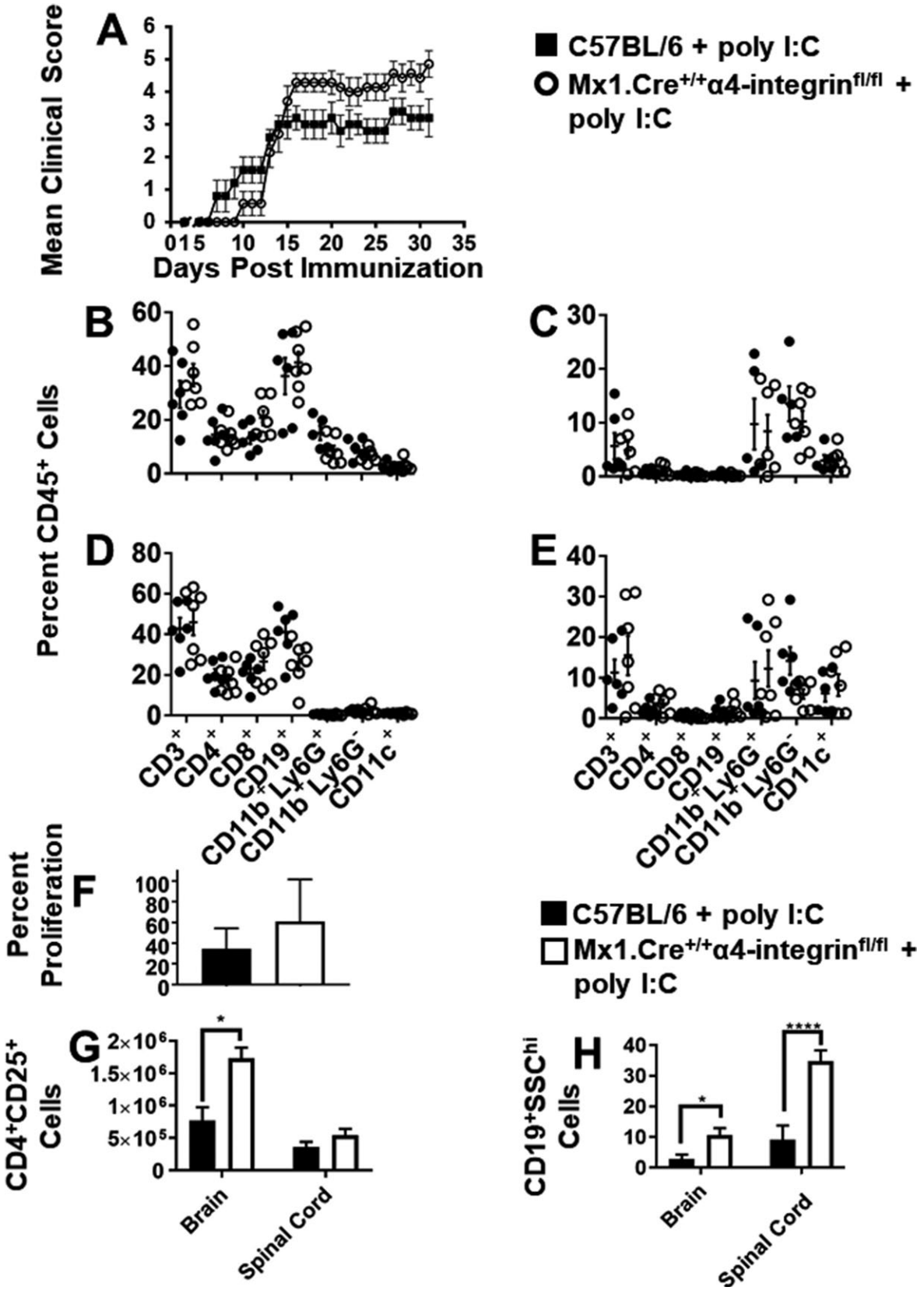


Figure 4. In vivo systemic Toll-like receptor 3 (TLR3) agonism through polyinosinic-polycytidylic acid (poly I:C) treatment reestablishes EAE disease susceptibility and CNS immune competence in the setting of relative α4-integrin deficiency. (A) When active EAE was induced in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice that were treated with poly I:C, EAE disease incidence, susceptibility, and severity were similar in both groups. Next, the percent of leukocytes in (B) lymph nodes, (C) spleen, (D) brain, and (E) spinal cord nodes was assessed by multi-parameter flow cytometry in mice that were actively immunized for EAE and treated with poly I:C on disease day 15. There were no differences in composition of leukocytes between the two strains, indicating a full cellular immune re-constitution. (F) Utilizing the CellTrace™ CFSE (5(6)-carboxyfluorescein N-hydroxysuccinimidyl ester) Cell Proliferation kit (Life Technologies, Carlsbad, CA), we detected no difference between the capacity of Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice that were treated with poly I:C to mount recall responses to MOG_{p35-55}. (G) The number of activated CD4^{+}CD25^{+} T cells was increased in the brain of Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice treated with poly I:C, and similar between both mouse strains in the spinal cord. (H) In the brain and spinal cord, we also observed a significant expansion of CD19^{+}SSC^{hi} B cells in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice treated with poly I:C.

animals of both mouse strains in whom active EAE had been induced in the absence or presence of poly I:C (Fig. 5B–E). The anatomical locations of BBB compromise indicated by EBD extravasation in the brain (Fig. 5F and G; Table 1), and spinal cord (Fig. 5H and I; Table 1) differed between Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice treated in vivo with poly I:C. Interestingly, we detected a preponderance of midbrain lesions in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice treated with poly I:C compared with controls, although this assessment was not quantitative. This observation may suggest a preferential migration into these anatomical locations by lymphocyte subsets that have a diminished requirement for α4-integrin to cross the BBB.[32–34]

In vivo TLR3 agonism through systemic poly I:C promotes diverse integrin usage in CNS-infiltrating leukocytes in the setting of relative α4-integrin deficiency

Our prior experiments indicated that TLR3 agonism through poly I:C reestablishes clinical and cellular immune competence in the CNS in the setting of relative α4-integrin deficiency. To determine the integrin usage required for leukocyte migration into the brain and spinal cords, we assessed the expression of Lymphocyte-function associated antigen-1 (LFA-1; β2-integrin; CD11a) (Fig. 6A–D), α5-integrin (CD49e) (Fig. 6E–H), and α4-integrin (CD49d) (Fig. 6I–L) on different lymphocyte and myeloid cell subsets in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice actively induced for EAE on day 15. The number of leukocytes subsets expressing CD11a, CD49e, and CD49d in all compartments was similar between mouse strains (Fig. 6A–L). These results suggest that TLR3 agonism with poly I:C permits full access of α4-integrin-expressing leukocytes to the brain, even in the setting of relative α4-integrin deficiency. A high prevalence of activated T cells in the CNS (Fig. 6G), and BBB-compromise (Fig. 5) may be contributing factors underlying this observation. In our experimental model, the ablation of α4-integrin in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice is incomplete (Fig. 2C–E). It is plausible that the frequency of α4-integrin positive leukocytes in primary and secondary lymphoid organs in these mice is sufficiently high to initiate and perpetuate CNS autoimmunity after treatment with poly I:C, possibly through the induction of soluble inflammatory mediators, including chemokines.

TLR3 agonism through systemic poly I:C administration differentially impacts cytokine expression in a compartment-specific manner in the setting of relative α4-integrin deficiency

Engagement of TLR3 results in the transcription and cellular expression of type I IFN. To confirm that systemic administration of poly I:C induces transcription of type I IFN within the CNS, we performed quantitative real time PCR for IFNβ in the brain and spinal cord of Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice 24 hours after administration of three doses of poly I:C on consecutive days. Enzymatic tissue dissociation and quantitative polymerase chain reaction (qPCR) are described in the Method section. In the spinal cord, there was an approximate 2-fold increase in IFNβ transcripts compared to tissue from untreated mice (Fig. 7A). There was no significant change in the transcription of IFNβ in the brain (data not shown). Transcription of IFNβ, and numerous pro-inflammatory cytokines were again assessed in the brain spinal cord on day 15 after active induction of experimental autoimmune encephalomyelitis (EAE), or 36 days after the last dose of poly I:C. In the spinal cord, we observed a significant increase in the transcription of interleukin (IL)-1 beta (β), and a substantial increase in the transcription of IL-1α, IL-6, IL-12, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon gamma (IFNγ) compared to mice not treated with poly I:C, confirming an altered inflammatory milieu (Fig. 7B). At this time point, there was not difference in the transcription of IFNβ between poly I:C-treated and untreated animals (Fig. 7B). There was also no significant change in the transcription of these cytokines in the brain (data not shown).

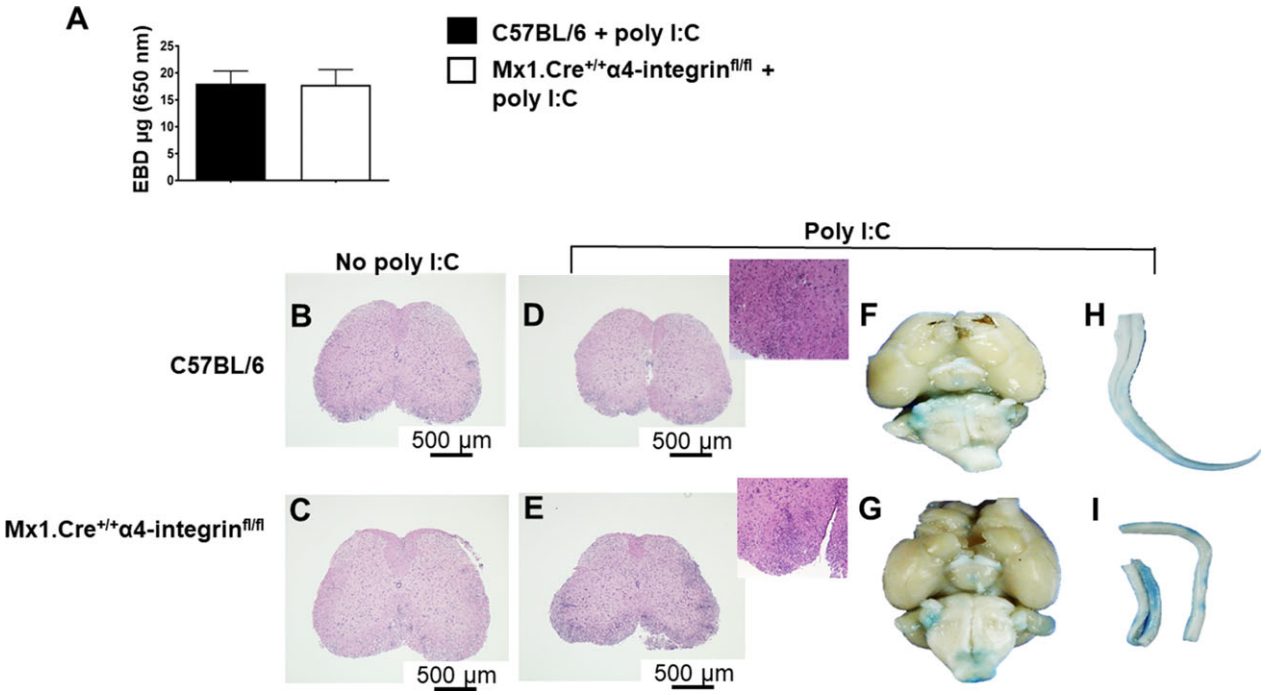


Figure 5. Toll-like receptor 3 (TLR3) agonism through polyinosinic-polycytidylic acid (poly I:C) compromises the blood-brain barrier. To test the effect of in vivo poly I:C treatment on blood-brain barrier BBB integrity, we performed an Evans Blue Dye (EBD) permeability assay. EBD has a high affinity for serum albumin. In the setting of BBB compromise, the serum-dye complex can penetrate the CNS parenchyma, and it can be visualized and quantified by spectrophotometry. (A) There was no difference in the amount of EBD detected in the CNS of Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice treated in vivo with poly I:C. (B–E) We also did not observe a difference in the absolute number of inflammatory infiltrates in the spinal cords between animals of both mouse strains in whom active EAE had been induced in the absence or presence of poly I:C. The anatomical locations of BBB compromise as indicated by EBD extravasation differed in the (F and G) brain, and (H and I) spinal cord differed between Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice treated in vivo with poly I:C.

Table 1. TLR3 agonism through poly I:C treatment leads to differential anatomical compromise of the blood-brain barrier demonstrated by Evans Blue Dye (EBD) in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice.

Genotype	Treatment	Tissue	Location of inflammation
Mx1.Cre$^{+/+}$a4-integrin$^{fl/fl}$	Poly I:C	Brain	Ventral anterior cochlear nucleus
	EAE		Middle and inferior cerebellar peduncles
		Spinal cord	Spinal cord white matter
Mx1.Cre$^{+/+}$a4-integrin$^{fl/fl}$	EAE	Brain	Optic tract
			Crus cerebri and pons
			Inferior cerebellar peduncle
		Spinal cord	Spinal cord white matter
C57BL/6	Poly I:C	Brain	Corticospinal tracts
	EAE		Sensory trigeminal tract
			Spinal trigeminal tract
		Spinal cord	Upper cervical spinal cord
			Spinal cord white matter
C57BL/6	EAE	Brain	Optic tracts
			Corticospinal tracts
			Spinal trigeminal tract
		Spinal cord	Spinal cord white matter

The differential expression of cytokines in brain and spinal cord is likely explained by the different inflammatory environment in both organs. EAE in C57BL/6 mice is predominantly a spinal cord disease,[35] and we show that poly I:C disruption of the BBB affects different anatomical sites in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control

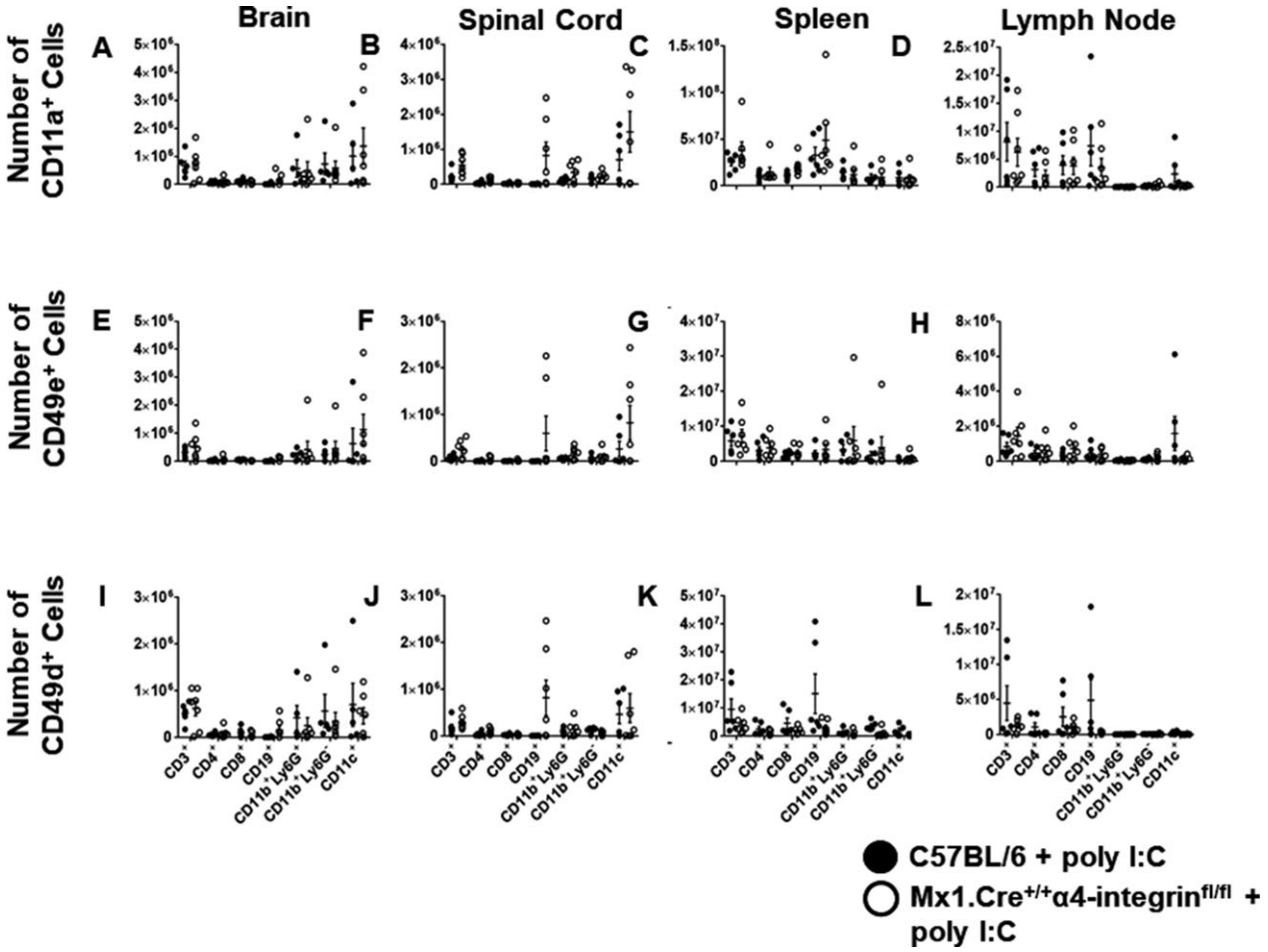


Figure 6. In vivo Toll-like receptor 3 (TLR3) agonism through systemic polycytidylic acid (poly I:C) promotes diverse integrin usage in CNS-infiltrating. To determine the integrin usage required for leukocytes migration into the brain and spinal cords, we assessed the expression of (A–D) Lymphocyte-function associated antigen-1 (LFA-1; β2-integrin; CD11a), (E–H) α5-integrin (CD49e), and (I–L) α4-integrin (CD49d) on different lymphocyte and myeloid cell subsets in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 control mice actively induced for EAE on day 15 by multi-parameter flow cytometry. (A–L) The number of leukocyte subsets expressing CD11a, CD49e, and CD49d in all compartments was similar between mouse strains.

mice (Table 1). The time-limited effect of poly I:C on IFNβ transcription is likely at least in part the result of its pharmacological properties. De Clercq demonstrated that poly I:C is rapidly degraded by nucleases present in human serum, and that the half-life in an in vitro experimental system was in the range of minutes.[36] Furthermore, the kinetics of IRF in response to poly I:C may be limiting the longevity of any effects on type I IFN expression. Marie and colleagues compared the magnitude and kinetics of IFN secretion.[37] Infection of WT embryo fibroblasts with Newcastle disease virus (NDV) or RNA resulted in the rapid production of high levels of IFNα with a peak at 6–8 h.

Discussion

The occurrence of PML during natalizumab treatment in patients with MS is a highly relevant and problematic issue for several reasons: (1) Natalizumab is a highly effective therapy that can greatly diminish the frequency of clinical relapses, and the accumulation of lesions in the brain on neuroimaging[38–40]; (2) the substantial risk of PML very likely reduces the number of patients and clinical providers who prescribe this highly effective agent; (3) there is currently no biological or biochemical marker that allows the identification of PML at-risk individuals with sufficient precision; (4) risk-stratification algorithms have not reduced the incidence of PML under natalizumab.[41–43]

One strategy to allow patients the benefits of natalizumab therapy without the fear of devastating outcomes from PML, CNS immune-reconstitution after its onset may be a feasible option. There is substantial evidence on persistency of JCV in the CNS.[44–47] A recent study in

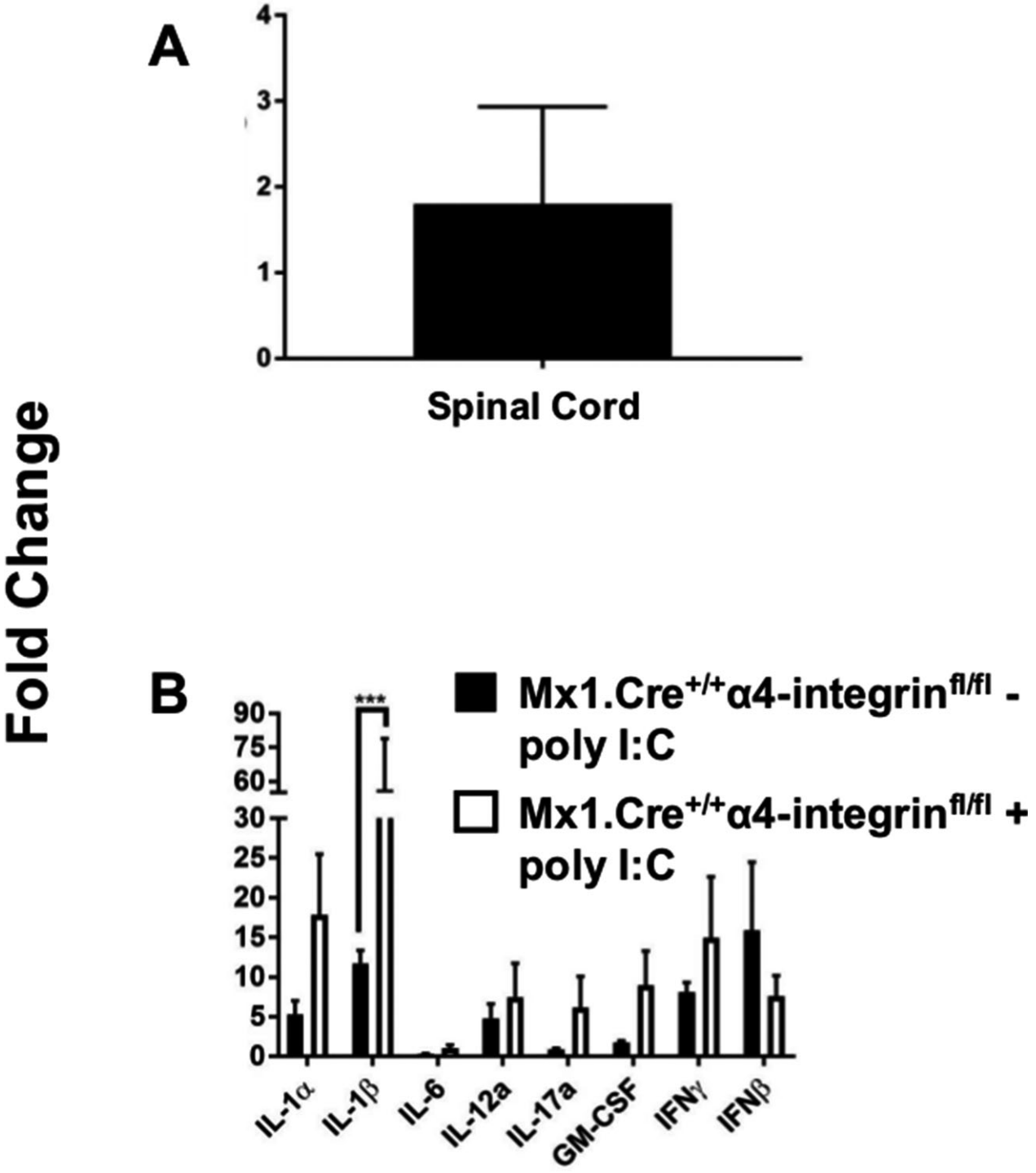


Figure 7. Systemic Toll-like receptor 3 (TLR3) agonism through polycytidylic acid (poly I:C) differentially impacts cytokine expression in a compartment-specific manner in the setting of relative α4-integrin deficiency. (A) Engagement of TLR3 results in the transcription and cellular expression of the type I interferon beta (IFN*β*) in spinal cord. To confirm that systemic administration of poly I:C induces transcription of type I IFN within the CNS, we performed quantitative real time polymerase chain reation (qPCR) for IFN*β* in the brain and spinal cord of Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice 24 h after administration of three doses of poly I:C on consecutive days. In the spinal cord, there was an approximate 2-fold increase in IFN*β* transcripts compared to tissue from untreated mice. (B) On day 15 after active induction of experimental autoimmune encephalomyelitis (EAE), or 36 days after the last dose of poly I:C, we observed a significant increase in the transcription of interleukin (IL)-1 beta (*β*), and a substantial increase in the transcription of IL-1α, IL-6, IL-12, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon gamma (IFNγ) compared to mice not treated with poly I:C in the spinal cord.

immunocompetent non-PML patients detected fragments of JCV DNA in brain oligodendrocytes and astrocytes, but not in neurons.[47] These findings suggest that JCV has access to the brain in immunocompetent individuals. In the setting of immunosuppression, it is conceivable that resident JCV initiates its lytic cycle in oligodendrocytes.[47]

Antagonism of α4-integrin interferes with immune competence of the CNS on multiple levels. Leukocyte migration from the periphery into the CNS involves multiple steps.[48–50] Integrins, including α4- integrin, facilitate leukocyte migration across the basement membrane of blood vessels and across the extracellular matrix.[51,52] In EAE, a model of MS, an early event is the presentation of antigen in the context of major histocompatibility complex (MHC) II in secondary lymphoid organs to $CD4^+$ T helper cells. These $CD4^+$ T cells become activated, clonally expand, and can be considered "autoimmune-prone". They then egress from the draining lymph nodes and are now capable of adhering to the endothelium of blood vessel walls and migrating into the CNS. Within the CNS, antigen-specific $CD4^+$ T cells are reactivated through the presentation of an identical or similar antigen by perivascular APCs, including hematopoietic macrophages[53] and DCs.[54] At this stage, these $CD4^+$ T cells are considered truly "autoimmune", and they can initiate and perpetuate CNS inflammation through the secretion of soluble inflammatory mediators, and through attracting other immune-competent leukocytes into the CNS. Antigen-recognition of neurotropic pathogens to T cells in secondary lymphoid organs, their migration to the CNS, and their re-activation in the CNS upon encounter of the pathogen antigen there relies on the same basic immunological principles as CNS autoimmunity.

In addition to its effects on CNS immune surveillance, natalizumab has biological effects in other organs that may impact host defense. Seeding of infected cells from other compartments to the CNS is biologically plausible. $CD34^+$ hematopoietic precursor cells are susceptible to JCV infection.[55] Interestingly, $CD34^+$ cells express high levels of α4-integrin.[56–59] Several groups of investigators recently showed that natalizumab mobilizes $CD34^+$ hematopoietic progenitor cells.[60,61] Antibody-mediated blockage of $CD34^+$ cell-homing into the bone marrow could contribute to their elevated numbers outside the bone marrow in natalizumab-treated patients.[62,63] Therefore, immune activation through TLR-3 agonism may provide additional beneficial effects in PML under natalizumab by diminishing the JCV reservoir outside the CNS.

As stated above, we intended to test whether TLR3 agonism leads to CNS immune-reconstitution in the setting of relative α4-integrin deficiency. Ideally, one would utilize an experimental model that measures adaptive immune responses against JCV. However, JCV susceptibility is species-specific and active replication is only permissive in humans.

Also, it appears that the effects of poly I:C on immune re-constitution in EAE could have been tested in combination with an anti-α4-integrin monoclonal antibody. This option was discarded based on observations made by Theien et al., who demonstrated that anti-α4-integrin treatment at maximum disease activity and during disease remission exacerbated disease relapses and increased the accumulation of $CD4^+$ T cells in the CNS.[3]

Instead, we utilized the EAE model to show that disease susceptibility can be fully reestablished when active EAE is induced in the setting of poly I:C-mediated conditional deletion of α4-integrin in the $Mx1.Cre^+$α4-$integrin^{fl/fl}$ mice. This model of CNS autoimmunity in EAE, as T cell-mediated anti-viral responses, is a valid functional readout that confirms intact adaptive immune responses within the CNS. Reestablishment of CNS immune surveillance in this model is associated with compromise of the BBB, an observation that is not surprising based on our knowledge of the effects of TLR3 in the brain and spinal cord.[64] Our data may suggest that this pathway could also be utilized to reestablish immune-competence during a CNS infection, including in patients with PML under natalizumab.

Theoretically, TLR3 agonists could also be administered to MS patients who are stable on natalizumab therapy, but who are at an increased risk for PML. Recent data reveal an estimated annual risk of 10/1000 in year six of treatment in MS patients with an anti-JCV index of >1.5.[65] This risk is substantially higher than 0.01/1000 patients in patients with an index of ≤0.9 in year one. Thus, an increased anti-JCV index, and a long treatment duration are risk factors for PML under natalizumab. If a decision is made by the patient at risk and the neurologist to continue natalizumab, TLR3 agonists could be administered. A limitation of this approach is that there currently appears to be no assay to reliably measure viral replication in the CNS directly or indirectly. The downstream effect of TLR3 agonism in viral diseases is the activation and expansion of T cells, and specific anti-JCV T cell responses[66] could be measured by tetramer analysis or other detection methods. However, these methods would have to be verified and standardized.

Perhaps one obvious question is why one would use TLR3 agonism instead of administering recombinant type I IFNs that are already approved for human disease, including for MS, hepatitis B, and hepatitis C. First, there are some biological limitations: One of the first MS patients that was diagnosed with PML under natalizumab was concomitantly treated with IFNβ-1β.[67] This was

interpreted by some as evidence that IFNβ possesses insufficient anti-viral potency to lower the risk of PML, or to ameliorate the disease course of PML. However, natalizumab has since been used exclusively as monotherapy, and any potential beneficial effect of IFNβ has therefore never been studied. As stated above, there are other type I IFNs, including IFNα, IFNκ, IFNω, of which IFNα appears to have the most potent anti-viral properties.[15–17] Secondly, there are pharmacological limitations. Exogenous type I IFNs do not have good CNS bioavailability in the brain.[68–71] In contrast, we confirm findings by Field et al. who demonstrated that systemic administration of poly I:C leads to upregulation of type I IFNs in the CNS of mice.[72]

One potential concern in pursuing TLR3-mediated immune re-constitution in the setting of PML under natalizumab is inflammatory immune reconstitution syndrome (IRIS) of the brain, which in itself can result in devastating neurological outcomes.[73,74] The induction of endogenous type I IFN expression through TLR3 agonism would possibly not only provide a strong anti-viral effect, but also some degree of immunomodulation. As stated above, several IFNβ-1a and IFNβ-1b preparations are currently approved for the treatment of MS because of their anti-inflammatory properties.[75] Interestingly, a synthetic complex of carboxymethylcellulose, polyinosinic-polycytidylic acid, and poly-L-lysine double-stranded RNA, poly IC:LC, was developed in the late 1960s as inducer of type I IFN by Hilton Levy.[76] At the time, recombinant IFN was not yet available, and naturally occurring type I IFN were expensive and inconsistent in quality. Eventually, an open phase I trials of poly IC:LC was conducted in patients with MS.[77,78] Monthly intravenous (i.v.) infusions at a dose of 100 μg/kg was followed by transient elevation of body temperature, and pseudoexacerbations.[78] All patients were hospitalized and managed with antipyretics and cooling blankets for the first 12 h. Most were discharged home the following day. Once the patient's body temperatures returned to normal they stabilized neurologically. Some patients also showed sustained neurological improvement. On most patients, blood was drawn after each infusion every 4 h for the 16 h, and there was a substantial increase in type I IFN, and to a lesser degree, of IFNγ.[79,80]

Finally, the concept of TLR3 agonism in the setting of PML or patients at risk for PML could now be tested clinically with agents that have extensively been utilized in a clinical setting. For instance, the TLR3 agonist rintatolimod has already been assessed in several phase III trials for the treatment of chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) and was found to be safe.

In conclusion, our data provide a biological rationale to test TLR3 agonists in MS patients on natalizumab who develop PML.

Acknowledgments

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Conflicts of Interest

O.S. serves on the editorial boards of the Multiple Sclerosis Journal and Therapeutic Advances in Neurological Disorders, has also served on data monitoring committees for Pfizer and TG Therapeutics without monetary compensation, advised EMD Serono and Genzyme. O.S. currently receives grant support from Sanofi Genzyme, and received travel support from Shire. D.O. received advisory and consulting fees from Celgene, Genentech, Genzyme, EMD Serono and Novartis, and research support from Biogen. R.Z.H, P.C.C, R.D. B.D., E.H, N.L., P.R, and M.K.R. report no conflict of interest.

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