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RESEARCH ARTICLE

Type I interferon-activated microglia are critical for neuromyelitis optica pathology

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Abstract

Neuromyelitis optica (NMO) is an inflammatory disease of the central nervous system (CNS) most frequently mediated by serum autoantibodies against the water channel aquaporin 4, expressed on CNS astrocytes, resulting in primary astrocytopathy. There is no cure for NMO, and treatment with Type I interferon (IFN α)-IFN β is ineffective or even detrimental. We have previously shown that both NMO lesions and associated microglial activation were reduced in mice lacking the receptor for IFN β . However, the role of microglia in NMO is not well understood. In this study, we clarify the pathomechanism for IFN α dependence of and the role of microglia in experimental NMO. Transcriptome analysis showed a strong IFN α footprint in affected CNS tissue as well as in microglial subpopulations. Treatment with IFN β led to exacerbated pathology and further microglial activation as evidenced by expansion of a CD11c⁺ subset of microglia. Importantly, depletion of microglia led to suppression of pathology and decrease of IFN α signature genes. Our data show a pro-pathologic role for IFN α -activated microglia in NMO and open new perspectives for microglia-targeted therapies.

KEYWORDS

CD11c⁺ microglia, depletion, microglia, neuromyelitis optica, Type I interferon

1 | INTRODUCTION

Neuromyelitis optica (NMO) is an immune-mediated chronic inflammatory disease of the central nervous system (CNS) that was long considered a clinical variant of multiple sclerosis (MS). Like the other

NMO spectrum disorders (NMOSD), NMO most often manifests with optic neuritis and transverse myelitis (Trebst et al., 2014). There is no cure for the disease. NMO is most frequently associated with serum autoantibodies specific for the water channel aquaporin 4 (AQP4), expressed by astrocytes (Lennon, Kryzer, Pittock,

Verkman, & Hinson, 2005). In clinical NMO lesions, AQP4 and glial fibrillary acidic protein (GFAP) expression are markedly reduced due to autoantibody and complement-mediated astrocyte damage (Misu et al., 2006; Misu et al., 2013) leading to secondary demyelination. Another characteristic feature of NMO lesions is microglial activation (Khoroshii, Wlodarczyk, Asgari, & Owens, 2013; Misu et al., 2013), but the specific role of microglia in this disease remains unclear. Activated microglia have been shown to produce and respond to Type I interferons (IFN β), one of which is IFN β (Kocur et al., 2015). Our previous findings showed that both NMO lesions and microglial reactivity were reduced in mice that were deficient in IFNAR, the receptor for IFN β , suggesting an important role for IFN β as well as microglia in the NMO pathogenesis. IFN β is used as a clinical therapeutic for MS and is beneficial in animal models for MS (Owens, Khoroshii, Wlodarczyk, & Asgari, 2014). There are conflicting data regarding the role of IFN β in NMO, showing beneficial, neutral, or even detrimental effects in experimental models (Khoroshii et al., 2013; Oji et al., 2016; Zhang, Bennett, & Verkman, 2011). Treatment with IFN β in clinical NMO is ineffective and is suspected to increase relapse rates (Kim, Kim, Li, Jung, & Kim, 2012; Palace, Leite, Nairne, & Vincent, 2010; Tanaka, Tanaka, & Komori, 2009). In this study, we aimed to clarify the role of IFN β in experimental NMO as well as to assess the contribution of microglia to NMO pathogenesis. Transcriptome analysis showed a strong IFN β footprint in experimental NMO. Moreover, pathology was exacerbated by treatment with IFN β . Interestingly, we observed expansion of a CD11c⁺ microglial subset in experimental NMO that was further enhanced by IFN β treatment. RNA-seq analysis revealed a clear IFN β footprint in CD11c⁺ as well as CD11c⁻ microglia. Importantly, depletion of microglia led to suppression of NMO pathology. This was accompanied by downregulation of IFN β -stimulated genes, but not IFN β , suggesting microglia as responders to IFN β . Taken together, our findings point to IFN β -stimulated microglia as contributing to development of lesions in experimental NMO, making them a potential target for future therapies.

2 | MATERIALS AND METHODS

2.1 | Human material

The use of human material was approved by the Committee on Biomedical Research Ethics for the Region of Southern Denmark (ref. No. S20080142) and the Danish Data Protection Agency (ref. No. 2008-41-2826). Human NMO-IgG and normal human IgG preparation was as described previously (Asgari, Khoroshii, Lillevang, & Owens, 2013). Human complement (HC') was purified from a pool of healthy serum donors.

2.2 | Mice

All mice used in this study were females aged 7–8 weeks, with a starting weight 18–22 g. The mice were maintained at 25°C with 55% humidity on a 12-hr light–dark cycle and given free access to food and

drinking water. All experiments were approved by the Danish Animal Experiments Inspectorate (approval number 2014-15-0201-00369).

C57BL/6J mice were obtained from Taconic Europe A/S. CX3CR1^{CreER} and iDTR mice were obtained from The Jackson Laboratory and maintained as a breeding colony (and crossed together to obtain CX3CR1 iDTR) in the Biomedical Laboratory, University of Southern Denmark (Odense). IFNAR1-KO mice on the C57BL/6 background were bred from mice originally provided by Prinz et al. (2008).

2.3 | Induction of experimental NMO

Experimental NMO was induced by stereotactic intracerebral injection of purified IgG isolated from a patient diagnosed with NMO, with HC'. Purified IgG isolated from healthy donor was used as a control. Intracerebral injections were performed on isoflurane-anesthetized animals. Mice were given Temgesic for pain relief. Animals were kept in a warm cabinet for 16–20 hr before they were transferred back to their cages. All injections were performed in the morning by the same investigator.

Stereotactic coordinates were 2 mm lateral from bregma and 0.2 mm anterior. A 30-gauge needle attached to a 50 μ l Hamilton syringe was inserted 3.5 mm in the right side of the brain to infuse a total of 10 μ l (300 μ g NMO-IgG + 288 μ g HC' / 300 μ g human IgG + 288 μ g HC' $\pm$ 2,000 U of carrier-free rIFN β (endotoxin level < 1 EU/ μ g, suitable for in vivo studies) (PBL Assay Science). Mice were sacrificed 2 days after the injection.

2.4 | Tissue processing and immunostaining

Brain sections were immunostained with primary rabbit antibodies: anti-AQP4 (Alomone Labs Ltd. Jerusalem, Israel), anti-GFAP (DAKO, Denmark), anti-Iba1 (Wako, Japan), secondary biotinylated goat anti-rabbit IgG (Abcam) antibody followed by streptavidin-HRP and developed with DAB (Sigma-Aldrich). Immunofluorescent stainings were performed with rabbit anti-Iba1 (Wako, Japan), anti-CD68 (Abcam); rat anti-TREM2 (Biotechne), anti-MAC1 (AbD Serotec) followed by secondary anti-rabbit Alexa 555 (Invitrogen), anti-rat Alexa 488 (Invitrogen), and DAPI. Hematoxylin and eosin staining was used to assess tissue viability, for example, to exclude necrotic lesions. Sections showing signs of necrosis related to needle damage were excluded. Semiquantitative evaluation of histological changes was performed by a blinded investigator. The histopathological changes included evaluation of brain sections for loss of AQP4- and GFAP staining as described in Asgari et al., 2013, Khoroshii et al. (2019), and Khoroshii et al. (2013). Based on four to five sections (with visible needle track) from each mouse, pathology was classified with regard to extent of lesions. Histological changes were graded as: (a) mild changes of limited extent; (b) moderate changes, loss of staining extended from the needle track; and (c) marked changes, loss of staining over an extensive cumulative area. Images were acquired using an Olympus DP71 digital camera mounted on an

Olympus BX51 microscope (Olympus, Ballerup, Denmark). Quantitative assessment of lesions was measured as cumulative area of loss of staining divided by total area of a hemisphere using ImageJ software.

2.5 | Microglia depletion

Then, 7 to 8-week-old C57BL/6 mice were fed at libitum with DietGel 76A (Clear H₂O) supplemented with PLX3397 (MedChemTronica, Sweden) (600 ppm) (Najafi et al., 2018) for 7–9 days.

2.6 | Fluorescence-activated cell sorting and flow cytometry

To isolate microglia from brain, mice were anaesthetized with 200 mg/kg of pentobarbital and intracardially perfused with ice-cold PBS. Brain tissue was collected, and a single cell suspension was generated by forcing through a 70 mm cell strainer (BD Biosciences). Mononuclear cells were collected after centrifugation on 37% Percoll (GE Healthcare Biosciences AB). They were first incubated with anti-Fc receptor (Clone 2.4G2; 1 mg/ml; BD Pharmingen) and Syrian hamster IgG (50 mg/ml; Jackson Immuno Research Laboratories Inc.) in PBS with 2% fetal bovine serum (FBS), then with anti-CD45 (Clone 30-F11; Biolegend), anti-CD11b (Clone M1/70; Biolegend) and biotin-conjugated anti-CD11c (Clone HL3; BD Pharmingen) antibodies in PBS with 1% FBS and finally with streptavidin-APC (Biolegend). Cell populations were gated based on isotype-matched control antibodies as CD45^{dim} CD11b⁺ CD11c[−] (CD11c[−] microglia), CD45^{dim} CD11b⁺ CD11c⁺ (CD11c⁺ microglia) and sorted on a FACSARIA III cell sorter (BD Biosciences) or data were collected on an LSRII flow cytometer (BD Biosciences) and analyzed using Flowlogic (Inivai, Australia).

2.7 | RNA extraction, quantitative real-time PCR

RNA from brain tissue was extracted using TRIzol (Invitrogen, Fisher Scientific) according to the manufacturer's protocol.

Fluorescence-activated cell sorting (FACS)-sorted CD11c⁺ and CD11c[−] microglia were placed in RLT buffer (Qiagen) and total RNA was extracted using RNeasy columns as per manufacturer's protocol (Qiagen).

Reverse transcription was performed with M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed with 1 µl cDNA in a 25 µl reaction volume containing Maxima Probe/ROX qPCR Master mix (Fermentas), TaqMan PreAmp Master Mix Kit: *Oas2*: Mm01201449_m1; *Oas2*: Mm00460961_m1; *Clec7a*: Mm01183349_m1; *Mx1*: Mm00487796_m1; *Ifi204*: Mm00492602_m1; *Trim14*: Mm01352552_m1 or with primers and probes (TAG Copenhagen A/S):

1. *Irf7*: Forward: CACCCCATCTTCGACTTCA;
Reverse: CCAAAACCCAGGTAGATGGTGTA;
Probe: CACTTTCTCCGAGAACT.

2. *Irf9*: Forward: ACAACTGAGGCCACCATTAGAGA;
Reverse: CACCACTCGGCCACCATAG;
Probe: TGAACCTCAGACTACTCGCT.
3. *Cxcl10*: Forward: GCCGTCATTTTCTGCCTCAT;
Reverse: GGCCCGTCATCGATATGG;
Probe: GGACTCAAGGGATCC.
4. *Ifnβ*: Forward: GCGTTCCTGCTGTGCTTCTC;
Reverse: TTGAAGTCCGCCCTGTAGGT;
Probe: CGGAAATGTCAGGAGCT.

PCR reactions were done on an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Results were expressed relative to 18S rRNA (2^{ΔCT} method) as endogenous control (TaqMan Ribosomal RNA control Reagents kit; Applied Biosystems). cDNA was diluted 1/1,000 for 18S rRNA analysis.

2.8 | RNA sequencing

2.8.1 | Whole tissue sequencing

Sequence libraries were prepared using the Illumina Stranded mRNA LT Kit, and RNA-sequencing (RNAseq) was performed using Illumina NextSeq with 2 × 75 bp paired end reads. Samples with RIN value <7 were excluded from the analysis. The raw sequencing data (BCL files) were first demultiplexed using Illumina software CASAVA thereby creating FASTQ files. The FASTQ files were subsequently processed using BBDOUK (<https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/>) from the BBTools software package for trimming remnant adaptor sequences, removal of low quality bases and reads, and for k-mer-filtering. The quality filtered reads were then first aligned against the murine transcriptome (Mus_musculus.GRCh38.89), and then to the mouse genome (GRCm38) using TOPHAT2 (Kim et al., 2013). Quantification was done using the HTseq-count python module embedded in the HTSeq tool package (Anders, Pyl, & Huber, 2015). The *edgeR* R-package (Robinson, McCarthy, & Smyth, 2010) was used for transformation of the raw counts into counts per million (cpm) and for filtration of very low expressed genes. Only genes which had cpm ≥1 cpm in at least four samples were kept for further analysis. After filtering, the data were normalized using trimmed mean of M-values normalization, and differential expression analysis comparing deletion and duplication was performed using the glmFit and glmLRT functions embedded in the *edgeR* package. False discovery rate (FDR) was used to correct for multiple testing and genes with FDR ≤0.05 were considered significantly differentially expressed.

2.8.2 | Sorted microglia sequencing

RNA quality check was done using an Agilent bioanalyzer, and only high-quality samples (RIN >7) were used for sequencing. Sequence libraries were prepared using the Illumina Truseq RNA sample

FIGURE 1 Type I interferon (IFN) fingerprint in experimental neuromyelitis optica (NMO). (a) A heatmap showing differentially regulated genes in NMO-IgG + HC' versus Hu-IgG + HC' injected brain hemispheres. Scale represents log2 fold change normalized counts per million (cpm) expression values. (b) Expression of *Mx1*, *Clec7a*, and *Ifi204* in brain hemispheres injected with Hu-IgG + HC' (blue bar) ($n = 3$) and NMO-IgG + HC' (green bar) ($n = 5$) and in unmanipulated brain (CTR = control; Black bar) ($n = 4$) was assessed by RT-qPCR. (c) Representative micrographs and quantitative assessment (% of area) of loss of AQP4 and glial fibrillary acidic protein (GFAP) immunoreactivity in corpus callosum and striatum of animals injected with Hu-IgG + HC' + rIFNβ ($n = 3$) and NMO-IgG + HC' ± rIFNβ ($n = 6/n = 8$). Data are based on at least two experimental repeats. Data are presented as means ± SEM; each n represents an individual mouse. p -Values were determined by two-tailed Mann-Whitney U test. * $p < .05$; ** $p < .01$

preparation kit. RNA sequencing was performed at the Genome Analysis Facility of the University Medical Center Groningen with an Illumina HiSeq 2500. Single read 50 bp sequencing was done, aligned to the ensemble reference genome using the Star 2.3.1l aligner (Dobin et al., 2013), allowing for two mismatches. Samtools 0.1.19 was used to

sort the aligned reads (Roussos, Katsel, Davis, Siever, & Haroutunian, 2012). Quantification was done using HT-seq count 0.5.4 (Anders et al., 2015). Between 6 and 11 million uniquely aligned reads per sample were generated. Count data were loaded in R and analyzed with the EdgeR BioConductor Package (Robinson et al., 2010).

Differential gene expression was done between groups of interest. Heatmaps were generated with heatmap2 function of package gplots.

2.9 | Statistics

No statistical methods were used to predetermine sample sizes, and exact group numbers were determined by animal availability. We ensured that the sample sizes were similar to those generally employed in the field. For each experiment, at least three mice were used per group (virtually all animals subjected to NMO show pathology). In order to collect sufficient amount of RNA for RNA-seq analysis from sorted cells, each sample consisted of 8–10 pooled mice.

To obtain unbiased data, histopathological analysis of the samples was performed blinded.

All experiments were repeated at least twice, and data are presented as means $\pm$ SEM. Statistical significance was assessed using the parametric or non-parametric two-tailed unpaired *t* test (GraphPad Prism 6, San Diego, CA) unless specified otherwise. Parametric test was used only for normally distributed data. Normal distribution was assessed by Shapiro–Wilk normality test. *p*-Values $< .05$ were considered significant.

3 | RESULTS

3.1 | Involvement of IFN β signaling in NMO-like pathology

To study the mechanism underlying NMO-like pathology we injected NMO-IgG with HC' to the striatum of wild-type (WT) C57BL/6 mice. We used RNA generated during experiments described in (Khoroshii et al., 2013) and compared transcriptomes of affected hemispheres 2 days postinjection with those from normal human-IgG (Hu-IgG) controls, which did not develop pathology. From 14,632 expressed genes, 20 were upregulated and 18 were downregulated (FDR ≤ 0.05) in NMO compared to Hu-IgG control (Figure 1a). Gene ontology (GO) analysis of genes upregulated in NMO versus Hu-IgG control showed enrichment for two categories: "defense response to virus" (Benjamini adjusted *p* = .037) and "cellular response to interferon-beta" (Benjamini adjusted *p* = .032). Further analysis in INTERFEROME (Rusinova et al., 2013) revealed that 75% of genes upregulated in NMO were regulated by IFN β . We confirmed significant upregulation of the IFN β -regulated genes *Mx1*, *Clec7a*, and *Ifi204* by RT-qPCR in NMO-IgG + HC' versus Hu-IgG + HC'-treated and naive mice (CTR) (Figure 1b). In addition, we compared transcriptomes of NMO-IgG + HC'-injected hemispheres from WT and IFNAR-1-KO that lack functional IFN β signaling and are resistant to NMO-pathology. GO analysis of 114 significantly upregulated genes revealed enrichment for 22 GO-terms including as expected categories related to immune responses ("innate immune response," "inflammatory response") as well as IFN β induction and response ("cellular response to Interferon-beta," "positive regulation of interferon-beta

production") but also phagocytosis ("positive regulation of phagocytosis," "response to bacterium") and cell death ("pyroptosis") (Table 1).

To clarify the role of IFN β we examined the effect of exogenous IFN β on NMO-like pathology. We performed histopathological analysis 2 days after intracerebral co-injection of rIFN β with NMO-IgG + HC'. We observed significantly more pronounced loss of AQP4 and GFAP staining in these mice in comparison to NMO-IgG + HC' without rIFN β (Figure 1c). As expected, loss of AQP4 and GFAP immunoreactivity was restricted to the needle track in brains injected with rIFN β with Hu-IgG + HC' (Figure 1c) showing that injection of rIFN β

TABLE 1 GO-enrichment analysis of upregulated genes in NMO + HC' injected-WT versus IFNAR1-KO brain hemispheres

NMO IgG+ HC' B6 vs. IFNAR1-KO		
GOTERM_BP_DIRECT term	<i>p</i> -Value	Benjamini corrected <i>p</i> -value
Defense response to virus	7.6E-30	4.5E-27
Immune system process	2.2E-22	6.4E-20
Innate immune response	1.3E-20	2.5E-18
Response to virus	2.5E-20	3.7E-18
Cellular response to interferon-alpha	2.0E-7	2.3E-5
Negative regulation of viral genome replication	3.3E-7	3.2E-5
Inflammatory response	4.8E-7	4.0E-5
Cellular response to interferon-beta	1.2E-6	8.7E-5
Positive regulation of interferon-beta production	4.9E-6	3.2E-4
Response to bacterium	1.9E-5	1.1E-3
Positive regulation of phagocytosis	6.9E-5	3.7E-3
Type I interferon biosynthetic process	2.1E-4	1.0E-2
Response to lipopolysaccharide	2.9E-4	1.3E-2
Response to Type I interferon	4.3E-4	1.8E-2
T cell activation	5.0E-4	2.0E-2
Response to interferon-beta	7.3E-4	2.7E-2
Pyroptosis	9.1E-4	3.1E-2
Positive regulation of interferon-alpha production	1.1E-3	3.6E-2
Positive regulation of NF-kappaB transcription factor activity	1.6E-3	4.4E-2
Immune response	1.6E-3	4.5E-2
Mast cell activation	1.6E-3	4.7E-2
Positive regulation of tumor necrosis factor biosynthetic process	1.6E-3	4.7E-2

Abbreviations: GO, gene ontology; HC', human complement; NMO, neuromyelitis optica; WT, wild type.

was not harmful to the brain unless NMO IgG + HC' was present. Of note, rIFN β did not induce leukocyte infiltration (not shown).

Taken together, these data point to an important role for Type I IFN signaling for initiation of NMO-like pathology and suggest that IFNAR signaling may mediate induction of phagocytosis and cell death in this model.

3.2 | IFN α signaling footprint in microglia

Since microglia can respond to IFN α and are major phagocytes in the brain, we next assessed their role in NMO-like pathology. We have previously shown activated morphology and increased IBA1 immunoreactivity of microglia in striatal lesions of NMO-IgG + HC'-injected brains.

FIGURE 2 Legend on next page.

Interestingly, such microglia activation (Khoroshii et al., 2013) as well as immunoreactivity of phagocytosis markers TREM2 and CD68 in microglia was not seen in NMO-IgG + HC'-injected IFNAR1-KO brains (Figure 2a). To further compare microglial phenotypes in NMO-IgG + HC'-treated WT and IFNAR1-KO mice as well as in WT mice treated with intrastriatal injection of rIFN β we used flow cytometry (Figure 2b). We observed a significant increase of CD11c⁺ microglia in WT mice with NMO when compared to controls. In IFNAR1-KO mice treated with NMO-IgG + HC' the percentage of CD11c⁺ microglia was significantly lower even than in WT Hu-IgG + HC' controls (Figure 2c). Of note, as reported earlier (Khoroshii et al., 2013), these mice do not develop NMO-like pathology. Importantly, co-injection of IFN β with NMO-IgG + HC' in WT mice led to significantly enhanced emergence of CD11c⁺ microglia (Figure 2c).

To assess the role of microglial subsets in NMO-like pathology, we compared transcriptomes of sorted CD11c⁺ and CD11c⁻ microglia from hemispheres affected by experimental NMO with naïve total microglia sorted from unmanipulated brains. Microglial markers (*Aif1*, *Irgam*, *Cx3cr1*, *Csf1r*) and signature genes (Butovsky et al., 2014) (*Sp1*, *Irf8*, *Olfml3*, *Hexb*, *Fcrls*, *Tgfb1*, *P2ry12*, *Siglech*, *Tmem119*) were similarly expressed in both CD11c⁺ and CD11c⁻ microglia populations as well as in naïve microglia showing high purity of cell isolation (not shown). From 13,628 genes expressed, 586 genes were upregulated and 135 decreased in CD11c⁻ microglia from experimental NMO, compared to naïve microglia. In CD11c⁺ microglia from experimental NMO brains, 699 genes were increased and 546 decreased, compared to naïve microglia, and 63 were increased and 208 decreased, compared to CD11c⁻ microglia. Although a multidimensional scaling plot showed that experimental NMO-derived CD11c⁺ and CD11c⁻ microglia and naïve microglia formed three separate and distinct global gene expression clusters (Figure 2d), GO enrichment analysis of upregulated and downregulated genes (FDR ≤ 0.05) in CD11c⁺ versus CD11c⁻ microglia showed either no enrichment or enrichment for "inflammatory response," "immune system process," respectively suggesting that overall CD11c⁺ and CD11c⁻ microglia were functionally similar in this context. CD11c⁺ and CD11c⁻ microglia compared to naïve microglia showed enrichment for 31 and 36 GO term

categories, respectively (Table S1). Both populations showed enrichment for categories associated with innate immune responses, response to IFN α and IFN β as well as positive regulation of IL-12 production, suggesting that both microglia populations were involved in immune response. Categories related to mitosis were significantly enriched in CD11c⁻ microglia, and categories related to migration and antigen presentation were enriched in the CD11c⁺ population. INTERFEROME analysis revealed that 43% of the upregulated genes in CD11c⁺ microglia and 50% in CD11c⁻ microglia were IFN α -regulated genes. In addition, we showed upregulations of two canonical IFN α signature genes *Irf7* and *Cxcl10* in microglial subsets from NMO-IgG + HC'-treated brains by RT-qPCR (Figure 2e). In order to shortlist candidate microglia-upregulated genes that are involved in experimental NMO we analyzed the overlap between upregulated genes in these microglial subsets and in affected brain tissue compared to two categories of unaffected controls, Hu-IgG + HC' in WT, and NMO-IgG + HC' in IFNAR1-KO. This revealed seven (*Ifi44*, *Ifi204*, *Mx1*, *Ifit3*, *Phf11b*, *Clec7a*, *AU020206*) overlapping genes in all the groups, six of which were IFN α -regulated genes. Interestingly, 45/114 upregulated genes in NMO IgG + HC' WT versus IFNAR1-KO overlapped with at least one of the microglia populations (Figure 2f), further supporting the importance of IFN α stimulation of microglia for induction of astrocytopathy.

3.3 | Microglia are necessary for NMO-like pathology

To directly assess the importance of microglia in experimental NMO, we depleted them. Several depletion strategies were attempted, but only the CSF1R antagonist PLX3397 (Elmore et al., 2014) was found to be useful.

In order to improve on reported PLX3397-induced microglial depletion rates (~90%) (Han, Harris, & Zhang, 2017) and to avoid microglia repopulation, we used a 7 days acute depletion protocol (Najafi et al., 2018). Since PLX3397 dissolves well in fat, we reconstituted it in fat-rich Diet Gel. Such feeding resulted in >99.9%

FIGURE 2 Type I interferon (IFN α) gene fingerprint in microglia in experimental neuromyelitis optica (NMO). (a) Representative micrographs showing immunoreactivity of IBA1, TREM2, MAC1, and CD68 in corpus callosum and striatum of wild-type (WT) and IFNAR1-KO animals injected with NMO-IgG + HC'. White line shows a needle track. (b) Representative flow cytometry dot plots showing gating strategy for identification of CD11c⁺ microglia. (c) Flow cytometry analysis showing proportions of CD11c⁺ microglia as a percentage of total microglia in WT brains injected with Hu-IgG + HC' $\pm$ rIFN β ($n = 7/n = 5$) and NMO-IgG + HC' $\pm$ rIFN β ($n = 11/n = 6$) as well as IFNAR-KO injected with NMO-IgG + HC' ($n = 7$). (d) Multidimensional scaling plot showing that subpopulations of microglia isolated from experimental NMO and naïve microglia have distinct transcriptional profiles. Colors indicate three different groups of samples: green represents CD11c⁺ microglia ($n = 2$), orange CD11c⁻ microglia ($n = 3$), and black naïve microglia ($n = 2$). Each n represents a pool of 8–10 mice from four individual cell sorts. (e) Expression of *Irf7* and *Cxcl10* in CD11c⁻ microglia and CD11c⁺ microglia isolated from ipsilateral hemisphere injected with Hu-IgG + HC' (blue bar) ($n = 4$) and NMO-IgG + HC' (green bar) ($n = 6$) was assessed by RT-qPCR. Each n represents a pool of four to six mice. (f) Venn diagram showing upregulated genes by CD11c⁺ microglia, CD11c⁻ microglia isolated from experimental NMO versus naïve microglia, and upregulated genes in NMO-IgG + HC'-injected hemisphere of brains from experimental NMO in WT versus unaffected controls (Hu-IgG + HC' in WT and NMO-IgG + HC' in IFNAR1-KO). Numbers of differentially expressed genes are indicated. Commonly upregulated genes from all the comparisons are marked with black outlining. Upregulated genes that were common in at least one microglia population and in experimental NMO in WT versus IFNAR1-KO are indicated by a pink line. Data are based on at least three experimental repeats. Data are presented as means $\pm$ SEM; each n represents an individual mouse. p -Values were determined by two-tailed Student's test. * $p < .05$

FIGURE 3 Depletion of microglia suppresses astrogliopathy. (a) Representative micrographs of brains injected with neuromyelitis optica (NMO)-IgG + HC' showing clear IBA1 immunoreactivity on control diet versus virtually no IBA1 immunoreactivity on PLX3397 diet. (b,c) Representative micrographs (b) and semiquantification scores (c) of loss of AQP4 and glial fibrillary acidic protein (GFAP) immunoreactivity in corpus callosum and striatum of animals injected with Hu-IgG + HC' (n = 3) and NMO-IgG + HC' (n = 8) fed with control (black bar) or PLX3397 diet (red bar) (n = 8). (d) Expression of *Irf7*, *Irf9*, *Cxcl10*, *Oas2*, *Clec7a*, *Mx1*, *Ifi204*, *Oas2*, and *Ifnβ* in brain hemispheres injected with Hu-IgG + HC' (n = 3) and NMO-IgG + HC' from mice that were fed with control (black bar) (n = 8) or PLX3397 diet (red bar) (n = 8) was assessed by quantitative real-time PCR (RT-qPCR). Data are based on at least two experimental repeats. Data are presented as means ± SEM; each n represents an individual mouse. p-Values were determined by two-tailed Mann-Whitney U test or two-tailed Student's (only if passed the normality test). ns—not significant; *p < .05; **p < .01; ***p < .005

depletion of microglia at Day 7 of treatment (not shown). We then induced NMO-like pathology for 2 days (the mice were fed with PLX3397 until the end of the experiment). We confirmed complete microglia depletion (Figure 3a). Interestingly, microglial depletion led to a significant reduction in NMO pathology, visible as reduced GFAP and AQP4 loss (Figure 3b,c). Moreover, we observed a decrease in IFN γ signature gene expression levels (*Irf7*, *Irf9*, *Cxcl10*, *Oas2*, *Clec7a*, *Mx1*, *Ifi204*, *Oas2*), and no difference in *IFNβ* expression (Figure 3d), all further suggesting microglia as primary responders to IFN γ .

4 | DISCUSSION

Here, we describe major importance of IFN γ -activated microglia in experimental NMO. We show a characteristic IFN γ gene expression footprint in affected tissue as well as in sorted microglia subpopulations. Moreover, we demonstrate that local exogenous application of IFN β leads to a further exacerbation of astrogliopathy. Interestingly, we show an activation of microglia and expansion of a CD11c⁺ microglial population and that this was enhanced by IFN β . In line with

these findings, deficiency in IFN β signaling suppressed experimental NMO, as well as prevented microglial activation (Khorrooshi et al., 2013) upregulation of phagocytosis markers and emergence of CD11c⁺ microglia. Importantly, depletion of microglia led to suppression of pathology and decrease of IFN β signature genes, but not of *Ifn β* . This all points to a pathomechanism whereby binding of NMO-IgG and HC' to astrocytes induces IFN β that activates microglia to initiate/facilitate NMO-like pathology.

The experimental system that we have used here models brain pathology induced by NMO-IgG and complement. As such, it is a model for pathology although not of actual disease. Injection of IgG purified from an NMO patient who was seropositive for anti-AQP4 together with HC' leads to induction of classical NMO-like astrocytopathology, which we have previously shown to be associated to secondary demyelination following intrathecal injection (Asgari et al., 2013). The 2-day intracerebral model that we used in this study models the initial phase of the astrocytopathology, with no evident leukocyte infiltration or demyelination but with strong microgliosis (Khorrooshi et al., 2013). The dependence on IFN β corresponds to clinical NMOSD and validates this as a useful model for induction of pathology.

There are conflicting data regarding the role of IFN β in NMO. We have previously shown that IFN β signaling was critical for experimental NMO pathology, in that mice deficient in IFNAR were protected (Khorrooshi et al., 2013). Another study showed that IFN β treatment had no effect on development of NMO lesions in a slice culture model (Zhang et al., 2011) but ameliorated disease in an EAE-based NMO model in rats (Oji et al., 2016). In the present study, we found a strong IFN β signaling footprint in experimental NMO, in line with Oji et al. (2016). Similarly, it has been recently shown that level of expression of IFN β signature genes positively correlated with disease severity in clinical NMOSD (Agasing et al., 2020). Moreover, treatment with IFN β dramatically exacerbated pathology. This is consistent with clinical observations that treatment with IFN β is either ineffective or detrimental in NMO (Kim et al., 2012; Palace et al., 2010). Differences between the in vivo experimental studies likely reflect differential roles of IFN β in T cell versus antibody + complement-mediated pathology (Berg, Khorrooshi, Asgari, & Owens, 2017; Khorrooshi et al., 2015).

Microglial activation is a hallmark of clinical and experimental NMO pathology (Khorrooshi et al., 2013; Misu et al., 2013). We have shown an increase of the CD11c⁺ microglia subpopulation. These cells have been shown to play a protective role in animal models of MS (Benmamar-Badel, Owens, & Wlodarczyk, 2020; Wlodarczyk et al., 2015; Wlodarczyk et al., 2018), where they express pro-myelinogenic insulin-like growth factor 1 (Wlodarczyk et al., 2015), as also shown in brain development (Wlodarczyk et al., 2017). Induction of this microglial subpopulation in adult mice has been shown to suppress EAE (Wlodarczyk et al., 2018). Here, we demonstrate that both CD11c⁺ and CD11c⁻ microglia showed strong IFN β signatures. Moreover, treatment with IFN β led to further expansion of CD11c⁺ microglia that correlated with exacerbated pathology. Of note, such emergence does not seem to be directly stimulated by IFN β , as

control animals treated with this cytokine did not show an increase in CD11c⁺ microglia. It has been recently demonstrated that one of the mechanisms for induction of CD11c⁺ microglia is microglial phagocytosis of necrotic or apoptotic cells (Anderson et al., 2019; Krasemann et al., 2017). IFN β has been shown to facilitate microglial phagocytosis (Kocur et al., 2015) and we showed enrichment of phagocytosis GO term as well as upregulation of phagocytosis markers TREM2 and CD68 in microglia in WT in comparison to IFNAR1-KO mice subjected to experimental NMO. Thus, it can be speculated that emergence of CD11c⁺ microglia is a result of IFN β -enhanced microglial phagocytosis of IgG + complement-marked or -damaged astrocytes. Accordingly, depletion of microglia led to suppression of pathology, and lesions were significantly smaller. Decrease of IFN-regulated genes that are normally expressed upon IFNAR activation, with no change in *Ifn β* expression, upon microglial depletion strongly suggests that microglia are responders to IFN β rather than producers. Whether CD11c⁺ microglia facilitate progression of the disease, and whether they are induced as a response to IFN β stimulation of phagocytosis of astrocytes by CD11c⁻ microglia, both require further investigation.

Several microglia depletion strategies were attempted during the course of this study. Although clodronate liposomes (Vinet et al., 2012) achieved robust microglial depletion in an ex vivo NMO spinal cord slice culture model the outcomes were inconclusive (not shown). Similarly, mixed results regarding microglia role in NMO pathology in this model have previously been shown, where LPS-activated microglia induced more pronounced lesions while microglia depletion did not have an impact for lesion development (Zhang et al., 2011). In addition, IFN β also had no effect on lesion development in that study (Zhang et al., 2011). These findings contradict our in vivo model as well as actual human disease findings. They could reflect artificially high content of horse serum (25%) in their culture medium as well as the age of tissue used. Both could influence microglial responsiveness, including to IFN β . We also used tamoxifen-induced expression of diphtheria toxin receptor (DTR) in microglia, using CX3CR1iDTR Tg mice as previously described (Bruttger et al., 2015). We confirmed that DTx efficiently depleted microglia in otherwise unmanipulated brains (~85% depletion) (Figure S1a). Induction of experimental NMO immediately and dramatically promoted microglial repopulation, despite continuous DTx administration (Figure S1b), possibly due to increased proliferation of the remaining 15% microglia that escaped depletion. Thus, caution is needed for use of this depletion model in investigation of microglial role in neuroinflammation.

Taken together, our data point to a detrimental role for IFN β -activated microglia in initiation of astrocytopathology and open new perspectives for microglia-targeted treatment strategies.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The datasets of RNA-seq from sorted microglia are deposited in Gene Expression Omnibus (GEO): GSE98784.

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