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Universidad del País Vasco | Euskal Herriko Unibertsitatea

Departamento de Neurociencias

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Purinergic P2X4 receptors modulate neuroinflammation and repair in experimental multiple sclerosis

DOCTORAL THESIS
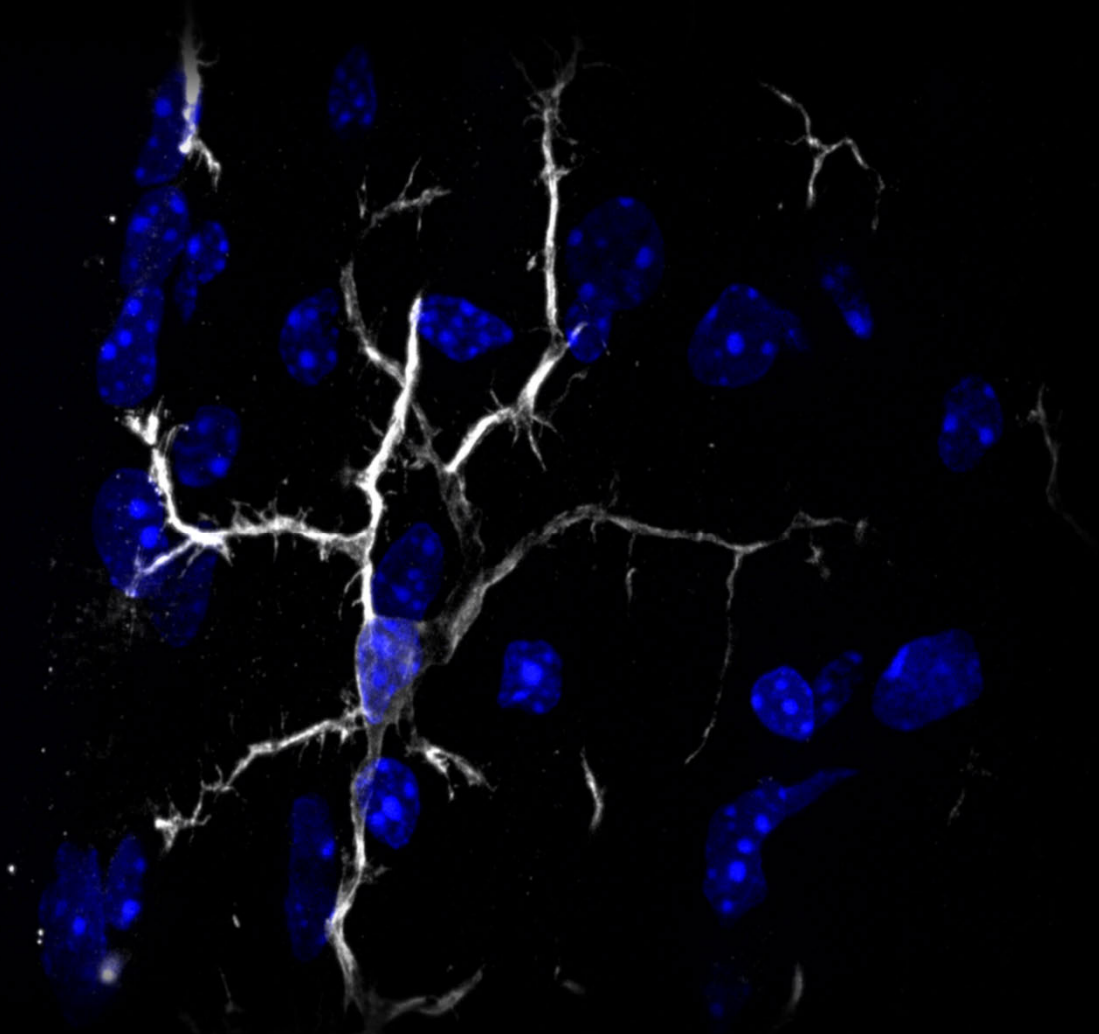### Alazne Zabala Olaizola
2019

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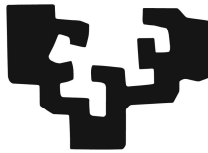
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Cover image: Immunolabelling of resting spinal cord microglia.
White: microglia. Blue: nuclear labelling.

DOCTORAL THESIS

Alazne Zabala Olaizola

2019

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Attitta eta amamai

ABBREVIATIONS

γδTCR	Gamma-delta T-cell receptor
5-BDBD	5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APC	Antigen presenting cell
APC (CC1)	Adenomatous polyposis coli clone CC1
Arg1	Arginase gen
ATP	Adenosine triphosphate
ATPγS	ATP-gammaS
B220	Cluster of differentiation 45 isoform B220
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
BzATP	2'(3')-O-(4-Benzoylbenzoyl)adenosine-5'-triphosphate tri (triethyl-ammonium) salt
C3	Complement protein 3
Calcein-AM	Calcein- acetoxymethyl
cAMP	Cyclic adenosine monophosphate
Ccl2	C-C motif chemokine ligand 2 gen
CCR2	C-C chemokine receptor type 2
CD	Cluster of differentiation
cDNA	Complementary DNA
CIS	Clinically isolated syndrome
CNS	Central nervous sistem
CNTF	Ciliary neurotrophic factor

DAMP	Damage associated molecular pattern
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle's Medium
DMT	Disease modifying treatment
DPI	Day postimmunization
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ERSI	Experimental Research in Stroke and Inflammation
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Antibody receptor
Foxp3	Forkhead box P3 gen
GABAergic	Gamma-aminobutyric acid-ergic
GDNF	Glial cell-derived neurotrophic factor
GM -CSF	Granulocyte macrophage colony-stimulating factor
HERV-W	Human endogenous retrovirus type W
IB4	Isolectin B4
Iba1	Ionized calcium binding adaptor molecule 1
IFN-ɣ	Interferon-γ
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IL	Interleukin
IMDM	Iscove´s Modified Dubelcco´s Medium
iNOS	Inducible oxide nitrite synthase
IRF	Interferon regulatory factor
IRF5	Interferon regulatory factor 5
IRF8	Interferon regulatory factor 8

II

IVM	Ivermectin
LPC	Lysolecithin
LPS	Lipopolysaccharide
Ly6G	Lymphocyte antigen 6 complex locus G6D
MBP	Myelin basic protein
MCM	Microglia conditioned medium
MCMS	Myelocortical multiple sclerosis
M-CSF	Macrophage colony-stimulating factor
MOG	Myelin oligodendrocyte glycoprotein
Mrc1	Mannose receptor gen
MRC1	Mannose receptor
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Multiple sclerosis
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG2	Neuron-glial antigen 2
NGF	Nerve growth factor
NIH	National Institutes of Health
NO	Nitric oxide
NOD	Non-obese diabetic
Nos2	Inducible oxide nitrite synthase gene
NP-1815-PX	(5-[3-(5-thioxo-4H-[1,2,4]oxadiazol-3-yl)phenyl]-1H-naphtho[1, 2-b][1,4]diazepine-2,4(3H,5H)-dione)
NT3	Neurotrophin-3
Olig2	Oligodendrocyte transcription factor 2
OPC	Oligodendrocyte progenitor cell
P2X4R	Purinergic P2X4 receptor

P2X7R	Purinergic P2X7 receptor
PAMP	Pathogen-associated molecular pattern
PB	Phosphate buffer
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PDL	Poly-D-Lysine
PFA	Paraformaldehyde
PLP	Proteolipid protein
PNI	Peripheral nerve injury
PPMS	Primary-progressive multiple sclerosis
PRR	Pattern recognition receptors
PVDF	Polyvinylidene difluoride
pPCR	Quantitative polymerase chain reaction
ROI	Region of interest
Ror	Retineic-acid-receptor-related orphan nuclear receptor gamma
RRMS	Relapsing-remitting multiple sclerosis
RT	Room temperature
SEM	Standard error of the mean
SMI-32	Neurofilament H Non-Phosphorylated protein
SNP	Single nucleotide polymorphisms
SPMS	Secondary progressive multiple sclerosis
T3	Tri-iodothyronine
T4	L -Thyroxine
TAK1	Transforming growth factor-beta-activated kinase 1
TBS-T	Tris buffer saline tween-20
TGF-β	Transforming growth factor-beta
Th	T helper cell

IV

TMEV   Theiler´s murine encephalomyelitis virus

TNF-α   Tumor-necrosis factor-alpha

TNP-ATP   2',3'-O-(2,4,6-Trinitrophenyl) adenosine-5'-triphosphate tetra(triethyl-ammonium) salt

Treg   Regulatory T cell

U   Unit

UDP   Uridine diphosphate

UTP   Uridine triphosphate

WM   White matter

WT   Wild type

YFP   Yellow fluorescent protein

INTRODUCTION

1. MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) which was first described clinically by Charcot in 1868. MS is the most common disabling neurological disease that typically affects young adults causing an irreversible physical and mental disability (*Dendrou et al., 2015*). Globally, the estimated number of people with MS is 2.3 million people worldwide (*Browne et al., 2014*) and about two-thirds of those affected are woman (*Alonso and Hernán, 2008; Giovannoni et al., 2016*).

MS is a heterogeneous disease clinically and pathologically (*Ntranos and Lublin, 2016*). The principal features of the disease are focal lesions with inflammatory reaction to myelin components leading to oligodendrocyte death and demyelination, and axonal damage (*Dendrou et al., 2015; Mahad et al., 2015*). The causes of the disease are still unclear, but it is widely accepted that both genetic and environmental factors converge in the manifestation of the disease (*Hafler et al., 2007; Huynh and Casaccia, 2013*). Demyelination in MS comes from an inflammatory process and, to a variable degree, from axonal damage and it may occur within any area of the CNS. Demyelination results in slower conduction or complete failure of transmission, leading to neuronal dysfunction and neurological symptoms and signs that might occur depending on the location of lesions in the CNS, especially in the white matter tracks, although lesions in the gray matter can also be found. The most common symptoms of MS include sensory and visual disturbances, spasticity, weakness, painful spasms, bladder dysfunction, tremor, ataxia, optic neuritis, fatigue, and dysphagia (*Compstom and Coles, 2008*). The progressive disability suffered by those affected by MS could be due to the accumulation of residual deficits during the outbreaks or to the inexorable progression of the disease, which is independent of the exacerbation.

1.1. MS subtypes

In 1996, Lublin and Reingold classified MS into four independent subtypes on the basis of the clinical course of the disease (*Lublin and Reingold, 1996*). However, the new findings in the understanding of MS and its pathology, showed the need to reexamine the classification of MS disease subtypes. The revised Lublin classification takes into account the magnetic resonance imaging (MRI) lesion activity and progression of disability to classify MS phenotypes in addition to the clinical activity (*Lublin, 2014*). The current four disease courses (types) of MS are (**Figure 1**):

o Clinically isolated syndrome, or CIS: this is the first clinical event that shows characteristics of inflammatory demyelination that could be MS but has not yet fulfill criteria of dissemination in time. When CIS is accompanied by magnetic resonance imaging (MRI)-detected brain lesions patients have 60-80% chance to develop MS, while when CIS is not accompanied by MRI-detected brain lesions, the probability is about 20% (*Miller et al., 2005*).

o Relapsing-remitting MS, or RRMS: this is the most common form, affecting approximately 85% - 90% of patients. It is characterized by unpredictable acute relapses followed by a period of recovery. Relapses coincide with focal CNS inflammation and demyelination that are typically discernible, using MRI, as white matter lesions (*Dendrou et al., 2015*). During this relapse, CNS repair mechanisms operate remodeling and compensating the damage (*Rocca et al., 2003; Rocca and Filippi, 2007*).

o Secondary progressive MS, or SPMS: this subtype is characterized by an initial RRMS, which later develops into a progressive stage with irreversible neurological decline. Approximately 80% of RRMS patients will evolve to SPMS. Inflammatory lesions are not characteristic of this subtype and progressive neurological decline is instead accompanied by CNS atrophy and increased axonal loss.

o Primary-progressive MS, or PPMS: 10-15% of the patients miss the relapsing phase of the disease and develop continuous disease progression from the onset.
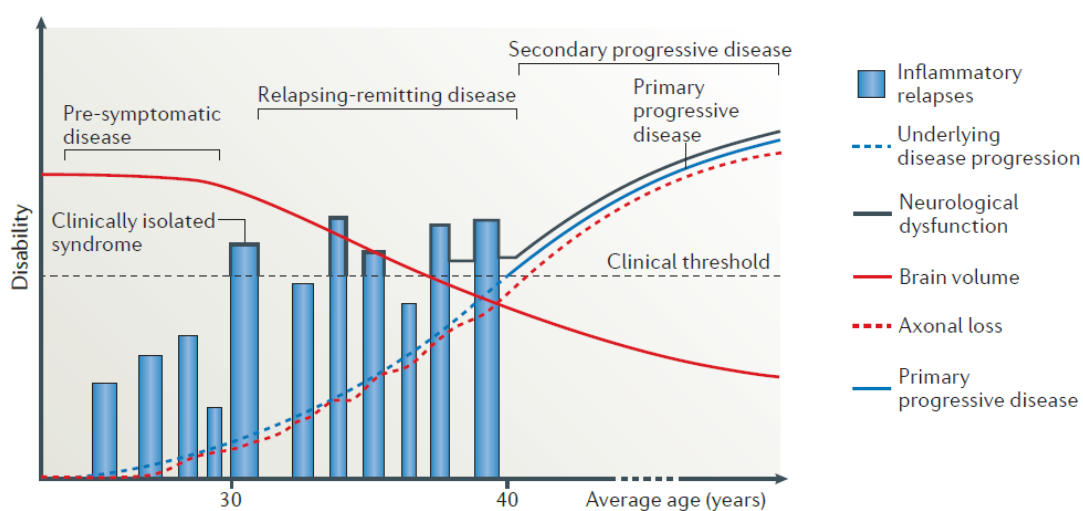
Figure 1. Representation of MS subtypes indicating the characteristic neurological dysfunction, brain atrophy and axonal loss in each subtype (*Dendrou et al., 2015*).

Recently a novel subtype of MS called myelocortical MS (MCMS) has been proposed. MCMS is characterized by demyelination of spinal cord and cerebral cortex, but not of cerebral white matter, although there is cortical neuronal loss (*Trapp et al., 2018*). This is the first study to provide pathological evidence that cortical neuronal degeneration can be independent of cerebral white matter demyelination.

1.2. Etiology of MS

Without a known predominant exogenous risk factor, it is an open question whether multiple sclerosis is triggered in the periphery ("outside-in" theory) or in the CNS ("inside-out" theory) (**Figure 2**) (*Tsunoda and Fujinami, 2002; Matute and Pérez-Cerdá, 2005*).

In the "**outside-in**" model, autoreactive T cells are activated in the periphery – probably through molecular mimicry (*Geginat et al., 2017*) – and enter in the CNS crossing the blood brain barrier (BBB) accompanied by activated B cells as well as innate immune cells including monocytes, dendritic cells and natural killer T cells leading to demyelination and axonal damage (*Lopez-Diego and Weiner, 2008*). The "outside-in" model supports the use of the animal model of experimental autoimmune encephalomyelitis (EAE) to study the MS. In this model, mice are immunized with myelin antigens, generating a peripheral immune response with activated CD4$^+$ T helper 1 (Th1) cells and Th17 cells. These activated cells cross the BBB (*Ajami et al., 2011*) and act against myelin inducing myelin destruction, oligodendrocyte death and clearance of damaged tissue by phagocytes (**Figure 2a**). As the disease progresses the immune cell infiltration decreases, and chronic inflammation is generated mainly due to microglia and astrocytes chronic activation.

Alternatively, the "**inside-out**" model argues that the initial event would be a primary cytodegeneration in the CNS. The most important evidence supporting this model is the histopathological finding of oligodendroglial apoptosis in the absence of immune cell infiltration at early stages of the disease described by Barnett and Prineas (*2004*) in patients who died during or shortly after the onset of a fatal relapse. A viral infection, glutamate and other agents can cause extensive oligodendrocyte apoptosis or primary axonal degeneration in tissue foci and as a result, myelin debris and highly antigenic constituents are generated, promoting autoreactive lymphocytes infiltration in the CNS as a secondary event (**Figure 2b**). There are several evidences associating viral infections in the CNS and MS. Theiler´s murine encephalomyelitis virus (TMEV) can infect the CNS and its infection is accompanied by axonal damage in mice (*Tsunoda and Fujinami, 2002*). Another example that supports the "inside-out"

model is the human endogenous retrovirus type W (HERV-W) which expresses syncytin, a protein that is cytotoxic to oligodendrocytes and shows increased expression in acute demyelinating lesions of MS patients (*Antony et al., 2004*). Oligodendrocyte apoptosis can also be caused by increased extracellular levels of glutamate that cause oxidative stress and excitotoxicity in oligodendrocytes and this response has been associated with MS (*Matute et al., 2001; Werner et al., 2001; Groom et al., 2003*).
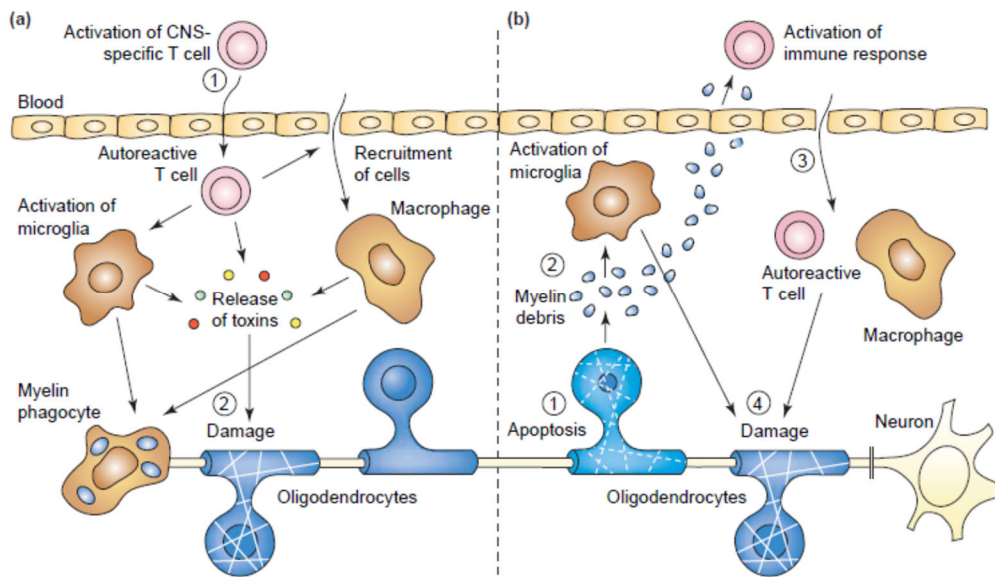
Figure 2. Alternative views of the mechanism of lesion formation in MS. (a) "Outside-in" model; activated T cells migrate into the CNS (1) and initiate inflammatory events, including recruitment of blood macrophages, activation of local microglia and release of toxins. This leads to myelin destruction, oligodendrocyte death and clearance of damaged tissue by phagocytes (2) (b) "Inside-out" model; viruses, glutamate and other agents can cause extensive oligodendrocyte apoptosis in tissue foci (1). Consequently, large amounts of myelin debris are generated (2), overwhelming the physiological mechanisms of elimination of apoptotic leftovers and, thus, triggering inflammation. Subsequently, T cells and macrophages invade the CNS (3) and initiate a stereotyped autoimmune attack of myelin (4), as described in (a). (*Matute and Pérez-Cerdá, 2005*).

Drawing support for either model of multiple sclerosis etiology warrants new studies. Even so, the "outside-in" and "inside-out" models could be acting in synergy and not be mutually exclusive.

1.3. Pathophysiology of MS

The pathological hallmark in MS is the presence of focal areas of inflammatory-mediated demyelination in the brain and spinal cord that are called plaques or lesions, indicative of myelin and oligodendrocyte loss (*Dendrou et al., 2015; Lassmann and Bradl, 2017*). Although axons and neurons are mostly preserved in the first stages of multiple sclerosis, disease progression results

in gradual neuroaxonal loss that correlates with patient disability and brain atrophy (*Dendrou et al., 2015*). However, there is also evidence for an early axonal damage (*Trapp and Nave, 2008*), resulting from a direct interaction with immune cells (*Zipp and Aktas, 2006*). Inflammation can be found at all stages of the disease, but it is more characteristic of the acute phase.

At the beginning of the disease, the BBB is disrupted and infiltrating immune cells are present in early lesions (**Figure 3**). Predominant infiltrating cells are the macrophages with lower amount of CD8$^+$ T cells and few CD4$^+$ T cells, B cells and plasma cells. Infiltrates are accompanied by the expression of immune associated molecules, such as major histocompatibility antigens, adhesion molecules or cytokines (*Dendrou et al., 2015*). Thus, there is a diffuse inflammatory process which is more accentuated within the focal lesions. Lesions are not exclusively found in the white matter, since demyelination can also be found in the grey matter. In the early phase of the MS there is little damage outside the lesion areas, termed normal-appearing white matter, although brain volume is already reduced. Microglia and macrophages are activated at the beginning of the EAE pathogenesis, event that could precede T cell activation and infiltration (*Ajami et al., 2011; Goldmann et al., 2013; Yamasaki et al., 2014; Yoshida et al., 2014*). In contrast, there are evidence that microglia/macrophage activation counteracts pathological processes by providing neurotrophic and immunosuppressive factors and by promoting oligodendrocyte differentiation and recovery (*Kotter et al., 2006; Miron and Franklin, 2014; Lampron et al., 2015*).

It is accepted that demyelinated areas can be partially repaired by remyelination, but at the chronic phase these repairing mechanisms are not as efficient as in the initial phases of the disease. A fail in the repairing mechanisms and an exhaustion of the neurological reserves determines the transition from RRMS to SPMS (*Ransohoff, 2012; Dendrou et al., 2015*). Accompanying disease progression, inflammatory T cell and B cell infiltrates, microglia and astrocyte activation, and diffuse myelin reduction and axonal injury are evident. Microglia and macrophages remain activated contributing to a chronic state of the disease. Moreover, the number of microglia and macrophages in the lesions correlates with the tissue damage, indicative of the contribution of these cells to the neurodegeneration (*Bitsch et al., 2000, Rasmussen et al., 2007; Fischer et al., 2013; Vogel et al., 2013*).

The understanding of the implication of the different cell populations at the different MS stages is difficult to clarify, due to the multicellular pathophysiology of the MS associated with infiltrating adaptive and innate immune cells, as well as CNS-resident microglial cells with inflammatory capacity. During last decades, several immunomodulatory therapies have resulted

in a great advance in the treatment of MS. The disease-modifying treatments (DMTs) target T and B cells activation and immune cell infiltration in the CNS. These treatments efficiently reduce relapses and their severity, but they are not able to stop disease progression and neuroaxonal damage continuous to accumulate resulting in an inevitable chronic phase of the disease (*Haghikia et al., 2013; Feinstein et al., 2015*). This supports the idea that there could be mechanisms involved in driving overt relapses different from those driving chronic progression. It is clear that peripheral immune system is implicated in this process, but there is an additional inflammatory component residing in the CNS that are microglial cells. These CNS-resident immune cells are less characterized and are not targeted with the DMTs. A goal for future treatment of multiple sclerosis should be a combination of modulation of peripheral immune cells and also, modulation of microglial cells, along with the provision of neuroprotective or neuroregenerative drugs (*Dendrou et al., 2015*).
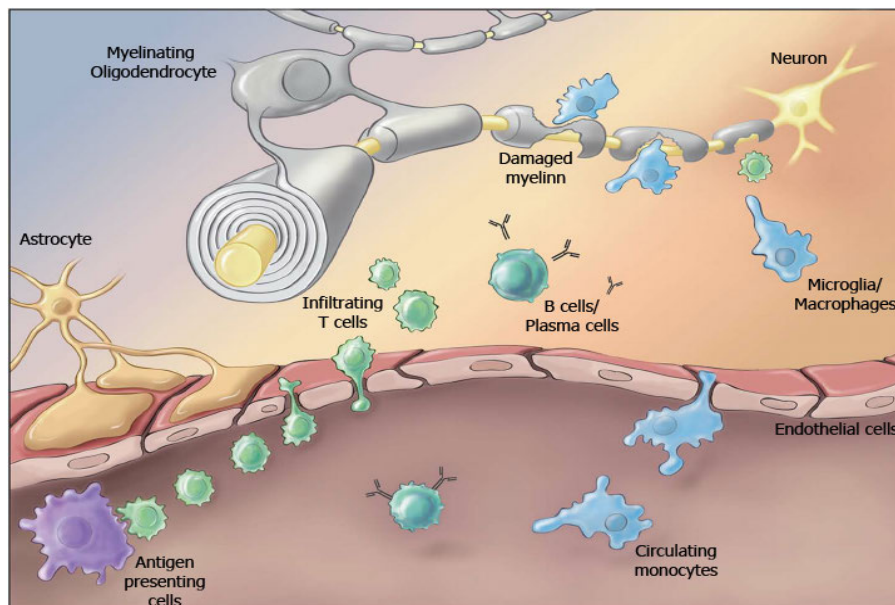
Figure 3. Pathophysiology of MS. In lymphoid organs autoreactive T cells interact with antigen-presenting cells and B cells and, after activation, are able to cross the blood brain barrier. In the CNS, reactivation of autoreactive T cells results in production of effector cytokines, attraction of macrophages and microglia, antibody production by plasma cells and attack by CD8^{+} T cells. Together these mechanisms lead to demyelination and axonal injury. Adapted from *Bittner and Meuth, 2013*.

1.4. Animal models of MS

Animal models are critical to study complex diseases as MS, a disease in which we have limited understanding of the etiology and pathophysiology. This complexity is also reflected in the animal models. There is currently no single animal model that can reflect the whole spectrum of the disease.

1.4.1. Experimental autoimmune encephalomyelitis (EAE)

The most commonly used animal model is called experimental autoimmune encephalomyelitis (EAE) and it is used to reproduce the inflammatory aspects of MS. The inoculation of an antigenic emulsion with an adjuvant, triggers an immune response. Due to the immunization regimen, the immune response is mediated by predominantly CD4$^+$ T cells directed against CNS myelin, whereas CD8$^+$ T cell responses dominate in MS, an aspect to consider when translating EAE results to MS. The pathological features are similar to MS, showing foci of inflammation and perivascular and periventricular demyelination. However, most EAE models show focused inflammation in the spinal cord, whereas MS is usually dominated by brain inflammation. The clinical course depends on the immunogen and mouse strain used. Immunization of SJL/J mice with an epitope of proteolipid protein (PLP) induces a clinical course with relapses (*Tuohy et al., 1989*), while EAE induced by myelin oligodendrocyte glycoprotein (MOG) in C57BL6/J mice results in chronic clinical course (*Tompkins et al., 2002*). Finally, immunization in non-obese diabetic (NOD) mice reproduces the chronic progression of the disease. The immunogen and the mouse strain need to be selected in concordance with the scientific question.

New genetic engineering technologies and transgenic mice, in combination with advances in imaging tools are now expanding the potential utility of EAE. Thus, EAE will remain an essential tool for preclinical and mechanistic research, enabling findings from simpler *in vitro* systems to be corroborated *in vivo*. However, EAE is a reductive model that needs to be used and interpreted with care (*Dendrou et al., 2015*).

1.4.2. Virus-induced demyelination

Many studies have put forward a hypothesis that a viral infection could be the initial event in MS pathology that results in immune response against CNS (*Tiwari et al., 2018*). Despite the current lack of evidence for an MS-specific viral infection, this model could be useful to provide insights into the basic mechanisms and to study the possible contribution of viruses in human MS (*Lassmann and Bradl, 2017*).

The best studied virus that induces demyelination in the CNS is Theiler's murine encephalomyelitis virus (TMEV). The BeAn and Daniel's TMEV strains are the most used ones that induce a chronic-progressive disease course in susceptible mice. The acute encephalitic phase is followed by a chronic demyelinating disease, which mainly affects the spinal cord. The lesions in this model are characterized by chronic inflammation, the formation of confluent

plaques of primary demyelination, axonal injury, and remyelination. Lesions show a mixture of CD4^{+} and CD8^{+} T cells, B cells, and plasma cells (*Tsunoda et al., 2007; Pachner, 2011*). Thus, the lesions share essential features with those present in MS.

1.4.3. Toxin-induced demyelination

Primary demyelination is a pathological hallmark of MS, distinguishing from other inflammatory diseases of the CNS. The discovery of new therapeutic strategies, targeting de- and remyelination requires a deep knowledge on the mechanisms involved in these processes (*Franklin and Gallo, 2014*). This information can be obtained better in toxin-induced demyelination models, which are not complicated by changes in the CNS due to inflammatory processes driven by adaptive immunity. The two most common demyelinating agents used are lysolecithin (LPC) and cuprizone (*Lassmann and Bradl, 2017*).

Cuprizone is a copper chelating reagent which acts as a toxin inducing oligodendroglial cell death and subsequent demyelination accompanied by astrocytes and microglia activation (*Matsushima and Morell, 2001*). The specific target are mature oligodendrocytes that undergo apoptosis due, probably, to extensive metabolic demand. The other cell populations are not affected (*Lucchinetti et al., 2000; Liu et al., 2010*), although the reasons remain unknown. Cuprizone addition to the diet in young adult mice results in demyelination of several white matter structures in the brain. After cuprizone removing from diet, new oligodendrocytes, generated from the pool of oligodendrocyte progenitor cells (OPC), form new myelin sheaths and remyelinate partially or totally brain white matter (*Matsushima and Morell, 2001*).

LPC is an activator of phospholipase A2 that is injected usually in the spinal cord inducing focal plaques of demyelination at a defined location. In this model, the demyelination occurs due to the toxic effect of the detergent on lipid membrane-rich myelin sheaths (*Jeffery and Blakemore, 1995*). LPC induced demyelination is independent of immune response and it works in immune-deficient mice too. Immediately after LPC injection, T cells, B cells and macrophages infiltrate the lesion site and this short-lived infiltration is proposed to be beneficial to initiate remyelination (*Bieber et al., 2003*). The lesion site is rapidly repaired, although the speed and degree of remyelination are age-dependent, showing complete remyelination in 5-6 weeks in young animals (*Franklin et al., 2012; Crawford et al., 2013*).

2. MICROGLIA

Mammalian CNS is composed of neurons and glial cells, being microglial cells the 5-12% of total glial population in healthy adult brain (*Lawson et al., 1990; Perry, 1998; Aguzzi et al., 2013*). Microglial cells, first characterized by Pío del Río-Hortega as a different cell type (*del Río-Hortega, 1919*), are the resident phagocytic cells of the CNS. These cells represent a unique population based on its origin and its functions.

Microglia origin had been controversial for many years, but recently it has been convincingly confirmed that microglia derives from yolk-sac myeloid progenitors, while the rest of the CNS cells are derived from the neuroectoderm (*Ginhoux et al., 2010; Kierdorf et al., 2013*). Myeloid progenitors migrate and proliferate in the CNS parenchyma during embryogenesis before the BBB is built (*Alliot et al., 1999; Ginhoux et al., 2010; Ginhoux et al., 2013; Prinz and Priller, 2014*) and later differentiate in microglial cells. Resident microglial cells in the healthy adult brain are not replaced by circulating blood progenitors, they persist during adulthood forming a robust long-lived CNS immune cell population by constant self-renewal (*Lawson et al., 1992; Ajami et al., 2007; Kierdorf et al., 2013*).

2.1. Role of microglia

Microglia are necessary at all stages of CNS development up to adulthood, and acquire different functions depending on developmental stage due to the evolving and maturing surrounding environment (**Figure 4**).

2.1.1. Microglia during development

Microglia seem to have several different functions during the development of the CNS, particularly as "architects", who organize and coordinate the patterns and wiring in the developing CNS (*Kierdorf and Prinz, 2017*). The functions of microglia during development are described below (**Figure 4**).

o Phagocytosis of apoptotic neurons. As professional phagocytes, microglia play a major role in removing apoptotic neurons from the developing CNS. During development, approximately 50% of the newborn neurons undergo apoptosis prior to adulthood (*Dekkers et al., 2013; Schafer and Stevens, 2015*) and microglia maintain the homeostasis by phagocytosing these newly generated non-surviving cells (*Ferrer et al., 1990; Peri and Nüsslein-Volhard, 2008; Sierra et al., 2010*).

o <u>Control of developing populations of neurons - Neurogenesis</u>. Microglia are necessary for proper assembly of neuronal networks (*Reemst et al., 2016*). Besides being scavengers, microglia support and promote neuronal survival and neurogenesis in the developing CNS by secretion of different neurotrophic factors such as insulin-like growth factor 1 (IGF-1) (*Ueno et al., 2013; Arno et al., 2014; Shigemoto-Mogami et al., 2014*). Moreover, they play active roles in the induction of neuronal cell death in the developing brain (*Frade and Barde, 1998; Marín-Teva et al., 2004; Wakselman et al., 2008*). Thus, microglia play a major part in the establishment of the correct number of neurons by regulating cell death and survival in a region-specific manner.

o <u>Guidance of the developing vasculature - Vasculogenesis</u>. One important event during the development of the CNS from a few neuronal layers to billions of neurons organized in different circuits within distinct brain regions is the vascularization of the CNS which provides nutrients and oxygen. Microglia are uniquely positioned to influence the early sprouting, migration, anastomosis, and refinement of the growing CNS vascular system (*Arnold et al., 2013*). Microglia can guide and connect sprouting vessels by secretion of soluble guidance factors (*Rymo et al., 2011*).

o <u>Maturation and refinement of neuronal circuits – Synaptic pruning</u>. Microglia are crucial for the synaptic maturation. Microglia have a dynamic role in the removal of unused dendritic spines during postnatal phases in a process called "synaptic pruning". This synaptic pruning is dependent on microglia number and on neuronal activity of the dendritic spines (*Paolicelli et al., 2011; Schafer et al., 2012*). Microglial synaptic pruning is also dependent on the labelling of developing synapses with complement protein 3 (C3), which leads to phagocytosis (*Stevens et al., 2007*). A deficiency in this function leads to a rather immature brain circuitry and is assumed to be involved in neurodevelopmental and neuropsychiatric disorders (*Paolicelli et al., 2011; Hoshiko et al., 2012; Schafer et al., 2012; Squarzoni et al., 2014; Zhan et al., 2014*).

o <u>Myelination</u>. Microglia support oligodendrogenesis by secreting specific cytokine mixtures into the subventricular zone of developing rat brains (*Shigemoto-Mogami et al., 2014*). *In vitro* studies also have shown that microglia enhanced oligodendrocyte survival by inhibition of apoptosis, by secretion of soluble factors such as PDGF (platelet-derived growth factor) and by inducing the expression of transcription factor NF-kB p65 in oligodendrocytes (*Nicholas et al., 2001*). In a new recent study, Wlodarczyk and colleagues uncover a transient

subset of CD11c^{+} microglia that regulate CNS myelinogenesis via the release of IGF-1, a factor essential in myelination (*Wlodarczyk et al., 2017*).

2.1.2. Microglia in adult brain

In the mature, healthy CNS so-called "resting" microglia is found in a ramified shape with long and highly branched processes maintaining nonoverlapping regions. However, this commonly used term "resting microglia" is therefore a misnomer, since *in vivo* two photon microscopy studies have shown that microglia is a highly motile cell in the healthy brain (*Nimmerjahn et al., 2005; Wake et al., 2009; Nimmerjahn, 2012*). Microglia are constantly and actively scanning their microenvironment by frequent process extension and retractions rather than just "resting". It is becoming clear that microglia make rapid contacts with all CNS components as nearby neurons, including synapses and Ranvier nodes, other glial cells and blood vessels, having an essential role in the correct brain functioning and maintenance of tissue integrity and homeostasis (*Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009; Kettenmann, 2011; Li et al., 2012; Zhang et al., 2018*). The functions of microglia in the adult brain are described in the following paragraphs (**Figure 4**).

o <u>Modulation of synaptic plasticity</u>. Microglial phagocytosis of synaptic elements occurs during postnatal development, adolescence but also in adulthood and during ageing (*Tremblay et al., 2010; Paolicelli et al., 2011; Schafer et al., 2012; Milior et al., 2016*), suggesting a critical role in maintaining synaptic plasticity and behavioral adaptation to the environment under physiological conditions (*Tremblay et al., 2010*). However, an excesive removal of synapses might aggravate several diseases such as Alzheimer´s disease, schizophrenia, or ageing (*Vilalta and Brown, 2017*). When synapses are removed from injured neurons, the process of phagocytosis is called "synaptic stripping" (*Kettenmann et al., 2013*). Healthy microglia additionally produce a broad spectrum of signaling molecules, from cytokines to neurotransmitters and extracellular matrix proteins which are capable of regulating neuronal activity, as well as synaptic activity and functional plasticity (*Bessis et al., 2007; Bechade et al., 2013; Ji et al., 2013*). These observations suggest that microglia are important effectors of structural plasticity in the healthy mature brain, mediating both the formation and elimination of synaptic elements.

o <u>Regulation of adult neurogenesis</u>. Neurogenesis continues in healthy mature brain in the subventricular zone and hippocampal subgranular zone where neural stem cells persist (*Sierra et al., 2014; Ribeiro Xabier et al., 2015; Fourgeaud et al., 2016*). Microglia serve

important functions regulating cellular density of neuronal progenitors in the neurogenic regions by phagocytosing apoptotic cells (*Sierra et al., 2014; Fourgeaud et al., 2016*). Whereas ageing seems to have a negative effect on microglia resulting in a decrease in neurogenesis, exercise and environmental enrichment physiologically prime microglia to support adult neurogenesis (*Choi et al., 2008; Vucovic et al., 2012; Gebara et al., 2013*). Because of their role in the maintenance of the hippocampal neurogenic niche during adulthood, microglia are essential components in learning and memory formation.

o <u>Remyelination.</u> In the case of demyelinating diseases such as MS, microglia play a pivotal role in regulating the remyelination process. Microglia are the responsible of phagocytosing myelin debris, which is essential to initiate remyelination because myelin debris stops OPC recruitment and differentiation in the lesion site (*Lampron et al., 2015*). In contrast, microglia may also exert a detrimental role in remyelination by the release of pro-inflammatory factors (*Domingues et al., 2016*).
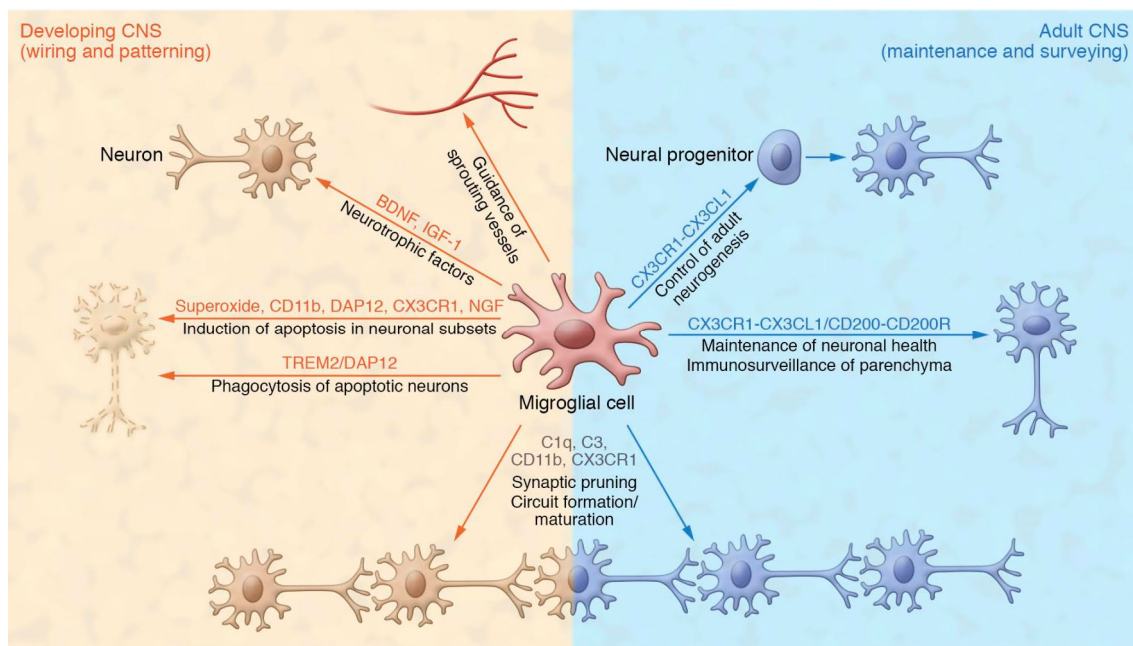
Figure 4. Schematic representation of microglial functional states in the healthy brain during development and during adulthood. In addition to their function as a resident immune cell in the CNS parenchyma, microglia display a variety of other functions to maintain tissue homeostasis. Microglia modulate wiring and patterning in the developing CNS by controlling neuronal populations inducing apoptosis and phagocytosing apoptotic cells, releasing neurotrophic factors, and guiding sprouting vessels. They also have principal role in the healthy adult brain modulating the plasticity of neuronal networks, and for regulating adult neurogenesis. (From *Kierdorf and Prinz, 2017*).

2.2. Microglia activation

One of the main characteristics of microglia is their rapid reaction to any exogenous or endogenous potential threat to the CNS homeostasis, such as infectious agents, trauma, or neurotransmitters released by damaged cells by undergoing morphological, genetic, and functional changes, usually referred as microglia "activation" (*Sousa et al., 2017*). These microglia changes include altered expression of cell surface markers and inflammation related genes, process retraction and acquisition of an amoeboid morphology, migration, proliferation and increased phagocytosis (*Kettenmann et al., 2011*). To be able to detect all this kind of signals and to properly react to them microglia express a great variety of receptors (Fc receptors, toll-like receptors, purinergic receptors, etc.) (*Domercq et al., 2013; Kigerl et al., 2014*). Some of them are classified as pattern recognition receptors (PRRs) that can recognize infections through pathogen-associated molecular patterns (PAMPs) and internal cellular disturbances through damage-associated molecular patterns (DAMPs) (*Kigerl et al., 2014*).

Activation of microglia is a natural response to CNS insults to eliminate cell debris/apoptotic cells after injury and to support tissue repair. Microglia are able to acquire distinct activation phenotypes depending on different stimulating cues or different stages of disease dynamic progression. Microglia can also act as antigen-presenting cells (APCs) and can release soluble factors such as cytokines and acute phase proteins that propagate and perpetuate the cerebral immune response (*Walter and Neumann, 2009; Yenari et al., 2010*). Microglia activation is present in most, if not all, pathological conditions in the CNS, but whether it plays a beneficial or destructive role or probably both is still under discussion.

A classically-activated or pro-inflammatory and an alternatively-activated or anti-inflammatory microglia terminology has been widely used to describe microglial activation (**Figure 5**). This definition came from the immunology field regarding macrophages stimulated by Th1 or Th2 cells cytokines, which express distinct arrays of pro-inflammatory or anti-inflammatory cytokines (*Boche et al., 2013*). Microglia are highly heterogeneous immune cells with a continuous spectrum of activation states (*Xue et al., 2014*) being the pro-inflammatory and anti-inflammatory microglia the opposite ends of this spectrum (*Mosser and Edwards, 2008; Murray et al., 2014; but see also Ransohoff, 2016*). Even if this classification underestimates the complexity of microglia plasticity, it still provides useful information regarding the diverse functions of the innate immune system in disease pathogenesis.

Pro-inflammatory microglia are activated in presence of lipopolysaccharide (LPS) and interferon gamma (IFN-ɣ) and releases large amounts of oxidative metabolites, proteases and pro-inflammatory cytokines. It has been associated with a pivotal role in CNS defence against pathogens, but it can also damage healthy neurons and glia by secreting a variety of neurotoxic factors such as inflammatory cytokines, reactive oxygen species, nitric oxide (NO) and glutamate, thus complicating the pathogenesis of neurodegenerative diseases (**Figure 5**) (*Walter and Neumann, 2009; Yenari et al., 2010*).
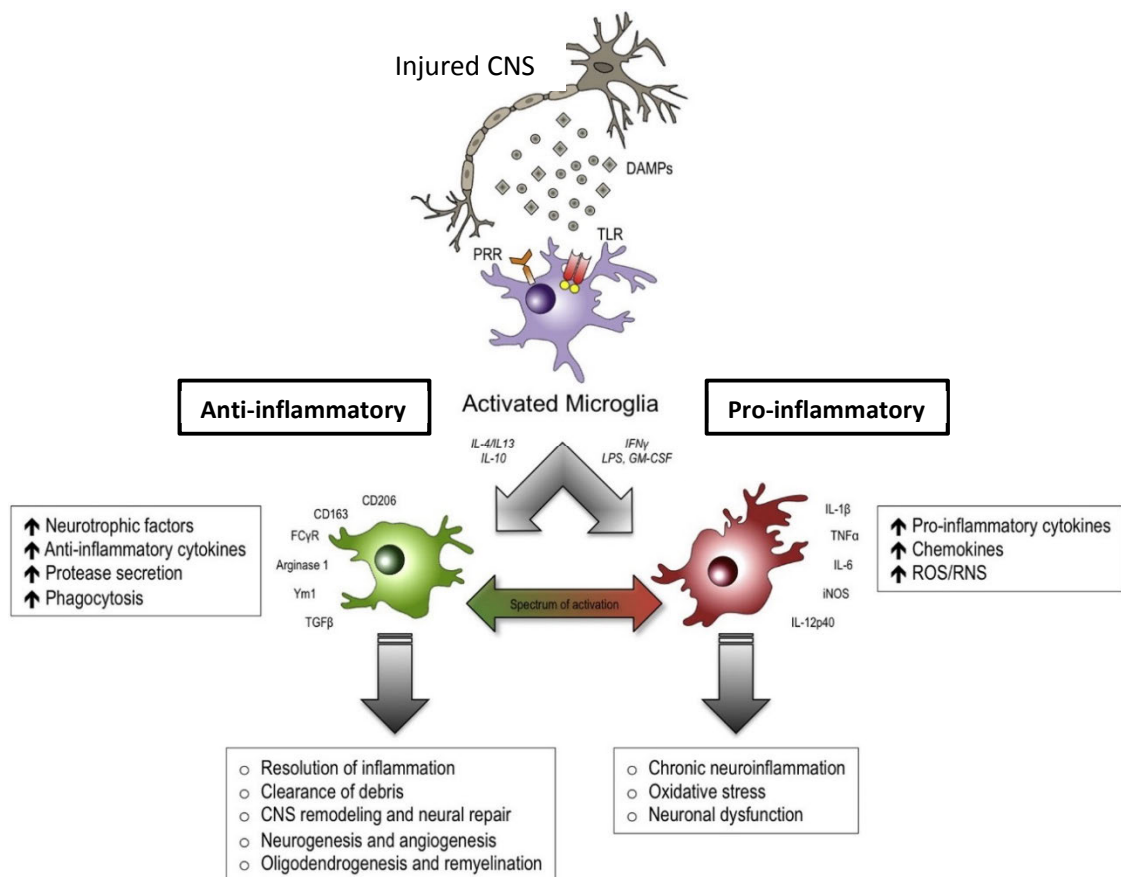
Figure 5. Microglia play important and different roles in neuroinflammation. Microglia can be neuroprotective (anti-inflammatory) by secreting neurotrophic and anti-inflammatory molecules or neurotoxic (pro-inflammatory) by releasing pro-inflammatory factors and cytotoxic substances (adapted from *Loane et al., 2016*).

In contrast, anti-inflammatory microglia are activated in presence of interleukin-4 (IL-4) and IL-13 and releases IL-10 and Arginase. It is also called neuroprotective or regenerative microglia due to its association with reduced inflammation, tissue repair and angiogenesis (*Czeh et al., 2011*). This phenotype blocks pro-inflammatory response, and produces neurotrophic factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell-

derived neurotrophic factor (GDNF), and anti-inflammatory cytokines, and shows enhanced phagocytosis (**Figure 5**) (*Czeh et al., 2011*).

2.3. Microglia/macrophages in MS

MS is an immune-mediated disease of the CNS, where the presence of activated microglia/macrophages in lesions is a hallmark of axonal damage (*Lucchinetti et al., 2000; Prineas et al., 2001*). Regarding its EAE model of disease, the number of activated monocytes from the bone marrow increases soon after immunization of mice, and these monocytes accumulate in the blood before entering the CNS (*King et al., 2009; Mildner et al., 2009*). The infiltration of the immune cells including monocytes into the spinal cord coincides with the clinical onset of disease (*King et al., 2009; Mishra et al., 2012*). Once BBB is disrupted, peripheral monocytes infiltrate the CNS and it is difficult to discriminate between activated microglia and monocytes. More recently, using a combination of parabiosis and myeloablation to replace circulating progenitors without affecting microglia, it has been demonstrated a strong correlation between monocyte infiltration and progression to the paralytic stage of EAE. Moreover, microglial cells die, and this cell death correlates with monocyte infiltration (*Ajami et al., 2011*). In addition, it has been demonstrated that endogenous microglia form perivascular clusters before demyelination or clinical signs become apparent (*Davalos et al., 2012*). Finally, in the chronic phase of EAE, when the density of T cells in lesions has subsided, accumulation of activated microglia/macrophages still persists (*Rasmussen et al., 2007; Vogel et al., 2013*). A great amount of studies are trying to elucidate how microglia/macrophages participate in the different phases of the disease.

The activation of microglia/macrophages may represent one of the initial steps in EAE pathogenesis, preceding and possibly triggering T cell development by antigen presentation and infiltration of blood-derived cells (*Heppner et al., 2005; Ajami et al., 2011; Goldmann et al., 2013; Yamasaki et al., 2014; Yoshida et al., 2014*). However, other studies indicate that microglia/macrophages activation counteracts pathological processes by providing neurotrophic and immunosuppressive factors and by promoting recovery (*Kotter et al., 2006; Miron and Franklin, 2014; Lampron et al., 2015*). This different response has been proposed to be due to different activation states of microglia/macrophages. The fact that T cells and microglia can reciprocally affect their phenotype is one of the major contributors in altering the complex balance in CNS.

In early, active inflammatory stage of the disease, pro-inflammatory microglia/macrophages are predominantly present and correlate to axonal damage. In contrast, anti-inflammatory phenotype densities undergo a gradual increase during the inflammatory process and is present in acute active lesions and in the rim of chronic active lesions where efficient remyelination occurs (*Miron et al., 2013*). Another study has recently confirmed these results using double transgenic mice expressing red fluorescent protein (tdTomato) under iNOS promoter (pro-inflammatory marker) and YFP under Arginase promoter (anti-inflammatory marker) (*Locatelli et al., 2018*). iNOS expressing microglia/macrophage accumulation was observed at earliest signs of EAE. The proportion of these cells in lesions decreased progressively over the disease course, while Arginase expressing cells increased. Moreover, in double positive cells, the ratio of iNOS versus Arginase shifted over time, in parallel of the entire microglia/macrophage population, suggesting that these cells represent an intermediate polarization state (*Locatelli et al., 2018*). Accordingly, in active MS lesions, although microglia/macrophages predominantly display pro-inflammatory markers, a major subset have an intermediate activation status (*Vogel et al., 2013*).

Pro-inflammatory microglia/macrophages are associated with enhanced antigen presentation properties and expression of high levels of inflammatory cytokines and reactive oxygen species that can contribute to pathology in disease states (*Benarroch, 2013*). IL-1β and TNFα are two of those cytokines produced by microglia during CNS inflammation. Both cytokines have been shown to be involved in the development of CNS inflammation through the disruption of the BBB, which facilitate the infiltration of leukocytes into the CNS (*Nishioku et al., 2010; Wang, 2014*). Moreover, TNFα has been associated with microglia-mediated neurotoxicity (*Block et al., 2007*).

Anti-inflammatory microglia/macrophages release several anti-inflammatory cytokines such as IL-4, IL-10, IL-13, IL-33, and TGF-β, which are involved in EAE suppression (*Jiang et al., 2012*), inflammation resolution and tissue repair (*Tierney et al., 2009; Mikita et al., 2011; Starossom et al., 2012; Laria et al., 2016*). In this activation state microglia/macrophages also release neurotrophic and growth factors that promote OPCs recruitment and differentiation and that protect neurons from damage (*Butovsky et al., 2006; Miron et al., 2013; Yu et al., 2015*). In addition, anti-inflammatory microglia/macrophages can mediate the differentiation of Th2 cells and regulatory T cells, which play an important role in regulating inflammation and controlling disease progression, as well as reducing Th1 cell activity and pro-inflammatory cytokine release (*Mosser and Edwards, 2008*). This pro-regenerative phenotype is associated with a higher

phagocytic capacity than the pro-inflammatory one being myelin debris phagocytosis necessary to OPC recruitment and differentiation to initiate remyelination (*Kotter et al., 2006*). Finally, it has been hypothesized that a block in the pro-inflammatory to anti-inflammatory switch would cause remyelination failure in chronic inactive MS lesions (*Miron et al., 2013; Sun et al., 2017*).

2.3.1. Microglia in remyelination

Contrary to the opinion often expressed that the CNS has little capacity for regeneration, lesions to oligodendrocytes or myelin, can be followed by a robust regenerative response that leads to the formation of new myelin sheaths in a process called remyelination (*Franklin and Ffrench-Constant, 2008; Franklin et al., 2012*). This regenerative response is most clearly seen in young animals following experimental demyelinating lesions and it is also seen in humans following lesions such as those caused by the MS disease (*Prineas et al., 1993; Patrikios et al., 2006; Patani et al., 2007; Franklin and Ffrench-Constant, 2017*). While remyelination is very efficient in the early stages of MS, it fails in the more chronic stages. It is thought that the loss of metabolic support to axons, normally provided by myelin sheaths, contributes to the axonal and neural degeneration and to the progressive disability that characterizes the later stage of MS (*Nave, 2010; Franklin et al., 2012*).

The key stages in remyelination are now well established. In response to demyelinating injury, OPCs that are in the vicinity are activated and enter the cell cycle (*Moyon et al., 2015*). Once activated, OPCs expand within areas of damage through a combination of proliferation and migration and finally, they undergo differentiation, a process culminating in the formation of new myelin sheaths (*Tripathi et al., 2010; Zawadzka et al., 2010*). These newly formed sheaths are often thinner than those formed during development, a characteristic widely used to distinguish areas of remyelination from normally myelinated axons (*Blakemore, 1974*).

The mechanisms controlling OPC activation are very diverse and not completely understand. An extensive literature now exists on the many factors that control both OPC division and migration, although only some studies relate to the study of OPCs in the context of demyelination (*Franklin and Ffrench-Constant, 2017*). One of mechanisms controlling OPC activation is the innate immune response triggered by tissue damage. Cells of the innate immune system, be they microglia or macrophages, are a major source of factors that enhance OPC activation, proliferation and migration (*Franklin and Ffrench-Constant, 2017*). Microglia/macrophages secrete a wide spectrum of signaling molecules that may modulate the reparative processes directly or indirectly. In recent years, many microglia/macrophage-derived

molecules have been identified that have direct effects on OPCs. IGF-1 and transforming growth factor-β (TGF-β) have long been known to promote OPC differentiation *in vitro* (*McMorris and Dubois-Dalcq, 1988; McKinnon et al., 1993; Butovsky et al., 2006*) and activin-A was found to be essential for OPC differentiation (*Miron et al., 2013*). The anti-inflammatory phenotype is more efficiently in releasing factors promoting OPC differentiation (*Miron et al., 2013*) and the timely transition from the pro-inflammatory to the anti-inflammatory state is critical for rapid and efficient remyelination.

In addition, other studies on the nature of the beneficial roles of microglia/macrophages focused on their ability to eliminate myelin debris generated during demyelination by phagocytosis (*Kotter et al., 2005; Döring et al., 2015; Lampron et al., 2015*). Studies in toxin-induced demyelination models, in which the inflammatory response is the consequence of demyelination and not its cause, have demonstrated a correlation between the abundance of debris-filled microglia/macrophages and the efficiency of remyelination (*Kotter et al., 2005; Lampron et al., 2015*). Myelin contains inhibitors of OPC differentiation which, in the intact CNS, are thought to prevent OPCs from differentiating in the absence of an exposed axon. In demyelinating diseases as MS, myelin debris generated by demyelination stop OPC differentiation and therefore they must be eliminated to facilitate OPC differentiation and remyelination. The efficacy of microglia/macrophages phagocytosing myelin debris therefore has a major influence on the reparative response.

In conclusion, through a combination of these mechanisms, microglia and macrophages can fashion a regenerative environment that maximizes the potential of OPCs to differentiate and remyelinate (**Figure 6**).

A common feature of the regenerative processes is that they become less efficient with ageing (*Goodell and Rando, 2015*). Remyelination undergoes a progressive slowing in rate throughout adult life (*Shields et al., 2000; Hampton et al., 2012; Pfeifenbring et al., 2015*). The effect of age is not only noticed in the delay of the mobilization of the microglia/macrophages, but also the ability to phagocytose myelin debris is diminished. It has been recently described that aged microglia/macrophages accumulate excessive amounts of cholesterol-rich myelin debris, which trigger cholesterol crystal formation and phagolysosomal membrane rupture. This fail in the myelin debris degradation process induces maladaptive immune response and impedes regeneration (*Cantuti-Castelvetri et al., 2018*). Moreover, it seems that the change of microglia/macrophage population from one that is predominantly pro-inflammatory to one that is predominantly anti-inflammatory is also delayed (*Franklin and Ffrench-Constant, 2017*).
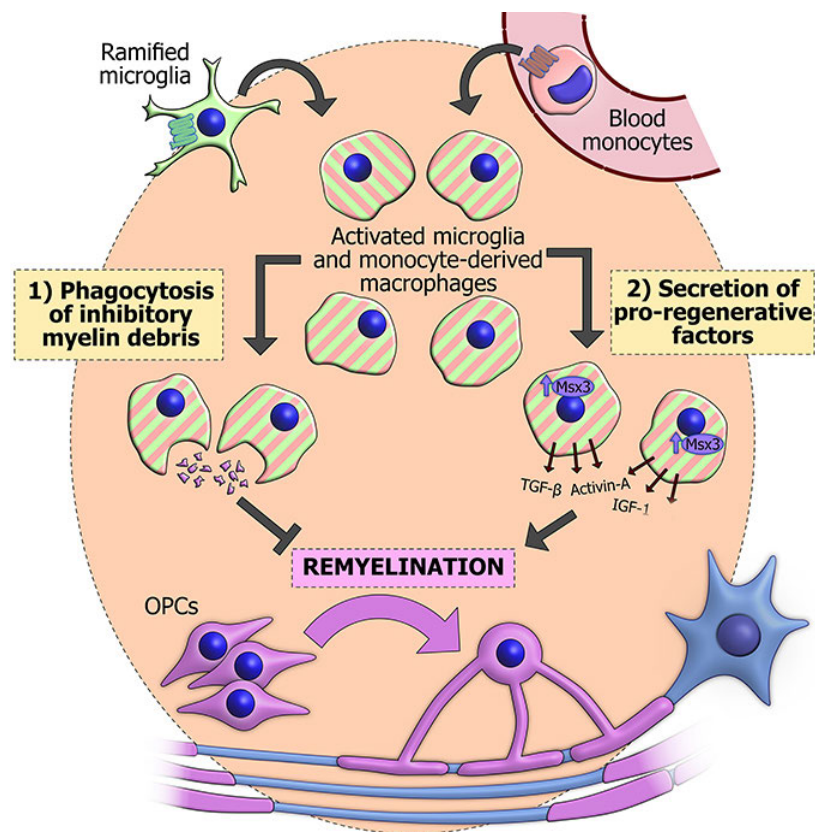
Figure 6. The actions of microglia/macrophages during remyelination. Microglia/macrophages can phagocytose inhibitory myelin debris and secrete an array of pro-regenerative factors. The combination of these functions promotes the differentiation of OPCs and subsequent reinvestment of new myelin sheaths around denuded axons (blue) (Adapted from *McMurran et al., 2016*).

As described above, microglia display an enormous plasticity in their responses to injury and they are able to promote resolution stages of inflammation and tissue regeneration too. Emerging strategies to drive microglia toward beneficial functions are appearing in the recent years, which provide insights into molecular mechanisms underlying the phenotypic switch. A variety of approaches have been proposed which rely on microglia treatment with pharmacological agents, cytokines, lipid messengers, or microRNAs, as well on nutritional approaches or therapies with immunomodulatory cells (*Fumagalli et al., 2018*). Reprograming microglia toward beneficial functions may provide new therapeutic opportunities to prevent the deleterious effects of inflammatory microglia and to control excessive inflammation in brain disorders.

3. PURINERGIC SIGNALING

One of the most outstanding facts of the purinergic system is the role of adenosine triphosphate (ATP) as energy source, as neurotransmitter and as immunomodulator. ATP molecule was first identified in 1929 and rapidly, its importance as a universal chemical energy source in biological systems was totally accepted. Later, the idea that purines could act as extracellular signaling molecules arose (*Holton, 1959*), and in 1972 Burnstock showed that ATP fit to perfection on the criteria that define a neurotransmitter (*Burnstock, 1972*). It is now widely recognized that extracellular ATP acts as either sole neurotransmitter or crucial co-neurotransmitter in most nerves in both the peripheral nervous system and CNS (*Burnstock, 2006; Khakh and North, 2012*). In healthy tissues, ATP released to extracellular space is tightly regulated by cell surface ectonucleotidases that degraded it and stop its signaling and at the same time generate adenosine and activate its purinergic signaling (*Cardoso et al., 2015*).

Purinergic signaling is a form of extracellular signaling mediated by adenosine and ATP. It involves the activation of purinergic receptors, thereby regulating several functions. This ATP and adenosine mediated purinergic signaling is essential for cell signaling and cell communication in the CNS (*Burnstock, 2017*).

In addition, ATP has been recently characterized as DAMP implicated in innate immunity (*Junger, 2011*). Immune cells, in the absence of pathogens, sense the injury by recognizing the release of molecules that are normally located inside the cell, known as DAMPs or "endogenous danger signals" (*Di Virgilio, 2007*). When ATP is released from damaged cells by pannexin opening as well as by other mechanisms, it can initiate inflammatory response and further amplify and sustain cell-mediated immunity through the activation of P2 receptors (*Domercq et al., 2013; Idzko et al., 2014*).

3.1. Purinergic receptors

Purines act as wide spread extracellular signaling molecules and their physiological effects are mediated through an extended family of purinoceptors (**Figure 7**). Two families of receptors for purines are recognized (*Burnstock, 1978*):

- Adenosine P1 receptors: a family of protein G-coupled metabotropic adenosine receptors. Currently it is established that there are four subtypes A1, A2A, A2B, A3.
- ATP P2 receptors that are subdivided into another two groups:

- P2X receptors: non-selective cation channels activated by ATP with high Ca^{2+} permeability that carry a depolarizing current under standard physiological conditions. Seven genes encoding P2XR have been identified, P2X1R-P2X7R.

- P2Y receptors: G protein-coupled metabotropic receptors sensitivity to ATP, adenosine diphosphate (ADP), uridine di- and triphosphate (UDP and UTP, respectively), or UDP-glucose depending on the receptor subtype. Upon activation, these receptors either activate phospholipase C and release intracellular calcium or affect adenylate cyclase and alter cAMP levels. There are eight P2Y receptors identified, P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14.
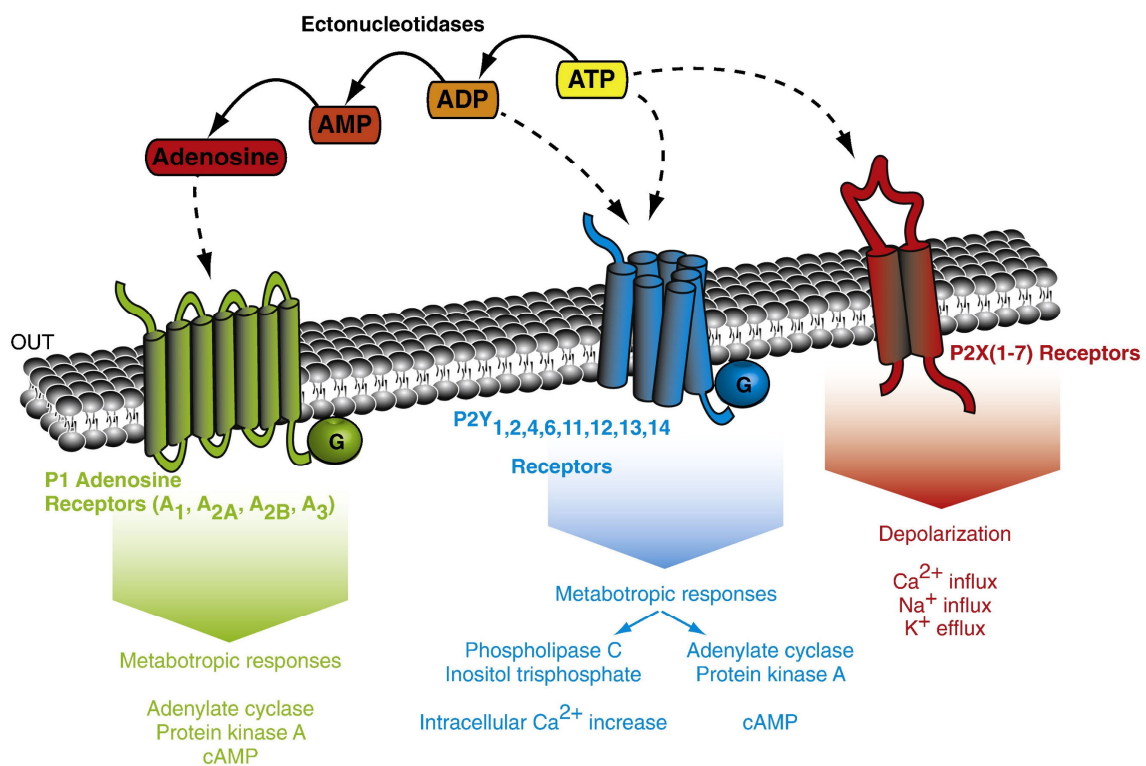
Figure 7. Purinergic receptors family: P1, P2Y and P2X. The extracellular ATP is an agonist of the P2X and P2Y receptors. Moreover, ATP is degraded to adenosine activating the P1 receptors and generating as intermediate molecule the ADP, agonist of P2Y receptors (*Baroja-Mazo et al., 2013*).

3.2. ATP receptors in microglia

Several neurological diseases and CNS injuries are characterized by initial microglia migration to lesion site and subsequent accumulation due to the proliferation of these cells. The early-phase signal responsible of this recruitment and accumulation is likely to be induced by

rapidly diffusible factors, such as ATP (*Domercq et al., 2013*). Microglial cells express a variety of purinergic receptors that control several functions including process extension and retraction, migration, proliferation, cytokine release and phagocytosis (**Figure 8**) (*Domercq et al., 2013*).

The P2X and P2Y receptors are particularly relevant in microglia, mainly the ionotropic P2X4 and P2X7 receptors and the metabotropic P2Y12 receptor. In healthy adult brain, microglia express low levels of P2X4R (*Ulmann et al., 2008*) and P2X7R (*Matute et al., 2007*) and high levels of P2Y12 receptor (*Haynes et al., 2006*). In pathological conditions, the expression pattern of purinergic receptors is altered. Microglial P2X7 receptor is upregulated in inflammatory sites of many neurodegenerative diseases (*Parvathenani et al., 2003; McLarnon et al., 2006; Arbeloa et al., 2012; Kimbler et al., 2012*). Similar to P2X7, P2X4 receptor is upregulated in activated microglia in inflammatory diseases (*Domercq et al., 2013*). In contrast to P2X4R and P2X7R, the expression of P2Y12 receptor is down-regulated in parallel to microglia activation (*Haynes et al., 2006; Beaino et al., 2017*).

In healthy brain, movement of the fine microglial processes is controlled primarily through the activation of P2Y12 receptors. In disease conditions, when ATP is released in the injury site, low ATP concentrations of surrounding areas activate P2X4 and P2Y12 receptors that mediate microglia chemotaxis characterized by cell body and process movement in order to recruit cells to the lesion site (*Honda et al., 2001; Ohsawa et al., 2007*). When microglia moved to the lesion site the loss of P2Y12 receptor occurs in parallel to microglial morphological change from ramified to ameboid state. This downregulation suggests that P2Y12 receptors are involved in the early, rather than late, responses of microglia to injury. In contrast, the upregulation of P2X4 receptor indicate that chemotaxis after injury could be mediated by this receptor (*Ohsawa et al., 2007*).

P2X7 receptors have low ATP affinity and ATP levels in the extracellular space are in the low nanomolar range due to its rapid inactivation by ecto-ATPases (*Abbracchio et al., 2009*), reason by which is not clear if this receptor is activated in physiological conditions. In the injury foci high ATP concentrations are available and can activate P2X7 receptors (*Di Virgilio et al., 2016*). P2X7 receptors have an important role in controlling microglial activation, proliferation, release of pro-inflammatory cytokines and cell death (*Burnstock, 2012*). In immune cells, P2X7 receptor activation promotes assembly of the "inflammasome" and caspase-1-dependent cleavage as well as the release of IL-1β and IL-18 pro-inflammatory cytokines, ultimately leading to a programmed pro-inflammatory cell death called pyroptosis. P2X7Rs do not desensitize and its prolonged activation trigger the opening of a big transmembrane pore, permeable to large

molecular weight molecules, which finally leads to cellular death (*Harada et al., 2011*). It has been suggested that P2X7R pore formation requires the involvement of another transmembrane molecule called Pannexin-1 (*Murphy et al., 2011*).

Although phagocytosis is activated primarily by the expression of "eat-me" signals on the surface of damaged or dead cells, injured neurons released UDP that activate P2Y6 receptor and in consequence the phagocytic signaling in microglia (*Koizumi et al., 2007*). UDP is also able to induce the expression of chemokines (*Kim et al., 2011*). In contrast, the activation of P2X7 or P1 receptors attenuates microglial phagocytic activity (*Fang et al., 2009; Bulavina et al., 2012*).
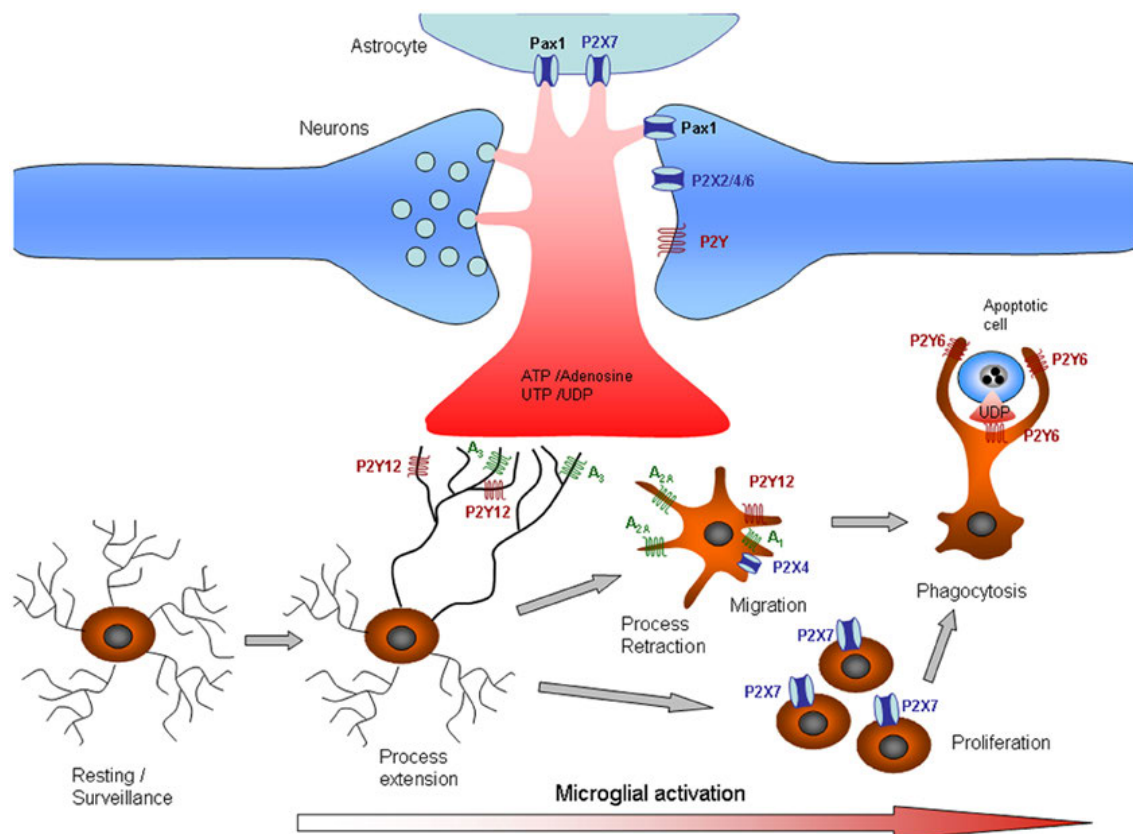
Figure 8. Purinergic signaling in microglia. Release or leakage of nucleotides/nucleosides from injured cells induces phenotypic alterations in microglia. Microglia undergo progressive changes, including altered expression of cell surface markers and inflammation-related genes, process retraction and the acquisition of an amoeboid morphology, cell body migration, and increasing phagocytic ability. The changes in microglial functions are partly associated with changes in purinergic receptors that determine different responses to ATP. Thus, process retraction is due to down regulation of P2Y12 receptors, whereas migration is mediated by P2X4 receptors and proliferation by P2X7 receptors. Phagocytosis signaling is also unmasked by the up regulation of P2Y6, which is activated by the release of UTP by dying cells. (*Domercq et al., 2013*).

3.3. P2X4R in the CNS

P2X4 receptors form homomeric or heteromeric assemblies with P2X6, P2X7 and P2X1. It is rapidly activated by ATP and similar to P2X7R, P2X4R has high permeability to Ca^{2+}, slowly desensitize and has higher affinity to ATP.

The receptor is abundantly expressed in the CNS (*Buell et al., 1996; Soto et al., 1996; Tsuda et al., 2003; Guo and Schluesener, 2005; Amadio et al., 2007; Vazquez-Villoldo et al., 2014*). Neuronal P2X4R has been implicated in physiological functions in the CNS including modulation of neurotransmission and synaptic strengthening (*Rubio and Soto, 2001; Sim et al., 2006; Baxter et al., 2011*). In glial cells, there is no evidence for P2X4R in astrocytes and oligodendrocytes. Electrophysiological recordings in acute slices demonstrated that astrocytes as well as oligodendrocytes lack P2X4R-mediated inward currents (*Lalo et al., 2008; Laboratory data*). In contrast, microglia have abundant P2X4R expression within brain and spinal cord and the role of P2X4R in these cells has received much attention in the last decades (*Tsuda et al., 2003; Ulmann et al., 2008*).

As it is mentioned above, P2X4R is up-regulated in microglial cells in EAE rats and in human MS optic nerve samples (*Guo and Schluesener, 2005; Vazquez- Villoldo et al., 2014*). Moreover, a specific phenotype of spinal microglia characterized by high expression of P2X4R has been described after peripheral nerve injury (PNI) (*Tsuda et al., 2003; Beggs et al., 2012*). Other studies demonstrate upregulation of P2X4R in microglia following hypoxia and ischemia (*Wixey et al., 2009; Li et al., 2011*) and in kainate-induced epilepsy model (*Ulmann et al., 2013*). It is described the implication of P2X4R in regulating the activation and migration of microglial cells to the injury site (*Guo and Schluesener, 2005; Schwab et al., 2005*).

The first evidence related to the pathological role of P2X4 was described in pain processing. Tsuda and colleagues described a major role for P2X4R in chronic pain and mechanical allodynia (*Tsuda et al., 2003*). P2X4R activation in spinal microglia leads to the release of BDNF, which communicates between microglia and spinal interneurons resulting in pain hypersensitivity via disinhibition of GABAergic input (*Tsuda et al., 2003; Coull et al., 2005; Ulmann et al., 2008; Trang et al., 2009*). Allodynia induced through PNI was reversed by pharmacological blockade of P2X4 receptors in the spinal cord (*Tsuda et al., 2003*) and P2X4^{-/-} mouse models show reduced inflammatory and neuropathic pain (*Ulmann et al., 2008*). These findings highlight the importance of P2X4R in microglial cells in the development of neuropathic pain (reviewed by *Inoue and Tsuda, 2012; Tsuda et al., 2013*). P2X4R is involved in the regulation of different microglia pathways, therefore may also play roles in controlling or exacerbating

inflammation in the CNS. Using LPS as a model of neuroinflammation the P2X4r antagonists TNP-ATP and 5-BDBD (5-(3-Bromophenyl)-1,3-dihydro-2*H*-benzofuro[3,2-*e*]-1,4-diazepin-2-one) were shown to reduce microglial activation *in vivo* and reduce the microglial loss in spinal cord (*Vazquez-Villoldo et al., 2014*).

P2X4 receptor has another peculiarity referring its localization in the cell; the receptor is mainly localized intracellularly, particularly in lysosomal compartments (*Qureshi et al., 2007; Toyomitsu et al., 2012*). This fact could be determinant for its function. P2X4R contains an additional C-terminal trafficking motif (YxxxG') which facilitates rapid internalization of cell surface P2X4R via clathrin-dependent endocytosis (*Royle et al., 2005*). Recently it was suggested that P2X4R functions as an ATP-gated channel in lysosomes (*Huang et al., 2014*) and regulates endolysosomal membrane fusion via Ca^{2+}-dependent activation of calmodulin (*Cao et al., 2015*).

3.3.1. IRF5-P2X4R axis

The interferon regulatory factor (IRF) family of transcription factors are implicated in diverse functions such as apoptosis, cell cycle, oncogenesis, and gene regulation in response to pathogen-derived signals. The mammalian IRF family comprise nine members (IRFs 1 -9) (*Zhao et al., 2014*). Among the IRF family members, interferon regulatory factor 5 (IRF5) plays a central role in inflammation. IRF5 mediates induction of pro -inflammatory cytokines such as IL-6, IL-12, IL-23, and tumor-necrosis factor-alpha (TNF-α) (*Takaoka et al., 2005; Krausgruber et al., 2011*). IRF5 is a key factor in defining the inflammatory macrophage phenotype and is highly expressed in monocytes and macrophages, but also in B cells and dendritic cells. Its expression in macrophages can be upregulated in response to the inflammatory environment and in particular to stimulation with GM -CSF and IFN-γ (*Krausgruber et al., 2011; Weiss et al., 2013*).

The role of IRFs in the CNS was entirely unknown, but Masuda *et al.* (*2014*) recently showed that within the spinal cord, IRF8 expression is selectively upregulated in microglia after PNI. Interestingly, IRF5 expression in activated microglia led to an induction of P2X4R expression by directly binding to the promoter region of the *P2rx4* gene (**Figure 9**). Consistent with this, IRF5-deficient mice did not upregulate spinal P2X4R after PNI. Thus, an IRF8-IRF5 transcriptional axis could contribute to shifting spinal microglia toward a P2X4R-expressing reactive state after PNI (*Masuda et al., 2014*).

On the other hand, the *Irf5* gene has been identified as a risk factor in several   ne diseases. IRF5 was originally found to be associated with systemic lupus erythematosus susceptibility (*Sigurdsson et al., 2005*). Other autoimmune diseases such as rheumatoid arthritis

(*Rueda et al., 2006; Shimane et al., 2009*), inflammatory bowel disease (*Dideberg et al., 2007*) or Sjögren's syndrome (*Miceli-Richard et al., 2007*) have been shown to share this genetic risk factor. High expression of IRF5 contributes to the development of immune-mediated diseases, including MS. In MS, two single nucleotide polymorphisms (SNPs) in the *Irf5* gene (rs4728142 and rs3807306) reached significant association in the three independent cohorts tested (*Kristjansdottir et al., 2008*).
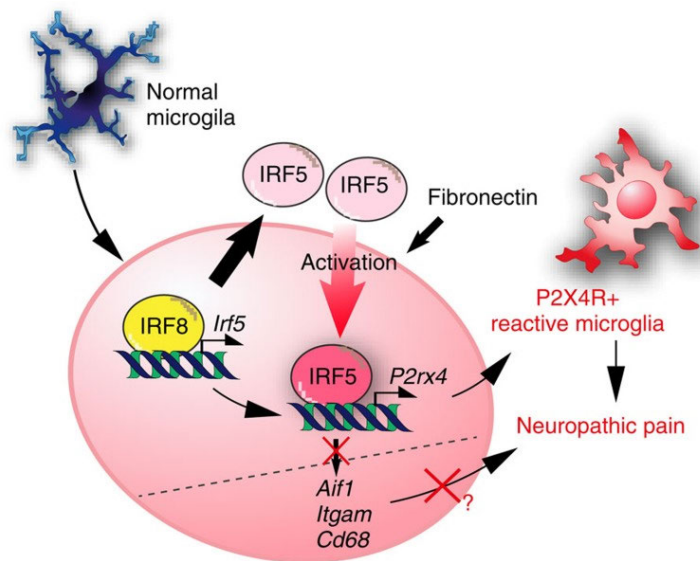

Figure 9. Schematic illustration and working models for determining P2X4R[+]-reactive microglia and neuropathic pain (*Masuda et al., 2014*).

HYPOTHESIS AND OBJECTIVES

Purinergic receptors expression is altered in multiple sclerosis (*Domercq et al., 2018*). In particular, P2X4 receptor is upregulated in inflammatory foci and in activated microglia in spinal cord of rats with experimental autoimmune encephalomyelitis (EAE), the acute model, as well as in the optic nerve of multiple sclerosis patients (*Vazquez-Villoldo et al., 2014*). In accordance, P2X4 receptor expression and activity is increased in LPS-activated microglia (*Vazquez-Villoldo et al., 2014*). More importantly, blockage of P2X4 receptor controls the fate of LPS-activated microglia *in vitro* and *in vivo*, including its survival (*Vazquez-Villoldo et al., 2014*) and exacerbates clinical symptoms in the chronic EAE model (preliminary data from Nuria Vazquez-Villoldo). In contrast, P2X4 receptor potentiation ameliorates EAE symptoms. In multiple sclerosis, spontaneous yet transient myelin repair can occur during the course of the disease. A major component of this regenerative process is a robust innate immune response consisting of peripherally-derived macrophages and CNS-resident microglia (*Kotter et al., 2005; Li J et al., 2005; Miron et al., 2013*). It has been described that microglial polarization to pro-inflammatory and anti-inflammatory activation state is differently regulated during the course of the disease in the EAE model and that a switch to an anti-inflammatory phenotype is necessary to favors remyelination (*Miron et al., 2013*). Based on all previous data, we hypothesized that P2X4Rs, by modulating microglial activation, could contribute to myelin repair in demyelinating diseases.

The general objective of this Doctoral Thesis has been to analyse the role of the P2X4 receptor and the transcription factor IRF5, which modulates its expression, in microglia activation and in the pathophysiology of MS using both *in vitro* and *in vivo* models.

For this purpose, we defined the following **specific objectives**:

1. To characterize the role of P2X4R in EAE pathogenesis
    1.1 P2X4R blockage:
        1.1.1 To analyse P2X4R expression after EAE immunization.
        1.1.2 The impact of P2X4R blockade in neurological signs.
        1.1.3 To study the role of P2X4R blockade on microglia activation *in vitro* and the effect on oligodendrocyte differentiation.
    1.2 P2X4R potentiation:
        1.2.1 To determine the impact of P2X4R potentiation in EAE neurological signs.
        1.2.2 To study the role of P2X4R potentiation on microglia activation *in vitro*.
2. To characterize the role of the transcription factor interferon regulatory factor 5 (IRF5)
    2.1 To analyse IRF5 expression after EAE immunization.
    2.2 To characterize the role of IRF5 in EAE model.
    2.3 To characterize the role of IRF5 in a toxin-induced demyelination model.

MATERIAL AND METHODS

1. ANIMALS

Experiments were performed in Sprague Dawley rats and in wild type (WT), P2X4^{-/-} (backcrossed for >15 generations onto C57BL6 background; *Sim et al., 2006*) and IRF5^{-/-} C57BL6 mice. All experiments were performed according to the procedures approved by the Ethics Committee of the University of the Basque Country (UPV/EHU). Animals were handled in accordance with the European Communities Council Directive. Animals were kept under conventional housing conditions (22 ± 2 temperature, 55 ± 10% humidity, 12-hour day/night cycle and with *ad libitum* access to food and water) at the University of the Basque Country animal unit. All possible efforts were made to minimize animal suffering and the number of animals used.

2. *IN VITRO* MODELS

2.1. Microglia culture

Primary mixed glial cultures were prepared from the cerebral cortex of neonatal rats and mice (P0-P2) following the protocol described by McCarthy and De Vellis (*1980*) with modifications. Cell suspension was obtained by mechanical and enzymatic dissociation of the cerebral cortex and was seeded in 75 cm^2 surface culture flasks previously treated with poly-D-Lysine (PDL). Flasks were maintained in Iscove´s Modified Dubelcco´s Medium (IMDM; Gibco) supplemented with 10% fetal bovine serum (FBS) at 37°C, with 5% of CO_2 in a humidified incubator.

Approximately 15 days after performing the culture, microglia were isolated by mechanical shaking. Free-floating microglia were collected from shaken astrocyte flasks and purified by pre-plating on bacterial plastic dishes (Sterilin). Microglia were maintained in petri dish 24-48 hours minimum with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. Microglia were tripsinized and seeded in PDL coated coverslips at 20,000 cells/well for immunocytochemistry, 100,000 cells/well for western blot, 200,000 cells/well for medium conditioning and for qPCR experiments. Cells were cultivated in DMEM + 10% FBS at 37°C, with 5% of CO_2 in a humidified incubator and were used after 24 hours.

Microglia cells were polarized according to previous protocols (*Durafort et al., 2012*) with minor modifications:

- Pro-inflammatory microglia: cells were treated with granulocyte macrophage colony-stimulating factor (GM-CSF, 5 ng/ml; Peprotech) in FBS-supplemented DMEM for 5 days followed by 24 hours treatment with lipopolysaccharide (LPS, 10 ng/ml; Sigma) and interferon-gamma (IFNγ, 20 ng/ml; Peprotech).

- Anti-inflammatory microglia: cells were treated with macrophage colony-stimulating factor (M-CSF, 20 ng/ml; Peprotech) in FBS-supplemented DMEM for 5 days followed by 24 hours treatment with interlekin-4 (IL-4, 20 ng/ml; Peprotech) and interleukin-13 (IL-13, 50 ng/ml; Peprotech).

To study the influence of microglia in oligodendrocyte development, medium conditioned from different polarized microglia was collected and added to oligodendrocyte progenitor cell (OPC) cultures for 3 days. Microglia cells were polarized in FBS-suplemented DMEM the first 5 days of the protocol and SATO medium without proliferating and differentiating factors (SATO-) (Table 1) was added to microglia cultures in the last 24h of the polarization protocol. Polarizing factors alone in SATO- were directly applied to OPCs as a control.

Table 1. SATO- medium composition.

Reagent	Concentration	Company
DMEM	**Base medium**	**Gibco**
BSA	100 µg/ml	Sigma
N-Acetyl Cysteine	6,3 ng/ml	Sigma
Glutamine	2 mM	Sigma
Penicillin- Streptomycin	100 U/ml	Invitrogen
Insulin	5 µg/ml	Sigma
Transferrin	100 µg/ml	Sigma
Progesterone	60 ng/ml	Sigma
Sodium Selenite	40 ng/ml	Sigma
Putrescine	16 µg/ml	Sigma

2.2. OPC culture

OPCs were obtained from the same primary mixed glial cultures used to isolate microglia. After microglia isolation from the flasks, the remaining OPCs present on the top of the confluent monolayer of astrocytes were dislodged by shaking flasks overnight. OPCs were purified from microglial cells by plating on bacterial Petri dishes. The non-adherent cells (OPCs) were further collected and seeded in PDL-coated coverslips at a density of 5,000 cells/well in complete SATO medium (SATO+) (Table 2) or SATO- (Table 1) for the experiments were oligodendrocyte differentiation was studied.

Table 2. Additional components added to SATO- medium for SATO+ medium preparation.

Reagent	Concentration	Company
Tri-iodothyronine (T3)	30 ng/ml	Sigma
L -Thyroxine (T4)	40 ng/ml	Sigma
CNTF	10 ng/ml	Peprotech
NT-3	1 ng/ml	Peprotech

2.3. Oligodendrocyte culture

Primary oligodendrocytes were obtained from Sprague-Dawley rat optic nerves (P10-12) as previously described (*Domercq et al., 2010*). Optic nerves were mechanically and enzymatically digested and seeded at 10,000 cells/well in PDL coated coverslips in SATO+ medium (Table 2). Cells were maintained at 37°C, with 5% of CO_2 in a humidified incubator 24 hours before corresponding treatments.

3. *IN VIVO* MODELS

3.1. Experimental autoimmune encephalomyelitis (EAE) induction

EAE was induced in 8-10 week old female P2X4^{-/-}, IRF5^{-/-} and WT C57BL6 mice by subcutaneous immunization with 300 μl of myelin oligodendrocyte glycoprotein 35-55 (MOG(35-55); 200 μg; Sigma) emulsioned in incomplete Freund's adjuvant (Sigma) supplemented with 8 mg/ml *Mycobacterium tuberculosis* H37Ra. Pertussis toxin (500 ng/0.1 ml;

Sigma) was injected intraperitoneally on the day of immunization and again 2 days later to facilitate and improve the development of the disease.

In this model mice begin to show neurological signs around 10-12 days post-immunization (dpi). The motor deficits increase with the time reaching a maximum peak around day 20 postimmunization, followed by a partial and slow recovery. Motor symptoms were recorded daily and scored from 0 to 8 as follows:

0, no detectable signs

1, weakness in the tail

2, paralysed tail

3, paralysed tail and weakness in hindlegs

4, paralysed tail and hemiparalysis of hindlegs

5, complete hindlimb paralysis

6, paralysis of hindlegs and rigidity in forelegs

7, tetraplegia

8, moribund

To analyse the role of P2X4R, EAE mice were treated daily with TNP-ATP (10 mg/kg), ivermectin (IVM, 1 mg/kg) or vehicle (saline) intraperitoneally from EAE onset (day 10 postimmunization) to the end of the experiment in order to do not interfere with immune priming. For the experiments designed to check the effect of TNP-ATP or IVM on immune priming, treatments were administered daily from day 0 to EAE peak. All mice were randomized before the immunization and when possible, before the appearance of EAE symptoms. Disease phases were assigned according to days after onset as follows: peak, 6-10 days after onset; recovery, score stabilized and 18-30 days after onset.

3.2. LPC-induced demyelination

LPC-induced demyelination was carried out in the spinal cord of 14-week-old male mice. Demyelinating lesions were induced by a stereotaxic injection of 0.5 µl of 1% LPC (Sigma) in saline solution. Prior to the surgery anesthesia was induced by intraperitoneal injection of a solution of ketamine (100 mg/kg)/ xylazine (10 mg/kg). Two longitudinal incisions into *longissimus dorsi* at each side of the vertebral column were performed, and the muscle tissue covering the column was removed. Animals were placed in a stereotaxic frame, the 13th thoracic vertebra was fixed in between the bars designed for manipulations on mouse spinal cord, and intravertebral space was exposed by removing the connective tissue. Dura mater was pierced

with a 30G needle, and LPC was injected via Hamilton syringe attached to a glass micropipette using a stereotaxic micromanipulator. The lesion site was marked with sterile charcoal so that the area of tissue at the lesion center could be unambiguously identified. Following LPC injection, the wound was sutured, and the animals were allowed to recover. Buprenorphine (0.1 mg/kg, subcutaneous injection) was administered as postoperative analgesic treatment. Mice were sacrificed 14 days after surgery, to analyse oligodendrocyte differentiation stage.

4. TECHNIQUES

4.1. Indirect myelin phagocytosis analysis in EAE mice

Myelin phagocytosis was quantified in MBP-Iba1 immunostained EAE spinal cord samples. Z-stacks were acquired in a LEICA TCS STED SP8 confocal microscope using 40x oil-immersion objective. Images were processed using *ImageJ* software (Fiji distribution) (NIH). Semi-automated phagocytosis of myelin debris was indirectly quantified based on colocalization between microglia/macrophages (including phagocytic pouches) and myelin debris. A single plane of the z-stacks was chosen for analysis based on the maximum labelling intensity for MBP. The "Threshold" tool was used to automatically identify myelin debris and microglia/macrophages. A preliminary analysis of all threshold filters was performed for each of the variables to determine which one best adjusted the original image: Yen filter after performing a "Gaussian blur" of radius = 1 for myelin debris; and Yen filter for microglia/macrophages. Upper and lower threshold levels were manually adjusted by comparing with the original image. All pixels above the threshold level were selected in a mask for myelin debris. To better detect phagocytic pouches in microglial images, a mask was created using the "Analyse Particles" tool with the "Include Holes" option checked. Then we used the "Measurements" tool to analyse the area of colocalization between microglia/macrophages and myelin debris.

4.2. Cell viability assay

Cell viability was assessed using Calcein-AM (Invitrogen). Cells were incubated with 0.5 µM calcein-AM for 30 minutes at 37°C. After washing viable cells were immediately quantified with the fluorimeter/spectrophotometer reader Synergy HT (Bio-tek Instruments Incl) at 485 nm excitation and 528 nm emission wavelength. Data was analysed using Gen5 (Bio-tek, Beverly,

MA, USA) software. The activity was expressed as the relative percentage of cellular death using respective values of non-treated oligodendrocytes as control (100% viability). Results were expressed as the mean± s.e.m. of at least three independent experiments performed in triplicate.

4.3. Western blot

Total protein was extracted by scraping the cells in sample buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.002% bromophenol blue and 5.7% β-mercaptoethanol in H_2O) supplemented with proteases inhibitor coctel (Roche). Samples were boiled at 95°C for 5 minutes, loaded and size separated by electrophoresis using Criterion TGX Precast 12% gels and transferred to Trans-Blot Turbo Midi PVDF Transfer Packs (Bio-Rad). Membranes were blocked in 5% skimmed milk, 5% serum in Tris-buffered saline/0.1% Tween-20 (TBS-T) for 1 hour at room temperature (RT). Proteins were detected by specific primary antibodies against BDNF (1:200; Santa Cruz), and β-actin (1:1000; Sigma) in 5% skimmed milk and 1% serum in TBS-T overnight at 4°C. After washing membranes were incubated with secondary peroxidase-coupled goat anti-rabbit antibodies (1:2000; Sigma) in 5% skimmed milk and 1% serum in TBS-T for 1 hour at RT. Finally, blots were developed using an enhanced chemiluminescence detection kit according to the manufacturer's instructions (Super Signal West Dura or Femto, Pierce). Images were acquired with a ChemiDoc MP system (BioRad) and quantified using ImageLab (Biorad). Values of BDNF were normalized to corresponding β-actin signal.

4.4. Pain assessment

Mechanical allodynia was assessed by an e-VF Electronic Von Frey aesthesiometer (Ugo Basile SRL) at different stages of EAE; before immunization, EAE preonset (5 dpi), onset (12 dpi) and before peak (16 dpi). Mice, placed upon an elevated wire mesh surrounded by a Perspex box, were exposed to increasing mechanical pressure to the plantar hind paw through a metal filament. Withdrawal threshold was measured automatically from the initiation of mechanical stimulus to withdrawal of the paw. Measurements were taken three times in both left and right hind paw separated by at least 10 minutes between each stimulus. Mean results for each animal were calculated.

4.5. Immunochemistry

Cells were fixed and processed for immunocytochemistry as previously described (*Domercq et al., 1999*). In all cases, cells were fixed in 4% paraformaldehyde (PFA) in phosphate buffer (PBS, 0.1 M, pH 7.4) for 20 minutes at RT and after washing with PBS, cells were processed for immunocytochemistry. For isolectin B4 (IB4) labelling, live cultures were incubated for 30 min with IB4 (1:100, Vector) in culture medium, washed with PBS, fixed in 4% PFA in PBS for 20 min and processed for conventional immunocytochemistry.

Mice from EAE experiments were deeply anesthetized with intraperitoneal injection of chloral hydrate (500 mg/kg) and transcardially perfused with 0.1 M sodium phosphate buffer, pH 7.4, followed by 4% PFA in the same buffer for 15-20 minutes. In some experiments, tissue was processed for multiple analysis. In this case, mice were anesthetized and decapitated and brain and spinal cord were only post-fixated in 4% PFA by immersion for 4 hours and cryoprotected in 20% sucrose solution in 0.1M PB for 72 hours at 4°C. Perfused samples were cut using a Microm HM650V vibratome (40 µm), while cryoprotected samples were embebed in Tissue-tek cryo embedding compound (Sakura) and cut using a cryostat CM3050 S (Leica) (10 µm).

LPC-injected mice were perfused with 2% PFA for 15-20 minutes and spinal cords were postfixed in 2% PFA for 20 minutes. Tissue was then cryoprotected in 15% sucrose for 72 hours and embedded in 7% gelatine/15% sucrose before cryostat cutting (12 µm).

Tissue sections from *in vivo* experiments or cells from *in vitro* experiments were permeabilized and non-specific labelling was blocked with 4% of serum of the specie in which secondary antibody was obtained in 0.1 M PBS and 0.1% Triton X-100 for 1 hour at RT. Next, samples were incubated with primary antibodies overnight at 4°C in 1% serum and 0.1% Triton X-100 (Table 3). After washing with PBS, corresponding fluorochrome-conjugated antibodies (Table 3) and Hoechst 33258 (1.5 µg/ml; Sigma) for nuclei labelling were applied and samples were incubated during 1 hour at RT. After washing, they were mounted using Glicergel mounting medium (Dako). All incubations and washing steps were performed with gently shaken in the case of free-floating vibratome sections.

Images were acquired with the same settings for all samples within one experimental group. Axiovision microscope (Zeiss) (Depatment of Neuroescience), Zeiss Axio Observer with Apotome microscope (Analytical and High-Resolution Microscopy in Biomedicine Service of the

UPV/EHU) and Leica TCS SP8 confocal microscope (Achucarro Basque Center for Neuroscience) were used to adquire the images.

Table 3. Antibodies used for immunochemistry.

Antibody	Host	Concentration	Company
APC (CC1)	Mouse	1:200	Calbiochem
Arginase	Goat	1:100	Santa Cruz
B220	Rat	1:200	BD Pharmingen
CD3	Rat	1:50	Serotek
Iba1	Rabbit	1:1000	Wako
iNOS	Mouse	1:500	BD Bioscience
IRF5	Rabbit	1:500	Abcam
Laminin	Rabbit	1:200	Sigma
MBP	Mouse	1:500	Covance
MBP	Rabbit	1:200	Millipore
MBP	Chicken	1:100	Millipore
MRC1	Rabbit	1:1000	Abcam
NG2	Rabbit	1:500	Abcam
Olig2	Mouse	1:1000	Invitrogen
P2X4R	Rabbit	1:400	Alomone
P2X7R	Rabbit	1:100	Alomone
SMI-32	Mouse	1:1000	Covance
IgG Rabbit-Alexa 488	Goat	1:400	Invitrogen
IgG Rabbit-Alexa 594	Goat	1:400	Invitrogen
IgG Rabbit-Alexa 633	Goat	1:400	Invitrogen
IgG Mouse-Alexa 488	Goat	1:400	Invitrogen
IgG Mouse-Alexa 546	Goat	1:400	Invitrogen
IgG Mouse-Alexa 594	Goat	1:400	Invitrogen

Olig2$^+$ cells in corpus callosum and in longitudinal sections of spinal cord and Iba1$^+$ cells in longitudinal sections of spinal cord were counted blindly in 40X images from an Axiovision microscope (Zeiss). To analyse microglia area, cells were outlined with the Iba1 labelling as the defining parameter for the region of interest (ROIs) using the Image J software (NIH). At least 4 different fields from three slices per animal were counted from 3 mice.

To quantify microglia polarization, immunoreactivity of inducible oxide nitrite synthase (iNOS), mannose receptor (MRC1) and Arginase was calculated in 40X images from an Axiovision microscope (Zeiss) with the *ImageJ* software (NIH) and normalized to the number of cells (8 fields per coverslip from at least 3 different experiments performed in triplicate). Results were expressed as changes in fluorescence intensity relative to those seen in control cells or to those seen in cells without TNP-ATP or IVM treatment.

To quantify microglia and oligodendrocyte expression of P2X receptors, ROIs were generated with Image J software in IB4^{+} and Olig2^{+} cells (12-15 cells per culture from three independent experiments) in images acquired using a 40X objective in a LEICA TCS STED SP8 confocal microscope.

To analyse oligodendrocyte occupied area, images were taken with 40X objective in Axiovision microscope (Zeiss). Oligodendrocytes were outlined with the MBP immunolabelling as the defining parameter for the region of interest (ROIs) using the Image J software (NIH).

To analyse the effect on oligodendrocyte differentiation, images were taken with 40X objective in Axiovision microscope (Zeiss). MBP^{+} cells were counted, and the results were expressed in percentage versus total cells (15 fields per coverslip were analysed by two observers from n = 3 different experiments performed in triplicate).

CD3^{+} cells, B220^{+} cells and Iba1^{+} cells in spinal cord sections from EAE mice were counted in 40X images acquired using a LEICA TCS STED SP8 confocal microscope and white matter (WM) area and lesion area were defined as ROI on the basis of MBP immunolabelling using the Image J software (NIH). At least 4 different fields from three slices per animal were counted from at least 6 mice.

To quantify axonal damage images were acquired using a LEICA TCS STED SP8 confocal microscope and SMI-32 immunoreactivity was calculated with the *ImageJ* software (NIH) and normalized to WM area defined as ROI. At least 4 different fields from three slices per animal were counted from at least 6 mice.

Lesion area, Olig2^{+} cells, CC1^{+} cells and CD3^{+} cells in LPC-injected mice spinal cord sections were quantified in 20X images acquired using a Zeiss Axio Observer with Apotome microscope. Cell number was normalized to lesion area defined as ROI on the basis of MBP immunolabelling using the Image J software (NIH). At least 3 different lesioned slices per animal were counted from at least 5 mice.

4.6. Fluorescence-activated cell sorting (FACS)

Animals were anesthetized by isoflurane and sacrificed by decapitation. The brains and spinal cords were extracted, homogenized and digested mechanically and enzymatically. Homogenates were centrifuged through a continuous 60% Percoll gradient to remove the myelin and other cell debris. To avoid non-specific binding of the antibodies, Fc receptors (antibody receptors) were blocked with TruStain FcX antibody (BioLegend) for 10 minutes at 4°C in FACS buffer (0.1% Bovine serum albumin, BSA and 1 mM EDTA in PBS). For cellular labelling, a mix of fluorochrome-conjugated antibodies (Table 4) was prepared and the incubation was simultaneous (30 minutes, 4°C). Stained cells were washed and resuspended in 300 µl FACS buffer for acquisition in the flow cytometer or for cell sorting.

For microglia sorting, samples were sorted using CD11b, CD45, Ly6G and CCR2 (Table 4) to distinguish between resident microglia (CD11b$^+$/CD45^{low}/Ly6G$^-$/CCR2$^-$) and invading macrophages (CD11b$^+$/CD45^{high}) (*Szulzewsky et al., 2015*) using a FACSAria IIIu (BD Bioscience).

For immune cell analysis, cells were measured using a BD LSR Fortessa and data were analysed with FlowJo software (Tree Star).

FACS experiments were performed in collaboration with the laboratory of Experimental Research in Stroke and Inflammation (ERSI) in the Department of Neurology of the University Medical Center of Hamburg-Eppendorf.

Table 4. Antibodies used for microglia sorting and for FACS analysis

Antibodies for microglia sorting	Fluorochrome	Clone	Company
CD11b	FITC	M 1/70	Biolegend
CD45	Bv421	30-F11	Biolegend
Ly6G	AF700	1A8	Biolegend
CCR2	PE	475301	R&D Systems
Antibodies for FACS	**Fluorochrome**	**Clone**	**Company**
CD4	Bv605	RM4-5	Biolegend
CD8a	perCP	53.6-7	Biolegend
γδTCR	Bv650	GL-3	Biolegend
CD3e	Bv421	17A2	Biolegend
CD45	APC-eFluor780	30-F11	eBioscience
CD11b	FITC	M 1/70	Biolegend

4.7. qPCR and Gene expression profiling

Total RNA from microglia cultures and from mice samples (lumbar spinal cord, lymph nodes and spleen) was isolated using Trizol (Invitrogen) according to manufacturer's instructions. Subsequently, from 1 µg of total RNA cDNA synthesis was conducted using SuperScript III retrotranscriptase (200 U/µl; Invitrogen) and random hexamers as primers (Promega). RNA from sorted microglia was isolated using RNeasy Plus Micro Kit (Quiagen) and cDNA synthesis was conducted using AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies Inc) and random hexamers as primers (Promega).

Real-time quantitative PCR reactions were performed with SYBR-Green using a BioRad CFX96 Real-time PCR detection system as described previously (*Domercq et al., 2016*). Specific primers were designed (Table 5) with Primer Express software (Applied Biosystems) in exon unions, in order to avoid the amplification of genomic DNA. The amount of cDNA was calculated by means of a standard curve elaborated from a pool of cDNA obtained from different microglia primary cultures. A normalization factor was calculated for each sample using GeNorm v3.5 free software (*Vandesompele et al., 2002*), based on the expression levels of three housekeeping genes (Table 5).

Gene expression of total RNA and microglia RNA from EAE experiments were further analysed using a 96.96 Dynamic Array™ integrated fluidic circuit (Fluidigm) real-time PCR and GenEx software. Results were depicted as relative gene expression according to the $\Delta\Delta C_q$ method ($2^{-\Delta\Delta Ct}$) and expressed in base 2 logarithmic scale.

Table 5. Primers used for qPCR

Sequences for rat primers

Target gene	Forward (5'->3')	Reverse (5'->3')
Arg1	GTGAAGAACCCACGGTCTGTG	GAGATGCTTCCAATTGCCATACTG
Ccl2	GTGCTGTCTCAGCCAGATGCA	GCTGCTGGTGATTCTCTTGTAGTT
Mrc1	AAGTTTAAGCACTGGCTGGCA	CAGGTTCTGATGATGGACTTCCTG
Nos2	GAGATTTTTCACGACACCCTTCAC	CATGCATAATTTGGACTTGCAAG
Housekeeping gene	**Forward (5'->3')**	**Reverse (5'->3')**
Cyclophilin A	CAAAGTTCCAAAGACAGCAGAAAA	CCACCCTGGCACATGAATC
Gapdh	GAAGGTCGGTGTCAACGGATTT	CAATGTCCACTTTGTCACAAGAGA
Hprt	ATGGACTGATTATGGACAGGACTGA	ACACAGAGGGCCACAATGTG

Sequences for mouse primers

Target gene	Forward (5'->3')	Reverse (5'->3')
Arg1	GGATTGGCAAGGTGATGGAA	CGACATCAAAGCTCAGGTGAA
Bdnf	TCCAAAGGCCAACTGAAGCA	CTGCAGCCTTCCTTGGTGTA
Ccl2	AGCAGCAGGTGTCCCAAA	TTCTTGGGGTCAGCACAGAC
Ccr7	GTGGTGGCTCTCCTTGTCA	GGTATTCTCGCCGATGTAGTCA
Chi3l3	GCCCACCAGGAAAGTACACA	CCTCAGTGGCTCCTTCATTCA
Clec7a	ACCACAAGCCCACAGAATCA	AGGAAGGCAAGGCTGAGAAA
Foxp3	ACCACACTTCATGCATCAGCTC	GGCTGGGTTGTCCAGTGGAC
Ifnγ	TAACTATTTTAACTCAAGTGGCATAGAGTG	GCCAGTTCCTCCAGATATCCAAG
Il10	AAAGGACCAGCTGGACAACA	TAAGGCTTGGCAACCCAAGTA
Il12a	AAACCAGCACATTGAAGACC	GGAAGAAGTCTCTCTAGTAGCC
Il1b	TGGCAACTGTTCCTGAACTCA	GGGTCCGTCAACTTCAAAGAAC
Il4	ACGGAGATGGATGTGCCAAA	GAAGCACCTTGGAAGCCCTA
Il6	CGATGATGCACTTGCAGAAA	ACTCCAGAAGACCAGAGGAA
Irf5	TGATGTCAAACCCCGAGAGAA	GAACATCTCCAGCAGCAACC
Irf8	GATATGCCGCCTATGACACA	CCCGTAGTAGAAGCTGATGAC
Jak3	CATAGAGGACGTGGACACTCAA	TGACATGTCTCCAGCCCAAA
Marco	TTCTGTCGCATGCTCGGTTA	TTGTCCAGCCAGATGTTCCC
Mbp	CCCTCACAGCGATCCAAGTA	CTCTGTGCCTTGGGAGGAA
Mr1	GCTCGCTGTATTCTTGGTGAA	ACCAGGATCGGAAACAGCTA
Mrc1	CACAAAGCCATGCTGTAGTACC	GTAAAACCCATGCCGTTTCCA
Msx3	CTCCAGTCGCGCACTCTT	CCGTGGTTTGCGATTGGTTT
Nos2	GAGGAGCAGGTGGAAGACTA	GGAAAAGACTGCACCGAAGATA
P2rx4	TTTGCGATTCAGACGCCAAC	ATGGAACACACCTTCCAGTCC
Ptgs2	CTTCTCCCTGAAGCCGTACA	TGTCACTGTAGAGGGCTTTCAA
Retnla	ATCCCTCCACTGTAACGAAGAC	ACAAGCACACCCAGTAGCA
Ror	ACTGAAAGCAGGAGCAATGGAAG	TTCAAAAAAGACTGTGTGGTTGTTG
Stat1	GCAGGTGTTGTCAGATCGAAC	ATGCACGGCTGTCGTTCTA
Stat3	TGGGCATCAATCCTGTGGTA	CCAATTGGCGGCTTAGTGAA
Stat6	TGACTTTCCACAACGCCTAC	CATCTGAACCGACCAGGAAC
Tgfb1	GCTGCGCTTGCAGAGATTAA	GTAACGCCAGGAATTGTTGCTA
Tnf	GGGTGATCGGTCCCCAAA	TGAGGGTCTGGGCCATAGAA

Housekeeping gene	Forward (5'->3')	Reverse (5'->3')
B2m	ACTGACCGGCCTGTATGCTA	ATGTTCGGCTTCCCATTCTCC
Gapdh	AGACGGCCGCATCTTCTT	TTCACACCGACCTTCACCAT
Hprt	CAGTACAGCCCCAAAATGGTTA	AGTCTGGCCTGTATCCAACA
Ppia	AGGGTTCCTCCTTTCACAGAA	TGCCGCCAGTGCCATTA

5. STATISTICAL ANALYSIS

Data are presented as mean ± s.e.m. with sample size and number of repeats indicated in the figure legends. Comparisons between two groups were analysed using paired Student's two-tailed t-test for data coming from *in vitro* experiments and unpaired Student's two-tailed t-test for data coming from *in vivo* experiments, except in MOG-EAE experiments where statistical significance in neurological score was determined by Mann-Whitney *U* test. Comparisons among multiple groups were analysed by one-way ANOVA analysis of variance followed by Bonferroni's multiple comparison tests for post hoc analysis. Statistical significance was considered at $p < 0.05$.

RESULTS

1. *P2x4r* expression is upregulated during EAE

It is known that after peripheral nerve injury, microglia in the spinal dorsal horn exhibit a reactive phenotype and upregulate expression of a variety of genes, including purinergic *P2x4r* (*Tsuda et al., 2003; Beggs et al., 2012*). Accordingly, in previews work from our group was described that the mRNA levels of *P2x4r* is upregulated in inflammatory foci and in activated microglia in the spinal cord of rats with acute experimental autoimmune encephalomyelitis (EAE) at the peak of the immune attack as well as in the optic nerve samples of multiple sclerosis (MS) patients (*Vazquez-Villoldo et al., 2014*).

We further analysed the expression and time course of the *P2x4r* in the chronic EAE model in mice. The chronic EAE model reproduces the inflammatory phase and the neurodegenerative phase of the MS disease. Mice were immunized with myelin oligodendrocyte glycoprotein (MOG) as described previously (*Matute et al., 2007*). Motor deficits of each animal were evaluated daily establishing a neurological score and the animals were sacrificed at the peak of the disease course, defined 6-10 days after onset, and at the recovery phase (30 days post-immunization) (**Figure 10A**).

Total mRNA was isolated from EAE mice spinal cords and analysed via Fluidigm real-time PCR. Levels of *P2x4r* expression were increased at the peak of the disease and remained elevated during the recovery phase (**Figure 10B**). Interestingly, there was a strong correlation between *P2x4r* expression and the neurological score, both at the peak and recovery (**Figure 10C**, r^2 = 0.99 and 0.61 respectively).

Next, we analysed *P2x4r* expression levels in microglia cells. We performed another EAE experiment and mice were sacrificed at the recovery phase (**Figure 10D**). We isolated microglia from spinal cord homogenates by fluorescence activated cell sorting (FACS) using CD11b, CD45, Ly6G and CCR2 antibodies (Cd11b$^+$CD45^{low}Ly6G$^-$CCR2$^-$; see gating strategy in **Figure 10E**). mRNA from sorted microglia was extracted and analysed by real-time PCR. *P2x4r* upregulation was also detected in FACS-isolated microglia in the spinal cord at EAE recovery phase (**Figure 10E, F**).
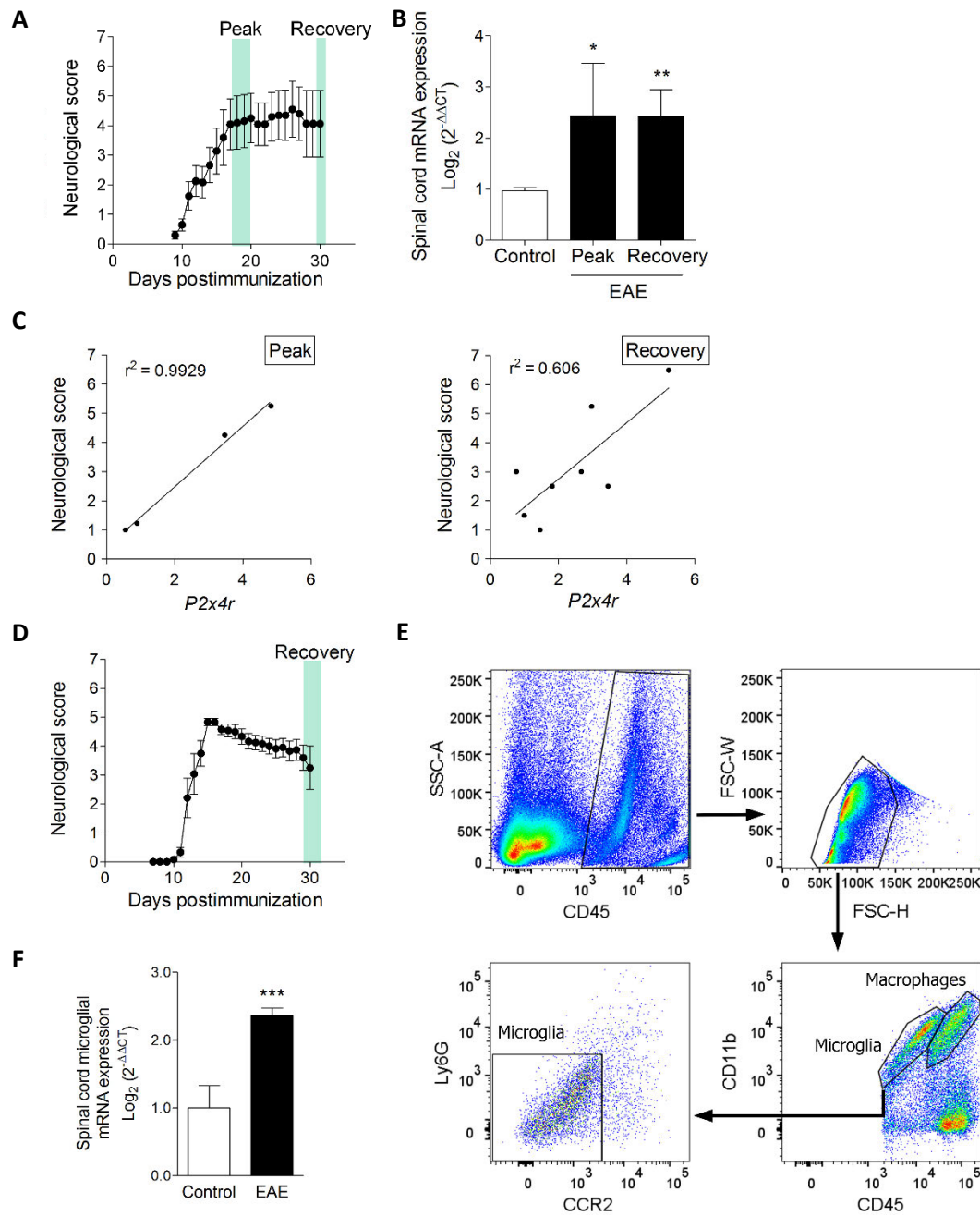
Figure 10. *P2x4r* is increased at peak and recovery phases in experimental autoimmune encephalomyelitis (EAE) mice.

A- Neurological score of EAE mice (n = 12).

B- Expression of *P2x4r* in the spinal cord of control (n = 7) and EAE mice at peak (n = 4) and recovery (n = 8) phases as analysed using qPCR.

C- Correlation of *P2x4r* expression with neurological score at EAE peak and recovery.

D- Neurological score of EAE mice used to microglia sorting (n = 6).

E- Plots depicting the strategy to distinguish resident microglia (Cd11b⁺CD45^low Ly6G⁻CCR2⁻) from invading macrophages (CD11b⁺CD45^high) in mice spinal cords.

F- *P2x4r* expression in microglia from control (n = 4) and EAE mice at the recovery phase (n = 6).

Data information: data are presented as mean ± s.e.m. and were analysed by one-way ANOVA (B) and Student's t-test (F). *P < 0.05, **P < 0.01, ***P < 0.001.

2. P2X4R blockade exacerbates EAE

We then tested the role of P2X4R in EAE pathogenesis in mice treated daily with P2X4R antagonist TNP-ATP (10 mg/kg) from the onset of the disease at 10 dpi. This time window is coincident with microglial activation, as previously reported (*Ajami et al., 2011*), and do not interfere with immune priming. Previous data have shown that microglia die at early stages of EAE induction, and that this population is replenished by infiltrating monocytes, promoting progression to paralysis (*Ajami et al., 2011*). Because P2X4R blockade was previously demonstrated to prevent LPS-induced microglia cell death (*Vazquez-Villoldo et al., 2014*), we reasoned that blockade of microglia cell death by TNP-ATP would prevent replacement by monocyte and improve clinical signs of EAE. In contrast, blockade of P2X4R with TNP-ATP exacerbated EAE disease (**Figure 11**).
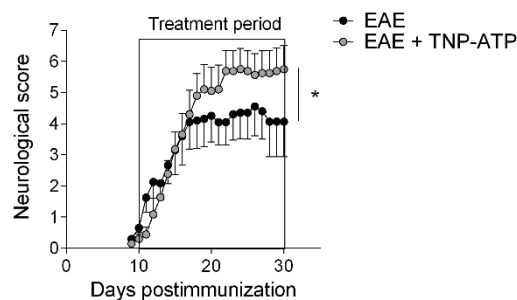

Figure 11. P2X4R blockade exacerbates experimental autoimmune encephalomyelitis (EAE).
Neurological score of vehicle and TNP-ATP (10 mg/kg) -treated mice after EAE induction (n = 10 mice/group). Mice were treated daily from 10th day postimmunization to the end of the experiment.

Data information: data are presented as mean ± s.e.m. Statistic was performed with Mann–Whitney U-test. *P < 0.05.

We next confirmed the role played by P2X4R in EAE pathogenesis using P2X4$^{-/-}$ mice. We first characterized the impact of P2X4R deficiency in microglia and oligodendrocytes development in normal conditions. We did not detect any change in the number or morphology of Iba1$^+$ cells in spinal cord nor in the number of Olig2$^+$ oligodendrocytes in the corpus callosum and spinal cord of two months old P2X4$^{-/-}$ mice (**Figure 12A, B**).

Once confirmed that the P2X4$^{-/-}$ mice do not have apparent alterations in microglia and oligodendrocytes in healthy conditions, we compared neurological score in wild type (WT) and P2X4$^{-/-}$ MOG-injected mice. In accordance with results obtained with TNP-ATP, P2X4$^{-/-}$ mice showed an exacerbated EAE (**Figure 13A**). To further assess that the effect of TNP-ATP was

P2X4R-dependent, we treated P2X4$^{-/-}$ mice with TNP-ATP (10mg/kg) from the onset of the disease. TNP-ATP failed to alter the course of EAE disease in P2X4$^{-/-}$ mice demonstrating the involvement of P2X4R in the effect observed in TNP-ATP-treated mice (**Figure 13B**). All these data confirmed the role played by P2X4R in EAE pathogenesis.
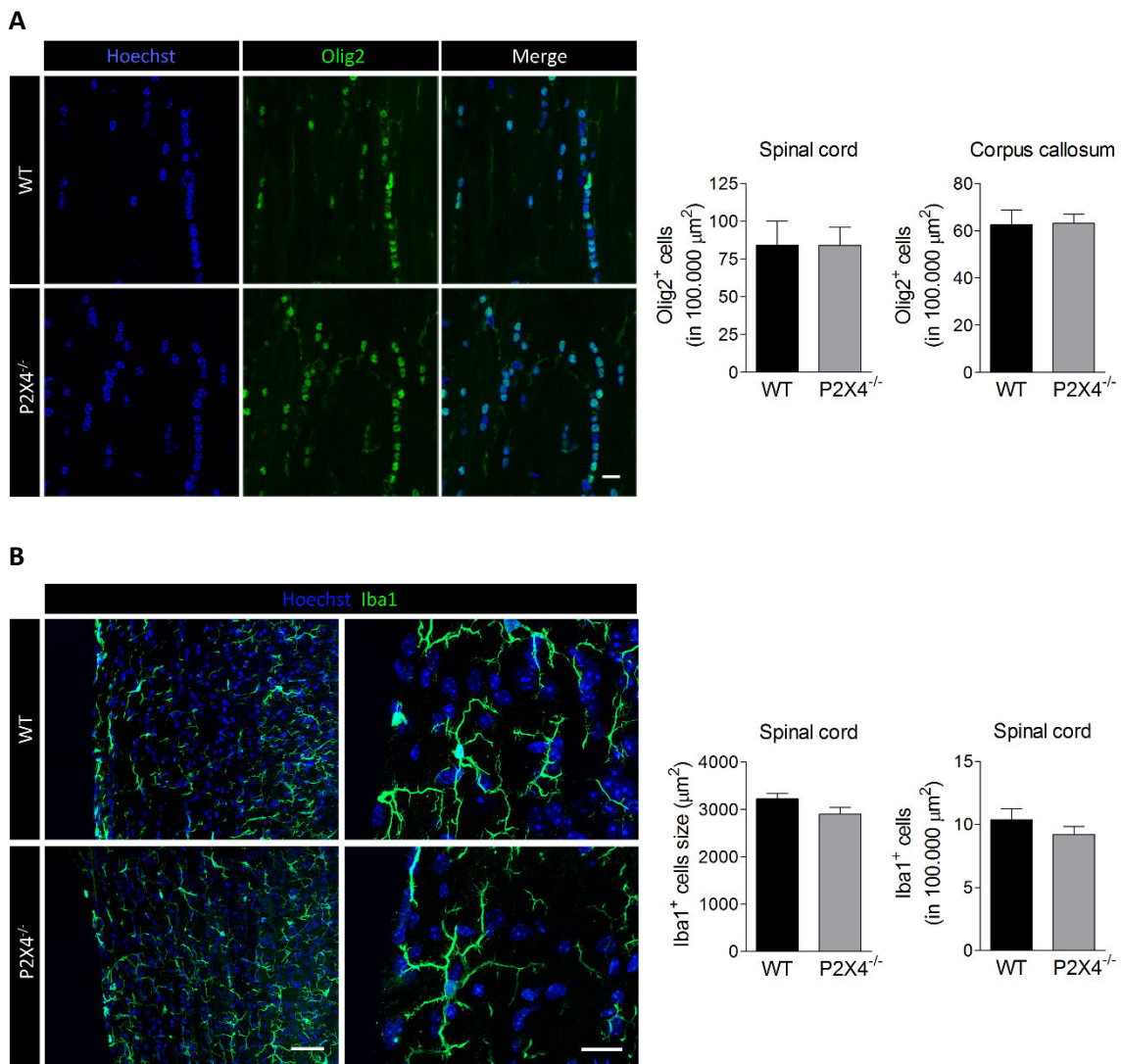
Figure 12. P2X4R deficiency does not alter normal development.

A- Immunofluorescent analysis of Olig2, a marker of oligodendrocytes, in corpus callosum and spinal cord of wild type (WT) and P2X4$^{-/-}$ mice. Images show representative fields in corpus callosum. Histograms represent the cell density calculated using *ImageJ* software. Cells were identified by nuclear Hoechst labelling. (n = 3 mice/group). Scale bar = 20 µm.

B- Immunofluorescent analysis of Iba1, a marker of microglia, in spinal cord of WT and P2X4$^{-/-}$ mice. Images show representative fields. Histograms represent the cell density and the area of the cells calculated using *ImageJ* software. Cells were identified by nuclear Hoechst labelling. (n = 3 mice/group). Scale bar = 50 µm (*left*) and 20 µm (*right*).

Data information: data are presented as mean ± s.e.m.

Figure 13. P2X4R deficiency exacerbates experimental autoimmune encephalomyelitis (EAE).

A- Neurological score of wild type (WT) and P2X4$^{-/-}$ mice after EAE induction (n = 10 mice/group). Mice were treated daily from 10th day postimmunization to the end of the experiment.
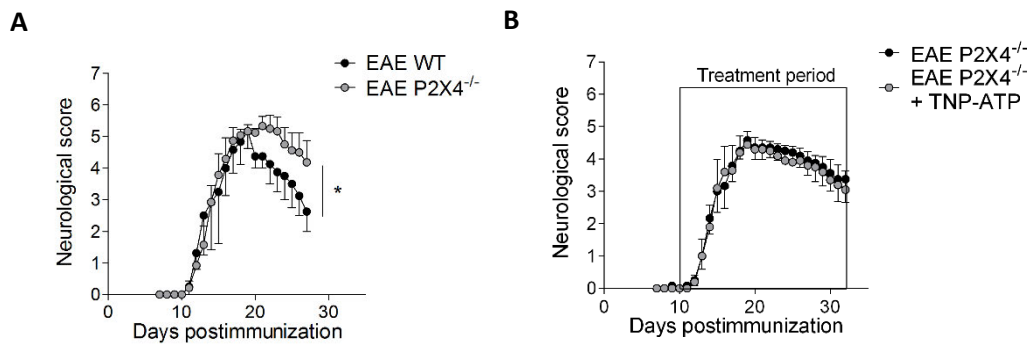
B- Neurological score after EAE induction in P2X4$^{-/-}$ mice treated with vehicle (n = 4) or TNP-ATP (n = 5).

Data information: data are presented as mean ± s.e.m. Statistic was performed with Mann–Whitney U-test. *P < 0.05.

3. P2X4R modulation do not affect immune priming in EAE

During EAE, T cells are primed in the mouse peripheral immune system before the onset of the clinical signs (*Stromnes and Goverman, 2006*), suggesting that treatment with TNP-ATP starting after the onset of the disease would have no impact on T cell infiltration. To exclude the involvement of P2X4R in adaptive immune system we performed an additional EAE experiment and treated mice with TNP-ATP during the priming phase (0 -17 dpi). TNP-ATP treatment during the priming phase did not affect disease development (**Figure 14A**). At the peak, we quantified by flow cytometry the immune response in periphery (spleen and lymph nodes) and in the spinal cord (see gating strategy in **Figure 14B**). Treatment with TNP-ATP did not change the number of CD4$^+$ T cells, CD8$^+$ T cells and γδ T cells in spleen, lymph nodes or in the spinal cord (**Figure 14C**). To further assess the CD4$^+$ T cell response, we measured mRNA for *Foxp3* and *Ror*, transcription factors that specifies regulatory T cells (Tregs) and T-helper 17 cells (Th17) respectively and *Ifnγ*, signature cytokine for T-helper 1 cells (Th1). We did not detect any change in transcript expression after TNP-ATP treatment (**Figure 14D**). These data suggest that the TNP-ATP treatment during the priming phase do not affect T cells infiltration and response in the spinal cord after EAE induction.
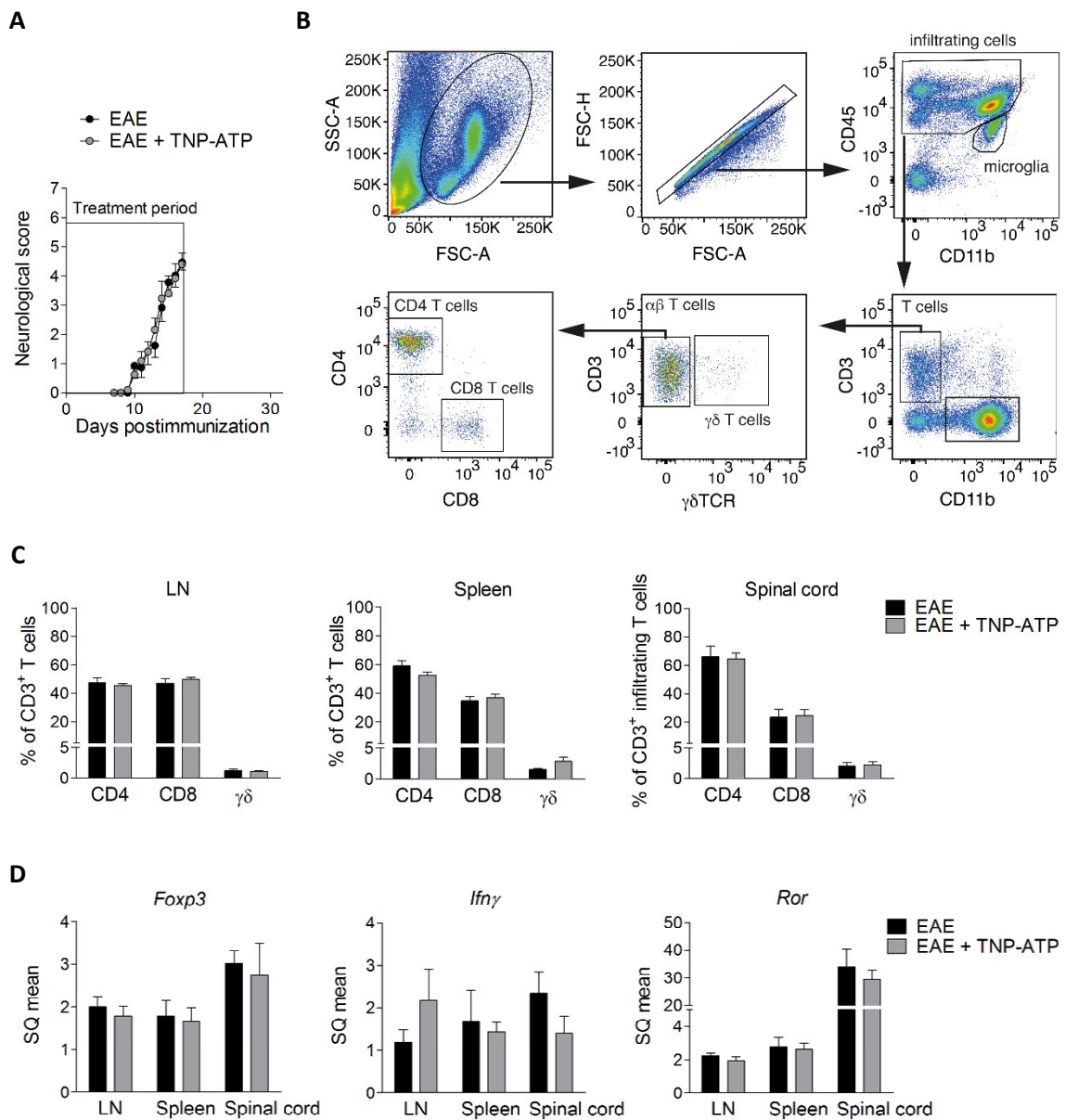
Figure 14. P2X4R do not interfere with immune priming.

A- Neurological score of vehicle (n = 13 from two independent experiments) and TNP-ATP-treated (n = 12 from 2 independent experiments) mice after experimental autoimmune encephalomyelitis (EAE) induction. Mice were treated daily from day 0 postimmunization to EAE peak.

B- Flow cytometry gating strategy for analysis of infiltrates in the spinal cord of EAE mice at peak.

C- Flow cytometric quantification of CD4+, CD8+ and γδ T cells of total CD3+ T cells in lymph nodes (LN), spleen and spinal cord at EAE peak of vehicle- (n = 7) and TNP-ATP-treated mice (n = 5).

D- Relative mRNA expression of *Foxp3* (Tregs), *Ifnγ* (Th17) and *Ror* (Th1) in LN, spleen and spinal cord at EAE peak of vehicle- (n = 6) and TNP-ATP-treated mice (n = 7).

Data information: data are presented as mean ± s.e.m.

4. Role of P2X4R on microglia polarization

Since myelin clearance is necessary for remyelination and recovery (*Li et al., 2005; Kotter et al., 2006; Neuman et al., 2009*) and phagocytosis and remyelination are modulated by microglia/macrophage polarization (*Miron et al., 2013*), we hypothesized that P2X4R could be involved in this process. We first checked the status of microglia/macrophage in TNP-ATP and vehicle-treated EAE mice. We performed gene expression profiling from the lumbar spinal cord of vehicle and TNP-ATP-treated EAE mice at the peak and recovery phase (**Figure 15A, B**). Expression of pro-inflammatory and anti-inflammatory genes involved in microglia/macrophage activation was analysed using a 96.96 Dynamic Array™ integrated fluidic circuit (Fluidigm). In accordance to previous data showing that macrophages and microglia showed an intermediate activation status in MS (*Vogel et al., 2013*), most pro-inflammatory and anti-inflammatory genes were significantly increased at EAE peak (**Figure 15A**) and recovery phase (**Figure 15B**). Blockade of P2X4R with TNP-ATP did not significantly alter anti-inflammatory gene expression, but it significantly increased pro-inflammatory gene expression at recovery phase (**Figure 15B**), but not at EAE peak (**Figure 15A**). A higher increase in pro-inflammatory gene expression was also detected on FACS-sorted microglia (Cd11b$^+$CD45^{low}Ly6G$^-$CCR2$^-$; see gating strategy in **Figure 10E**) from EAE P2X4$^{-/-}$ mice versus EAE WT (**Figure 15C**). Then, we analysed the expression of iNOS (pro-inflammatory marker) in spinal cord sections of TNP-ATP-treated mice and P2X4$^{-/-}$ mice after EAE induction. Accordingly, we found an increase of iNOS expression in microglia/macrophages after EAE induction in TNP-ATP-treated mice and in P2X4$^{-/-}$ mice (**Figure 16A, B**). These data suggest that P2X4R could be influencing microglia/macrophage activation.

A Peak

	Control	EAE	EAE + TNP-ATP	p values	
				*	#
Ccl2				0.0012	0.2537
Ccr7				0.0086	0.2853
Cd40				0.0137	0.3679
Cd86				0.0187	0.3818
Il12a				0.0116	0.3722
Il1b				0.0001	0.2603
Il6				0.0047	0.3210
Marco				0.0000	0.0117
Mr1				0.1145	0.4858
Nos2				0.0054	0.4577
Ptgs2				0.0005	0.1911
Stat1				0.0081	0.3448
Tnf				0.0016	0.3424
Arg1				0.0014	0.4686
Chi3l3				0.0011	0.2001
Clec7a				0.0028	0.3330
Il10				0.0028	0.2765
Il4				0.0001	0.1561
Jak3				0.0486	0.1860
Mrc1				0.0743	0.3072
Msx3				0.4348	0.0987
Retnla				0.3203	0.1171
Stat3				0.0616	0.2511
Stat6				0.0622	0.2860
Tgfb1				0.0192	0.4668

B Recovery

	Control	EAE	EAE + TNP-ATP	p values	
				*	#
Ccl2				0.0002	0.0014
Ccr7				0.0160	0.1126
Cd40				0.0213	0.0184
Cd86				0.0105	0.0472
Il12a				0.0077	0.0051
Il1b				0.0003	0.0009
Il6				0.1259	0.0013
Marco				0.0393	0.4322
Mr1				0.0197	0.3372
Nos2				0.0021	0.0002
Ptgs2				0.0108	0.0047
Stat1				0.0007	0.0125
Tnf				0.0006	0.0132
Arg1				0.0777	0.0109
Chi3l3				0.0009	0.0911
Clec7a				0.0095	0.1054
Msx3				0.0117	0.2143
Il10				0.0129	0.1158
Il4				0.0519	0.0977
Jak3				0.0307	0.2421
Mrc1				0.4501	0.1327
Retnla				0.3078	0.2373
Stat3				0.0243	0.0223
Stat6				0.0314	0.1732
Tgfb1				0.0483	0.1258

C FACS-isolated microglia

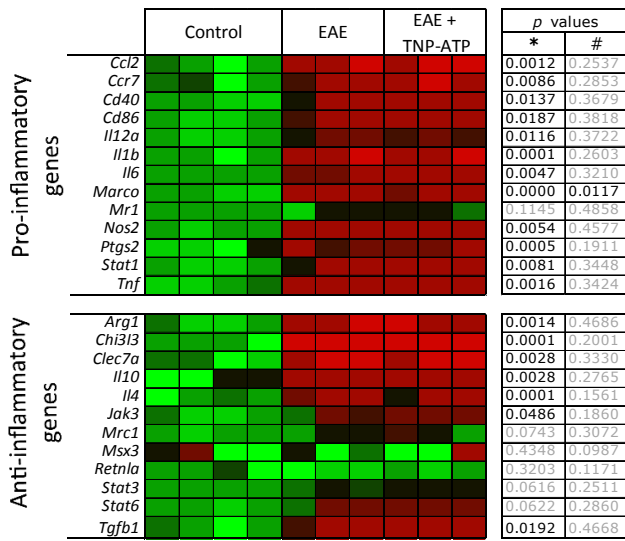|	Control WT	Control P2X4-/-	EAE WT	EAE P2X4 -/-	p values	
					*	#
Ccl2					0.0047	0.0163
Ccr7					0.3895	0.0017
Cd86					0.275	0.357
Il1b					0.0304	0.0219
Il6					0.038	0.2109
Marco					0.0902	0.0545
Mr1					0.376	0.0259
Nos2					0.0348	0.0253
Ptgs2					0.0066	0.027
Stat1					0.0148	0.4689
Tnf					0.2838	0.0094
Arg1					0.4111	0.0119
Chi3l3					0.4698	0.0991
Clec7a					0.0016	0.0326
Il10					0.1169	0.3244
Il4					0.1651	0.0024
Jak3					0.0916	0.0108
Mrc1					0.0097	0.1684
Msx3					0.0013	0.002
Retnla					0.0016	0.0008
Stat3					0.4006	0.1522
Stat6					0.1715	0.0843
Tgfb1					0.0681	0.0232

Figure 15. P2X4R blockade increases pro-inflammatory gene expression after experimental autoimmune encephalo-myelitis (EAE) induction.

A-B- Heatmaps showing changes in mRNA levels of pro-inflammatory and anti-inflammatory markers in the spinal cord at EAE peak (A) and at EAE recovery (B) in the presence or absence of TNP-ATP (n = 3).

C- Heatmap showing changes in pro-inflammatory and anti-inflammatory markers in FACS-isolated microglia from control, EAE wild type (WT) and EAE P2X4$^{-/-}$ mice at recovery phase (n = 2 in duplicate).
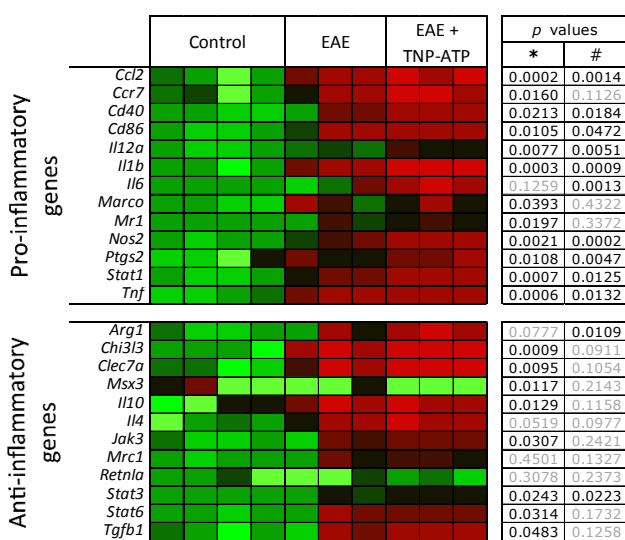
Data information: tables indicate statistical significance between control and EAE (*) and between EAE and EAE + TNP-ATP (#) (A, B) or between EAE WT and EAE P2X4$^{-/-}$ (#) (C). Data were analysed by Student's t-test.
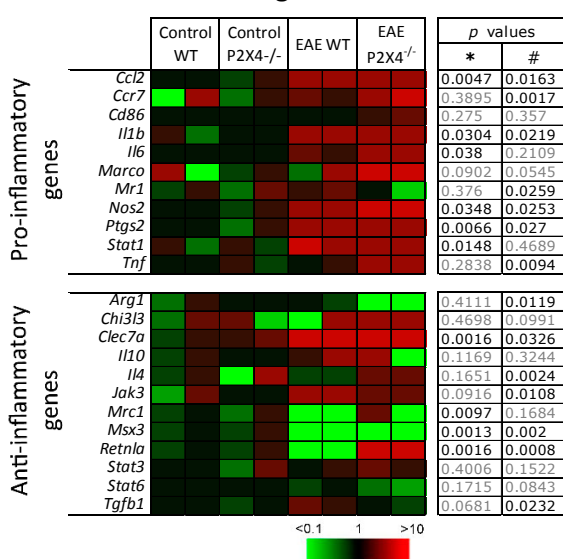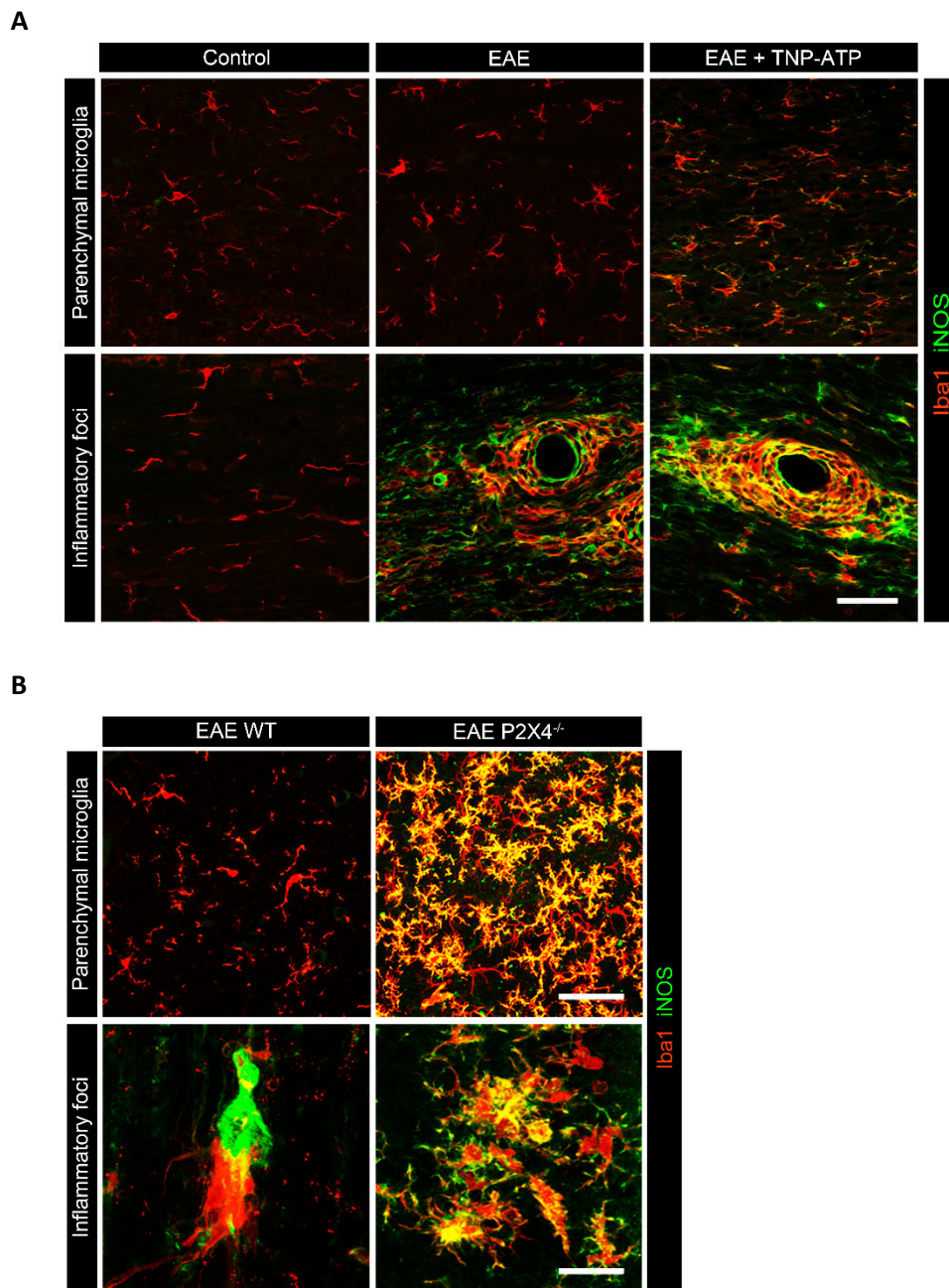
Figure 16. P2X4R blockade increases iNOS expression after experimental autoimmune encephalomyelitis (EAE) induction.

Immunofluorescence iNOS (pro-inflammatory marker; green) expression was increased in Iba1^{+} cells (red) in the spinal cord of TNP-ATP-treated versus vehicle-treated mice (A) and in P2X4^{-/-} mice versus wild type (WT) mice (B) after EAE induction (n = 3). Analysis was performed at the recovery phase of the EAE. Scale bars = 50 (A; B *top*) and 25 (B *bottom*) μm.

To further analyse the influence of P2X4R on microglia activation *in vitro*, cells were primed with colony stimulating factors and cytokines or LPS to differentiate into pro-inflammatory and anti-inflammatory microglia according to a previous protocol (*Durafort et al., 2012*; **Figure 17**, see details in methods). Activation was analysed by immunocytochemistry using pro-inflammatory (iNOS) and anti-inflammatory (mannose receptor, MRC1 and Arginase) markers. Blockade of P2X4R with TNP-ATP induced a significant increase in iNOS expression and a significant reduction in MRC1 and Arginase expression (**Figure 18A, B**). Accordingly, qPCR analysis revealed an increase in pro-inflammatory genes and a decrease in anti-inflammatory genes after TNP-ATP treatment during polarization (**Figure 19**). Then, we characterized microglia activation in P2X4$^{-/-}$ cells. P2X4$^{-/-}$ microglia cultures showed a significant increase in iNOS expression and a significant decrease in MRC1 expression in pro and anti- inflammatory microglia respectively (**Figure 20**). Altogether, these data suggest that P2X4Rs modulate microglial activation.
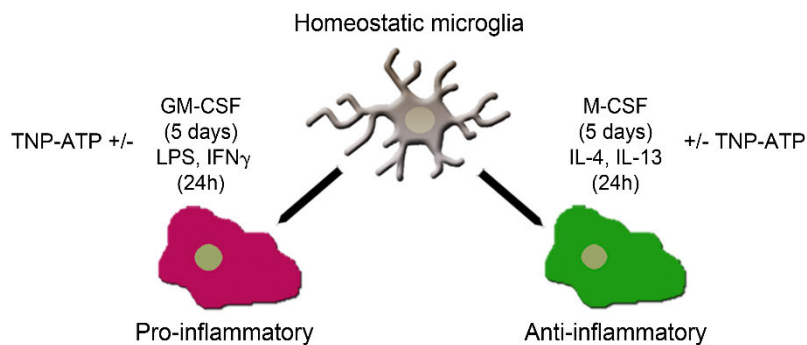

Figure 17. Schematic representation of microglia polarization protocol.

- Pro-inflammatory microglia: cells were treated with GM-CSF (5 ng/ml) for 5 days followed by 24 hours (h) treatment with LPS (10 ng/ml) and IFNγ (20 ng/ml).

- Anti-inflammatory microglia: cells were treated with M-CSF (20 ng/ml) 5 days followed by 24 hours (h) treatment with IL-4 (20 ng/ml) and IL-13 (50 ng/ml).

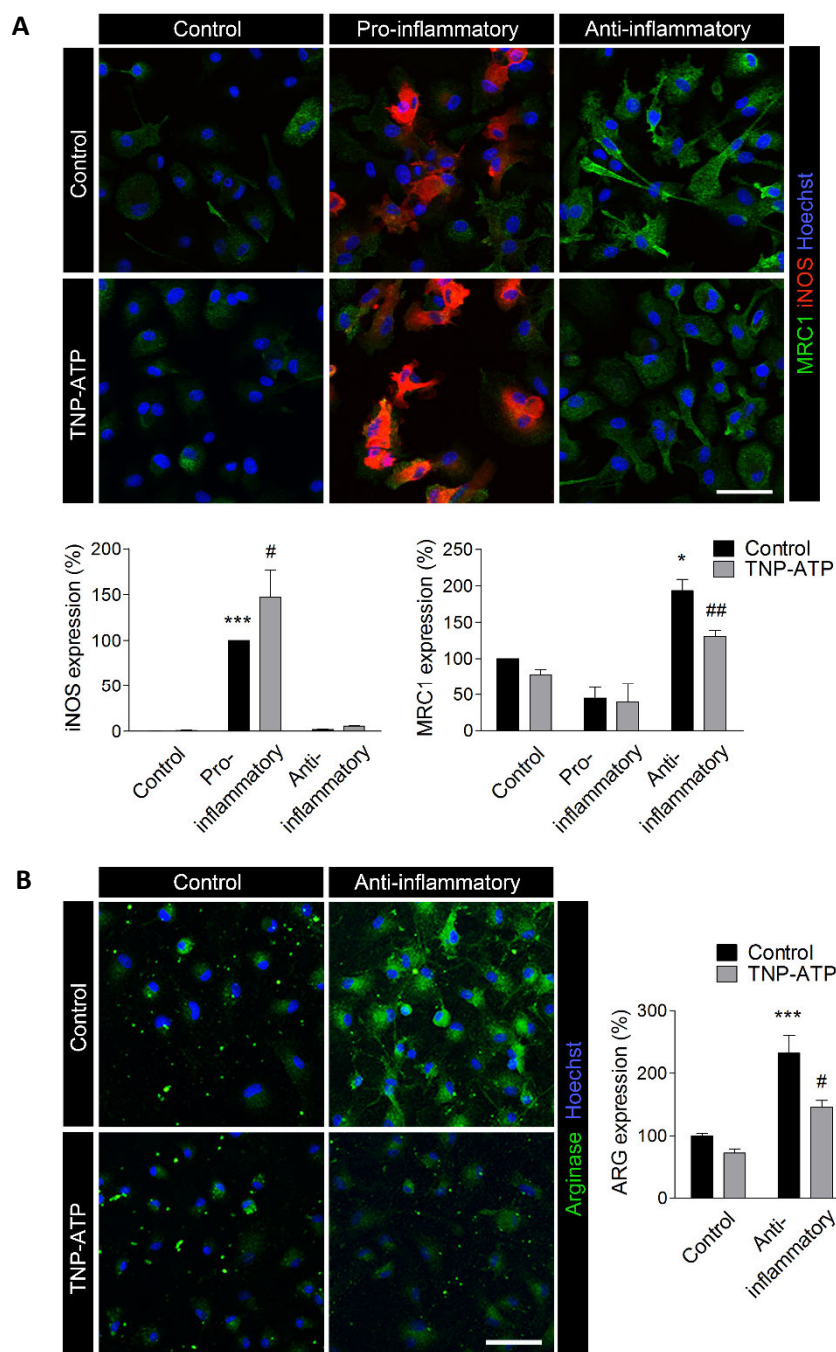Polarizing protocol was performed in the presence or absence of TNP-ATP (10 µM) all the time.

Figure 18. P2X4R modulates microglia activation.

A- Immunofluorescent labelling for iNOS (red) (n = 7) and mannose receptor (MRC1, green) (n = 4) in control microglia and after different polarization protocols (see **Figure 17**) in the absence or presence of TNP-ATP (10 µM). Histograms represent immunoreactivity per cell. Cells were identified by nuclear Hoechst labelling. Scale bar = 50 µm.

B- Immunofluorescent labelling for Arginase (green) (n = 3) in anti-inflammatory microglia in the absence or presence of TNP-ATP (10 µM). Histograms represent immunoreactivity per cell. Cells were identified by nuclear Hoechst labelling. Scale bar = 50 µm.

Data information: data are presented as mean ± s.e.m. and were analysed by one-way ANOVA. */#P < 0.05, ##P < 0.01, ***P < 0.001 versus control (*) or versus pro-/anti-inflammatory microglia (#).
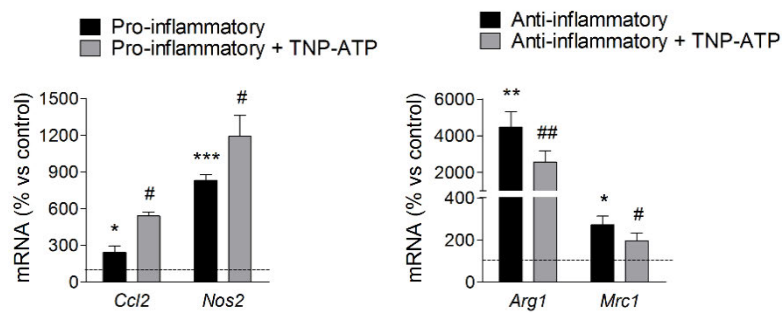
Figure 19. P2X4R modulates mRNA expression of microglia activation markers.

qPCR quantification of pro-inflammatory genes (*Ccl2* and *Nos2*) and anti-inflammatory genes (*Arg1* and *Mrc1*) in different activated microglia in the absence or presence of TNP-ATP (10 µM) (n = 3).

Data information: data are presented as mean ± s.e.m. and were analysed by one-way ANOVA. */#P < 0.05, **/##P < 0.01, ***P < 0.001 versus control (*) or versus pro-/anti-inflammatory microglia (#).
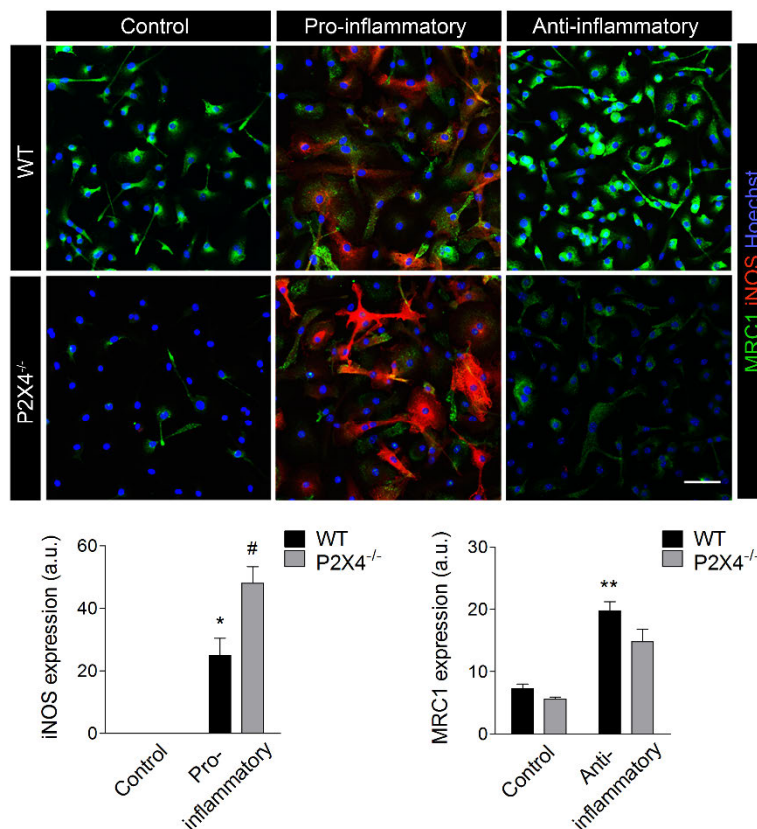

Figure 20. Pro-inflammatory and anti-inflammatory polarization in wild type (WT) and P2X4$^{-/-}$ microglia.

Immunofluorescent labelling for iNOS (red) and mannose receptor (MRC1, green) in control, pro-inflammatory and anti-inflammatory microglia from WT and P2X4$^{-/-}$ mice. Histograms represent immunoreactivity per cell (n = 4). Cells were identified by nuclear Hoechst labelling. Scale bar = 50 µm.

Data information: data are presented as mean ± s.e.m. and were analysed by one-way ANOVA. */#P < 0.05, **P < 0.01 versus control (*) or versus pro-/anti-inflammatory microglia (#).

5. Effect of P2X4R on oligodendrocyte differentiation

A switch from a pro-inflammatory to an anti-inflammatory phenotype occurs in microglia and peripherally derived macrophages during remyelination in MS and this change is essential for efficient remyelination (*Miron et al., 2013*). These data led us to the hypothesis that P2X4R blockade in microglia could be indirectly affecting oligodendrocyte differentiation and remyelination. We first characterized the expression and function of P2X4R in oligodendrocytes and microglia in mixed culture. Triple immunofluorescent analysis showed that P2X4R expression is virtually absent from Olig2$^+$ oligodendrocytes progenitor cells and in mature oligodedrocytes and highly enriched in isolectin B4$^+$ microglia cells in microglia-oligodendrocytes progenitor cells (OPCs) mixed cultures (**Figure 21A**). On the contrary, P2X7 receptors are expressed in both cell populations (**Figure 21B**).

To further exclude any direct role of P2X4R on oligodendrocyte differentiation, we stimulated culture oligodendrocytes with pharmacological agents. Oligodendrocytes were stimulated with ATPγS (10 µM, 3 days) at low concentrations to avoid P2X7R activation. Oligodendrocyte differentiation was analysed by quantifying the surface area of the cells and the number of MBP$^+$ cells. ATPγS did not induce any change in both parameters (**Figure 22A**). Previous data in the laboratory have demonstrated the expression of functional P2X7 receptors in oligodendrocytes and that their overactivation induces oligodendrocyte cell death (*Matute et al., 2007*). So, we analysed whether P2X4R, if present in oligodendrocytes, could affect oligodendrocyte viability. Long term incubation (3 days) with BzATP (broad-spectrum agonist of purinergic receptors) induced oligodendrocyte cell death but TNP-ATP (10 µM) did not inhibit cell death. In addition, long term incubation with ATPγS in the absence or presence of the positive modulator ivermectin (IVM, 3 µM) did not induce oligodendrocyte cell death (**Figure 22B**). Altogether, these results suggested that oligodendrocytes lack P2X4Rs.
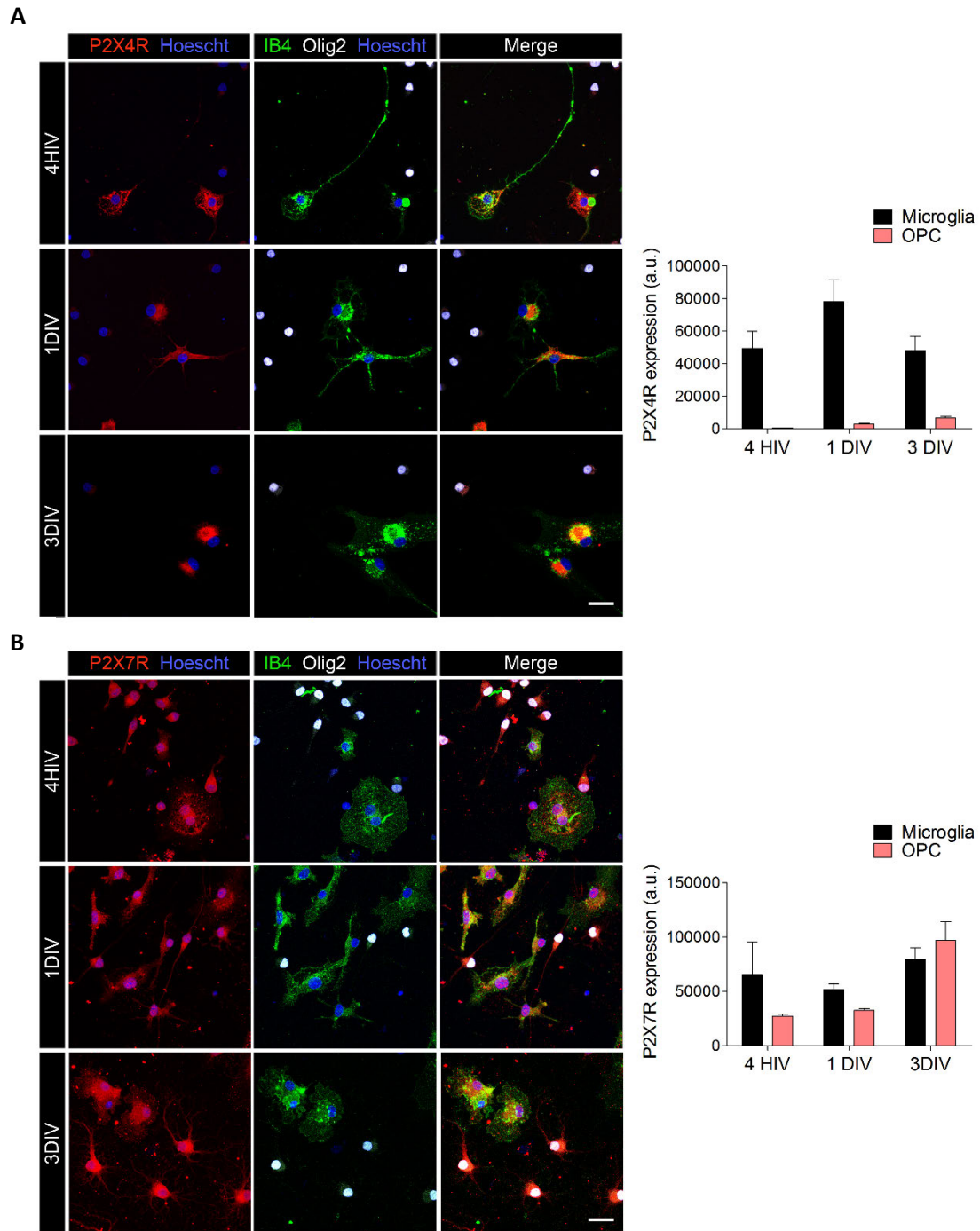
Figure 21. Oligodendrocytes lack P2X4Rs.

Representative images of microglia-OPCs cocultures immunofluorescent labelling for P2X4R (A; red) or P2X7R (B; red), Olig2 (oligodendrocyte lineage marker; white), isolectin B4 (microglia marker; IB4; green) and Hoechst (blue) at different stages of oligodendrocyte development, 4 hours *in vitro* (HIV), 1 and 3 days *in vitro* (DIV). Scale bar = 20 μm. Notice the presence of P2X4R on IB4+ cells and the absence in Olig2+ cells. Histograms show quantification of P2X4R (A) and P2X7R (B) in microglia and OPCs at different stages of oligodendrocyte development in cocultures (n = 3).
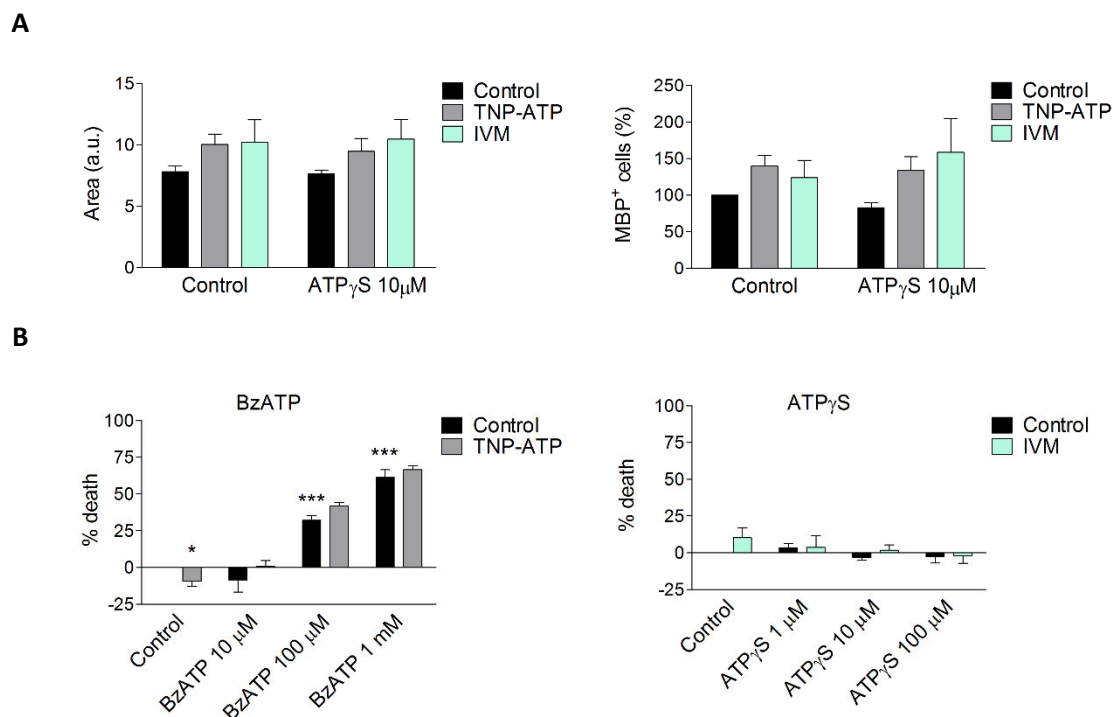
Data information: data are presented as mean ± s.e.m.

A

B

Figure 22. No functional role of P2X4R in oligodendrocytes *in vitro*.

A- Effect of ATPγS (10 µM) in the absence or presence of ivermectin (IVM) or TNP-ATP (10 µM) on oligodendrocyte differentiation quantified based on the area of the cells (*left*) and the number of MBP+ cells/total cell number (*right*). Area was calculated with *Image J* software. (n = 3).

B- Oligodendrocyte cell viability after 24h incubation with BzATP, broad-spectrum agonist of purinergic receptors, in the absence or presence of TNP-ATP (10 µM) (*left*) or ATPγS (*right*) in the absence or presence of IVM (3 µM). (n = 3).

Data information: data are presented as mean ± s.e.m. and were analysed by Student's t-test. *P < 0.05, ***< 0.001 versus control.

Next, we analysed whether microglial P2X4R could play a role on oligodendrocyte differentiation. To this end, we polarized microglia to control, pro-inflammatory or anti-inflammatory state in the presence or absence of TNP-ATP (10 µM). SATO- medium was added to microglia cultures in the last 24h of the polarization protocol (see methods). Microglia conditioned medium (MCM) was collected and added to OPC cultures for 3 days (see cartoon in **Figure 23A**). All microglia-conditioned media induced an increase in the number of mature MBP+ oligodendrocytes as compared to polarization factors alone (**Figure 23B**). However, anti-inflammatory microglia induced a higher increase in oligodendrocyte differentiation, an effect that was blocked in the presence of TNP-ATP (**Figure 23B**).
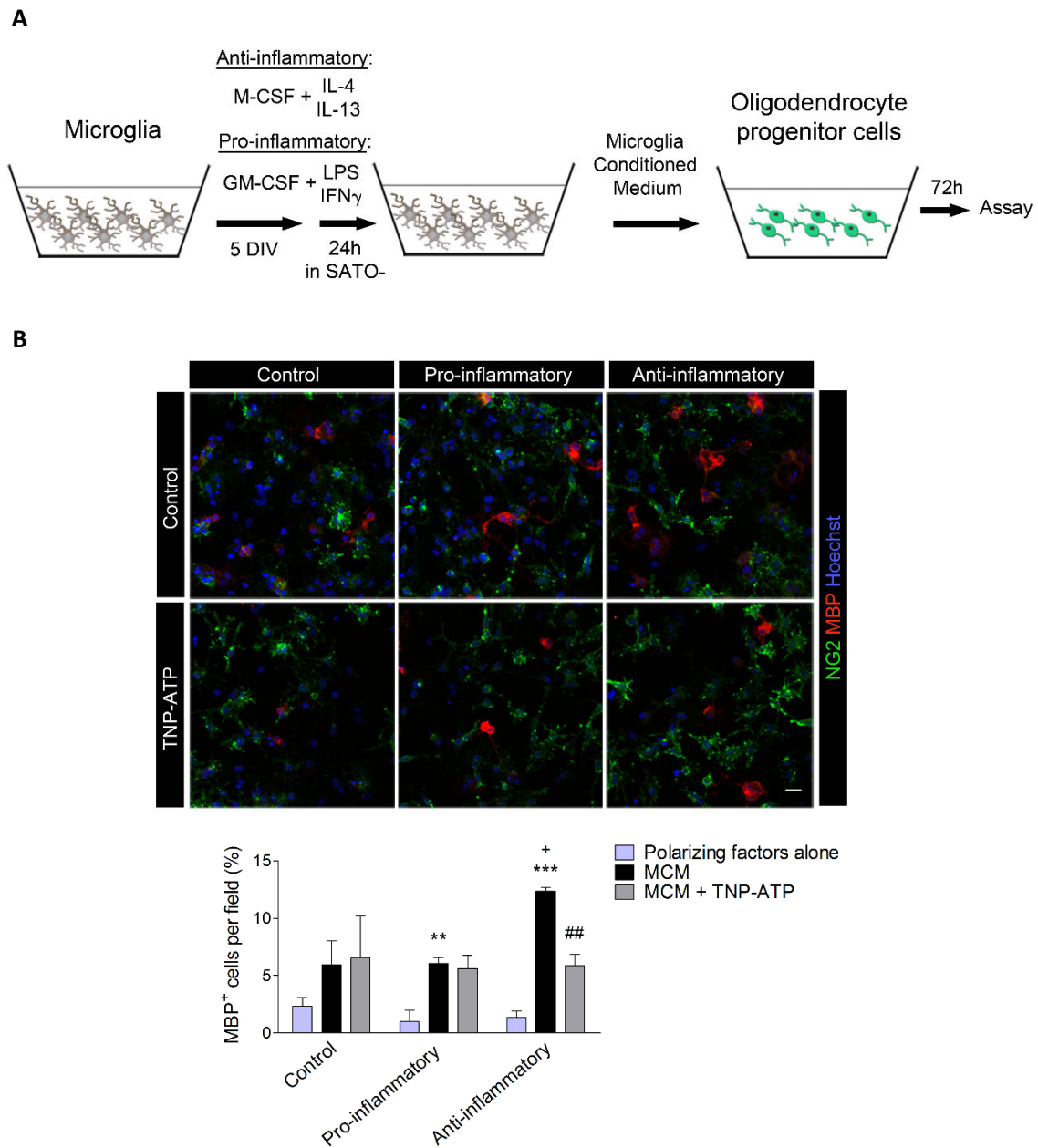
A

B

Figure 23. Microglial P2X4R modulates oligodendrocyte differentiation.

A- OPCs were treated with control, anti-inflammatory and pro-inflammatory microglia-conditioned media (MCM).

B- Immunofluorescent labelling for NG2 (green) and MBP (red) in OPCs treated with MCM from differentially activated microglia. Histogram represents the quantification of MBP+ cells per field (n = 3). Cells were identified by nuclear Hoechst labelling. Scale bar = 20 μm.

Data information: data are presented as mean ± s.e.m. and were analysed by one-way ANOVA. +P < 0.05, **/##P < 0.01, ***P < 0.001. Symbols indicate significance versus polarizing factors (*), versus MCM (#) or versus Control MCM (+).

Since BDNF enhances oligodendrocyte differentiation and myelination (*Wong et al., 2013*) and P2X4R stimulation in microglia has been linked to BDNF release (*Tsuda et al., 2003; Coull et al., 2005*), we asked whether BDNF was involved in the anti-inflammatory microglia response. We first corroborate the involvement of P2X4 receptor in BDNF release. To activate P2X4R we stimulated cells with IVM (3 µM). Western blot analysis revealed that IVM enhanced BDNF release in WT microglia but not in P2X4$^{-/-}$ microglia, confirming the role of P2X4R (**Figure 24A**). Then, we analysed BDNF production in differentially polarized microglia. Western blot analysis showed a significant increase in BDNF production in anti-inflammatory microglia, which was significantly reduced in the presence of TNP-ATP (**Figure 24B**).
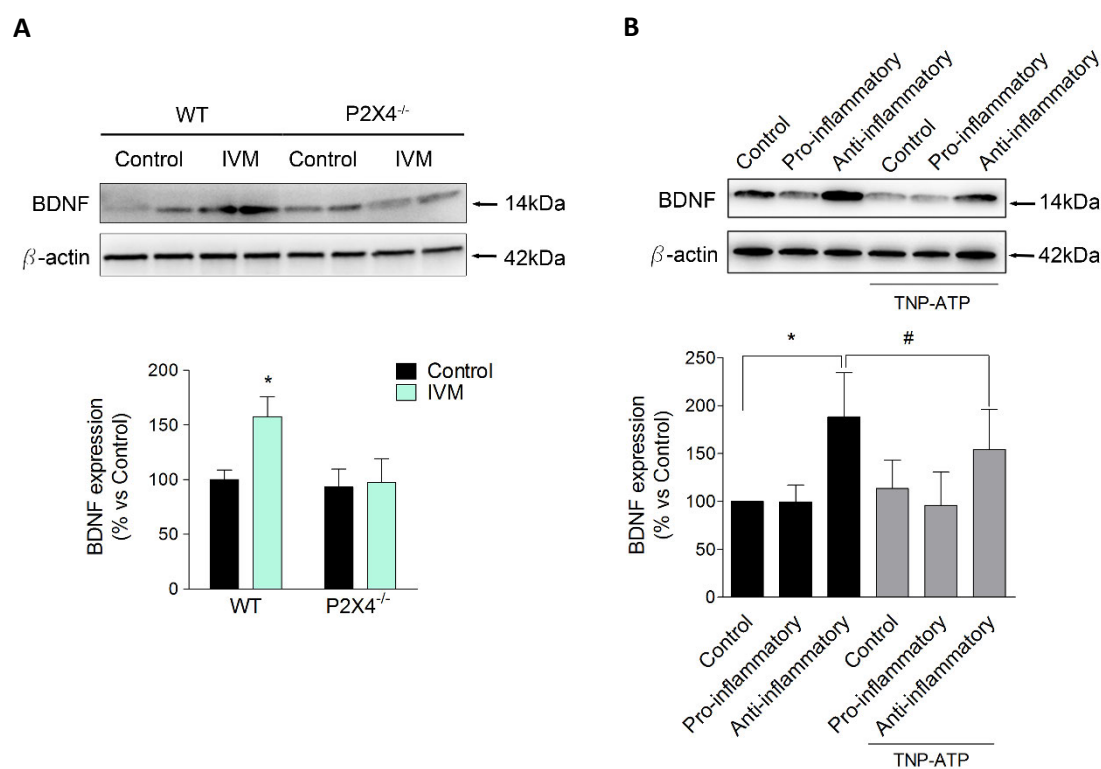

Figure 24. Increased BDNF production in anti-inflammatory microglia.

A- Western blot analysis of BNDF in control and ivermectin (IVM)-treated wild type (WT) and P2X4$^{-/-}$ microglia. Histogram shows BDNF expression normalized to β-actin (n = 3).

B- Representative immunoblots of BDNF levels in differentially activated microglia *in vitro* in the absence or presence of TNP-ATP (10 µM) and densitometry quantification of BDNF normalized to β-actin. (n = 10).

Data information: data are presented as mean ± s.e.m. and were analysed by Student's t-test. */#P < 0.05.

After these *in vitro* results, we checked *Bdnf* mRNA levels in EAE samples at the recovery phase. Accordingly, *Bdnf* levels were significantly reduced in the presence of TNP-ATP, a fact that correlated with the downregulation of *Mbp* expression (**Figure 25A**). *Bdnf* mRNA was also reduced in FACS isolated microglia from control and EAE P2X4^{-/-} mice (**Figure 25B**). These data suggested that P2X4R blockade in microglia inhibit oligodendrocyte differentiation by shifting microglia towards a pro-inflammatory phenotype, an effect that could impede remyelination *in vivo*.
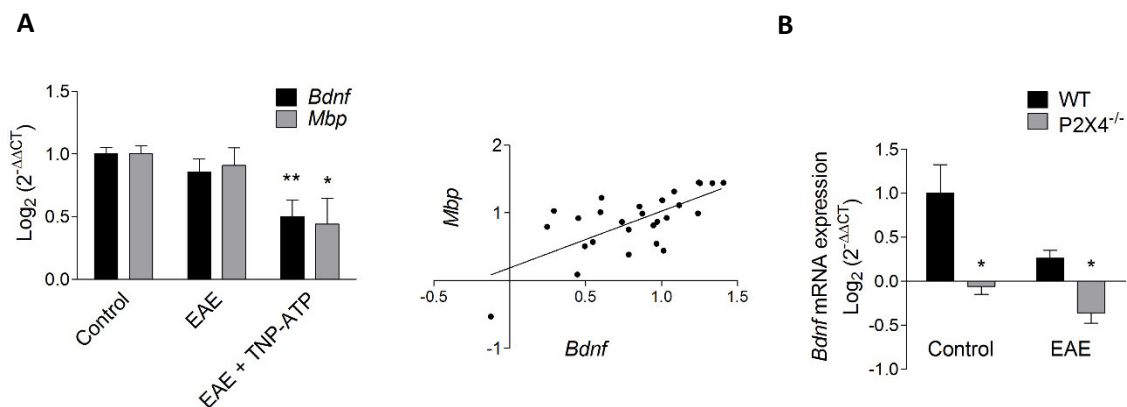

Figure 25. P2X4R deficiency induces a reduction in *Bdnf* levels after experimental autoimmune encephalomyelitis (EAE) induction.

A- *Bdnf* and *Mbp* mRNA expression (*left*) and correlation of the expression (*right*) at EAE recovery phase in control mice, EAE mice and TNP-ATP-treated EAE mice. (n = 10 mice/group).

B- *Bdnf* mRNA levels in the spinal cord of wild type (WT) and P2X4^{-/-} mice in control conditions and at the recovery phase of EAE (n = 4 mice/group).

Data information: data are presented as mean ± s.e.m. and were analysed by one-way ANOVA. *P < 0.05, **P < 0.01.

6. P2X4R potentiation ameliorates EAE

We then explored the therapeutic potential of P2X4R potentiation. To that aim, we used Ivermectin (IVM), an FDA-approved semi-synthetic macrocyclic lactone used in veterinary and clinical medicine as an anti-parasitic agent. IVM allosterically modulates both ion conduction and channel gating of P2X4Rs (*Priel and Silberberg, 2004*). We first analysed the role of P2X4R potentiation in EAE pathogenesis in mice treated daily with IVM (1 mg/kg) after the onset of the disease (10 dpi). IVM induced a significant amelioration of the motor deficits of EAE (**Figure 26A**).

To further assess that the effect of IVM was P2X4R-dependent, we treated P2X4$^{-/-}$ mice with IVM from the onset of the disease. IVM failed to alter the course of EAE disease in P2X4$^{-/-}$ mice confirming the specificity of the treatment (**Figure 26B**). This data indicates that IVM treatment effectively promoted recovery.
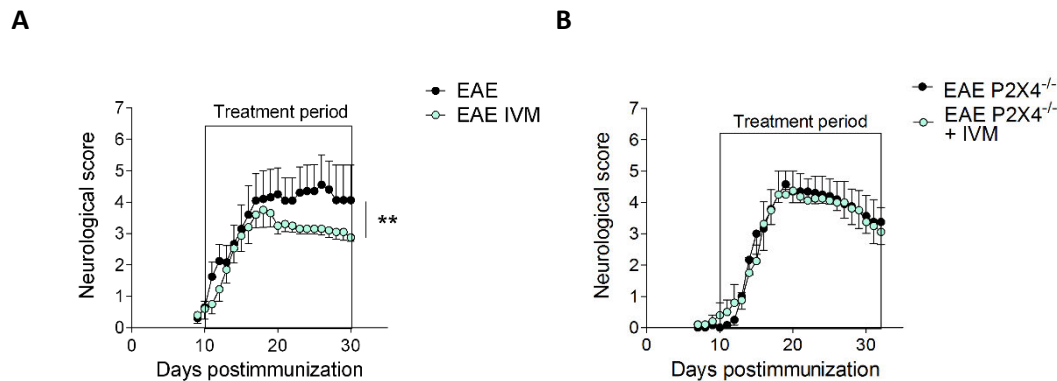

Figure 26. P2X4R potentiation ameliorates experimental autoimmune encephalomyelitis (EAE).

A- Neurological score of EAE and ivermectin (IVM)-treated EAE mice (1 mg/kg) (n = 5 mice/group). Mice were treated daily from day 10 postimmunization to the end of the experiment.

B- Neurological score after EAE induction in P2X4$^{-/-}$ mice treated with vehicle (n = 4) or IVM (1 mg/kg) (n = 5). Mice were treated daily from day 10 postimmunization to the end of the experiment.

Data information: data are presented as mean ± s.e.m. Statistic was performed with Mann-Whitney U-test. **P < 0.01.

As we did for TNP-ATP treatment, to further exclude the involvement of P2X4R in adaptive immune system we performed an additional EAE experiment and treated mice with IVM during the priming phase (0 -17 dpi). IVM treatment did not affect disease development (**Figure 27A**). Mice were sacrificed at the peak of symptoms and immune response was quantified by flow cytometry in periphery (spleen and lymph nodes) and in the spinal cord (see gating strategy in **Figure 14B**). The number of CD4$^+$ T cells, CD8$^+$ T cells and γδ T cells was not altered after IVM treatment during the priming phase (**Figure 27B**). Next, CD4$^+$ T cell response was characterized more in detail by qPCR and we did not detect any change in transcript expression of *Foxp3* (T regs), *Ror* (Th17) and *Ifnγ* (Th1) after IVM treatment (**Figure 27C**).
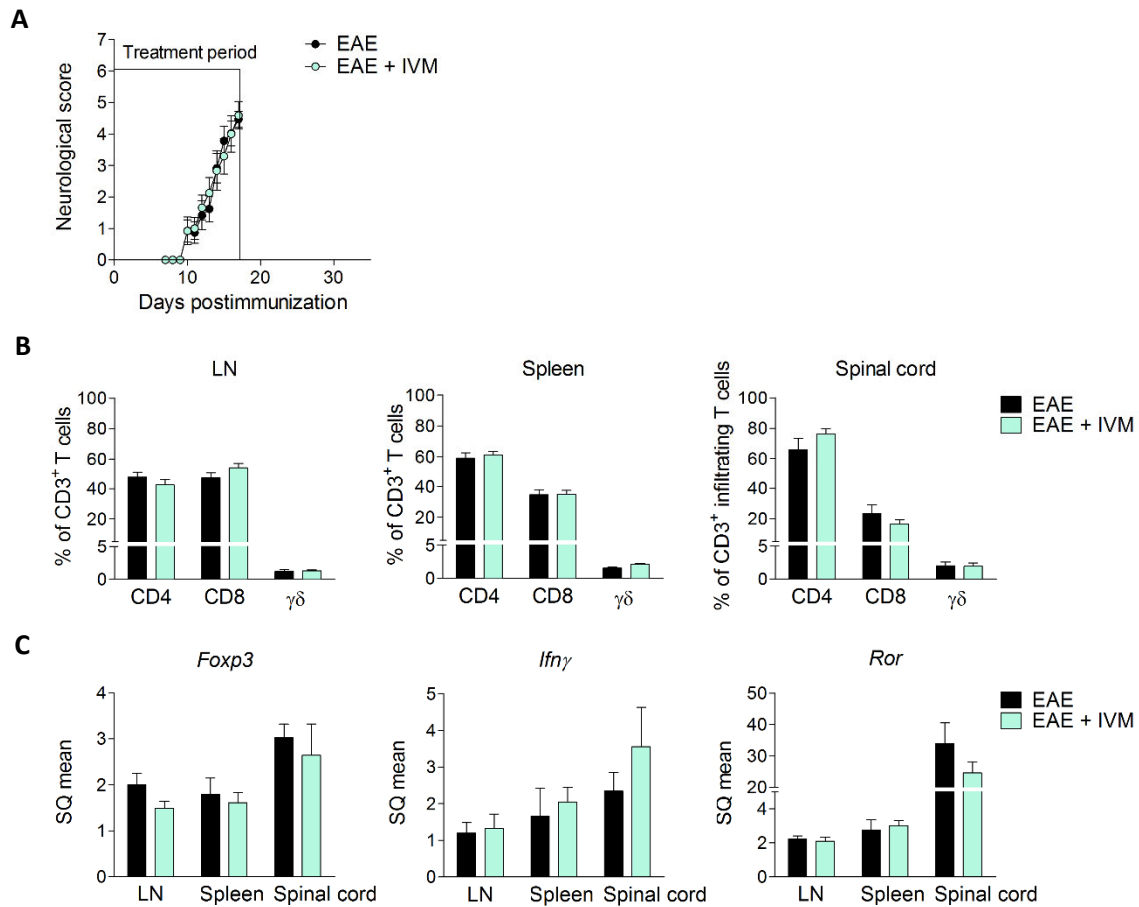
Figure 27. P2X4R do not interfere with immune priming.

A- Neurological score of vehicle and ivermectin (IVM)-treated (1mg/kg) mice after experimental autoimmune encephalomyelitis (EAE) induction (n = 13 mice/group from two independent experiments). Mice were treated daily with IVM from day 0 postimmunization to EAE peak.

B- Flow cytometric quantification of CD4$^+$, CD8$^+$ and γδ T cells in lymph nodes (LN), spleen and spinal cord at EAE peak of vehicle- and IVM-treated mice (n = 7 mice/group) (gating strategy as described in **Figure 5B**).

C- Relative mRNA expression of *Foxp3*, *Ifnγ* and *Ror* in LN, spleen and spinal cord at EAE peak of vehicle- (n = 6) and IVM-treated mice (n = 7).

Data information: data are presented as mean ± s.e.m.

Then we analysed the effect of IVM on microglial polarization *in vitro*. Microglia cells were polarized in the presence or absence of IVM (3 μM) and treatment induced a significant increase in polarization to an anti-inflammatory phenotype (**Figure 28A**). Accordingly, qPCR analysis revealed a decrease in pro-inflammatory genes and an increase in anti-inflammatory genes after IVM treatment during polarization (**Figure 28B**). Altogether, these data suggest that P2X4R potentiation by IVM could favour microglia switch to an anti-inflammatory phenotype necessary to efficient remyelination.
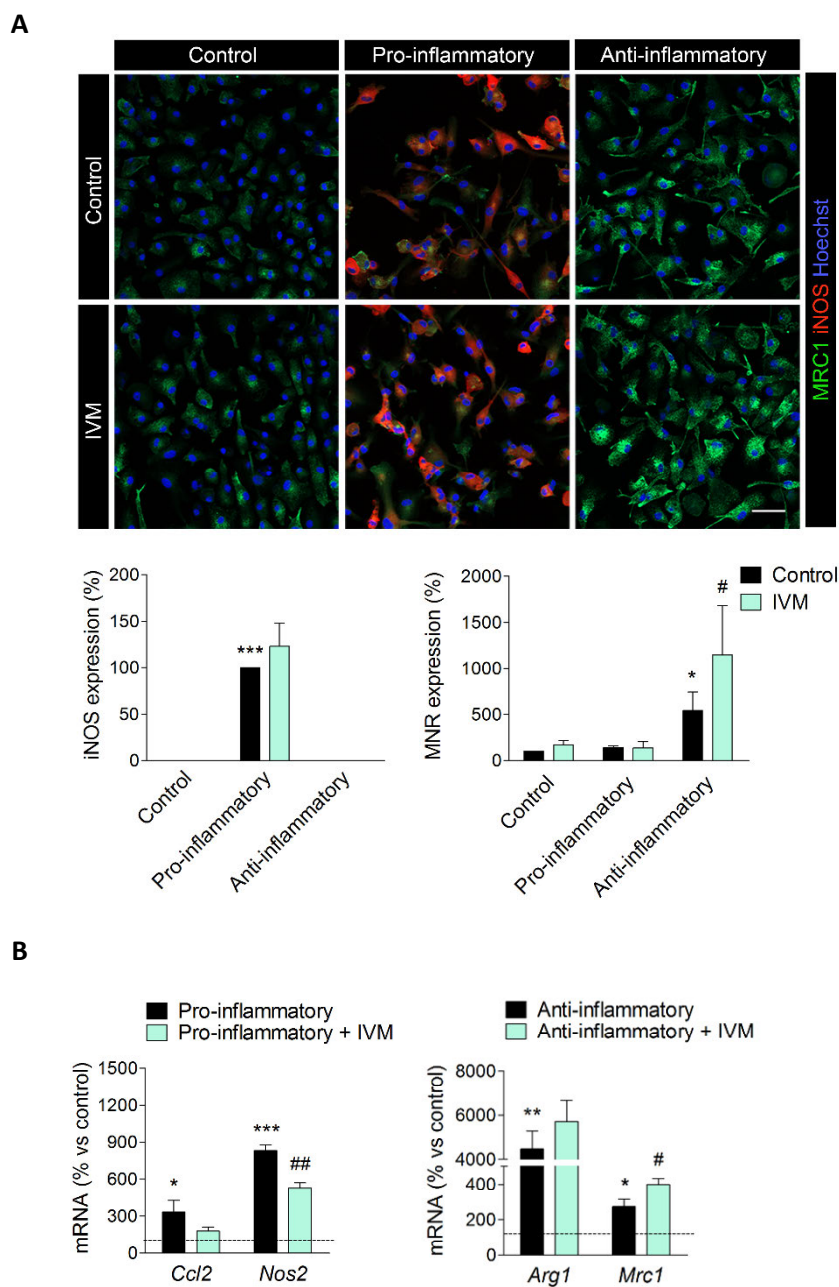
Figure 28. P2X4R modulates microglia polarization.

A- Immunofluorescent labelling for iNOS (red) and mannose receptor (MRC1, green) in different activated microglia *in vitro* in the absence or presence of ivermectin (IVM) (3 µM). Histograms represent immunoreactivity per cell (n = 3). Cells were identified by nuclear Hoechst labelling. Scale bar = 50 µm

B- qPCR quantification of pro-inflammatory genes (*Ccl2* and *Nos2*) and anti-inflammatory genes (*Arg1* and *Mrc1)* in different activated microglia in the absence or presence of IVM (3 µM) (n = 3).

Data information: data are presented as mean ± s.e.m. and were analysed by one-way ANOVA. */#P < 0.05, **/##P < 0.01, ***P < 0.001 versus control (*) or versus pro-/anti-inflammatory microglia (#).

7. Treatment with TNP-ATP or IVM do not change mechanical allodynia in EAE mice

The therapeutic potential of IVM in MS could be compromised by its possible influence in neuropathic pain. Thus, a recent study reported the anti-allodynic effect of a novel P2X4R antagonist in mice with traumatic nerve damage, although the antagonist did not affect acute nociceptive pain and motor function (*Matsumura et al., 2016*). Moreover, pain occurs in MS patients with a wide prevalence (*O'Connor et al., 2008*) although it does not correlate with disease severity.

To clarify the role of P2X4R, we performed an EAE experiment where mice were treated daily from day 0 to the end of the experiment with vehicle, TNP-ATP or IVM and mechanical allodynia was analysed before the immunization, at preonset (5 dpi), onset (12 dpi) and before peak (16 dpi). We evaluated mechanical withdrawal by electronic Von Frey at different stages of EAE before the appearance of severe motor deficits. Withdrawal thresholds were significantly diminished in vehicle-treated mice at EAE onset (12 dpi) and before peak (16 dpi) relative to baseline responses, indicative of an increased pain sensitivity during EAE. However, there were no significant differences between vehicle-treated mice and TNP-ATP- or IVM-treated mice (**Figure 29**).
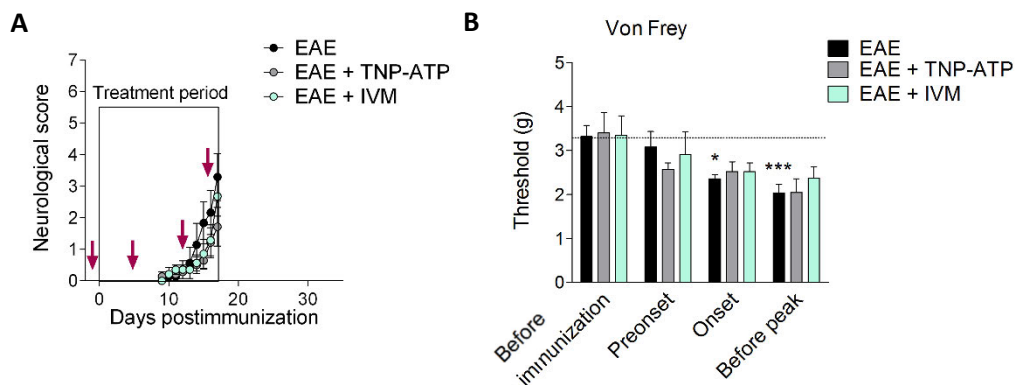

Figure 29. P2X4R potentiation do not enhance allodynia in experimental autoimmune encephalomyelitis (EAE).

A- Neurological score of vehicle (n = 6), TNP-ATP- (n = 7) and ivermectin (IVM)-treated mice (n = 7) after EAE induction. Mice were treated daily from day 0 to 17 postimmunization (dpi). Arrows indicate mechanical allodynia measurements before the immunization, at preonset (5 dpi), onset (12 dpi) and before peak (16 dpi).

B- Withdrawal threshold (grams) of mechanical stimulation by von Frey filament applied to the mouse hindpaw in vehicle- (n = 6), TNP-ATP- (n = 7) and IVM-treated mice (n = 7) before EAE immunization, at preonset, at onset and before peak of EAE neurological symptoms.

Data information: data are presented as mean ± s.e.m. Statistics were performed with Students t-test. *P < 0.05, ***P < 0.001 versus EAE before immunization.

8. *Irf8* and *Irf5* transcription factors expression is upregulated during EAE

Interferon regulatory factor 5 (IRF5) is a transcription factor involved in innate immune response and previous data have demonstrated that interferon regulatory factor 8 (IRF8)-IRF5 transcriptional axis is a critical regulator for shifting microglia cells towards a P2X4R[+]-reactive phenotype (*Masuda et al., 2014*). IRF5 is upregulated in microglia after GM-CSF exposure (*Krausgruber et al., 2011*) and is critical for pro-inflammatory gene expression (*Takaoka et al., 2005; Krausgruber et al., 2011*). Moreover, genetic polymorphisms in human IRF5 that lead to the expression of various unique isoforms or higher expression of *Irf5* mRNA have been linked to immune-mediated diseases, including MS (*Kristjansdottir et al., 2008; Tang et al., 2014*).

We first analysed the expression and time course of *Irf8* and *Irf5* in the same EAE samples used to analyse *P2x4r* expression (**Figure 10A**). Accordingly, we observed that *Irf8* and *Irf5* transcription factors were upregulated at the peak and recovery phase of the disease (**Figure 30A**) and their expression correlated well with *P2x4r* expression (**Figure 30B**). IRF5 expression, as visualized by immunohistochemistry, was also increased in EAE chronic lesions (**Figure 31**).
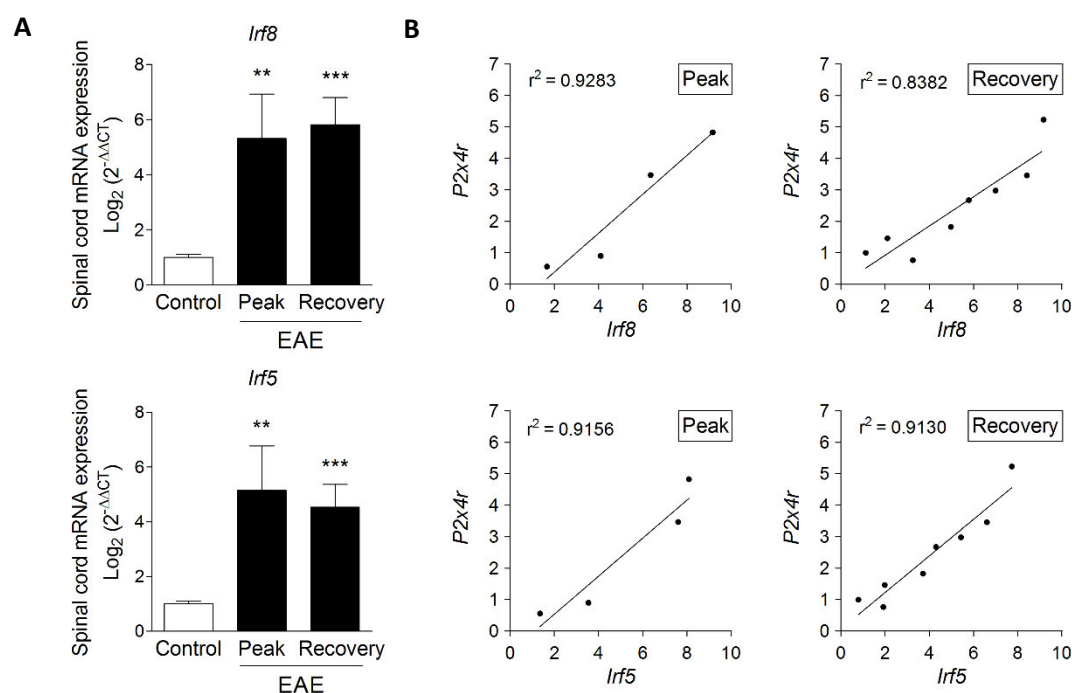
Figure 30. *Irf8* and *Irf5* are increased in experimental autoimmune encephalomyelitis (EAE) mice at peak and at recovery phase.

A- Expression of *Irf8* (*top*) and *Irf5 (bottom)* in the spinal cord of control (n = 7) and of EAE mice at peak (n = 4) and at recovery (n = 8) phase as analysed using qPCR.

B- Correlation of *P2x4r* expression with *Irf8* (*top*) and *Irf5* (*bottom*) expression at EAE peak and recovery phases.

Data information: data are presented as mean ± s.e.m. and were analysed by one-way ANOVA. **P < 0.01, ***P < 0.001.

Figure 31. IRF5 is overexpressed in EAE lesions.

Representative images showing spinal cord immunofluorescent labelling for IRF5 (green) in EAE mice at recovery phase. Note the accumulation of IRF5⁺ cells in the lesion area. In blue, nuclear Hoechst labelling. Scale bar = 50 μm.
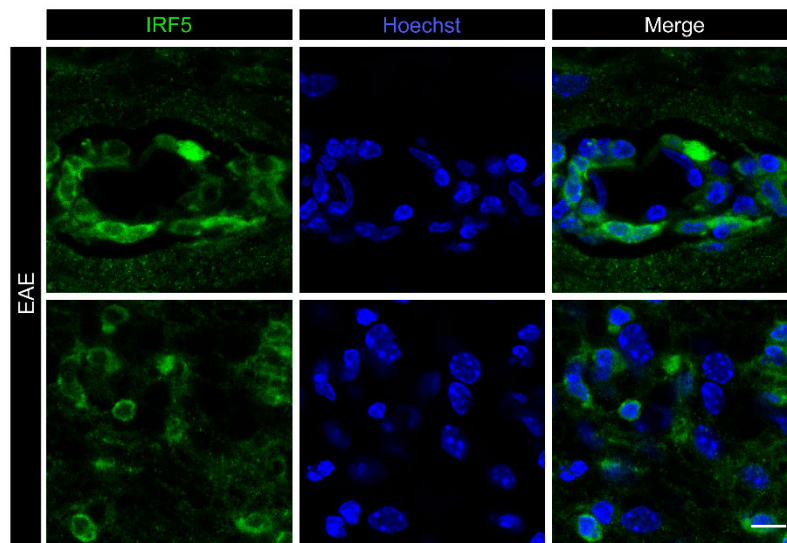
9. Role of IRF5 transcription factor in EAE pathogenesis

To analyse the role of IRF5 in EAE pathogenesis we performed an EAE experiment in IRF5$^{-/-}$ mice. IRF5$^{-/-}$ mice showed a significant delay in the appearance of neurological symptoms, indicating that immune priming could be postponed in IRF5$^{-/-}$ mice (**Figure 32A, B**). However, similarly to the blockage of P2X4R, IRF5$^{-/-}$ mice showed an exacerbation of clinical signs in the recovery phase (**Figure 32A**). When analysed individually, we observed that EAE maximal score was similar in both genotypes (**Figure 32A, C**) but IRF5$^{-/-}$ mice stayed longer at maximal score before the initiation of recovery (**Figure 32D**) than WT mice and that recovery was smaller (**Figure 32A**). These results suggest that IRF5 have a dual role in EAE pathogenesis and that IRF5 deletion, similarly to P2X4R deletion, could impair EAE remyelination at the recovery phase.
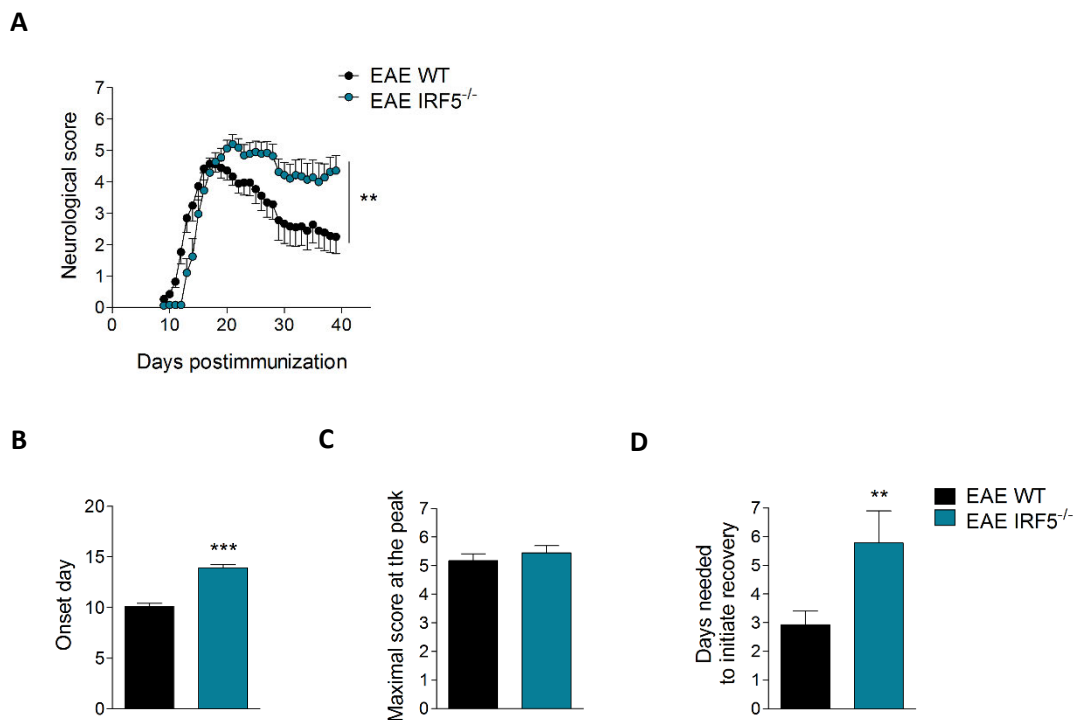
Figure 32. IRF5$^{-/-}$ mice have a delay at the onset, but impairment at the recovery.

A- Neurological score of wild type (WT) (n = 14) and IRF5$^{-/-}$ (n = 12) mice after experimental autoimmune encephalomyelitis (EAE) induction.

B, C, D- Onset day of neurological symptoms (B), maximal score at the peak of the disease (C) and number of days needed to initiate recovery (D) in WT (n = 14) and IRF5$^{-/-}$ (n = 12) mice after EAE induction.

Data information: data are presented as mean ± s.e.m. from two independent experiments. Statistics were performed with Mann–Whitney U-test (A) and Student' t-test (B, D). **$P < 0.01$, ***$P < 0.001$.

Next, we checked the activation status of microglia/macrophages in IRF5$^{-/-}$ mice after EAE induction as we did for TNP-ATP-treated EAE mice. We performed gene expression profiling from the lumbar spinal cord of WT and IRF5$^{-/-}$ EAE mice at the recovery phase (score showed in **Figure 32A; Figure 33**). Expression of pro-inflammatory and anti-inflammatory genes were analysed via Fluidigm qPCR. We corroborate the upregulation of both phenotype markers in WT EAE mice. IRF5$^{-/-}$ mice showed a modest but significant upregulation of most pro-inflammatory and anti-inflammatory markers versus WT mice (**Figure 33**). These data are surprising since IRF5 is involved in microglia activation and regulates pro-inflammatory gene expression.
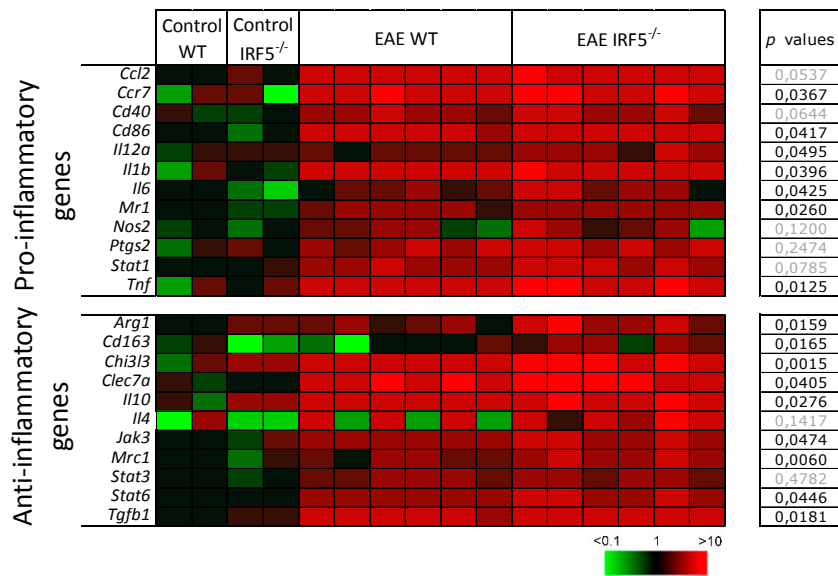
Figure 33. IRF5 deficiency increases gene expression of microglia activation markers after experimental autoimmune encephalomyelitis (EAE) induction.

Heatmap showing significant changes in pro-inflammatory and anti-inflammatory mRNA expression in the spinal cord at EAE recovery phase in wild type (WT) and IRF5$^{-/-}$ mice (n = 6).

Data information: tables indicate statistical significance between EAE WT and EAE IRF5$^{-/-}$ mice. Data were analysed by Student's t-test.

10. IRF5 deletion has no effect in immune response

IRF5 plays a central role in inflammation and, in addition to microglia, it is also expressed in peripheral immune cells. First, we checked the immune cell infiltration by immunohistochemistry, and we did not detect differences in T cell (CD3$^+$ cells) and B cell (B220$^+$ cells) infiltration in the spinal cord after EAE induction in WT versus IRF5$^{-/-}$ mice (**Figure 34A**). We further analysed the role of IRF5 in adaptive immune system by qPCR for *Cd4* and *Cd8* mRNA and IRF5 deficiency did not alter transcript expression in the spinal cord (**Figure 34B**). Finally, transcript expression of *Foxp3*, *Ror* and *Ifnγ* was analysed to characterize CD4$^+$ T cell response. The only significant change was an increase in *Ror* expression (Th17 response) in lymph nodes in IRF5$^{-/-}$ mice compared to WT mice at the recovery phase of EAE (**Figure 34C**).
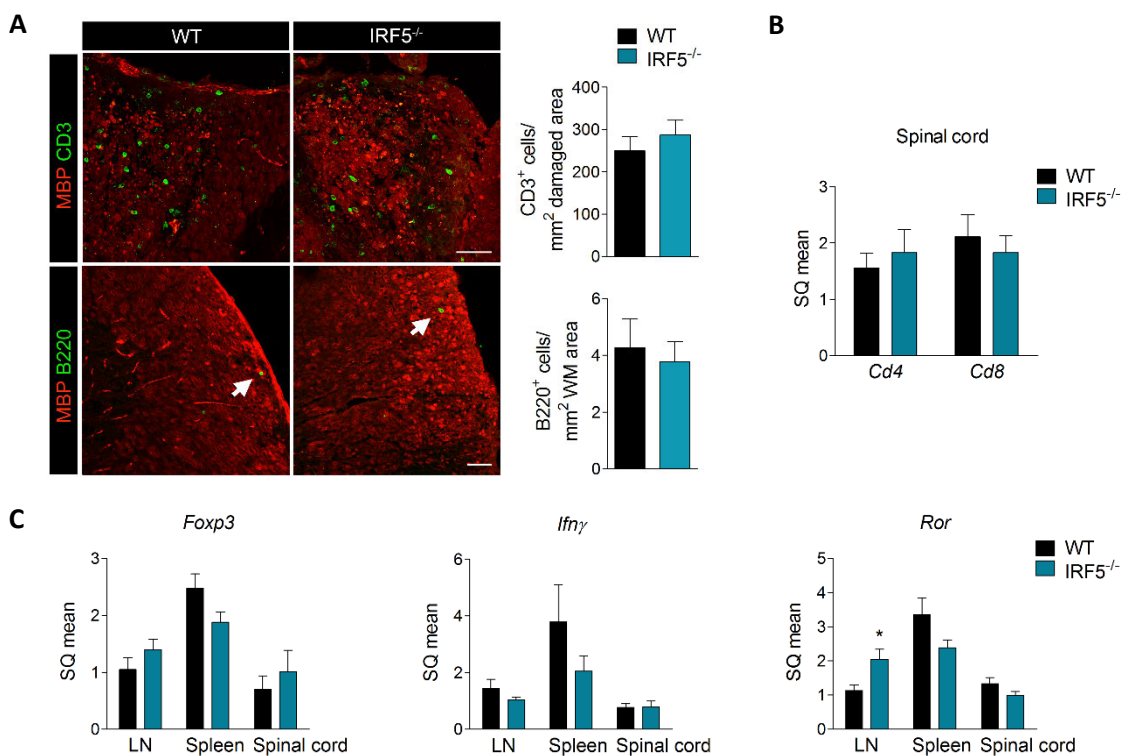
Figure 34. Absence of IRF5 has no impact on immune response.

A- Spinal cord immunolabelling for MBP (red) and CD3 (T cell marker; green; *top*) and B220 (B cell marker; green, *bottom*) in wild type (WT) (n = 7) and IRF5$^{-/-}$ (n = 6) mice at EAE recovery phase. Histograms showing CD3$^+$ cells normalized to lesion area (*top*) and B220$^+$ cells normalized to white matter (WM) area (*bottom*). Arrows point to B220$^+$ cells. Scale bar = 50 μm.

B- Relative mRNA expression of *Cd4* and *Cd8* in the spinal cord of WT (n=14) and IRF5$^{-/-}$ (n=12) mice at EAE recovery phase.

C- Relative mRNA expression of *Foxp3* (Tregs), *Ifnγ* (Th1) and *Ror* (Th17) in lymph nodes (LN), spleen and spinal cord of WT (n = 14) and IRF5$^{-/-}$ (n = 12) mice.

Data information: data are presented as mean ± s.e.m. from two independent experiments (B, C). Statistics were performed with Student' t-test. *P < 0.05.

11. IRF5 deficiency increased tissue damage in EAE mice

To further analyse the role of IRF5 in EAE pathogenesis histological characterization of WT and IRF5$^{-/-}$ mice was performed after EAE induction. Spinal cord sections were stained at the end of the experiment for MBP. Lesions in white matter (WM) were defined on the basis of MBP immunolabelling, as the absence of labelling or the accumulation of myelin debris, together with the presence of inflammatory cells accumulation, as revealed by Hoechst. Lesion area was slightly increased in IRF5$^{-/-}$ mice although the difference was not significant (**Figure 35A**). Myelin debris has higher immunoreactivity for MBP than the undamaged myelin likely due to the unmasking of protein epitopes. Taking advantage of this peculiarity, we quantified myelin debris

and IRF5$^{-/-}$ EAE mice showed a significant increase in myelin debris in the lesions than WT mice (**Figure 35B**). Microglia/macrophage number was also quantified using Iba1 labelling and IRF5$^{-/-}$ mice showed a significant increase in Iba1$^+$ cells in damaged area versus WT EAE mice (**Figure 35C**). Finally, axonal damage was measured using SMI-32 immunolabelling and higher immunoreactivity was detected in IRF5$^{-/-}$ versus WT mice (**Figure 35D**). SMI-32 was evaluated in total WM because it is present also outside the lesions. These results suggest that IRF5 deletion is detrimental at EAE recovery phase.
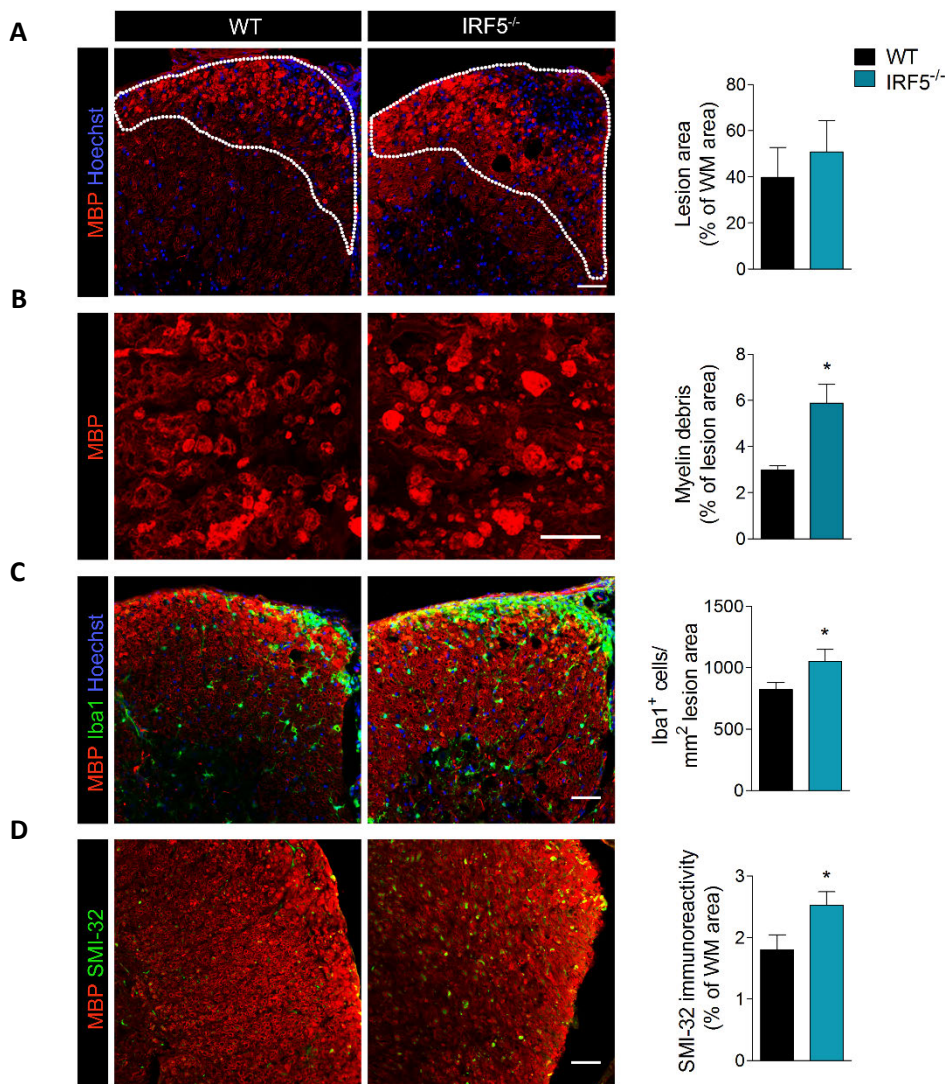
Figure 35. IRF5 deletion increases myelin and axonal damage in EAE.

Immunofluorescent MBP analysis of spinal cord sections to define lesion area (A) or MBP debris accumulation for myelin damage (B); with Iba1, for microglia/macrophages (C), and SMI-32 for axonal damage (D). In A and C nuclear Hoechst labelling in blue. White dash line in A indicates lesion border. *Right*, quantification of the lesion area (A), myelin debris (B), microglia/macrophages number (C) and axonal damage (D) in the spinal cord of wild type (WT) (n=7 (A, C, D); n=3 (B)) and IRF5$^{-/-}$ (n=6 (A, C, D); n=3 (B)) mice at EAE recovery phase. WM = white matter. Scale bar = 50 µm.

Data information: data are presented as mean ± s.e.m. Statistics were performed with Student' t-test. *P < 0.05.

12. Impaired phagocytosis in IRF5$^{-/-}$ mice after EAE induction

Phagocytosis of myelin debris by microglia is essential for an efficient regenerative response (*Kotter et al., 2006; Ruckh et al., 2012*). We observed a higher accumulation of disrupted or fragmented myelin after EAE induction in IRF5$^{-/-}$ mice (**Figure 35**). Because of that, we hypothesis that these features could be the consequence of a failure on microglia/macrophage phagocytosis. Phagocytosis was quantified at EAE recovery as the colocalization of Iba1 and MBP immunolabelling in lesion areas (see methods). IRF5$^{-/-}$ microglia/macrophages showed higher damaged myelin accumulation than WT microglia/macrophages (**Figure 36**). Moreover, myelin debris size was bigger and located preferentially in phagocytic processes in IRF5$^{-/-}$ mice. In contrast, WT mice showed more abundance of partially degraded myelin into the cytoplasm (**Figure 36**). These data suggest that IRF5 deficiency could impaired microglia/macrophages phagocytic capacity.
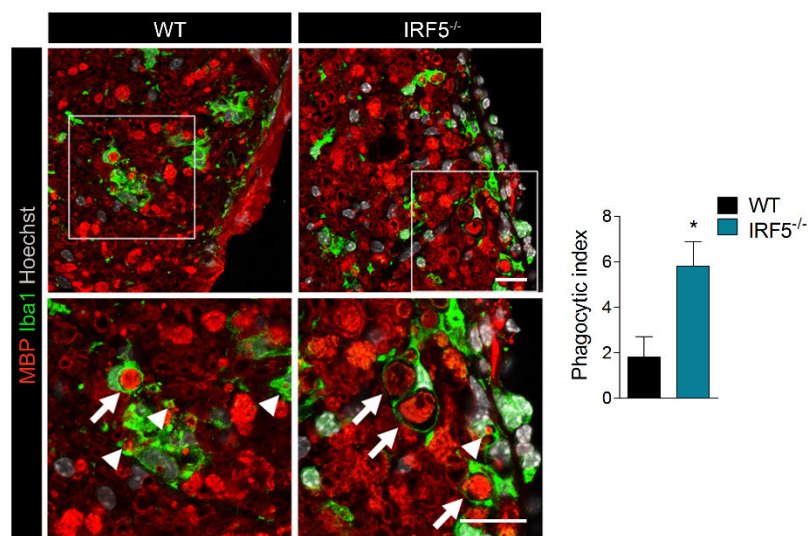
Figure 36. IRF5 deletion impairs myelin debris phagocytosis after experimental autoimmune encephalomyelitis (EAE) induction.

Spinal cord sections of wild type (WT) (n = 3) and IRF5$^{-/-}$ (n = 3) mice at EAE recovery phase immunolabelled for MBP (red) and Iba1 (green). Nuclear Hoechst labelling (gray) was also performed. Notice the higher presence of degraded myelin in cytoplasm (arrowheads) in WT mice and the abundance of bigger myelin debris located in phagocytic processes (arrows) in IRF5$^{-/-}$ mice (bottom images are the insets in top ones). Histogram shows the phagocytosis index calculated on the basis of MBP accumulation in Iba1$^+$ cells using *ImageJ* in WT and IRF5$^{-/-}$ mice. Scale bar = 20 μm.

Data information: data are presented as mean ± s.e.m. Statistic was performed with Student' t-test. *P < 0.05.

13. Decreased OPC recruitment in IRF5$^{-/-}$ mice after lysolecithin (LPC)-induced demyelination

In order to analyse whether IRF5 deficiency impairs remyelination, we used another MS animal model, LPC-induced demyelination model. In this model, demyelination occurs due to the toxic effect of LPC on myelin sheaths (*Jeffery and Blakemore, 1995*) and it is independent of the immune response, a characteristic that makes it a suitable model to analyse remyelination.

LPC was injected in the spinal cord of WT and IRF5$^{-/-}$ mice and remyelination was analysed by immunohistochemistry at 14 days after demyelination. At that stage OPCs have migrated to the lesion and they have differentiated into mature myelinating oligodendrocytes. We observed that LPC induced higher lesions in IRF5$^{-/-}$ mice than in WT mice, defined on the basis of MBP immunolabelling as absence (**Figure 37**). Next, we quantified total cell number from the oligodendrocyte linage (Olig2$^+$ cells) and the adult oligodendrocytes (CC1$^+$ cells) in the lesion area. IRF5$^{-/-}$ mice showed a decrease in the total number of Olig2$^+$ cells inside the lesion (**Figure 38A**). However, there was no difference in the percentage of Olig2$^+$ cells differentiated into mature oligodendrocytes CC1$^+$ in the lesions (**Figure 38B**). These results suggest that remyelination deficiency was due to a failure in recruitment more than a failure in differentiation.
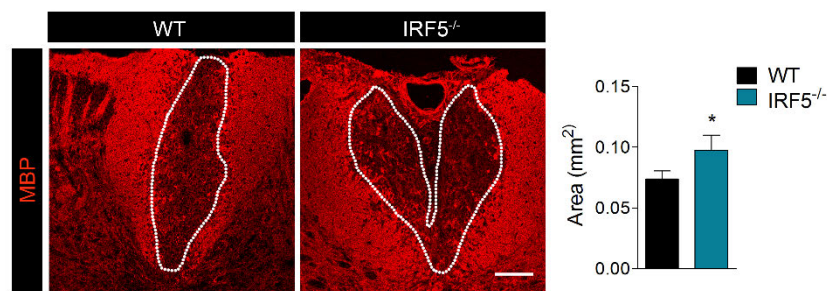
Figure 37. Increased damage in IRF5$^{-/-}$ mice after lysolecithin (LPC)-induced demyelination.

Spinal cord sections of wild type (WT) and IRF5$^{-/-}$ mice immunolabelled for MBP (to define the lesion area; red) 14 days after LPC injection. Histogram shows the quantification of the lesion area in the spinal cord of WT (n=5) and IRF5$^{-/-}$ (n=6) mice. White dash line indicates lesion border. Scale bar = 100 μm.

Data information: data are presented as mean ± s.e.m. Statistic was performed with Student' t-test. *P < 0.05.
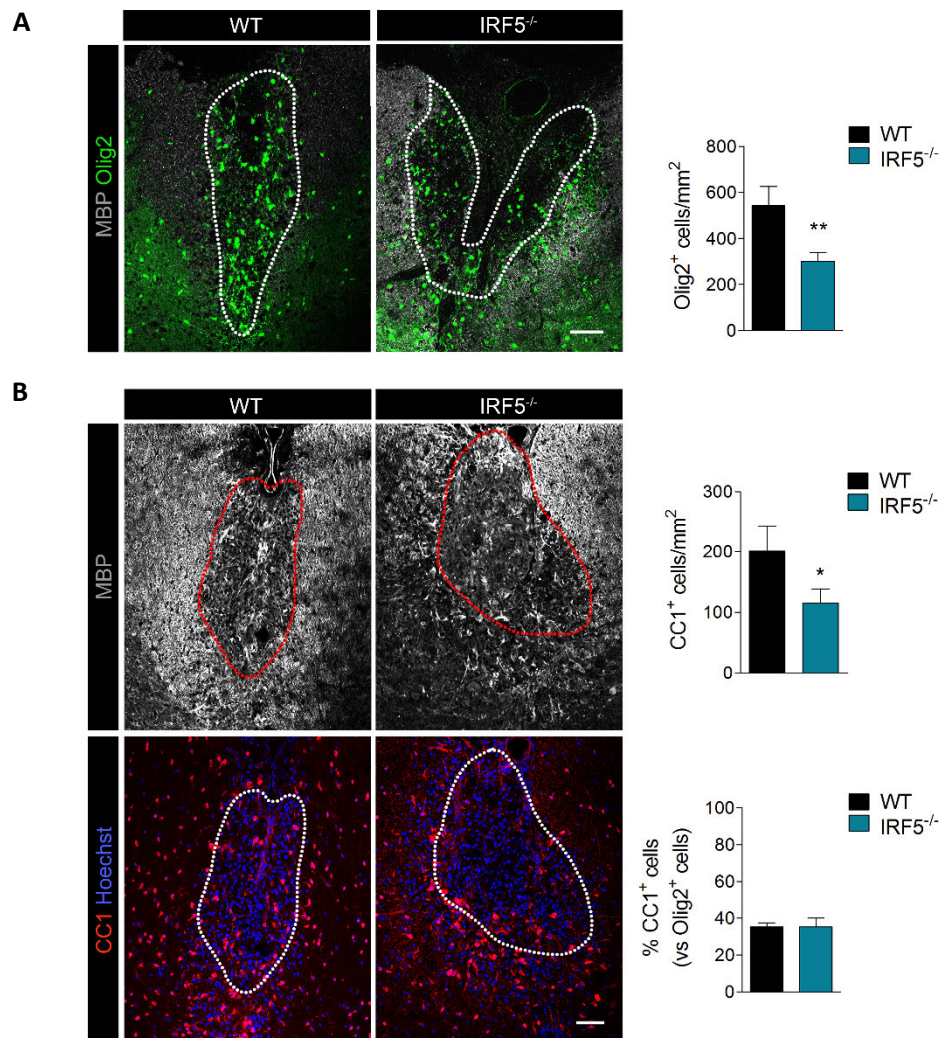
Figure 38. IRF5 deletion decreases OPC recruitment after lysolecithin (LPC)-induced demyelination.

A- Spinal cord sections of wild type (WT; n = 5) and IRF5$^{-/-}$ (n = 6) mice immunolabelled for Olig2 (oligodendrocyte linage marker; green) and MBP (to define the lesion area; grey) 14 days after LPC injection. Histogram show quantification of Olig2$^+$ cells in lesion area. White dash line indicates lesion border. Scale bar = 50 μm.

B- Spinal cord sections of WT (n = 5) and IRF5$^{-/-}$ (n = 6) mice immunolabelled for MBP (to define the lesion area; grey; *top*) and CC1 (for mature oligodendrocytes; red; *bottom*) 14 days after LPC injection. Nuclear Hoechst labelling was also performed (blue). Histograms show quantification of CC1$^+$ cells normalized to lesion area (*top*) and the percentage of CC1$^+$ cells versus Olig2$^+$ cells (*bottom*). Red dash line (*top*) and white dash line (*bottom*) indicates lesion border. Scale bar = 50 μm.

Data information: data are presented as mean ± s.e.m. Statistics were performed with Student' t-test. *P < 0.05, **P < 0.01.

14. Altered inflammatory response in IRF5$^{-/-}$ mice after LPC injection

Although demyelination in this model is immune response independent, immediately after LPC injection T cells, B cells and macrophages infiltrate in the lesion site and this short-lived infiltration is proposed to be beneficial to initiate remyelination (*Bieber et al., 2003*).

As detailed before, we observed that lesions were higher at 14 days in IRF5$^{-/-}$ mice than in WT mice (**Figure 37**). Moreover IRF5$^{-/-}$ mice showed significant inflammation of the meninges, (**Figure 39**), as detailed with laminin immunolabelling. No meningitis has been previously described in the literature in this animal model and accordingly, we did not detect any change in WT mice. To further analyse the role of IRF5 in the immune response after LPC injection, we quantified by immunohistochemistry T cells (CD3$^+$ cells). In WT mice few CD3$^+$ cells were detected mainly in the lesion site, but also outside the lesion (**Figure 40**). In contrast, IRF5$^{-/-}$ mice showed a higher infiltration of T cells in lesion site (**Figure 40**). All these data suggest that IRF5$^{-/-}$ mice have an exacerbated immune response. Whether this altered response is responsible of the delay in remyelination remains to be determined.
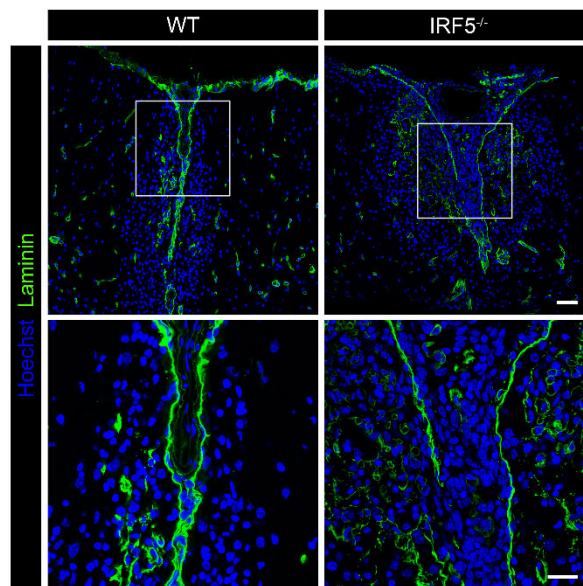
Figure 39. IRF5 deficiency alters inflammatory response after lysolecithin (LPC)-induced demyelination.
Top, spinal cord sections of wild type (WT; n = 5) and IRF5$^{-/-}$ (n = 6) mice immunolabelled for laminin (to meninges visualization; green) 14 days after LPC injection. Nuclear Hoechst labelling was also performed. *Bottom*, higher magnification of the area in the white box. Scale bar = 50 μm (*top*) and 25 μm (*bottom*).
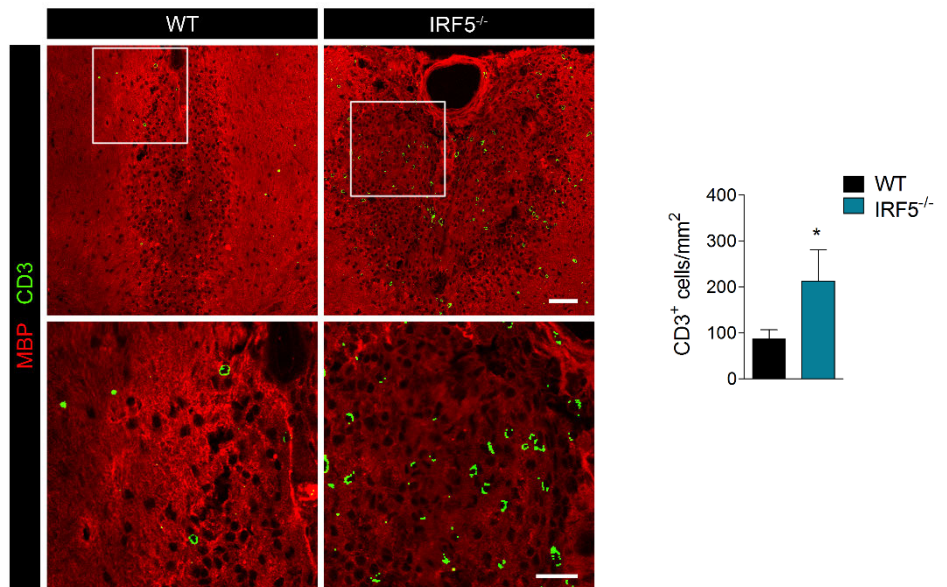
Figure 40. IRF5 deficiency alters T cells response after lysolecithin (LPC)-induced demyelination.

*Top, s*pinal cord sections of wild type (WT; n = 5) and IRF5$^{-/-}$ (n = 6) mice immunolabelled for MBP (red) and CD3 (T cell marker; green) 14 days after LPC injection. *Bottom*, higher magnification of the area in the white box. Scale bar = 50 µm (*top*) and 25 µm (*bottom*).

Data information: data are presented as mean ± s.e.m. Statistic was performed with Student' t-test. *P < 0.05.

DISCUSSION

Several pathological conditions of the CNS have in common the upregulation of the purinergic P2X4 receptor (*Tsuda et al., 2003; Wixey et al., 2009; Li et al., 2011; Beggs et al., 2012; Ulmann et al., 2013; Vazquez-Villoldo et al., 2014*). In brain injury it is described an upregulation of P2X4R that shifts microglia towards a P2X4R-expressing reactive state through an IRF8-IRF5 transcriptional axis (*Beggs et al., 2012*). In the present study we showed that IRF8-IRF5-P2X4R is upregulated at the peak and recovery phase of EAE. Moreover, we demonstrated that blockade of P2X4R exacerbates EAE whereas potentiation with IVM ameliorates this autoimmune experimental disease. Mechanistically, P2X4 receptor signalling potentiation in microglia/macrophage favours a switch to an anti-inflammatory phenotype that by secreting factors such as BDNF improves recovery. In parallel IRF5 deletion delay EAE onset but impairs EAE remyelination similar to EAE P2X4$^{-/-}$ mice.

1. P2X4 receptor controls microglia activation and favours remyelination in experimental autoimmune encephalomyelitis

1.1. Microglial *P2x4r* is upregulated in EAE mice

ATP is a multifunction signaling molecule copiously released into the extracellular space during activation, stress or cell damage. It is known that purinergic signaling in the CNS, mediated by ATP or molecules derived from its metabolism, participate in neurodegenerative and neuroinflammatory diseases such as MS (*Amadio et al., 2011*). Purinergic receptors are abundantly expressed in the CNS (*Tsuda et al., 2003; Ulmann et al., 2008*) and in microglia cells, purinergic receptors are involved in several functions as motility, cytokine release and phagocytosis (*Domercq et al., 2013*). Microglial P2X4R upregulation seems to be common in most acute and chronic neurodegenerative diseases associated with inflammation (reviewed in *Domercq et al., 2013*). P2X4R overexpression is described in microglial cells in EAE rats and in human MS optic nerve samples (*Vazquez- Villoldo et al., 2014*). These data are in accordance with the *P2x4r* upregulation observed in our EAE experiments.

1.2. Treatments specificity

In this work TNP-ATP treatment was able to exacerbate a clinical course of mice after EAE induction and in contrast IVM was able to improve the development of the disease at the recovery phase. TNP-ATP is a non-selective P2X antagonist, but there is no a selective and potent

antagonist of P2X4R with solubility in water. 5-BDBD works as a selective P2X4 receptor antagonist, but it displays a very low water-solubility, which limits its application using systemic injection. An exception is the new compound NP-1815-PX which is a potent and selective antagonist of P2X4R (*Matsumura et al., 2016*). However, the compound is not commercial. In order to corroborate the specificity of TNP-ATP and IVM treatments in our experiments, we tested them in P2X4^{-/-} mice after EAE induction. Neither TNP-ATP nor IVM induced any change in the neurological score in P2X4^{-/-} mice after EAE induction, thus corroborating that the effects observed are P2X4R-dependant and further supporting the specificity of the pharmacological tools used in the study.

1.3. P2X4R modulation affects mainly microglia cells

In brain, P2X4Rs are highly enriched in microglia compared to oligodendrocytes and other CNS cells (gene expression data from *Zhang et al., 2014*). However, P2X4R is also present in neurons and in healthy conditions the receptor activation contributes to synaptic strengthening in the CA1 area of the hippocampus (*Sim et al., 2006*). In inflammatory pain it has been recently shown that P2X4R expressed by sensory neurons controls neuronal BDNF release that contributes to hyper-excitability during chronic inflammatory pain (*Lalisse et al., 2018*). We can not exclude effects in neuronal P2X4R, but our *in vitro* experiments support the role of microglial P2X4R in the results obtained in our experimental model. In addition, our immunocytochemical and pharmacological characterization of P2X4R showed selective microglia expression versus oligodendrocytes. Accordingly, the lack of P2X4R-mediated inward currents in electrophysiological recordings in astrocytes and oligodendrocytes corroborate that microglia are the main P2X4R expressing cells in the CNS (*Lalo et al., 2008; Zabala et al., 2018*). Interestingly, although oligodendrocytes lack P2X4 receptor, a recent study has described upregulation of P2X4R in Schwann cells following nerve crush injury. Moreover, overexpression of P2X4R in Schwann cells by genetic manipulation promoted recovery and remyelination via BDNF release after nerve injury (*Su et al., 2018*). These results are in accordance with the beneficial role described for P2X4R in this work.

1.4. P2X4R modulation does not affect immune priming

P2X4R could also be expressed in peripheral T cells and could modulate immune response. In fact, several studies have shown the involvement of P2X4R in T cells activation. Pannexin-1 hemichannels and P2X1 and P2X4 receptors facilitate ATP release and autocrine

feedback mechanisms that control T-cell activation at the immune synapse between T cell and APCs (*Woehrle et al., 2010; Manohar et al., 2012*). T cells also require ATP release and purinergic P2X4R signaling for their migration to APCs (*Ledderose et al., 2018*). On the other hand, APCs, including CNS microglia and perivascular macrophages, play pivotal roles in initiating Th17-cell development and transmigration through the BBB leading to EAE (*Bartholomäus et al., 2009; Goldman et al., 2013; Xiao et al., 2013; Yoshida et al., 2014*). However, our results did not substantiate a direct (T-cell mediated) or indirect (APC-dependent) role of P2X4R for the development of T cell response and recruitment to the CNS (*Zabala et al., 2018*). Altogether we proposed that P2X4R blockage or potentiation does not interfere with the efficacy of immunization and the cellular immune response against MOG.

1.5. P2X4R potentiation switch microglia to an anti-inflammatory phenotype and ameliorates EAE symptoms at the recovery phase

The role of inflammation in promoting neural repair is gaining increasing recognition. Products of macrophages as well as of microglia, their CNS counterparts, facilitate the regeneration of axons (*David et al., 1990; Yin et al., 2006*) and promote remyelination in animal models of demyelination as their deficiency retards the process of remyelination (*Kotter et al., 2005; Kondo et al., 2011; Miron et al., 2013; Sun et al., 2017; Cantuti-Castelvetri et al., 2018*). However, the innate immune system capacity to restore myelination in the context of MS depends on microglia/macrophage polarization state. Thus, pro-inflammatory microglia/macrophage deactivation suppress EAE acute phase (*Starossom et al., 2012*), whereas microglia/macrophage polarization to an anti-inflammatory phenotype is essential for efficient remyelination later on (*Butovsky et al., 2006; Miron et al., 2013; Sun et al., 2017*). Thus, a switch from a pro-inflammatory to an anti-inflammatory dominant polarization of microglia/macrophage is critical in the repair process and therefore manipulating polarization phenotypes of microglia/macrophage might be a promising therapeutic strategy for treating MS. We here demonstrate that blocking P2X4R or its deficiency exacerbated a switch to a pro-inflammatory phenotype and increased neurological deterioration in the recovery phase whereas its potentiation with IVM increased anti-inflammatory polarization and ameliorate clinical signs. A role of P2X4R in recovery is also supported by the beneficial/detrimental effect of P2X4R manipulation in remyelination in LPC-treated slices, a model lacking adaptive immune activation. Nowadays the DMTs available to treat MS target the adaptive immune cells activation, being efficient reducing relapses, but they do not stop disease progression to a

chronic phase (*Haghikia et al., 2013; Feinstein et al., 2015*). In this sense, it is relevant the fact that P2X4R potentiation could be a therapeutic approach to the treatment of the chronic phases of MS.

Emerging evidence indicate that the activation states of microglia/macrophages, is a continuum with pro-inflammatory and anti-inflammatory phenotypes on either end of this spectrum. This idea is in line with studies describing that macrophages and microglia showed an intermediate activation state in MS (*Ponomarev et al., 2007; Vogel et al, 2013*) and that different populations of myeloid cells exists along the different phases in EAE mice (*Ajami et al., 2018; Locatelli et al., 2018*). Moreover, the movement of microglia/macrophage population along this spectrum appears to be unidirectional. At early stages pro-inflammatory microglia/macrophages are mainly detected in lesions, which over time are replaced by intermediate-activated ones and ultimately by anti-inflammatory microglia/macrophages (*Locatelli et al., 2018*). We detected an upregulation of both pro-inflammatory and anti-inflammatory markers at EAE peak and chronic phase, a finding consistent with the existence of a variable spectrum of microglia/macrophage phenotypes depending on the environmental signals inside or around the lesions.

Resident microglia and monocytes contribute differentially to EAE induction (*Ajami et al., 2011; Yamasaki et al., 2014*) whereas few studies have addressed their specific contribution to remyelination (*Lampron et al., 2015*). We could not discriminate between the role of macrophages which express high levels of P2X4R and microglia. Although both cell types show different properties in EAE induction (*Ajami et al., 2011; Yamasaki et al., 2014*), both type of cells are similarly polarized and participate in remyelination in MS model (*Miron et al., 2013*). Further experiments deleting specifically P2X4R in microglia and/or macrophages are necessary to define the role played by P2X4R in the two cell populations.

1.6. P2X4R potentiation enhances oligodendrocyte differentiation

Previous data on literature demonstrated that OPC differentiation and myelination in the CNS are controlled by highly regulated sequences of molecular interactions with neurotransmitters released by axons, growth factors, neuregulins, integrins and cell adhesion molecules. Among all, it is well known that BDNF enhances oligodendrocyte differentiation and myelination (*Wong et al., 2013*). A main source of BDNF promoting oligodendrogenesis after white matter ischemic insults are astrocytes (*Miyamoto et al., 2015*). However, microglia are

also another important source of BDNF in physiological conditions and after injury (*Dougherty et al., 2000; Parkhurst et al., 2013*). Microglial P2X4R activation has been linked to BDNF release, causing tactile allodynia (*Ferrini et al., 2013*) and accordingly, we detected increased BDNF production after IVM treatment on microglia cells, an effect that was not detected in P2X4$^{-/-}$ microglia. We showed here that all microglia conditioned medium induced oligodendrocyte differentiation, but the differentiation was significantly higher with anti-inflammatory microglia conditioned medium. After analysing BDNF production, an increased production was detected in anti-inflammatory microglia and this effect was significantly reduced by TNP-ATP treatment. In addition, *Mbp* levels correlated strongly with *Bdnf* levels at EAE peak and recovery and were dramatically reduced after TNP-ATP treatment. However, these data are only correlative, so we not exclude the role of other factors secreted by microglia in oligodendrocyte differentiation.

1.7. P2X4R potentiation with IVM does not enhance allodynia in EAE

It is well established that P2X4 receptors are involved in neuropathic pain after peripheral nerve injury and P2X4 receptor blockers have been proposed as potential therapeutic drugs to treat neuropathic pain (*Tsuda et al., 2003; Trang et al., 2009, Beggs et al., 2012; Matsumura et al., 2016*). Chronic pain is a common symptom associated with demyelinating autoimmune diseases of the CNS, such as MS. In MS, chronic neuropathic pain is one of the most frequent symptoms that dramatically reduces the quality of life of MS patients (*Ferraro et al., 2018*). It has been reported that approximately 50-80% of patients with MS feel pain (*Osterberg et al., 2005*), but the underlying pathological mechanism are still poorly understood. The possible involvement of P2X4 receptor in neuropathic pain is a relevant point to consider before performing further research and investment in IVM as a potential drug for MS. In EAE models of MS, pro-nociceptive behaviours may be evoked reliably in the lower extremities, tail and hindpaws, effectively recapitulating the predominantly lower extremity pain reported by patients with MS (*Svendsen et al. 2005b; Olechowski et al., 2013; Serizawa et al., 2018*). Hence, EAE mice are used for investigating the pathophysiology of MS-associated neuropathic pain. In accordance, our results indicate that mechanical sensitivity was increased during EAE, but P2X4R blockage or potentiation did not alter this sensitivity, indicating that we were not modifying MS-associated pain mechanisms.

2. Dual role of IRF5 during EAE

2.1. *Irf5* is upregulated in EAE mice

IRF5 drives de novo expression of P2X4R by directly binding to the promoter region (*Masuda et al., 2014*). Genome-wide SNP analysis identified IRF8 as a susceptibility factor for multiple sclerosis (*De Jager et al., 2009*). In addition, genetic polymorphisms in human IRF5 that lead to the expression of various unique isoforms or higher expression of *Irf5* mRNA have been linked to immune-mediated diseases, including MS (*Kristjansdottir et al., 2008*). IRF5 and IRF8 play a key role in the induction of pro-inflammatory cytokines, contributing to the polarization of macrophages to a pro-inflammatory phenotype and initiation of a potent Th1-Th17 response that boost EAE disease progression (*Krausgruber et al., 2011; Yoshida et al., 2014*). Here we showed that *Irf5*, *Irf8* and *P2x4r* mRNA expression are increased and are correlated at the peak as well as in the recovery phase of the EAE. IRF5 was also detect at protein level in lesion areas of EAE mice at the recovery phase. In accordance, in vitro polarization of microglia towards a pro-inflammatory phenotype, not to an anti-inflammatory, upregulate P2X4R expression and function (*Zabala et al., 2018*). However, the risk factor for MS of IRF5 and IRF8 contrast with the protective role described here for P2X4R. Thus, although this receptor is activated during pro-inflammatory polarization, it is conceivable that P2X4R overexpression may help to resolve or counterbalance the inflammatory reaction by priming a subsequent anti-inflammatory response. Indeed, the presence of pro-inflammatory macrophages is a prerequisite for the successive emergence of anti-inflammatory macrophages and tissue homeostasis during wound healing and *Listeria monocytogenes* infections (*Chazaud, 2014; Bleriot et al., 2015*).

2.2. IRF5 deficiency is beneficial at initial phases of EAE, delaying the onset of the disease

Previous data have demonstrated that IRF8-IRF5 transcriptional axis is a critical regulator for shifting microglia towards a P2X4R[+]-reactive phenotype (*Masuda et al., 2014*). On the other hand, IRF8^{−/−} mice are resistant to EAE, since the T cell response is not generated in these mice upon MOG immunization. This data is in accordance with the studies that described IRF8 as MS risk factor (*De Jager et al., 2009; Disanto et al., 2012*). As both transcription factors, IRF8 and IRF5, are implicated in the same transcriptional axis, in this work we analyzed the role of IRF5 in the EAE model. First we detected a significant delay in the appearance of neurologic symptoms in IRF5^{-/-} mice after EAE induction. Accordingly, IRF5 deficiency has beneficial effects in several autoimmune diseases such as systemic sclerosis (*Saigusa et al., 2015*) and systemic lupus

erythematosus (*Ban et al., 2016*). IRF5 is a transcription factor involved in innate immune response, although it also play a role in B cell response (*Ban et al., 2018*). The beneficial effects of IRF5 deficiency described previously are related to the modulation of the innate immune system response. The delay observed in the onset of symptoms after EAE induction in IRF5$^{-/-}$ mice indicate that immune priming in the periphery or the infiltration of T cells into the spinal cord is postponed. However, we could not discard the hypothesis that IRF5 depletion in microglia/macrophages leads to the delay in T cell response at EAE onset. Previous data on literature have demonstrated that the specific depletion of transforming growth factor (TGF)-b-activated kinase 1 (TAK1) in microglia cells suppresses EAE development (*Goldmann et al., 2013*). They demonstrated that microglia at early stages of disease provide a permissive cytokine microenvironment that potentiates the immune response locally. Thus, IRF5 deficient microglia could be involved in the onset delay of EAE and more experiments are needed to corroborate this hypothesis.

2.3. Absence of recovery in IRF5$^{-/-}$ mice at chronic EAE

Individual neurological scores indicated that IRF5$^{-/-}$ mice stayed longer at maximal score before the initiation of recovery and that the recovery was smaller. Because IRF5 is present only in immune cells, not in oligodendroctytes, we hypothesized that this failure could be due to a failure in microglia/macrophage function. Indeed, we did not detect any alteration in T cell infiltration or response at EAE chronic phase. The benefits of microglia/macrophage are also attributed to being required in clearing myelin debris after a demyelinating episode (*Kotter et al., 2006; Neuman et al., 2009; Lampron et al., 2015; Cantuti-Castelvetri et al., 2018*). Indeed, P2X4R blockage impairs myelin endocytosis and degradation in microglia cells and P2X4R deficient mice show higher accumulation of myelin debris after EAE induction (*Zabala et al., 2018*). In accordance with these data, IRF5$^{-/-}$ mice showed a higher accumulation of myelin in microglia/macrophage at chronic EAE. Whether this accumulation represents deficient or excessive phagocytosis is currently under investigation. *In vitro* preliminary data in our laboratory indicate that IRF5 deficient microglia exhibit impaired myelin degradation but no change in myelin endocytosis (data not shown). Further experiments will be needed to clarify this point and the mechanism involved. Together, our results indicate that IRF5 is necessary for the correct myelin clearance and degradation after EAE induction.

P2X4R blockade or potentiation modulates the effect of polarization on phagocytosis (*Zabala et al., 2018*). However, the opposite interpretation is also possible. Thus, phagocytosis

of myelin controls microglia/macrophage inflammatory response (*Kroner et al, 2014*). Phagocytosis of myelin in vitro promotes anti-inflammatory polarization (*Boven et al., 2006; Liu et al., 2006*). However, this shift could be altered in pathological conditions. After spinal cord injury, microglia retain a predominantly pro-inflammatory state, a fact detrimental for recovery (*Kroner et al, 2014*). Two of the factors that prevents phagocytosis-mediated conversion from pro-inflammatory to anti-inflammatory microglia are TNF alpha and iron accumulation into microglia/macrophages. Other factor determinant in this shift could be the age of the cells. Thus, it has been recently described that phagocytosis of myelin in aged microglia/macrophages after demyelination results in cholesterol accumulation in these cells, leading to a maladaptive inflammatory response with inflammasome activation and impaired remyelination (*Cantuti-Castelvetri et al., 2018*). Relative to this idea, IRF5$^{-/-}$ mice showed a modest but significant upregulation of some pro-inflammatory markers at EAE chronic phase. These data are surprising since IRF5 is involved in microglia/macrophage activation and regulates pro-inflammatory gene expression (*Krausgruber et al., 2011*). Whether this upregulation is the secondary consequence of a deficient phagocytosis and not the direct consequence of the transcriptional activity is an hypothesis currently under investigation.

3. Role of IRF5 in LPC-demyelination model

Studies in toxin-induced demyelination models showed that myelin debris generated by demyelination and containing inhibitors of OPC differentiation must be eliminated for an efficient remyelination process. Microglia/macrophages are the responsible of phagocytosing myelin debris, a necessary process to initiate the reparative response. Apart from their phagocytic function, they release several factors into the injured CNS that influence oligodendrocyte recruitment and differentiation (*Kotter et al., 2005; Döring et al., 2015; Lampron et al., 2015*). Our results after LPC-induced demyelination indicate that IRF5 deficiency inhibits OPC recruitment to lesion site delaying the reparative process and this could explain the bigger lesion areas observed in IRF5$^{-/-}$ mice. Further studies are needed to conclude whether this impaired recruitment is due to a delay in myelin debris phagocytosis, an effect that would be in accordance with the results observed in EAE model.

The demyelination after LPC injection is independent of the immune response since it is not alter in immune-deficient mice (*Bieber et al., 2003*). However, immediately following the LPC injection, lesion sites are often infiltrated with T cells, B cells and macrophages. This immune

response is proposed to have a beneficial role in CNS repair (*Bieber et al., 2003; Nielsen et al., 2011; Dombrowski et al., 2017*). Our results indicate that the immune response in IRF5^{-/-} mice after LPC-demyelination is exacerbated as there is higher infiltration of CD3^{+} T cells and severe inflammation of the meninges. This altered response has probably detrimental consequences as lesions in IRF5^{-/-} mice are higher. Although toxin-induced demyelination models are mainly independent of the immune response, previous evidence suggest that under pathological or altered situations demyelination in these models could induce a secondary immune activation. For example, subtle myelinopathy induced by abbreviated cuprizone treatment coupled with subsequent immune activation can initiate inflammatory demyelination similar to MS lesions (*Caprarielo et al., 2018*). Thus, the myelin injury drives the immune activation. Biochemical alteration of myelin, in particular, deimination of myelin basic protein (a major myelin constituent that influences its compaction), exposes an immunodominant epitope (*Musse et al., 2006*). The extent of modified myelin biochemistry correlates with the severity of MS lesion subsets (*Moscarello et al., 1994; Wood et al., 1996; Bradford et al., 2014*). Thus, LPC-induced damage to myelin together with problems in myelin clearance could lead to longer exposion of antigenic myelin epitopes initiating an altered immune response in IRF5^{-/-} mice. The contribution of the immune cells to higher lesion area or to the fail in OPC recruitment must be analyzed in further experiments.

CONCLUSIONS

The conclusions of this work are as follows (see also **Figure 41**):

1. Expression of *P2x4r* and the transcription factors controlling its expression, *Irf8* and *Irf5*, was increased in EAE.

2. EAE clinical signs were exacerbated by P2X4R blockage and ameliorated by P2X4R potentiation with the allosteric modulator ivermectin.

3. P2X4R blockage or potentiation does not interfere with the immune priming after EAE immunization.

4. P2X4R activation favors a switch of microglia/macrophage to an anti-inflammatory phenotype and increases BDNF release, that promotes oligodendrocyte differentiation.

5. IRF5 deletion delays EAE onset but exacerbate clinical signs at the recovery phase.

6. IRF5$^{-/-}$ mice showed an accumulation of myelin debris and a deficient microglia/macrophage phagocytosis.

7. IRF5$^{-/-}$ mice showed a deficient recruitment of oligodendrocyte progenitors and an exacerbation of the immune reaction after toxin-induced demyelination.
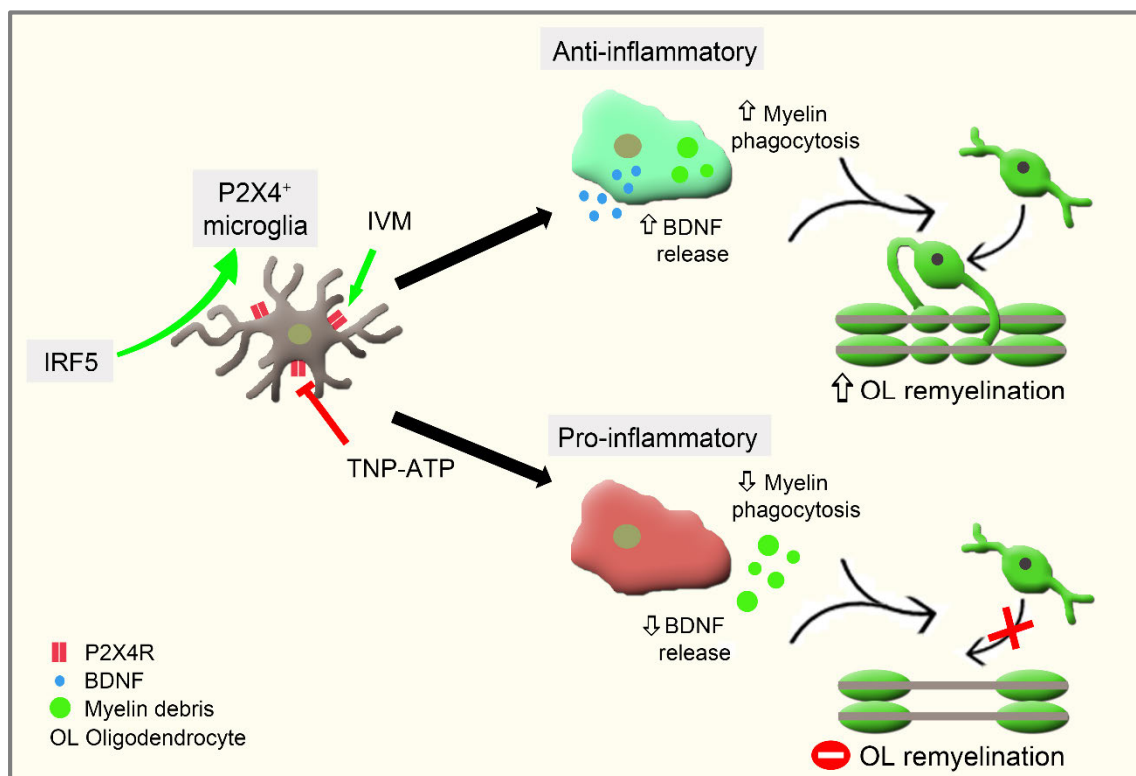

Figure 41. Scheme showing how modulating IRF5 expression and microglia/macrophage P2X4R activation determines clinical outcome in the experimental autoimmune encephalomyelitis (EAE) model of MS (modified from *Zabala et al., 2018*).

Manipulating the innate immune system to promote repair might be a promising therapeutic strategy for treating MS. Altogether data here suggest that IRF5-P2X4R axis upregulation could be a marker of neuroinflammatory response in MS and that P2X4R is a key modulator of microglia/macrophage polarization. The results obtained in this work support the use of IVM to potentiate a microglia/macrophage switch to a reparative phenotype that favours remyelination in MS. It is important to note that anti-helminthic host responses are based on anti-inflammatory macrophage polarization (*Satoh et al., 2010*) and thus, it is conceivable that the mechanism described here could be added to the IVM therapeutic effects against helminths. The fact that IVM is already in use as an anti-parasitic agent in humans will facilitate challenging this drug in clinical trials in demyelinating diseases.

IRF5 transcriptional factor is implicated in many autoimmune diseases, including MS. The results obtained in this work point to a complex role of IRF5 that evolves during EAE development from detrimental to beneficial, a finding in correlation with the complex and dual role of microglia/macrophages in the pathology. Different mechanisms and cell types could be involved in this dual effect. More studies are planned to clarify i) the role of IRF in immune priming and ii) the potential of boosting its activity to promote remyelination.

In conclusion, the results obtained in this thesis have made a great advance in understanding the role of P2X4 receptor and IRF5 transcription factor in MS pathophysiology. Because P2X4 receptor and IRF5 modulate macrophage/microglia response, we have also made important advances in understanding the role of microglia/macrophage in the recovery phase of EAE. Understanding chronic progression of MS, more than autoimmune-driven relapses, and understanding the disease pathways that allow recovery in the animal models is certainly relevant to design alternative interventions to treat specifically primary and secondary progressive MS.

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