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Interferon-β regulates the production of IL-10 by toll-like receptor-activated microglia

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Abstract

Pattern recognition receptors, such as toll-like receptors (TLRs), perceive tissue alterations and initiate local innate immune responses. Microglia, the resident macrophages of the brain, encode TLRs which primary role is to protect the tissue integrity. However, deregulated activation of TLRs in microglia may lead to chronic neurodegeneration. This double role of microglial responses is often reported in immune-driven neurologic diseases, as in multiple sclerosis (MS). Consequently, strategies to manipulate microglia inflammatory responses may help to ameliorate disease progression. In this context, the anti-inflammatory cytokine interleukin (IL)-10 appears as an attractive target. In this study, we investigated how activation of microglia by TLRs with distinct roles in MS impacts on IL-10 production. We found that activation of TLR2, TLR4, and TLR9 induced the production of IL-10 to a greater extent than activation of TLR3. This was surprising as both TLR3 and IL-10 play protective roles in animal models of MS. Interestingly, combination of TLR3 triggering with the other TLRs, enhanced IL-10 through the modulation of its transcription, via interferon (IFN)-β, but independently of IL-27. Thus, in addition to the modulation of inflammatory responses of the periphery described for the axis TLR3/IFN-β, we now report a direct modulation of microglial responses. We further show that the presence of IFN-γ in the microenvironment abrogated the modulation of IL-10 by TLR3, whereas that of IL-17 had no effect. Considering the therapeutic application of IFN-β in MS, our study bears important implications for the understanding of the cytokine network regulating microglia responses in this setting.

KEYWORDS
anti-inflammation, cytokines, innate immunity, multiple sclerosis, neurodegeneration

Abbreviations: CNS, central nervous system; DAMP, damage-associated molecular patterns; DMEM, Dulbecco's modified Eagle medium; EAE, experimental autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GSK-3, glycogen synthase kinase; HEPES, 4-(2-hydroethyl)-1-piperazineethanesulfonic acid; HPRT, hypoxanthine-guanine phosphoribosyl transferase; IFN, interferon; IFNAR, interferon alfa/beta receptor; IL, interleukin; IRF, IFN regulatory factor; LCCM, L929-cell conditioned medium; LPS, lipopolysaccharide; MS, multiple sclerosis; MyD88, myeloid differentiation primary response gene 88; P, postnatal; PAMP, pathogen-associated molecular patterns; PRR, pattern-recognition receptors; rt-PCR, real-time PCR; Th, T helper cells; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

1 | INTRODUCTION

The contribution of myeloid cells to the central nervous system (CNS) function is increasingly appreciated, particularly during inflammation (Gertig & Hanisch, 2014). Neuroinflammation initiated by astroglial cells plays cardinal roles in the CNS, including protective responses against infection and restorative responses to CNS injury. However, the same components that participate in host protective responses are observed in neuroinflammatory disorders (Sochocka, Diniz, & Leszek, 2016).

Indeed, pathologic neuroinflammation is reported in many CNS diseases, including multiple sclerosis (MS), Alzheimer's disease, Parkinson's disease, stroke, and traumatic brain injury (Czirr & Wyss-Coray, 2012; Shastri, Bonifati, & Kishore, 2013). The critical balance between neuroprotection and neurodegeneration is largely dependent on microglia immune responses (Ransohoff & Brown, 2012).

Microglia, the resident macrophages of the CNS (Hanisch & Kettenmann, 2007), play an important role in patrolling the parenchymal tissue, contributing to the maintenance of neuronal homeostasis and the initiation of innate immune responses in the brain (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011; Perry & Teeling, 2013; Ransohoff & Brown, 2012). In line with their myeloid origin, microglia express numerous pattern recognition receptors (PRRs), among which toll-like receptors (TLRs) (Eggen, Raj, Hanisch, & Boddeke, 2013; Shastri et al., 2013), involved in the recognition of pathogen- or damage-associated molecular patterns (PAMPs and DAMPs, respectively). Upregulation and activation of several TLRs in microglia has been described as a protective mechanism during infection by viruses, bacteria and parasites (Sochocka et al., 2016). In the context of neurodegeneration, the initial activation of microglia (and other glial cells) by DAMPs plays a pivotal role in repairing injury and maintaining the CNS homeostasis, but with the increase in neuronal death, this very same activation further potentiates cell death, thus enhancing neurodegeneration (Hanke & Kielian, 2011; Hayward & Lee, 2014; Kigerl, de Rivero Vaccari, Dietrich, Popovich, & Keane, 2014; Shastri et al., 2013; Su, Bai, Zhou, & Zhang, 2016). Consequently, it is conceived that regulating the activation of microglia, for example, through TLRs, may allow the regulation of several neurological diseases (Pedras-Vasconcelos, Puig, & Verthelyi, 2009).

TLR responses are mediated by the intracellular adaptor molecules myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) (Kawai & Akira, 2010; Pandey, Kawai, & Akira, 2014). Most TLRs signal uniquely via MyD88, the exceptions being TLR4, which signals via both MyD88 and TRIF, and TLR3, which signals uniquely through TRIF (Kawai & Akira, 2010). Both MyD88 and TRIF initiate a series of intracellular signaling cascades that culminate with the production of several immune mediators, as cytokines, chemokines, reactive oxygen species, and nitric oxide (Kawai & Akira, 2010). MyD88-dependent signals efficiently activate the NF-kB family of transcription factors, leading to the production of many cytokines, as tumor necrosis factor (TNF), interleukin (IL)-6, IL-1β, and also IL-10 (Pandey et al., 2014). TRIF-mediated signals induce interferon regulatory factor (IRF)-3, leading to the production of type I interferons (IFNs) and other IFN-responsive factors (Pandey et al., 2014). Of all these cytokines, two have been associated with protection during neurodegeneration: type I IFNs and IL-10.

Type I IFNs comprise a family of cytokines with prominent roles during viral resistance (McNab, Mayer-Barber, Sher, Wack, & O'Garra, 2015). In the context of CNS infections, protective type I IFN responses are also observed, for example, during intracerebral infection with lymphatic choriomeningitis virus (Merigan, Oldstone, & Welsh, 1977; Nayak et al., 2013) or with La Cross virus or coronavirus mouse

hepatitis virus, where microglial cells were identified as type I IFN sources (Kallfass et al., 2012; Roth-Cross, Bender, & Weiss, 2008). A role for microglial type I IFN signaling was also described during the resolution of sterile injury (Khorooshi & Owens, 2010). Furthermore, the induction of type I IFNs is critical for limiting experimental autoimmune encephalomyelitis (EAE) as mice deficient for type I IFN receptor (IFNAR) suffer from a higher disease course, increased macrophage, T-cell and B-cell infiltration, and greater demyelination (Prinz et al., 2008). Importantly, administration of IFN-β is the first-line drug for the treatment of MS (Inoue & Shinohara, 2013; Marrie & Rudick, 2006). Despite all these protective effects, chronically increased levels of IFN are linked to various diseases, including encephalitis or other type I interferonopathies (Goldmann, Blank, & Prinz, 2016).

IL-10 is a powerful anti-inflammatory cytokine produced by most immune cells that plays the important role of preventing exacerbated immune responses and subsequent tissue immunopathology (O'Garra & Vieira, 2007). The production of IL-10 by TLR-activated microglia is well documented (Butchi, Du, & Peterson, 2010; Jack et al., 2005; Ledeboer et al., 2002; Mizuno, Sawada, Marunouchi, & Suzumura, 1994; Olson & Miller, 2004). The potential of IL-10 in controlling inflammatory responses in the brain has gained recent importance (Kwilasz, Grace, Serbedzija, Maier, & Watkins, 2015). IL-10 has been tested as a therapeutic approach to a series of neurologic pathologies, where excessive and persistent neuroinflammation acts as a driver of neurodegeneration. Examples of this include the administration of IL-10 in animal models of brain ischemia (Perez-de Puig et al., 2013), Parkinson's disease (Joniec-Maciejak et al., 2014), and MS (Cua, Hutchins, LaFace, Stohlman, & Coffman, 2001). There are, however situations, such as in Alzheimer's disease, where a detrimental role for IL-10 has been shown (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015). In either case, modulating IL-10 in the CNS appears as an attractive approach to rewire otherwise detrimental immune responses. This calls for a deep understanding on the molecular mechanisms regulating IL-10 production in the brain, something that remains poorly studied (Lobo-Silva, Carriche, Castro, Roque, & Saraiva, 2016).

Owing to the potential of IL-10 in restoring the immune balance in the brain, targeting the molecular mechanisms that regulate IL-10 production by brain resident cells is an interesting area that deserves attention. In this study, we investigated the induction of IL-10 upon TLR activation in microglia and some of the operating mechanisms that can enhance it. We also probed how microenvironmental cues further modulate IL-10.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All animal experiments were performed in strict accordance with the recommendations of the European Union Directive 2010/63/EU. Animals were kept and bred with water and food *ad libitum*, according with the Portuguese National Authority for Animal Experimentation guidelines. Newborn mice were humanely euthanized by decapitation and every effort was made to minimize suffering.

2.2 | Mice

Adult wild-type, IL-10-deficient, and type I interferon receptor (IFNAR)-deficient mice, all in C57BL/6 background, were kept and bred at ICVS. The IFNAR deficient mice were a kind gift of Dr Anne O'Garra (Francis Crick Institute, London, UK). Postnatal day 0 (P0)–P3 mice were used to perform primary microglia cell cultures. Adult mice (8–12 weeks) were used to generate bone marrow-derived macrophages.

2.3 | Media and stimuli

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), HEPES solution, sodium piruvate, L-glutamine, and penicillin–streptomycin were purchased from Invitrogen. Cells were cultured in DMEM supplemented with 10% FBS, 1% HEPES solution, 1% sodium piruvate, 1% L-glutamine, and 1% penicillin–streptomycin (cDMEM). Pam3CSK4 (TLR2/TLR1 ligand), polyI:C (TLR3 ligand), and CpG (TLR9 ligand) were obtained from Invivogen. Lipopolysaccharide (LPS) from *Escherichia coli* (TLR4 ligand) was bought from Sigma. Mouse recombinant IFN-β, IL-27, IFN-γ, and IL-17 were bought from R&D systems. Neutralizing IFN-γ antibody (clone XMG1.2) was a kind gift of Dr Rui Appelberg (IBMC, Porto). All media was prepared using endotoxin-free plastics and all stimuli were suspended in endotoxin-free media or water.

2.4 | Primary microglial cell cultures

Primary microglia cultures were established from P0-P3 C57BL/6 mice brains. Brains were aseptically removed, washed with cDMEM to remove blood leftovers, and homogenized using a 40 μm cell strainer. 8.5×10^6 cells were plated in cDMEM onto 75 cm^2 flasks and cultured for 2 weeks in a humidified atmosphere at 37°C with 5% CO_2. The media was changed on days 3, 6, 9, and 12. On day 14, flasks were shaken for 4 h at 240 rpm to detach microglial cells from the mixed cultures. On day 14, around 90% of the collected cells were CD11b+, as detected by flow cytometry. Cells were collected and 1×10^5 cells plated in 100 μL of cDMEM per well in 96-well plates for further stimulation. At different times poststimulation, the cells were harvested for RNA analysis and the supernatants for cytokine detection.

2.5 | Generation of primary bone marrow-derived macrophages

Bone marrow-derived macrophages were differentiated from bone marrow precursors cultured in cDMEM, supplemented with 20% L929-cell conditioned media (LCCM), as previously described (Teixeira-Coelho et al., 2014). Briefly, total bone marrow cells were cultured in microbiological Petri dishes (Sterilin) and kept at 37°C and 5% CO_2. Cells were fed on day 4 with equal volume of cDMEM containing 20% LCCM. On day 7, macrophages were harvested, counted and seeded into 24-well tissue culture plates at 5×10^5 cells in 500 μL per well in culture medium. Cells were stimulated as indicated below and the culture supernatants harvested 24 hr later for IL-10 detection.

2.6 | Cell stimulation

Purified microglial cells recovered on day 14 or bone marrow-derived macrophages recovered on day 7 were stimulated for different time points as appropriate. Unless specified in the figure legends, TLR stimuli were used at a concentration of 2 μg/mL for Pam3CSK4, 20 ng/mL for polyI:C, 25 ng/mL for LPS, and 1 μM for CpG. Recombinant cytokines were supplemented at the following concentrations: 20 ng/mL for IFN-β; 10 ng/mL for IL-27; 50–100–250 U/mL for IFN-γ; and 10–20–50 ng/mL for IL-17.

2.7 | Cytokine detection

Cytokine production was measured 24 hr poststimulation in the supernatant of microglial cell cultures by enzyme-linked immunosorbent assay (ELISA) or by Multiplex, following the manufacturer's instructions (eBioscience and Procarta, respectively).

2.8 | RNA extraction, cDNA, and quantitative real-time PCR (Rt-PCR)

Total RNA from stimulated and nonstimulated cells was extracted and precipitated using TRIzol 143 Reagent (Invitrogen) and reverse transcribed into cDNA using the manufacturer (Thermo Scientific) instructions. *Il10* and *Ifnb* gene expression were assessed by rt-PCR using TaqMan MasterMix (Applied Biosystems) and normalized against *hypoxanthine phosphoribosyltransferase 1* (*Hprt1*) expression, as previously described (Teixeira-Coelho et al., 2014).

2.9 | mRNA stability determination

mRNA stability was determined as described before (Teixeira-Coelho et al., 2014). Microglia cultures were TLR-stimulated for 1 hr and at that time-point actinomycin D added to the cultures. After 30, 60, or 90 min, the cells were lysed and the expression of IL-10 was analyzed by RT-PCR as indicated above.

2.10 | Statistical analysis

Data are expressed as mean ± SD and analyzed by one or two-way analysis-of-variance (ANOVA) tests or student's *t* test, as indicated in the figure legends. The *p* values considered as statistically significant were *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, and ****$p \leq .0001$.

3 | RESULTS

3.1 | TLR stimulation modulates the inflammatory landscape of microglia

To obtain an overview of the inflammatory landscape of microglia triggered by different TLRs, mouse primary microglia cell cultures were stimulated with chemical agonists for TLR2 (Pam3CSK4), TLR3 (polyI:C), TLR4 (LPS), or TLR9 (CpG). These TLRs are representative of two surface TLRs (TLR2 and 4) mainly associated with the recognition of bacterial products and two intracellular (TLR3 and 9), mainly activated by viruses (Kawai & Akira, 2010). Twenty-four hours poststimulation

with the aforementioned TLR agonists, the culture supernatants were assayed for a panel of 13 cytokines by multiplex or ELISA (Figure 1). Of the 13 cytokines tested, IL-9 and IL-17 were consistently below detection level for all the TLR agonists studied. Also below detection level were all the tested cytokines in nonstimulated cells. We observed that whereas certain cytokines (IL-10, IL-6, IL-12, IFN-β, IFN-γ, IL-4, and IL-5; Figure 1a–g) were greatly dependent on the TLR triggered, others (TNF, IL-23, IL-27, and IL-22; Figure 1h–k) were produced in equivalent amounts, independently of the stimulus. Within the first group, it was interesting to note that TLR3 was the poorest inducer of cytokine production (Figure 1a–g), with the exception of IFN-β (Figure 1d), which was induced at higher levels upon TLR3 stimulation of microglia. Furthermore, the production of anti-inflammatory IL-10 (Figure 1a) by microglia was among the most affected by the type of TLR stimulated. Indeed, IL-10 production varied from the highest amounts induced downstream of TLR9 triggering, to barely detectable ones downstream of TLR3 (Figure 1a). These differential responses were not dependent on the dose of agonist used to stimulate microglia, as increasing the doses of TLR ligands did not result in enhanced secretion of IL-10, nor of TNF (Supporting Information, Figure 1). In particular, increasing the doses of TLR3 agonist did not improve IL-10 secretion, which was for most of the cases below detection level (data not shown). We then tested whereas non-TLR stimuli would also induce IL-10 production by microglia. We found that IL-10 production was also triggered by fungal ligands for the PRR dectin-1 (Supporting Information, Figure 2a). The combined stimulation dectin-1/TLR2 was a more potent stimulus than either independent one (Supporting Information, Figure 2a), being the same pattern was observed for TNF production (Supporting Information, Figure 2b). Finally, we compared the production of IL-10 upon TLR stimulation of microglia to that of myeloid cells of the periphery, namely primary mouse bone marrow-derived macrophages. The pattern of IL-10 secretion upon TLR stimulation of macrophages for 24 hr (Supporting Information, Figure 3) was similar to that observed for microglia. Interestingly, for both cell types, TLR3 stimulation remained the poorer inducer of IL-10 (Supporting Information, Figure 3).

Taken together, we show that microglia respond to different TLR stimulation with the production of an array of cytokines. We highlight IL-10 as a molecule which production is highly dependent on the stimuli and TLR3 as a poor inducer of IL-10. Nevertheless, the pattern of IL-10 production is similar for myeloid cells of distinct embryonic origins.

3.2 | Modulation of IL-10 production by TLR3

Because both the activation of TLR3 and the presence of IL-10 have been linked to neuroprotection in MS and EAE (Gooshe, Abdolghaffari, Gambuzza, & Rezaei, 2014; Kwilasz et al., 2015), we were surprised to see that activation of TLR3 in microglia was uncoupled from IL-10 secretion. We next questioned if TLR3 signaling could potentially interfere with other TLRs, in what respects the induction of IL-10 in microglia. It was interesting to observe that co-stimulation of TLR2-, TLR4-, or TLR9-activated microglia with TLR3 significantly enhanced IL-10 production (Figure 2a–c). So, although TLR3 triggering of microglia does not lead to productive IL-10 secretion, it is an enhancing signal for IL-10 production in these cells.

To further understand the molecular events underlying the modulation of IL-10 upon TLR3 co-stimulation of microglia, we focused on the combination TLR2/TLR3. We measured the transcription of the *Il10* gene over time in microglia stimulated via TLR2 alone, TLR3 alone or the combination TLR2/TLR3. Consistent with the decreased amounts of IL-10 protein, the transcription of the *Il10* gene upon TLR3 stimulation was significantly reduced as compared to that induced by TLR2 activation (Figure 2d). This suggested that the signaling downstream TLR3 is not productive in what concerns IL-10 transcription. Co-stimulating microglia with TLR2 and TLR3 did not enhance the amount of *Il10* mRNA early poststimulation, when compared with TLR2 stimulation alone (Figure 2d). However, whereas upon TLR2 activation a peak of *Il10* gene expression was detected at 1 hr poststimulation and progressively decreased during the analyzed 6 hr, upon TLR2/TLR3 activation, the level of *IL-10* mRNA peaked at 1 hr and then again at 6 hr poststimulation (Figure 2d). This suggested that in the combined stimulation a second wave of *Il10* transcription may be occurring, which would justify the higher amounts of IL-10 protein detected in this case. Alternatively, maintenance of the *Il10* mRNA could result from increased *Il10* mRNA stability, as previously shown for macrophages (Teixeira-Coelho et al., 2014). To investigate this hypothesis, microglia were stimulated with TLR2 or TLR2/TLR3 and at 1 hr poststimulation actinomycin D was added to the cultures and the *IL-10* transcription followed for an extra 90 min. A similar decline in the detection of IL-10 either in single or combined stimulation was observed (Figure 2e). Therefore, the combination of TLR3 with TLR2 triggering led to enhanced IL-10 transcription, rather than enhanced mRNA stability, thus explaining the higher levels of IL-10 produced by microglia upon TLR2/TLR3 co-stimulation.

3.3 | TLR3 activation enhances IL-10 production via type I IFN receptor triggering

As the effect of TLR3 on IL-10 occurred after the initial wave of gene transcription mediated by TLR2, we questioned whether a feedback loop resulting from cytokines downstream of TLR3 could contribute to the higher IL-10 production observed upon TLR2/TLR3 co-stimulation of microglia. Considering the cytokine landscape obtained upon TLR3 triggering of microglia (Figure 1), if such mechanism was operating, the best candidate molecule would be IFN-β, as it is strongly induced by TLR3 (Figure 1g) and it has been described to potentiate IL-10 in studies performed with bone marrow-derived macrophages and dendritic cells (Iyer, Ghaffari, & Cheng, 2010; F. W. McNab et al., 2014; Wang et al., 2011). Furthermore, we observed an early transcription of the *Ifnb* gene downstream of TLR3 signaling in microglia (Figure 3a), which suggested that IFN-β would be produced early enough to modulate the second *Il10* transcriptional wave. Also, in line with the protein data (Figure 1g), no *Ifnb* transcription was observed upon TLR2 stimulation of microglia (Figure 3a). To test the role of IFN-β in inducing IL-10 in microglia, we generated microglia from WT or IFNAR deficient mice, which do not respond to type I IFNs (IFN-β or IFN-α). Although IL-10 production in response to single TLR2 or TLR3 stimulation was not affected by the absence of IFNAR, microglia deficient for this receptor failed to upregulate IL-10 production 24 hr poststimulation with TLR2
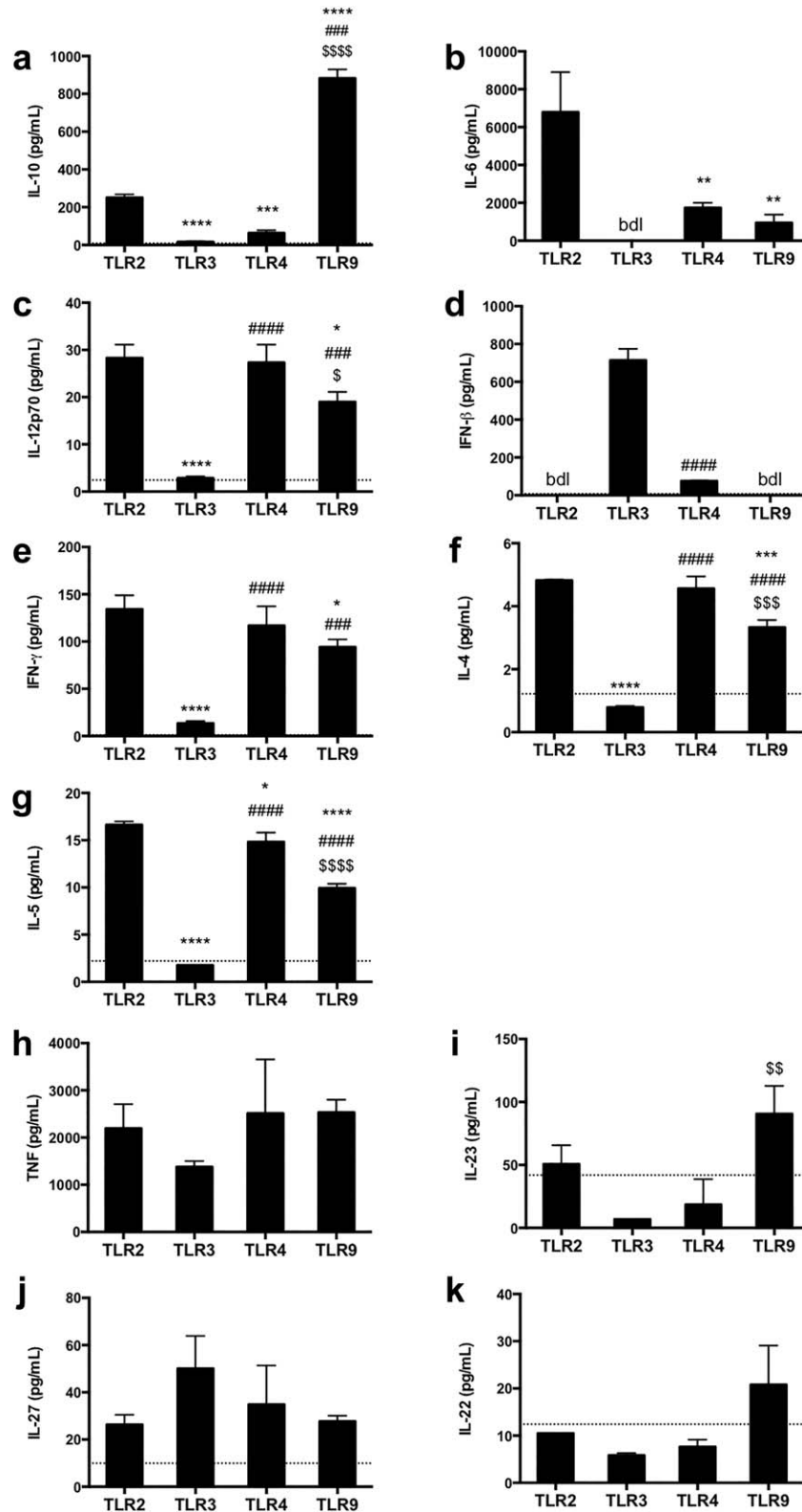
FIGURE 1 Inflammatory landscape of TLR-stimulated microglia. (a–k). Primary microglial cell cultures were left unstimulated or stimulated for 24 hr with chemical TLR agonists for TLR2, TLR3, TLR4, or TLR9, as described in Section 2. Cell culture supernatants were collected and cytokine production was measured by multiplex assay (IL-10, TNF, IL-12p70, IL-23, IL-27, IL-22, IFN-γ, IL-4, and IL-5) or ELISA (IL-6 and IFN-β). Unstimulated cells did not produce detectable amounts of cytokines. The detection limit for each cytokine is represented as a dotted line in each graph. Represented are the mean ± SD for triplicate wells per condition set after mixed cultures generated from independent mice. Statistical differences were assessed by one-way ANOVA or student's *t* test. Significant statistical differences relative to TLR2 are represented by *; to TLR3 by #; to TLR4 by $. One symbol, $p < .05$; two, $p < .01$; three, $p < .001$; and four, $p < .0001$. bdl, below detection level
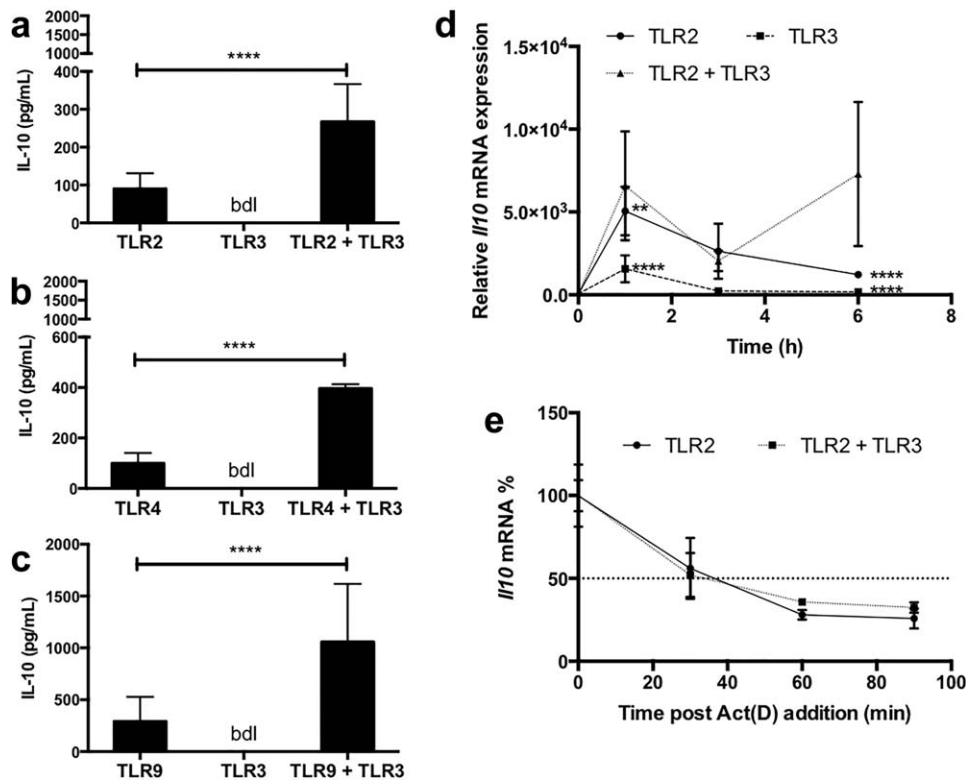
FIGURE 2 TLR3 potentiates IL-10 production by TLR-stimulated microglia, by modulating the transcription of the *Il10* gene. (a–c) WT microglial cells were left unstimulated or stimulated for 24 hr with chemical agonists for TLR2, TLR4, or TLR9 alone or in combination with a TLR3 agonist. Cell culture supernatants were collected and IL-10 production measured by ELISA. IL-10 production by unstimulated cells was undetectable. (d) WT microglial cells were left unstimulated or stimulated with TLR2 (open circle) and TLR3 (close square) agonists alone or in combination (close circle). RNA samples were collected at the indicated time-points and rt-PCR was performed to evaluate *Il10* gene expression normalized to that of *Hprt*. (e) WT microglial cells were stimulated for 1 hr with the TLR2 agonist alone (open circle) or in combination (close circle) with the TLR3 agonist and then actinomycin D was added to cells. RNA samples were collected at 30, 60, and 90 min after actinomycin D addition and rt-PCR was performed to evaluate *Il10* gene expression as before. The dotted line represents 50% of the RNA detected at 1 hr poststimulation. Represented are the mean ± SD for triplicate wells per condition for two independent experiments. Statistical differences were assessed by student's *t* test or two-way ANOVA. Significant statistical differences are represented by ** to $p < .01$; *** to $p < .001$ and **** to $p < .0001$. ActD, actinomycin D; bdl, below detection level

and TLR3 (Figure 3b). Thus, our findings strongly suggest that the production of type I IFN downstream of TLR3 triggering is a key event for the modulation of IL-10 production by this receptor. To further validate these findings, we compared the amount of IL-10 secreted by microglia co-stimulated with TLR2/TLR3 or with TLR2 in combination with recombinant IFN-β. As shown in Figure 3c, treatment of microglia with recombinant IFN-β did not induce IL-10 on its own, but enhanced it when combined with TLR2 stimulation. Furthermore, the transcriptional profile of the *Il10* gene upon co-stimulation of microglia with TLR2 and IFN-β was similar to that observed for TLR2/TLR3 (Figure 3d) and the *Il10* mRNA stability was not altered by IFN-β (Figure 3e).

3.4 | Induction of IL-10 upon combined TLR2/TLR3 stimulation is not accompanied by an overall deregulation of microglia

Co-stimulation of microglia with TLR3 enhanced IL-10 production via IFN-β, thus opening a novel mechanism underlying its beneficial role in

MS and being a possible tool to locally regulate inflammatory responses. For this, it is however important that TLR3 co-activation does not promote an overall cytokine storm. To investigate if this was the case, we run the multiplex panel in supernatants from microglia stimulated with TLR2 in combination with TLR3 (Figure 4). Co-stimulation of microglia via TLR2 and TLR3 did not lead to an overall deregulation of microglia responses, with many cytokines remaining at a level identical to that observed for TLR2 single stimulation (Figure 4). It was interesting to note that IL-1β secretion, which was undetectable upon single stimulation of TLR2 or TLR3, became detectable upon co-stimulation of microglia (Figure 4k).

In addition to IL-10, another molecule found to be markedly upregulated in the case of TLR2/TLR3, as compared to TLR2 alone, was IL-27 (Figure 4e). This cytokine has previously been implicated in the regulation of IL-10 production by macrophages in some reports (Iyer et al., 2010). We thus questioned whether IL-27 could play a role in inducing IL-10 by TLR2-activated microglia. Because IL-10 enhancement upon TLR3 triggering depended on IFNAR (Figure 3b), we first measured IL-27 production in WT or IFNAR-deficient microglia activated via TLR2/
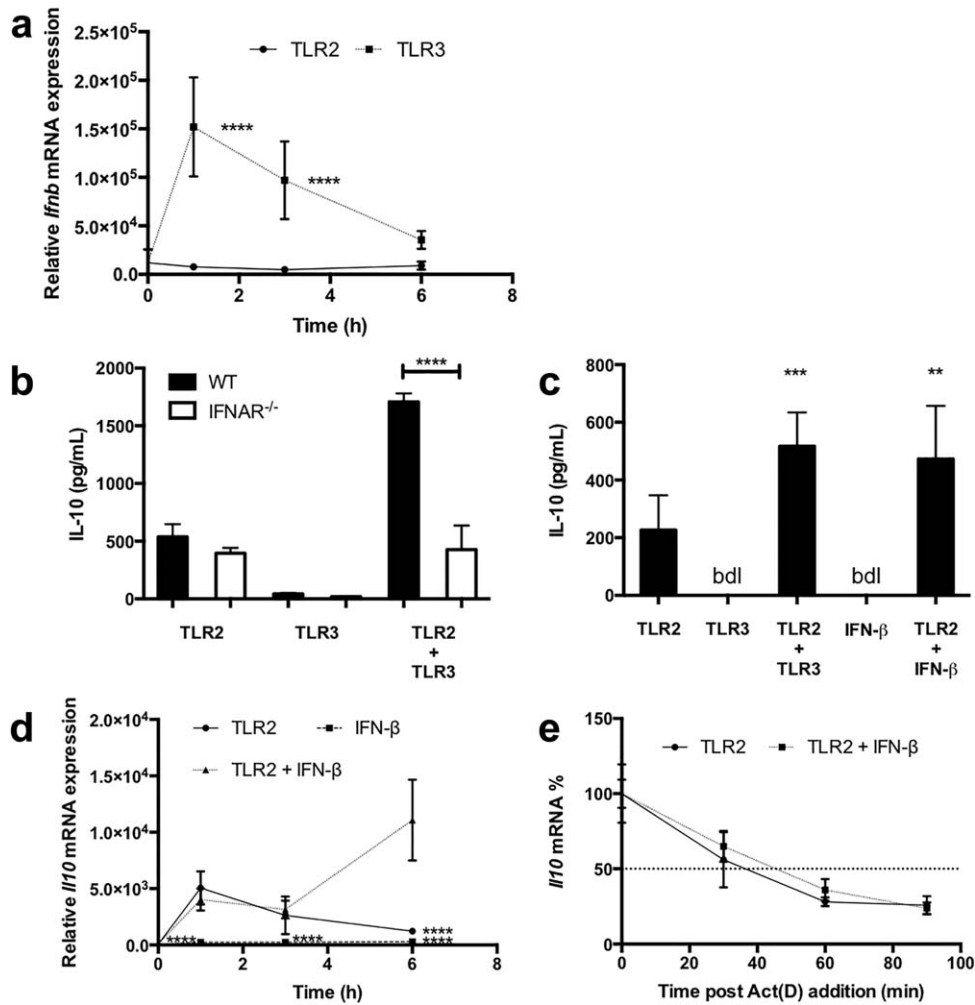
FIGURE 3 IFN-β directly enhances IL-10 transcription in TLR-activated microglia. (a) WT microglial cells were stimulated for 6 hr with TLR2 (open circle) or TLR3 (close square) agonists. RNA samples were collected at the indicated time-points poststimulation and rt-PCR was performed to evaluate *Ifnb* gene expression. (b) Microglial cells generated from WT and IFNAR−/− mice were stimulated with TLR2 and TLR3 agonists alone or in combination. Cell culture supernatants were collected 24 hr poststimulation and IL-10 production was measured by multiplex. (c) WT microglial cells were stimulated with TLR2 and TLR3 agonists in the presence or absence of recombinant IFN-β. Cell culture supernatants were collected and IL-10 production was measured by ELISA. (d) WT microglial cells were stimulated for 6 hr with the TLR2 agonist (open circle), recombinant IFN-β (close square) or with their combination (close circle). RNA samples were collected at the indicated time-points poststimulation and rt-PCR was performed to evaluate *Il10* gene expression. (e) WT microglial cells were stimulated for 1 hr with the TLR2 agonist alone (open circle) or in combination with recombinant IFN-β (close circle) and then actinomycin D was added to cells. The *Il10* mRNA stability was measured as before. The dotted line represents 50% of the RNA detected at 1 hr after stimulation. Represented are the mean ± SD for triplicate wells per condition for two independent experiments. Statistical differences were assessed by one- or two-way ANOVA. Significant statistical differences are represented by ** to $p < .01$; *** to $p < .001$; and **** to $p < 0.0001$. ActD, actinomycin D; bdl, below detection level

TLR3. IL-27 production by TLR2/TLR3 stimulated microglia was diminished in the absence of IFNAR (Figure 4l). We then assessed whether IL-27 would augment IL-10 production by TLR2-stimulated microglia. As before, stimulation of microglia with TLR2 and IFN-β augmented the production of IL-10 (Figure 4m). However, this was not observed when IFN-β was replaced by IL-27, nor did the addition of both IFN-β and IL-27 to the cultures further increased IL-10 production (Figure 4m). Of note, stimulation of microglia with either recombinant cytokine in the absence of TLR2 did not result in IL-10 production (Figure 4m). Therefore, the increase in IL-10 secretion by TLR3/TLR2-stimulated

microglia seems to be directly potentiated by IFN-β, with no major role for IL-27.

3.5 | IL-10 enhancement by TLR3 is modulated by microenvironmental factors

Altogether, our data show that TLR3 potentiates IL-10 production in microglia stimulated with a variety of PRR agonists and that this involves the production of IFN-β and the activation of the IFNAR. Furthermore, this IL-10 enhancement is not accompanied by an overshoot
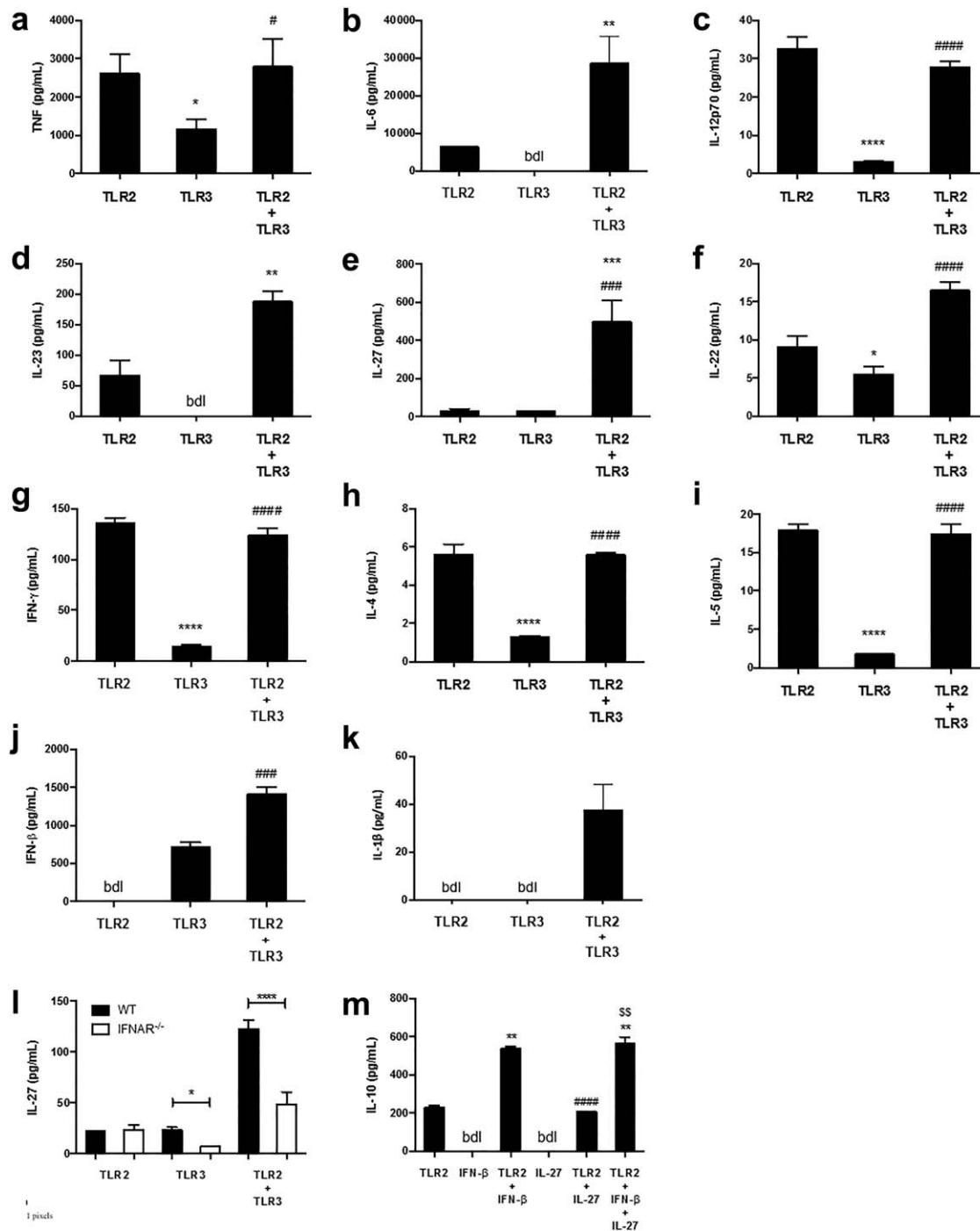
FIGURE 4 TLR3 co-stimulation of microglia does not lead to overshooting responses. (a–k) WT microglial cells were stimulated for 24 hr with TLR2 or TLR3 agonists alone or in combination. Cell culture supernatants were collected and cytokine production was measured by multiplex assay (IL-12p70, IL-23, IL-27, IL-22, IFN-γ, IL-4, and IL-5) or ELISA (TNF, IL-6, IFN-β, and IL-1β). Significant statistical differences relative to TLR2 are represented by *; to TLR3 by #. (l) WT and IFNAR−/− microglial cells were stimulated for 24 hr with the TLR2 or TLR3 agonists alone or in combination. Cell culture supernatants were collected and IL-27 production was measured by multiplex. (m) WT microglial cells were stimulated for 24 hr with the TLR2 agonist, IFN-β or IL-27 alone or in combination. Cell culture supernatants were collected and IL-10 production was measured by ELISA. Significant statistical differences relative to TLR2 are represented by *; to TLR2 + IFN-β by #; to TLR2 + IL-27 by $. The detection limit for each cytokine is represented as a dotted line in each graph. Represented are the mean ± SD for triplicate wells per condition set after mixed cultures generated from independent mice. Statistical differences were assessed by student's t test or one-way ANOVA. Significant statistical differences are represented by one symbol, $p < .05$; two, $p < .01$; three, $p < .001$; and four, $p < .0001$. bdl, below detection level
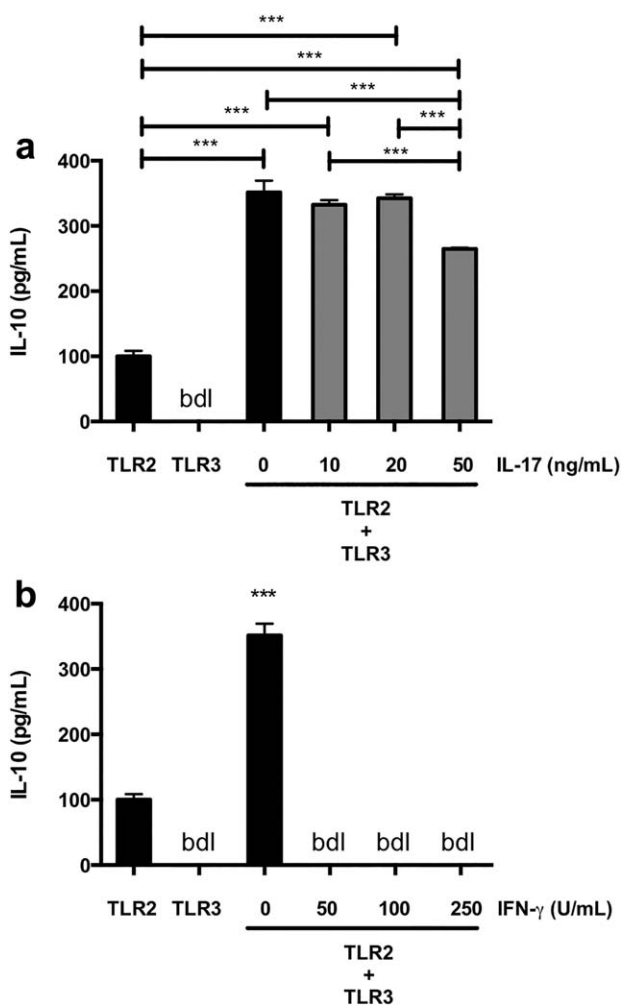
FIGURE 5 The cytokine milieu impacts the TLR3-driven enhancement of IL-10. WT microglial cells were stimulated for 24 hr with TLR2 or TLR3 agonists alone or in combination in the absence or presence of increasing doses of (a) IL-17 or (b) IFN-γ. Cell culture supernatants were collected and IL-10 production was measured by ELISA. Represented are the mean ± SD for triplicate wells per condition for two independent experiments. Statistical differences were assessed by student's *t* test or one-way ANOVA. Significant statistical differences are represented by *** $p < .001$. bdl, below detection level

of the microglia response. These findings are interesting because IL-10 expression in the brain has been proposed as a therapeutic approach for a series of neurological diseases with an immune component, such as MS, Parkinson's disease, and brain injury (Kwilasz et al., 2015). Furthermore, because IFN-β is currently used in the clinics to treat MS patients, it is tempting to speculate that it may exert its local action by promoting IL-10 secretion. However, in the case of complex diseases such as MS, the immune environment generated in the CNS also comprises T-cell-derived cytokines, notably IFN-γ and IL-17. To understand whether the presence of these cytokines would impact on the modulation of IL-10 by TLR3 signaling, we stimulated microglia via TLR2 and TLR3 and combined this with increasing doses of recombinant IL-17 or IFN-γ. Interestingly, whereas the IL-17-enriched environment did not

alter the modulation of IL-10 secretion by TLR3 (Figure 5a), the IFN-γ-enriched milieu totally abrogated it (Figure 5b). Therefore, the successful modulation of IL-10 production in microglia by TLR3 or IFN-β will clearly depend on the immune composition of the tissue microenvironment, a factor that needs to be taken into account in IL-10-based therapies.

4 | DISCUSSION

Surveillance of the CNS by innate immune cells, such as resident microglia, occurs in both physiological conditions and pathological states (Ousman & Kubes, 2012) and is critical for the maintenance of the CNS homeostasis. Pathways for initiating inflammation include an expanding number of cellular sensors, namely the TLR family. Members of this family are expressed on innate immune cells, including microglia cells and astrocytes (Holley et al., 2012; Kawai & Akira, 2010). Activation of microglia through TLRs, and other PRRs, plays an important role in initiating innate immune responses that protect the CNS from aggressions, such as the presence of pathogens, and that promote tissue regeneration after injury (Okun et al., 2009). However, cumulative evidence show that persistently activated microglia, and reactive astrocytes, can also contribute to pathogenesis of several types of CNS diseases, such as MS (Goverman, 2011; Okun et al., 2009). Therefore, microglial cells may contribute to either neuroprotection or neurodegeneration, depending on the setting and the context they are in. Consequently, switching microglial responses toward neuroprotection may be beneficial in different diseases, for example, in MS (Weiner, 2008). In this setting, the modulation of IL-10 production by activated microglia might prove of interest.

Several studies addressed the importance of TLRs in MS pathology, showing that the expression of these receptors is increased in brain lesions of both MS and EAE (Bsibsi, Ravid, Gveric, & van Noort, 2002). Interestingly, whereas activation of TLR2, TLR4, and TLR9 is detrimental in MS and EAE (Prinz et al., 2006; Visser et al., 2005), that of TLR3 protects from disease (Gooshe et al., 2014; Touil, Fitzgerald, Zhang, Rostami, & Gran, 2006). We started this study by looking at the inflammatory landscape of microglia triggered by these TLRs. We found that, overall, TLR3 was the least inflammatory TLR, as only low cytokine levels were detected upon activation of microglia with polyI: C, a TLR3 agonist. This poor reactivity of TLR3 may underlie its protective nature in EAE (Gooshe et al., 2014). Furthermore, TLR3 triggering led to pronounced IFN-β secretion, a molecule that is currently used to treat MS patients (Plosker, 2011; Trojano et al., 2009) and which presence is protective in EAE (Goldmann et al., 2016; Touil et al., 2006). However, in apparent contrast with these data, we found that another protective cytokine in EAE (Bettelli et al., 1998), the anti-inflammatory cytokine IL-10, was poorly induced downstream of TLR3. In the context of MS or EAE, tissue inflammation is accompanied by cellular death, which releases several DAMPs and propagates microglia reactivity. It is therefore most likely that several TLRs, and indeed other PRRs, get activated. When TLR3 activation was combined with that of the other tested TLRs, a significant increase on the production of IL-10

was observed. Importantly, this increase of IL-10 production as a result of combined TLR2/TLR3 stimulation of microglia has a functional impact. Indeed, whereas the production of IL-12 downstream of TLR2 or TLR3 stimulation is only mildly increased in IL-10-deficient microglia, it is markedly enhanced in co-stimulated microglia, in the absence of IL-10 (Supporting Information, Figure 4). Thus, the enhancement of IL-10 in TLR2/TLR3 co-stimulated microglia appears to hamper proinflammatory responses. We narrowed the regulation of IL-10 by TLR3 down to a positive feedback loop involving IFN-β that mechanistically induced a second wave of *Il10* gene transcription, but did not affect the stability of the *Il10* mRNA. This observation mirrors the effect of IFN-β in innate immune cells of the periphery (Iyer et al., 2010; McNab et al., 2014; Wang et al., 2011), supporting the existence of transversal mechanisms operating in circulating versus resident macrophages, such as microglia. We herein also show that the production of IL-10 by TLR-activated microglia follows a pattern similar to that observed in conventional macrophages derived from the bone marrow. TLR3 is the sole TLR signaling only via the TRIF pathway (Kawai & Akira, 2010), which has been previously involved in sustaining the stability of the *Il10* mRNA in bone marrow-derived macrophages (Teixeira-Coelho et al., 2014). Thus, it is tempting to speculate that cell-specific mechanisms are also in place, to ensure specificity in the regulation of IL-10. Pinpointing the common, and the cell-specific, mechanisms regulating this cytokine is critical if tailor-made interventions are to be envisaged.

The protective role for TLR3 in MS and EAE appears to be associated with the triggering of neuroprotective responses in astrocytes (Bsibsi et al., 2010), but also with the release of IFN-β (Touil et al., 2006) and of IL-27 (Fitzgerald et al., 2007) by innate immune cells of the periphery. In EAE, lack of endogenous IFN-β in the CNS leads to augmented microglia activation, resulting in a sustained inflammation, cytokine production, and tissue damage with consequent chronic neurological deficits (Teige et al., 2003) in support of an anti-inflammatory role for this molecule. Furthermore, expression of IFN-β within the CNS (Khorooshi et al., 2015), including in microglia (Kocur et al., 2015), plays a protective role in EAE. Other studies further link IFN-β therapy with an enhancement of IL-10 in MS and an accompanying inhibition of T helper (Th) 17 cell responses (Krakauer, Sorensen, Khademi, Olsson, & Sellebjerg, 2008; Kvarnstrom, Ydrefors, Ekerfelt, Vrethem, & Ernerudh, 2013; Ramgolam, Sha, Jin, Zhang, & Markovic-Plese, 2009; Sweeney et al., 2011), but all do so in immune cells of the periphery. We now reveal another protective action of IFN-β, by directly targeting microglia leading to enhanced IL-10 and IL-27, both protective cytokines in MS. At least in microglia, these events seem to be unrelated, with the IFN-β potentiation of IL-10 being independent of IL-27. This is in line with other studies performed in macrophages (McNab et al., 2014) and contrasts with a possible role of IL-27 in promoting IL-10 production by myeloid cells (Iyer et al., 2010) and effector Th cells (Freitas do Rosario et al., 2012). As mentioned above, the response to IFN-β therapy in MS has been correlated with an inhibition of Th17 cells (Krakauer et al., 2008; Kvarnstrom et al., 2013; Ramgolam et al., 2009; Sweeney et al., 2011). As herein shown, the direct effect of IFN-β in potentiating IL-10 secretion by TLR-activated microglia would still

occur in the context of IL-17, but would be largely compromised in the presence of IFN-γ. The role of IFN-γ in modulating IL-10 production by microglia is still not fully understood, but it is likely dependent on the activation of glycogen synthase kinase (GSK)-3 (Green & Nolan, 2012). It is interesting to note that low amounts of IFN-γ were detected upon stimulation of the microglia cultures with TLR2, 4, and 9 agonists, but not upon TLR3 triggering. Yet, this pattern of endogenous IFN-γ production was not related to that of IL-10 expression. The fact that absence of IFN-γ under TLR3 stimulation did not license IL-10 production fits with the mechanism we propose, where low IL-10 triggering in response to TLR3 stimulation is related to deficient basic transcriptional activity. In further support of an independent endogenous IFN-γ vs IL-10 production, blocking of IFN-γ with a neutralizing antibody did not alter IL-10 secretion by TLR2-stimulated microglia (Supporting Information, Figure 5). In sum, by using an in vitro model of neonate microglia activation, our study illustrates the complexity of the events regulating microglial responses, providing hints to possible mechanisms operating in the adult brain in the context of EAE. It will be important to now probe these mechanisms in vivo, by resorting to various genetically modified mice and to also address the effects of microglia modulation on adjacent cells, notably on neurons.

Our data showing that IFN-β is a direct modulator of IL-10 in TLR-activated microglia also has implications in the study of animal models of MS. In a recent study, the most common mouse backgrounds used in laboratory research (Balb/c and C57BL/6) were shown to bear important differences in the ability of their macrophages to produce IL-10 in response to TLR triggering (Howes et al., 2016). These differences were related to a differential induction of IFN-β in either background (Howes et al., 2016). If the same differences apply to microglia, the use of either mouse strain to induce EAE may lead to distinct results. In line with a possible impact of the mouse genetic background in the response of microglia to TLR activation, previous studies reported IFN-β production downstream of TLR9 activation in microglia generated from IRW mice (Butchi et al., 2010). This was not the case in our study, where C57BL/6 mice were used (Figure 1d).

The interaction between the brain and the immune system is now accepted to play pivotal roles in health and disease, particularly in pathologies involving a strong neuroimmune component. Microglia are key pieces in this interaction. As such, it is critical to understand the network of events that drive microglia responses. Our study contributes to this understanding in what regards the intrinsic orchestration of IL-10 expression and the impact of the cytokine microenvironment in this regulation. Understanding these processes is chief to the targeted manipulation of IL-10 in the brain.

AUTHORS' CONTRIBUTIONS

DS and GMC performed the experiments. DS, AGC, SR, and MS designed the study. DS, AGC, SR, and MS analyzed and interpreted the data. All authors read and approved the final manuscript.

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