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Astrocytes Produce IL-19 in Response to Bacterial Challenge and are Sensitive to the Immunosuppressive Effects of this IL-10 Family Member

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Abstract

There is growing appreciation that resident glial cells can initiate and/or regulate inflammation following trauma or infection in the central nervous system (CNS). We have previously demonstrated the ability of microglia and astrocytes to respond to bacterial pathogens or their products by rapid production of inflammatory mediators, followed by the production of the immunosuppressive cytokine interleukin (IL)210. IL-19, another member of the IL-10 family of cytokines, has been studied in the context of a number of inflammatory conditions in the periphery and is known to modulate immune cell activity. In the present study, we demonstrate the constitutive and/or inducible expression of IL-19 and its cognate receptor subunits, IL-19R α and IL-19R β (also known as IL-20R1 and IL-20R2, and IL-20RA and IL-20RB), in mouse brain tissue, and by primary murine and human astrocytes. We also provide evidence for the presence of a novel truncated IL-19R α transcript variant in mouse brain tissue, but not glial cells, that shows reduced expression following bacterial infection. Importantly, IL-19R functionality in GLIA is indicated by the ability of IL-19 to regulate signaling component expression in these cells. Furthermore, while IL-19 itself had no effect on glial cytokine production, IL-19 treatment of bacterially infected or Toll-like receptor ligand stimulated astrocytes significantly attenuated pro-inflammatory cytokine production. The bacterially induced production of IL-19 by these resident CNS cells, the constitutive expression of its cognate receptor subunits, and the immunomodulatory effects of this cytokine, suggest a novel mechanism by which astrocytes can regulate CNS inflammation.

Keywords

inflammation; bacterial meningitis; IL-20R; innate immunity; glia

Introduction

Resident cells of the central nervous system (CNS) play a critical role in the initiation and progression of damaging inflammation following infection. Microglia and astrocytes express an array of cell surface and cytosolic pattern recognition receptors, either constitutively or

following activation, to perceive microbial and viral motifs (as reviewed in Furr and Marriott, 2012; Hanke and Kielian, 2011). We, and others, have demonstrated that glial cells utilize these sensors to respond to clinically relevant CNS pathogens such as *Borrelia burgdorferi*, *Neisseria meningitidis*, *Staphylococcus aureus*, and herpes simplex virus-1 (HSV-1) and produce immune mediators to precipitate lethal CNS inflammation (Chauhan et al., 2009; Furr et al., 2011; Kielian et al., 2002; Liu et al., 2010; Rasley et al., 2002). However, we have shown that astrocytes and microglia also produce significant quantities of the immunosuppressive cytokine IL-10 in a delayed manner following challenge with either *B. burgdorferi* or *N. meningitidis* (Rasley et al., 2006). This finding, coupled with the demonstration that IL-10 can inhibit glial inflammatory responses (Rasley et al., 2006), suggests a mechanism whereby astrocytes and microglia could play an important role in either inflammation resolution following pathogen clearance, or in limiting damage during chronic infections.

IL-10 is the namesake and best characterized of a family of cytokines that also includes IL-19, IL-20, IL-24 (also known as Mda-7), IL-26, IL-28A/B, and IL-29. Many of these cytokines are related by homologous intron/exon structures and signal through common heterodimeric transmembrane receptor subunits to exert their biological actions (as reviewed in Commins et al., 2008; Gallagher, 2010; Ouyang et al., 2011). Of these, IL-19 (first described in Gallagher et al., 2000) has been further classified as a member of an IL-10 family subgroup that also includes IL-20 and IL-24 (Ouyang et al., 2011). Like its other subgroup members, IL-19 has been shown to signal through a heterodimeric receptor composed of alpha and beta subunits (IL-19R α and IL-19R β , respectively; also known as IL-20RA and IL-20RB, and IL-20R1 and IL-20R2) and binds this receptor complex with higher affinity than either IL-20 or IL-24 (Dumoutier et al., 2001; Parrish-Novak et al., 2002). One study suggests that IL-19 can promote monocyte inflammatory mediator production (Liao et al., 2002) and local expression of this cytokine and its receptor has been associated with inflammatory disease states including psoriasis, rheumatoid arthritis, asthma, and septic shock (Blumberg et al., 2001; Hsing et al., 2008; Liao et al., 2004; Sakurai et al., 2008). However, a growing body of evidence indicates that IL-19 promotes immunosuppressive cytokine expression (Gallagher et al., 2004) while limiting pro-inflammatory mediator production and Th1 lymphocyte polarization (Cuneo et al., 2010; England and Autieri 2012; Gabunia et al., 2011). These findings suggest that IL-19 production during disease states represents an attempt by the host to limit inflammatory damage and promote tissue repair (Azuma et al., 2011).

In the present study, we have assessed the ability of primary astrocytes and microglia to produce IL-19 in response to clinically relevant CNS pathogens, and the responsiveness of these cells to this IL-10 family member. We demonstrate the constitutive and/or inducible production of IL-19, and the functional expression of its cognate receptor subunits in brain tissue and isolated glial cultures. Importantly, we demonstrate that IL-19 can significantly attenuate bacterially induced inflammatory astrocyte responses suggesting another means by which this major glial cell population can regulate CNS inflammation.

Materials and Methods

Reagents

Flagellin (isolated from *Salmonella typhimurium* strain 14028) was purchased from Enzo Life Sciences (Farmingdale, NY). Peptidoglycan, lipopolysaccharide (LPS; from *E. coli*) and polyinosinicpolycytidylic acid sodium salt (polyI:C) were purchased from Sigma-Aldrich (St. Louis, MO). Doses and exposure durations used represent those that were empirically determined to elicit optimal responses.

Murine and Human Glial Cell Cultures

Primary murine glia were isolated and cultured as described previously (Bowman et al., 2003; Rasley et al., 2002). Briefly, a cell suspension was prepared from six to eight neonatal C57BL/6 mouse brains per preparation. Microglia were obtained from this mixed glial culture by shaking flasks in an orbital shaker and subsequent adherence to plastic culture vessels while astrocytes were isolated using trypsin-EDTA. Astrocytes were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and microglia were grown in RPMI 1640 and 10% FBS supplemented with 20% conditioned medium from LADMAC cells (ATCC number CRL-2420) as a source of monocyte colony stimulating factor (O'Keefe et al., 2001). Cells isolated in this manner were demonstrated to be >95% authentic microglia or >97% astrocytes as assessed by their characteristic morphology and expression of CD11b and F4/80 (Rasley et al., 2002), or glial fibrillary acidic protein (GFAP) (Bowman et al., 2003), respectively, as determined by confocal microscopy. The murine microglia-like cell line EOC 13.31 (ATCC no. CRL-2468) was used in some experiments as previously described (Rasley et al., 2002). Primary human astrocytes were purchased and maintained in a supplemented medium provided by the vendor (Scien-Cell, Carlsbad, CA) with 2% FBS. These cells have been characterized as authentic astrocytes by the vendor because of their expression of GFAP as determined by immunofluorescent analyses.

Bacterial and Viral Propagation and Antigen Preparation

In these studies, we have utilized *B. burgdorferi* strain N40 (Barthold et al., 1993), *N. meningitidis* strain MC58, *S. aureus* strain UAMS-1, and *S. pneumoniae* strain CDC CS109, a clinical isolate from a patient with meningitis. *S. pneumoniae* was grown on tryptic soy agar with 5% defibrinated sheep blood and cultured overnight in Todd-Hewitt broth at 37°C with 5% CO₂ as previously described by our laboratory (Liu et al., 2010). A low clonal passage of *B. burgdorferi* was grown in Barbour-Stoenner-Kelly (BSK) II medium (Barbour et al., 1984) as we have described (Rasley et al., 2002). *N. meningitidis* was grown overnight (12–15 hours) in 25 ml of Columbia broth on an orbital shaker at 37°C with 5% CO₂ as previously described (Chauhan et al., 2008). For cell lysates, *B. burgdorferi* or *N. meningitidis* were centrifuged at 10,000g, washed three times with phosphate buffered saline (PBS) prior to pulsing three times with a cell sonicator. *B. burgdorferi* antigen isolates generated in this manner have previously been demonstrated to be free of detectable levels of LPS (Rasley et al., 2002).

Viral stocks of vesicular stomatitis virus (VSV) Indiana strain and herpes simplex virus-1 (HSV-1) MacIntyre strain from a patient with encephalitis (ATCC, VR-539) were prepared

as previously described by our laboratory and viral titers were quantified using standard plaque assays (Chauhan et al., 2010; Furr et al., 2011).

In Vitro Stimulation of Glial Cells

For *in vitro* exposure of isolated glia to *S. aureus*, *B. burgdorferi*, or *N. meningitidis* bacteria were harvested by centrifugation and washed twice in PBS. Confluent cell layers of glia (2×10^6 cells) were washed twice with 2 mL of PBS to remove growth media and then exposed to bacteria at multiplicities of infection (MOI) of between 1:1 and 100:1 bacteria to glial cells, as indicated, in media without antibiotics for 90 min at 37°C. Following this period, cell cultures were washed and incubated in media with 10% FBS supplemented with gentamicin (25 µg/mL) to kill remaining extracellular bacteria as we have described (Chauhan et al., 2008). In experiments featuring bacterial antigens or TLR agonists as the stimulus, 2×10^6 glial cells were exposed to *B. burgdorferi* or *N. meningitidis* cell lysates, bacterial flagellin, LPS, peptidoglycan, or polyI:C, as indicated. In some experiments, microglia or astrocytes were infected with HSV-1 or VSV at MOIs of between 0.01 and 10 plaque forming units per cell and the viruses were allowed to adsorb for 1 hour prior to washing to remove nonadherent viral particles as we have previously described (Chauhan et al., 2010; Furr et al., 2011). At 2–24 hours following bacterial or viral infection or exposure to bacterial lysates, culture supernatants and/or total RNA were collected. Experimental results presented represent the time points at which optimal or maximal responses were obtained.

Intracranial Bacterial Administration

For *in vivo* administration of *S. pneumoniae*, bacteria were harvested by centrifugation and washed in PBS. Viable bacteria were administered via intracerebral (i.c.) injection at a nonlethal dose (1×10^7 bacteria) into wild type 6–8 week-old female C57BL/6 mice (Jackson Laboratories) as previously described (Liu et al., 2010) using an approach that allows bacteria in an aqueous solution to reflux into the subarachnoid space via the needle tract leading to meningitis formation. At 48 hours postinfection, animals were euthanized and brain, heart, lung, skin, and muscle tissues were isolated for analysis. All studies were performed in accordance with relevant federal guidelines and institutional policies regarding the use of animals for research purposes.

Isolation of RNA, Semi-Quantitative PCR, Quantitative Real-Time PCR, and Sequencing

Total cellular RNA was isolated from microglia and astrocytes with Trizol reagent (Invitrogen, Grand Island, NY) and reverse transcribed as previously described (Bowman et al., 2003). Semi-quantitative PCR was performed to determine expression or transcript length/composition of mRNA encoding murine and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), IL-19, IL-19R α , and IL-19R β as we have described (Bowman et al., 2003). In addition, quantitative real-time PCR was also performed to determine the level of expression of mRNA encoding murine IL-19, IL-19R α , IL-19R β , and GAPDH using the SYBR Green approach on a 7500 Fast Real-Time PCR machine (Applied Biosystems, Grand Island, NY) as described previously (Chauhan et al., 2008). Melting curve analysis was performed to determine the melting temperature of each sample as an indicator of the

purity of the reaction product and to denote the absence of primer dimers or other nonspecific PCR products.

PCR primers used to amplify cDNA derived from cellular mRNA are shown in Table 1. All primers were designed using commercial software (Integrated DNA Technologies, Coralville, IA) based on their location in different exons of the genomic sequences and their lack of significant homology to sequences present in Gen-Bank (National Center for Biotechnology Information, Bethesda, MD), except for the human IL-19 primer pair, which was based on previously published sequences (Hsing et al., 2012). The identity of each PCR amplified fragment was verified by size comparison with DNA standards (Promega, Madison, WI). Where indicated, PCR products were extracted using spin columns according to manufacturer's instructions (Bio-Rad, Hercules, CA) and their sequences were determined using a commercial sequencing service (Davis Sequencing, Davis, CA). All RNA expression levels are reported as relative levels normalized to the expression of the housekeeping gene GAPDH determined in parallel real time PCR reactions.

Quantification of IL-6, TNF- α , and IL-19 Secretion

Specific capture ELISAs were performed to quantify TNF- α secretion by glial cells using a commercially available ELISA kit (R&D Systems, Minneapolis, MN) as described previously by our laboratory (Chauhan et al., 2008). IL-6 secretion was measured using a rat anti-mouse IL-6 capture antibody and a biotinylated rat anti-mouse IL-6 detection antibody (Clones MP5-20F3 and MP5-C2311, respectively, BD Pharmingen, San Diego, CA). Human IL-6 secretion was measured using a rat anti-human IL-6 capture antibody and a biotinylated rat anti-human IL-6 detection antibody (Clones MQ2-13A5 and MQ2-39C3, respectively, BD Pharmingen). IL-19 secretion was measured using a rat anti-mouse IL-19 capture antibody (Clone RN19, eBioscience, San Diego, CA) and a biotinylated rabbit anti-mouse IL-19 polyclonal antibody (LifeSpan BioSciences, Seattle, WA). IL-19 levels could not be determined in experiments featuring *S. aureus* as this bacterium was found to contain a cross-reactive antigen (data not shown).

Immunoblot Analyses for IL-19R α and IL-19R β

Immunoblot analyses for the presence of IL-19R α and IL-19R β in glial cells were performed essentially as previously described by our laboratory (Furr et al., 2011). After incubation with a rabbit polyclonal antibody against IL-19R α (Bioss, Woburn, MA) or IL-19R β (Bioss) for 24 hours at 4°C, blots were washed and incubated in the presence of a horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling, Danvers, MA). Bound enzyme was detected with the Super Signal system (Thermo Scientific, Rockford, IL). To assess total protein loading in each well, immunoblots were reprobed with a goat anti-mouse β -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblots shown are representative of at least three separate experiments.

Statistical Analysis

All results are presented as the mean $\pm$ SEM and were tested statistically by Student's two-tailed t-test or one- or two-way analyses of variance (ANOVA) with Tukey's post-hoc test as appropriate using commercially available software (GraphPad Prism, GraphPad

Software Inc. La Jolla, CA). Where appropriate, the Bonferroni correction was used to account for multiple hypothesis testing. Results were considered to be statistically significant at a *P*-value of less than 0.05.

Results

IL-19 is Expressed by Astrocytes Following Bacterial Challenge

To begin to assess whether glial cells can express IL-19, we have determined whether mRNA encoding IL-19 is present in the CNS either constitutively or following infection. As shown in Fig. 1, only very low levels of mRNA encoding IL-19 were detectable in the brains of uninfected mice as determined by semi-quantitative RT-PCR and quantitative real-time PCR. Interestingly, all mice showed increased IL-19 mRNA in whole brain tissue at 72 hours following intracranial administration of *S. pneumoniae* (Fig. 1).

To begin to determine whether the *in vivo* increase in IL-19 mRNA expression is attributable, at least in part, to expression of this IL-10 family member by resident CNS cell types, we have performed *in vitro* studies employing isolated cultures of primary astrocytes and microglia. As shown in Fig. 2, resting astrocytes constitutively expressed very low, but detectable, levels of mRNA encoding IL-19, consistent with our *in vivo* studies. Such mRNA expression was reflected in our detection of only modest levels of IL-19 protein release by unstimulated cells (Fig. 2C). Importantly, stimulation of astrocytes with bacterial LPS elicited marked increases in IL-19 mRNA expression and protein release in a time-dependent manner (Fig. 2). Furthermore, this effect was not restricted to this TLR4 agonist as the TLR3 ligand polyI:C and the TLR5 ligand flagellin similarly induced IL-19 production by murine astrocytes at the level of mRNA expression (Fig. 3A) and protein release (Fig. 3B), while the TLR2 ligand peptidoglycan (50 ug/mL) elicited a more modest release of IL-19 (517 versus 145 pg/mL by unstimulated cells: *P*<0.05, *n*=3).

Importantly, we have determined whether clinically relevant bacterial pathogens of the CNS can induce IL-19 expression. As shown in Fig. 3A,B, the disparate Gram-negative bacteria *N. meningitidis* and *B. burgdorferi* induced IL-19 mRNA and protein expression by primary murine astrocytes. Such induction was in contrast with the expression of other IL-10 family members by astrocytes. While antigens derived from either pathogen induced robust IL-19 mRNA expression in this time frame, *B. burgdorferi* antigens failed to induce expression of mRNA encoding IL-20, IL-22, or IL-24, and the highest dose of *N. meningitidis* antigens induced only modest levels of IL-24 mRNA and barely detectable levels of mRNA encoding IL-20 (Fig. 3C). The ability of these pathogens to induce IL-19 expression was not restricted to murine cells as both bacterial species were found to elevate IL-19 mRNA expression in primary human cells from the very low levels seen in unstimulated cells (Fig. 3A). Furthermore, induction of IL-19 in murine astrocytes was not limited to Gram-negative organisms as the Gram-positive bacterium *S. aureus* similarly induced the expression of mRNA encoding IL-19 in these cells and did so in a dose and time-dependent manner (Fig. 3D and 3E). However, these data were in contrast to the effect of viral pathogens as neither the RNA virus VSV nor the DNA virus HSV-1 induced detectable IL-19 secretion by astrocytes (data not shown).

Finally, we have also determined whether microglia can constitutively express IL-19 or can be induced to produce this cytokine. As shown in Fig. 3F, resting primary murine microglia did not express mRNA encoding IL-19. Exposure of microglia to bacterial LPS or infection with *N. meningitidis* elicited modest levels of IL-19 mRNA expression (Fig. 3F). However, such expression could be attributable to even a small number of contaminating astrocytes. This notion is supported by our studies using the EOC13.31 microglia-like cell line in which these stimuli failed to elicit detectable IL-19 production (data not shown). Taken together, our data indicate that astrocytes, but not microglia, are a significant source of IL-19 following bacterial challenge.

Astrocytes Express Cognate IL-19 Receptor Subunits

To begin to determine whether glial cells can respond to IL-19, we have investigated whether mRNA encoding the alpha and beta receptor subunits (IL-19R α and IL-19R β) that comprise the cognate heterodimeric IL-19 receptor are expressed in the CNS, either constitutively or following infection. As shown in Fig. 4A, both IL-19R α and IL-19R β are readily detectable in uninfected mouse brains and are not increased following intracranial bacterial administration.

Consistent with this finding, resting cultures of isolated murine astrocytes express mRNA encoding IL-19R β at robust levels that show very little change following bacterial infection (Fig. 4B,C). In contrast, levels of mRNA encoding IL-19R α in murine astrocytes were rapidly elevated from relatively low constitutive levels following *S. aureus* challenge (Fig. 4B,C), but levels of mRNA encoding IL-19R α or IL-19R β were not significantly changed following treatment with rIL-19 (30 and 300 ng/mL) for 2, 8, or 15 hours (data not shown). Murine astrocytes were found to exhibit robust IL-19R α and IL-19R β protein expression constitutively, but bacterial challenge failed to significantly elevate such expression (Fig. 4D).

The ability of *S. aureus* to induce IL-19 receptor mRNA expression was not restricted to murine cells as this pathogen elevated IL-19R β and, to a lesser extent, IL-19R α mRNA expression in primary human cells from the negligible levels seen in unstimulated cells (Fig. 4E). Finally, we have also determined whether microglia constitutively express IL-19R α or IL-19R β , or can be induced to express these subunits. As shown in Fig. 4F, resting primary murine microglia expressed low but detectable levels of mRNA encoding IL-19R β , but did not express mRNA encoding IL-19R α . In contrast to monocytes/macrophages (Sakurai et al., 2008), exposure of microglia to rIL-19 or infection with *N. meningitidis* did not elicit demonstrable changes in either IL-19R α or IL-19R β mRNA expression (Fig. 4F).

Expression of a Novel Truncated IL-19R α mRNA Variant in Brain Tissue

Surprisingly, PCR primers specific for a transcript encoded by IL-19R α exons 3 to exon 5 generated a prominent second product with brain tissue cDNA that was approximately 150 base pairs smaller than that predicted by the published sequence (Fig. 5A). This product was not observed with skin, heart (Fig. 5A), or muscle (data not shown) cDNA and was not detected in experiments using isolated astrocytes or microglia (data not shown). Using a primer set specific for the anticipated sequence of a direct exon 3–exon 5 junction (Table 1),

PCR yielded a product at the predicted size indicating the expression of a novel transcript in brain tissue that excludes IL-19R α exon 4 (Fig. 5A). Interestingly, the level of expression of this truncated transcript was significantly reduced in brain tissue following intracranial bacterial administration (Fig. 5B). The composition of this IL-19R α variant was further defined with additional primer sets that predominantly yielded products predicted for a transcript encoded by exons 1 through 3 and exons 5 through 7 (Fig. 5C). Finally, the presence of an IL-19R α transcript variant lacking exon 4 was confirmed by DNA sequence analysis (data not shown). A schematic representation of the exon composition of full-length IL-19R α and the transcript variant is shown in Fig. 5D, and the results of the sequence analysis corresponding to the exon 3–exon 5 junction is indicated.

IL-19 Suppresses Inflammatory Cytokine Production by Activated Primary Astrocytes

To determine the functional significance of IL-19R expression by resident CNS cells, we have assessed the effect of rIL-19 on glial immune functions. Exposure of astrocytes or microglia to rIL-19 (30 or 300 ng/mL) for 2 or 8 hours had no significant effect on expression of mRNA encoding the inflammatory cytokines IL-6 or TNF- α , or the immunosuppressive cytokine IL-10 (data not shown). However, treatment of murine astrocytes with rIL-19 rapidly induced the expression of mRNA encoding the negative regulator of cytokine signaling, SOCS3 (Fig. 6A). Furthermore, pretreatment with rIL-19 markedly attenuated the IL-6 and TNF- α responses of astrocytes to *N. meningitidis* infection or exposure to the TLR agonists flagellin and polyI:C (Fig. 6C).

Recombinant IL-19 treatment was capable of influencing SOCS3 expression in murine microglia despite an apparent absence of IL-19R α expression by this cell type. However, in contrast to its effect on astrocytes, rIL-19 elicited a rapid down-regulation of SOCs mRNA expression in microglia (Fig. 6A). While pretreatment of microglia with a high dose of rIL-19 reduced polyI:C-induced TNF- α production, this effect appears to be attributable to the presence of astrocyte contamination as no attenuation was observed in parallel experiments utilizing the EOC13.31 microglial cell line (Fig. 6C). Taken together, our data suggest that IL-19 can significantly attenuate the immune responses of activated astrocytes but not microglia.

Discussion

The IL-10 family of cytokines is comprised of a number of pleiotropic cytokines that can impact immune function and inflammation (as reviewed in Ouyang et al., 2011). While several family members appear to promote potentially damaging inflammation, both IL-10 and IL-19 have been shown to be immunosuppressive and protective in some inflammatory diseases (Azuma et al., 2011). We have previously demonstrated that murine microglia and astrocytes show the delayed production of IL-10 in response to exposure to clinically relevant bacterial pathogens or their components, and we have shown that this cytokine can suppress the inflammatory responses of these glial cell types (Rasley et al., 2006). In the present study, we demonstrate that IL-19 is up-regulated in the CNS following bacterial infection. Importantly, we demonstrate that isolated murine and human astrocytes can be induced to express robust levels of this cytokine, but not IL-20, IL-22, or IL-24, in response

to disparate Gram-negative and Gram-positive bacterial pathogens. Furthermore, we have shown that bacterial motifs recognized by members of the TLR family of pattern recognition receptors are a sufficient stimulus to induce IL-19 production by astrocytes.

Interestingly, neither RNA nor DNA viruses were capable of inducing astrocyte IL-19 expression despite the ability of a specific TLR3 ligand to elicit IL-19 release. It is not clear why an agonist of a TLR that is generally considered to play a major role in viral recognition induces such production while infectious viral particles do not. However, the ability of bacterial rather than viral pathogens to promote IL-19 release by astrocytes might be beneficial to the host given previous reports that this IL-10 family member promotes Th2 type T-cell responses while inhibiting Th1 cytokine production and cell-mediated immunity (Oral et al., 2006; Wahl et al., 2009; Yeh et al., 2011). As such, local IL-19 production by this major glia cell type could serve to promote protective host immune responses while limiting potentially damaging Th1 cytokine-mediated inflammation. The ability of astrocytes to elicit robust IL-19 production was in contrast to our findings in primary microglia and a microglia-like cell line that did not release this cytokine. This result also differs from studies in monocytes and macrophages that produce IL-19 following activation (Sakurai et al., 2008; Wolk et al., 2002), further illustrating the marked phenotypic differences that exist between these myeloid cell types.

In addition to demonstrating that astrocytes are capable of producing this IL-10 family member, we have shown that both subunits of the cognate receptor for IL-19 are constitutively expressed in brain tissue. Interestingly, we have also detected the presence of a novel IL-19R α transcript variant in brain tissue in which exon 4 is spliced out, while the other six exons are retained. This variant is dissimilar to the elongated IL-19R α transcript reported by Wei and Chang (2008) and was detected in infected and uninfected brain tissues, but not in skin, heart, lung, or muscle tissues. The fact that this variant appears to be expressed in a tissue specific manner suggests that it may mediate physiological actions unique to the CNS. The abundance of this unexpected transcript suggests that it is either present in a major cell type or is expressed at a high level in a relatively small population of cells. To begin to establish the cellular source(s) of this variant, we used specific primer sets to assess mRNA expression in isolated glial cultures. Astrocytes, the most abundant glial cell type, were found to constitutively express IL-19R β and IL-19R α transcripts of the predicated length, and such expression was elevated following bacterial challenge of both murine and human cells. However, astrocytes failed to express the truncated IL-19R α variant. In contrast, microglia constitutively expressed IL-19R β but did not express either IL-19R α form, either at rest or following bacterial challenge. Such a finding is consistent with previous reports indicating that other myeloid immune cell types do not express IL-19R α (Kunz et al., 2006; Nagalakshmi et al., 2004; Wolk et al., 2002; Wolk et al., 2008).

While the identity of CNS population expressing the IL-19R α variant remains to be determined, nucleotide translation and protein domain analyses indicate that the expressed variant would contain the extracellular domains but not the cytoplasmic signaling tail. Such cytokine receptor truncation is reminiscent of IL-1R2 and decoy receptor 3 (DcR3) which function as decoy receptors for IL-1 α/β and TNF-related apoptosis-inducing ligand, respectively (Lin and Hsieh, 2011; Peters et al., 2013). However, in contrast with IL-1R2

and DcR3 that mediate their functions as a result of strong affinities for their respective ligands, IL-19R α does not bind IL-19 tightly (Dumoutier et al., 2001; Parrish-Novak et al., 2002; Pletnev et al., 2003). Instead, it is essential for the initiation of cell signaling events (Blumberg et al., 2001; Dumoutier et al., 2001; Logsdon et al., 2012; Parrish-Novak et al., 2002; Pletnev et al., 2003) and so a truncated IL-19R α form has the potential to associate with IL-19R β to complex with IL-19 but not initiate cell responses. Given that the IL-19R α variant is down regulated following *in vivo* infection, it is tempting to speculate that decreased levels of the truncated form of IL-19R α could act in concert with increased IL-19 production to promote cellular responses to this cytokine in a similar manner to that seen for the TNF family of ligands and receptors (Ashkenazi and Dixit, 1999).

Importantly, we demonstrate that astrocytes functionally express IL-19R and show that IL-19 can regulate the immune responses of this major glial cell type to clinically relevant CNS pathogens. Exposure of isolated primary astrocytes to recombinant IL-19 induces the expression of SOCS3, a key negative regulator of cytokine signaling, by these cells. Consistent with this finding, and in contrast to the reported ability of IL-19 to induce monocyte inflammatory cytokine production (Liao et al., 2002), recombinant IL-19 treatment markedly decreased astrocyte immune responses to bacterial challenge or specific TLR ligands. Such an effect resembles the previously documented effects of IL-10 on glia and other immunologically active cells following microbial challenge (Fiorentino et al., 1989; Rasley et al., 2006). While the immunosuppressive effects of IL-19 occur at relatively high doses, they resemble the concentrations required to elicit biological effects in non-CNS cell types (e.g. Gabunia et al., 2012). This might be explained by the relatively low reported binding affinity of the IL-19R (Kd of approximately 1×10^{-7} M as determined by surface plasmon resonance) that is almost 100 fold lower than the binding affinities of IL-10 and IL-22 receptors for their respective ligands (Logsdon et al., 2012).

In contrast to the effects of IL-19 on astrocyte inflammatory mediator production, this cytokine did not significantly alter the immune responses of activated microglia. However, we have found that microglia are functionally responsive to IL-19, as determined by the ability of this cytokine to induce rapid reductions in the level of SOCS3 expression in these cells. In this regard, microglia appear to be similar to immune cells such as macrophages that respond to IL-19 despite the absence of the IL-19R α subunit. It is presently unknown how IL-19 might mediate IL-19R α -independent effects because this cytokine, unlike IL-20 and IL-24, is not known to signal through any other heterodimeric receptor combination (Dumoutier et al., 2001; Parrish-Novak et al., 2002). It remains to be determined whether an as yet unidentified receptor subunit associates with IL-19R α to bind IL-19 (as suggested by Gallagher et al., 2004), or if some cell type-specific factor(s) permits IL-19R β to be functional in the absence of other receptor subunits.

Taken together, the present study indicates that astrocytes produce IL-19 in response to bacterial pathogens, functionally express the cognate IL-19R receptor subunits, and are sensitive to the immunosuppressive effects of this IL-10 family member. The ability of IL-19 to modulate pro-inflammatory cytokine production following bacterial challenge suggests a negative feedback mechanism whereby TLR stimulation induces IL-19 release in addition to IL-6, TNF- α , and other proinflammatory cytokines, which acts to limit further

inflammatory mediator production. Such an effect could help to regulate and/or resolve CNS inflammation following infection in order to limit neuronal damage. Finally, we provide evidence for a novel IL-19R α transcript variant in brain tissue might function as a non-signaling decoy receptor for IL-19. Given the apparent decrease in receptor variant expression in infected brain tissue, it is tempting to speculate that decreased levels of truncated IL-19R α act in concert with increased IL-19 production to promote the immunosuppressive actions of this cytokine.

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FIGURE 1.

IL-19 mRNA expression is upregulated in the CNS following bacterial infection. Mice received vehicle solution (CON; animal numbers 1 through 3) or were infected by direct intracranial administration with 1×10^7 *S. pneumoniae* (INF; animal numbers 4 through 6). At 72 hours following challenge, whole brain tissue was processed and analyzed for IL-19 mRNA expression by semi-quantitative RT-PCR (Panel A) and quantitative real-time PCR (Panel B). Positive (LPS stimulated macrophages) and negative (no input cDNA) PCR controls (+ and –, respectively) are shown. Real-time PCR data is presented as relative levels of IL-19 mRNA expression normalized to expression of the housekeeping gene GAPDH.

FIGURE 2.

IL-19 expression by primary murine astrocytes is upregulated after exposure to bacterial LPS. Cells (2×10^6) were unstimulated or challenged with LPS (5 ng/mL) and 3, 6, or 12 hours following treatment, expression of mRNA encoding IL-19 was determined by semi-quantitative RT-PCR (Panel A) and quantitative real-time PCR (Panel B). Real-time PCR data are presented as relative levels of IL-19 mRNA expression normalized to expression of the housekeeping gene GAPDH. Levels of IL-19 protein were determined in supernatants after 3, 6, and 12 hours of culture in the absence (-) or presence (+) of LPS by specific capture ELISA (Panel C). Asterisks indicate a statistically significant difference from unstimulated cells at each time point ($P < 0.05$, $n=3$).

FIGURE 3.

Astrocytes demonstrate robust IL-19 production following exposure to disparate clinically relevant bacterial CNS pathogens and their components. Panel A: Murine astrocytes (mAST; 2×10^6 cells) were unstimulated (0) or challenged with *N. meningitidis* (Nm; MOI of 30:1, 24 hours), *B. burgdorferi* (Bb; MOI of 5:1, 8 hours) flagellin (Fl; 500 ng/mL, 24 hours) or polyI:C (pIC; 1 μ g/mL, 24 hours) and expression of mRNA encoding IL-19 was determined by semi-quantitative RT-PCR. Human astrocytes (hAST) were unstimulated (0) or challenged with *N. meningitidis* (MOI of 30:1) or *B. burgdorferi* (MOI of 30:1) for 12

hours prior to analysis of IL-19 mRNA expression. Panel B: Murine astrocytes (2×10^6 cells) were unstimulated (0) or challenged with *N. meningitidis* (Nm; MOI of 30:1), flagellin (Fl; 500 ng/mL) or polyI:C (pIC; 1 μ g/mL) for 24 hours and IL-19 protein release was determined by specific capture ELISA. Asterisks indicate a statistically significant difference from unstimulated cells ($P < 0.05$, $n=3$). Panel C: Murine astrocytes (2×10^6 cells) were unstimulated (0) or challenged with mixed *B. burgdorferi* (Bb; 1 or 5 μ g/mL) *N. meningitidis* (Nm; 1 or 10 μ g/mL) antigens for 4 hours prior to determination of mRNA expression encoding the IL-10 family members IL-19, IL-20, IL-22, and IL-24, and the inflammatory cytokine IL-6. Positive PCR controls for IL-19 (LPS stimulated murine macrophages), IL-20 (LPS stimulated murine macrophages), IL-22 (unstimulated mouse splenocytes), and IL-24 (unstimulated mouse thymocytes) are shown (+). Panels D and E: Murine astrocytes (2×10^6 cells) were unstimulated (0) or challenged with *S. aureus* (MOI of 1:1, 10:1, and 100:1) for 4 or 8 hours and IL-19 mRNA expression was determined by semiquantitative RT-PCR (Panel D) and quantitative real-time PCR (Panel E). Real-time PCR data are presented as relative levels of IL-19 mRNA expression normalized to expression of the housekeeping gene GAPDH. Asterisks indicate a statistically significant difference from unstimulated cells ($P < 0.05$, $n = 3$). Panel F: Primary murine microglia (2×10^6 cells) were unstimulated (0) or challenged with *N. meningitidis* (Nm; MOI of 1:1, 10:1 or 30:1), or LPS (5 ng/mL) for 12 hours and expression of mRNA encoding IL-19 was determined by semi-quantitative RT-PCR. A positive PCR control (LPS stimulated murine astrocytes) is shown (+).

FIGURE 4.

Astrocytes express mRNA encoding cognate IL-19 receptor subunits. Panel A: Mice received vehicle solution (C) or were infected by direct intracranial administration with 1×10^7 *S. pneumoniae* (I). At 48 hours following challenge, whole brain tissue was processed and analyzed for IL-19R α and IL-19R β mRNA expression by semi-quantitative RT-PCR. Panels B, C, and D: Murine astrocytes (2×10^6 cells) were unstimulated (0) or challenged with *S. aureus* (MOI of 1:1, 10:1, and 100:1) and IL-19R mRNA expression was determined at 4 and 8 hours by semi-quantitative RT-PCR (Panel B) and quantitative real-time PCR

(Panel C), and IL-19R protein levels were determined at 24 hours post-infection by immunoblot analysis (Panel D). Real-time PCR data are presented as relative levels of IL-19R α and IL-19R β mRNA expression normalized to expression of the housekeeping gene GAPDH. Asterisks indicate a statistically significant difference from unstimulated cells ($P < 0.05$, $n=3$). Representative immunoblots for IL-19R α and IL-19R β are shown and average levels of expression were determined by densitometric analysis normalized to housekeeping gene expression, and are presented in the bar graphs ($n=3$). Panel E: Human astrocytes (2×10^6 cells) were unstimulated (0) or challenged with *S. aureus* (MOI of 1:1 and 10:1) for 4 hours prior to analysis of IL-19 mRNA expression. Panel F: Primary murine microglia (2×10^6 cells) were unstimulated (0) or challenged with recombinant IL-19 (30 and 300 ng/mL) for 2 or 15 hours and expression of mRNA encoding IL-19R α and IL-19R β was determined by semi-quantitative RT-PCR.

FIGURE 5.

Expression of a novel truncated IL-19R α transcript variant in brain tissue. Panel A: Mice received vehicle solution (0) or were infected by direct intracranial administration with 1×10^7 *S. pneumoniae* (INF). At 48 hours following challenge, brain (B), skin (S), heart (H), and lung (L) tissue was processed and analyzed for IL-19R α transcript length by RT-PCR using a primer set designed to anneal to sequences in exon 3 and exon 5. B* indicates PCR performed on cDNA derived from brain tissue using a primer designed to anneal to a sequence predicted following exon 3–exon 5 junction. Panel B: Quantification of truncated

IL-19R α mRNA expression in uninfected (0) and infected (INF) brain tissue by real-time PCR. Data are presented as relative levels of variant IL-19R α mRNA expression normalized to expression of the housekeeping gene GAPDH. Asterisk indicates a statistically significant difference from unstimulated cells ($P < 0.05$, $n=3$). Panel C: PCR products yielded from uninfected brain tissue (B) and skin (S) cDNA by primer sets designed to span exons 1 to 3, 5 to 7, and 3 to 4. A negative PCR control (no input cDNA) is shown (-). Panel D: Proposed exon composition and sequence of the brain tissue-associated truncated IL-19R α transcript variant corresponding to the exon 3–exon 5 junction.

FIGURE 6.

IL-19 suppresses inflammatory cytokine production by activated primary astrocytes. Panel A: Murine astrocytes (Ast) or microglia (Mg) (2×10^6 cells) were unstimulated (0) or exposed to recombinant IL-19 (rIL-19; 30 or 300 ng/mL) for 2 hours and SOCS3 mRNA expression was determined by semi-quantitative RT-PCR. Panel B: Murine astrocytes (2×10^6 cells) were untreated or exposed to rIL-19 (30, 100, or 300 ng/mL) for 4 hours prior to challenge with *N. meningitidis* (Nm; MOI of 30:1), flagellin (Fl; 500 ng/mL), polyI:C (pIC; 1 μ g/mL) or vehicle control (0) for 24 hours and IL-6 and TNF- α protein release was

determined by specific capture ELISA. Panel C: Primary murine microglia (Mg) or EOC 13.31 microglia-like cells (EOC) (2×10^6 cells) were untreated or exposed to rIL-19 (30, 100, or 300 ng/mL) for 4 hours prior to challenge with polyI:C (pIC; 1 μ g/mL) or vehicle control (0) for 24 hours and TNF- α protein release was determined by specific capture ELISA. Asterisks indicate a statistically significant difference from unstimulated cells ($p < 0.05$, $n=3$).

TABLE 1

PCR Primers Employed

mRNA	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	CCATCACCATCTTCCAGGAGCGAG	CACAGTCTTCTGGGTGGCAGTGAT
mIL-19	ATGACCAACAACCTGCTGACATTC	CAGTTCTCCTAGAGACTTAAGGG
hIL-19	GGCAATGTCAGGAACAGAGG	AGCGGAATAAGACAGCCTGA
mIL-19R α exons 1–3	AACTGACAGCCCATGCAC	GTCTCAACAGAAAGGTCACAGTAG
mIL-19R α exons 3–4	GGCAGAAGAAATGGCTGAATG	CTGTCAGGGCAATAGAGATGG
mIL-19R α exons 3–5	AAAGTGAAGGCCATTTGGGAAGCC	TGGACACAATACAGAGTGTGGGC
mIL-19R α exons 5–7	CCCAACACTCTGTATTGTGTCC	GTCAAAGACTCTTGTGCCAATTTC
mIL-19R α variant	CTTTCTTGGAATGGTCCCAG	GTGAGGAAGACATACCAGAAGA
hIL-19R α	AAACACAAATTGGCCACCAGAGG	TCCAAAGTCCTGGCACACTGCTTC
mIL-19R β	AACCGAAATGCAACTGTCCTCACC	ACAAACGCAAACAGAGCTAGTGCC
hIL-19R β	ATGGCTTCCACCTGGTTATTGAGC	TGAAGCCAACAAAGGCAAACAGGG
mSOCS3	TTTCGCTTCGGGACTAGC	CGCTCAACGTGAAGAAGTG