The Role of IL-33 in the Brain During Neonatal Hypoxic Injury

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Abstract

This study investigated whether Interleukin (IL)-33 exerts anti-inflammatory effects on BV2 microglial cells exposed to hypoxic conditions. It was hypothesized that IL-33 would reduce pro-inflammatory cytokine expression under hypoxia. To test this, BV2 microglia were subjected to either hypoxic or normoxic conditions and treated with IL-33 at different time points. Gene expression and cytokine release were analyzed to assess inflammatory responses. Contrary to our hypothesis, IL-33 did not reduce inflammation under hypoxia. Instead, it appeared to enhance the expression of pro-inflammatory markers in some conditions, particularly under normoxia. These findings suggest that IL-33 may not have a consistent anti-inflammatory role in microglia and that its effects are highly context-dependent. Further research is needed to clarify the mechanisms by which IL-33 modulates microglial activation and to explore potential therapeutic implications in neuroinflammatory conditions.

Keywords: interleukin-33 (IL-33), microglia, hypoxia, proinflammatory cytokines, normoxia, neuroinflammation

The Role of IL-33 in the Brain During Neonatal Hypoxic Injury

Microglia, the resident immune cells of the central nervous system (CNS), are critical mediators of neuroinflammation and neuroprotection. Similar to other tissue-resident immune cells of the body, microglia continuously survey the brain for any signs of injury, stress or cell damage. Hypoxia, which is characterized by low oxygen levels, can activate microglia, resulting in the release of pro-inflammatory mediators that can either attenuate or contribute to neuronal damage. Neurons are particularly sensitive to any change in homeostasis, including hypoxia or ischemia, decreased oxygen or blood flow, or any increase in pro-inflammatory immune molecules. Recent studies suggest that interleukin-33 (IL-33), a member of the IL-1 cytokine family, plays a multifaceted role in modulating microglial activation under hypoxic conditions. This review explores the dual roles of IL-33 as a neuroprotective agent and pro-inflammatory mediator, highlighting its potential therapeutic implications. The goal of the proposed experiments was to determine whether IL-33 can attenuate the activation of microglia and production of other pro-inflammatory molecules following oxygen deprivation (hypoxia) *in vitro*, and what inflammatory markers are most significantly upregulated or downregulated, with the goal of understanding IL-33's potential role in neuroinflammation and as a potential therapeutic agent in conditions like hypoxia.

Literature Review

Microglial Activation and Neuroinflammation

Microglia play a central role in the immune response of the central nervous system (CNS). Microglia are capable of transitioning between pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes depending on certain environmental cues. During hypoxic

conditions, microglia tend to shift towards an M1 phenotype, characterized by the release of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β). The goal of this pro-inflammatory response is to recruit additional microglia or peripheral immune cells to the injured area to help clear away dead and dying cells. However, these cytokines can also contribute to neuronal injury and exacerbate neurodegeneration (Hoogland et al., 2015).

Hypoxia-Ischemia and Neuroinflammation

Hypoxic ischemic encephalopathy (HIE) is a neonatal brain injury characterized by a lack of oxygen or blood flow to the newborn's brain. Hypoxia-ischemia is one of the most serious causes of neurological deficits in children and can lead to significant long-term deficits including cerebral palsy, epilepsy, learning deficits, and vision/hearing impairments. The only current treatment of hypoxia-ischemia is therapeutic hypothermia, where the baby's body is cooled a few degrees, but this treatment must be initiated within six hours following birth, and up to 40% of treated infants still experience negative neurodevelopmental outcomes. This points to the need for additional therapeutic targets that might help eliminate the brain damage caused by hypoxia-ischemia.

In hypoxia-ischemia with encephalopathy (HIE), microglial activation is particularly pronounced. Bobbo et al. (2019) , found that hypoxia induces the upregulation of IL-6 in microglia. IL-6, in particular, is an interesting cytokine that can be up-regulated as part of a pro- or anti-inflammatory response, highlighting the pleiotropic or dual role of various cytokines in neuroinflammation versus neuroprotection. This dynamic response underscores the importance of understanding the molecular pathways underlying microglial activation, particularly those influenced by IL-33.

IL-33: A Multifunctional Cytokine

IL-33 is primarily expressed in astrocytes, oligodendrocytes, and endothelial cells in the CNS, and serves dual roles. Intracellularly, it functions as a nuclear factor that regulates gene expression. Extracellularly, it acts as an alarmin that signals tissue damage and initiates immune responses through the ST2 receptor (Italiani et al., 2018).

In multiple sclerosis models, IL-33 has been shown to promote M2 polarization in microglia, which in turn reduces demyelination and supports tissue repair (Chen et al., 2019). However, in Alzhemier's disease (AD) models, Il-33 exacerbated neuroinflammation by enhancing the M1 activation and increasing amyloid-beta deposition (Fu et al., 2016). This duality makes Il-33 an interesting target for studying its effects under hypoxic conditions.

IL-33 in Hypoxia-Induced Microglial Activation

Hypoxia significantly alters Il-33 signaling in the CNS. Under hypoxic conditions, IL-33 expression is upregulated, potentially increasing the inflammatory response. In a study on traumatic brain injury, Wicher et al (2017) discovered in his studies that IL-33 enhanced microglial involvement and changed the cytokine's expression, meaning it exacerbated inflammation in the acute phase of hypoxia. Similarly, Ravizza et al. (2008) found that hypoxia activates IL-1β pathways, which would induce neuroinflammation that could ultimately result in epilepsy following the initial hypoxic event.

These findings suggest that IL-33 may amplify the hypoxic activation of microglia by promoting M1 polarization and increasing the release of pro-inflammatory cytokines. For example, Tamagawa-Mineoka et al. (2014) discovered in his studies that high IL-33 levels correlate with increased inflammation severity in atopic dermatitis, a pattern that could potentially be present in hypoxia-induced neuroinflammation.

Anti-Inflammatory and Neuroprotective Roles

Despite IL-33's proinflammatory role, it can also promote neuroprotection under certain conditions. IL-33-induced M2 polarization of microglia has been seen in models of organ fibrosis, where it modulated inflammation and tissue remodeling (Kotsiou et al., 2018). In the CNS, this effect could mitigate hypoxia-induced neuroinflammation by helping to clear debris and support the survival of neurons.

In a study of IL-33 in pancreatic cancer, Kieler et al. (2018) has shown that IL-33/ST2 signaling modulates immune responses, which goes to suggest a more complex role in balancing the pro- and anti-inflammatory pathways. These findings suggest that IL-33's effects may be context-dependent, and may potentially reduce hypoxia-induced neuroinflammation by changing microglial activation states.

Upregulation of Pro-Inflammatory Cytokines

IL-33's interactions with microglia in hypoxic environments show that hypoxia induced microglial activation is associated with increased roles of IL-33 in neuroinflammation and neuroprotection. By examining findings from various studies, this review highlights IL-33's potential as both a therapeutic target and mediator of inflammatory responses.

Microglial Activation and the Role of IL-33

Under hypoxic conditions, microglia are activated to release inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-alpha (TNF-α). IL-33, which is released in response to cellular stress, can amplify or suppress these responses. For instance, IL-33 was shown to increase IL-6 production in microglia during hypoxia, highlighting its role in modulating inflammatory pathways (Bobbo et al., 2019). This interplay between hypoxia, IL-33, and

microglial activation is central to understanding the neuroinflammatory princesses underlying hypoxia-ischemia with encephalopathy (HIE).

The IL-33/ST2 Axis in Inflammatory Regulation

IL-33 signals through its receptor ST2, which exists in both membrane-bound (ST2L) and soluble (sST2) forms. IL-33/ST2 has been found to be associated with various inflammatory and fibrotic diseases throughout the body. In the brain, IL-33 binding to ST2L on microglia induces the release of cytokines, chemokines, and other inflammatory mediators. In an endometriosis mode, the upregulation of IL-33 was correlated with how severe the inflammation was, reinforcing its role in immune cell activation and cytokine production (Mbarik et al., 2015). Increased sST2 levels in the gut have also been linked to inflammation associated with chronic infections, highlighting the importance of IL-33 signaling in a number of inflammatory disease states (Mehraj et al., 2016).

In hypoxic conditions, the IL-33/ST2 pathway could potentially play a critical role in modulating microglial responses. Hypoxia-induced upregulation of IL-33 may worsen neuroinflammation by enhancing ST2-mediated signaling. However, IL-33 has also been shown to promote tissue repair through M2 polarization microglia, which are associated with anti-inflammatory and neuroprotective effects (Francos-Quijorna et al., 2016). This dual role reflects the complexity of IL-33/ST2 signaling in hypoxia-driven neuroinflammation.

IL-33 and Cytokine Regulation in Microglia

The release of proinflammatory cytokines is an indication of microglial activation under hypoxic conditions. IL-33 influences this process by regulating the expression of key cytokines such as IL-1β, IL-6, and TNF-α. In a study on systemic inflammation, IL-6 and TNF-α levels were found to be significantly elevated in cerebrospinal fluid, indicating central inflammation

(Enache et al., 2019). IL-33 has been shown to upregulate these cytokines in various models in both hypoxic and neurodegenerative conditions.

IL-33's effects on cytokine expression appear to depend on its concentration and the cellular context. In allergic asthma models, IL-33 was found to stimulate pro-inflammatory responses, which supports its potential as a therapeutic target (Salter et al., 2016). However, IL-33 signaling through ST2 was shown to reduce cytokine release in certain fibrotic conditions, indicating its potential for anti-inflammatory effects (Kotsiou et al., 2018). These findings suggest that IL-33 may act as a molecular switch, capable of amplifying or suppressing inflammation depending on the surrounding conditions.

Therapeutic Potential of IL-33 in Hypoxia-Induced Neuroinflammation

The therapeutic potential of IL-33 lies in its ability to modulate microglial responses and cytokine expression. In traumatic brain injury models, IL-33 reduced neuroinflammation by promoting M2 polarization of microglia and also enhancing tissue repair (Wicher et al., 2017). These findings suggest that IL-33-based therapies could be effective in reducing hypoxia-induced neuroinflammation.

However, IL-33's pro-inflammatory effects in other contexts must also be considered. In Alzheimer's disease models, IL-33 exacerbated amyloid-beta deposition and neuroinflammation, highlighting the need for context-specific interventions (Italiani et al., 2018). The small molecule activators of antioxidant pathways in microglia have shown to be active in reducing oxidative stress and inflammation. This suggests potential combinatorial approaches with IL-33-based therapies (Foresti et al., 2013).

Methodological Approaches to Study IL-33 and Hypoxia

Understanding the role of IL-33 in hypoxia-induced neuroinflammation requires strong methodological approaches. In vitro models using microglial cell lines such as BV2 cells are effective in isolating the effects of hypoxia and IL-33, without the effects of confounding variables. Researchers can examine changes in cytokine production and gene expression through hypoxia chambers that can simulate low oxygen conditions.

Studies have also used enzyme-linked immunosorbent assays (ELISA) to measure cytokine levels in cell culture media and provide insights into IL-33's effects on inflammatory markers (Bobbo et al., 2019). Additionally, RNA extraction and reverse transcription quantitative PCR (RT-qPCR) can be utilized to analyze the expression of inflammatory genes such as IL-1β and IL-6. These techniques allow for precise measurement of IL-33-mediated changes in microglial activation and inflammation.

Challenges and Future Directions

Despite its therapeutic potential, IL-33 presents several challenges as a target for neuroinflammation. Its dual role in promoting and also suppressing inflammation makes the development of IL-33-based therapies more complex. The lack of in vitro models for studying IL-33 in hypoxia-induced neuroinflammation also limits the understanding of its effects in complex biological systems.

Future research should focus on shedding light on the specific molecular mechanisms that underlie IL-33's dual role in microglial activation. Investigating the relationship between IL-33 and other cytokines, such as IL-4 and IL-10, could provide insights into its potential for shifting the microglia towards a neuroprotective phenotype (Francos-Quijorna et al., 2016). Additionally, exploring the effects of IL-33 in animal models of HIE could bridge the gap between in vitro findings and clinical applications.

Conclusion

IL-33 plays an important role in modulating microglial activation during hypoxia, with implications for neuroinflammatory and neurodegenerative diseases. Its dual role as a neuroprotective and pro-inflammatory mediator highlights its complexity as a therapeutic target. By combining findings from studies on IL-33/ST2 signaling, cytokine regulation, and microglial activation, this review underscores the need for context-specific interventions to harness IL-33's therapeutic potential. Further research is essential to translate these findings into effective therapies for hypoxia induced neuroinflammation and related conditions.

Methods

Research Approach and Research Method

A quantitative research approach was selected to investigate the effects of IL-33 on microglial activation under hypoxic conditions. This approach allows the measurement of the changes in cytokine levels and relative gene expression levels from microglial cells to be shown through statistical analysis, which provides objective and replicable data. By focusing on numerical outcomes, such as the cytokine concentrations and relative gene regulation, it also makes it so that a quantitative approach is the most appropriate method to test the hypothesis and draw reliable conclusions on IL-33's impact on microglial behavior in the presence of hypoxia.

The rationale behind a quantitative approach lies directly in its ability to analyze specific and measurable outcomes, like cytokine levels in pg/mL, which directly address the research question. A qualitative approach would not allow for the measurement of precise and numerical data that are needed to characterize the changes in inflammatory markers and microglial phenotypes. A quantitative approach ensures that the results are valid and generalizable within a controlled environment.

BV2 microglial cell culture

The study utilized an *in vitro* experimental approach with BV2 microglial cell lines (Cytion Cat. No. 305156, LOT16072). BV2 microglial cells are widely recognized as reliable substitutes for microglia, as they are derived directly from mouse microglia. They were chosen because they replicate the key aspects of microglial function under stress conditions or infection. The cells were rapidly thawed, counted and plated at a density of 7,600 cells per well in a 96-well plate, and fed with 300 µl of Dulbecco's Modified Eagle Medium (DMEM) that was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. This approach allows for multiple replicates and treatments within a single experiment.

BV2 cells are widely used in neuroinflammation research to isolate the specific response of microglial cells, which has been shown by Bobbo et al. (2019) and Wicher et al. (2017), which both used an experimental method with BV2 cells. This experimental method allows for precise control over *in vitro* conditions, such as hypoxia and IL-33 treatment, which would be more difficult to conduct *in vivo*. This approach also allows for the isolation of IL-33's specific effects under hypoxic conditions without the interference of other neural cell types and external factors, while also being able to quickly manipulate the timing and dose of either treatment or hypoxia without the use of any animals.

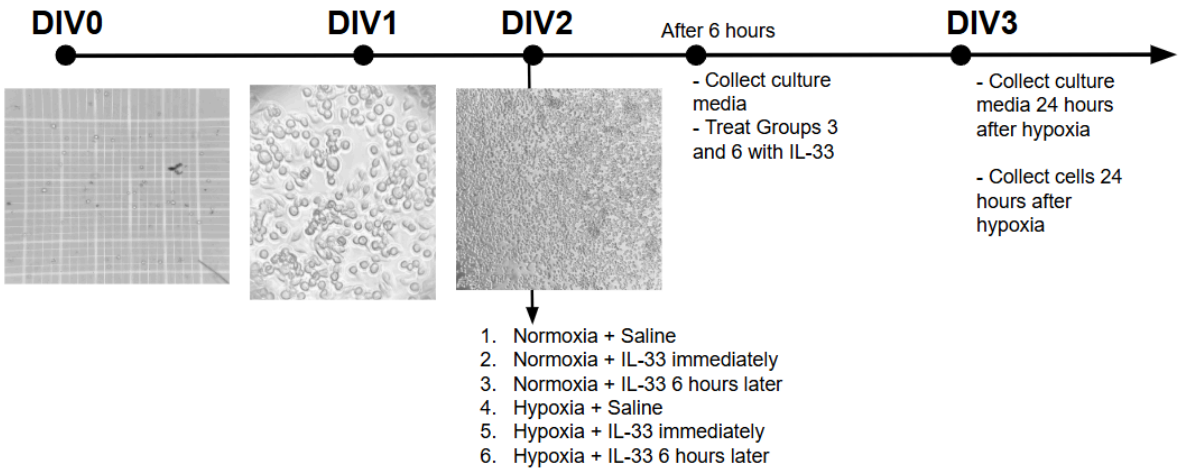
Hypoxia procedure and IL-33 treatment

To simulate hypoxia, BV2 cells were placed in a hypoxia chamber, which lowers oxygen levels to replicate oxygen deprivation. Cells were treated with IL-33 (2 ng/ul) after an adherence period of 48 hours. Supernatant media were collected for cytokine analysis at 6 and 24 hours after the start of hypoxia. Cells were harvested 24 hours after the start of hypoxia for gene

expression analysis utilizing reverse transcription quantitative polymerase chain reaction (RT-qPCR). For a full timeline of the experimental procedure see **Figure 1** below.

Figure 1

Experimental Timeline



Bobbo et al. showed that RT-qPCR is a highly sensitive method for recognizing cytokine changes and gene expression in microglia under hypoxic conditions. Similarly, Hoogland et al. (2015) showed that hypoxia induces microglia to release inflammatory cytokines, which emphasized the need for methods like RT-qPCR to record the markers accurately.

BV2 cells were used because they are a well established and widely used model for neuroinflammation research, and they closely replicate the behavior of microglial cells under stressful conditions, like hypoxia in this experiment. Unlike primary microglia, which require extraction from animal brains, BV2 cells provide a consistent and reproducible model that allows for controlled experimentation. Their established use in neuroinflammation research, including studies on IL-33 and hypoxia, ensures that findings are relevant to understanding microglial responses in disease conditions.

Content validity is ensured by using RT-qPCR, which is a standard and widely accepted method for measuring cytokine levels via their mRNA expression. This technique directly measures the ongoing transcription of important inflammatory markers that are relevant to the research question. Internal validity is maintained by conducting all experiments under controlled conditions, including regulation of oxygen levels, temperature, and IL-33 treatment concentrations. Using biological replicates also increases experimental validity. External validity is supported by using BV2 cells, which are commonly used in neuroinflammation studies, allowing results to be applicable to broader research on neuroinflammatory diseases.

Culture Procedure:

1. BV2 microglial cells are cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.
2. The cells are counted and seeded into 96 well plates at a density of 10,000 cells per well.
3. Hypoxia is induced by placing the cells in a hypoxia chamber set to 1% oxygen for a designated period.
4. Cells are treated with or without IL-33 (2ng/mL) following a 48 hour adherence period.
5. After treatment, RNA is extracted from cells for gene expression analysis using RT-qPCR.

Quantitative Real-Time PCR (qRT-PCR) analysis of the cytokine response

RNA was extracted from the cells using phenol-chloroform extraction, and RNA quality and concentration were measured on a Nanodrop. 250 ng RNA from each sample was converted to cDNA using Qiagen's reverse transcriptase kit. The samples were stored at -20 °C in the freezer until use. For the qRT-PCR, a master mix was prepared by adding all the components of the reaction, including SYBR Green Mix, Forward and Reverse primers, DNA template, and

Rnase Free water. Three negative controls were also prepared: one with no reverse transcriptase, one without template RNA, and just Rnase free water. The PCR plates or tubes were run according to the Qiagen or IDT protocols with the predetermined annealing temperatures (**Table 1**).

Table 1

Primer sequences/source and their corresponding annealing temperatures.

Genes of Interest	Catalog Number	Annealing Temp	NCBI Reference Sequence
18s rRNA	Qiagen Cat No. QT02448075	60℃	NR_003278
IL-1B	Qiagen Cat No. QT01048355	60℃	NM_008361
IL-6	Qiagen Cat No. QT00098875	60℃	NM_031168
TNFα	Qiagen Cat No. QT00104006	60℃	NM_013693
ST2	Qiagen Cat No. QT01761088	60℃	NM_001025602

Statistical analyses

The 18s gene served as the internal control in this study. For every reaction, the cycle threshold (Cq) value was measured to assess gene expression levels. Relative expression of the target genes was quantified using the 2ΔCq approach, which normalization performed against the sample with the lowest expression level. Samples were excluded if their normalized relative gene

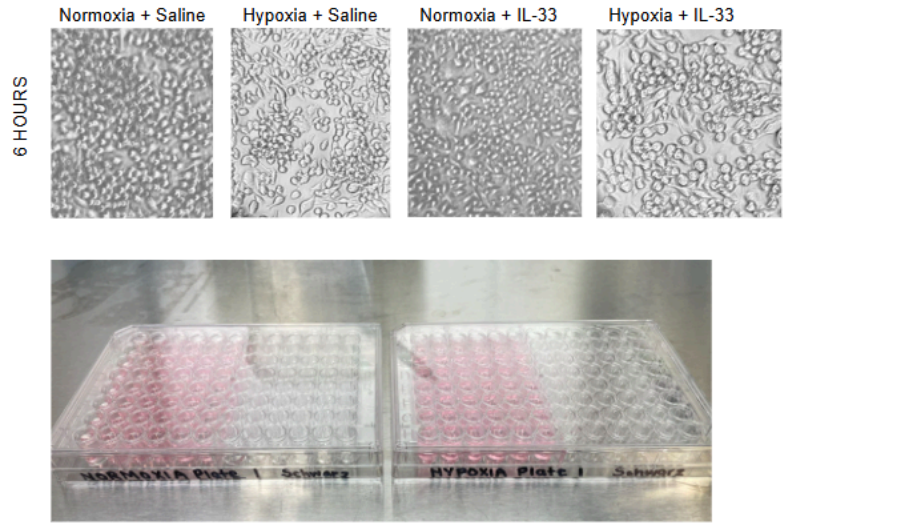
expression was two standard deviations greater than other samples within the same treatment group. However, no more than one sample was ever excluded from specific treatment groups at a given time point. Two-way ANOVA was used to compare the effects of hypoxia vs normoxia, and IL-33 treatment (saline, IL-33 immediately, or IL-33 6 hours after hypoxia) on cytokine expression. This statistical test is appropriate for analyzing interactions between two independent variables (hypoxia and IL-33) that can have multiple levels (e.g. time point of IL-33 treatment) on the dependent variables (cytokine levels and gene expression). Significant main effects and significant interactions of these factors were reported using $p < 0.05$. Significant interactions were followed up with Tukey's post hoc test ($p < 0.05$) to analyze individual differences between groups.

Results

BV2 microglial cells were plated and exposed to 1% hypoxia for 24 hours to determine the effect of the hypoxia on inflammatory gene expression. A subset of cells were treated with IL-33 just prior to the start of hypoxia. A second subset of cells were treated with IL-33 6 hours after the initiation of hypoxia. At the 6 hour time point, culture media was collected for future analysis of cytokine proteins that would be released into the media and images of the cells were taken (**Figure 2**).

Figure 2

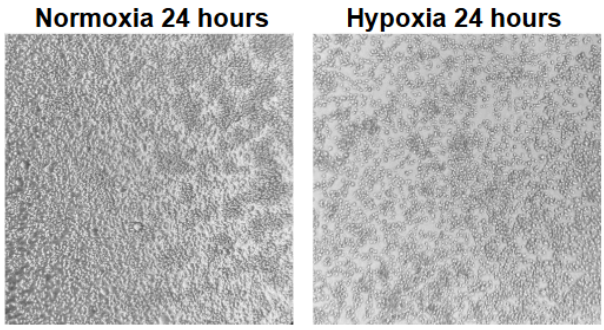
Experimental Setup & Methods



The cells were lysed and collected at 24 hours after the start of hypoxia to assess inflammatory gene expression, and images of the cells were collected just prior (**Figure 3**).

Figure 3

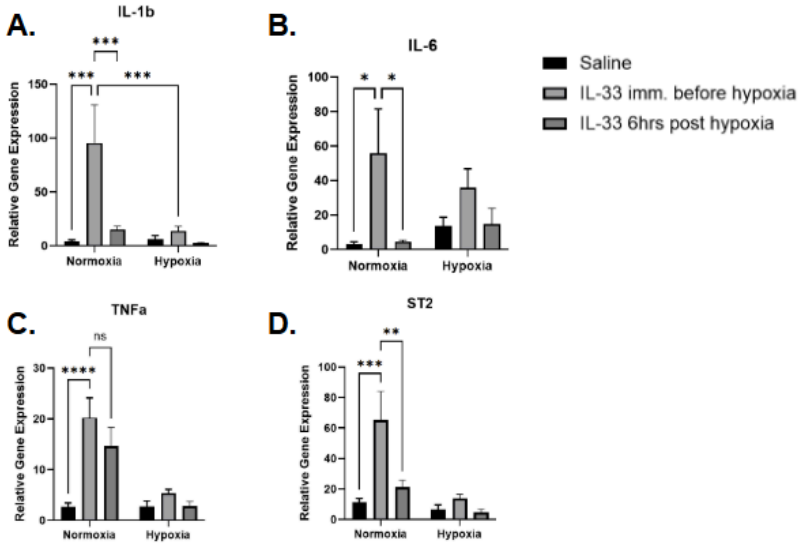
Microglial Morphology in Normoxia vs. Hypoxia (24 Hours)



The results of this gene expression analysis are represented in **Figure 4**.

Figure 4

IL-33 Alters Microglial Gene Expression in Normoxia but Not in Hypoxia



Analysis of the proinflammatory cytokine IL-1β gene expression in the microglial cells revealed a significant main effect of hypoxia ($F_{1,36}$=6.636, p=0.0142) and a significant main effect of IL-33 treatment ($F_{2,36}$=6.727, p=0.003). That said, the analysis also revealed a significant interaction of hypoxia and IL-33 treatment ($F_{2,36}$=4.42, p=0.019), where IL-33 significantly increased IL-1β expression in the microglia 24 hours after treatment compared to the saline treated control (p=0.0003) and compared to the cells that received IL-33 6 hours after the start of the experiment (p=0.008). There were no significant effects of IL-33 in the cells that had hypoxia for 24 hours (**Figure 4A**).

Analysis of the proinflammatory IL-6 gene expression in the microglial cells revealed no significant main effect of hypoxia ($F_{1,37}$=0.01, p=0.980) or interaction of hypoxia and IL-33 treatment ($F_{2,37}$=0.887, p=0.424). There was a significant main effect of IL-33 treatment alone ($F_{2,37}$=5.14, p=0.010) such that IL-33 significantly increased the expression of IL-6 in microglia (**Figure 4B**). Post hoc comparisons revealed that IL-33 significantly increased the expression of IL-6 in the normoxia cells 24 hours after exposure (p=0.016), but that IL-33 did not significantly

increase IL-6 in the normoxia cells after only 18 hours (p=0.997). Despite the main effect of IL-33 treatment, post hoc comparisons revealed that the IL-33 treatment did not significantly increase IL-6 in the hypoxia treated cells (p=0.497 vs saline).

Analysis of proinflammatory TNFα gene expression in the microglial cells revealed a significant main effect of hypoxia ($F_{1,39}$=19.27, p<0.0001) and a significant main effect of IL-33 treatment ($F_{2,39}$=8.169, p=0.001); however there was also a significant interaction of hypoxia and IL-33 treatment ($F_{2,39}$=5.15, p=0.014). Post hoc analyses revealed that IL-33 significantly increased TNFα in the normoxia cells both after 24 hours of treatment (p<0.0001) and after 18 hours of treatment (p=0.002). In contrast, IL-33 did not significantly increase TNFα in the hypoxia cells (p=0.776 saline vs IL-33 immediately; and p=0.999 saline vs IL-33 6 hours after the start of hypoxia; **Figure 4C**).

Analysis of ST2, which is the receptor for IL-33, revealed a significant main effect of hypoxia ($F_{1,37}$=10.87, p<0.000) and a significant main effect of treatment ($F_{2,37}$=6.78, p=0.003). There was, however, a significant interaction of hypoxia and IL-33 treatment ($F_{2,37}$=3.56, p=0.038). Post hoc comparisons revealed that IL-33 significantly increased the expression of ST2 after 24 hours of treatment, only in the normoxia cells (p=0.0003). In contrast, IL-33 did not significantly increase ST2 expression after only 18 hours of treatment in the normoxia cells (p=0.698). IL-33 also had no effect on ST2 expression in hypoxia cells, either 24 hours after treatment (p=0.841) nor 18 hours after treatment (p=0.989, **Figure 4D**).

Discussion

In summary, the results of these experiments indicate that contrary to the initial hypothesis, IL-33 was not anti-inflammatory in the context of hypoxia. Rather, these results

indicate that in control microglia that had normoxia for 24 hours, IL-33 significantly increased the expression of a variety of proinflammatory cytokines.

Neuroinflammation plays a major role in many neurological disorders, and hypoxia has been shown to exacerbate the inflammatory response microglia exhibit. Microglia are the resident immune cells of the central nervous system that are capable of responding to a variety of tissue injury and insults. IL-33 is an alarmin cytokine that has been shown to influence microglia gene expression and function, but how it plays a role in hypoxic conditions remains unclear. The main objective of this research study was to investigate how microglia are affected after being exposed to oxygen deprivation and whether treatment with IL-33 exerts anti-inflammatory cytokines following these hypoxic conditions. Contrary to the hypothesis, the results show that IL-33 did not provoke an anti-inflammatory response in hypoxic conditions. Instead, it significantly increased the expression of IL-1□, TNF-α, IL-6, and ST2 in cells exposed to normoxic conditions, but had little to no effect in cells exposed to hypoxic conditions. This suggests that IL-33's effects on inflammation is dependent on the conditions, and that hypoxia alters the microglial response to IL-33.

These results challenge the idea that IL-33 plays an anti-inflammatory role in microglia, particularly under hypoxic conditions. While prior studies have suggested that IL-33 can act as an anti-inflammatory mediator in certain neuroinflammatory contexts (Gadani et al., 2015), these findings suggest that hypoxia may change the role of IL-33 as a regulatory cytokine. Since the inflammatory markers IL-1□, IL-6, and TNF-α increased in normoxic cells, it indicates that IL-33 could enhance the inflammatory response under normal oxygen conditions instead of suppressing it. This reveals many implications for the IL-33 based therapeutic solutions for hypoxia related neuroinflammatory conditions. If IL-33 doesn't reduce inflammation caused by

hypoxia, treatments that target IL-33 signaling may need to redirect and be tailored towards other disease contexts (Fu et al., 2016).

Furthermore, ST2, IL-33's receptor, was significantly upregulated in normoxia but not hypoxia, which suggests that hypoxia could interfere with IL-33 receptor signaling, thereby potentially limiting its effects. ST2 is the primary receptor that IL-33 acts through, and its reduced expression in hypoxic conditions could potentially explain the lack of response to the IL-33 treatment in low oxygen conditions. Hypoxia-induced metabolic shifts toward glycolysis have been previously shown to influence immune signaling pathways (He et al., 2022), which could further contribute to IL-33's signaling being changed. This raises important questions about how exactly hypoxia affects IL-33 signaling pathways in microglia, warranting further investigation.

Interpretations and Limitations of the Current Study

While the data suggests that hypoxia alters IL-33 signaling in microglia, alternative explanations should also be taken into consideration. One possibility could be the timing of IL-33 administration. In the study conducted, IL-33 was administered either immediately or six hours after hypoxia, and its effects were measured after 24 hours. It is possible that different time points of IL-33 or different observation periods could lead to different results.

Another potential explanation for our current findings is the severity of the hypoxia that was used in the current study. The cells were exposed to 1% oxygen deprivation for 24 hours, which was a very severe level of hypoxia, and an extended period of time compared to other studies which use between 1-5% oxygen for 2-24 hours of time. When extracting the RNA from the hypoxic cells, many of them had very low levels of RNA, with poor RNA quality measurements. This suggests potential cell death of the hypoxic cells prior to collection, which

would mean the hypoxia might have been too strong. That said, we took images of the cells just prior to collection, after 24 hours of hypoxia, and found no over differences in how the cells looked (**Figure 3**). Nonetheless, the hypoxic cells may have been in an earlier state of dying that resulted in degradation of the RNA prior to collection. We would predict that different strengths of oxygen deprivation, perhaps levels that are less severe, could yield different results about how IL-33 affects microglial function.

Additionally, hypoxia-induced metabolic changes in microglia could have affected their ability to to respond to IL-33. Hypoxia is a cellular stressor, and hypoxia is known to shift cellular function toward glycolysis, as a method to cope with the metabolic stress, which can indirectly influence inflammatory signaling pathways by diverting cellular energy away from these other functions. If hypoxia-induced metabolic changes interfered with the IL-33 receptor expression or even downstream signaling, it could explain why IL-33 had little to no effect on hypoxia-exposed cells, as the cells worked harder to cope with the metabolic stress of the oxygen deprivation.

While this study provides valuable insights into the role of IL-33 in hypoxia-induced neuroinflammation, several limitations should be acknowledged. One limitation is that the study used an *in vitro* model of microglial activation, which may not fully capture the complexities of the brain going through IL-33 treatment. The brain includes a variety of interactions between neurons, astrocytes, microglia and many other immune cells that might ultimately influence IL-33 signaling in ways that were not accounted for in this study using microglial cells alone.

Another limitation is the lack of protein level analysis of the inflammatory cytokines. While the gene expression changes of IL-1β, IL-6, TNF-α, and ST2 were measured, and we predict that these changes in mRNA are likely to be translated into proteins, it is possible that

post-transcriptional mechanisms could have influenced the final protein levels of these cytokines that are produced and secreted by the cells. Future studies could include ELISA or Western blot analyses, which could add additional information that could help to better understand the gene expression results demonstrated here.

Future Directions and Considerations

Based on the current findings of this study, there are several opportunities for future research. An important area that should be addressed is investigating the molecular mechanisms of how hypoxia changes IL-33 signaling in microglia. Future studies could explore whether and how hypoxia will affect IL-33's receptor expression, downstream signaling pathways, or metabolic states that influence microglial responsiveness to IL-33.

Another potential direction is exploring IL-33's role in hypoxia-induced neuroinflammation using animal models. In vivo studies could help determine whether the effects observed in the cultured microglia hold true just outside of the cell culture and can be translated to a whole-organism context. Also, studies seeing how IL-33 interacts with other inflammatory markers in hypoxic conditions could provide insights into its broader role in neuroinflammation.

Finally, future research should examine whether IL-33's effects differ across various other disease models such as stroke, traumatic brain injury, and also neurodegenerative disorders. Understanding the context-dependent nature of IL-33 signaling could inform therapeutic strategies for modulating neuroinflammation across different pathological conditions.
By addressing these questions, future studies can build on the findings of this research and further elucidate IL-33's role in hypoxia-induced neuroinflammation.

Conclusion

This study shows that IL-33 does not have an anti-inflammatory effect on hypoxia exposed microglia, which challenges the notion that it acts as an anti-inflammatory agent. Instead, IL-33 significantly raised inflammatory cytokines IL-1β, IL-6, TNA-α and ST2 under normoxia, but had a negligible effect on them during hypoxia, suggesting oxygen levels influence its signaling. This reveals a critical area of a gap in knowledge regarding the function of IL-33 in neuroinflammation and suggests that hypoxia could change its receptor expression or downstream signaling pathways. These findings have profound implications for IL-33-mediated therapies, predicting that its therapeutic potential would be oxygen-dependent and requires reconsideration in the setting of hypoxia-related neurological diseases. Further research must address the molecular causes of such changes and ascertain whether these actions persist in in vivo models, potentially informing the basis for the development of more targeted neuroinflammatory therapies.

References

Bobbo, V. C. D., Jara, C. P., Mendes, N. F., Morari, J., Velloso, L. A., & Araújo, E. P. (2019). Interleukin-6 Expression by Hypothalamic Microglia in Multiple Inflammatory Contexts: A Systematic Review. BioMed research international, 2019, 1365210. https://doi.org/10.1155/2019/1365210

Chen, Z., Xu, N., Dai, X., Zhao, C., Wu, X., Shankar, S., Huang, H., & Wang, Z. (2019). Interleukin-33 reduces neuronal damage and white matter injury via selective microglia M2 polarization after intracerebral hemorrhage in rats. Brain research bulletin, 150, 127–135. https://doi.org/10.1016/j.brainresbull.2019.05.016

Enache, D., Pariante, C. M., & Mondelli, V. (2019). Markers of central inflammation in major depressive disorder: A systematic review and meta-analysis of studies examining cerebrospinal fluid, positron emission tomography and post-mortem brain tissue. Brain, behavior, and immunity, 81, 24–40. https://doi.org/10.1016/j.bbi.2019.06.015

Foresti, R., Bains, S. K., Pitchumony, T. S., de Castro Brás, L. E., Drago, F., Dubois-Randé, J.-L., Bucolo, C., & Motterlini, R. (2013). Small molecule activators of the Nrf2-HO-1 antioxidant axis modulate heme metabolism and inflammation in BV2 microglia cells. *Pharmacological Research*, *76*, 132–148. https://doi.org/10.1016/j.phrs.2013.07.010

Francos-Quijorna, I., Amo-Aparicio, J., Martinez-Muriana, A., & López-Vales, R. (2016). IL-4 drives microglia and macrophages toward a phenotype conducive for tissue repair and functional recovery after spinal cord injury. *Glia*, *64*(12), 2079–2092. https://doi.org/10.1002/glia.23041

Fu, A. K., Hung, K. W., Yuen, M. Y., Zhou, X., Mak, D. S., Chan, I. C., Cheung, T. H., Zhang, B., Fu, W. Y., Liew, F. Y., & Ip, N. Y. (2016). IL-33 ameliorates Alzheimer's disease-like

pathology and cognitive decline. Proceedings of the National Academy of Sciences of the United States of America, 113(19), E2705–E2713. https://doi.org/10.1073/pnas.1604032113

Gadani, S. P., Walsh, J. T., Smirnov, I., Zheng, J., & Kipnis, J. (2015). The glia-derived alarmin IL-33 orchestrates the immune response and promotes recovery following CNS injury. Neuron, 85(4), 703–709. https://doi.org/10.1016/j.neuron.2015.01.013

He, D., Xu, H., Zhang, H., Tang, R., Lan, Y., Xing, R., Li, S., Christian, E., Hou, Y., Lorello, P., Caldarone, B., Ding, J., Nguyen, L., Dionne, D., Thakore, P., Schnell, A., Huh, J. R., Rozenblatt-Rosen, O., Regev, A., & Kuchroo, V. K. (2022). Disruption of the IL-33-ST2-AKT signaling axis impairs neurodevelopment by inhibiting microglial metabolic adaptation and phagocytic function. Immunity, 55(1), 159–173.e9. https://doi.org/10.1016/j.immuni.2021.12.001

Hong, H., Liao, S., Chen, F., Yang, Q., & Wang, D. (2020). Role of IL‐25, IL‐33, and TSLP in triggering united airway diseases toward type 2 inflammation. *Allergy*, *75*(11), 2794–2804. https://doi.org/10.1111/all.14526

Hoogland, I. C. M., Houbolt, C., van Westerloo, D. J., van Gool, W. A., & van de Beek, D. (2015). Systemic inflammation and microglial activation: systematic review of animal experiments. *Journal of Neuroinflammation*, *12*(1). https://doi.org/10.1186/s12974-015-0332-6

Italiani, P., Puxeddu, I., Napoletano, S., Scala, E., Melillo, D., Manocchio, S., Angiolillo, A., Migliorini, P., Boraschi, D., Vitale, E., & Di Costanzo, A. (2018). Circulating levels of IL-1 family cytokines and receptors in Alzheimer's disease: new markers of disease

progression? *Journal of Neuroinflammation*, *15*(1). https://doi.org/10.1186/s12974-018-1376-1

Kieler, M., Unseld, M., Johann Wojta, Kaider, A., Bianconi, D., Svitlana Demyanets, & Prager, G. W. (2018). Plasma levels of interleukin-33 and soluble suppression of tumorigenicity 2 in patients with advanced pancreatic ductal adenocarcinoma undergoing systemic chemotherapy. *Medical Oncology*, *36*(1). https://doi.org/10.1007/s12032-018-1223-3

Kotsiou, O. S., Gourgoulianis, K. I., & Zarogiannis, S. G. (2018). IL-33/ST2 Axis in Organ Fibrosis. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.02432

Mbarik, M., Kaabachi, W., Henidi, B., Sassi, F. H., & Hamzaoui, K. (2015). Soluble ST2 and IL-33: Potential markers of endometriosis in the Tunisian population. *Immunology Letters*, *166*(1), 1–5. https://doi.org/10.1016/j.imlet.2015.05.002

Mehraj, V., Jenabian, M.-A., Ponte, R., Lebouché, B., Costiniuk, C., Thomas, R., Baril, J.-G., LeBlanc, R., Cox, J., Tremblay, C., & Routy, J.-P. (2016). The plasma levels of soluble ST2 as a marker of gut mucosal damage in early HIV infection. *AIDS*, *30*(10), 1617–1627. https://doi.org/10.1097/qad.0000000000001105

Osimo, E. F., Pillinger, T., Rodriguez, I. M., Khandaker, G. M., Pariante, C. M., & Howes, O. D. (2020). Inflammatory markers in depression: A meta-analysis of mean differences and variability in 5,166 patients and 5,083 controls. *Brain, Behavior, and Immunity*, *87*. https://doi.org/10.1016/j.bbi.2020.02.010

Ravizza, T., Gagliardi, B., Noé, F., Boer, K., Aronica, E., & Vezzani, A. (2008). Innate and adaptive immunity during epileptogenesis and spontaneous seizures: Evidence from experimental models and human temporal lobe epilepsy. *Neurobiology of Disease*, *29*(1), 142–160. https://doi.org/10.1016/j.nbd.2007.08.012

Salter, B. M., Oliveria, J. P., Nusca, G., Smith, S. G., Tworek, D., Mitchell, P. D., Watson, R. M., Sehmi, R., & Gauvreau, G. M. (2016). IL-25 and IL-33 induce Type 2 inflammation in basophils from subjects with allergic asthma. Respiratory research, 17, 5. https://doi.org/10.1186/s12931-016-0321-z

Tamagawa-Mineoka, R., Yasutaro Okuzawa, Masuda, K., & Norito Katoh. (2014). Increased serum levels of interleukin 33 in patients with atopic dermatitis. *Journal of the American Academy of Dermatology*, *70*(5), 882–888. https://doi.org/10.1016/j.jaad.2014.01.867

Wicher, G., Wallenquist, U., Lei, Y., Enoksson, M., Li, X., Fuchs, B., Abu Hamdeh, S., Marklund, N., Hillered, L., Nilsson, G., & Forsberg-Nilsson, K. (2017). Interleukin-33 Promotes Recruitment of Microglia/Macrophages in Response to Traumatic Brain Injury. *Journal of Neurotrauma*, *34*(22), 3173–3182. https://doi.org/10.1089/neu.2016.4900

Yeon Sik Hong, Su Jin Moon, Young Bin Joo, Chan Hong Jeon, Mi La Cho, Ji Hyeon Ju, Oh, H., Yu Jung Heo, Sung Hwan Park, Ho Youn Kim, & Jun Ki Min. (2011). Measurement of Interleukin-33 (IL-33) and IL-33 Receptors (sST2 and ST2L) in Patients with Rheumatoid Arthritis. *Journal of Korean Medical Science*, *26*(9), 1132–1132. https://doi.org/10.3346/jkms.2011.26.9.1132