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Glia Maturation Factor Induces Interleukin-33 Release from Astrocytes: Implications for Neurodegenerative Diseases

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Abstract

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Multiple sclerosis (MS) involve activation of glial cells and release of inflammatory mediators leading to death of neurons. Glia maturation factor (GMF) is up-regulated in the central nervous system (CNS) in these neurodegenerative diseases. Interleukin-33 (IL-33) is highly expressed constitutively in the CNS. We have treated mouse astrocytes, mixed culture with glial cells and neurons, and only neurons with GMF and/or IL-33 *in vitro*. Both GMF and IL-33-induced chemokine (C-C motif) ligand 2 (CCL2) release in a dose and time-dependent manner. We report that GMF induced IL-33 release, and that IL-33 augments GMF-induced TNF- α release from mouse astrocytes. IL-33 induces CCL2, TNF- α and nitric oxide release through phosphorylation of ERK in mouse astrocytes. Incubation of mixed culture containing glial cells and neurons or only neuronal culture with IL-33 reduced the number of neurons positive for microtubule-associated protein 2. In conclusion, IL-33 augments GMF-mediated neuroinflammation and may provide a new drug target for neurodegenerative and autoimmune diseases.

Keywords

CCL2; Glia maturation factor; Interleukin-33; Microtubule-associated protein 2; Neurodegenerative diseases; TNF- α

Introduction

Inflammatory response of glial cells could exacerbate pathogenesis of neurodegenerative and autoimmune diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Multiple sclerosis (MS). In these diseases, inflammation occurs in order to clean up the lesion and to limit disease progression. However, prolonged and sustained inflammatory reactions could cause cytotoxic effects and increase the severity of the disease. Immunoreactive glial cells release proinflammatory cytokines, chemokines and deleterious free radicals in neurodegenerative diseases (Monnet-Tschudi et al. 2011). High levels of proinflammatory mediators in the brain cause neuronal damage leading to cognitive impairment.

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Conflicts of interest: none.

Glia maturation factor (GMF), a potent proinflammatory factor was first discovered, purified, sequenced and sub-cloned from bovine brain in our laboratory (Lim et al. 1989; Kaplan et al. 1991). We have previously shown the up regulation of GMF expression in the central nervous system (CNS) of neurodegenerative and autoimmune diseases (Zaheer et al. 2011). GMF is involved in the proinflammatory mediator release from glial cells (Zaheer et al. 2007). Interleukin-33 (IL-33) is a multifunctional cytokine belonging to the IL-1 cytokine family. Currently, there is increasing focus on the pathophysiological role of IL-33 in the CNS, where it is expressed in astrocytes but not in the cortical neurons or microglia (Yasuoka et al. 2011). Recently, IL-33 has been reported to be increased in MS (Christophi et al. 2012) and its animal model experimental autoimmune encephalomyelitis (EAE) (Li et al. 2012).

IL-33 function as a novel alarmin (released after cell injury) and altering the immune system to infections, tissue damage and necrosis (Hsu et al. 2010). IL-33 is highly expressed and is proinflammatory in the CNS as glial cells express IL-33 receptors (ST2), and respond by proliferating and releasing inflammatory molecules such as tumor necrosis factor- α (TNF- α), IL-1 β and nitric oxide (Yasuoka et al. 2011). IL-1 β and TNF- α are known to be elevated in the brains of neurodegenerative diseases, and TNF- α and IL-1 β in turn are potent inducers of IL-33 expression. In the CNS, IL-33 acts on microglia to proliferate, secrete cytokines and chemokines, and enhance phagocytosis (Yasuoka et al. 2011). The present study was carried out to demonstrate the association of GMF and IL-33 in the exacerbation of inflammatory factors release from mouse primary astrocytes.

Methods

Mouse astrocytes culture

Pregnant C57BL/6 mice were purchased (Charles River, Wilmington, MA) and sacrificed on 16–17th day to get the embryos. Primary cultures of astrocytes were developed as we have described previously (Zaheer et al. 2001; Zaheer et al. 2002) but with slight modifications. Astrocytes were grown in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Ham) (DMEM F12; Life Technologies, Grand Island, NY) containing 5–10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD) and 1% penicillin/streptomycin (Life Technologies) at 37°C in a 5% CO₂ and 95% air atmosphere in tissue culture flasks (Costar, Corning Incorporated, Corning, NY). Mouse astrocytes grown in this procedure were >98% positive for glial fibrillary acidic protein (GFAP), a specific marker for astrocytes. Animals were cared and used according to the guidelines of Institutional Animal Care and Use Committee (IACUC).

Mouse mixed cell (glia and neurons) culture

Primary mixed glia and neuronal cell cultures were established from the mouse embryonic brain by trypsin dissociation as reported previously (Lee et al. 2004) but with slight modifications. These cells were cultured in DMEM, 5% FBS, 5% horse serum and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO₂ and 95% air atmosphere. In this procedure, we obtained about 55% of glia (mostly astrocytes) and 45% of neurons in the culture.

Mouse neuronal culture

Primary cerebral cortical neuronal cultures were prepared from 15 or 16-day fetal mouse brain and cultured in Neurobasal medium (Life Technologies) containing B27 (Life Technologies), 2 mM L-glutamine (Sigma, St. Louis, MO) and 1% penicillin/streptomycin at 37°C in a humidified 5% CO₂ and 95% air atmosphere (Zaheer et al. 2007). Neurons were cultured on poly-D-lysine (Sigma) coated cover glass in 24 well tissue culture plates

(Costar, Corning Incorporated) to use for Microtubule-associated protein 2 (MAP2) immunocytochemical staining for the neuronal morphologic analysis. This culture represents a nearly pure neuronal population.

Effect of IL-33 on mouse astrocytes - Chemokine (C-C motif) ligand 2 (CCL2), IL-33 and TNF- α measurement by enzyme-linked immunosorbent assay (ELISA) and Nitric acid assay

Confluent astrocytes (2 weeks-cultured) were plated at 0.5×10^6 cells /500 μ l complete medium/well in 24 well tissue culture plates. Then the cells were incubated with recombinant mouse GMF- β (99% homologous with human GMF- β), a custom made protein (Kaplan et al. 1991) or recombinant mouse IL-33 (R&D System, Minneapolis, MN) for dose-response and time-course studies as indicated in the results and legend sections. GMF used in this experiment was purified by Gel filtration and was endotoxin free. In the dose-response and time-course studies, we first incubated the longer time point group, and the shorter time point group was stimulated lastly. This incubation procedure was followed to stop the reaction of all time point groups at the same time to avoid the difference in cell conditions such as differentiation and releasing capacity. After the incubation period was over, the culture supernatant media were collected from the wells, centrifuged and the media from the tubes were collected and stored at -80°C for the proinflammatory mediator assays. In another set of experiments, mouse astrocytes were also incubated with various concentrations of GMF or IL-33 for TNF- α release. Additionally, mouse astrocytes were also incubated with GMF with or without IL-33 and TNF- α release was measured in the supernatant media. The working concentrations of GMF and IL-33 were prepared in sterile 0.1% BSA PBS freshly just before use on the day of experiment for the stimulation. The vehicle control treatment was carried-out with plain medium containing equal amount 0.1% BSA in PBS. IL-33, TNF- α and CCL2 were assayed in the media using commercial ELISA kits (DuoSet kits, R&D Systems). Total nitric oxide was measured in the supernatant media using commercial assay kit (Enzo Life Sciences, Plymouth Meeting, PA) and read with a Microplate reader at 540 nm as per the kit procedure.

Effect of IL-33 on mixed cell culture and neuronal culture

In order to study the direct effect of IL-33 on brain cells, we have incubated mixed glia culture, and neuronal culture over the cover-glass with recombinant mouse IL-33 for 48 hrs. Then the cover glass with cells were removed from the culture plate and washed in phosphate buffered saline (PBS; Life Technologies). Following this step, the cells (mixed culture and neuronal culture) were immunostained for MAP2 to evaluate the neuronal morphology as previously reported (Marx et al. 2001). Briefly, cover glass slides with cells were fixed with 4% paraformaldehyde in PBS (USB Corporation, Cleveland, OH) for 15 min at room temperature. Then the slides with cells were blocked (5% normal goat serum) and then incubated with mouse MAP2 (1:500 dilutions) monoclonal antibody (Chemicon International, Temecula, CA) at 4°C overnight, goat anti-mouse biotinylated IgG secondary antibody for 1 hr at room temperature, avidin-biotin-complex (ABC) reagent for 30 min and finally with ImmPACT DAB Peroxidase Substrate (both were from Vector Laboratories, Burlingame, CA) solution for 5 min. Development of brown color indicated positive reaction for MAP2 in the neurons. The slides were washed in PBS in between the incubations. The slides were then mounted with Permount (Fisher Scientific, Fair Lawn, NJ) and observed under the microscope and photographed. In another set of cultures, neurons were also grown on poly-D-lysine (Sigma) coated 25 cm^2 tissue culture flasks (Costar, Corning Incorporated) and incubated with IL-33 (10 ng/ml) for 48 hrs. After this incubation period was over, the neurons in the flasks were directly observed under the microscope and photographed (un stained pictures).

Effect of IL-33 on ERK phosphorylation in the mouse astrocytes

Mouse astrocytes were seeded at 1×10^6 cells/ml in the tissue culture flasks (25cm^2) and stimulated with IL-33 (20 ng/ml) for 0 to 60 min at 37°C . Then the cells were lysed in RIPA cell lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% NP-40, 0.5% deoxycholate) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA) and then 25 μg proteins was subjected to SDS-PAGE on 12% gels. The membrane was probed for phospho ERK using rabbit polyclonal phospho-p44/42 MAPK (ERK1/2) antibody (Cell Signaling Technology,) followed by total ERK using rabbit polyclonal p44/42 MAPK (ERK1/2) antibody (Cell Signaling Technology) and goat anti-rabbit-IgG-HRP secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were also probed for β -actin to confirm equal protein loading. Densitometry of immunoblots was performed using Image J software (National Institutes of Health, Bethesda, MD). We then determined densitometric ratios of phospho ERK to the total ERK and normalized to unstimulated control cells. Control untreated cell densitometric value was considered as 1 and then compared with IL-33 treated cells.

Statistical analysis

All the results were analyzed using GraphPad InStat 3 software. Data were presented as mean $\pm$ SEM and analyzed using One-way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison test to evaluate significant differences between groups. Only ANOVA and Tukey-Kramer were used unless otherwise mentioned. Unpaired t-test was used when comparing only two groups/conditions. A p-value less than 0.05 was considered statistically significant.

Results

Dose-response and time-course effect of GMF and IL-33 on CCL2 release from mouse astrocytes

Among molecules produced during neuroinflammation associated with neuronal death, CCL2 seemed to be a very important chemotactic chemokine. Therefore, we first examined if GMF and IL-33 stimulates CCL2 release from cultured mouse primary astrocytes *in vitro*. In dose-response and time-course studies, astrocytes were incubated with recombinant mouse GMF at 5 to 200 ng/ml concentrations for 2 to 24 hrs at 37°C and assayed the culture supernatant media for CCL2 release by ELISA. We report that GMF induces astrocytes to release significantly ($p < 0.05$) increased amount of CCL2 (Fig. 1A, $n=3$) in a dose-dependent and time-dependent manner as compared to unstimulated control astrocytes. GMF as low as 25 ng/ml concentration significantly ($p < 0.05$) induced CCL2 release (266.6 ± 56.5 pg/ml) from astrocytes when compared with untreated control cells (88.1 ± 1.6 pg/ml) at 24 hrs incubation period (Fig. 1A). Next, in another dose-response and time-course studies (2 to 48 hrs), mouse primary astrocytes were incubated with various concentrations of recombinant mouse IL-33 (1 to 200 ng/ml) *in vitro* and the culture supernatant media were assayed for CCL2 release by ELISA. We report that IL-33 induces mouse primary astrocytes to release significantly ($p < 0.05$) increased levels of CCL2 (Fig. 1B) as compared to unstimulated control cells ($n=3$) in a dose-dependent manner. Interestingly, IL-33 as low as 1 ng/ml concentration significantly induced CCL2 release (299.1 ± 29.9 pg/ml) when compared to unstimulated control astrocytes (79.2 ± 17.7 pg/ml) at 6 hrs incubation time period (Fig. 1B).

GMF and IL-33 induces TNF- α release from mouse astrocytes

We then studied if GMF and IL-33 independently induces TNF- α release from mouse astrocytes in vitro. Mouse astrocytes were treated with various concentrations of GMF (25 to 200 ng/ml) or IL-33 (1 to 50 ng/ml) for 24 hrs and the TNF- α release was measured in the supernatant media by ELISA (n=4). GMF as well as IL-33 -induced significant release of TNF- α from astrocytes in a dose-dependent manner. GMF significantly ($p<0.05$) induced TNF- α release when compared to control cells (Fig. 2A) from 50 ng/ml concentration onwards. In this experiment, IL-33 induced significant TNF- α release ($p<0.05$) at 10, 25 and 50 ng/ml concentrations in a dose-dependent manner when compared to untreated control cells (Fig. 2B). In another separate experiment, mouse astrocytes were first pre-incubated with GMF (50 ng/ml) for 15 min followed by incubation with IL-33 (10 ng/ml) for 24 hrs (n=4) in vitro and the supernatant media were assayed for TNF- α (Fig. 2C). We report that IL-33 augments GMF-induced TNF- α release from 441.3 ± 59.4 pg/ml to 752.4 ± 43.0 pg/ml from the astrocytes ($p<0.001$) (Fig. 2C). IL-33 alone induced significant release of TNF- α (76.3 ± 9.2 pg/ml) when compared to the release from untreated control cells (21.6 ± 4.3 pg/ml, $p<0.05$; unpaired t test) (Fig. 2C).

GMF induces IL-33 expression in mouse astrocytes

We then investigated to know if GMF induces the release of IL-33 from mouse primary astrocytes in vitro. In this experiment, astrocytes were incubated with recombinant mouse GMF (50 ng/ml) for 24 hrs and IL-33 release was measured in the cell culture supernatant media by ELISA. We report that GMF significantly induced high amount of IL-33 release (583 ± 69 pg/ml) from astrocytes when compared to unstimulated control cells (0 pg/ml) (Fig. 3A, n=6).

IL-33 induces nitric oxide release from mouse astrocytes

Incubation of mouse astrocytes with IL-33 (25 ng/ml) for 2 hrs significantly ($p<0.05$) induced (67.3 ± 7.0 μ mole/L) the release of nitric oxide when compared to the release from untreated control cells (19.9 ± 0.83 μ mole/L; Fig. 3B, $p<0.05$; unpaired t test).

IL-33 mediates neuronal damage and degeneration

Mouse mixed (glia and neuronal) culture and pure neuronal cultures were grown on cover slips in the tissue culture plates and incubated with recombinant mouse IL-33 (10 ng/ml) for 48 hrs. Then we removed the cover glass with cells and washed in PBS. These cells on the cover slips were then fixed and then immunostained these cells for MAP2 distribution. Development of brown color indicated positive reaction for MAP2 in the neurons. The slides were mounted, observed under the microscope and photographed. IL-33 treatment-induced loss of neuronal projections and network of neurons, indicative of neuronal damage when compared with untreated control neurons (Fig. 3C). Moreover, images of neurons in the culture flask (unstained picture) also show that IL-33 (10 ng/ml) decreased the neuronal number as well as induced neuritis like appearance when compared to untreated control neuronal cells without IL-33 treatment (Fig. 3C).

IL-33 induces phosphorylation of ERK in mouse astrocytes

In order to study IL-33 signaling pathways, mouse astrocytes were incubated with recombinant mouse IL-33 (20 ng/ml) for 0 to 60 min at 37°C and total ERK1/2 and phospho ERK1/2 were detected by immunoblotting. Representative results showed that IL-33 stimulation induced ERK phosphorylation as early as 5 min (2.3-fold increase) when compared to unstimulated control cells (n=3, Fig. 3D). This increase continued until 60 min (1.8 fold increase) when compared to untreated control cells. Values shown were the densitometric ratio of phospho ERK to the total ERK and normalized to the control. Control

(C) densitometric value was considered as 1. We have used 2 different antibodies, and both produced similar band appearance of ERK, and total ERK. β -actin was used as a loading control.

Discussion

In the present study, we report that GMF as a novel inducer of IL-33 release as well as IL-33 augments GMF-mediated TNF- α release from mouse primary astrocytes. Our present study also report that both IL-33 as well as GMF induces the release of chemokine CCL2 in a dose-dependent and time-dependent manner from the primary mouse astrocytes and that IL-33 induce the release of nitric oxide from these cells. By MAP2 immunocytochemistry of neurons, and direct microscopic observation of neurons in the tissue culture flasks, we further report that IL-33 mediates neuro toxic effects causing neuronal damage and neurodegeneration changes.

IL-33 and its receptor ST2 show both protective (physiologic) and anti-inflammatory activities depending upon the concentration and cell types/organ (Yasuoka et al. 2011; Jiang et al 2012; Li et al. 2012; Milovanovic et al. 2012). IL-33 could enhance the development of inflammatory autoreactive T cells independent of ST2 in EAE model. A previous study reported that IL-33 as low as 0.1 ng/ml induced the release of cytokines from mast cells (Hudson et al. 2008). High concentration of IL-33 is known to be released from activated inflammatory cells, necrotic and damaged cells in a proinflammatory microenvironment. Physiologic or protective dose of IL-33 may be lesser than the proinflammatory activity dose in the specific cell types. Our present study shows that IL-33, even as low as 1 ng/ml significantly induces potent chemo attractant CCL2 release from mouse astrocytes in vitro. Our present study also demonstrated that IL-33 induces nitric oxide as well as TNF- α release from the astrocytes which could be neuro toxic, and therefore reduce the neuronal density in the culture. It is previously reported that nitric oxide and TNF- α in excess cause neuronal as well as oligodendrocyte damage (Jana et al. 2005). IL-33 augments or synergistically acts with other inflammatory mediators in proinflammatory micro environment. Others and we have previously shown that IL-33 augments inflammatory mediator release from macrophages (Espinassous et al. 2009) and mast cells (Theoharides et al. 2010). Here, in the present investigation, we report that IL-33 augments GMF-induced TNF- α release from mouse astrocytes. Previous studies have demonstrated that IL-33 induces glial cells to release inflammatory mediators causing either neuro protective or neuro toxic effects. Another previous study has shown decreased IL-33 expression in the brain of AD patients compared to control subjects and also reported that IL-33 expression was consistently restricted to vascular capillaries in the brain (Chapuis et al. 2009). Further, it has been also reported that IL-33 induces microglia and enhance phagocytosis (Yasuoka et al. 2011) suggesting a protective role of IL-33 in AD. However, IL-33 has been implicated in the EAE (Han et al. 2011) and suggested to cause demyelination by activating various inflammatory cells (Christophi et al. 2012). Moreover, IL-33 blockade has been shown to inhibit the development of EAE (Li et al. 2012). Another recent study indicates that ST2 deficiency attenuates resistance of BALB/c mice to EAE induction through enhancing differentiation of proinflammatory antigen presenting cells and the differentiation of encephalitogenic T cells in the lymph node rather than affecting their action in the target tissue (Milovanovic et al. 2012).

IL-33 is localized to the nucleus of astrocytes and acts intracellularly as a transcription factor and extracellularly as nuclear factor kappa B (NF- κ B) activation cytokine. mRNA and protein of IL-33 are expressed in the astrocytes but not by cortical neurons and microglia (Han et al. 2011; Yasuoka et al. 2011). IL-33 receptor constitute a transmembrane form (ST2 or ST2L) and a soluble form (sST2) (Han et al. 2011). ST2L and sST2 are expressed in

both astrocytes and microglia (Yasuoka et al. 2011). IL-33 binds with ST2 and IL-1RAcP (Chackerian et al. 2007). IL-1RAcP is expressed by the neurons, astrocytes and microglia (Yasuoka et al. 2011). Other studies report that ST2 is expressed in astrocytes but not in neurons or microglia (Andre et al. 2005; Han et al. 2011). Our present study supports the proinflammatory role of IL-33 as it induces astrocyte to release proinflammatory and neuro toxic factors. Though the expression and its role of IL-33 in the CNS diseases are known to some extent, the exact mechanism of regulation of IL-33 production in the CNS is not yet clearly investigated specifically in association with other proinflammatory factors such as GMF. IL-33 can induce autocrine or paracrine inflammatory reactions in glial cells.

GMF, another proinflammatory factor is mainly localized in the neurons, astrocytes, microglia and oligodendrocytes in the CNS. GMF in excess, causes proinflammatory reactions in the CNS, leading to the death of neurons in the neurodegenerative and neuroinflammatory diseases. Up-regulation of GMF expression by the glial cells (Thangavel et al. 2012), could augment or synergize with other inflammatory mediators in the CNS to cause chemoattraction, proliferation and activation of glial cells and release of inflammatory mediators and exacerbate disease severity in neuroinflammatory diseases. IL-33 along with other inflammatory mediators may also up-regulate GMF expression in the CNS. GMF in turn, may act through autocrine and/or paracrine signaling manner in the CNS to exacerbate neuroinflammatory reactions. We have previously reported that GMF induces inflammation related genes such as granulocyte macrophage-colony stimulating factor (GM-CSF), IL-1 β and CCL4 (Zaheer et al. 2002). The present study demonstrates that GMF induces significant amount of IL-33 release from mouse astrocytes, showing a close relationship of GMF and IL-33 in the activation of glia such as astrocytes. High level of CCL2 was found in the cerebrospinal fluid (CSF) of AD patients (Farfara et al. 2008) and MS patients, and EAE (Conductier et al. 2010). CCL2 is mainly released from astrocytes in the brain and cause chemotaxis of astrocytes around senile plaques (Wyss-Coray et al. 2003) and is implicated in the cognitive decline of AD patients (Westin et al. 2012). CCL2 accelerates glial cell mediated neurocognitive dysfunction in the mice model (Kiyota et al. 2009). CCL2 is increased in the early stage of AD pathogenesis (Galimberti et al. 2006) and further suggested that CCL2 could be an additional biomarker for monitoring the disease progression. In addition to recruiting leukocytes at the sites of inflammation, CCL2 also facilitate 'opening' the blood-brain-barrier (BBB) (Stamatovic et al. 2005). Although expression of cytokines and chemokines is limited in the normal CNS, aberrant expression occurs in the CNS diseases including AD, MS and PD. The lack of either CCL2 and/or CCR2 prevents the entry of inflammatory cells that are important for the CNS tissue damage and demyelination (Dong and Benveniste 2001). Our present study showed that both IL-33 and GMF-induced CCL2 release from astrocytes in a dose-dependent as well as time-dependent manner, which could attract large number of glial cells at the site of inflammation in the neurodegenerative diseases.

A previous study (Marx et al. 2001) has reported that incubation of rat mixed glial culture as well as neuronal culture with IL-1 β and TNF- α for 48 hrs decreased the number of neurons that were immunoreactive for MAP2. Our present study with IL-33 and MAP2 immunocytochemistry showed a similar decrease of neurons when the mixed culture and neuronal culture were incubated with IL-33 for the same 48 hrs period as previously reported by Marx et al. (2001). IL-33 can act on astrocytes through ST2 and on neurons through IL-1RAcP. TNF and IL-6 are known to decrease the number of neurons in the culture (Venters et al. 2000; Marx et al. 2001) and IL-33 is known to release these cytokines from specific cell types. Our current study reports that both GMF and IL-33 induce the release of TNF- α from astrocytes which could further mediate neuronal damage and affect neuronal survival. Our present results suggest that IL-33 could cause neuronal damage through the activation of glial cells, as also seen in the mixed culture and neuronal culture.

Our previous studies in rat astrocytes have shown that GMF activates mitogen-activated protein kinase (MAPK) and NF- κ B, release TNF- α and IL-1 β (Zaheer et al. 2001; Zaheer et al. 2002). IL-33 has been shown to activate extracellular signal-regulated kinases (ERK) and p38 MAPK in endothelial cells, but only ERK in epithelial cells to release chemokine IL-8 (Yagami et al. 2010). In the present study, we demonstrate that IL-33 activate ERK that could release inflammatory mediators including CCL2, TNF- α and nitric oxide from mouse astrocytes.

Astrocyte activation and its proliferation is associated with human neurodegenerative and autoimmune diseases such as AD and MS/EAE (Miljkovic et al. 2011). Astrocytes are emerging as an important innate immune cell in the demyelinating and neurodegenerative diseases. It seems that up-regulated expression of IL-33 in the CNS, may contribute to the endothelial dysfunction and vascular permeability through the nitric oxide (Choi et al. 2009) and other proinflammatory mediator release, leading to further BBB disruption in the neurodegenerative and autoimmune diseases. Further, *in vivo* studies including models where IL-33 and GMF are produced endogenously are required to elucidate the role of IL-33 and GMF in the CNS inflammatory diseases. In addition to the studies using rodent astrocytes, the present findings should be evaluated using human primary CNS cells *in vitro*.

In summary, our present study shows that GMF induces IL-33 release from mouse astrocytes and that IL-33 augments GMF-mediated TNF- α release from these cells. We also report that IL-33 induces the release of proinflammatory mediators such as chemokine CCL2, cytokine TNF- α and neuro toxic substance nitric oxide and mediate neuronal damage and neuronal death. In conclusion, GMF along with IL-33 may exacerbate neuroinflammation and may provide a new therapeutic target for neurodegenerative and autoimmune diseases.

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Fig. 1.

Effect of IL-33 and GMF on chemokine CCL2 release from mouse astrocytes *in vitro*. In dose-response and time course studies, mouse astrocytes were incubated with (A) recombinant mouse GMF (5–200 ng/ml, 2–24 hrs) or (B) recombinant mouse IL-33 (1–200 ng/ml, 2–48 hrs) in tissue culture plates at 37°C. After the incubation period was over, the cell culture supernatant media were collected and CCL2 level was assayed by ELISA (n=3). The control cells were incubated with equal volume of medium containing 0.1% BSA in PBS. Results were presented as mean $\pm$ SEM (*p<0.05 control vs treated, ANOVA with Tukey-Kramer post test).

Fig. 2.

Mouse astrocytes were incubated with various concentrations of **(A)** recombinant mouse GMF (25–200 ng/ml) or **(B)** recombinant mouse IL-33 (1–50 ng/ml) for 24 hrs for dose-response effect and measured TNF-α release in the cell culture supernatant media (n=4) by ELISA. In another experiment, mouse astrocytes were pre incubated with **(C)** GMF (50 ng/ml) for 15 min before adding IL-33 (10 ng/ml) for further 24 hrs. Astrocytes were also incubated either with GMF or with IL-33 for 24 hrs **(C)**. After the incubation period was over, the supernatant media were collected and TNF-α release was assayed by ELISA (n=4). The control cells were treated with equal volume of medium containing 0.1% BSA in PBS in all the experiments. Results were presented as mean ± SEM (*p<0.05 control vs treated, ANOVA with Tukey-Kramer post test).

Fig. 3.

Mouse astrocytes were incubated with (A) recombinant mouse GMF (50 ng/ml) for 24 hrs in tissue culture plate and the culture supernatant media were assayed for IL-33 release (n=6). We then incubated mouse astrocytes with (B) recombinant mouse IL-33 (25 ng/ml) for 2 hrs in tissue culture plate and the total nitric oxide release was measured in the supernatant media using commercial kit (n=3). Results were presented as mean $\pm$ SEM (*p<0.05 control vs treated, Unpaired t-test). (C) Representative photomicrograph showing the effect of 48 hrs incubation with recombinant mouse IL-33 (10 ng/ml; n=3) on mixed (glial and neuronal cells) or pure neuronal cultures and immunostained with neuronal marker MAP2 monoclonal antibody. Brown color indicates the positive reaction for MAP2. Note that IL-33 induced loss of neuronal projections as well as network of neurons when compared to untreated control cells. Images of neuronal culture in the tissue culture flasks (un stained picture) observed directly under the microscope show that IL-33 (10 ng/ml) induced neuritis and damage when compared to control untreated cells. Original magnification = $\times 200$ and $\times 400$. (D) Representative immunoblots of phosphorylated ERK in mouse astrocytes after incubation with recombinant mouse IL-33 (20 ng/ml) for 0–60 min (n=3). The cells were lysed and the protein was subjected to immunoblotting. The membrane was probed for phospho ERK using rabbit polyclonal phosphop44/42 MAPK (ERK1/2) antibody and total ERK using rabbit polyclonal p44/42 MAPK (ERK1/2) antibody. β -actin was used as a loading control. Representative time-course (0 to 60 min) study of IL-33 stimulation showed ERK phosphorylation at 5 min in the mouse astrocytes. We further performed densitometric ratios of pERK to the tERK and normalized to unstimulated control cells. Normalized densitometric value of control (C) was considered as 1. The control cells were treated with equal volume of medium containing 0.1% BSA in PBS in all the experiments.