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Interleukin-33 upregulation in peripheral leukocytes and CNS of multiple sclerosis patients

George P. Christophi[2,4,*], **Ross C. Gruber**[1,3,*], **Michael Panos**[1,*], **Rebecca L. Christophi**[2], **Burk Jubelt**[1,2], and **Paul T. Massa**[1,2,$]

[1]Department of Neurology, Albert Einstein College of Medicine, Bronx, NY, USA

[2]Department of Microbiology & Immunology, Albert Einstein College of Medicine, Bronx, NY, USA

[3]Department of Pathology, Albert Einstein College of Medicine, Bronx, NY, USA

[4]Department of Medicine, Washington University School of Medicine, St. Louis, MO, USA

Abstract

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS). Here we document for the first time that the cytokine IL-33 is upregulated in both the periphery and the CNS of MS patients. Plasma IL-33 was elevated in MS patients compared to normal subjects and a three-month treatment of MS patients with interferon β-1a resulted in significant decrease of IL-33 levels. Similarly, stimulated cultured lymphocytes and macrophages from MS patients had elevated IL-33 levels compared to normal subjects. In parallel, the transcription factor NF-κB that mediates IL-33 transcription was also elevated in leukocytes of MS patients. IL-33 was elevated in normal-appearing white matter and plaque areas from MS brains and astrocytes were identified as an important source of IL-33 expression in the CNS. In summary, IL-33 levels are elevated in the periphery and CNS of MS patients, implicating IL-33 in the pathogenesis of MS.

Keywords

multiple sclerosis; autoimmunity; demyelination; cytokines; IL-1; IL-33; st2; interferon therapy; inflammation; NF-kappa B; macrophages; T-cells; macrophages; mast cells; plasma; central nervous system; astrocytes; microglia; EAE; TMEV

1. INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) characterized by multifocal white matter lesions and progressive neurologic disability (1). Several studies have demonstrated that MS lesions contain multiple leukocyte cell types including lymphocytes, macrophages, and dendritic cells, all of which are believed to contribute to lesion formation by various distinct and interacting

$Corresponding Author: Mailing Address: Department of Neurology, Upstate Medical University, State University of New York, 750 East Adams Street, Syracuse, NY 13210, Phone Number: (315) 464-7606, Fax: (315) 464-6402, massap@upstate.edu.
*Contributed equally to the manuscript

mechanisms (2, 3). Interestingly, both leukocytes and CNS lesions of MS patients have elevated levels of cytokines and contain activated transcription factors including NF-κB (4, 5), STAT1 (5–7) and STAT6 (8–10), which can lead to enhanced expression of inflammatory genes and toxic molecules known to promote demyelination (11–17).

Interleukin-33 (IL-33) is a novel cytokine of the IL-1 cytokine family that has been recently implicated in several inflammatory and autoimmune diseases like Lupus Erythematosus, rheumatoid arthritis, ulcerative colitis, psoriasis, and cardiovascular disease (18–20). IL-33 transcription is mediated by NF-κB activation secondary to Toll-like receptor (TLR) signaling or stimulation with TNF-α or IL-1β. (21–24). The receptor for IL-33, ST2L is differentially expressed on multiple leukocyte subsets and signals to activate both MAPK and NF-κB pathways through primarily TNF receptor-associated Factor 6 (TRAF6) (21, 25). ST2L is expressed on endothelial cells, mast cells, basophils, macrophages, and dendritic cells *in vivo* and are thus believed to be main targets for IL-33 inflammatory activity, including upregulation of the cytokines IL-6, IL-8, and IL-13 (18, 19, 23, 26–29).

Administration of exogenous IL-33 in vivo leads to pronounced splenomegaly and eosinophilia, increased mucous production, and hypertrophy of the digestive and respiratory tracts. These pathological changes occur in conjunction with increases in Th2 molecules such as IL-4, IL-5, and IL-13 and IgE (23). Other studies emphasize the importance of IL-33 in amplifying innate immunity and possibly serving as an alarmin to activate the immune system following cell necrosis or apoptosis (30, 31)

IL-33 has been shown to have important biologic functions in several immune mediated diseases including asthma, dermatitis, rheumatoid arthritis, inflammatory bowel disease, autoimmune hepatitis, and cardiovascular disease among others (18, 19, 31). IL-33 can act to modulate several pathways including Th2 skewing and mast cell, eosinophil, and macrophage activation (32). Regarding CNS disease, our group was the first to show that IL-33 is expressed in mouse CNS astrocytes and that CNS-derived IL-33 is functionally active (33). Recently, another study showed that IL-33 is induced in the CNS in the experimental allergic encephalomyelitis EAE mouse model of MS (34). Specifically, mouse IL-33 is predominantly expressed in the CNS by endothelial cells and astrocytes where it affects astrocyte activation and ultimately results in microglia proliferation and secretion of cytokines and chemokines (34). Alternatively, IL-33 can activate CNS mast cells through degranulation and secretion of other pro-inflammatory mediators including IL-1, IL-6, and MCP-1 (33, 35–37).

This is the first study that explores the expression of the cytokine IL-33 in MS. Here we demonstrate that IL-33 is elevated in the plasma of patients with active relapsing remitting (RR) MS. Interestingly, *in vivo* therapeutic treatment with interferon β-1a suppresses IL-33 levels in MS patients, suggesting a possible mode of interferon action. Furthermore, stimulation of IL-2 expanded PBMCs (peripheral blood mononuclear cells) and macrophages from MS patients resulted in significantly increased levels of IL-33 compared to normal controls. Furthermore, we demonstrate that IL-33 elevation correlates with increased activation of the transcription factor NF-κB, which has been previously shown to induce IL-33 expression (21–23). Lastly, we show that the CNS – both in normal appearing white matter (NAWM) and plaque tissue – of MS patients, expresses higher levels of IL-33 compared to normal subjects. In summary, these findings suggest that IL-33 may play a potential role in the pathogenesis of MS both in the periphery and within the CNS.

2. MATERIALS AND METHODS

2.1. Patient selection for blood

All patients selected were clinically diagnosed as having definite relapsing-remitting (RR) MS (38), and none received any disease modifying treatment including IFN-β, glatiramir acetate, steroids, or other immunosuppressive agents for at least two months prior to donating blood. RR MS group donated blood before and after a three-month treatment with recombinant interferon β-1a (39). Table I provides additional information on the patients and normal subjects used in this study. The Institutional Review Board of SUNY Upstate University approved all studies, and both patients and normal controls granted informed consent before providing blood.

2.2. PBMC isolation and expansion

Patients and normal subjects donated 60 ml of blood which was collected in heparinized tubes. Blood was diluted 1:1 with HBSS and overlaid onto lymphocyte separation medium (Cellgro, Herndon, VA). After centrifugation, the plasma was collected and used to quantify cytokine levels, while the 10 ml of the interface containing the PBMCs was collected and washed twice with HBSS. For the *in vivo* studies several samples of the freshly isolated cells were either resuspended in STAT- 60 (Tel-Test, Friendswood, TX) for RNA analysis, RIPA buffer for protein analysis (40), or fixed for intracellular flow cytometry analysis. For the *in vitro* studies, the remaining PBMCs were cultured for one week in RPMI media with 20 units/ml IL-2 (R & D Systems), and 10% fetal bovine serum. After one week, cells were treated with cytokines and analyzed as outlined in the text. Cultured PBMCs were stained with antibodies against CD3, CD19, and CD14 were used to for differentiating T-cells, B-cells, and monocytes respectively. The proportion of cell types making up the PBMCs of MS patients and controls were the same (80% T-cells, 5% B-cells, 3% monocytes, and 12% of negative cells). A majority of negative cells probably represent natural killer (NK) cells based on their scatter properties (41).

2.3. Human peripheral blood monocyte-derived macrophages

Patients and normal subjects donated 50 ml of blood collected in heparinized tubes. Blood was diluted 1:1 with Hank's balanced salt solution (HBSS) and overlaid onto lymphocyte separation medium (Cellgro, Herndon, VA). After centrifugation, the 10 ml of the interface containing the PBMCs was collected and washed twice with HBSS. Adherent monocytes were cultured for one week in RPMI media, 15% fetal bovine serum, and 50ng/mL GM-CSF as previously described (42, 43). Medium was replenished every 3 days and non-adherent cells were removed at the second feeding (at 6 days). For experiments, cells were cultured for an additional day in the absence of GM-CSF, washed twice with PBS, and adherent cells were lysed in STAT- 60 (Tel-Test, Friendswood, TX) for RNA analysis or in RIPA buffer (44) for protein analysis. Flow cytometry cells were detached by incubation in 5.0mM EDTA for 10 minutes at 37 degrees C. The method consistently yielded more than 95% pure macrophages assessed by both morphological criteria as described (45) and expression of the myeloid lineage marker CD14 as determined by flow cytometric analysis.

2.4. Brain samples

Fresh frozen tissue specimens were obtained from the Human Brain and Spinal Fluid Resource Center, Veteran's Administration, West Los Angeles Healthcare Center, Los Angeles, CA 90073, which is sponsored by NINDS/NIMH, the National Multiple Sclerosis Society, and the Department of Veterans Affairs. Frozen tissue samples from normal controls and MS patients were screened by a neuropathologist to confirm the clinical diagnosis of MS and exclude confounding pathologies. None of the subjects had ischemic

brain injury. Tissue from MS patients consisted of either normal appearing white matter (NAWM) that had no apparent histopathologic changes, or plaque that contained white matter tissue with areas of focal demyelination and infiltration of immune cells. The tissue quality was determined with tissue pH with a cutoff pH of 6.7 (Table 2). The quantity of the purified RNA was assessed using the RNA Pico Lab Chip Kit with the Agilent Technologies Bioanalyzer and agarose gel electrophoresis. In addition, RNA was quantified spectrophotometrically and only samples with sufficient quantity (500ng total yield) and appropriate optical density (OD 260/280 ratio = 1.7–2.1) were used for subsequent analysis (46).

2.5. Real-Time RT-PCR

Total RNA was isolated using STAT-60 RNA isolation kit (Tel-test). RNA was quantified spectrophotometrically and 0.5 μg of total RNA was converted into cDNA. Briefly, to generate cDNA 0.5μg total RNA and random primers (Invitrogen, Carlsbad, CA) were incubated at 72 degrees for 10 minutes. Reverse transcription was performed using the Superscript II RT enzyme (Invitrogen, Carlsbad, CA) and following the manufacturer's specifications. cDNA was diluted to 200 μL with water and 4μL was used for quantitative real time PCR using a SYBR Green kit (Abgene, Epson, UK). The following forward and reverse primers were used at 10 nM: IL-33 AAGGCAAAGCACTCCACAGT and GAAAGAAGTTTGCCCCATGT, MCP-1 GCTCATAGCAGCCACCTTC and GCTTCTTTGGGACACTTGC, IP-10 TTCAAGGAGTACCTCTCTCTAG and CTGGATTCAGACATCTCTTCTC, TARC CGAGGGACCAATGTGGGC and GGGTGAGGAGGCTTCAAGACC, GAPDH ACCACCATGGAGAAGGC and GGCATGGACTGTGGTCATGA, β-Actin AGGCACCAGGGCGTGAT and GCCCACATAGGAATCCTTCTGAC. The PCR parameters were 15 minutes for 95 degrees, 35 cycles at 95 degrees for 15 seconds, and 60 degrees for 1 minute in an ABI Prism 700 thermocycler (Applied Biosystems, Foster City, CA). Serial dilutions of cDNA containing a known copy number of each gene were used in each quantitative PCR run in order to generate a standard curve relating copy number to threshold amplification cycle (47). Gene expression levels were calculated during the logarithmic amplification phase by determining the initial mRNA copy number using the standard curve. Amplification of each gene specific fragment was confirmed both by examination of melting peaks and by agarose gel electrophoresis.

2.6. Cytokine ELISA

The levels of the cytokine IL-33, IL-6, IFN-γ, and IL-13 were measured using R&D Systems DuoSet ELISA kits (R&D Systems) following the manufacturer's protocol.

2.7 NF-κB DNA-binding activity assay

NF-κB DNA-binding activity was analyzed using the TransAMNF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions and as previously described (48–50). Briefly, nuclear extracts were prepared (51) from macrophages of normal subjects and MS patients that were treated with media alone or 5μg/mL of LPS for 1 hour. Alternatively, nuclear extracts were prepared from freshly isolated PBMCs that were isolated from normal subjects and MS patients before and after a three-month treatment with IFN-β1a. Protein levels of the nuclear extracts were quantified with the Bradford assay (Pierce Chemicals, Rockford, IL) and 10 μg were incubated in a 96-well plate coated with oligonucleotide containing the NF-κB consensus-binding sequence 5′-GGGACTTTCC-3′. Bound NF-κB was then detected by a p65-specific primary antibody. An HRP-conjugated secondary antibody was then applied to detect the bound primary antibody and provided the basis for colorimetric quantification. The enzymatic product was measured at 450 nm with a reference wavelength of 650 nm by a microplate reader. To

quantify the amount of NF-κB, serial dilutions of purified p65 recombinant protein (20ng - 0.16ng) were measured to provide a calibration curve between p65 binding and absorbance. The specificity of the assay was further tested by the addition of wild type or mutated NF-κB consensus oligonucleotide in the competitive or mutated competitive control wells before the addition of nuclear extracts. The addition of the wild-type NF-κB consensus oligonucleotide completely abolished NF-κB binding.

2.8 Immunohistochemistry

Fresh frozen tissue was sectioned at 7 micrometers, placed onto glass slides and fixed in −20 °C acetone for 15 minutes. Endogenous peroxidase was inactivated using 0.5% hydrogen peroxide in PBS for 10 minutes. Endogenous biotin was quenched using the Dako Biotin blocking system (Dako, Carpinteria, CA). Tissue was blocked for 2 hours in PBS containing 10% horse serum at room temperature (RT). After blocking, tissue sections were incubated with primary mouse monoclonal antibodies to IL-33 (Nessy-1, Axxora, San Diego, USA) or control mouse IgG antibodies (Dako, Carpinteria, CA) in PBS containing 10% horse serum overnight at 4°C. Subsequently, tissue sections were incubated with 5ug/mL of the biotinylated anti-mouse IgG antibody followed by streptavadin-HRP (Dako, Carpinteria, CA). Red color precipitate was produced using AEC according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Sections were coverslipped using aqueous mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA). Images were taken at 200X magnification on a Nikon Eclipse E800 microscope equipped with a spot camera. Bar in the figure represents 40 micrometers.

2.9 Double Immunofluorescence of IL-33 and GFAP

Fresh frozen tissue was sectioned at 7 micrometers, placed onto glass slides and fixed in -20 °C acetone for 15 minutes. Sections were incubated with the primary antibodies, IL-33 (Axxora, San Diego, USA), at 4°C overnight followed by GFAP (Serotec, Oxford, England) for 1 h room temperature. Secondary antibodies (Jackson Immunoreasearch, West Grove, PA, USA) were incubated for 2 hr at room temperature. Slides were mounted with gelvatol and kept at 4° C in the dark. Images were taken on a SPOT RT Slider CCD camera using a Nikon Eclipse TE 2000-U microscope.

2.10 Statistical Analysis

Histograms contain statistical means with standard error values. The number of MS patients and normal subjects used in each experiment are shown in Table I & II. The p-values were generated using the unpaired Student's t-test and a p-value of less than 0.05 was chosen to indicate statistical significance between two sample means.

3. RESULTS

3.1. Plasma IL-33 is elevated MS patients

The levels of IL-33 protein were measured in plasma of RRMS patients and normal subjects (Figure 1). Plasma was isolated from blood of 32 normal subjects and 32 untreated RRMS patients, and the levels of IL-33 protein were quantified with ELISA (Table I). The average level of IL-33 in normal subjects was 60 pg/mL. In contrast, active RRMS patients had significantly higher levels of IL-33 with an average concentration of 280 pg/mL (Figure 1A). Interestingly, *in vivo* treatment with recombinant IFN-β 1a for three-months significantly suppressed the levels of plasma IL-33 compared to untreated RRMS patients. In order to corroborate the ELISA findings and examine whether IL-33 was transcriptionally regulated, we also quantified IL-33 mRNA in freshly isolated PBMCs by real-time RT-PCR (Figure 1B). Freshly isolated PBMCs demonstrated a significant 15-fold higher level of

IL-33 compared to normal subject PBMCs. Importantly, as with protein levels in the blood, IFN-β-1a significantly suppressed IL-33 RNA levels in PBMC. To control for mRNA loading we examined the expression of the housekeeping genes GAPDH and β-actin by real time PCR in the samples and demonstrated similar expression levels (data not shown).

Additionally we quantified the expression of the plasma cytokines IL-6, IFN-γ, and IL-13 in the same samples of normal subjects, and RRMS before and following *in vivo* treatment with IFN-β-1a (Figure 1C–E). IL-6, IFN-γ, and IL-13 were elevated in untreated RRMS patients compared to normal subjects (52–54). IFN-β 1a treatment significantly reduced the levels of the NF-κB-responsive cytokine but not the levels of IFN-γ. Importantly, IL-13 that has been shown to be induced following IL-33 stimulation was also reduced in plasma of RRMS following in vivo IFN-β 1a treatment (23, 29).

3.2. IL-33 expression in cultured lymphocytes and macrophages of MS patients

Next we examined the expression of IL-33 in cultured PBMCs from untreated RRMS patients and normal control subjects. First PBMCs were cultured in the presence of IL-2 for one week to expand the lymphocyte population (Figure 2A). The proportion of cell types making up the PBMCs of MS patients and controls were 80% T cells, 5% B cells, and 3% monocytes as previously documented (10). We further stimulated cultured PBMCs with phorbol myristate acetate (PMA) and ionomycin for 24 hours. Following stimulation, the levels of IL-33 measured by ELISA were increased, and importantly PBMCs from untreated RRMS patients secreted significantly higher levels compared to normal subjects (Figure 2A).

Additionally, we examined the expression of IL-33 in macrophages of normal subjects and RRMS patients (Figure 2B). Blood monocytes were expanded in the presence of GM-CSF for one week resulting in more than 95% adherent macrophages determined by morphology and CD14 staining (55). Macrophages were then stimulated with LPS for 24 hours and secreted IL-33 protein levels were determined with ELISA (Figure 2B) as previously shown (56). Importantly, the stimulated macrophages from MS patients secreted significantly higher levels of IL-33 compared to macrophages from normal subjects.

3.3. NF-κB expression activation correlates with IL-33 levels

To examine possible mechanisms of elevated IL-33 in leukocytes of MS patients, we quantified the activation of the transcription factor NF-κB, which has been shown previously to mediate IL-33 induction (21–24). Nuclear extracts from freshly isolated PBMCs or cultured macrophages were prepared and allowed to bind an NF-κB consensus oligonucleotide sequence. Bound NF-κB was then detected by a p65 (RelA)-specific antibody and quantified based on a calibration using purified p65 recombinant protein. NF-κB DNA binding activity was significantly increased in PBMCs of MS patients compared to normal subjects and IFN-β treatment resulted in a significant decrease in NF-κB activation (Figure 3A) (4, 5, 54, 55). Furthermore, LPS treatment significantly induced NF-κB activation and was significantly more elevated in macrophages from MS patients compared to normal subjects (Figure 3B). Thus, NF-κB activation correlated with IL-33 expression levels suggesting a possible transcriptional mechanism by which IL-33 is elevated in MS.

3.4. IL-33 is elevated in the CNS of MS patients

In mice, in addition to immune cells, CNS resident cells, and especially astrocytes express high levels of IL-33 (19, 33). Also, IL-33 is induced in the CNS of EAE and TMEV mouse models of MS. Therefore, it was important to examine the expression of IL-33 in the CNS of MS patients (Figure 4). We used fresh frozen sectioned brains in which both the diagnosis and differentiation of normal appearing white matter (NAWM) or plaque was confirmed by

a neuropathologist (Table II). The tissue quality was determined by tissue pH with a cutoff of 6.7 and appropriate RNA quality was determined as previously described (46). First, we examined the tissue expression of IL-33 protein by ELISA and mRNA by RT-PCR in MS NAWM that contained CNS resident cells but no apparent infiltrating immune cells. IL-33 protein was significantly elevated in the NAWM of MS patients compared to normal subject white matter (Figure 4A). IL-33 mRNA was correspondingly increased in the NAWM from MS patients compared to white matter of normal subjects (Figure 4B). Additionally, both the IL-33 mRNA and protein were elevated in white matter containing areas of focal demyelination and immune cell infiltration (plaque) of MS patients compared to white matter from normal subjects.

Furthermore, we quantified the expression of the cytokines IL-6, IFN-γ, and IL-13 by ELISA (Figure 5C–E) and the mRNA expression of the chemokines MCP-1, IP-10, and TARC by RT-PCR (Figure 5F–H) in the same samples to examine how expression correlates with IL-33 and evaluate the degree of inflammation in MS brain NAWM or plaque areas. In NAWM there was significant elevated levels of IL-6, IL-13, MCP-1, IP-10, and TARC suggesting that moderate inflammation was present and was originated from CNS resident cells. IFN-γ was not elevated in NAWM suggesting that the primary source was infiltrating immune cells. In the plaque areas involving active areas of demyelination, dense leukocyte infiltrate, and peri-plaque white matter, showed increase levels of IL-6, IFN-γ, IL-13 and MCP-1, IP-10, and TARC compared to normal subjects.

3.5 CNS IL-33 immunohistochemistry and localization in astrocytes

Furthermore, to determine the distribution and cellular localization of IL-33, brain tissue was immunohistochemically stained with IL-33 antibody (Figure 5). For negative control we used a control mouse IgG that did not reveal any staining pattern. IL-33 was present in nuclei of cells both in plaques, periplaque regions, and in normal appearing white matter cells of MS brains. In distinct contrast, IL-33 staining of cells in normal subject white matter showed only trace amounts of IL-33 as well as light staining around blood vessels (Figure 5). Double immunofluorescence staining showed a high incidence of IL-33 within GFAP+ astrocytes of MS patients confirming previous findings in mouse astrocytes both *in vivo* and *in vitro* (19, 33). Taken together, IL-33 expression in CNS glia of MS subjects is much higher than in normal subject brain and may therefore play an important role in the pathophysiology of MS (Figure 6).

4. DISCUSSION

This is the first study to describe the expression of the cytokine IL-33 in MS. We have shown that the levels of IL-33 are elevated in the plasma and in freshly isolated PBMCs of RRMS patients. Furthermore, therapeutic treatment with IFN-β-1a significantly suppressed IL-33 expression in the blood of MS patients. *In vitro*, activation of IL-2-expanded PBMCs and cultured macrophages led to a significantly higher secretion of IL-33 in MS patients compared to normal subjects. Importantly, we have shown that both NAWM and plaques of MS patients have significantly higher levels of IL-33 compared to white matter in brains of normal subjects. These data suggest a possible role for IL-33 in the immune response in the periphery and within the CNS in MS subjects that may be relevant to the demyelinating process.

Several cytokines are elevated in MS, which can lead to elevated transcription factors including NF-κB in blood leukocytes (54). Importantly, NF-κB signaling plays a central role in MS pathogenesis in modulating neural cell survival and by inducing numerous inflammatory genes including cytokines, proteases, and reactive oxygen species (57, 58) in CNS resident cells and infiltrating immune cells. We have previously shown that a

deficiency in the protein tyrosine phosphatase SHP-1, a master negative regulator of inflammatory signaling, is at least partly responsible for enhanced NF-κB activation in PBMCs, macrophages, and CNS resident cells of MS patients (54, 55). Importantly, IL-33 transcription is mediated by NF-κB activation (21–24) and here we demonstrate that NF-κB activation correlates with IL-33 expression suggesting one of the possible mechanisms of the transcriptional upregulation of IL-33 in leukocytes of MS subjects.

An important consideration arising from these studies is the source and functional role for IL-33 in the pathogenesis of MS. The present studies indicate two potential sites from which IL-33 may be derived. The first is CNS tissue in which we have previously demonstrated nuclear IL-33 is induced and released by astrocytes treated with various TLR ligands (59). As with other IL-1-family cytokines, astrocytes required both a priming step with TLR ligands and subsequent triggering step such as treatment with ATP (60) for maximal release of IL-33 (59). IL-33 release occurs by an incompletely defined mechanism in which these cytokines are liberated from the nucleus and/or cytosol to the extracellular environment through the plasma membrane. In the case of IL-1β and IL-18, proteolytic processing of the pro-forms in the cytoplasm occurs via caspase-1 activity within assembled inflammasomes (61). However, in the case of IL-33, release does not require proteolytic processing by inflammasomes (62). Rather, nuclear IL-33 release from cells occurs following necrosis in which the plasma membrane becomes extensively damaged (63). Thus, increased IL-33 activity in tissues follows a pattern of induction and release that combines mechanisms seen with IL-1 family cytokines (ie. priming) and that of alarmins such as HMGB1 which require cell necrosis for release (62). Alarmins such as IL-33 signal tissue damage to innate effectors that promote further inflammation for elimination of necrotic tissue.

In addition to having an important role as a pro-inflammatory NF-κB-inducing cytokine, IL-33 is extensively involved in promoting Th2 and allergic immune responses and mediating activation of mast cells and eosinophils (33, 64–66). There is a documented elevation in both Th1 and Th2 cytokines in MS and IL-33 secretion in the periphery and from activated astrocytes in the CNS of MS patients which might be partially responsible for promoting the Th2 response (52, 54, 55, 67). IL-33 has also been shown to activate mast cells, which is an important inflammatory cell in MS (36, 68–70). Therefore, the elevated IL-33 expression of IL-33 in MS might play a critical role in mast cell activation that can mediate secretion of neurotoxic molecules, enhance blood-brain barrier entry, and modulate activation of CNS and immune cells. Importantly, and additional function of IL-33 is to facilitate trafficking of immune cells into the CNS by specifically attracting Th2 cells and increasing adhesion to fibronectin of human mast cells (71, 72).

Macrophages play an important role in MS through direct phagocytosis of myelin and through secretion of inflammatory molecules that cause oligodendrocyte and neuronal death (55, 73, 74). IL-33 has been shown to act on monocytes, dendritic cells, and macrophages to induce the expression of cytokines, chemokines, and other proinflammatory genes (56, 75, 76). Therefore, elevated IL-33 expression levels documented here are expected to contribute to inflammatory demyelination through macrophage and dentritic cell activation. More work is needed to examine if macrophages from MS subjects have altered sensitivity to IL-33 stimulation.

We demonstrated that *in vivo* three-month IFN-β treatment significantly decreases IL-33 expression in plasma and fresh PBMCs of MS patients and correlated with significant reduction in NF-κB activation and IL-6 and IL-13 expression. We have previously shown that IFN-β treatment both *in vivo* and *in vitro* induces the expression of the protein tyrosine phosphatase SHP-1 that can negatively regulate NF-κB activation and NF-κB responsive gene expression (51, 54, 55). Additionally, we have previously shown that anti-SHP-1

siRNA prevented IFN-β mediated NF-κB suppression. Based on our findings, it is possible that IFN-β suppresses IL-33 expression by reducing NF-κB activation, but further experiments are required to establish the exact mechanism.

The CNS has been found to have extremely high expression of IL-33 at the mRNA level and indeed the CNS has the highest expression of IL-33 of all organs in the body (23). Here we demonstrate that IL-33 protein is significantly elevated in brain white matter areas of active demyelination containing dense leukocyte infiltrate along with high levels of inflammatory infiltrate. Importantly, IL-33 is elevated both at the protein and mRNA level in normal appearing white matter of MS patients compared to normal subjects, suggesting that CNS resident cells are an important source of IL-33 in the CNS. Additional inflammatory mediators like IL-6, IL-13, MCP-1, IP-10, and TARC are also elevated in NAWM of MS patients further suggesting a significant inflammatory reaction in CNS resident cells despite absence of infiltrating immune cells (8, 9).

We have previously described the expression of biologically active IL-33 protein by CNS cells, particularly mouse astrocytes (33). Astrocytes are nonhematopoietic epithelial-like cells of the CNS and are known to express both subunits of the IL-33R, ST2L and IL-1R accessory protein (IL-1Rap) (23, 77). Additionally, studies in mice have shown that IL-33 is expressed by both astrocytes and endothelial cells *in vivo,* which may act on microglia expressing ST2L (34). Within the CNS, microglia are an important target of IL-33, which increases microglia proliferation, secretion of inflammatory cytokines and chemokines, and phagocytosis (34). Importantly, activated microglia are important mediators of demyelination in MS (78). These data point to an possible inflammatory role of IL-33 in the CNS that can contribute to immune cell activation and augment signaling pathways mediating oligodendrocyte and neuronal injury.

Although speculative, a primary role for IL-33 in MS pathogenesis can be easily envisioned based on our present observations and known requirements for IL-33 release from cells. For instance, intracellular IL-33 may be both increased and subsequently released from astrocytes and macrophages by viruses infections (59, 61, 79). This is an attractive pathway as viruses have long been implicated in the pathogenesis of MS and many excellent animal models exist (80). Thus, virus infection provides both priming via viral PAMPs (eg. dsRNA) and release via virus-induced necrosis (81–84). Cell necrosis further results in extracellular increases in danger-associated molecular patterns (DAMPs) (eg. ATP) in which extensive plasma membrane leakage (85, 86) and IL-33 release to the extracellular space is amplified (87, 88).

The targets for liberated IL-33 in MS is another key unanswered question but various sites can be considered that are consistent with MS lesion development. For instance, activation of mast cells by released IL-33 (89, 90) which have been implicated to play a key role in MS pathogenesis(91, 92), secrete large quantities of TNF-α and IL-6 (69). Moreover, IL-33 may be particularly proinflammatory in the CNS as both microglia and astrocytes express IL-33 receptors and respond by proliferating and producing TNF-α and IL-1β (34). These archetypical proinflammatiory cytokines further promote inflammatory mediators and recruitment of lymphocyte and macrophage effectors to the CNS instigating intense inflammatory responses in the white matter (93). Finally, MS lesion development may relate to enhanced systemic IL-33 expression in plasma and peripheral blood leukocytes described in the present studies. As in the CNS, microbial infections may be inducers of IL-33 production and release by peripheral myeloid and lymphoid cells in which IL-33 expression has been described (79, 94). While the exact role of IL-33 in MS remains to be determined, our present data allow consideration of potential pathways in which IL-33 may participate in MS that will be better understood once complex aspects of IL-33 expression, feedback

mechanisms (95), and IL-33 responsiveness of immune cell subsets (18) are completely known.

Acknowledgments

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Highlights

The first study documenting IL-33 expression in multiple sclerosis

IL-33 was elevated in plasma and activated leukocytes of MS patients

Three-month treatment with interferon β-1a suppressed plasma IL-33 levels

IL-33 was elevated in brains of MS patients and astrocytes highly expressed IL-33

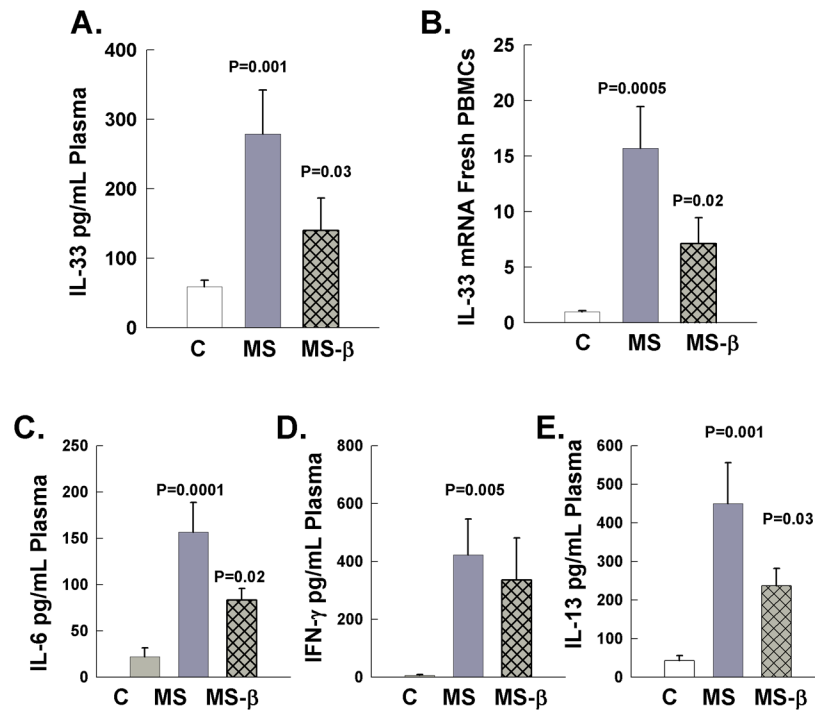IL-33 is elevated in the periphery and CNS, implicating IL-33 in MS pathogenesis

Figure 1.
IL-33 protein in plasma and mRNA expression in freshly drawn PBMCs and plasma levels of IL-6, IFN-γ, and IL-13. Cytokines levels were quantified in plasma PBMCs of normal subjects (C), untreated RR MS patients (MS), and *in vivo* in RR MS patients following three month treatment with interferon β-1a (MS-β). **A.** IL-33 was quantified by ELISA in plasma. **B.** Il-33 mRNA levels were quantified in freshly isolated PBMCs. The absolute mRNA transcript copy numbers per 10ng of total RNA were quantified using real time RT-PCR and were normalized to the levels of normal subjects that are shown as 1. **C – E:** The levels of IL-6, IFN-γ, and IL-13 were quantified in the plasma of the same samples of normal subjects and MS patient before and after three month treatment with interferon β-1a.
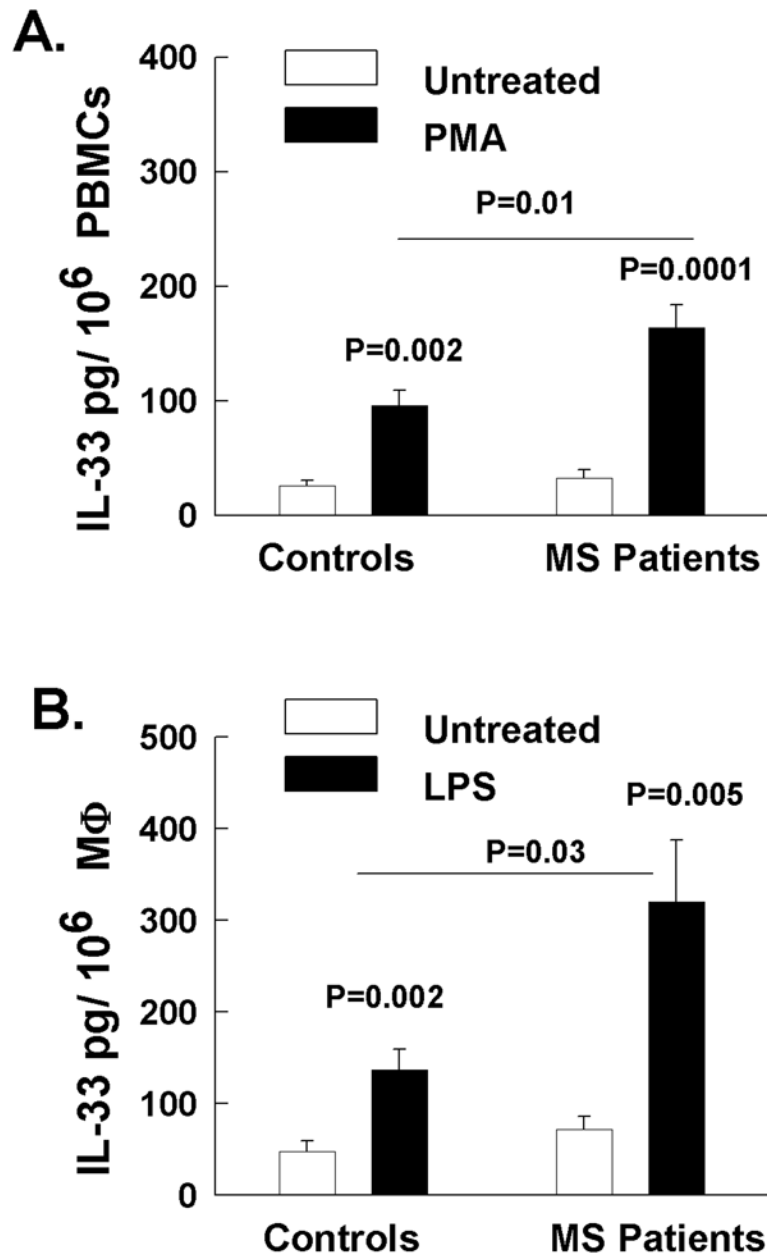
Figure 2.
IL-33 was quantified by ELISA in the supernatants of cultured PBMCs or macrophages of normal subjects and MS patients. **A.** PBMCs were cultured in the presence of IL-2 for one week and then stimulated with Phorbol myristate acetate and and ionomycin (PMA) for 24 hours or media alone. Then IL-33 was quantified in the supernatants **B.** Adherent monocytes were expanded in the presences of GM-CSF for one week. Macrophages were then stimulated with LPS for 24 hours and secreted IL-33 protein levels were determined with ELISA.
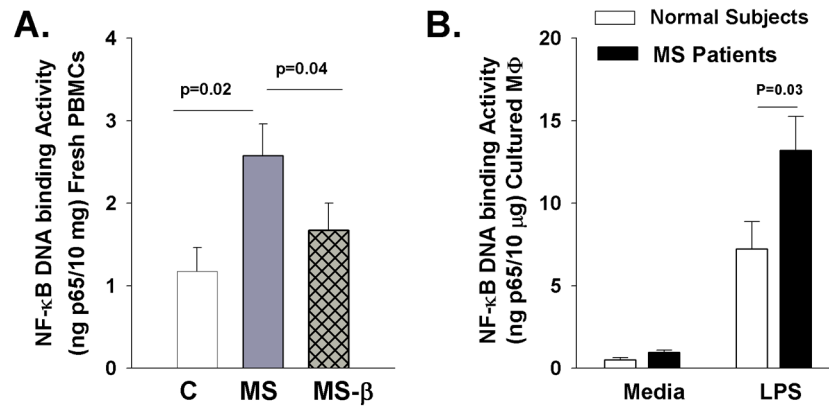
Figure 3.
NF-κB activation in macrophages of MS patients and normal subjects. Nuclear extracts were isolated from freshly isolated PBMCs or cultured macrophages and allowed to bind an NF-κB consensus-binding sequence. Bound NF-κB was then detected by a p65 specific antibody and quantified based on a calibration curve generated by using a purified p65 recombinant protein. The NF-κB DNA binding activity is reported as ng of bound p65 protein per 10μg of total protein in nuclear extracts. **A.** NF-κB activation in freshly isolated PBMCs from normal subjects (C), untreated MS patients (MS), and MS patients following three month *in vivo* IFN-β treatment (MS-β). **B.** NF-κB activation in cultured macrophages from normal subjects and MS patients following treatment with media alone or LPS for 1 hour.
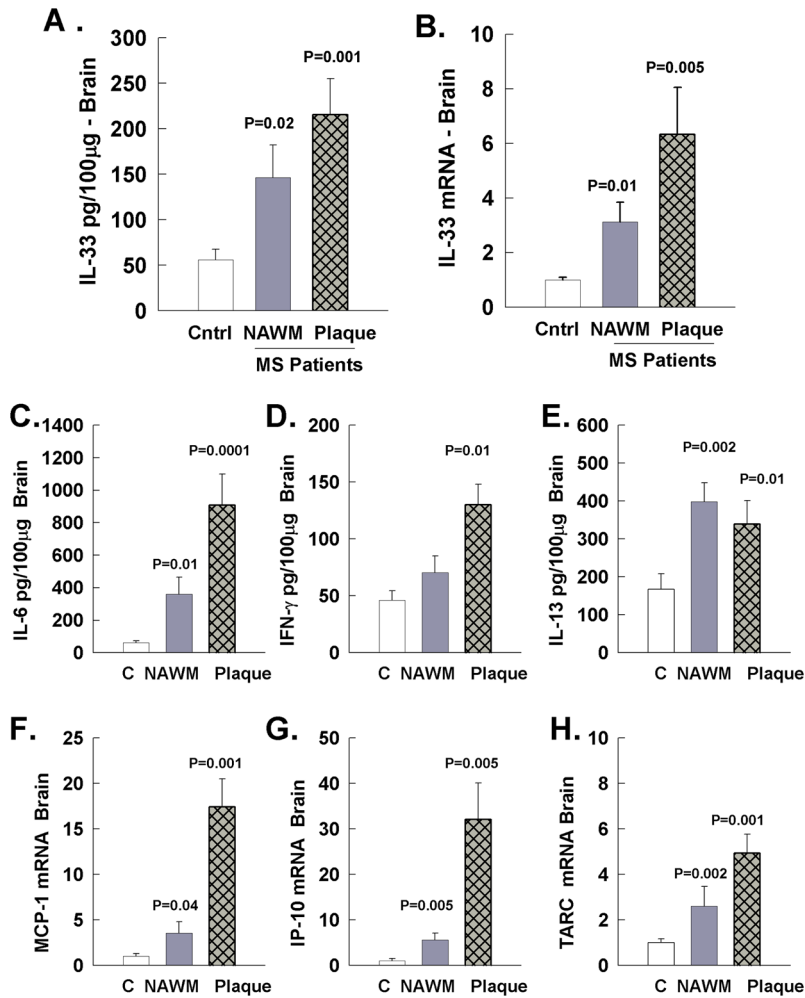
Figure 4.
IL-33 expression along with cytokine and chemokine expression in white matter from brains of normal subjects (C), normal appearing white matter of MS patients (NAWM), and white matter plaque areas of MS patients (Plaque). **A.** IL-33 protein expression by ELISA quantified as pg Il-33/100μg of total protein in brain lysate. **B.** IL-33 mRNA expression determined by real time RT-PCR and results were normalized to the levels of normal subjects that are shown as 1. **C – E:** The levels of IL-6, IFN-γ, and IL-13 were quantified in the brain of the same samples by ELISA and displayed as pg cytokine/100μg of brain protein. **F – H:** The levels of the chemokines MCP-1, IP-10, and TARC were also quantified by real time RT-PCR and results were normalized to the levels of normal subjects that are shown as 1
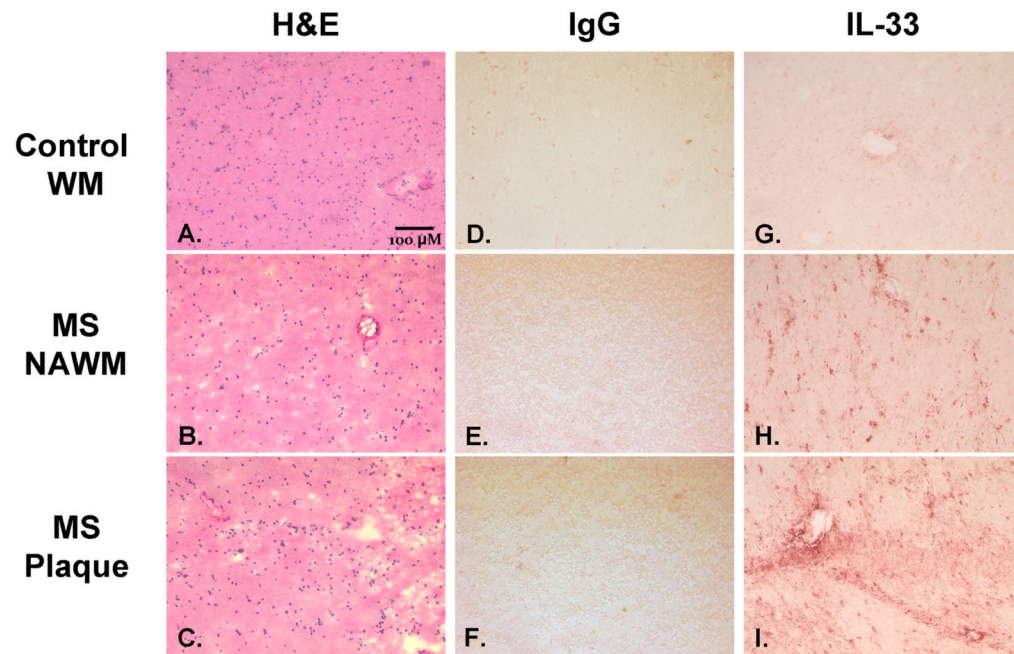
Figure 5.
Imunohistochemical staining of IL-33 the CNS of MS patients. **A–C**: Hematoxylin and eosin stain of **A.** white matter from normal subjects, **B.** normal appearing white matter from MS patients, and **C.** plaque region with focal demyelination and immune cell infiltration from MS patient. **D–F:** Negative control IgG immunohistochemical staining with red precipitate of **D.** white matter from normal subjects, **E.** normal appearing white matter from MS subject, and F. plaque region with focal demyelination and immune cell infiltration from MS patient. **G–I:** Representative immunohistochemical staining of IL-33 with red precipitate of **G.** white matter from normal subjects, **H.** normal appearing white matter from MS patients, and **I.** plaque region with focal demyelination and immune cell infiltration from MS patient. Images were taken at 200x magnification and the bar indicates a length of 40uM.
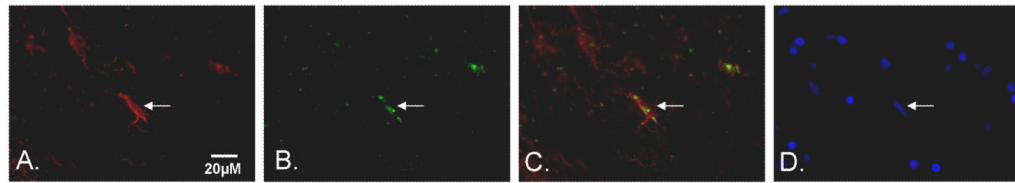
Figure 6. Double immunofluorescence of IL-33 and GFAP in MS brain
Representative images from fresh-frozen normal appearing white matter of MS patients.
Tissue was stained with **A.** glial fibrillary acidic protein (GFAP) a specific marker for
astrocytes and appears red in the image, **B:** IL-33 cytokine staining that appears green. **C.**
The images for GFAP and IL-33 were merged. **D.** DAPI staining showing cell neuclei
Arrow indicates the identical cell through the images. Bar indicates 20 μM.

Table I

Biometric data of MS patients and normal subjects who donated blood for the study

Il-33 protein and mRNA was quantified in the plasma, and freshly isolated PBMCs, cultured IL-2 expanded PBMCs, and cultured macrophages of those subjects. RR MS are untreated Relapsing Remitting multiple sclerosis patients. The data are shown in mean value ± SD. For the gender, F stands for females and M stands for males. The age at onset of clinically diagnosed MS and disease duration are shown in years. The clinical symptoms were measured on Kurtzuke's Expanded Disability Status Scale (EDSS) at the time the initial blood was drawn. Patients with RR MS donated blood before and after a three-month treatment with recombinant interferon β-1a and EDSS scores shown here were calculated just before treatment with interferon β-1a.

Patient Category	Number of Subjects	Age (Years)	Gender	Age at Onset	Disease Duration	EDSS Score
Normal Subjects	32	41±12	22F, 10M	---	---	---
RR MS	32	41±9	22F, 10M	36±9	5.9± 5	2.5±1.4
IFN β-1a Tx	20	40±8	14F, 6M	34±9	5.4±4	2.4±1.2

Table II

Biometric data of MS patients and normal subjects that were used to examine Il-33 expression in the CNS

Brain tissue was used to quantify IL–33 mRNA and protein and brain IL–33 expression and localization was determined by immunohistochemisty and immunofluorescence. Fresh frozen brain sections were received from the Brain Bank. Brain white matter was used from normal subjects. The brains of MS patients had two district separate blocks from normal appearing white matter (NAWM) and plaque areas. The data are shown as the mean value ± SD. For the gender, F stands for females and M stands for males.

Patient Category	Number of Subjects	Age (Years)	Gender	Lysis Time	Tissue pH
Normal Subjects	20	63±13	11F, 9M	15±4	7.1±0.2
MS	9	60±12	6F, 3M	19±7	7.0±0.3