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Research Paper

Long-term interleukin-33 treatment delays disease onset and alleviates astrocytic activation in a transgenic mouse model of amyotrophic lateral sclerosis

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ABSTRACT

Inflammation is a prominent feature of the neuropathology of amyotrophic lateral sclerosis (ALS). Emerging evidence suggests that inflammatory cascades contributing to the disease progression are not restricted to the central nervous system (CNS) but also occur peripherally. Indeed, alterations in T cell responses and their secreted cytokines have been detected in ALS patients and in animal models of ALS. One key cytokine responsible for the shift in T cell responses is interleukin-33 (IL-33), which stimulates innate type 2 immune cells to produce a large amount of Th2 cytokines that are possibly beneficial in the recovery processes of CNS injuries. Since the levels of IL-33 have been shown to be decreased in patients affected with ALS, we sought to determine whether a long-term recombinant IL-33 treatment of a transgenic mouse model of ALS expressing G93A-superoxide dismutase 1 (SOD1-G93A) alters the disease progression and ameliorates the ALS-like disease pathology. SOD1-G93A mice were treated with intraperitoneal injections of IL-33 and effects on disease onset and inflammatory status were determined. Spinal cord (SC) neurons, astrocytes and T-cells were exposed to IL-33 to evaluate the cell specific responses to IL-33. Treatment of SOD1-G93A mice with IL-33 delayed the disease onset in female mice, decreased the proportion of CD4+ and CD8 + T cell populations in the spleen and lymph nodes, and alleviated astrocytic activation in the ventral horn of the lumbar SC. Male SOD1-G93A mice were unresponsive to the treatment. In vitro studies showed that IL-33 is most likely not acting directly on neurons and astrocytes, but rather conveying its effects through peripheral T-cells. Our results suggest that strategies directed to the peripheral immune system may have therapeutic potential in ALS. The effect of gender dimorphisms to the treatment efficacy needs to be taken into consideration when designing new therapeutic strategies for CNS diseases.

Abbreviations: ALS, amyotrophic lateral sclerosis; fALS, familial ALS; sALS, sporadic ALS; CNS, central nervous system; IL-33, interleukin-33; SOD1, superoxide dismutase 1; IL-33R, interleukin-33 receptor; TNF, tumor necrosis factor; IL-6, interleukin-6; EAE, experimental autoimmune encephalomyelitis; TG, transgenic; WT, wildtype; SC, spinal cord; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adaptor molecule-1; RT, room temperature; PBS, phosphate buffered saline; DMEM, Dulbecco's minimum essential medium; RT, room temperature; IL-1RAcP, interleukin-1 receptor accessory protein; IL-10, interleukin-10; MCP-1, monocyte chemoattractant protein-1; IFN-γ, interferon gamma; Arg-1, arginine-1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; *NFE2L2*, the gene encoding Nrf2; HO-1, hemeoxygenase-1; SD, standard deviation; ANOVA, analysis of variance; CM, conditioned medium

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Introduction

Amyotrophic lateral sclerosis (ALS) is the most common motoneuron disease with adult-onset. It results in progressive degeneration of motoneurons in the primary motor cortex, brain stem, and spinal cord (SC) (Rowland and Shneider, 2001). Subsequently, ALS leads to rapidly advancing paralysis of skeletal muscles and ultimately to death due to respiratory failure usually within 3–5 years after disease onset. The only approved drug for treating ALS is riluzole, which delays the onset of ventilator-dependence or tracheostomy in selected patients by 3–5 months, possibly by a mechanism related to glutamate toxicity (Lacomblez et al., 1996). The majority of ALS cases are considered to represent the sporadic disease type (sALS), while only 10–15% of ALS cases are inherited (fALS) and caused by mutations in several genes (Rowland and Shneider, 2001; Ince et al., 2011). Superoxide dismutase 1 (SOD1) gene mutations have been estimated to underlie approximately 20 percent of fALS cases (Rosen, 1993; Renton et al., 2011). The etiology of sALS is unclear and is currently considered multifactorial and polygenic in the majority of cases (Eisen, 2009).

While the in depth molecular and cellular mechanisms of ALS pathology have remained obscure, several molecular pathways have been shown to induce or contribute to motoneuron dysfunction and injury in both fALS and sALS. These include mitochondrial dysfunction, protein aggregation, excitotoxicity, aberrant RNA processing, altered axonal transport, oxidative stress and toxicity of nonneuronal (glial) cells (Lin et al., 1998; Williamson and Cleveland, 1999; Nagai et al., 2007; Ilieva et al., 2009; Barber and Shaw, 2010; Cova et al., 2010; Cozzolino and Carri, 2012). Whilst most, if not all of these pathological processes are clearly interdependent, the role of glial cells as well as local and systemic inflammation appear to play a crucial role in the progression of ALS (Kawamata et al., 1992; Raibon et al., 2008). For example, astrocytic dysfunction to the pathogenesis of ALS has been reported (Pellerin and Magistretti, 1994; Phatnani et al., 2013): astrocytes derived from mSOD1 mice show altered metabolic activity (Pellerin and Magistretti, 1994; Phatnani et al., 2013), and astrocytic secretion of pro-inflammatory mediators is a significant contribution to motoneuron degeneration in ALS (Hensley et al., 2006; Zhao et al., 2013).

Interleukin-33 (IL-33), a member of the IL-1 family of cytokines, is one of the key regulators of inflammatory and immune processes. In fact, IL-33 is a dual-function protein as it mediates inflammatory responses when released outside the cell but is also known to be associated with chromatin and to act as a transcriptional regulator with potential to dampen nuclear factor kappaB activity (Schmitz et al., 2005; Carriere et al., 2007; Ali et al., 2007; Haraldsen et al., 2009). IL-33 binds to a heterodimeric receptor complex comprising of IL-1 receptor accessory protein (IL-1RAcP) and ST2. The soluble form of ST2 acts as a decoy receptor harnessing the functions of IL-33 (Hayakawa et al., 2007; Molofsky et al., 2015). The IL-33 receptor is expressed on a variety of cell types, including leukocytes and mast cells and upon binding to IL-33, stimulates the innate type 2 immune cells to produce Th2 cytokines, which are thought to be beneficial and enhance the recovery processes in many CNS injuries and neurodegenerative diseases (Schmitz et al., 2005; Moro et al., 2010; Neill et al., 2010; Price et al., 2010). On the other hand, IL-33 can also enhance LPS-mediated production of tumor necrosis factor (TNF) and interleukin-6 (IL-6) by macrophages, suggesting promotion of proinflammatory processes in some conditions (Miller, 2011). Although IL-33 mediated Th2 responses are well known to promote the pathogenesis of asthma by expanding Th2 cells and to mediate joint inflammation, atopic dermatitis and anaphylaxis by mast cell activation (Miller, 2011), recent studies suggest that IL-33 is particularly important in brain functions and mediates protection in neurological diseases (Yasuoka et al., 2011; Jiang et al., 2012; Lin et al., 2012; Milovanovic et al., 2012; Korhonen et al., 2015; Pomeshchik et al., 2015). Indeed, the highest levels of IL-33 in the body are found in the CNS where it is mainly produced by astrocytes (Yasuoka et al., 2011). Since IL-33 has been shown to ameliorate the progression of experimental autoimmune encephalomyelitis (EAE), a rodent model of neuroinflammation (Jiang et al., 2012), improve the outcome from cerebral stroke (Korhonen et al., 2015) and spinal cord injury (SCI) (Pomeshchik et al., 2015), and the levels of IL-33 have been reported to be reduced and the levels of soluble ST2 increased in ALS (Lin et al., 2012), we aimed to investigate whether administration of recombinant IL33 alters the disease progression of SOD1-G93A transgenic mouse model of ALS.

Experimental procedures

Animals

Transgenic (TG) SOD1-G93A mice (B6.Cg-Tg-(SOD1-G93A)1Gur/J, The Jackson Laboratory, Bar Harbot, ME, USA) with a high copy number of the human SOD1-G93A gene were maintained on C57Bl/6 J congenic background. Motor deficits and progressive paralysis start in these hemizygous mice at the age of 17–19 weeks and the end stage of the disease manifests at the age of 24–26 weeks (Naumenko et al., 2011; Pollari et al., 2011). Disease onset was determined by the wire-hang test where mouse was placed hanging upside-down on a wire grid and latency to fall was recorded (Miana-Mena et al., 2005). Disease onset was determined by the inability to keep hold on the wire grid for three minutes. The test was repeated three times per week to detect the onset age. TG and WT littermates were used for experiments and these littermates were randomly and equally divided into treatment groups by using GraphPad QuickCalcs. All animal experiments were conducted according to the national regulations of the usage and welfare of laboratory animals and approved by the Animal Experiment Committee in the State Provincial Office of Southern Finland. Altogether 54 animals were used in the study from which 14 mice died for reasons unrelated to the disease. The n number used in each experiment is indicated in the legend of each figure.

IL-33 treatment

Mice received recombinant mouse IL-33 (Biolegend, SanDiego, CA, USA) twice per week i.p. at a dose of 1 μg/mouse for a month and 0.5 μg/mouse for the following weeks according to our published (Korhonen et al., 2015; Pomeshchik et al., 2015) and unpublished data of the effective and safe dose. IL-33 was diluted into phosphate buffered saline (PBS) right before use. PBS injections served as vehicle controls. The treatment was started pre-symptomatically at the age of 80 days and continued for 9 weeks until the mice reached the age of 20 weeks. The mice were sacrificed at the age of 22 weeks and tissues dissected for flow cytometry of spleen and lymph node leukocytes, qPCR and immunohistochemical analysis of the SC.

Flow cytometry

The spleen and lymph nodes were homogenized using 70 μm cell strainers (BD Biosciences San Jose, CA, USA) to produce single cell suspensions. Red blood cells from spleens were lysed with Pharmlyse (BD Biosciences) and then washed with D-PBS (Sigma, St. Louis, MO, USA) + 1% FBS buffer. Nonspecific antigen binding was blocked using 5 μg/ml CD16/32 (clone 2.4-G2, BD Bioscience). One million cells were stained for 30 min on ice with fluorochrome-conjugated FITC-Ly6C (BD Biosciences,), PE-CD11b (1:400) and PE-Gr-1 (1:200) to gate out monocytic cells and FITC-CD4 (1:300) and PerCP-eFluor710-CD8 (1:600) (eBioscience, San Diego, CA, USA). Samples were washed twice in buffer and analyzed on a FACSCalibur (BD Biosciences) equipped with a single 488-nm argon laser. Data analysis was performed using Cellquest Pro software (BD Biosciences).

Immunohistochemistry

The mice were anesthetized with an overdose of sodium pentobarbital (Mebunat, Orion, Espoo, Finland) and transcardially perfused with heparinized saline (2500 IU/l) to remove blood from the tissues. The meninges were removed from the SC and the mid lumbar area was cut in half longitudinally. Tissues were post-fixed in 4% PFA for 21 h at 4 °C, prepared as paraffin-embedded sections and cut with a microtome (Leica SM 2000R, Leica Instruments GmbH, Nussloch, Germany) into 5-µm sections. The SC sections were immunostained with antibodies to glial fibrillary acidic protein (GFAP 1:500, Chemicon / Millipore, Billerica, MA, USA), ionized calcium binding adaptor molecule-1 (Iba-1, 1:250 Wako, Osaka, Japan), Arginase-1 (Arg-1, 1:200, Santa Cruz Biotechnology, Dallas, TX, USA) and p38 (1:200, Cell signaling technology, Danvers, MA, USA) followed by Alexa Fluor 568- or 488-conjugated secondary antibody (Life Technologies, Carlsbad, CA, USA) or biotinylated rabbit anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA, USA) followed by avidin-biotin complex (Vectastain Elite kit, Vector Laboratories Inc.) and visualization using nickel enhanced diaminobenzidine (Sigma, St. Louis, MO, USA) as a substrate. The sections were imaged with a microscope using 10X magnification (Olympus BX51, Olympus, NY, USA) with an attached digital camera (Color View 12 or F-View; Soft Imaging System, Muenster, Germany) running Analysis Software (Soft Imaging System). The immunopositivity in the SC ventral horn was quantified from 10 sections spanning at 50 µm intervals using Image-Pro 6.2 software (Media cybernetics, Rockville, MD, USA).

Embryonic SC neuronal culture

SC neurons from C57BL76j E14 mouse embryos were obtained using a protocol modified from that of previously described (Vartiainen et al., 1999). Briefly, embryos were decapitated, the SCs were dissected out, and the meninges and the dorsal root ganglia were removed. SCs were digested in 0.5 mg/ml papain (Sigma), 0.04 mg/ml DNase (Roche) in PBS 5–10 min at 37 °C. Papain solution was replaced with 1 mg/ml BSA, 0.04 mg/ml DNase and 10 mM glucose in PBS and gently triturated and centrifuged. The pellet was resuspended into Dulbecco's minimum essential medium (DMEM), 10% FBS, 2 mM glutamine, penicillin-streptomycin (all from Gibco) and plated at the density of 2.37×10^5 cells/cm2 onto poly-D-ornithine-coated (Sigma) multiwell plates. Cells were maintained in humidified atmosphere at 37 °C in 5% CO2. On next day, the medium was replaced with Neurobasal, L-glutamine, penicillin-streptomycin supplemented with B27 (all from Gibco), 25 µM glutamic acid (Sigma) and 10 µM AraC (Sigma). After 24 h, the medium was replaced with Neurobasal with supplements excluding AraC. One third of the medium was changed every three days and the cells were used for experiment after 7 days in culture. The experiments were done in the supplemented medium. The SC of each embryo was processed separately and genotyped for human SOD1. Approximately 90% of cells in the culture were neuronal and rest glial, mainly astrocytic, cells. On day 7 in culture cells were co-treated with 10 µM glutamate and 1, 10 or 100 ng/ml IL-33 (Biorbyt Ltd, Cambridge, United Kingdom). After 24 h of exposure, cell viability was measured with LDH assay.

Cortical neuronal culture

Cortices from embryonic day 14 fetal brains of C57Bl/6 J mice were dissected and suspended in Krebs buffer containing trypsin (20x, Sigma). After 15 min incubation at 37 °C, fresh buffer with DNAse was added. The tissue suspension was gently mixed and centrifuged at 375 g for three minutes. The cell pellet was resuspended into Neurobasal/B27/L-glutamine/Gentamycin medium. The viable cells were plated on 48-well culture plates precoated with poly-D-lysine (0.5 µg/µl, Sigma) at 200 000 cells/well. Four days after plating 50% of medium was changed to fresh culture medium.

MTT assay

The MTT (3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide) assay was used to explore the effect of IL-33 in glutamate exposed cortical neurons. In the reaction, reductases of metabolically active, viable cells reduce the yellow, soluble MTT to the dark, water-insoluble MTT-formazan which is detected by a spectrophotometer at 570 nm. Cortical neurons were plated onto a 48-well plate pre-coated with poly-D-lysine in neurobasal media supplied with B27, L-Glutamine and Gentamycin. The neurons were exposed to 100 µM glutamate and different concentrations of recombinant IL-33 (1, 10, 100 ng/ml) at day 6 after plating. Twenty-four hours later, 200 µl fresh medium containing 20 µl of MTT solution (5 mg/ml) was added into the wells, and the cells were incubated for 2 h at 37 °C after which 200 µl of SDS–DMF buffer (200 µg/ml SDS, 50% dimethylformamide, pH 4.7) was used to dissolve the blue crystals. The absorbance was read with a Bio-Tek Elx-800 microplate reader. The data are expressed as % of control.

LDH assay

Relative cell viability was measured from SC neuronal cultures by the LDH assay according to manufacturer's protocol (Roche, Basel, Switcherland). Shortly, after 24 h exposure to glutamate and different concentrations of IL-33 (1, 10 and 100 ng/ml), supernatant was collected from SC neurons and mixed with reaction solutions. Solutions were incubated in RT until sufficient colour development was reached and absorbance was measured at 490 nm with Victor microplate reader reader (PerkinElmer, Waltham, MA, USA).

Human T cell culture

Human peripheral blood mononuclear cells (PBMC) were isolated from the blood samples received from healthy adult volunteers at the Finnish Red Cross Blood Service (approved by the Research Ethics Committee of Northern Savo Hospital district, license no. 42//2010) using Leucosep® tubes (Greiner bio-one, Germany) according to the manufacturer's instructions. Shortly, the blood samples were diluted 1:3 in 2 mM EDTA in PBS and transferred to Leucosep® tube. The samples were centrifuged at 1000 × *g* for 10 min and the interphase containing PBMCs was collected and washed once with PBS and once with T-cell medium [RPMI 1640 (Sigma-Aldrich, USA) containing 1 mM sodium pyruvate (Sigma-Aldrich), 10% heat-inactivated FBS (BioWest, France), 2 mM Glutamax (Life Technologies), 50 IU / ml Penicillin (Life Technologies), 50 µg / ml Streptomycin (Life Technologies), 1 x non-essential amino acids (Life Technologies), 10 mM HEPES (Life Technologies), 20 µM 2-mercaptoethanol (Life Technologies) and 25 IU / ml of recombinant human IL-2 (Peprotech, Rocky Hill, NJ, USA). The CD3 + T cells were enriched for 5 days in dishes pre-coated with mouse anti-human CD3 antibody (5 µg / ml in PBS; BD Biosciences, USA) (Brown et al., 2010). The medium was changed every other day and the stimulation with CD3 was repeated every two weeks. The culture contained approximately 90% T cells as confirmed by flow cytometry with staining against CD3 antibody (data not shown). T-cells were exposed to IL-33 (10 ng/ml) for 24 h and the conditioned medium (CM) was applied to astrocytes as described below.

Astrocytic culture

Astrocyte cultures were prepared as described previously (Pihlaja et al., 2011) with some modifications. Briefly, cortices were isolated from 6-8-week-old WT or TG mice, the tissue suspended in Hank's Balanced Salt Solution (Lonza, Allendale, NJ, USA) and centrifuged at 375 x g for 5 min at room temperature. After the addition of 0.25% trypsin-EDTA (Invitrogen / Life Technologies, Great Island, NY, USA), the suspension was incubated at 37 °C for 30 min with occasional shaking. Fresh culture medium was added to neutralize the effect of trypsin and

the suspension was centrifuged at 375 x g for 5 min. The cell suspension was added on top of Percoll (Sigma) and centrifuged at 375 x g for 10 min. The supernatant was discarded and the layer of glial cells was washed once with fresh culture medium. The cells were plated onto poly-L-lysine pre-coated flask in DMEM/F12 containing 10% heat-inactivated FBS, 2 mM L-Glutamine, 100 U / ml penicillin-streptomycin and G5 supplement (Invitrogen) (Pihlaja et al., 2011). The microglia were removed by shaking the plates for 2 h prior the experiments. The passages 4–8 were used for the experiments. Astrocytes were exposed to different concentrations of IL-33 (1, 10 and 100 ng/ml) and to LPS (10 µg/ml) and the expression of *IL-33* and the receptor components *ST2* and *IL-1RAcP* was determined by using qPCR. The cytokine secretion was measured from the cell culture medium using cytometric bead array (CBA, BD Pharmingen, San Jose, CA, USA). CM from IL-33 treated T-cells was applied to LPS or vehicle exposed astrocytes for 24 h and CBA (BD Pharmingen) was used to determine the cytokine concentrations secreted to the medium.

Cytometric bead array immunoassay

A panel of cytokines was analyzed by using CBA (BD Pharmingen) according to manufacturer's instructions. Briefly, 10 µl of CM or cytokine standards were added to the mixture of the capture beads and PE labeled detection antibodies. The samples were incubated for 2 h at room temperature, protected from light, washed and acquired on FACSCalibur. FCAP Array v2 (SoftFlow, Hungary) software was used to analyze the data. The data are expressed as ng/ml.

RT-PCR

Total RNA was extracted from gastrocnemius muscle samples of WT and TG mice and cultured primary astrocytes by TRIzol reagent (Invitrogen) and DNAeasy MiniKit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions. cDNA was synthesized from 500 ng of total RNA using random hexamer primers as a template and Maxima reverse transcriptase (all from Fermentas). The relative mRNA expression levels of *IL-33* and IL-33 receptor complex subunits *ST2L* and *IL1RAP* were measured from cultured astrocytes and the levels of *Arg-1*, Nuclear factor (erythroid-derived 2)-like 2 (Nrf2; the gene encoding Nrf2 protein: *NFE2L2*) and hemeoxygenase-1 (*HO-1*) from muscles by qPCR (StepOnePlus, Applied Biosystems / Life Technologies) using specific Assays-on-demand (Applied Biosystems/ Life Technologies). The expression levels were normalized to 18S ribosomal RNA and represented as fold change in the expression ± standard deviation (SD).

Statistical analysis

The data are expressed as mean + standard deviation (SD). The data were analyzed with GraphPad Prism software using Mantel-Cox survival statistics with a log-rank test for testing differences in the disease onset proportions. All other data were analyzed with one-way analysis of variance (ANOVA) or two-way ANOVA, followed by Sidak's or Tukey's multiple comparisons post hoc test when appropriate. P-values below 0.05 were considered as significant. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$)), **** ($p < 0.0001$).

Results

Long-term IL-33 treatment delayed the disease onset in TG female mice as measured by wire hang test

The mean age of the disease onset in both male and female vehicle-treated TG mice was 135 days as measured by wire hang test. The 9-week treatment with IL-33 significantly delayed the disease onset in female TG mice compared to their vehicle treated controls (Fig. 1A).

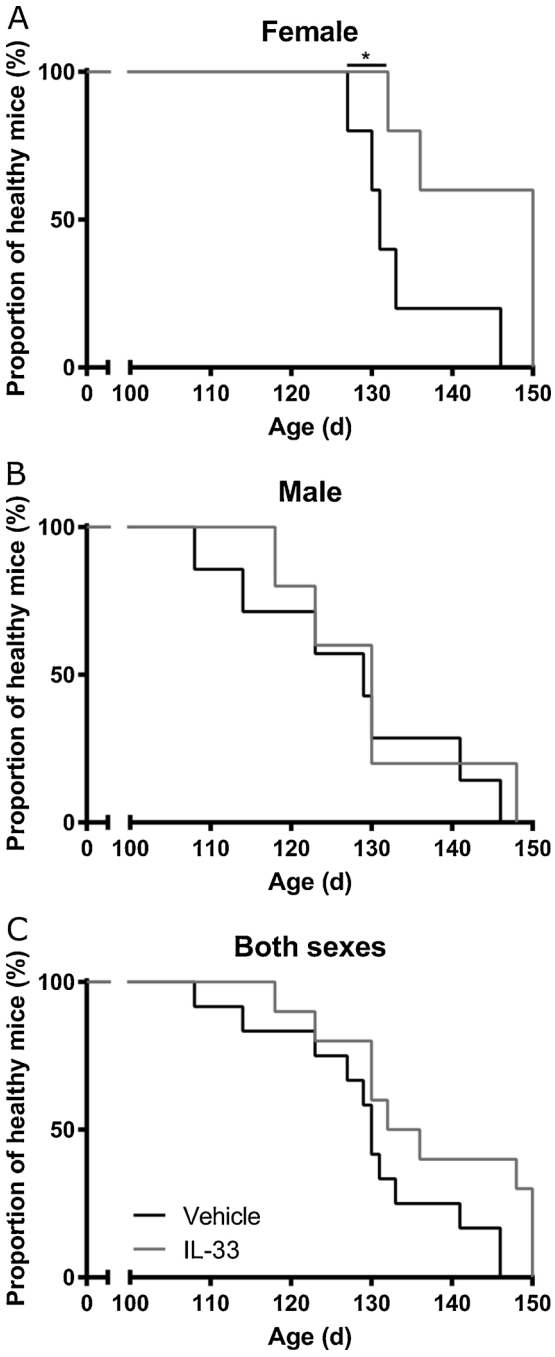

Fig. 1. Long-term IL-33 treatment delayed the disease onset of female TG mice. The treatment was initiated before the disease onset and it was continued until the sacrifice at the symptomatic phase. IL-33 treatment delayed the disease in the female mice (A) whereas male mice were unresponsive to the treatment (B). When the results from both sexes were combined the significance was lost (C). n = 5-7. Mantel-Cox Log-rank test. * $p < 0.05$.

Interestingly, male mice were unresponsive to the treatment as shown in Fig. 1B. When both sexes were pooled, the significance of the treatment effect was lost (Fig. 1C), thereby illustrating the gender difference in the treatment response.

IL-33 treatment reduced leukocyte counts in spleen and lymph nodes of TG female mice

Upon sacrifice at the age of 22 weeks, the spleens and lymph nodes of the mice were harvested for flow cytometry analysis of different

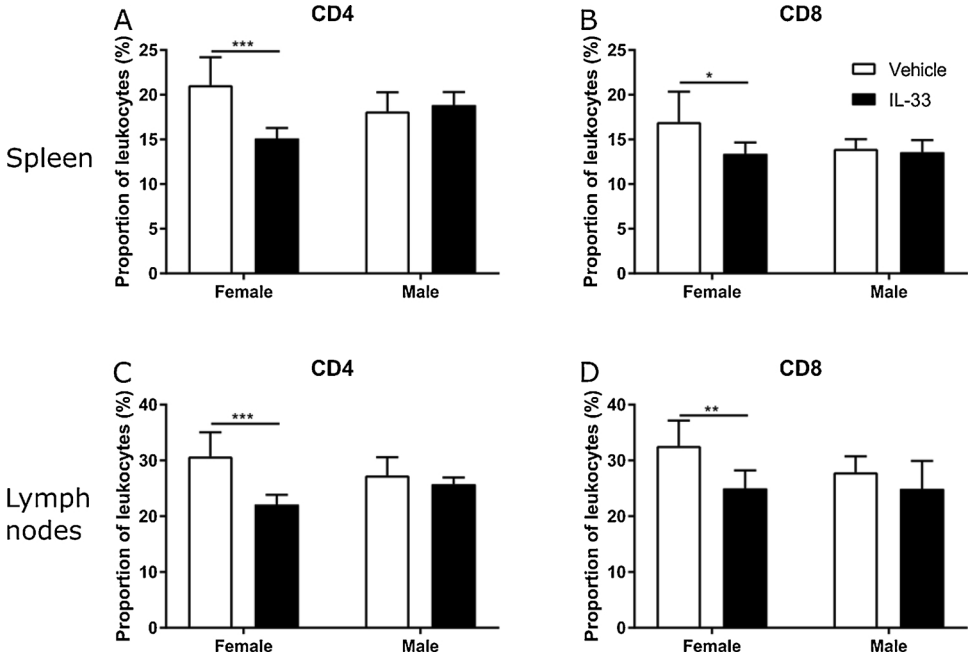

Fig. 2. IL-33 altered CD4 + and CD8 + T-cell populations in the spleen and lymph nodes. The relative numbers of lymphocyte populations were measured from the spleen and lymph nodes using flow cytometry. IL-33 treatment significantly decreased both CD4 + and CD8 + populations in both spleen (A–B) and lymph nodes (C–D), respectively, of SOD1-G93A female mice. The males were unresponsive to the treatment. Data are presented as mean ± SD. n = 5-7. Two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

leukocyte populations. Staining against CD4 and CD8 revealed that the ALS-like disease progression in TG mice did not induce alterations in the relative amount of CD4 + and CD8 + cells in the spleen compared to WT littermates (data not shown). IL-33 treatment significantly decreased the proportion of CD4 + cells in the spleen of TG female mice, however, it failed to cause significant alterations in TG male mice (Fig. 2A). Similarly, IL-33 failed to induce any alterations in the percentage of splenic CD8 + cells in male mice, whereas in females the treatment induced a significant reduction of CD8 + cells (Fig. 2B)

Similar to spleen, ALS-like disease progression did not induce any alterations in the percentage of CD4 + and CD8 + cells in the lymph nodes of the vehicle treated TG mice compared to their WT controls (data not shown). Long-term IL-33 treatment significantly decreased the proportion of lymphoid CD4 + cells in the TG female mice; however, it did not alter the relative amount of CD4 + cells in the lymph nodes of male TG mice (Fig. 2C). Accordingly, IL-33 treatment had no effect on the proportion of CD8 + cells in the lymph nodes of male TG mice (Fig. 2D), but reduced the proportion of CD8 + cells in the lymph nodes of the female mice (Fig. 2D).

IL-33 treatment reduced astrocytic activation in the spinal cords of TG female mice

The extent of ALS-related astrogliosis was analyzed in the ventral horn of the lumbar SC of the symptomatic TG mice and their WT controls by immunohistochemical staining against a marker of astroglial activation, GFAP. In accordance to published literature (Hall et al., 1998; Pollari et al., 2011; Gerber et al., 2012) all TG mice showed increased astrocytic activation (Fig. 3A,B). Long-term treatment with IL-33 significantly decreased the astrocytic activity in female TG mice (Fig. 3A), whereas it failed to alter GFAP immunoreactivity in male mice (Fig. 3B). Astrocytes were evenly distributed over the analyzed area and there were no drastic differences between the vehicle and IL-33 treated groups in astrocyte morphology, although this was not assessed quantitatively.

IL-33 treatment did not affect to SC microgliosis in TG mice but instead polarized the macrophages towards alternative activation

As expected, all vehicle-treated TG mice showed significant upregulation in the Iba-1 immunoreactivity compared to their WT controls (Fig. 4A, B). Long-term treatment with IL-33 did not alter microgliosis in either gender (Fig. 4A,B). IL-33 failed to significantly increase Arg-1 immunoreactivity in the ventral horn of the SC of female mice (Fig. 4I), yet it significantly increased Arg-1 immunoreactivity in TG male mice (Fig. 4J).

IL-33 treatment decreased the MAP kinase p38 immunoreactivity in the TG female mice

The extent of p38 immunoreactivity was assessed by immunohistochemistry. All vehicle treated TG animals exhibited elevated p38 immunoreactivity in the ventral horn of the SC sections (Fig. 5A,B). IL-33 treatment significantly reduced the levels of p38 in TG female mice (Fig. 5A) but failed to alter the p38 immunoreactivity in male mice (Fig. 5B).

IL-33 increased the mRNA expression of Arg-1, NFE2L2 and HO-1 in the muscles of TG mice

ALS is a disease affecting motoneuronal junctions and in the TG model used in this study, is apparent as hind limb muscle atrophy and paralysis (Brooks et al., 2004). To evaluate the impact of ALS pathology and peripheral IL-33 treatment on the inflammatory and anti-oxidant responses in the gastrocnemius muscle, we measured the mRNA expression of *Arg-1*, *NFE2L2* and *HO-1* in the muscle samples. ALS pathology did not alter the expression levels of *Arg-1* and *NFE2L2*, however, TG male mice showed mild, yet significant increase in the levels of *HO-1* (Fig. 6A-F). IL-33 treatment increased the expression levels of *Arg-1* in female TG mice (Fig. 6A), but failed to significantly increase the expression levels of *NFE2L2* and *HO-1* compared to vehicle treated TG female mice (Fig. 6C, E). IL-33 significantly increased the expression of all these genes in TG male mice (Fig. 6B,D,F).

IL-33 is not directly neuroprotective against glutamate-induced excitotoxicity in primary spinal cord or cortical neurons

To evaluate whether IL-33 is directly neuroprotective against glutamate induced toxicity, E14 cortical neurons from WT mice and E14 spinal cord neurons from WT and TG mice were exposed to glutamate

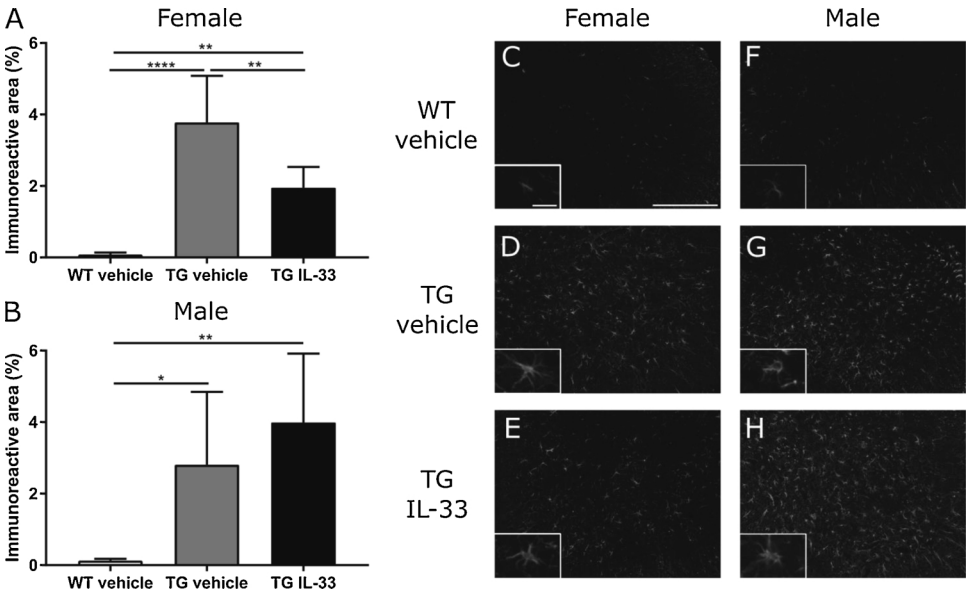

Fig. 3. IL-33 treated female mice showed decreased astrocytic activation in the affected spinal cord. Increased GFAP immunoreactivity was evident in the ventral horn of SC in all TG animals (A–B). IL-33 treatment significantly reduced the immunoreactivity in the TG female mice (A), however, it failed to reduce the extent of GFAP immunoreactivity in the TG male mice (B). Representative images of WT vehicle (C, F), TG vehicle (D, G) and TG IL-33 treated (E, H) females and males, respectively. High magnification inserts show cell morphology. Scale bar 100 μm, 10 μm in inserts. Data are presented as mean + SD. n = 5-7. One-way ANOVA. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Female df = 17, F = 33.28; Male df = 16, F = 8.590.

(100 or 10 μM, respectively) and increasing concentrations of recombinant IL-33 (1 ng/ml, 10 ng/ml and 100 ng/ml). Cell death was measured by MTT or LDH assay 24 h after the exposure. IL-33 alone was not toxic to neurons (data not shown) and was unable to prevent the glutamate induced neuronal death in cortical or spinal cord neurons (Fig. 7A and B, respectively).

Since astrocytes contribute to neuron death in ALS (Nagai et al., 2007) and we observed decreased astrocytic immunoreactivity in the IL-33 treated TG female mice, we aimed to investigate whether astrocytes respond to IL-33 by changing their cytokine secretion profile. Adult astrocytes isolated from WT and TG mice were exposed to increasing concentrations of recombinant IL-33 (1 ng/ml, 10 ng/ml and 100 ng/ml) and astrocytic cytokine production was measured in the culture media using CBA at 24 h after the exposure. TG astrocytes secreted IL-6 to a significantly higher degree compared to WT astrocytes (Fig. 7C). IL-33 exposure of TG astrocytes induced a further significant increase in the secretion of IL-6 compared to vehicle-treated TG astrocytes (Fig. 7C).

Expression levels of IL-33 and its receptors differ between WT and TG astrocytes

We next evaluated the expression levels of *IL-33* and its receptor subunits in primary adult astrocytes isolated from WT and TG mice. The basal expression of *IL-33* was statistically not significant between the genotypes, yet the highest concentration of IL-33 induced higher *IL-33* expression in WT than TG astrocytes (Fig. 8A). IL-33 treatment-induced increase in the expression levels of *ST2* receptor subunit in TG astrocytes but had no effect on the astrocytes isolated from WT mice (Fig. 8B). There was no genotype effect in the expression level of *IL1-RAcP* between TG and WT astrocytes and IL-33 altered the expression levels of *IL1RAcP* only in WT astrocytes (Fig. 8C). The levels of IL-33 as well as its receptor (both ST2 and IL1RAc) were analysed by qPCR also in spinal cord samples. There were no significant differences between the groups (data not shown).

The expression of IL-6 in the WT and MCP-1 in TG astrocytes is decreased by the conditioned media derived from T cells exposed to IL-33

In cerebral stroke the neuroprotection of IL-33 may be mediated by T cell-secreted anti-inflammatory cytokines that reduce the proinflammatory status of astrocytes (Korhonen et al., 2015). To detect whether this is also true with astrocytes isolated from TG mice, T cells were first exposed to IL-33 or vehicle after which the T-cell derived conditioned media (CM) was applied to the WT and TG astrocytes in the presence or absence of LPS. After 24 h incubation, the culture medium was removed and the cytokine levels in the media measured using CBA. As expected, CM derived from IL-33 treated T cells reduced the level of IL-6 protein in the WT LPS treated astrocytes (Fig. 9A). However, this decrease was not detected in the TG astrocytes, which exhibited higher levels of IL-6 compared to WT astrocytes (Fig. 9A). Instead, the levels of MCP-1, a chemokine participating to the loosening of the blood-CNS barrier (Stamatovic et al., 2005) were lower in TG astrocytes and further significantly decreased by CM derived from IL-33 treated T-cells (Fig. 9B).

Discussion

Here we show for the first time that a long-term treatment with IL-33 delays the disease onset in female SOD1-G93A TG mice. We report that IL-33 treatment modulated the peripheral inflammatory response, which may have reduced astrocytic activation in the affected SC.

Neuroinflammation is a well characterized pathological feature of ALS. Based on vast amount of literature, micro- and astroglial activation, together with a prominent increase in toxic inflammatory mediators contribute to the disease progression and neuronal death (Ilieva et al., 2009). The initial aim of glial activation is to protect neurons, but in chronic diseases such as ALS, the failure of glial cells to eradicate or detoxify the initial stimuli leads to persistent inflammatory processes contributing to the vicious cycle of inflammation and stand by neuronal death (Liao et al., 2012). Treatments with the ability to modulate the inflammatory processes in the CNS have proven efficacy in various models of neurodegeneration, including ALS (Gordon and Martinez, 2010). T cell responses contributing to the increased levels of proinflammatory cytokines have been named as the Th1 type response, whereas the Th2 shifted profile in inflammatory mediators is thought be cytoprotective. IL-33 has been shown to induce a Th2 shift in immune responses by stimulating type 2 innate immune cells in various tissues (Molofsky et al., 2015) as well as monocytes in the spleen and mesenteric lymph nodes (Price et al., 2010) eventually resulting in the production of a variety of cytokines, such as IL-4, IL-10 and GM-CSF (Rosen, 1993; Eisen, 2009; Miller et al., 2008; McLaren et al., 2010).

Dysfunctions in T cell responses have been identified in ALS patients and T cell infiltrates have been detected in ALS patient SC (Engelhardt et al., 1993). ALS patients have elevated levels of CD8+ cytotoxic T cells and natural killer T cells (Mantovani et al., 2009; Rentzos et al.,

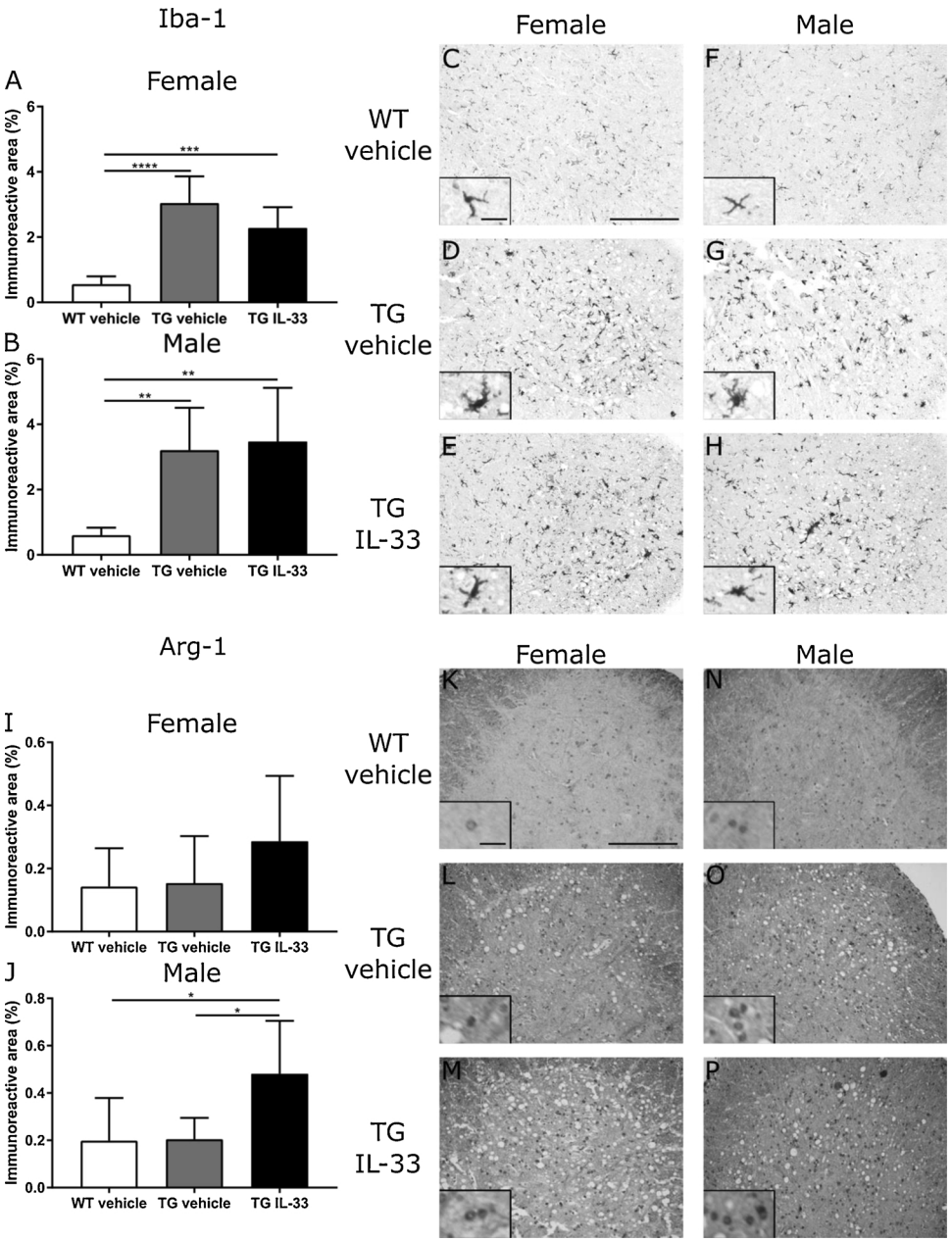

Fig. 4. IL-33 treatment failed to alter Iba-1 immunoreactivity but increased Arg-1 in TG male mice. Both female (A) and male (B) TG animals showed increased Iba-1 immunoreactivity in the ventral horn of the SC compared to WT controls. IL-33 treatment failed to alter Iba-1 immunoreactivity in both genders (A, B). Representative images of WT vehicle (C, F), TG vehicle (D, G) and TG IL-33 treated (E, H) females and males, respectively. The immunoreactivity for Arg-1, a marker for neuroprotective microglia/macrophages, was similarly measured from the spinal cord ventral horn. IL-33 treatment failed to alter the amount of Arg-1 positive cells in female TG animals (I), whilst in males IL-33 increased the extent of Arg-1 immunoreactivity (J). Representative images of WT vehicle (K, N), TG vehicle (L, O) and TG IL-33 treated (M, P) females and males, respectively. High magnification inserts show cell morphology. Scale bar 100 μm, 10 μm in inserts. Data are presented as mean ± SD. n = 5-7. One-way ANOVA. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. Female df = 18, F = 33.86; Male df = 17, F = 10.49 for Iba1. Female df = 18, F = 1.474; Male df = 16, F = 5.058 for Arg1.

2012) and importantly, the number of T regulatory cells is decreased compared to healthy controls (Mantovani et al., 2009; Rentzos et al., 2012). Similarly, peripheral markers of inflammation in ALS patients are altered and the disease is associated with an increase in CD4 + T cell activation (Mantovani et al., 2009). The fact that T cells isolated from ALS patients secrete IFNγ upon stimuli (Holmoy et al., 2006) and that T cell associated pro-inflammatory mediators correlate with the disease severity (Graves et al., 2004; Shi et al., 2007) suggest skewed T cell responses in the disease progression of ALS. However, total depletion of T cells has been showed to be deleterious. Thus, the observed neuroprotection may be mediated via Th2 subset of CD4 + T cells (Banerjee et al., 2008; Beers et al., 2008, 2011). Interestingly, the plasma levels of IL-33 are decreased in ALS patients (Lin et al., 2012) implying defects in IL-33 signaling. Indeed, IL-33 has been shown to ameliorate other neurological diseases: autoimmune encephalomyelitis (EAE), stroke and spinal cord injury through inducing the peripheral cytokine profile towards the Th2 direction (Jiang et al., 2012; Korhonen et al., 2015; Pomeshchik et al., 2015). Moreover, the lack of IL-33 signaling pathway induces a shift in CD4+ cell phenotype towards proinflammatory in a mouse model of EAE (Milovanovic et al., 2012). Our data show that IL-33 treated female mice had lower relative levels of CD4+ and CD8 + T-cells in the peripheral organs responsible for storage and release of T-cells. In addition, CM from IL-33 treated T-cells induced a significant reduction in the expression of MCP-1 in TG astrocytes. Thus, we hypothesize that IL-33 treatment in the current study setup delayed the disease onset by affecting peripheral T-cells, possibly by altering their subset specification towards anti-inflammatory direction.

Astrocytes are the main neurosupportive glia in the CNS and are capable of responding to CNS pathological conditions by secreting a plethora of pro-inflammatory cytokines. Whereas IL-33 treated mice showed no changes in the extent of Iba-1 immunoreactivity and increased levels of Arg-1 did not explain the delay in the disease onset in female mice, IL-33 treatment significantly reduced astrogliosis in specifically the female TG mice. Astrocytic activation has been implicated as one of the major mechanism of neurotoxicity in models of ALS. Expression of mutated human SOD1 specifically in astrocytes has been shown to be toxic to neurons, whereas expression in microglia,

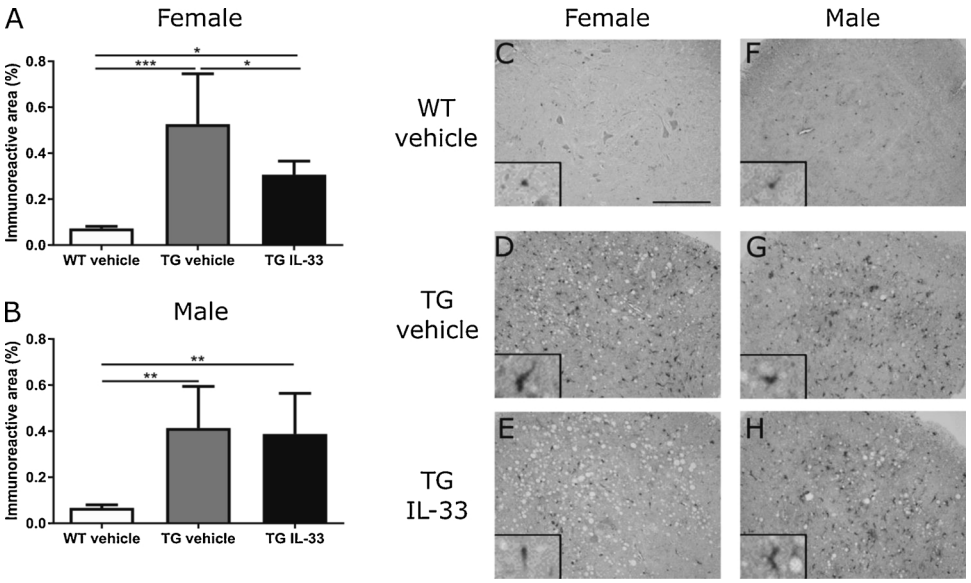

Fig. 5. IL-33 treatment reduced the p38 levels in female mice. The extent of p38 immunoreactivity was quantified from the ventral horn of SC. p38 immunoreactivity was significantly increased in the TG female mice and downregulated by IL-33 treatment of female TG mice (A). IL-33 failed to reduce p38 in TG male mice (B). Representative images of WT vehicle (C, F), TG vehicle (D, G) and TG IL-33 treated (E, H) females and males, respectively. High magnification inserts show cell morphology. Scale bar 100 µm, 10 µm in inserts. Data are presented as mean ± SD. n = 5-7. One-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Female df = 16, F = 17.03; Male df = 17, F = 10.56.

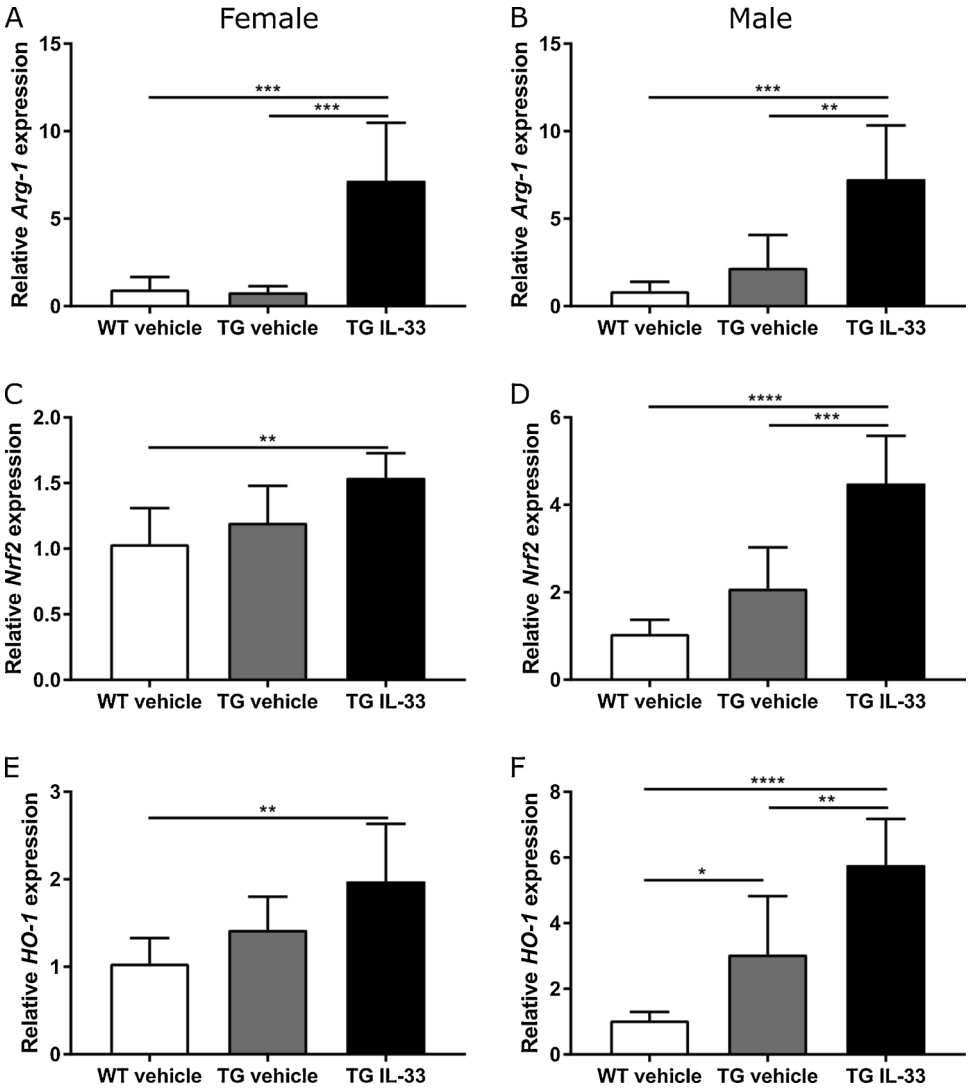

Fig. 6. IL-33 treatment increased *Arg-1, NFE2L2* and *HO-1* gene expression in the muscles of TG male mice. Gene expression was measured from gastrocnemius muscle samples of WT and TG mice by using qPCR. IL-33 treatment significantly increased the expression levels of *Arg-1* in the muscles of both female and male TG mice (A and B, respectively) compared to vehicle treated controls. In TG female mice IL-33 induced a modest increase in *NRE2L2* (C) and *HO-1* (D), which failed to reach statistical significance when compared to TG vehicle treated controls. Instead, in male TG mice, IL-33 significantly increased the levels of *NRE2L2* (D) and *HO-1* (F). Data are presented as mean ± SD. n = 4–8. One-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

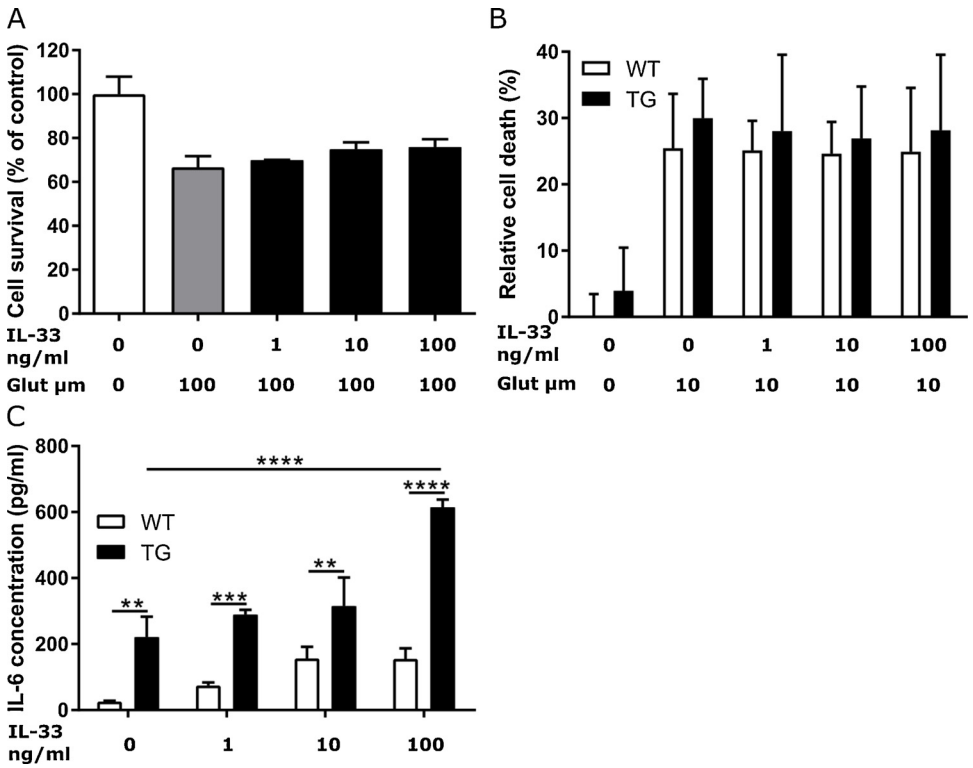

Fig. 7. IL-33 did not prevent glutamate induced death in primary neurons. Spinal cord and cortical neurons were exposed to 100 µM glutamate and increasing concentrations of IL-33 (1, 10 and 100 ng/ml). Glutamate exposure induced significant neuron death as measured by MTT and LDH assays, which was unaltered by IL-33 treatment. (A and B). n = 3. One-way ANOVA. *** $p < 0.001$. IL-33 induced a dose dependent secretion of IL-6 in TG astrocytes, and the levels were constantly higher in TG cells compared to WT cells (C). Both experiments were repeated 3 times with similar data. Data are presented as mean ± SD. n = 3. Two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

fibroblasts, cortical neurons and myocytes is not (Nagai et al., 2007; Haidet-Phillips et al., 2011; Fritz et al., 2013). Astrocyte secreted cytokines have been shown to aggravate the disease pathology (Endo et al., 2015) and on the other hand, neuroprotection in TG ALS mice is often associated with diminished astrocyte activation (Pollari et al., 2011; Miquel et al., 2012). Astrocytes are in close contact with the BBB making them highly susceptible to interaction with peripheral leukocytes. Although the BBB effectively protects the CNS from the invading leukocytes, ALS pathology has been shown to increase T-cell infiltration into the affected SC (Engelhardt et al., 1993; Mantovani et al., 2009; Rentzos et al., 2012; Nardo et al., 2016). Once T-cells are recruited to the CNS, they encounter astrocytic end-feets as one of the first cellular structures. A number of in vitro evidence indicates that T-cells can alter the function of astrocytes. Specifically, T-cell secreted INFγ (Yong et al., 1991; Lee et al., 2013) and IL-19 (Zhou et al., 2011) potentiate astrocytic inflammatory activation. In the light of these findings, it is not surprising that T-cell secreted cytokines can modulate astrocytic activation (Korhonen et al., 2015) as evident also in the current study.

IL-33 mediates signaling through IL-33 receptor complex formed by ST2 (IL-1RL1) and IL-1RAcP (Schmitz et al., 2005; Chackerian et al., 2007). At the baseline there was a tendency for lower expression of *IL-33* and higher expression of *ST2* subunit TG astrocytes compared to WT astrocytes and this difference reached statistical significance upon exposure to 100 ng/ml IL-33. These findings are in line with the study showing that plasma levels of IL-33 are decreased in ALS patients (Lin et al., 2012) indicating an imbalance in the Th1–Th2 immune axis and increased proinflammatory milieu. The increased level of ST2 may be a compensatory mechanism to combat against the decreased levels of IL-33. The increased expression of ST2 might on the other hand explain the increased IL-6 secretion by TG astrocytes upon exposure to IL-33. Earlier studies have demonstrated that IL-33 may induce the expression of proinflammatory cytokines including IL-6 in acinar cells (Kempuraj et al., 2013), hepatic stellate cells (Tan et al., 2017), peripheral blood mononuclear cells and human bronchial epithelial cells (Shang et al., 2015), and thus it is not surprising that direct IL-33 exposure has similar effects on cultured astrocytes. Considering that IL-33 was unable to protect neurons from glutamate excitotoxicity, our data indicate that the delay in the disease onset in IL-33 treated TG females is not due to direct binding of IL-33 on the cells of the CNS. Instead, the CM derived from IL-33 treated T-cells decreased the expression of *MCP-1*, a chemokine that has been shown to increase the permeability of the blood-CNS barrier (Stamatovic et al., 2005) in TG astrocytes, thereby supporting the hypothesis that IL-33 exerts its effect through peripheral T-cell mediated cytokines, acting especially on astrocytes, thus decreasing their inflammatory status. This is similar to conditions of stoke, where peripheral IL-33 administration elicited neuroprotection via peripheral T-cells, altering their phenotype towards Th2 direction (Korhonen et al., 2015).

p38 belongs to the group of mitogen-activated protein kinases (MAPK) that are activated by phosphorylation. It has been shown that p38 is activated by mSOD1 and it contributes to axonal transport in squid axoplasm (Bosco et al., 2010; Morfini et al., 2013). Aberrant mSOD1-related activation of p38 has also been seen in transgenic mouse models of ALS (Tortarolo et al., 2003; Holasek et al., 2005; Morfini et al., 2013). p38 is found in the intracellular inclusions of mSOD1 transgenic mice as well as in human ALS patients. These inclusions are located especially in degenerating motor neurons and reactive astrocytes, thus indicating a role for p38 in the pathology of ALS (Bendotti et al., 2004). IL-33 has been shown to be protective and to inhibit p38 in myocardial infarction (Yin et al., 2014). In this study, we found that decreased p38 immunoreactivity correlated with the delay in the disease onset and reduction of GFAP immunoreactivity.

In the current study a long-term treatment of TG male mice with IL-33 elevated the levels of *NRE2L2* in the muscles. In support of this, the mRNA levels of *HO-1*, an antioxidant known also as Hsp32 (Sharp et al., 2013), were increased in the muscle of IL-33 treated TG male mice. Nrf2 is a nuclear transcription factor regulating the expression of the antioxidant genes, such as CD36, and the genes participating in glutathione homeostasis (Ishii et al., 2004; Petri et al., 2012). Nrf2 has also been shown to participate in the pathology of ALS by decreasing the oxidative stress (Neymotin et al., 2011; Petri et al., 2012). HO-1 is activated by the Nrf2 pathway and is neuroprotective in a TDP-43 mouse model of ALS (Duan et al., 2010; Kanno et al., 2012) although some contradictory results about the function of HO-1 in ALS have also

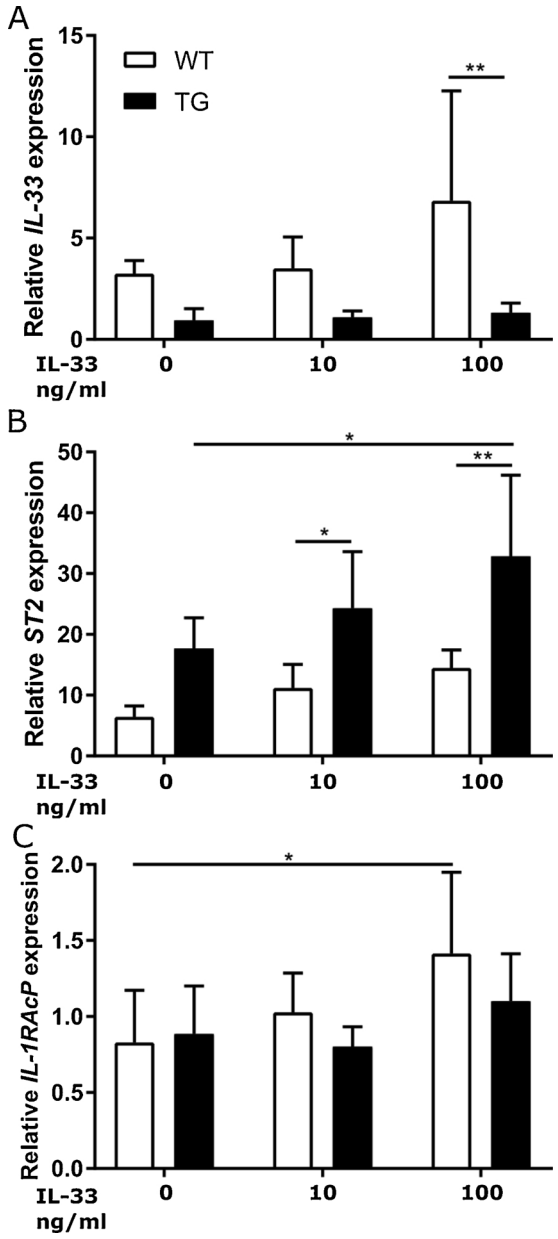

Fig. 8. IL-33 altered the expression levels of *IL-33* and *ST2*. TG and WT primary astrocytes were treated with increasing concentrations of IL-33 (10 and 100 ng/ml). The expression of *IL-33* and the receptor components *ST2* and *IL-1RAcP* were measured by qPCR. IL-33 induced the expression of *IL-33* in a concentration dependent manner in WT astrocytes (A). The expression levels of *ST2* receptor were higher in TG astrocytes compared to WT astrocytes (B). IL-33 failed to alter the expression levels of *IL-1RAcP* and there were no significant differences between the genotypes (C). Data are presented as mean ± SD. n = 4-5. Two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

been reported (Xu et al., 2011; Guo et al., 2012). Taken together, these results indicate that IL-33 may also participate in oxidative stress by preventing lipid peroxidation and by regulating the antioxidant pathways. However, these findings cannot explain the delay in the disease onset in female TG mice.

Gender-dependent alterations in ALS pathology have been previously described in the literature (Azari et al., 2003; Vercelli et al., 2008; Acevedo-Arozena et al., 2011; Alves et al., 2011; Heiman-Patterson et al., 2011). Epidemiological studies have shown that ALS is more common in men than in women (McCombe and Henderson, 2010). Gender has been reported to also impact in mouse models of ALS: the disease onset in SOD1-G93A mice (Hegedus et al., 2009; Alves et al., 2011) and rats (Suzuki et al., 2007) is delayed in females compared to males. In the current study, we detected the same delay in the disease onset between the genders; in male mice the disease was initiated some days earlier than female disease (on average 128 and 133 days, respectively). We recognize that the female mice received the treatment a few days longer than the male mice thus possibly contributing to the treatment response in female mice. However, some neuroprotective treatments have been reported to have sex-dependent effects (Azari et al., 2003; Naumenko et al., 2011). These gender-dependent outcomes may be due to various differences in immune functions of mice. In fact, the possibility that the endogenous levels or regulation mechanisms of IL-33 and its downstream anti-inflammatory mediators are different in male and female mice cannot be currently excluded. Even though the mechanisms for this intriguing phenomenon are still under investigation, our current study reinforces the impact of gender-dependent treatment responses in ALS models.

Taken together, long-term IL-33 treatment delays the disease onset in the SOD1-G93A female mice, while the males remain unresponsive to the treatment. Our results indicate that rather than being directly neuroprotective, IL-33 provides therapeutic benefit by reducing the percentage of CD4+ and CD8+ lymphocytes and by reducing astrocytic dysfunctions. We do acknowledge that we carried out analysis only on late-stage animals, and thus are not able to show whether the observed changes in T-cell numbers and astrocytic activation were evident at the time when the treatment was started. However, prior studies have shown that SOD1-G93A mice do not exhibit extensive astrogliosis prior the disease onset around the age of 11 weeks (Reviewed in Vargas and Johnson, 2010), when the IL-33 treatment was started

The results obtained suggest that IL-33 treatment alters T cell responses in the periphery and via cross talk with CNS endogenous glia, diminishes harmful astrocytic activation, thereby delaying the disease onset in female mice. Our results strengthen the emerging literature of the importance of T cell regulation and dysfunction in ALS disease pathogenesis and support the hypothesis that immune system directed strategies may be potential therapeutic approaches for ALS.

Author's contributions

All authors read and approved the final manuscript
Study conception and design: PK, EP, KMK, GG, JK, TM
Acquisition of data: PK, EP, KMK, SL, YP, ES, SW, GG, VK-G,
Analysis and interpretation of data: PK, EP, KMK, SL, SW, GG, VK-G, GG, JK, TM
Drafting of manuscript: PK, EP, TM
Critical revision: PK, EP, KMK, TM

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Ethical approval

All animal experiments were carried out according to national regulation of the usage and welfare of laboratory animals and approved by the Animal Experiment Committee in State Provincial Office of Southern Finland.

Consent for publication

Not applicable

Availability of data and materials

All data acquired during the study is available from the

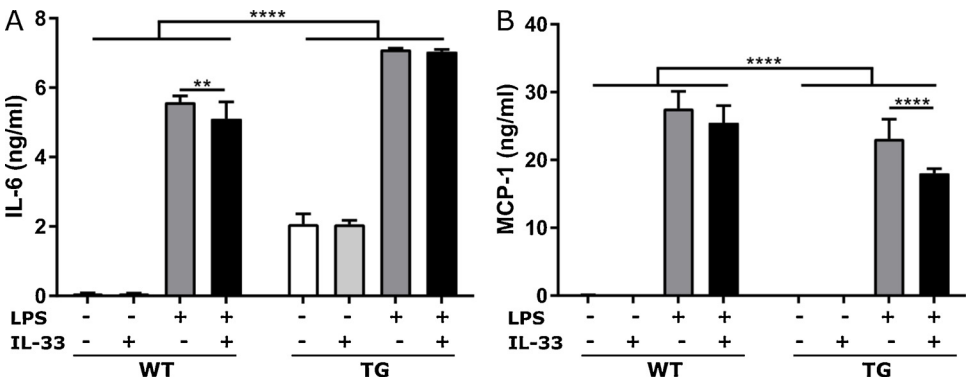

Fig. 9. Conditioned medium derived from IL-33 treated T-cells reduced astrocytic proinflammatory activation. WT and TG astrocytes were exposed to LPS and the conditioned medium derived from IL-33 or vehicle treated T-cells. After 24 h exposure the cytokine levels were measured from the culture medium by CBA. In WT astrocytes, the conditioned medium derived from IL-33 stimulated T-cells decreased the levels of proinflammatory cytokine IL-6 (A). In TG astrocytes the levels of IL-6 were significantly higher compared to the WT astrocytes and unaltered by the T-cell derived conditioned medium (A). Instead, the levels of MCP-1 were lower in TG astrocytes compared to WT astrocytes and significantly downregulated in TG cells by the conditioned medium derived from IL-33 stimulated T-cells (B). Data are presented as mean ± SD. n = 6. Two-way ANOVA. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

corresponding author upon reasonable request.

Conflict of interests

EP, TM, KMK and JK are the inventors of the patent.

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