**IL-22 promotes Fas expression in oligodendrocytes and inhibits
FOXP3 expression in T cells by activating the NF- κ B pathway in
multiple sclerosis**

Jin Zhen^a, Jun Yuan^b, Yongwang Fu^b, Runxiu Zhu^b, Meiling Wang^b, Hong Chang^b,
Yan Zhao^b, Dong Wang^b, Zuneng Lu^{a*}

a. Department of Neurology, Renmin Hospital of Wuhan University, Wuhan University, Wuhan, Hubei 430060, P.R. China.

b. Department of Neurology, Inner Mongolia Autonomous Region People's Hospital, Hohhot, 010000, Inner Mongolia, P.R. China.

Running head: IL-22 functions in MS

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E-mail address for each author:

zhenjin2015@yeah.net

hospital2000@aliyun.com

*Corresponding author: Professor Zuneng Lu, Department of Neurology, Renmin Hospital of Wuhan University, Wuhan University, No. 99, Jiefang Road 238, Wuchang District, Wuhan, Hubei 430060, China E-mail address:
hospital2000@aliyun.com

Abbreviations: multiple sclerosis, MS; experimental autoimmune encephalomyelitis, EAE; interleukin, IL; transcription factor forkhead box P3, FOXP3; healthy control, HC

Abstract

Multiple sclerosis (MS) is characterized by an increase in interleukin-22 and Fas, and a decrease in FOXP3, among other factors. In this study, we examined patients with MS and healthy control subjects and used the experimental autoimmune encephalomyelitis (EAE) animal model to identify the effects of IL-22 on oligodendrocytes and T cells in MS development. In MS, the expression of Fas in oligodendrocytes and IL-22 in CD4⁺CCR4⁺CCR6⁺CCR10⁺ T cells was enhanced. Ikaros and FOXP3 were both decreased in T cells. Depending on exogenous IL-22, Fas increased the phosphorylation of mitogen- and stress-activated protein kinase 1 and activated the nuclear factor- κ B pathway in oligodendrocytes, leading to an increase in Fas and oligodendrocyte apoptosis. IL-22 decreased FOXP3 expression by activating NF- κ B, and it further inhibited PTEN and Ikaros expression. Tregs reversed the functions of IL-22. Taken together, these findings help to elucidate the mechanisms of IL-22 in MS development.

Keywords: Multiple sclerosis, FOXP3, Ikaros, IL-22, Fas, NF- κ B.

1. Introduction

Multiple sclerosis (MS) is a common cause of chronic neurological disability beginning in early to middle adult life and manifests as an inflammatory demyelinating disease of the human central nervous system (CNS). Oligodendrocytes are glial cells in the CNS, and their death occurs via the upregulation of Fas expression in MS (Patel and Balabanov, 2012). The increased expression of Fas in oligodendrocytes results in an interaction with Fas ligand (FasL) on CD8⁺ T cells, rendering them direct targets of CD8⁺ T cells and leading to oligodendrocyte injury. In the search of what most likely triggers MS, two mechanisms have become prominent: the molecular mimicry between self-antigens and infectious agents, and the bystander activation of auto-reactive immune T cells (Libbey et al., 2007; Sospedra and Martin, 2005).

Molecular mimicry occurs when self-antigens and infectious pathogens share similar peptide sequences or structural motifs. When a relevant pathogenic microbe infects an organism, the immune system attacks the common epitope shared between self- and nonself-antigens. Additionally, bystander activation is a potential mechanism resulting in MS. This mechanism requires the primary activation of tissue-resident antigen presenting cells (APCs) within the CNS. These APCs enable T cells to initiate an autoimmune response against CNS epitopes (Munz et al., 2009). Self-antigens involved in MS include myelin oligodendrocyte glycoprotein (MOG), myelin proteolipid protein (PLP), myelin-associated glycoprotein (MAG), myelin basic protein (MBP), 2',3'-cyclic-nucleotide-3'-phosphodiesterase (CNPase), B crystalline

and S100b protein (McCarthy et al., 2012; Sospedra and Martin, 2005). Experimental autoimmune encephalomyelitis (EAE) is an extensively studied animal model that is commonly used to model the inflammatory aspects of MS and is induced using MOG, MBP or PLP (Denic et al., 2011; Grigorian et al., 2011; Krishnamoorthy and Wekerle, 2009).

Th22, a new subtype of CD4⁺ Th cells, is involved in the development of MS. Th22 cells are characterized by the secretion of IL-22, a member of the IL-10 family. Although the precise role of IL-22 has not been fully elucidated, it appears to be highly relevant to MS (Pan et al., 2013; Sabat et al., 2014), with IL-22 expression being increased in MS (Pan et al., 2013; Rolla et al., 2014). Additionally, IL-22 is also involved in the pathogenesis of EAE (Grigorian et al., 2011). IL-22 signals via a receptor complex consisting of the IL-10R β chain and IL-22 receptor (IL-22R). Furthermore, IL-22R belongs to the class II cytokine receptor family and consists of two subunits, IL-22R1 and IL-10R2 (or IL10RB) (Wolk et al., 2004; Wolk and Sabat, 2006). IL-22R1, a risk gene for MS, is expressed in a variety of non-immune tissues, including the skin, lung, kidney, pancreas and other organs, although its expression in glial cells remains unclear (Beyeen et al., 2010; Lill et al., 2014). IL-22 binding to IL-22R1 activates the signal transducer and activator of transcription 3 (STAT3) pathway and the Akt-mitogen activated protein kinase (MAPK) pathway in different cell lines (Brand et al., 2006; Wolk et al., 2006). Oligodendrocytes in MS lesions exhibit increased expression of Fas (Dowling et al., 1996; Hoftberger et al., 2004), and the expression is controlled by IFN- γ and is dependent on interferon

regulatory factor 1 (IRF-1) (Pouly et al., 2000; Stang et al., 2007). Increased Fas on glial cells interacts with FasL on CD8⁺ T cells, leading to glial cell death (Hovelmeyer et al., 2005). In the present study, to understand the roles of IL-22 and IL-22R1 in MS, we examined Fas expression in glial cells treated with IL-22 and detected the effect of increasing IL-22 levels on the nuclear factor- κ B (NF- κ B) pathway and Fas expression in oligodendrocytes in MS.

CD4⁺CD25⁺FOXP3⁺ regulatory T cells (Tregs) are a subset of cells that are specialized for the suppression of the activation and proliferation of effector T cells. Thus, this subset is of particular importance in limiting autoimmunity. Tregs are characterized by the expression of cellular marker CD25 and transcription factor forkhead box P3 (FOXP3). Abnormal expression patterns of microRNA and genes have been previously described in MS and have been suggested as disease biomarkers and potential therapeutic targets (Arruda et al., 2015). CD4⁺ and CD8⁺ T cells obtained from MS patients exhibit upregulated expression of miR-16, miR-155 and miR-142-3p, and downregulated expression of FOXP3, FOXO1, programmed cell death 1 and IRF2 binding protein 2 (Arruda et al., 2015). FOXP3 may also be regulated by additional microRNAs and proteins. Recent data have revealed a particularly important protein in Tregs, namely, the Ikaros transcription factor. Ikaros upregulates FOXP3 expression via its attachment to the *foxp3* promoter (Heller et al., 2014). However, in MS, the function of IL-22 in regulating Ikaros and FOXP3 requires further study.

In the present study, we analyzed the expression of IL-22 in patients with MS and the

percentage of Th22 cells. Both IL-22 and Th22 were increased in MS compared with healthy controls, and additionally, Fas was upregulated in oligodendrocytes in MS. Increasing IL-22 could activate the NF- κ B pathway to positively regulate Fas in oligodendrocytes and negatively regulate phosphatase and tensin homolog (PTEN), Ikaros and FOXP3 in T cells. We used IL-22 to treat mouse primary glial cells *in vitro* and found that IL-22 promotes Fas expression. Tregs purified from EAE or control mice were co-cultured with oligodendrocytes after IL-22 treatment, indicating that Tregs could reverse IL-22 function.

2. Materials and Methods

2.1 Cells and reagents.

B104 cells, rat neuroblastoma cells, were obtained from OTWO BIOTECH INC. (Shenzhen, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) and F12 (1:1) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA). When cell growth reached 60 to 70% confluence, 10% FBS was replaced with a 1% N2 supplement (Invitrogen; Thermo Fisher Scientific, Inc., Grand Island, NY, USA).

The CG4 cell line, the rodent oligodendrocyte progenitor cell line, was obtained from BioVector NTCC Inc. (Beijing, China) and propagated or proliferated on poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) coated 35-mm or 100-mm dishes. Rat CG4 cells were cultured in growth medium consisting of 70% DMEM and F12 (1:1) and 30% B104-conditioned medium. For the preparation of B104-conditioned medium, B104 confluent cells were cultured for 4 days in DMEM without FBS, but

with the addition of the N2 supplement. The CG4 cells differentiated after the withdrawal of B104-conditioned medium.

IL-22, APC-anti-CD3, fluorescein isothiocyanate (FITC)-anti-CD4, phycoerythrin (PE)-anti-IL-22, PerCP-CD25 and PE-FOXP3 were purchased from BioLegend, Inc. (San Diego, CA, USA). The primary antibodies for Western blotting analyses were obtained from Abcam (Cambridge, MA, USA). The secondary antibodies were obtained from ProteinTech Group, Inc. (Chicago, IL, USA). The transfection reagent jetPRIME[®] was purchased from Polyplus Transfection (New York, NY, USA). The NF- κ B inhibitor JSH-23 was purchased from Sigma-Aldrich. H89 was obtained from EMD Millipore (Billerica, MA, USA). LY294002 was purchased from Cell Signaling Technology (Danvers, MA, USA). Caspase-Glo[®] 3/7 Assay kit was obtained from Promega (Madison, WI, USA).

Experiments involving patients and animals were approved by the Ethics Committee of Wuhan University (Wuhan, China).

2.2 Flow cytometry analysis.

Peripheral blood was obtained from patients with MS (n=30), and peripheral blood samples from healthy control subjects (n=25) were provided by the Inner Mongolia Autonomous Region People's Hospital (Hohhot, China). Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll gradient centrifugation (Fresenius Kabi Norge AS, Østfold, Norway). PBMCs were adjusted to a final concentration of 10^6 cells/ml in RPMI-1640 medium supplemented with 10% FBS. PBMCs were stimulated with phorbol myristate acetate (PMA, 50 ng/ml) and

ionomycin (1 µg/ml, BioLegend, Inc.) for 12 h. Subsequently, 10 µg/ml of Brefeldin A (Sigma-Aldrich) was added, and the cells were cultured for an additional 4 h. These cells were stained with APC-CD3 and FITC-CD4 or PE-IL22. After surface staining, PBMCs were fixed and permeabilized with Fix & Perm (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. PBMCs were incubated with anti-human PE-IL-22.

After PBMC culturing, CD4⁺ T cells were purified using human CD4⁺ T Lymphocyte Enrichment Sets-DM via negative selection (BD Bioscience). Purified CD4⁺ T cells were stained using FITC-C-C chemokine receptor (CCR) 4, APC-CCR6, PerCP-CCR-10 and PE-IL-22. Flow cytometry was subsequently performed.

For the detection of Fas in oligodendrocytes, 10⁶ cells were suspended in phosphate-buffered saline (PBS) and incubated with either 10 µg/ml FITC anti-human Fas antibody (clone DX2, BioLegend, Inc.) or rabbit anti-mouse Fas polyclonal antibody (Abcam) for human and mouse cells, respectively. FITC-isotype IgG antibodies were used as negative controls. Samples were analyzed using an Accuri C6 Flow Cytometer (BD Biosciences).

2.3 Enzyme-linked immunosorbent assay (ELISA).

IL-22 in the serum was detected using a human IL-22 ELISA kit (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions.

2.4 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) detection.

PBMCs from patients with MS and healthy controls were obtained. IL-22 was used to

treat PBMC or CG4 cells for 72 h. Total RNA was extracted using the TRIzol reagent. For cDNA synthesis, 1 µg of total RNA was reverse-transcribed using the PrimeScript RT Reagent Kit (Toyobo, Saito City, Japan). qPCR was performed using cDNA with the SYBR Green Real-Time PCR Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) and specific primers using the ABI Prism7100 Fast Cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The level of mRNA was normalized against GAPDH, and the primer sequences were as follows:

FOXP3, forward	5'-GAACGCCATCCGCCACAACCTGA-3'	and	reverse
	5'-CCCTGCCCCCACCACCTCTGC-3';	IL-22,	forward
	5'-CAACAGGCTAAGCACATGTCA-3'	and	reverse
	5'-ACTGTGTCCTTCAGCTTTTGC-3';	Ikaros,	forward
	5'-CCAATGTGCTCATGGTTCAC-3'	and	reverse
	5'-TAGCTTCGGCCACAATATCC-3';	human Fas,	forward
	5'-GGCTATAGATCACCTTCATGTA-3'	and	reverse
	5'-GCAGTTAACTCAGGGACCAAG-3'	(Koshkina et al., 2011);	GAPDH,
forward	5'-CGAGATCCCTCCAAAATCAA-3'		and
reverse	5'-TTCACACCCATGACGAACAT-3';	rat Fas,	forward
	5'-ATGGCTGTCCTGCCTCTGGT3'	and	reverse
	5'-ACGCTCCTCTTCAACTCCAAA3'	(Gui et al., 2013);	mouse β-actin, forward
	5'-AATTCCATCATGAAGTGTGA3'	and	reverse
	5'-ACTCCTGCTTGCTGATCCAC3';	mouse Fas,	forward
	5'-TCTGGTGCTTGCTGGCTCAC3'	and	reverse

5'-CCATAGGCGATTCTGGGAC3' (Singh et al., 2008).

2.5 Isolation and culture of mouse oligodendrocyte precursor cells.

This experiment was performed as previously described (Chen et al., 2007). Oligodendrocyte precursor cells were obtained from embryos from timed-pregnancy mice. Precursor cells were cultured in a growth medium for 14 days and subsequently differentiated into oligodendrocytes in oligodendrocyte differentiation medium.

2.6 Caspase 3 activity detection.

Caspase 3 is a target molecule of Fas and triggers cell apoptosis. To test whether IL-22 could induce apoptosis in oligodendrocytes, the Caspase-Glo® 3/7 Assay kit was used to determine oligodendrocyte apoptosis after cells were treated with IL-22 according to the manufacturer's instructions.

2.7 Plasmid construction.

The mouse IL-22 gene was amplified and cloned into the pcDNA3.1(-) vector. The specific primers were P1 (AT GGATCC ATGGCTGTCCTGCAGAAATCTATG) and P2 (TT AAGCTT TCAGACGCAAGCATTCTCAGAG). The underlined bases in the oligonucleotide pairs P1 and P2 represent *Bam*HI and *Hind*III sites, respectively, which facilitated cloning into the *Bam*HI and *Hind*III sites of pcDNA3.1(-). The recombinant plasmid was sequenced prior to transfection. To identify the effect of endogenous IL-22 on oligodendrocytes, pcDNA3.1-IL-22 was transfected into mouse primary oligodendrocytes using the jetPRIME® reagent. After 72 h, the cells were harvested, and Fas was detected using RT-qPCR and Western blotting analyses.

2.8 Western blot detection.

Anti-phospho-mitogen and stress-activated protein kinase-1 (MSK1) (Thr581) (1:1,000), anti-MSK1 (1:1,000), anti-phospho-NF- κ B p65 (Ser468) (1:1,000), anti-NF- κ B p65 (1:1,000), anti-PTEN (1:2,000), anti-p110a (PI3K) (1:1,000), anti-Akt (1:2,000) and anti-FOXO1 (1:1,000) antibodies were purchased from Cell Signaling Technology. Anti-Fas (1:1,000), anti-Ikaros (1:1,000) and anti-FOXP3 (1:2,000) were obtained from Abcam, and anti- β -actin (1:10,000) was purchased from Sigma-Aldrich. Mouse oligodendrocyte precursor cells were isolated from mice and were cultured into oligodendrocytes in oligodendrocyte differentiation medium. Primary glial cells and CG4 cells were treated with IL-22 (0-80 ng/ml) for 60 min. Cell line cultures and cells from EAE animals were harvested and lysed in cell lysis buffer [Tris (pH 7.5) 20 mM, NaCl 150 mM, Triton X-100 1%, protease inhibitor 1%]. A total of 30 μ g of protein was used to perform Western blot analysis. MSK-1, p65 and Fas protein expression levels were determined. Inhibitors of NF κ B p65, MSK-1, JSH-23 and H89 were used to treat CG4 cells at a concentration of 10 μ M. For T-cell detection, CD4⁺ T cells were purified from the blood of healthy donors and patients with MS using human CD4⁺ T Lymphocyte Enrichment Sets-DM via a negative selection kit. An inhibitor of PI3K, LY294002, was used to treat CD4⁺ T cells at a concentration of 10 μ M. CD4⁺ T cells were lysed using lysis buffer, and 20 μ g of protein was used to detect p65, PTEN, PI3K, AKT, FoxO1, Ikaros and FOXP3 via Western blotting.

2.9 Purification of mouse Tregs.

For Treg cell function assays *in vitro*, Treg cells were isolated from spleens and sorted

using a magnetic bead cell purification kit according to the manufacturer's instructions (Miltenyi Biotech, Inc., San Diego, CA, USA). In addition, Tregs separated from EAE or control mice were co-cultured with oligodendrocytes either after IL-22 treatment or control treatment. In addition, Fas in oligodendrocytes was determined using RT-qPCR.

2.10 Statistical analysis.

Data are expressed as the mean $\pm$ SD. Statistical analyses were performed using Student's t-test or one-way analysis of variance.

3. Results

3.1 Levels of IL-22 in PBMCs are increased in MS.

To detect IL-22 in MS, flow cytometry (FCM) was performed. We collected PBMCs from 20 MS patients and 20 healthy controls using Ficoll density centrifugation, and CD3⁺CD4⁺IL-22⁺ cells were detected using FCM. The proportion of IL-22 within the CD3⁺CD4⁺ T-cell population was increased in patients with MS (Fig. 1A and B). In addition, the IL-22 levels in the serum were analyzed using ELISA. These results demonstrated that the IL-22 levels in the peripheral blood of patients with MS were increased compared with healthy controls (Fig. 1C).

Th22 cells are the main source of secreted IL-22 and function as key regulators in various autoimmune diseases (Eyerich et al., 2010; Wolk et al., 2006). However, the cells' function in EAE and MS is not clearly understood. The percentage of Th22 cells was determined using FCM, and CCR4⁺CCR6⁺CCR-10⁺ cells in CD4⁺ T cells were gated (Fig. 2A), with IL-22 expression also detected.

CD4⁺CCR4⁺CCR6⁺CCR-10⁺IL-22⁺ T cells were increased in MS (Fig. 2B). It has been previously reported that CD4⁺CCR4⁺CCR6⁺CCR-10⁺IL-22⁺ T cells represent Th22 cells (Rolla et al., 2014). These findings suggest that circulating Th22 was increased in MS patients compared with healthy controls.

3.2 Fas expression in rat CG4 cells and mouse primary oligodendrocytes is increased after IL-22 stimulation.

Fas is upregulated in oligodendrocytes. Mouse oligodendrocyte precursor cells were cultured as described by Chen Y (Chen, et al., 2007). Subsequently, the differentiated CG4 cells and mouse oligodendrocytes were treated with different concentrations of IL-22 (0, 10, 20, 40 and 80 ng/ml) for 48 h. Then, total RNA was extracted and used in RT-qPCR. IL-22 enhanced Fas expression in both cell types (Fig. 3A and B). Similar results were obtained using FCM detection, with increased Fas expression on the oligodendrocyte surface (Fig. 3D). To further investigate the effect of IL-22 on Fas, pcDNA3.1-IL-22 was transfected into primary mouse oligodendrocytes and the Fas RNA was analyzed. This result showed that endogenous IL-22 enhanced Fas expression in mouse oligodendrocytes (Fig. 3C), which suggested that increased IL-22 may upregulate Fas expression and promote oligodendrocyte apoptosis.

To confirm activation of apoptosis induced by IL-22 in oligodendrocytes, caspase 3 activity was detected using a Caspase-Glo® 3/7 Assay kit. The results showed that 80 µg/ml of IL-22 increased the luminescence intensity in CG4 cells and primary mouse oligodendrocytes, which suggested that caspase 3 activity was enhanced by IL-22 in oligodendrocytes (Fig. 3E, F). These results hinted that IL-22 could cause

oligodendrocyte apoptosis by the Fas-caspase pathway.

3.3 Proportion of regulatory T cells is decreased in PBMCs in MS.

As regulatory T cells play an important role in maintaining inflammation, we measured the proportion of Tregs within total PBMCs in MS patients and healthy controls. Natural Tregs were quantified by analyzing the epigenetic status of FOXP3 using flow cytometry. CD3 and CD4 were used to gate the marked T cells, and CD25⁺ T cells were gated on CD3⁺CD4⁺ T cells (Fig. 4A). In addition, the CD4⁺CD25⁺FOXP3⁺ T cell percentages were analyzed. The proportion of CD4⁺CD25⁺FOXP3⁺ Tregs was reduced in MS patients compared with healthy controls (Fig. 4A and B). The RT-qPCR results also showed that the FOXP3 mRNA levels were reduced in MS patients (Fig. 4C). Taken together, these results indicate that Tregs were significantly decreased in patients with MS.

3.4 IL-22 promotes Fas expression in oligodendrocytes via the MSK-1-NFκB pathway.

To investigate the effect of IL-22 on Fas expression in the development of MS, we first isolated oligodendrocyte precursor cells and cultured them into mature oligodendrocytes. In addition, we cultured CG4 cells with B104-conditioned medium to obtain differentiated cells. *In vitro*, 0 or 80 ng/ml IL-22 with or without JSH-23 and H89 was used to treat oligodendrocytes and CG4 cells for 60 min. The cells were lysed and used for the detection of phosphorylated MSK-1, NFκB-p65 and Fas. *In vivo*, oligodendrocytes were isolated from EAE and control mice and used to determine the levels of phosphorylated MSK-1, NFκB-p65 and Fas. After IL-22

treatment, the phosphorylation of MSK-1 was increased, whereas total MSK-1 expression did not change. The phosphorylation of p65 was also promoted in IL-22-treated cells. Fas expression was also elevated in oligodendrocytes and CG4 cells (Fig. 5A). Similar results were obtained in the mouse model. The phosphorylation levels of MSK-1 and p65 were both increased in EAE animals compared with control animals (Fig. 5B). However, JSH-23 or H89 inhibited Fas expression by inhibiting p65 directly or indirectly (Fig. 5C). The data suggest that increasing IL-22 resulted in the phosphorylation and activation of MSK-1, which subsequently activated p65, an important molecule of the NF- κ B pathway, and further increased Fas expression in oligodendrocytes. Together, IL-22 increased Fas expression by activating the MSK-1/NF κ B pathway *in vitro* and *in vivo*.

3.5 IL-22 inhibits FOXP3 expression in T cells via the NF κ B-PTEN-PI3K pathway.

To investigate the effect of IL-22 on FOXP3 expression in the development of MS, we prepared CD4⁺ T cells from patients with MS and healthy donors using a kit. CD4⁺ T cells were lysed, and total protein was used to detect p65, PTEN, PI3K, AKT, FoxO1, Ikaros and FOXP3 by Western blotting. The levels of phosphorylation of NF- κ B p65 in MS patients was increased compared with healthy donors, and PTEN expression was decreased in patients with MS, which suggests that the activation of NF- κ B inhibited PTEN expression (Fig. 6A). This result was consistent with a previous study by Escrivá *et al.* (Escrivá et al., 2008). In addition, the reduction in PTEN promoted P110 α , a subunit of PI3K. As a downstream signaling molecule, Akt was phosphorylated (Fig. 6A), which led to the inhibition of FOXO1 expression (Fig.

6A). The downregulation of FOXO1 inhibited Ikaros and FOXP3 expression in the T cells from MS patients (Fig. 6A).

Ikaros upregulates FOXP3 expression. Ikaros mRNA was measured using RT-qPCR analysis. Total RNA was obtained from PMBCs from MS patients and healthy controls and was subsequently reverse transcribed into cDNA and used for RT-qPCR. These results showed that the Ikaros mRNA levels were decreased in MS patients (Fig. 6C). The alterations in Ikaros levels were consistent with those found in FOXP3, which suggested that a decrease in Ikaros may result in a decrease in FOXP3. Furthermore, similar results were observed in T cells from healthy donors treated with 0 or 80 ng/ml of IL-22 (Fig. 6B). However, when T cells were treated with IL-22 and a PI3K inhibitor, LY294002, the FOXP3 expression level was increased, which indicated that NF- κ B-PTEN-PI3K was involved in the IL-22-mediated pathological process (Fig. 6D). These data suggest that IL-22 decreased FOXP3 expression by activating the NF- κ B-PTEN-PI3K pathway in CD4⁺ T cells.

3.6 Tregs from healthy individuals inhibit Fas upregulation induced by IL-22.

To explore Treg function in IL-22-induced Fas expression in oligodendrocytes, Fas expression in EAE and control mice was measured, and Treg cells were subsequently separated from EAE or control mice and co-cultured with oligodendrocytes (two cell ratio was 100:1) after IL-22 or PBS treatment. The Fas levels were determined using RT-qPCR. The expression of Fas in oligodendrocytes from EAE mice was increased compared with control mice (Fig. 7A). Furthermore, Tregs from healthy mice demonstrated normal FOXP3 expression, which could reverse Fas expression induced

by IL-22. However, EAE mouse Tregs did not exhibit this function (Fig. 7B). Taken together, these results suggest that IL-22 may stimulate Fas expression and that Tregs could effectively reverse the function of IL-22.

4. Discussion

MS is a T cell-mediated autoimmune disease. Th22 cells, a new subtype of T cells, play an important role in the pathogenesis of MS and EAE (Qin et al., 2011; Zhang et al., 2012).

In the present study, we reported that the proportion of Th22 cells and levels of IL-22 in patients with MS were increased compared with healthy controls. The serum IL-22 levels were also increased in patients with MS. These results are consistent with those of a previous study by Xu (Xu et al., 2013) in which the IL-22 levels in CD3⁺CD4⁺T cells in patients with MS were increased. To investigate whether IL-22 was secreted from Th22 cells, flow cytometry was performed. These results indicated that the proportion of Th22 cells was significantly enhanced in patients with MS. The present study further identified that IL-22 in MS was predominantly produced by CD4⁺CCR4⁺CCR6⁺CCR10⁺ T cells. In addition, these cells differed from Th17 cells given that these cells also produced IL-22 and were identified as Th22.

We observed that in MS, the proliferation of Treg cells *in vivo* was reduced, which was identified using direct *ex vivo* analysis of peripheral blood Treg cells (Carbone et al., 2014). It has been previously reported that FOXP3 was decreased in MS, which is consistent with the findings obtained in the present study. The current study also found that Ikaros was reduced in MS. As a regulator of FOXP3, Ikaros may be a

critical factor in the development of MS. Ikaros contributed to a significant decrease in the function of CD4⁺CD25⁺FOXP3⁺ Treg cells in patients with MS.

Fas is widely expressed in normal and neoplastic tissues. Fas-mediated apoptosis plays an important role in various biological processes, including activation-induced cell death, T-cell-induced cytotoxicity, immune privilege and tumor surveillance (Restifo, 2000). Additionally, oligodendrocytes in MS lesions demonstrate increased Fas expression. In the present study, Fas was detected in oligodendrocytes, with the Fas levels enhanced in MS patients compared with healthy controls. Additionally, high concentrations of IL-22 increased the Fas levels in rat CG4 cells and mouse primary oligodendrocytes. IL-22 promoted the activity of caspase 3, an apoptotic marker. The suggested mechanism is that IL-22 activated the MSK-1/NF-κB pathway to increase Fas expression. JSH-23 and H89 are inhibitors of p65 and MSK-1. In the present study, both inhibited Fas expression induced by IL-22, which suggests that MSK-1/NFκB participated in the IL-22 induction of Fas.

In MS patients and healthy donors, IL-22 reduced the FOXP3 levels and decreased Treg cells. We further explored the mechanism and used IL-22 to treat CD4⁺ T cells from healthy donors, indicating that IL-22 increased the phosphorylation of NF-κB P65 and inhibited PTEN expression. Additionally, PI3K and Akt phosphorylation were inhibited, leading to a reduction of FOXO1, Ikaros and FOXP3. We obtained the same results in CD4⁺ T cells from MS patients and healthy controls, with IL-22 reducing FOXP3 expression by activating the NFκB-PTEN-PI3K pathway. An inhibitor of PI3K recovered the FOXP3 expression reduced by IL-22. This result

further demonstrated that NFκB-PTEN-PI3K was important in IL-22 regulation of FOXP3 expression.

In this study, it was observed that IL-22 played two important roles. In MS, inflammation factors and immune cells play important roles. Tregs can modulate the immune response. However, in MS patients, the expression levels of IL-22 and FOXP3 were incongruent. Cell experiment showed that IL-22 decreased FOXP3 expression in Tregs by the mechanism of IL-22 inhibiting the PTEN-FOXO1 pathway. On the other hand, IL-22 induced Fas expression *in vivo* and *in vitro* by the mechanism of IL-22 promoting the MSK-1/NFκB pathway in oligodendrocytes. These are two independent functions in two cell types, T cells and oligodendrocytes, which indicates that IL-22 has different roles in different cells, which may be associated with different receptors expressed on different cells. These diverse roles need to be studied further.

The EAE model of MS is often used to study neuroinflammatory disease mechanisms and was used in the present study to investigate MS. When Tregs from EAE or healthy control mice were co-cultured with mouse primary oligodendrocytes treated with or without IL-22, the results indicated that Tregs from control mice could reverse IL-22-induced Fas expression in oligodendrocytes. This finding was not observed in Tregs obtained from EAE mice. Taken together, these results suggest that Tregs play a critical role in the development of MS.

5. Conclusions:

The current study demonstrated the important roles of IL-22 in MS. In patients with

MS and in EAE animals, IL-22 expression was increased compared with healthy donors and control mice. This finding suggests that IL-22 participates in the pathological process of MS. Additionally, Tregs were investigated in this disease, with the percentages of Tregs being increased. In particular, Fas expression was enhanced in glioma cells. These results may represent a new pathway by which to treat this disease.

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7. Conflicts of interest

The authors declare no conflicts of interest.

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Figures

Figure 1. IL-22 expression in patients with multiple sclerosis (MS) and healthy controls.

A. Flow cytometry plots (percentage of CD4⁺IL-22⁺ T cells indicated) generated from a representative control subject and a subject with MS. B. Percentage of CD4⁺IL-22⁺ T cells in freshly isolated peripheral blood mononuclear cells from control subjects (n=20) and subjects with MS (n=20). C. IL-22 concentration in the serum from patients with MS and the healthy controls. * $P < 0.0001$ by Mann-Whitney U-test (two tails) (mean $\pm$ standard error).

Figure 2. IL-22 expression in Th22 cells from patients with multiple sclerosis (MS) and healthy controls.

A. Gating of flow cytometry. B. Flow cytometry plots (percentage of CD4⁺CCR4⁺CCR6⁺CCR10⁺IL-22⁺ T cells indicated) generated from a representative control subject and a subject with MS.

Figure 3. Fas expression and caspase 3 activity in oligodendrocytes. A. Fas mRNA in CG4 cells treated with different concentrations of IL-22. B. Fas mRNA in primary mouse oligodendrocytes treated with different concentrations of IL-22. C. Fas mRNA in primary mouse oligodendrocytes transfected with or without pcDNA3.1-IL-22. D. Fas expression in GC4 cells and primary mouse oligodendrocytes treated with 0 or 80 ng/ml of IL-22 was detected *in vitro* and in EAE mouse model. E. Caspase 3 activity

in CG4 cells treated with different concentrations of IL-22. F. Caspase 3 activity in primary mouse oligodendrocytes treated with different concentrations of IL-22.

Figure 4. FOXP3 expression in patients with multiple sclerosis (MS) and healthy controls.

A. FOXP3 in the PMBCs from MS patients and healthy controls. Flow cytometry plots (percentage of $CD4^+CD25^+FOXP3^+$ T cells indicated) generated from a representative control subject and a subject with MS. B. Percentage of $CD4^+CD25^+FOXP3^+$ T cells in freshly isolated PBMCs from control subjects (n=20) and subjects with MS (n=20). C. FOXP3 mRNA levels in patients with MS and healthy controls. PBMCs, peripheral blood mononuclear cells.

Figure 5. Expression of phosphorylated MSK-1 and p65 in oligodendrocytes and EAE mice.

A. The expression of phosphorylated MSK-1 and p65 were analyzed by Western blotting in CG4 and mouse primary oligodendrocytes following a 60-min treatment with IL-22. B. The expression levels of phosphorylated MSK-1 and p65 were analyzed by Western blotting in EAE and control mice. C. Fas expression was detected in GC4 cells treated with inhibitors of NFkB p65, MSK-1, JSH-23 and H89.

Figure 6. Expression of p65, Akt, PTEN and FOXO1 in patients with multiple sclerosis (MS).

A. Expression of phosphorylated p65, Akt, PTEN and FOXO1 in CD4⁺ T cells from healthy controls and MS patients. B. Expression of phosphorylated p65, Akt, PTEN and FOXO1 in CD4⁺ T cells from healthy controls following a 60-min treatment with IL-22. C. Ikaros mRNA levels in MS patients and healthy controls. D. FOXP3 expression was detected in CD4⁺ T cells treated with an inhibitor of PI3K, LY294002.

Figure 7. Fas expression in oligodendrocytes after co-culture with Tregs.

A. Fas mRNA levels in EAE and control oligodendrocytes. B. Fas mRNA levels in oligodendrocytes treated with IL-22 and co-cultured with Tregs obtained from EAE or control mice.

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