Role of Cathepsin C and Cystatin F in demyelinating diseases

Wilaiwan Wisessmith

Doctor of Philosophy

Department of Physiological Sciences

School of Life Science

The Graduate University for Advanced Studies

2014

Acknowledgement

I would like to express my gratitude to my major advisor Prof. Kazuhiro Ikenaka for his advice, support, understanding and encouragement. He always patiently and kindly teaches and conducts me. His way of thinking and teaching made me feel comfortable and very happy in the study and research. He gave the good opportunity and guidance me through this academic success. I very appreciate for everything that you have done to me, thank you very much.

I gratefully thank Dr. Kenji Tanaka, who has been my supervisor during the beginning of my study, for his many support and advice. I had a great experience to do research at Division of Neurobiology and Bioinformatics, National Institute for Physiological Sciences. He gave me supports and valuable comments and kind suggestion throughout my study.

I appreciate thank to the members of my thesis committee Prof. Suzumura Akio, Prof. Minokoshi Yasuhiko and Prof. Fukata Yuko, who gave me supports, helpful recommendation and kind suggestion.

I appreciate all teachers and colleagues in Prof. Ikenaka's lab for their assistance and guidance, and being my best friends. They made laboratory sweet, warm and lively place to me. Without their help, my thesis would not have been successful. I wish to thank all friends that made my life in Japan wonderful and feel like the second home. I would like to specially thank Division of Neurobiology and Bioinformatics, National Institute for Physiological Sciences where I discovered what I want to be and fulfill my dream.

Finally, I am grateful my precious family; my mother, father and my lovely brothers for their trust, love, understanding and infinite support given to me all the way. This achievement could not even have been possible without them. Thank you a lot for always standing by my side.

Contents

Abbreviations

APC: Antigen-presenting cell

ARG-1:Arginase 1

ATRA: All-trans retinoic acid

BBB: Blood brain barrier

c-fms: Colony stimulating factor 1 receptor

CatC: Cathepsin C

CatG: Cathepsin G

CD4: Cluster of differentiation 4

CNS: Central nervous system

CTLs: Cytotoxic T-lymphocyte

CysF: Cystatin F

DAB: Diaminobenzidine

EAE: Experimental autoimmune encephalomyelitis

FAST: Flexible accelerated STOP tetracycline operator tetO-knockin system

GrA: Granzyme A

GrB: Granzyme B

Iba1: Ionized calcium-binding adapter molecule 1

ICE: Interleukin 1 beta converting enzyme

IFN-γ: Interferons gamma

IL-1β: Interleukin 1 beta

TNF-α: Tumor necrosis factor alpha

KD: Knock down

MBP: Myelin basic protein

MOG: Myelin oligodendrocyte glycoprotein

MS: Multiple sclerosis

NBT/BCIP: Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate

NE: Neutrophil elastase

NK: Natural killer cells

NO: Nitric oxide

OE: Overexpression

PBS: Phosphate buffered saline

PFA: Paraformaldehyde

Plp: Proteolipid protein

PMNs: Polymorphonuclear neutrophils

PR3: Proteinase 3

SSC: saline-sodium citrate

Th: T-helper cells

TNF-α: Tumor necrosis factors alpha

TPA: 12-O-tetradecanoylphorbol-13-acetate

General summary

There are many type of diseases that damage myelin in the central nervous system (CNS). Multiple sclerosis (MS) is the most common demyelinating disease in the CNS. In this disorder, the immune system attacks the myelin sheath and causes inflammation and injury to the sheath and ultimately to the nerve fibers that it surrounds. The results are multiple areas of scarring (sclerosis), where premyelinating oligodendrocytes fail to remyelinate axon at affected area. The studies on therapeutic attack on MS have been significantly increased in number in recent years and improved therapeutic outcome. Animal models have been critically important for addressing and establishing MS treatment. There are several animal models that can be used for the study. In this study, the heterozygous transgenic 4e ($plp^{4e/-}$) mouse and the experimental allergic encephalomyelitis (EAE) model were selected to study different demyelination phases.

The chronic phase study used the $plp^{4e/-}$ *mouse*, in which the proteolipid protein gene is overexpressed. This mouse model starts demyelination accompanied by myelin regeneration from 2 months old. The regeneration of myelin will stop at 6 months old but demyelinating still proceeds. At an early phase, $Plp^{4e/-}$ mouse model shows pathology with no immune response involvement, therefore the inductive event is different from that of MS. However, at later phase, they have similar pattern of pathological events, in which premyelinating oligodendrocytes fail to remyelinate axons. This is similar to the observation in chronic phase of MS. We previously found that Cathepsin C (CatC) is upregulated in microglia of demyelination models, especially in chronic demyelinated lesions in $Plp^{4e/-}$ mouse. Additionally, its inhibitor Cystatin F (CysF), which is a cysteine protease inhibitor and is also induced during

early phase of demyelination, ceased expression in chronic demyelinated lesions. The myelin oligodendrocyte glycoprotein (MOG) induced EAE is most commonly used as a laboratory model for MS. Inductive phase of EAE shows pathology and progression of disease similar to those observed in MS, but in the final phase mouse gain full recovery. Thus, I selected EAE mouse to study acute demyelination phase. The study on the role of CatC and CysF played in demyelinating diseases should be useful for development of effective new therapeutic target of MS or other demyelinating diseases.

To study the role of CatC and CysF in demyelinating disease, we generated mouse line to manipulate CatC and CysF expression by using Flexible Accelerated-STOP-Tetracycline Operator Knockin (FAST) system. The homozygotes knock-in mouse ($CatC^{STOP/STOP}$, $CysF^{STOP/STOP}$) showed no expression of CatC or CysF, which can be considered as CatC or CysF knockdown (CatCKD or CysFKD) mice. CatCKD and CysFKD transgenic mice were crossed with $Iba^{tTA/-}$ and $Plp^{4e/-}$ mouse to overexpress CatC or CysF in microglia (CatCOE and CysFOE) of $Plp^{4e/-}$ mouse for the study in the chronic phase. From previous studies, CysF is upregulated in ongoing remyelinating phase but CatC conversely tends to dominate CysF in the chronic phase of PLP transgenic mouse. In ongoing remyelination phase of $Plp^{4e/-}$ mouse at age 4 months, CysFKD mouse significantly enhanced demyelination, while no effect were found in CatC deprived $Plp^{4e/-}$ mouse at the same time point of an early phase. Conversely, during the chronic demyelination phase at age 8 months, CatCKD mouse diminished demyelination in the $Plp^{4e/-}$ mouse. Therefore, CysF plays a main role during early remyelinating phase. I confirm this result by changing the balance of CatC and CysF expression by overexpressing CatC in microglia of $Plp^{4e/-}$ mouse. I

found that CatCOE mouse also resulted in a similar phenotype with CysFKD mouse showing early demyelination appearance.

For acute phase, MOG-EAE model was used. I found CatC mRNA expressing cells in the grey horn and anterior median fissure of spinal cord at an early phase of MOG-EAE models. These CatC mRNA expressing cells were identified as microglia and neutrophils, respectively. From this result, I concluded that CatC should have an important role in pathogenesis of EAE because of their earier expression at target sites than other molecules. In the late phase of MOG-EAE, CatC was expressed at chronic demyelinating regions but not CysF. CatCKD expression showed less demyelination in the EAE mouse. Conversely, CatC overexpression in microglia significantly enhanced its demyelination. This result is similar to the finding in the chronic demyelination model, $PLP^{4e/-}$ mouse. Therefore balance of CatC and CysF expression plays an important role in demyelinating diseases in both acute and chronic phases.

Part I

Plp$^{4e/-}$ transgenic mouse;

Chronic demyelination model

Introduction

Demyelination is characterized by destruction of normal myelin. Important consequences of demyelination are imposed first on the axon in the form of disturbed impulse conduction (Shivane and Chakrabarty 2007). Multiple sclerosis (MS) is an inflammatory demyelinating disease, which is associated with focal destruction of myelin sheaths and neurodegeneration of the central nervous system (CNS). It is the most common demyelinating disease in young adult. MS affects on patients of all ages but mostly develop symptoms between 18 and 50 years old (Adams and Kubik 1952). Clinically, MS is characterized by relapses and remissions of neurological disturbance. The neurological symptoms depend on the site of the specific CNS pathways that are involved, such as optic neuritis (optic nerve); paraesthesia, numbness, weakness, clumsiness of the limbs, bowel and bladder disturbance (spinal cord); vertigo, diplopia and oscillopsia (brainstem); and ataxia (cerebellum) (Graham and Lantos 2002).

The progression course of MS varies among individuals and is unpredictable. In most patients, the disease is characterized initially by episodes of reversible neurological deficits. MS mostly begins as a clinical worsening (relapsing) illness with episodes of neurological dysfunction lasting several weeks, followed by substantial or complete improvement (remitting). The acute phase lesion generally are described as actively demyelinating lesions in which myelin phagocytes directly engage normal appearing myelin sheaths in the presence of infiltrating T-cells (Barnett and Prineas 2004). Some patients remain stable for many years because of either prolonged periods of clinical remission or substantial recovery from repeated episodes of relapses. The recovery is often less complete in the late phase with time pass and too many repeated relapse (Noseworthy 1999).

In the chronic phase, the lesions are composed of areas of myelin and oligodendrocyte loss along with infiltrates of inflammatory cells, including lymphocytes and macrophages by focal inflammation. Oligodendrocytes are unable to completely remyelinate destroyed myelin sheath. However, the recruited oligodendrocyte progenitor cells are capable of proliferation, and differentiation into mature myelinating oligodendrocytes. The newly formed myelin sheaths are thinner and often not as effective as the original ones. The repeated attacks of MS pathology result in ineffective remyelination and the focal plaques of demyelination is built up around the damaged axons (Chang et al., 2002)

The remyelination failure is a critical impediment to recovery in MS. Several factors and molecules are report to affect remyelination, such as chondroitin sulfate proteoglycans, PSA-NCAM, notch pathway and hyaluronan (Lau et al., 2012, Charles et al., 2002, Wang et al., 1998, Sloane et al., 2010). The brain environment is also important to support the recovery. Under the normal physiological conditions in the adult CNS, microglia/macrophage monitor the CNS environment for pathogens, maintain CNS homeostasis, phagocytosis of dead and dying cells, and respond rapidly to perturbations in the local environment. These cells are referred to as macrophages or microglia (macrophages/microglia) (Stollg and Jander 1999). The activated microglia was found to accumulate in all plaque regions in the MS brain (Tanuma et al., 2006). The activated microglia and macrophages at the injured CNS are unable to be distinguished by their morphology or antigenic markers. Microglia and macrophages are able to convey both cytotoxic or protective responses based on environmental signals, the cell surface receptors they express and the intracellular signaling pathways that are activated. The polarization of macrophages and microglia

underlie the differing functional properties of macrophages/microglia, which results in cells with either pro- or anti-inflammatory properties (Samuel and Antje 2011). The "classically activated" microglia are defined as M1 microglia, which express proinflammatory cytokines and mediators, such as nitric oxide (NO) and TNF-α, to promote inflammation and antigen presentation. The "alternatively activated" microglia (M2) produce anti-inflammatory mediators, including IL-4 and IL-10 to induce tissue repair. The collective evidence indicated that M1 and M2 microglia have distinct functions in MS (Gao and Tsirka 2011).

The study from Kagawa et al. (1994) attempted to rescue hypomyelination phenotype in the Jimpy mouse (X-linked recessive mutant, characterized by abnormal myelin formation in the CNS) by introducing extra wild-type mouse *Plp* (proteolipid) genes to produce *plp* gene overexpressing transgenic mice (Kagawa et al., 1994). The heterozygous transgenic 4e ($plp^{4e/-}$) mouse has overexpression of *Plp* gene about 2 times in oligodendrocytes that resulted in CNS demyelination along with myelin regeneration from 2 months of age but myelin regeneration terminates after 6 months of age. In the late phase (chronic demyelinating phase), remyelination process is impaired and results in the appearance of naked axons. Demyelination in the $Plp^{4e/-}$ mouse was caused by arrested oligodendrocyte maturation. The processes of oligodendrocyte extended parallel with axons but failed to form myelin structure. The presence of premyelinating oligodendrocytes in the demyelinated lesion of $plp^{4e/-}$ mouse are also found in MS (Ma et al., 2006). The cDNA microarray analysis was employed to study altered gene expression in the $plp^{4e/-}$ mouse brain at 2, 4, 6 and 8 months of age. They found cystatin F *(CysF)* gene was elevated during acute demyelinating and active remyelinating phase but was reduced rapidly at the chronic

demyelination phase when myelin repair ability was terminated in the *plp*$^{4e/-}$ mouse (Ma et al., 2007).

CysF is a type II cysteine protease inhibitor, which belongs to the cystatin superfamily (Ni et al., 1998). Type II cystatins have been implicated in the control of proteolysis under normal or pathological conditions. The secreted form of CysF is a functionally inactive disulphide-linked dimer. The activation of CysF in living cells relies on the proteolytic process by cleaving N-terminal linking peptide (Schuttelkopf et al., 2006). Under the normal condition, CysF is expressed selectively in immune cells such as T cells, natural killer (NK) cells and dendritic cells (Halfon et al., 1998). CysF has been shown to be strongly upregulated in LPS-stimulated monocyte-derived dendritic cells or downregulated in 12-O-tetradecanoylphorbol-13-acetate (TPA) or all-trans retinoic acid (ATRA) stimulated U937 cells. Both TPA and ATRA are usually used as the apoptotic stimuli to induce apoptosis. CysF levels are controlled according to the physiological state of the cells (Nathanson et al., 2002, Hashimoto et al., 2000). The major target of CysF in different immune cell types is the amino-peptidase cathepsin C (CatC), which regulates the activation of effector serine proteases in T cells, natural killer cells, neutrophils and mast cells. CatC is a physiological target of CysF but only once CysF has undergone post-translational proteolytic processing (Hamilton et al., 2008).

CatC or dipeptidyl aminopeptidase 1 is a unique lysosomal cysteine protease because of its oligomeric structure. Other cysteine proteases are monomeric proteins with a molecular mass of 20–30 kDa, whereas CatC have oligomeric structure with a molecular mass of 200 kDa (McDonald et al., 1972). The enzyme is constitutively expressed in many tissues with the highest levels in lung, kidney, liver, spleen, and

placenta but relatively low in the brain (Pham et al., 1997). CatC was thus revealed to be one of the major processing machineries known so far. Its function is to activate many granule-associated serine proteases in T cell, NK cells, neutrophils and mast cells that are related to immune and inflammatory processes, such as neutrophil-derived cathepsin G (CatG), neutrophil elastase (NE), proteinase 3 (PR3) and the cytotoxic lymphocyte-associated protease granzymes A and B (GrA and GrB). Activation of target enzymes is achieved by the removal of an 'activation dipeptide' at the N terminus. CatG, NE, and PR3 are polymorphonuclear neutrophils (PMNs) specific granule-associated enzymes, whose main function is to degrade various components of the extracellular matrix, due to their proteolytic properties (Adkison, et al. 2002, Eyles, et al. 2006). Granzymes are natural serine protease that is expressed in granules of activated cytotoxic T-lymphocytes (CTLs) and natural killer cells (NK). CatC cleaves progranzymes A and B (Pro-GrA and GrB) to proteolytically active form, which will cascade several pathway (Pham and Ley 1999, Kam et al., 2004). GrA induces cell death processes and lead to the cleavage of DNA into large fragments in the target cell nucleus and activate interleukin 1 beta converting enzyme (ICE), which can cleave proIL-1β to generate IL-1β (Irmler et al., 1995, Fen et al., 2003). GrB induces cell death through the activation of caspase dependent and independent pathways. GrB can directly cleave the proapoptotic protein Bid, which generates an active form of Bid that translocates to the mitochondria and induces permeability of this organelle. This Bcl-2 regulated pathway has rapid kinetics and causes the release of cytochrome c, which leads to caspase activation. GrB also acts on the mitochondria by inducing caspase-independent cell death through the generation of reactive oxygen species (ROS) (Boivin et al., 2009, Choy 2010).

In the *plp*$^{4e/-}$ mouse both CatC and CysF was found to be upregulated in a specific manner. CysF expresssion was induced during acute demyelinating and active remyelinating process but was decreased rapidly at the chronic demyelinating area when the ability to repair myelin was lost. CysF was expressed by activated microglia mostly in the white matter during acute demyelination and active remyelination phase and continues its expression only in regions where myelin still remains in chronic demyelinating phase 8 months of age. CysF was also found only at the edge of the plaques (remyelinating area) in MS, but not inside the plaques that was consistent with animal demyelination model, *plp*$^{4e/-}$ mouse (Ma et al., 2011). Conversely, the study in our groups showed CatC was expressed at low level during acute demyelination but dominated CysF expression in the chronic demyelinating phase. We generated the transgenic mice, in which CatC or CysF level can be manipulated by the use of Flexible Accelerated STOP Tetracycline Operator tetO-knockin (FAST) system to use in this study (Tanaka et al., 2010).

The demyelinating diseases are untreatable and result in disability and reduce quality of life. The earlier treatment would be expected to produce better results than later treatment of MS (Comi et al., 2001). The study about demyelinating process is very important for find missing pieces to cure disease. Thus, even more so than in most other human diseases, demyelination research depends on the availability of suitable experimental models to elucidate the pathogenic mechanisms and to develop effective and specific therapies (Wekerle and Kurschus 2006). The study for discovery of the master molecules and key cell type that affect myelin regeneration like CatC and CysF is my aim of this study. This should be helpful to fulfill development of

some part of new effective treatments for MS and other demyelinating diseases. In this study, I investigated roles of CatC and CysF in demyelinating diseases.

Materials and Methods

Experimental Animals

Hemizygote Plp transgenic (*Plp*$^{4e/-}$) mouse from the 4e line (mixed background with C57BL/6 and DBA2; Kagawa et al., 1994) and CatCSTOPtetO and CysFSTOPtetO mouse (generated by FAST system) were used (Tanaka, et al. 2010, Shimizu 2012). *Plp*$^{4e/-}$ was crossed with CatCSTOPtetO and CysFSTOPtetO mice to generate homozygous CatCSTOPtetO-Plp$^{4e/-}$ (*CatC*$^{STOP/STOP}$*Plp*$^{4e/-}$; CatCKD), CysFSTOPtetO-Plp$^{4e/-}$ (*CysF*$^{STOP/STOP}$*Plp*$^{4e/-}$; CysFKD), heterozygous CatCSTOPtetO-Plp$^{4e/-}$ (*CatC*$^{STOP/-}$*Plp*$^{4e/-}$) and CysFSTOPtetO-Plp$^{4e/-}$ (*CysF*$^{STOP/-}$*Plp*$^{4e/-}$). To generate CatC overexpression mouse, CatCSTOPtetO and CysFSTOPtetO mouse were crossed with heterozygous *Iba1*$^{tTA/-}$ mouse to generate *CatC*$^{STOP/-}$*Iba*$^{tTA/-}$ (CatCOE) and *CysF*$^{STOP/-}$*Iba*$^{tTA/-}$ (CysFOE) mouse, respectively. The heterozygous CatCSTOPtetO-Plp$^{4e/-}$ (*CatC*$^{STOP/-}$*Plp*$^{4e/-}$), CysFSTOPtetO-Plp$^{4e/-}$ (*CysF*$^{STOP/-}$*Plp*$^{4e/-}$) and *Plp*$^{4e/-}$ were used as control mice in this study. All animal procedures were conducted in accordance with the guidelines described in the National Institutes of Health Guide for the care and use of laboratory animals and by the National Institute for Physiological Sciences Animal Care and Use Committee. Mice were sacrifice at different time point: 2.5, 4, 6 and 8 months of age.

Mouse genotyping

Mouse fingers were cut off and collected 7-12 days post natally, and placed in a 1.5 ml microfuge tube. NaOH solution consisting of 25 mM NaOH, 2 mM EDTA (100 μl) was added to the fingers in the microfuge tube. After incubation at 100°C for

45 min, 100 µl of 40mM Tris-HCl pH 7.5 was added. The following PCR primer sets were used in mouse genotyping: two pairs of primers: CXRF2 Forward: (5'-TACTCAAGTGCCCCTAGGAAG-3'); CRXR2 Reverse: (5'-ATTGATCTTAAGAACCTC-3') and 4e-primer-2: (5'-CAATGCGCTTACTG-ATGCGG-3'); 4e-primer-3: (5'-CGCACAGAAGCTAT-TATGCG-3') for $Plp^{4e/-}$ mouse. CatC205L: (5'-AAGGCAAGGACTCAGGGACAGAAA-3') and tetOup: (5'-AGCAGAGCTCG-TTTAGTGAACCGT-3') for knock in allele of CatCSTOP-tetO mouse. CatC361U: (5'-TTTGGCGTTCCTTGAAAGGCAGAG-3') and CatC205L: (5'-AAGGCAAGGACTCAG-GGACAGAAA-3') for wide type allele of Cathepsin C. CysF708L: (5'-TCTCAGGGTTCCAAGAGTGTCC-3') and tetOup: (5'-AGCAGAGCTCGTTTAGTGAACCGT-3') for knock in allele of CysFSTOP-tetO mouse. CysF202U: (5'-TTTCTTCACATCAGCATCCC-3') and CysF708L: (5'-TCTCAGGGTTCCAAGAGTGTCC-3') for wide type allele of Cystatin F. Iba1552U: (5'-ATGCCTGGGAGTTAGCAAGGGAAT-3') and mTA24L: (5'-CGGAGTTGATCACCTTGGACTTGT-3') for Iba1-mtTA mouse.

Genotyping PCR for the Plp gene was carried out for 35 cycles in a programmable heat block as follows: 95°C for 2 min, 56°C for 30 sec, and 72°C for 30 sec, with a final extension step of 5 min. PCR products were fractionated by 2% agarose gel electrophoresis and stained with ethidium bromide. Genotyping PCR for CatC and CysF gene was carried out for 35 cycles as follows: 95°C for 2 min, 59°C or 63 for 15°C sec, and 72°C for 30 sec, with a final extension step for 2 min. PCR products were fractionated by 1.2% agarose gel electrophoresis and stained with ethidium bromide.

Mouse tissue preparation

Mice were anesthetized with chloral hydrate and perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde solution (PFA). Brains were removed and stored in the same fixative overnight. Then, the brains were cryoprotected in PBS containing 20% sucrose overnight, embedded in OCT compound (Sakura Finetechnical Co., Tokyo, Japan), and cut into 20 μM slices by cryostat (Leica CM3050, Germany).

In Situ Hybridization

Cryosections from 4% PFA perfused brain were used. Frozen sections were fixed in 4% PFA for 20 min, followed by two washes with PBS for 5 min, proteinase K (20 μg/ml) treatment for 30 min. Sections were washed with PBS and fixed with 4% PFA for 15 min to inactivate the proteinase, and then washed with 0.1 M triethanolamine-HCl for 15 min followed by washing with PBS for 5 min 2 times. Prehybridization was carried out for 2-4 hours at 65°C in the hybridization buffer containing 50% formamide, 5X saline-sodium citrate (SSC), 0.2 mg/ml yeast tRNA and 200 μl/ml heparin. After removing prehybridization buffer, hybridization buffer containing 300 ng/ml digoxigenin-labeled cRNA probe was added and hybridization was performed at 65°C overnight. After washing with SSC and blocking with the blocking buffer, sections were incubated with alkaline-phosphatase-conjugated anti-digoxigenin antibody for 1 hour at room temperature. Unbound antibody was removed by washing with MABT for 30 min 3 times. The color development was performed by

incubation with Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) for up to 24 hours at room temperature and the reaction was stopped by immersing into PBS. The sections were counterstained with Nuclear Fast Red (Vector Lab, Burlingame, CA). Images were captured using Olympus digital camera system (DP70) in combination with microscopy, Olympus BX51.

Immunohistochemical Analysis

Cryosections from 4% PFA perfused brain were used. After washing the fixed sections with PBS one time for 5 min, antigen retrieved was performed. Sections were treated with heat in a microwave oven (550W) for 3 min in 10 mM citrate buffer (pH 6.0) and cooled down to room temperature. The sections were incubated with 0.03% H_2O_2 in PBS-T (0.1% Triton-X100) for 30 min. The sections were blocked by incubating them with 10% normal goat serum in PBS-T for 1 hour at room temperature. Sections were incubated with primary antibodies at 4°C overnight followed by incubation with secondary antibodies for 1 hour at room temperature. After washing with PBS-T for 5 min three times, sections were incubated with ABC solution (horseradish peroxidase-streptavidin-biotin complex, Vectastain ABC kit; Vector laboratories, CA, USA). The sections were colored with 3,3'-diaminobenzidine (DAB) solution with 0.03% H_2O_2. The sections were dehydrated with serial concentration of ethanol (70%, 80%, 90%, 100%), cleared with clear plus and were mounted with permount solution. Images were captured using Olympus digital camera system (DP70) in combination with microscopy, Olympus BX51.

The following antibodies were used: rabbit anti-Iba1 polyclonal antibody (1:1000; Wako, Osaka, Japan), mouse anti-CNPse monoclonal antibody (1:1000; Sigma, St. Louis, MO), goat anti-arginase-1 polyclonal antibody and rat anti-CD16/32 monoclonal antibody (1:200; SantaCruz, CA, USA). Secondary antibodies were labeled with biotin (Vectastain).

Image Analysis and Statistical Analysis

The myelin stained area was quantified by measuring the density using densitometry analysis with Image J program (National Institutes of Health, Bethesda, MD, USA). Data were expressed as mean $\pm$ S.E.M. Significance was assessed by o unpaired two-tailed Student's *t*-test using the scientific statistic software Graphpad Prism version 5.0b (GraphPad Software, Inc., La Jolla, CA, USA). *P*-values of less than 0.05 were considered significant.

Result

CatC and CysF gene manipulation mouse

To study the role of CatC and CysF in demyelinating diseases, we generated transgenic mouse lines in which CatC or CysF expression can be manipulated by the FAST system in this study (Tanaka, et al. 2010). CatCKD, CysFKD, CatCOE and CysFOE mice were observed for any defects that arrise from gene manipulation. None of the transgenic mice showed any physical phenotype or clinical symptoms. I further examined histological phenotype. In wild type mouse, *CatC* mRNA was found only in the CA2 region of hippocampus and choroid plexus (Shimizu 2012), and is not found in any other area of the brain (Figure 1A). *CysF* mRNA was not found in any area of the brain (Figure 1B). Activated microglia, which are strongly labeled by *c-fms* mRNA ISH, was not prominent (Figure 1C). Microglia morphology as detected by Iba-1 IHC showed normal "resting" shape; small cells body and numerous very thin and highly branched processes (Figure 1D).

CatCKD mouse showed no *CatC* mRNA positive cells resulting from the STOP sequence of FAST system, which inhibits CatC gene transcription, and behaved like knockout mouse (Figure 2A). No *CysF* mRNA was found in CatCKD mouse as in the wild type mouse (Figure 2B). The microglia activation was not evident when its *c-fms* expression was compared with that of the wild type mouse (Figure 2C) and microglia morphology as judged by the Iba-1 staining remains at the resting stage (Figure 2D). CysFKD mouse showed no CatC or CysF mRNA positive cells (Figure 3A-B) and a similar number of *c-fms* positive cells with the number in the wild type mouse (Figure 3C). However, the "activated" microglia phenotype, which is

characterized by shorter and thicker processes with swollen cell body, was found in the CysFKD (Figure 3D). CatCOE and CysFOE mice showed *CatC* and *CysF* mRNA respectively overexpressed (Figure 4A, 5B). CatCOE mouse brain did not expressed *CysF* mRNA (Figure 4B), but CysFOE mouse showed a few *CatC* mRNA-positive cells in some area of the brain (Figure 5A). Both CatCOE and CysFOE mice showed increased level of *c-fms* mRNA (Figure 4C, 5C), suggesting activation of microglia (Figure 4D, 5D). The result showed that gene manipulation by FAST system is appropriate to use in this study. Complete knock down and overexpression CatC and CysF were achieved by the FAST system. The gene manipulation to increase CatC and CysF expression resulted in microglia activation in both cases. Previous study showed the CatC and CysF gene expression manipulated mice do not have any symptoms but this result from detailed histological study suggested some abnormality in microglia activation.

CysF deprivation enhanced demyelination in chronic demyelinating model.

Previous study showed CysF expression was dominated that of CatC at an early phase of demyelination in the $Plp^{4e/-}$ mouse. At this stage, CysF should be inhibiting CatC activity. To observe the role of CysF in this phase, I observed CysFKD-$Plp^{4e/-}$ and the control mouse at 4 months of age, which is the phase that shows demyelination together with remyelination. The result showed less myelin remaining in CysFKD-$Plp^{4e/-}$ mouse when compare with control mice (Figure 6A-C). I quantified the myelin stained area from total area of single cerebellar folium of sagittal cryosection. The percentage of total myelin stained area of CysFKD-$Plp^{4e/-}$ mouse significantly decreased to 5.15 ± 0.86 % (n = 6) when compared with control group;

10.63 ± 0.49 % (n = 5) (Figure 6D). The result showed that the gene manipulation to deprive CysF results in aggravated demyelination at an early phase of chronic demyelinating model, *Plp*$^{4e/-}$ mouse. This result suggests that the balance of CatC and CysF is very important for maintaining myelin regeneration.

The excess of CatC early terminated myelin regeneration.

Since CysF is a CatC inhibitor, CatC activity might increase when the mouse was deprived of CysF. The result above showed worsens demyelination by CysFKD. If balance of CatC and CysF expression plays a critical role in myelin regeneration at an early phase of chronic demyelinating diseases, I used CatCOE-*Plp*$^{4e/-}$ and *Plp*$^{4e/-}$ mouse to observe the effect of CatC overexpression in microglia on demyelination. At the same time point when I observed CysF knockdown mouse at 4 month of age, CatCOE-*Plp*$^{4e/-}$ mouse showed severe symptoms and died by that time (data not shown). Thus, I chose to study CatCOE-*Plp*$^{4e/-}$ and *Plp*$^{4e/-}$ mouse at more early time point; at 2.5 months of age. The result showed less myelin remaining in CatCOE-*Plp*$^{4e/-}$ mouse when compared with *Plp*$^{4e/-}$ mouse (Figure 7A-B). I quantified the myelin stained area from total area of single cerebellar folium of sagittal cryosection. The percentage of total myelin stained area of CatCOE-*Plp*$^{4e/-}$ mouse significantly decreased to 5.10 ± 0.36 % (n = 8) when compared with the control group (7.86 ± 0.64 %, n = 4) (Figure 7C). The result was similar to CysF knockdown mouse. These results demonstrate that changing CysF-dominated expression over CatC expression to CatC dominant expression, either by decreasing CysF expression or by enhancing CatC expression in microglia (CatCOE mouse), results in enhanced demyelination and eliminated myelin regeneration much earlier in the *Plp*$^{4e/-}$ mouse. These results

confirmed the balance of CatC and CysF is very important for maintaining myelin regeneration at an early phase of demyelinating diseases.

CatC play a role in a chronic demyelinating lesion

CysF has been shown to play an important role at an early phase of chronic demyelinating diseases, but not in the late phase when CatC expression becomes dominant over CysF expression. Therefore, CatC seems to have prior role than CysF in the late phase of chronic demyelination. To understand the role of CatC in this phase, I used CatCKD-*Plp*$^{4e/-}$ and control mice (CatC-*Plp*$^{4e/-}$ and *Plp*$^{4e/-}$), and observed their phenotype at 4, 6 and 8 months of age. The myelinated area did not differ between both groups at 4 months of age, which is the early phase of chronic demyelination (data not shown). The control groups gradually lost myelin at 6 months of age (Figure 8B-C) and clearly showed severe damage of myelin structure at 8 months of age (Figure 8E-F). However, CatCKD-*Plp*$^{4e/-}$ mouse clearly had more remaining myelin when compared with the control groups (Figure 8A, B). I quantified the myelin stained area from total area in single cerebellar folium of sagittal cryosection. The percentage of total myelin stained area of CatCKD-*Plp*$^{4e/-}$ mouse significantly increased myelin level to 14.11 $\pm$ 1.25 % (n = 9) when compared with control groups (7.90 $\pm$ 2.97 %, n = 3) at 8 months of age (Figure 8H), while no significant difference was found at 6 months of age (Figure 8G). The result showed that even in the late stage of chronic demyelinating diseases, the balance of CatC and CysF is important for maintaining myelin regeneration process. The late phase, in which CatC expression dominated CysF expression, CatC deprivation enhanced myelin regeneration ability.

M1 and M2 microglia polarization in *Plp*$^{4e/-}$ mouse

Previous study showed that CatC and CysF were expressed in activated microglia of *Plp*$^{4e/-}$ mouse (Shimizu 2012). The difference in macrophage/microglia polarization is thought to be the cause for the differing functional properties that results in cells with either pro-inflammatory (M1) or anti-inflammatory (M2) properties (Samuel and Antje 2011). CysF was expressed in the activated microglia, but it is not known how they were polarized (M1 or M2 properties). The result showed CD16/32 positive M1 cells was mostly expressed in the white matter (Figure 9A) and seemed to have similar expression pattern to *CysF* mRNA positive cells (Figure 9C). M2 microglia as assessed by ARG-1 staining was expressed like small dots at the gray matter, which is located on the edge of white matter of cerebellum (Figure 9B) and did not co-localize with *CysF* mRNA (Figure 9D). This result concluded that CysF might be expressed in some of M1 microglia, but not in M2 microglia.

Discussion

In the chronic lesion of MS, remyelination process is inadequate, which results in demyelinated lesion. The most crucial question is what factors govern the recruitment and differentiation phases of remyelination, and whether an inappropriate regulation of any of these factors can account for remyelination failure? Several studies have identified several pathways that would be expected to have therapeutic efficacy (Fancy, et al. 2010). This study demonstrated CysF and CatC are possible crucial factors and the main trigger to inhibit myelin regeneration. I found microglia in CysFKD, CatCOE and CysFOE mice were activated. However, CatC knockdown mouse did not show any physical and histological phenotype. In CysFKD and CatCOE mice, the increment of CatC and deprivation of its inhibitor, CysF, might increase CatC activity in microglia causing microglia itself to be activated. Maintenance of brain environment is very important for recovery process from any injury, diseases or infection. Microglial cells monitor the well being of their environment, and are able to respond to signs of homeostatic disturbance with a program of supportive and protective activity, to safeguard innate defense mechanisms, or to assist in specific immune reactions (Uwe-Karsten 2002). The activated microglia was found to persist in chronic demyelinating lesions (Taupin, et al. 1997). Activated microglia suppression promotes myelin regeneration by supporting survival and function of oligodendrocyte progenitors (Zhang, Goetz and Duncan 2003). CatC over-activation in the CNS cause of microglia activation and its activity to maintain environment. Surprisingly, I found CysF overexpression also activated microglia and induced CatC expression. CysF should serve a protective function by inhibiting activities of such endogenous proteinases, which otherwise may cause uncontrolled proteolysis and

tissue damage (Hamilton, et al. 2008). I was not able to explain why upregulation of CysF also induced microglia and CatC activation. It seems the abnormality in CatC or CysF levels beyond physiological level causes brain environment disturbance and activate microglia. CatC and CysF might send some crucial signal that alerts CNS to prepare microglia ready for response with any kind of disturbance in the CNS.

In the $Plp^{4e/-}$ mouse, CysF and CatC had similar of expression pattern at an early phase. However, CysF was found to be expressed earlier than CatC during active demyelination and remyelination period process and dominated CatC expression. In this phase, myelin regeneration process is very important to maintain the myelin component, which is similar to the early phase of MS, which show relapse-remission symptoms (Niehaus, et al. 2000). $Plp^{4e/-}$ mouse showed no paralysis or any muscular dystrophy defect but showed sign of nerve conduction disturbance and a tonic seizure. CysF induction in microglia is dependent on the phagocytosis of compact myelin membranes (Ma, et al. 2011). CysF might have some role in supporting myelin regeneration process. Here I show that CysF knockdown in $Plp^{4e/-}$ mouse result in decreased myelin regeneration and impaired myelination at an early phase of chronic demyelinating disease. CysF inhibits CatC protease activity and disturb all downstream signals that are able to cause disturbance of myelin regeneration process. (Hamilton, et al. 2008, Irmler, et al. 1995, Pham and Ley 1999). The balance of CatC and CysF expression is seemed to have a critical role in myelin regeneration at the early phase of chronic demyelinating diseases. This conclusion was confirmed by CatC overexpression in microglia showing severe demyelination at a very early phase of chronic demyelinating disease. In the early phase of chronic demyelinating disease, CysF play dominant role to support myelin regeneration by interfering CatC function.

I found M1 microglia was present in the white matter, which was similar to that of CysF positive cells in *Plp*$^{4e/-}$ mouse. In contrast, M2 microglia was only found in the gray matter was surrounding demyelinating regions and did not co-localize with CysF expressing cells. Several studies revealed important role of microglia/macrophage activation into different cell types, referred to as microglia/macrophage polarization, that results in cells with either pro-inflammatory (M1 Cells) or anti-inflammatory properties (M2 cells) (Samuel and Antje 2011). M1 microglia/macrophages have a negative impact on axon regeneration by expressing chondroitin sulphate proteoglycan, which is a potent inhibitor of axonal growth, 17-fold higher than in M2 polarized cells (Martinez et al. 2006). The microenvironment of the injured spinal cord contains factors that actively prevent or block M2 polarization and tends to polarize to M1 cells. In the late phase *Plp*$^{4e/-}$ mouse, microglia tends to polarize to pro-inflammatory M1 cells. CysF seemed to relate with M1 polarization cells.

During the late phase of chronic demyelinating disease, CatC was found to dominate CysF and full activation is expected. This might cause impaired myelin regeneration and appearance of naked axons in the *Plp*$^{4e/-}$ mouse (Kagawa et al. 1994). I found CatC knockdown result in more intact myelin in the late phase of chronic demyelination. This gives strong evidence that CatC is one of the main players to induce demyelination. The conclusion of this study determined that balance of CatC and CysF expression plays an important role in chronic demyelinating disease.

Figure Legend

Figure 1. Identified CatC, CysF and microglia expression in wild type mouse

The adult wild type mouse was observed for *CatC*, *CysF*, *c-fms* mRNA expression (A-C) and microglia morphology (D) at the cerebrum. The brain sagittal cryosection was performed in situ hybridization by using *CatC*, *CysF* and *c-fms* mRNA probes and immunohistochemistry by using rabbit polyclonal anti-Iba1 antibody for microglia staining.

Figure 2. Identified CatC, CysF and microglia expression in CatC knockdown mouse

The adult CatCKD mouse was observed for *CatC*, *CysF*, *c-fms* mRNA expression (A-C) and microglia morphology (D) at the cerebrum. The brain sagittal cryosection was performed in situ hybridization by using *CatC*, *CysF* and *c-fms* mRNA probes and immunohistochemistry by using rabbit polyclonal anti-Iba1 antibody for microglia staining.

Figure 3. Identified CatC, CysF and microglia expression in CysF knockdown mouse

The adult CysFKD mouse was observed for *CatC*, *CysF*, *c-fms* mRNA expression (A-C) and microglia morphology (D) at the cerebrum. The brain sagittal cryosection was performed in situ hybridization by using *CatC*, *CysF* and *c-fms* mRNA probes and immunohistochemistry by using rabbit polyclonal anti-Iba1 antibody for microglia staining.

Figure 4. Identified CatC, CysF and microglia expression in CatC overexpression mouse

The adult CatCOE mouse was observed for *CatC*, *CysF*, *c-fms* mRNA expression (A-C) and microglia morphology (D) at the cerebrum. The brain sagittal cryosection was performed in situ hybridization by using *CatC*, *CysF* and *c-fms* mRNA probes and immunohistochemistry by using rabbit polyclonal anti-Iba1 antibody for microglia staining.

Figure 5. Identified CatC, CysF and microglia expression in CysF overexpression mouse

The adult CysFOE mouse was observed for *CatC*, *CysF*, *c-fms* mRNA expression (A-C) and microglia morphology (D) at the cerebrum. The brain sagittal cryosection was performed in situ hybridization by using *CatC*, *CysF* and *c-fms*

mRNA probes and immunohistochemistry by using rabbit polyclonal anti-Iba1 antibody for microglia staining.

Figure 6. CysF knockdown mouse enhanced demyelination in *Plp*$^{4e/-}$ mouse

CysFKD-*Plp*$^{4e/-}$ mouse (A), CysF-*Plp*$^{4e/-}$ (B) and *Plp*$^{4e/-}$ (C) transgenic mice were observed for myelin in the brain at 4 months. CysF-*Plp*$^{4e/-}$ and *Plp*$^{4e/-}$ mouse were used as control. The transgenic mice brain sagittal cryosection were performed immunohistochemistry by using mouse monoclonal anti-CNPase antibody for myelin staining, which showed in dark brown color. The percentage of myelin stained area was quantified by measuring the density using densitometry analysis with Image J program and their differences are represented in the graph (D). Values represented as mean $\pm$ SEM. The unpaired two-tailed Student's *t*-test was performed for statistical analysis. (*$p < 0.05$ compared with control group)

Figure 7. CatC overexpression mouse induced early demyelination in *Plp*$^{4e/-}$mouse

CatCOE-*Plp*$^{4e/-}$ (A) and *Plp*$^{4e/-}$ (B) transgenic mice were observed for myelin in the brain at 2.5 months of age. The transgenic mice brain sagittal cryosection were performed IHC by using mouse monoclonal anti-CNPase antibody for myelin staining, which showed in dark brown color. The percentage of myelin stained area was quantified by measuring the density using densitometry analysis with Image J program

and their differences are represented in the graph (C). Values represented as mean ± SEM. The unpaired two-tailed Student's *t*-test was performed for statistical analysis. (*$p < 0.05$ compared with control group)

Figure 8. CatC knockdown mouse diminished demyelination in *Plp*$^{4e/-}$ mouse

CatCKD-*Plp*$^{4e/-}$ mouse (A and D), CatC-*Plp*$^{4e/-}$ (B and E) and *Plp*$^{4e/-}$ (C and F) transgenic mice were observed the amount myelin in the brain at 6 and 8 months of age. CatC-*Plp*$^{4e/-}$ and *Plp*$^{4e/-}$ were used as the control. The transgenic mice brain sagittal cryosection were performed IHC by using mouse monoclonal anti-CNPase antibody for myelin staining, which showed in dark brown color. The percentage of myelin stained area was quantified by measuring the density using densitometry analysis with Image J program and their differences are represented in the graph (G and H). Values represented as mean ± SEM. The unpaired two-tailed Student's *t*-test was performed for statistical analysis. (*$p < 0.05$ compared with control group)

Figure 9. CysF related with M1 but not M2 Microglia/Macrophage cells in chronic phase of *Plp*$^{4e/-}$ mouse

Plp$^{4e/-}$ mouse at 8 months of age was identified for microglia/macrophage polarity related CysF at the late phase of chronic demyelinating disease. CD16/32 positive cells (A) and ARG-1 Positive cells (B) was labeled. The double labeling of *CysF* mRNA with CD16/32 (C) and *CysF* mRNA with ARG-1 (D) was showed. The

brain sagittal cryosection was performed in situ hybridization by using *CysF* mRNA probe and immunohistochemistry by using rat monoclonal anti-CD16/32 for M1 cells and goat polyclonal anti-Arginase1 (ARG-1) antibody for M2 cells staining.

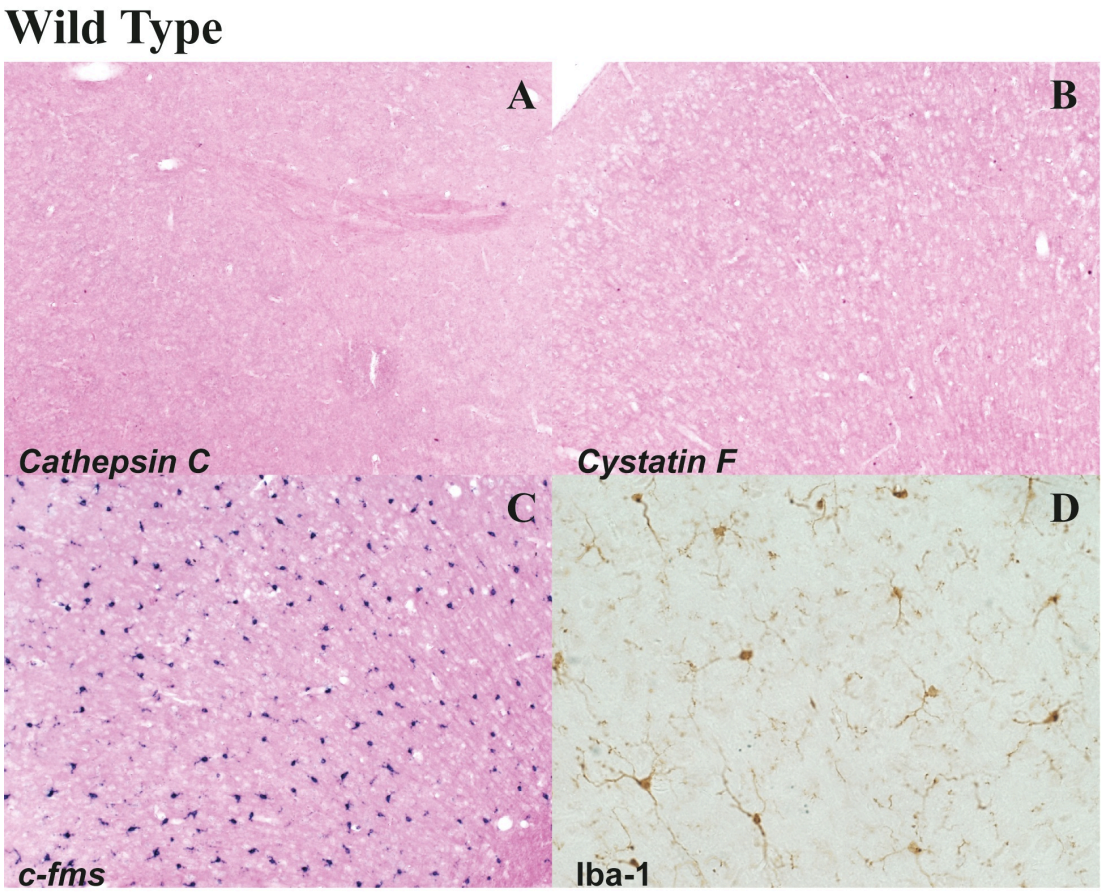


Figure 1

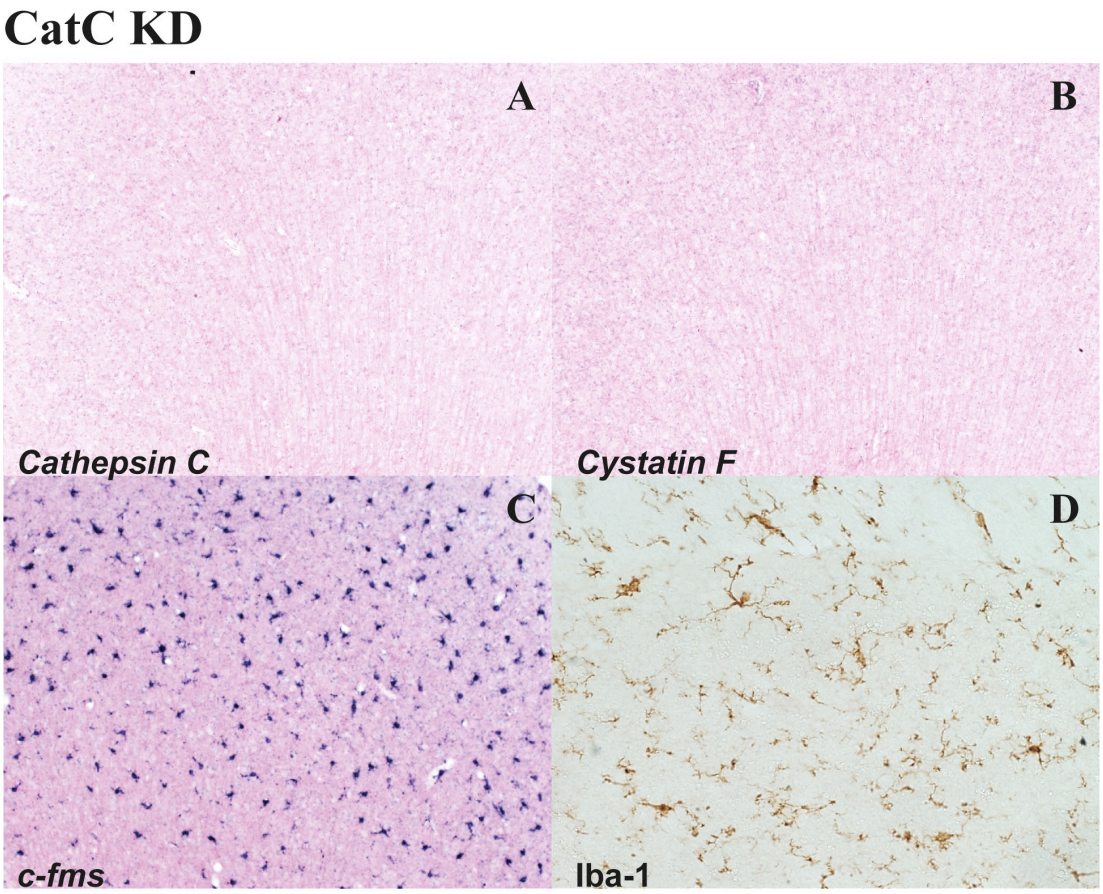


Figure 2

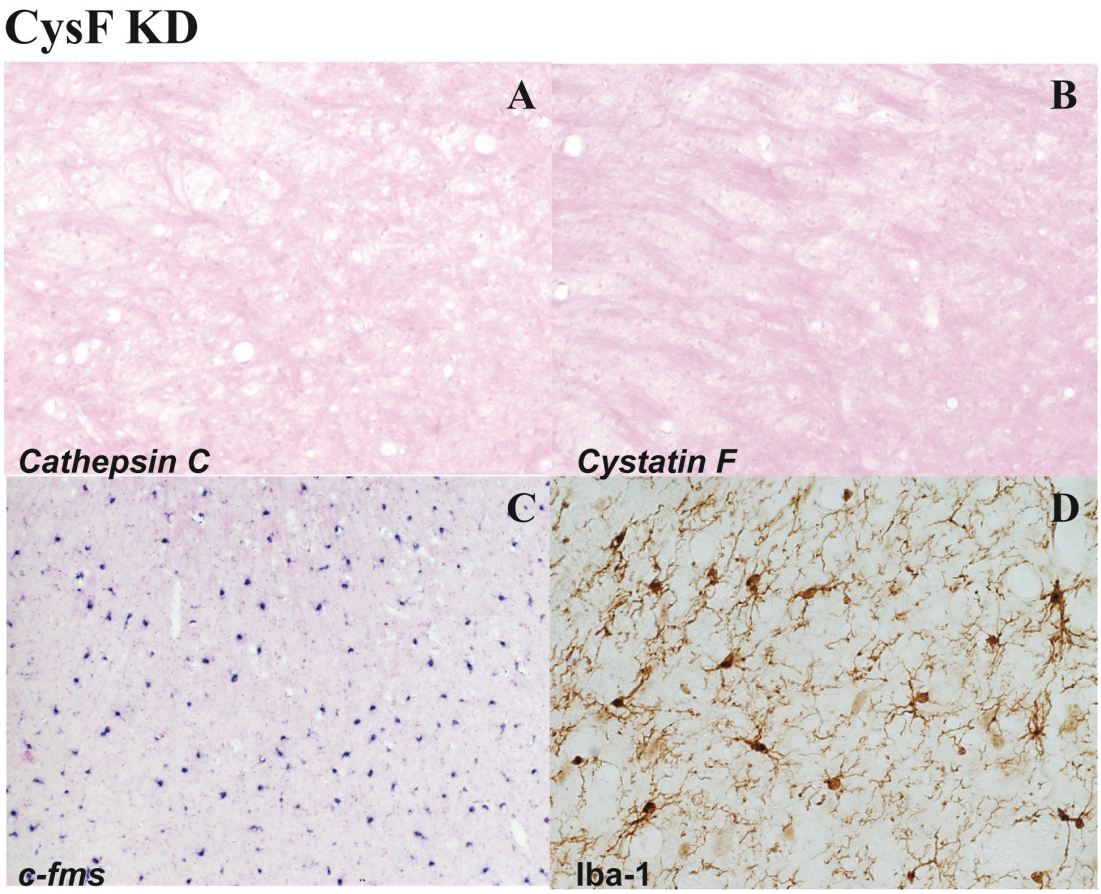


Figure 3

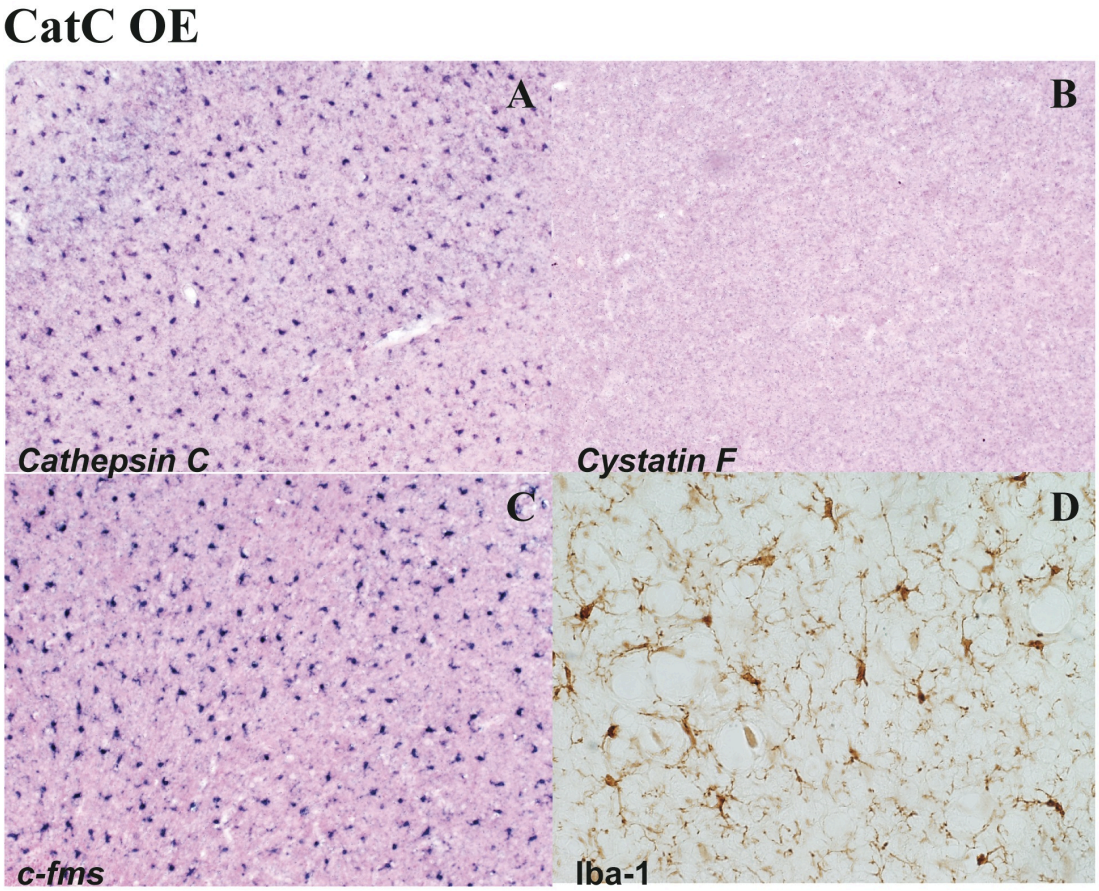


Figure 4

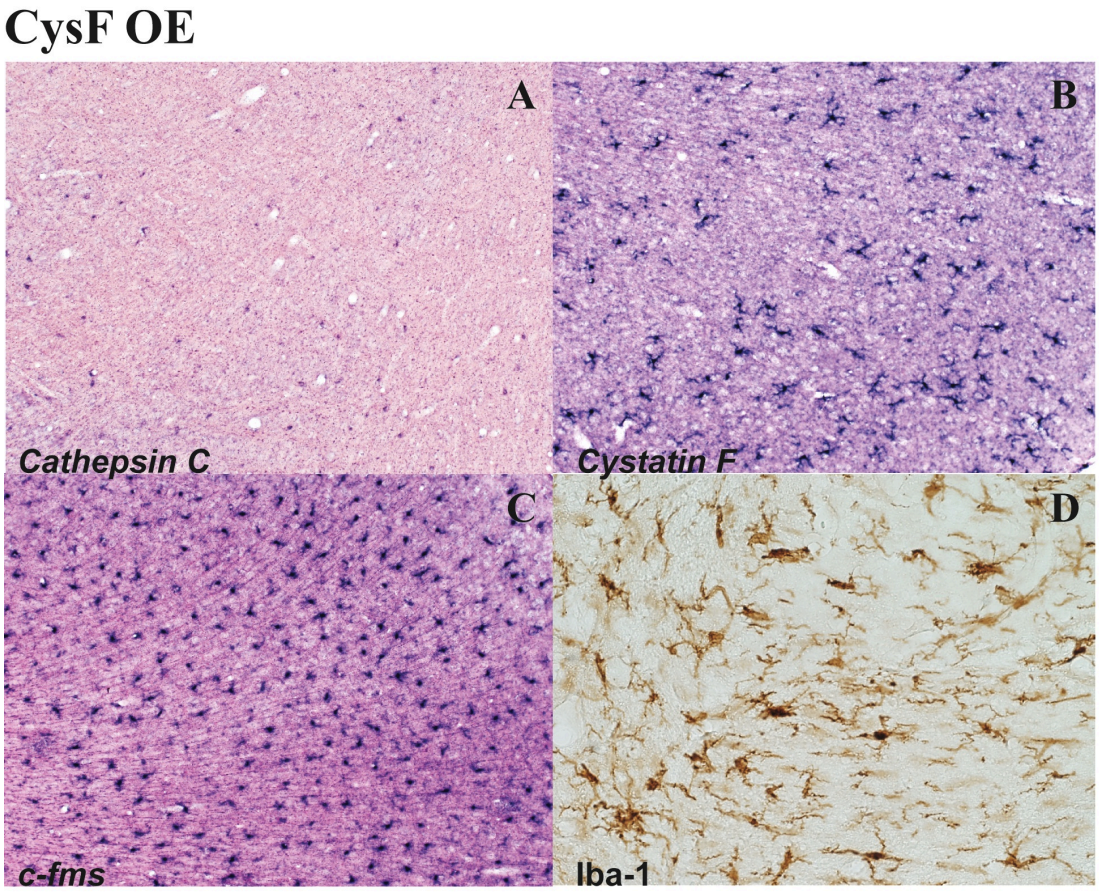


Figure 5

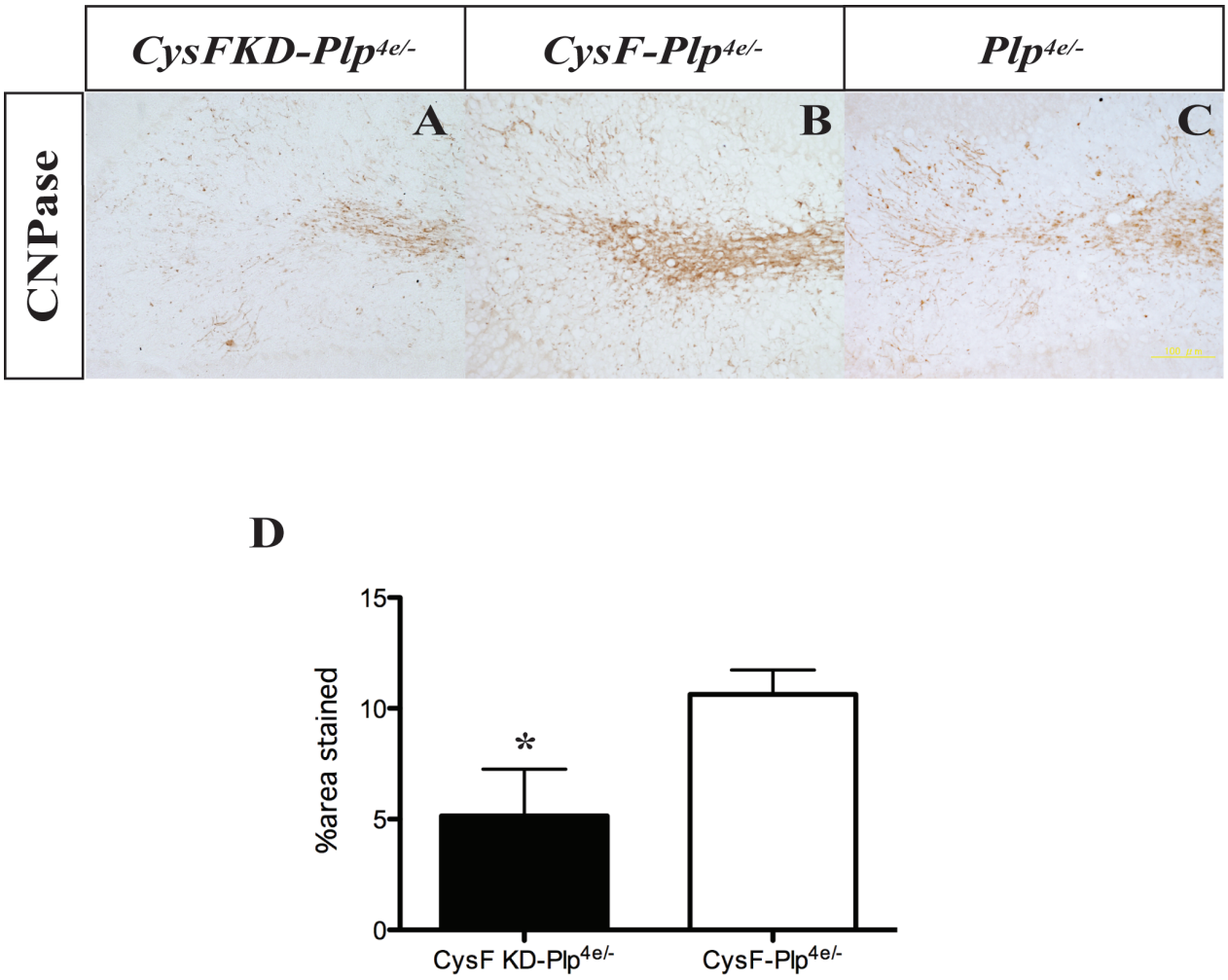
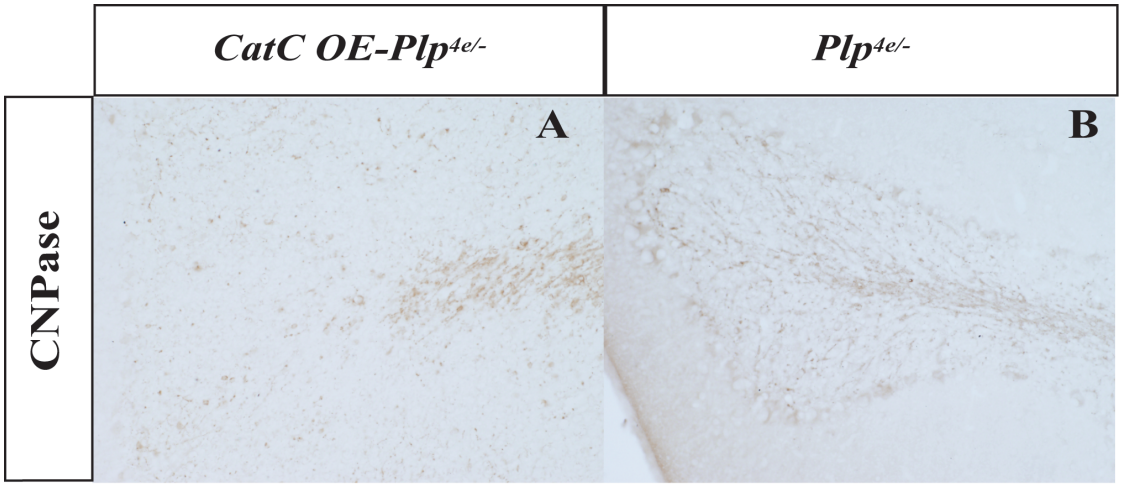

CysFKD-Plp4e/-
CysF-Plp4e/-
Plp4e/-
CNPase
A
B
C
D
%area stained
15
10
5
0
*
CysF KD-Plp4e/-
CysF-Plp4e/-

Figure 6


CatC OE-Plp4e/-
Plp4e/-
CNPase
A
B

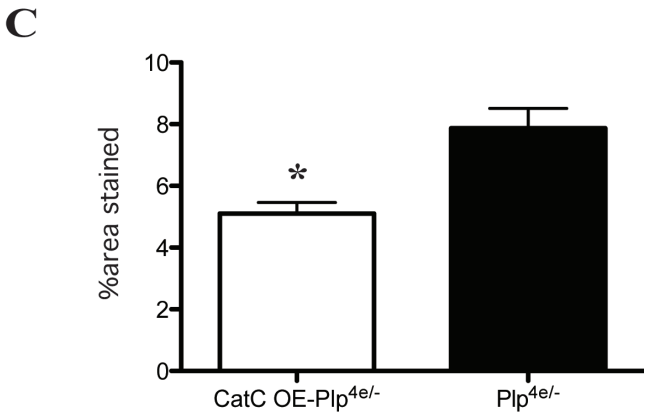

C
%area stained
10
8
6
4
2
0
*
CatC OE-Plp4e/-
Plp4e/-

Figure 7

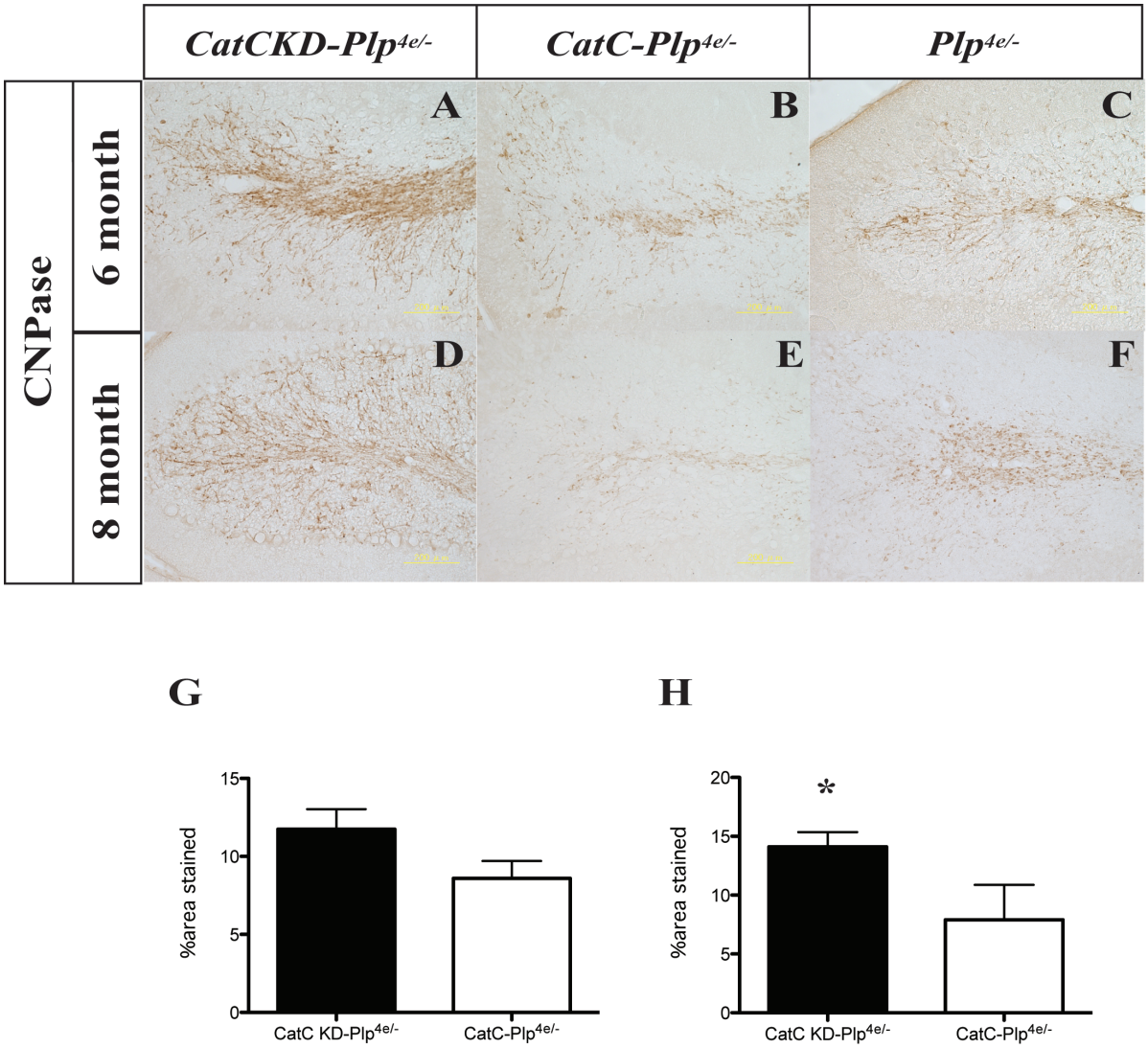

CatCKD-Plp4e/-
CatC-Plp4e/-
Plp4e/-
CNPase
6 month
8 month
A
B
C
D
E
F
G
H
%area stained
15
10
5
0
CatC KD-Plp4e/-
CatC-Plp4e/-
20
*

Figure 8

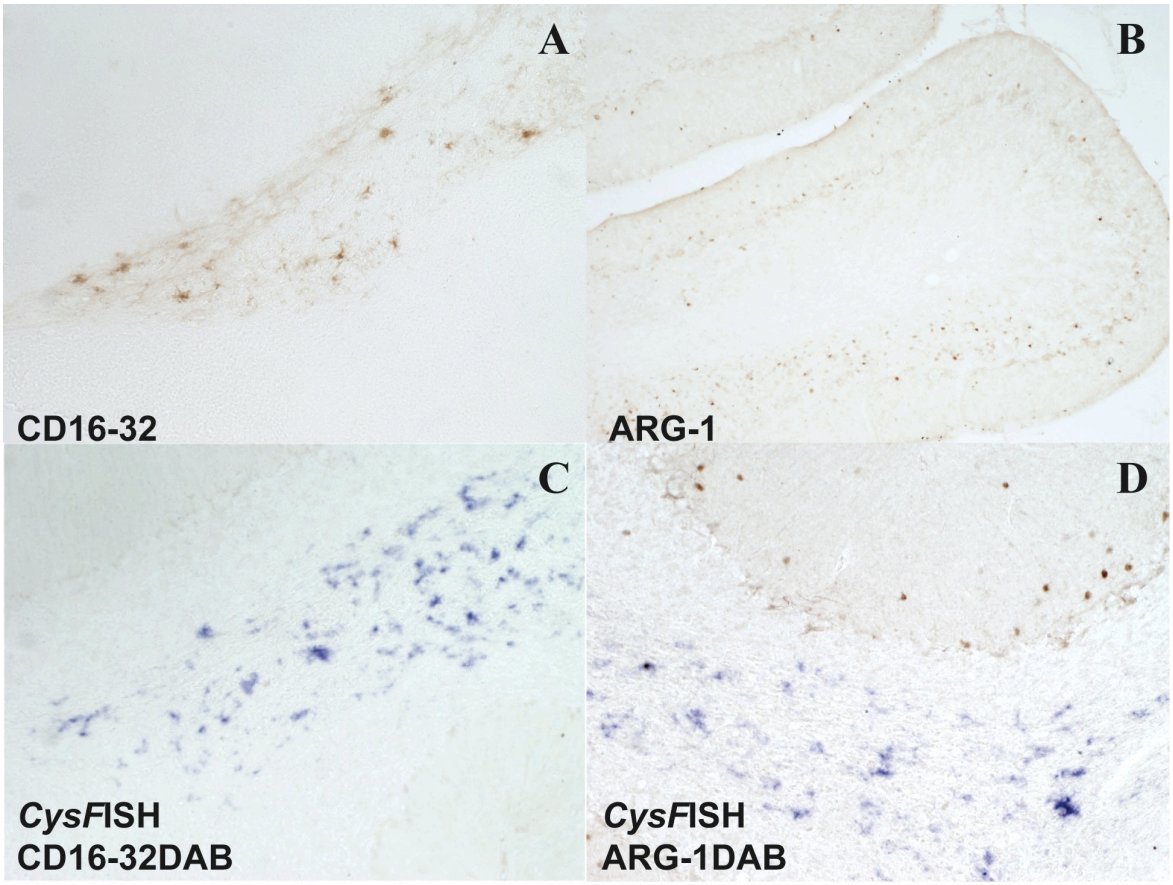


Figure 9

Part II

Expression and function of CatC-CysF system at an early phase of inflammatory demyelination

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an experimental inflammatory demyelinating disease, which is most widely used as an animal model for human demyelinating disease such as MS. This disease model exhibits many clinical and histological features of MS, and is caused by the induction of autoimmunity to antigens. Disease can be monophasic, involving an acute paralytic episode (relapsing-remitting) followed by complete recovery. In the monophasic and relapse-remitting forms, recovery from disease is associated with clearance of inflammatory infiltrates from the CNS (Baker and Jackson 2007). The symptoms of EAE in mice are variable and mimic different clinical manifestations seen in human MS. Thus EAE have same cause and progressive of disease similar to those of MS but in the final phase mouse gain full recovery, which is suitable for studing acute demyelination phase. EAE can be actively induced by the injection of whole spinal cord preparations, or proteins derived from myelin. Myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG) are the major known CNS antigens that elicit an immune response and cause paralytic disease in mice (Benveniste 1997).

The ability of CD4+ MBP-reactive T cells to induce paralytic signs in mice established the immunological basis of EAE. Demyelination and paralytic episodes are associated with infiltration of myelin-specific inflammatory Th1 CD4+ T cells into the CNS (Hickey et al., 1991). The activation of the autoreactive CD4+ T cells that are specific for initiating antigen epitope occurs in the draining lymph node. Following activation, the effector CD4+ T cells enter the circulation and extravasate into the CNS. Once in the CNS, the autoreactive CD4+ T cells initiate myelin destruction

through the activation of resident and infiltrating antigen-presenting cells (APCs). The activated infiltrating immune cells secrete cytokines and chemokines that not only recruit immune cells into the CNS, but also help to open the blood–brain barrier (BBB) (Engelhardt 2006). Besides the re-activation of the CD4+ T cells that are specific for the initiating antigen activation, myelin antigens are released, phagocytosed, processed, and presented principally within the CNS by peripherally derived myeloid dendritic cells (DCs) to naive CD4+ T cells, both of which can enter through the compromised BBB. During the acute phase of disease the destruction of myelin allows for the release of both PLP and MBP. Due to antigen availability and CD4+ T-cell precursor frequency, the activation of the secondary population of CD4+ T cells specific for PLP occurs during the primary relapse (known as intramolecular epitope spreading). During the secondary relapse, CD4+ T cells specific for MBP are activated (known as intermolecular epitope spreading) (Miller et al., 2007, Shin et al., 2012).

The polarization of macrophages and microglia underlie their differing functional properties. Their beneficial effects by modulating their polarization take advantage opportunity for the treatment of CNS demyelinating diseases. The M1, is classically-activated macrophages, which are induced by the prototypical Th-1 cytokine interferon-γ (IFN-γ) or lipopolysaccharide (LPS). M1 macrophages/ microglia mainly secrete pro-inflammatory cytokines such as TNF-α, IL-12, IL-23, IL-1β, IL-6 and chemotactic factors, as well as inducing cytotoxic mediators. Conversely, M2 macrophages/micrglia are usually induced by the Th-2 cytokines IL-4 or IL-10, and IL-13. M2 cells secreting large amounts of the anti-inflammatory cytokine IL-10, enhancing arginase-1 (Arg-1) activity and specifically expressing

mannose receptor (MR; CD206) and macrophage chitinase 3-like protein 3 (Chi3l3). They are involved in killing extracellular pathogens, debris removal, angiogenesis, tissue remodeling and wound healing (Martinez et al., 2009). The polarization of macrophages has been analyzed during EAE. In acute EAE, more M1 than M2 cells are found at the early stage. The proportion of M2 macrophages increases and more M2 cells are seen at the EAE peak and in the recovery stage, indicating that they are associated with disease remission (Ahn et al., 2012). Recent studies suggest that the microenvironment of severe relapsing EAE favors M1 polarization and increasing the proportion of M2 cells would be supportive in the treatment of this disease. Therefore, balancing M1/M2 in an appropriate ratio, and modulating the levels of cytokines and other factors in the microenvironment of the demyelinated CNS to reduce excessive or prolonged M1 polarization and enhance M2 polarization may be useful for innervention of new therapeutic goal (Li and Cheng 2013).

From previous study in our group, we found CatC and CysF are expressed in the spinal cord of MOG induced EAE mouse. CatC is expressed at chronic remyelinating area, where myelin is totally lost. Conversely CysF is expressed at surrounding area of chronic demyelinated region with still remaining intact myelin. We also found CysF KD mouse showed worsen EAE symptoms (Shimizu 2012). These results are similar with the observation in the chronic demyelination model, $plp^{4e/-}$ mouse; even though the cause of disease is different. The $plp^{4e/-}$ mouse is a very good model for studying the chronic demyelination phase of MS because it has similar pathology; 1) The pathological phenotype appear in adulthood, 2) Remyelination process is terminated and appearance of naked axon in the final phase and 3) The premyelinating oligodendrocytes are present in the demyelinated lesion but fail to

form myelin structure on axons. However, *plp*$^{4e/-}$ mouse is not appropriate for studying the acute demyelination phase because the initiation and mechanism of the disease is different. EAE model is more commonly used for the research toward therapeutic application and appropriate to study acute demyelination phase. To fulfill the study in the role of CatC and CysF in demyelinating diseases, I used EAE as a model for studying acute demyelinating phase.

Materials and Methods

The most procedure is similar to the part one: the chronic demyelination model. Some different or additional procedure was added.

Myelin Oligodendrocyte Glycoprotein induced Experimental Autoimmune Encephalomyelitis (MOG-EAE)

Mice aging between 7-10 weeks and body weight between 18-22 g were used for the EAE experiments. The $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLY-RNGK; Peptide Institute Inc., Osaka, Japan, 150 μg) emulsified in complete Freund's adjuvant (1:1), consisting of incomplete Freund's adjuvant (Sigma-Aldrich, Missouri, USA) with 4 mg/ml Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI, USA). Mouse were immunized subcutaneously with MOG/CFA into pelvic region followed by injection intraperitoneally with 400 ng pertussis toxin (List Biological Labs, Inc., CA, USA) immediately (Day 0) and 48 hours later (Day 2). The severity of EAE was daily monitored and graded as the clinical score of 0-5: 0, no clinical symptom; 1, limb tail; 1.5, Limp tail and impaired righting reflex; 2, Limp tail and hind legs weakness; 2.5, Limp tail and paralyzed of one of hind leg; 3, both hind leg paralysis or one front and hind leg paralysis or all of: severe head tilting, walking only along edges of the cage, pushing against the cage wall, spinning when picked up by the tail; 3.5, both hind leg complete paralysis; 4, both hind leg complete paralysis and

one front leg paralysis; 5, Complete quadriplegia, moribund or death of the animal after proceeding clinical defect.

Immunohistochemical Analysis

For immunofluorescence, Cryosections from 4% PFA perfused spinal cord was used. After washing the fixed cells with PBS one time for 5 min, antigen retrieve was performed. After three times wash with PBS, the sections were treated with the blocking buffer containing 0.1% Triton X-100 for 1 hour at room temperature. Samples were then incubated with primary antibodies, followed by appropriate Alexa-conjugated secondary antibodies. Secondary antibodies were labeled with biotin (Vectastain) or Alexa 568-conjugated anti-goat IgG (Invitrogen), Alexa 568-conjugated anti-rat IgG (Invitrogen).

Result

CatC and CysF expression in relation with the clinical feature in inflammatory demyelinating diseases.

In the part one of my study, I described about the possible interactive roles of CatC and CysF in myelin regeneration process in chronic demyelinating diseases model. To further study towards clinical application, EAE was chose as an experimental model for the early phase of inflammatory demyelination. Clinical symptom of EAE is due to the autoimmune response and attack against myelin proteins, which is similar to that of MS. From the studies on the chronic demyelination model, *Plp*$^{4e/-}$ mouse, CatC and CysF was shown to have unique expression pattern: CysF expression dominated CatC expression in the early phase, and then, CatC expression dominated CysF expression in the late phase of demyelination. Thus, I examined whether CatC and CysF also have unique expression pattern or not. I induced MOG-EAE in the wild type mouse and observed at several time points (1, 2, 3 and 4 weeks after immunization). The clinical symptoms of MOG-EAE mouse was observed and its clinical score was recorded everyday after immunization until the day of sacrifice. The cross cryosection at the thoracic level of spinal cords was used to observe CatC, CysF and c-fms mRNA by in situ hybridization. The demyelinated area was demonstrated by immunostaining with anti-PLP antibody. After 7 days of immunization (Figure 10A), mice have not shown any clinical symptom (clinical score = 0) and no demyelinating area was observed. Surprisingly, CatC mRNA positive cells were found infiltrating at the anterior median fissure of spinal cord and also found that microglia activation at surrounding area, which is determined by c-fms mRNA positive signal. Conversely, CysF has not been expressed in this stage. Two weeks

after immunization (Figure 10B), mice start to exhibit clinical symptoms that were evident by tail drop and gradually progressive weakness to lower limb paresis and balance disturbance (clinical score = 2/3). The myelin start to show demyelination, which is presented as loss of PLP immunostaining (red dot surrounding area). CatC and c-fms mRNA increased expression and accumulated at demyelinating area. Few of CysF mRNA signal was observed at the edge of demyelinating area, where myelin still remains. After 3 to 4 weeks of immunization (Figure 10C-D), several demyelinating region were clearly observed and mice presented severe clinical symptoms by showing weakness in both limb and/or moribund (clinical score = 3/4). CatC and c-fms mRNA were largely accumulated at demyelinating and surrounding area. CysF expression still remained weak and was observed only nearby demyelinating area. The clinical symptoms gradually recovered after 6 weeks of immunization (Figure 10E). The demyelinating area was also smaller. CatC and c-fms expression remained only in a small region that still showed demyelination. CysF also showed a similar expression pattern to CatC with expression found only at the edge of demyelinating area where myelin still remained.

CatC was expressed in the infiltrating cells at a very early phase without any clinical defects and kept its expression to the late demyelinating phase. CysF expression was totally different from that of CatC. CysF was expressed much later than CatC and its expression was found only at the edge of demyelinating regions, which are refer to active myelin regeneration regions.

CatC is first expressed by infiltrating cells in an early phase of inflammatory demyelination.

At early phase of inflammatory demyelination (MOG-EAE mouse, 1 week after immunization), CatC expression was found in infiltrating cells at the anterior median fissure and in the cells of the anterior gray horn of the spinal cord (Figure 11A). To identify the CatC expressing cells, cryosections at T-level of MOG-EAE 1 week after immunization were analyzed. From the previous study, CatC was found to be expressed in microglia, hence the CatC expressing cells at the anterior gray horn might be microglia. I performed double labeling of CatC ISH and Iba-1 IHC. The result showed CatC expressing cells in the anterior gray horn of the spinal cord is microglia (Figure 11B). However, the infiltrating cells were found at anterior median fissure, which is not a part of CNS and based on its anatomical structure, the CatC expressing might belong to one of the hematopoietic cell lineage, which crossed BBB after triggering EAE. The hematopoietic cell type was identified with cell morphology after H&E stain. I performed double labeling of CatC ISH and H&E stain. The result showed CatC expressing cells at the median anterior fissure of spinal cord had polymorphonuclear (Figure 11C-D), which identify them as neutrophils. CatC expressing cells infiltrated at an early phase of inflammatory demyelination to the anterior median fissure of spinal cord. CatC was expressed by microglia in the anterior gray horn and also in the neutrophil at the anterior median fissure of spinal cord. CatC should play some role in an early phase of EAE induced demyelination by autoimmune attack.

CatC and CysF expression in myelin degenerated area.

The phagocytosis of myelin debris by microglia and macrophages in acute MS lesions is well documented, which is the major mechanism used to remove pathogens and cell debris by immune system (Liu et al., 2006). In an early phase of EAE, we found only CatC expressing cells infiltrating the CNS before triggering myelin damage by the immune attack but did not find CysF involvement in this phase. I further observed CatC and CysF expression in the later phase of EAE, when myelin degeneration is induced by immune attack and myelin debris is phagocytosed. The myelin phagocytosing microglia were detected by Galectin 3 (Gal3) staining (Sano, et al. 2003). Sagittal cryosection of thoracic level of spinal cords 2 weeks of immunization was used to detect CatC, CysF and Gal3 mRNA by in situ hybridization. The demyelinated area was demonstrated by immunostaining with anti-PLP antibody. The demyelinated area at the anterior funiculus region (Figure 12B) also expressed CatC and Gal3 (Figure 12A, C). A low level of CysF was found at chronic demyelinating area (Figure 12D). This result suggested that CatC plays a role during myelin phagocytosis by microglia/macrophage. CysF may play a minor role in the late inflammatory demyelinating phase.

M1 and M2 microglia polarization in inflammatory demyelination, MOG-EAE

The difference in macrophage/microglia polarization underlies the different functional properties, that result in cells with either pro-inflammatory (M1) or anti-inflammatory (M2) properties. The most macrophages/microglia are M1 cells, with only a transient and small number showing M2 polarization. Several studies suggest

that the microenvironment of the injured spinal cord favors M1 polarization with only a transient appearance of M2 macrophages early after injury (Samuel and Antje 2011). I observed M1 and M2 macrophage/microglia expression in the MOG-EAE mouse. Sections at thoracic level of the spinal cord 6 weeks after immunization were used to observe M1 and M2 cells. M1 cells were found to accumulate at the demyelination area (Figure 13A-C) but only few M2 cells were found (Figure 13D-F).

CatC plays a role in an early phase of inflammatory demyelinating model.

From a previous study in our group, we found worsening of the symptom of MOG-EAE mouse when CysF expression was eliminated, indicating that CysF plays a supportive role in MOG-EAE. In Part one of this study, the balance of CatC and CysF expression is important for maintaining myelin regeneration in both early phase and late phase of $Plp^{4e/-}$ mouse. Thus, CatC might also play an important role in demyelination or myelin regeneration in an early phase of inflammatory demyelinating model, MOG-EAE. In this phase, CatC seems to play more important role than CysF. To clarify the role of CatC in this phase, I used CatCKD and wild type mouse to induce MOG-EAE. The clinical score of CatCKD mouse after induction of MOG-EAE was significantly reduced when compared with wide type at 10 days after immunization (Figure 14), suggesting that CatC expression is toxic to the mouse during MOG-EAE. To confirm this conclusion, I used CatCOE and wild type mouse to induce MOG-EAE. CatC overexpression mouse significantly increased clinical score when compare with wide type at 7-12 days after immunization (Figure 15).

CatCKD, CatCOE and wild type mouse at 2 weeks after immunization was further examined for histological analysis. The sagittal cryosections at the thoracic level of spinal cords was used to detect CatC, CysF and c-fms mRNA by in situ hybridization. The demyelinated area was demonstrated by immunostaining with anti-PLP antibody (Figure 16A-C4). CatC expression seems to be similar between CatCOE mouse and the wild type mouse. However, when I observed CatC expression much earlier, CatC expressing cells was found to infiltrate the CNS (Figure 16A-C1). MOG-EAE-induced CatCOE mouse significantly increased demyelinating area when compare with the control group. Conversely, MOG-EAE induced CatCKD mouse significantly decreased demyelinating area when compared with the control group. Microglia activation identified by strong *c-fms* mRNA positive signal was found to accumulate in demyelinating area (Figure 16A-C3). CysF mRNA was found at low level in the area surrounding demyelinating region (Figure 16A-C2). The percentage of demyelinating area of CatCOE mouse significantly increased to 4.23 $\pm$ 0.24 % (n = 4) when compare with the control group; 2.88 $\pm$ 0.49 % (n = 4) but no significant different was found in CatCKD mouse (1.18 $\pm$ 0.38 %, n = 3) (Figure 16D). These results correlated with chronic demyelinating model, $Plp^{4e/-}$, which suggest that CatC also have a role in an early phase of inflammatory demyelinating disease.

Discussion

In this study we demonstrated that CatC-CysF system plays an important role in inflammatory demyelination by using MOG-EAE. First, I found CatC expressing cells, which are neutrophil and microglia, infiltrated CNS in an early phase of EAE, before clinical symptoms was observed. Previous study found the number of neutrophils was significantly increased during onset of the disease. Neutrophils are one of the major sources of inflammatory cells to initiate EAE, which suggest that neutrophils may contribute to demyelination in the acute phase of EAE and play a greater role in the pathogenesis of EAE (Wu et al., 2010). Neutrophils were found to promote the blood-brain barrier breach and together with T cells lead to further inflammatory cell influx and myelin damage (Sayed et al., 2010). Neutrophil-derived serine proteases; Cathepsin G (CatG), neutrophil elastase (NE), and proteinase 3 (PR3) are expressed specifically in mature neutrophils, which required CatC for activating their function and play an important role in inflammation (Adkison et al., 2002). CatC was also predominantly expressed in the activated microglial cells throughout the entire brain after 24 hours, which is an early phase, of lipopolysaccharide (LPS)-induced neuroinflammation (Fan et al., 2012). These observations suggest that CatC could participate in the development of neuroinflammation and progression to demyelination in the CNS.

I also found CatC-CysF maintain expression along the progression of demyelination in MOG-EAE. CatC was expressed at the chronic demyelination area, where myelin regeneration process is terminated, but CysF was expressed at the edge of demyelination area, where myelin still remains intact. CatC is a one of the two genes that was found to be consistently up regulated at first few weeks of MOG-EAE

among 12,000 genes (Mix et al., 2004). T lymphocyte as the mediator of EAE enters into the parenchyma of the CNS and is a critical early feature in the pathogenesis of immune-mediated illnesses (Zamvil and Steinman 1990). CatC has been implicated in the processing of granzymes, which are neutral serine proteases exclusively expressed in the granules of activated cytotoxic lymphocytes (Kummer et al., 1996, Pham and Ley 1999). Especially, granzyme is a natural serine protease after activated by CatC, and will activate downstream cascade several of pathways, such as cell death pathway or induction of pro-inflammatory cytokine TNF-α and IL-1β generation (Shresta et al., 1999, Irmler et al., 1995). TNFα induces pro-inflammatory M1 macrophages (Sindrilaru et al., 2011). I found M1 cells are present in the chronic demyelination area, which correlates with CatC expression. CatC might induce M1 polarization and cause inhibition of regeneration process (Samuel and Antje 2011). Modulating the levels of cytokines and other factors in the microenvironment of the damaged spinal cord to reduce excessive or prolonged M1 polarization may therefore be a desirable therapeutic goal.

In chronic demyelination model, $Plp^{4e/-}$ mouse, CysF was found to show similar expression pattern as in EAE. *CysF* mRNA positive cells was found to be decreased at 8 months of age, when remyelination is severely affected. CysF indicates the occurrence of ongoing remyelination (Ma et al., 2011). CysF as endogenous inhibitor of CatC attenuate the activation of a wide range of downstream serine proteases involved in inflammation and immunity (Hamilton et al., 2008). CatC and CysF seem to be strongly related to the inflammatory demyelination. This conclusion was confirmed in MOG-EAE-induced CatC knockdown and CatC overexpression mice in this study. The absence of CatC result in easing the severity of EAE,

conversely the increment of CatC in microglia enhanced severity in MOG-EAE. These strongly suggest CatC-CysF interaction have an important role in both pathogenesis of inflammatory demyelination in EAE.

EAE model is most commonly used as a model in research for intervention therapeutic for MS or other demyelination diseases. In this study, I examined the possible role of CatC and CysF in inflammatory demyelinating model. The discovery of CatC-CysF system strongly related to inflammatory demyelination might be the key molecules for further development of the new treatment in the future.

Figure legends

Figure 10. Several mRNA expressed at various time points of MOG-EAE mouse

Several mRNA expression were examined on wild type mouse induced MOG-EAE at several time point after MOG peptide immunization at 1, 2, 3, 4 and 6 weeks (A-E). The spinal cord at T-level section was performed ISH by using *CatC*, *CysF*, *c-fms* (for microglia activation) probe for observed *CatC*, *CysF*, *c-fms* mRNA and IHC by using rat monoclonal anti-PLP antibody for myelin staining (A-E). The clinical symptoms were observed everyday after immunization and record as clinical score. [0 = Normal, 1 = limp tail, 2 = hind legs weakness, 3 = both hind leg paralysis, 4 = both hind leg complete paralysis and one front leg paralysis, 5 = Moribund or death]

Figure 11. *CatC* mRNA expressing cells infiltrated at an early phase of MOG-EAE

An early phase after immunization wide type mouse with MOG-EAE (1 week after immunization) was observed. The spinal cord at T-level section was performed ISH by using *CatC* probe for observed mRNA (A). The differentiate blood cell types was identified by hematoxylin and eosin staining (C). The picture showed high magnification of *CatC* mRNA infiltrated cells identification by double labeling *CatC* mRNA with IHC by using rabbit anti-Iba1 polyclonal antibody for microglia staining

from anterior gray horn of spinal cord (B) and Hematoxylin and eosin staining for blood cell differentiation from anterior median fissure of spinal cord (D).

Figure 12. CatC and CysF expresses in phagocytosis myelin degenerated area.

The wild type mouse was induced MOG-EAE and sacrificed at 2 weeks after immunization. The expression of *CatC*, *CysF*, *c-fms* mRNA were examined. The spinal cord at T-level section was performed ISH by using *CatC*, *CysF*, *c-fms* probe (C, D) and IHC by using rat monoclonal anti-PLP antibody for myelin staining (B). The myelin phagocytosis by immune response detected by Galectin 3 (Gal3) IHC (A).

Figure 13. Identified M1 and M2 Microglia/Macrophage polarity in MOG induced EAE

The wild type mouse was induced MOG-EAE and sacrificed at 6 weeks after immunization. ARG-1 positive cells (A) and CD16/32 Positive cells (B) was labeled. The cross spinal cord cryosection at T-level was performed immunohistochemistry by using rat monoclonal anti-CD16/32 for M1 cells and goat polyclonal anti-Arginase1 (ARG-1) antibody for M2 cells staining that defined by red color followed by nuclear staining with Hoechst (B, E) and Merge picture was showed (C, F).

Figure 14. *CatC* knockdown mouse showed less severity in MOG-EAE

The clinical score of $CatC^{STOP/STOP}$ (CatCKD) and control mouse, which was induced MOG-EAE and sacrificed at 2 weeks after immunization. The clinical symptoms were observed everyday after immunization and record as clinical score. [0 = Normal, 1 = limp tail, 2 = hind legs weakness, 3 = both hind leg paralysis, 4 = both hind leg complete paralysis and one front leg paralysis, 5 = Moribund or death]. Values represented as mean $\pm$ SEM. The unpaired two-tailed Student's *t*-test was performed for statistical analysis. (*$p < 0.05$ compared with control group)

Figure 15. *CatC* overexpression mouse showed higher severity in MOG-EAE

The clinical score of $CatC^{STOP/STOP}Iba^{tTA/-}$ (CatCOE) and control mouse, which was induced MOG-EAE and sacrificed at 2 weeks after immunization. The clinical symptoms were observed everyday after immunization and record as clinical score. [0 = Normal, 1 = limp tail, 2 = hind legs weakness, 3 = both hind leg paralysis, 4 = both hind leg complete paralysis and one front leg paralysis, 5 = Moribund or death]. Values represented as mean $\pm$ SEM. The unpaired two-tailed Student's *t*-test was performed for statistical analysis. (*$p < 0.05$ compared with control group)

Figure 16. CatC gene manipulation affect MOG-induced EAE pathology

CatCKD (A), CatCOE (B) and wild type mouse (C), which was induced MOG-EAE and sacrificed at 2 weeks after immunization. *CatC*, *CysF*, *c-fms* mRNA were examined. The spinal cord at T-level section was performed ISH by using *CatC*, *CysF*, *c-fms* (for microglia activation) probe for observed *CatC*, *CysF*, *c-fms* mRNA and IHC by using rat monoclonal anti-PLP antibody for myelin staining. The percentage of demyelination area was quantified by measuring the density using densitometry analysis with Image J program and their differences are represented in the graph (D). Values represented as mean $\pm$ SEM. The unpaired two-tailed Student's *t*-test was performed for statistical analysis. (*$p < 0.05$ compared with control group)

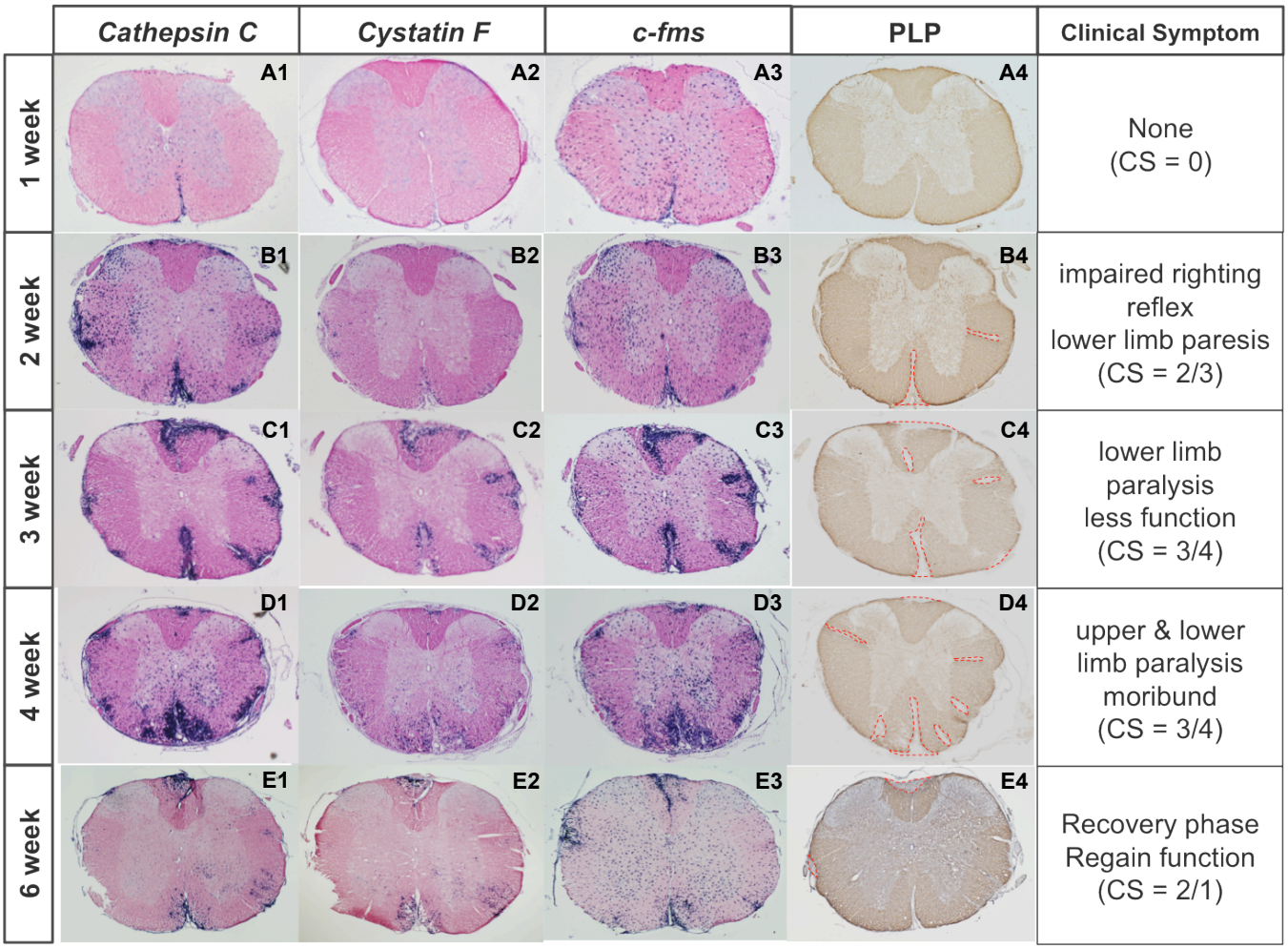

Cathepsin C
Cystatin F
c-fms
PLP
Clinical Symptom
1 week
2 week
3 week
4 week
6 week
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
None
(CS = 0)
impaired righting reflex
lower limb paresis
(CS = 2/3)
lower limb paralysis
less function
(CS = 3/4)
upper & lower limb paralysis
moribund
(CS = 3/4)
Recovery phase
Regain function
(CS = 2/1)

Figure 10

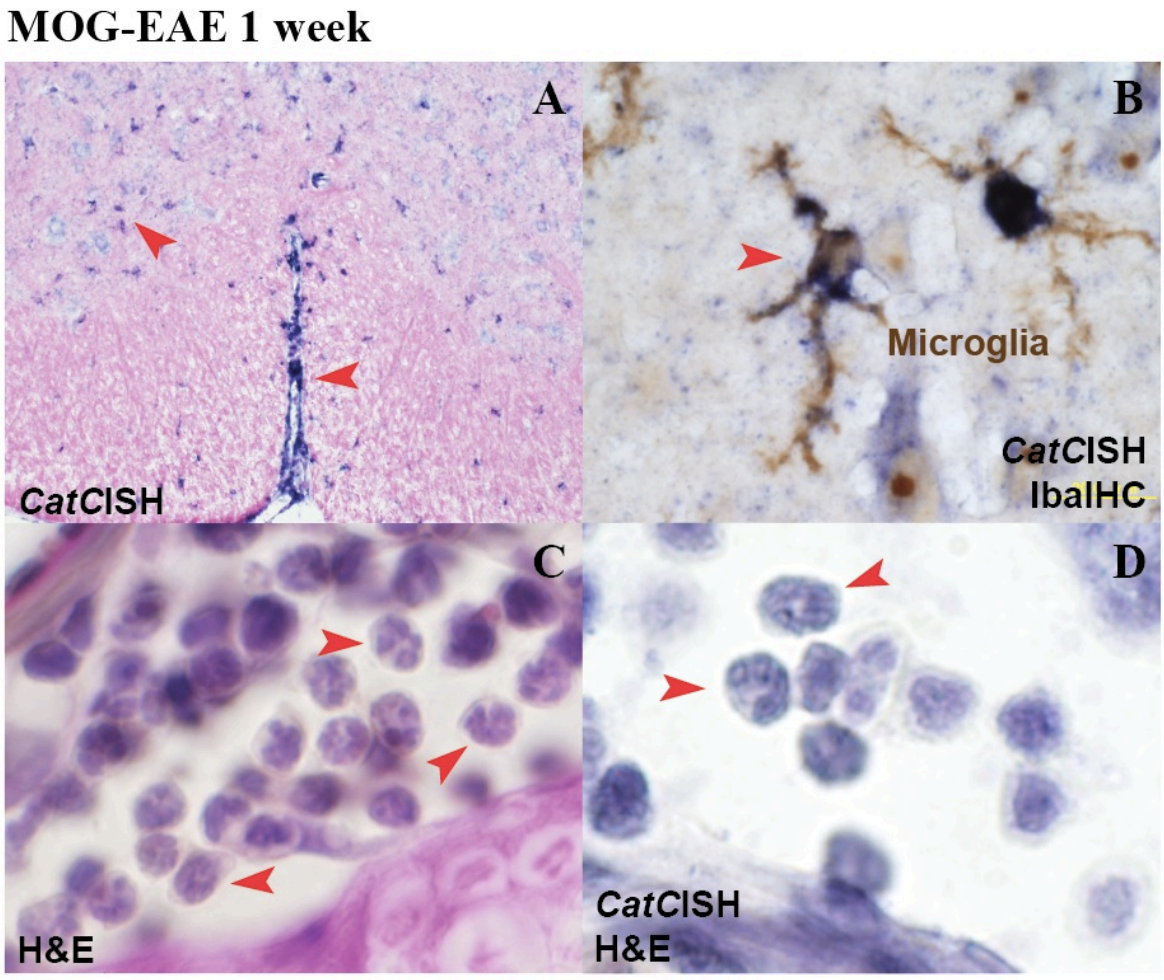

MOG-EAE 1 week
A
CatClSH
B
Microglia
CatClSH
IbaIHC
C
H&E
D
CatClSH
H&E

Figure 11

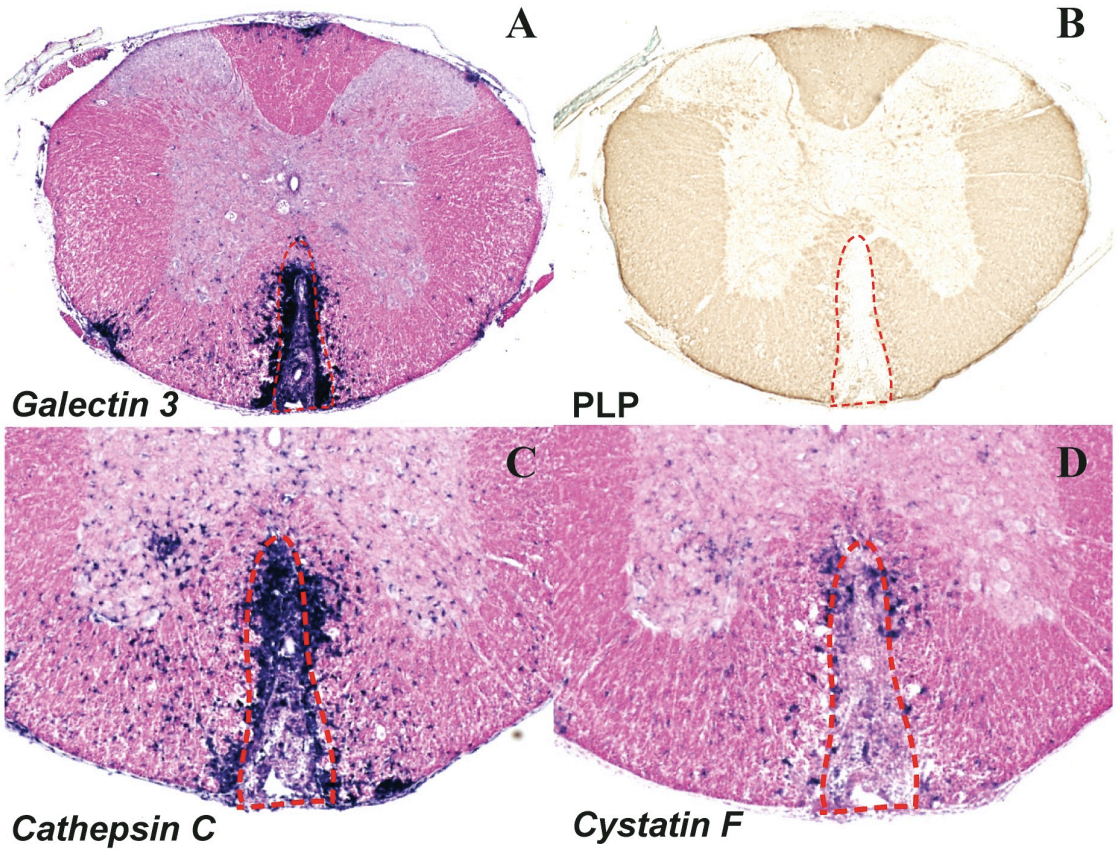


Figure 12

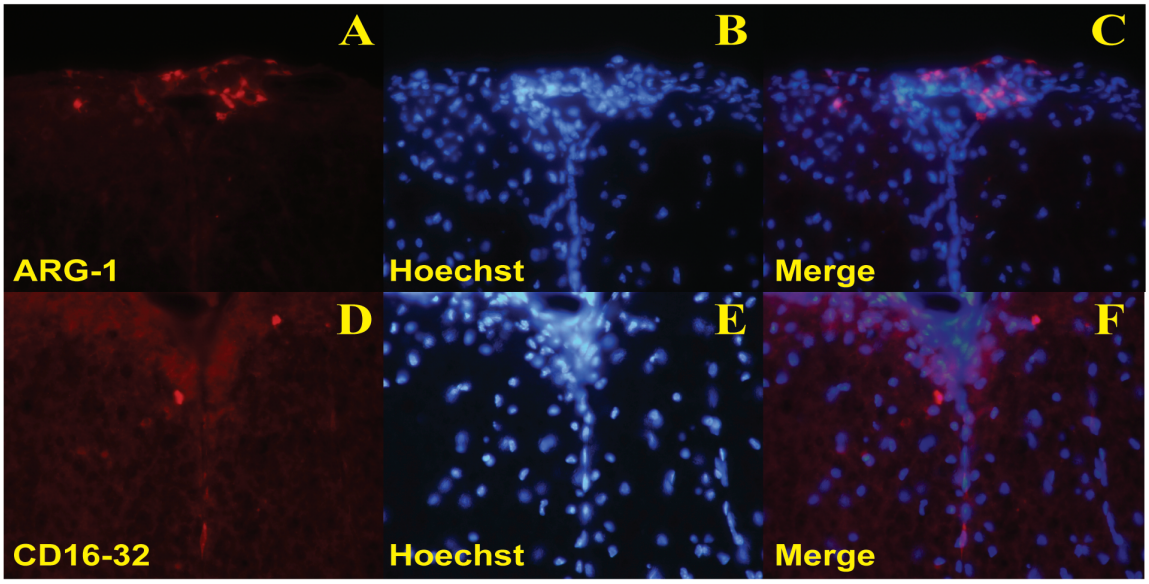


Figure 13

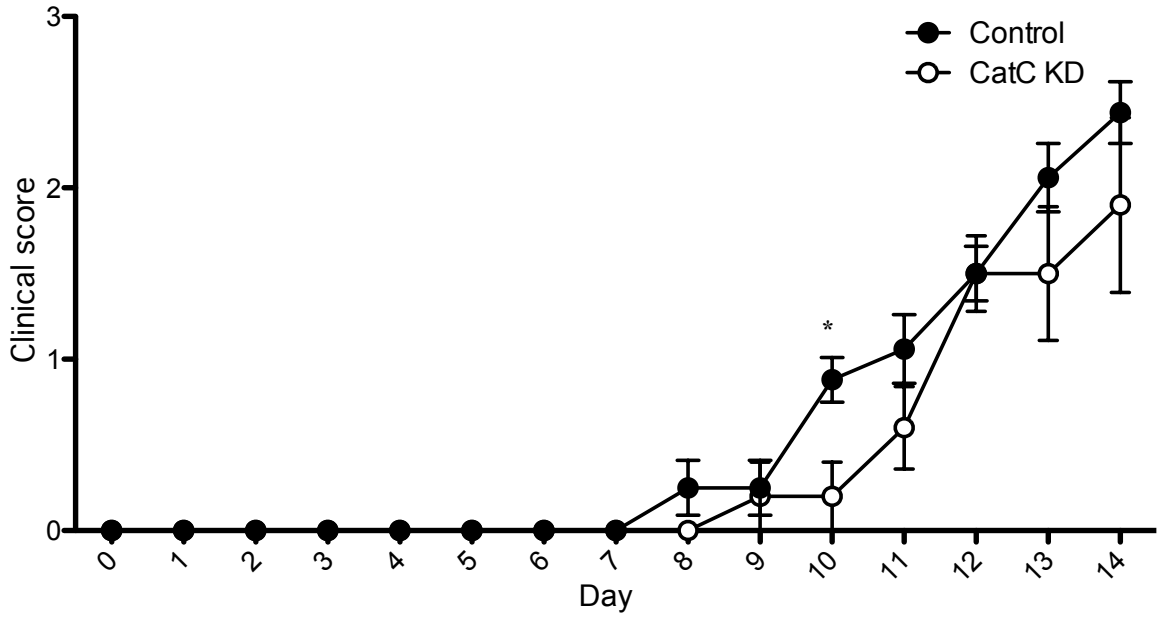


Figure 14

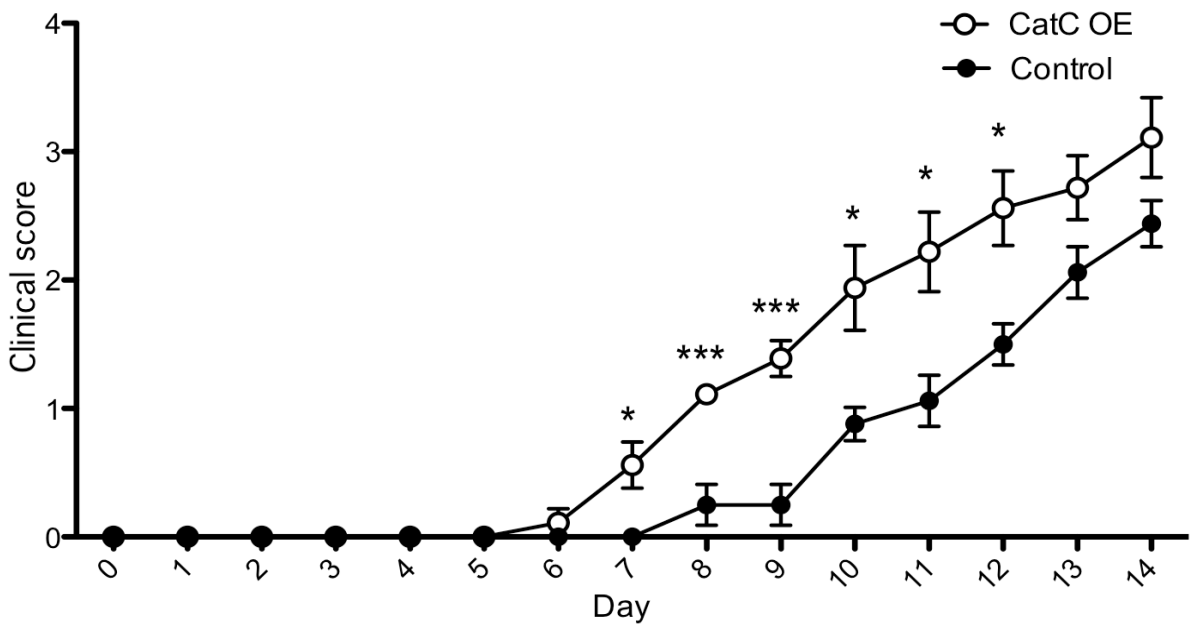
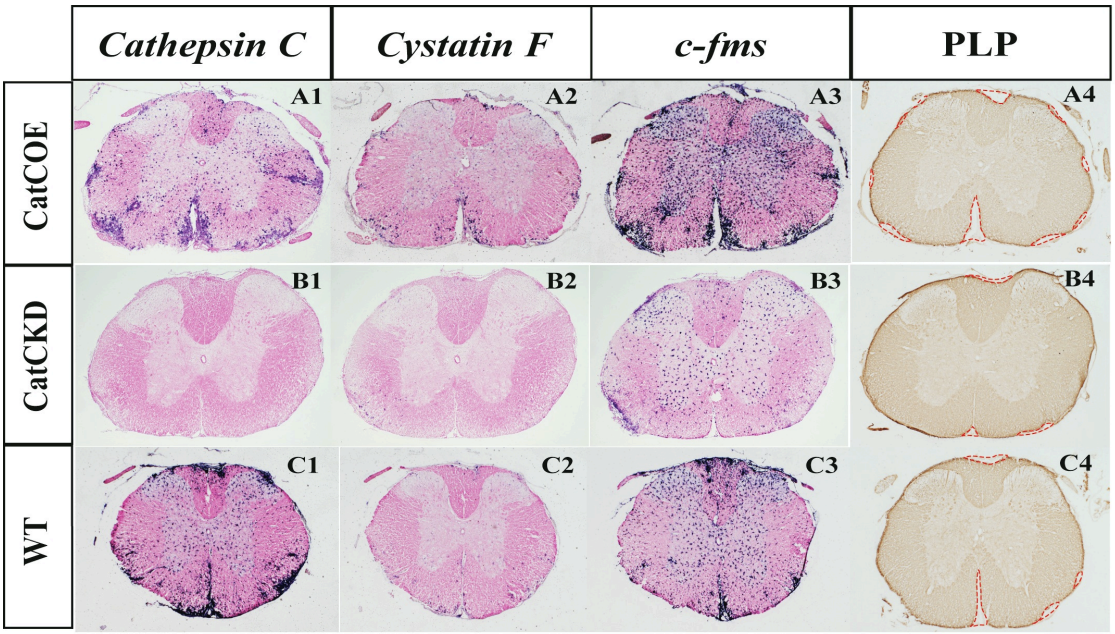

CatC OE
Control
Clinical score
Day
0
1
2
3
4
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
*
***
***
*
*
*

Figure 15


Cathepsin C
Cystatin F
c-fms
PLP
CatCOE
CatCKD
WT
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4

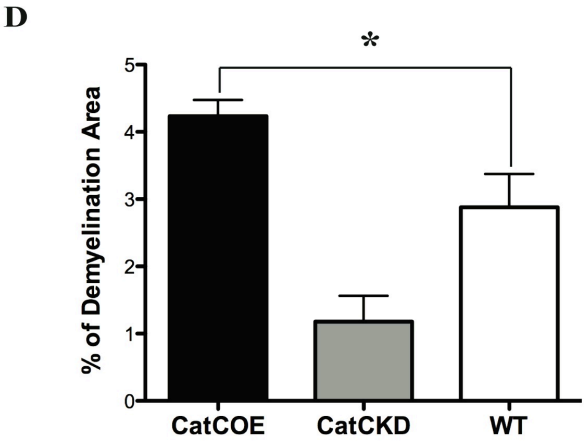

D
*
% of Demyelination Area
0
1
2
3
4
5
CatCOE
CatCKD
WT

Figure 16

Bibliography

Adams, R. D., & Kubik, C. S. (1952). The morbid anatomy of the demyelinative diseases . *The American Journal of Medicine* , 12, 510-546.

Adkison, A. M., Raptis, S. Z., Kelley, D. G., & Pham, C. T. (2002). Dipeptidyl peptidase I activates neutrophil-derived serine proteases and regulates the development of acute experimental arthritis. *The Journal of Clinical Investigation* , 109, 363-371.

Ahn, M., Yang, W., Kim, H., Jin, J., Moon, C., & Shin, T. (2012). Immunohistochemical study of arginase-1 in the spinal cords of Lewis rats with experimental autoimmune encephalomyelitis. *Brain research* , 1453, 77–86.

Baker, D., & Jackson, S. J. (2007). Models of Multiple Sclerosis. *Advances in clinical neuroscience and rehabilitation* , 6, 10-12.

Barnett , M. H., & Prineas, J. W. (2004). Relapsing and remitting multiple sclerosis: Pathology of the newly forming lesion. *Annals of Neurology* , 55, 458-468.

Benveniste, E. N. (1997). Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. *Journal of Molecular Medicine* , 75, 165-173.

Boivin, W. A., Cooper, D. M., Hiebert, P. R., & Granville, D. J. (2009). Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma. *Laboratory Investigation* , 89, 1195-1220.

Chang, A., Tourtellotte, W. W., Rudick, R., & Trapp, B. D. (2002). Premyelinating Oligodendrocytes in Chronic Lesions of Multiple Sclerosis. *The New England Journal of Medicine* , 346, 165-173.

Charles, P., Reynolds, R., Seilhean, D., Rougon, G., Aigrot, M. S., Niezgoda, A., et al. (2002). Re-expression of PSA-NCAM by demyelinated axons: an inhibitor of remyelination in multiple sclerosis? *Brain* , 125, 1972-1979.

Choy, J. C. (2010). Granzymes and perforin in solid organ transplant rejection. *Cell Death and differentiation* , 17, 567-576.

Comi, G., Filippi, M., Barkhof, F., Dureli, L., Edan, G., Fernandez, O., et al. (2001). Effect of early interferon treatment on conversion to definite multiple sclerosis: a randomised study. *The Lancet* , 357, 1576-1582.

Ebers, G. C., & D., D. A. (1998). Genetics of Multiple Sclerosis. *Seminar in Neurology* , 18, 295-299.

Engelhardt, B. (2006). Molecular mechanisms involved in T cell migration across the blood–brain barrier. *Journal of Neural transmission* , 113, 477-485.

Eyles, J. L., Roberts, A. W., Metcalf, D., & Wicks, I. P. (2006). Granulocyte colony-stimulating factor and neutrophils-forgotten mediators of inflammatory disease. *Nature Clinical Practice Rheumatology* , 2, 500-510.

Fan, K., Wu, X., Fan, B., Li, N., Lin, Y., Yao, Y., et al. (2012). Up-regulation of microglial cathepsin C expression and activity in lipopolysaccharide-induced neuroinflammation. *Journal of Neuroinflammation* , 9, 1-13.

Fancy, S. P., Kotter , M. R., Harrington , E. P., Huang, J. K., Zhao, C., Rowitch , D. H., et al. (2010). Overcoming remyelination failure in multiple sclerosis and other myelin disorders. *Experimental Neurology* , 225, 18-23.

Fen, Z., Beresford, P. J., Oh, D. Y., Zhang, D., & Lieberman, J. (2003). Tumor Suppressor NM23-H1 Is a Granzyme A-Activated DNase during CTL-Mediated Apoptosis, and the Nucleosome Assembly Protein SET Is Its Inhibitor. *Cell* , 112, 659-672.

Gao, Z., & Tsirka, S. E. (2011). Animal Models of MS Reveal Multiple Roles of Microglia in Disease Pathogenesis. *Neurology Research International* , 2011, 1-9.

Graham, D. I., & Lantos, P. L. (2002). Greenfield's neuropathology, 7th edition. London: Arnold.

Halfon, S., Ford, J., Foster, J., Dowling, L., Lucian, L., Sterling, M., et al. (1998). Leukocystatin, A New Class II Cystatin Expressed Selectively by Hematopoietic Cells. *The Journal of Biological Chemistry* , 273, 16400-16408.

Hamilton, G., Colbert, J. D., Schuettelkopf, A. W., & Watts, C. (2008). Cystatin F is a cathepsin C-directed protease inhibitor regulated by proteolysis. *The EMBO Journal* , 27, 499-508.

Hashimoto, S.I., Suzuki, T., Nagai, S., Yamashita, T., Toyoda, N., & Matsushima, K. (2000). Identification of genes specifically expressed in human activated and mature dendritic cells through serial analysis of gene expression. *Blood* , 96, 2206-2214.

Hickey, W. F., Hsu, B. L., & Kimura, H. (1991). T-lymphocyte entry into the central nervous system. *Journal of Neuroscience Research* , 28, 254-260.

Irmler, M., Hertig, S., MacDonald, R. H., Sadoul, R., Becherer, J., Proudfoot, A., et al. (1995). Granzyme A Is an Interleukin 1 beta-converting Enzyme. *The Journal of Experimental Medicine* , 181, 1917-1922.

Kagawa, T., Ikenaka, K., Inoue, Y., Kuriyama, S., Tsujii, T., Nakao, J., et al. (1994). Glial cell degeneration and hypomyelination caused by overexpression of myelin proteolipid protein gene. *Neuron* , 13, 427-442.

Kam, C.M., Gotz, M. G., Koot, G., McGuire, M., Thiele, D., Hudig, D., et al. (2004). Design and evaluation of inhibitors for dipeptidyl peptidase I (Cathepsin C). *Archives of Biochemistry and Biophysics* , 427, 123-134.

Kummer, A. J., Kamp, A. M., Citarella, F., Horrevoets, A. J., & Hack, E. C. (1996). Expression of human recombinant Granzyme A Zymogen and its activation by the cysteine proteinase Cathepsin C. *The journal of biological chemistry* , 271, 9281–9286.

Lau, L. W., Keough, M. B., Haylock-Jacobs, S., Cua, R., Doring, A., Sloka, S., et al. (2012). Chondroitin sulfate proteoglycans in demyelinated lesions impair remyelination. *Annals of Neurology* , 72, 419-432.

Li, C., & Cheng, H. (2013). Polarization of macrophages and microglia in inflammatory demyelination. *Neuroscience Bulletin* , 29, 189–198.

Liu, Y., Hao, W., Letiembre, M., Walter, S., Kulanga, M., Neumann, H., et al. (2006). Suppression of microglial inflammatory activity by myelin phagocytosis: role of p47-PHOX-mediated generation of reactive oxygen species. *Neurobiology of Disease* , 26, 12904-12913.

Ma, J., Matsumoto, M., Tanaka, K. F., Takebayashi, H., & Ikenaka, K. (2006). An animal model for late onset chronic demyelination disease caused by failed terminal differentiation of oligodendrocytes . *Neuron Glia Biology* , 2, 81-91.

Ma, J., Tanaka, K. F., Shimizu, T., Bernard, C. C., Kakita, A., Takahashi, H., et al. (2011). Microglial Cystatin F expression is a sensitive indicator for ongoing demyelination with concurrent remyelination. *Journal of Neuroscience Research* , 89, 639-649.

Ma, J., Tanaka, K. F., Yamada, G., & Ikenaka, K. (2007). Induced expression of Cathepsins and Cystatin C in a murine model of demyelination. *Neurochemical Research* , 32, 311-320.

Martinez, F. O., Gordon, S., Locati, M., & Mantovani, A. (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: New molecules and patterns of gene expression. *The journal of immunology* , 117, 7303-7311.

Martinez, F. O., Helming, L., & Gordon, S. (2009). Alternative activation of macrophages: An immunologic functional perspective. *Annual Review of Immunology* , 27, 451-483.

McDonald, K. J., Callahan, P. X., & Ellis, S. (1972). Preparation and specificity of dipeptidyl aminopeptidase I. *Methods in Enzymology* , 25, 272-281.

Miller, S. D., Turley, D. M., & Podojil, J. R. (2007). Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. *Nature review Immunology* , 7, 665-677.

Mix, E., Ibrahim, S., Pahnke, J., Koczan, D., Sina, C., Bottcher, T., et al. (2004). Gene-expression profiling of the early stages of MOG-induced EAE proves EAE-resistance as an active process. *Journal of neuroimmunology* , 151, 158-170.

Nathanson, C.-M., Wasselius, J., Wallin, H., & Abrahamson, M. (2002). Regulated expression and intracellular localization of cystatin F in human U937 cells. *European Journal of Biochemistry* , 269, 5502–5511.

Niehaus, A., Shi, J., Grzenkowski, M., Diers-Fenger, M., Archelos, J., Hartung, H.P., et al. (2000). Patients with active relapsing-remitting multiple sclerosis synthesize antibodies recognizing oligodendrocyte progenitor cell surface protein: Implications for remyelination. *Annals of Neurology* , 48, 362-371.

Noseworthy, J. H. (1999). Progress in determining the causes and treatment of multiple sclerosis. *Nature* , 399, A40-A47.

Pham, C. T., & Ley, T. J. (1999). Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B in vivo. Proceedings of the National Academy of *Sciences* , 96, 8627-8632.

Pham, C. T., Armstrong, R. J., Zimonjic, D. B., Popescu, N. C., Payan, D. G., & Ley, T. J. (1997). Molecular cloning, chromosomal localization, and expression of murine Dipeptidyl Peptidase I. *The Journal od Biological Chemistry* , 272, 10695-10703.

Samuel, D., & Antje, K. (2011). Repertoire of microglial and macrophage responses after spinal cord injury. *Nature reviews neuroscience* , 12, 388-399.

Sano, H., Hsu, D. K., Apgar, J. R., Yu, L., Sharma, B., Kuwabara, I., et al. (2003). Critical role of galectin-3 in phagocytosis by macrophages. *American Society for Clinical Investigation* , 112, 389–397.

Sayed, B. A., Christy, A. L., Walker, M. E., & Brown, M. A. (2010). Meningeal mast cells affect early T cell central nervous system infiltration and blood-brain barrier integrity through TNF: A role for neutrophil recruitment? *The journal of immunology* , 184, 6891-6900.

Schuttelkopf, A. W., Hamilton, G., Watts, C., & Aalten, D. M. (2006). Structural basis of reduction-dependent activation of human Cystatin F. *The Journal of Biological Chemistry* , 281, 16570-16575.

Shimizu, T. (2012). Analysis of fate of oligodendrocyte and progenitor cells in chronic demyelinatined lesion and identification of a factor that influences their cell fate determination; PhD thesis, The Graduate University for Advanced Studies, Kanagawa

Shin, T., Ahn, M., & Matsumoto, Y. (2012). Mechanism of experimental autoimmune encephalomyelitis in Lewis rats: recent insights from macrophages. *Anatomy and cell biology* , 45, 141-148.

Shivane, A. G., & Chakrabarty, A. (2007). Multiple sclerosis and demyelination. *Current Diagnostic Pathology* , 193-202.

Shresta, S., Graubert, T. A., Thomas, D. A., Raptis, S. Z., & Ley, T. J. (1999). Granzyme A initiates an alternative pathway for granule-mediated apoptosis. *Immunity* , 10, 595-605.

Sindrilaru, A., Peters, T., Wieschalka, S., Baican, C., Baican, A., Peter, H., et al. (2011). An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *The Journal of Clinical Investigation* , 121, 985-997.

Sloane, J. A., Batt, C., Ma, Y., Harris, Z. M., Trapp, B., & Vartanian, T. (2010). Hyaluronan blocks oligodendrocyte progenitor maturation and remyelination through TLR2. *Proceedings of the National Academy of Sciences of the United States of America* , 107, 11555-11560.

Stollg, G., & Jander, S. (1999). The role of microglia and macrophages in the pathophysiology of the CNS. *Progress in Neurobiology* , 58, 233-247.

Tanaka, K. F., Ahmari, S. E., Leonardo, D. E., Richardson-Jones, J. W., Budreck, E. C., Scheiffele, P., et al. (2010). Flexible Accelerated STOP Tetracycline Operator-Knockin (FAST): A versatile and efficient new gene modulating system. *Biological Psychiatry* , 67, 770-773.

Tanuma, N., Sakuma, H., Sasaki, A., & Matsumoto, Y. (2006). Chemokine expression by astrocytes plays a role in microglia/macrophage activation and subsequent neurodegeneration in secondary progressive multiple sclerosis. *Acta Neuropathologica* , 112, 195-204.

Taupin, V., Renno, T., Bourbonniere, L., Peterson, A. C., Rodriguez, M., & Owens, T. (1997). Increased severity of experimental autoimmune encephalomyelitis, chronic macrophage/microgIiaI reactivity, and demyelination in transgenic mice producing tumor necrosis factor-alpha a in the central nervous system. *European Journal of Immunology* , 27, 905-913.

Uwe-Karsten, H. (2002). Microglia as a source and target of cytokines. *Glia ,* 40, 140-155.

Wang, S., Sdrulla, A. D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., et al. (1998). Notch Receptor Activation Inhibits Oligodendrocyte Differentiation. *Neuron* , 21, 63-75.

Wu, F., Cao, W., Yang, Y., & Liu, A. (2010). Extensive inWltration of neutrophils in the acute phase of experimental autoimmune encephalomyelitis in C57BL/6 mice. *Histochemistry and Cell Biology* , 133, 312-322.

Zamvil, S. S., & Steinman, L. (1990). The T Lymphocyte in Experimental Allergic Encephalomyelitis. *Annual Review of Immunology* , 8, 579-621.

Zhang, S.-C., Goetz, B. D., & Duncan, I. D. (2003). Suppression of activated microglia promotes survival and function of transplanted oligodendroglial progenitors. *Glia* , 41, 191-198.