University of Alberta

Endoplasmic Reticulum Stress Induction by an Endogenous Retrovirus Glycoprotein During Neuroinflammation: Regulation by a free radical scavenger

by

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Examining Committee members

- Dr. Christopher Power
- Dr. Larry Guilbert
- Dr. Fabrizio Giulinani
- Dr. Marek Michalak

Abstract

Endoplasmic reticulum (ER) stress is a cellular homeostatic mechanism, which is utilized by cells to adapt to inter- and intra-cellular changes. There is a burgeoning literature showing that the human endogenous retroviral envelope glycoprotein, Syncyin-1, oxidative stress and reactive oxygen species (ROS) participate in the pathogenesis of multiple sclerosis (MS). I investigated the contribution of Syncytin-1-induced ER stress in MS and its animal model, experiment autoimmune encephalomyelitis (EAE). The prototypic ER stress biomarker, XBP-1 spliced variant (XBP-1/S), was increased in cerebral white matter of MS patients compared to non-MS controls and was correlated with Syncytin-1 expression. Syncytin-1 over-expression caused glia cytotoxicity but was mitigated by the ROS scavenger, crocin. Treatment with crocin on day 7 post-EAE induction ameliorated EAE disease severity in mice by reducing EAE pathology. Herein, I demonstrate that crocin attenuates Syncytin-1-induced ER stress in astrocytes while also diminishing disease severity in EAE in conjunction with suppression of neuroinflammation.

Acknowledgements

I am truly grateful to all those who have supported me throughout my Master's program.

I would like to acknowledge every past and present member of the BrainPowerLab. First and foremost, thank you to Dr. Chris Power for the many opportunities you have provided to me, the expertise you have shared with me and your constant guidance. Thank you to Farshid, Kris, Ferdinand, Rakesh, Leah, Krista, Shaona, Hong, Yu Zhu, Maria, Liz, Amber and Tarn for the many conversations including scientific thought-provoking and social interactions. Thank you for all your help in my experiments, data analyses, patience and making me laugh. This would not have been possible without your support.

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I am grateful towards my committee members, Dr. Larry Guilbert, Dr. Marek Michalak and Dr. Fabrizio Giuliani for your time and effort towards my project. I truly appreciate your constructive criticisms and guidance during my committee meetings.

Thank you to all my friends, who gave me their support and encouragement during these last two years. This list is too long and I wish not to leave anyone out. I am truly grateful to all my closest friends. Thank you to anyone I shared a laugh, smile or a simple 'hi'.

I would like to thank my family: Mom, Dad, Monique and Rob for their constant support. Thanks Dad for supporting me in everything I do. Thanks Mom for your love, I wish you could have been here, but I know you're here in spirit. Monique, as a sister you have always been there for me. To Cheryl, thank you for coming into my life. Your love and support has been invaluable to me during this endeavor.

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Table 1: List of primers used for real time RT-PCR

Gene	Species		Sequence (5'-3')	Annealing temperature
GAPDH	Hm/Ms	Fwd	AGC CTT CTC CAT GGT GGT GAA GAC	50-60
		Rev	CGG AGT CAA CGG ATT TGG TCG	
TNF-α	Hm	Fwd	ACC TCA TCT ACT CCC AGG TCC	58
		Rev	CTC TTG ATG GCA GAG AGG AGG	
TNF-α	Ms	Fwd	ATG CTG GGA CAG TGA CCT GG	60
		Rev	CCT TGA TGG TGG TGC ATG AG	
F4/80	Ms	Fwd	GCC ACC TGC ACT GAC ACC	54
		Rev	GCT GCA CTT GGC TCT CC	
XBP-1/S	Hm	Fwd	CGT GAG TCC GCA GCA GGT	51
		Rev	GGA GGC TGG TAA GGA ACT AGG T	
PERK	Hm	Fwd	GGG ACT ATG GAT GGC AAT GAT GAG	55
		Rev	CTG GGC TGG AGT TTT TCT GTG GT	
BiP	Hm	Fwd	GCG CGG GCC GAG GAG GAG	61
		Rev	ATA GGA CGG CGT GAT GCG GTT GC	
CHOP	Hm	Fwd	AAC CAG CAG AGG TCA CAA GC	55
		Rev	AGC CGT TCA TTC TCT TCA GC	
Calreticulin	Hm	Fwd	TTA CGC ACT GTC CGC CAA A	55
		Rev	GCT CAT GCT TCA CCG TGA ACT	
GRP58	Hm	Fwd	TCA AGG GTT TTC CTA CCA TCT ACT TC	59
		Rev	TTA ATT CAC GGC CAC CTT CAT	
HLA-DR	Hm	Fwd	GGA CAA AGC CAA CCT GGA AA	55
		Rev	AGG ACG TTG GGC TCT CTC AG	
IFNα	Hm	Fwd	GTG ATC TCC CTG AGA CCC AC	54
		Rev	GAT CTC ATG ATT TCT GCT CTG ACA ACC	
IL-6	Hm	Fwd	GCA GAA AAA GGT GGG TGT GT	56
		Rev	AGC TGC GCA GAA TGA GAT GAG TTG T	
IL-12p35	Hm	Fwd	AGC CTC CTC CTT GTC GCT ACC	55
		Rev	GCC TCC ACT GTG CTG GTT TTA TC	
Rantes	Hm	Fwd	CTT TGT CAC CCG AAA GAA CC	55
		Rev	GTT TCA TCA TGT TGG CCA GG	
TGF-β	Hm	Fwd	TTC AAC ACA TCA GAG CTC CGA	55
		Rev	GGA GAG CAA CAC GGG TTC AG	

Table 1 (continued): List of primers used for real time RT-PCR

Gene	Species		Sequence (5'-3')	Annealing temperature
XBP-1/S	Ms	Fwd Rev	CGT GAG TCC GCA GCA GGT CTA GAG GCT TGG TGT ATA C	59
PERK	Ms	Fwd Rev	AAG TAG ATG ACT GCA ATT ACG CTA TCA A TTT AAC TTC CCG CAT TAC CTT CTC	59
BIP	Ms	Fwd Rev	TCA TCG GAC GCA CTT GGA A CAA CCA CCT TGA ATG GCA AGA	59
GRP58	Ms	Fwd Rev	TCA AGG GTT TTC CTA CCA TCT ACT TC TTA ATT CAC GGC CAC CTT CAT	59
OASIS	Ms	Fwd Rev	GAG CCG CAA GAA GAA GGA GTA AAG GGA GCC CAG GAC CAG AAC G	59
CHOP	Ms	Fwd Rev	GTC CCT AGC TTG GCT GAC AGA TGG AGA GCG AGG GCT TTG	59
CD3ε	Ms	Fwd Rev	TCT CGG AAG GTC GAG GAC AGT TTG AGG CTG GTG TGT AGC AG	59
NOS2	Ms	Fwd Rev	CAG CGG GAT GAC TTT CCA A AGG CAA GAT TTG GAC CTG CA	59
Arginase	Ms	Fwd Rev	GGA ATC TGC ATG GGC AAC CTG TGT AGG GTC TAC GTC TCG CAA GCC A	59
PAR-2	Ms	Fwd Rev	TGG CCA TTG GAG TCT TCC TGT T TAG CCC TCT GCC TTT TCT TCT C	59

Table 2 - Genes correlated with HERV-W *env* in the brains of MS and non-MS patients.

Host genes	Spearman correlation with HERV-W *env* (R-value)
FoxP3/CD3	-0.64 *
TGF-Beta	0.74 **
CD3	0.63 *
HLA-DRA	0.61 *
IL-12p35	0.69 *
5aReductase	-0.70 *
Granzyme A	0.90 ***
CD11c	0.52 ns
GFAP	0.18
IL-10	0.73 *
IL-23p19	0.68 **
CD8b	0.60 *
HLA-DQA1	0.32 ns

Figures

Figure 1 – XBP-1/S and Syncytin-1 transcripts in the brains of MS patients and its correlation to HERV-W *env*. (A) A 26 bp-intron is spliced out of XBP-1 unspliced (left) to yield XBP-1 spliced (right), which lacks the pstI restriction digestion site. (B) Real time RT-PCR transcript levels of *XBP-1* spliced transcript variant and Synctyin-1 in the brains of MS patients compared to the brains of non-MS patients. Data are presented as median. *P<0.05. This experiment was done in technical triplicates. N=5 and 8 for control and MS groups respectively. (C) Spearman correlation analysis between the transcript levels of *XBP-1 spliced* and *HERV-W env* (P<0.05). (D) Heat map of immune gene transcript levels in the brains of MS and Non-MS patients.

Figure 2 – Crocin diminishes syncytin-1 induced XBP-1 splicing in human astrocytes. (A) Western blot of human astrocytes transfected with vector containing ERVWE1 or empty vector. (B) Dual luciferase assay of human astrocytes transfected with syncytin-1 or empty vector treated with either PBS or crocin for 16 hours, starting 8 hours post transfection. ERVWE1 or empty vector were co-transfected with *Renilla* luciferase and *Firefly* luciferase promoters. *Renilla* luciferase is constitutively expressed and was used as an internal control. *Firefly* luciferase is only expressed when XBP-1 is spliced during ER stress. *Firefly* luciferase was normalized to *Renilla* luciferase. Thapsigargin (TG) is a chemical that induces ER stress and was used as a positive control. Data are presented as mean ± SEM. (C) BiP, (D), NOS2 and (E) HIF-1α transcript levels in astrocytes transfected with ERVWE1-containing vector or empty vector and treated with varying concentrations of crocin for 24hours. Experiment was repeated twice. *$P<0.05$.

Figure 3 – The effect of supernatants from astrocytes transfected with Syncytin-1 on Oligodendrocyte viability. (A) Astrocytes cultured for 6 days, fixed with 4% formaldehyde, stained with galactocerebroside or CNPase and viewed under fluorescent microscopy. In-cell western analysis of rat oligodendrocytes treated with astrocytes transfected with either syncytin-1 or empty vector and treated with crocin. Human astrocytes were transfected with either syncytin-1 or empty vector. Medium was changed 6 hours post transfection and supernatants were collected 24 hours after changing the medium. Supernatants were applied to oligodendrocytes cultured in a 96-well plate with increasing concentrations of crocin for 24 hours. Cells were fixed and stained for CNPase. CNPase immunoreactivity was measured semi-quantitatively using Licor in-cell western. Data are presented as mean ± SEM. (D) Oligodendrocytes were differentiated for one week and exposed to supernatants form astrocytes or microglia with or without crocin, fixed with 4% formaldehyde, stained with 4',6-diamidino-2-phenylindole(DAPI) and measured semi-quantitatively using a luminometer. This experiment was repeated once. Ns: not significant. **P<0.01. ***P<0.001.

Figure 4 – Sodium Nitroprusside-induced toxicity on rat oligodendrocytes is inhibited by crocin in a concentration-dependant manner. (A) Confocal imaging of rat oligodendrocytes stained with CNPase. Oligodendrocytes were isolated from Sprague Dawley rats and allowed to differentiated for 6 days on poly-ornithine coated 96-well plates. Oligodendrocytes were treated with sodium-nitroprusside (SNP), an NO donor, for 24 hours to induce cytotoxicity and varying concentrations of crocin. (B) In-cell western analysis probed with CNPase of rat oligodendrocytes treated with SNP and increasing concentrations of crocin. Data are presented as mean ± SEM. *P<0.05.

Figure 5 – The effect of tunicamycin on ER stress transcripts in human microglia *in vitro.* Human microglia were treated with either PBS or tunicamycin (TM) for 2 hours. RNA was isolated and a cDNA library was synthesized using random primers. Real-time RT-PCR was performed using primers to specifically amplify (A) *XBP-1/S*, (B) *PERK*, (C) *BiP*, (C) *CHOP*, (D) *calreticulin* and (E) *GRP58*. Data are presented as mean of technical replicates ± SEM.

Figure 6 – The effect of tunicamycin on immune genes in human microglia *in vitro*. Human microglia were treated with either PBS or tunicamycin (TM) for 2 hours. RNA was isolated and a cDNA library was synthesized using random primers. Real-time RT-PCR was performed using primers to specifically amplify (A) *HLA-DR*, (B) *INFα*, (C) *IL-6*, (D) *IL-12p35*, (E) *RANTES* and (F*) TGFβ*. Data are presented as mean of technical replicates ± SEM.

Figure 7 – ER stress gene transcript levels in the spinal cords of EAE animals. (A) Lumbar spinal cords were isolated from EAE and control mice at peak disease severity. RNA was isolated from the lumbar spinal cord and a cDNA library was synthesized. RT-PCR using primers designed to amplify XBP-1 spliced and unspliced transcripts was performed. Amplicons were cut with pstI. XBP-1 unspliced transcript variant has a pstI restriction site and gets cut into 2 smaller fragments. PstI restriction site gets spliced out during XBP-1 splicing and therefore XBP-1 spliced transcript variant does not have a pstI restriction site. During PstI digestion, XBP-1 spliced transcript variant does not get spliced and therefore the amplicon exists as one large amplicon. Sizes of digested amplicons were measured on a 2% agarose gel using gel electrophoresis. This experiment was repeated 3 times (B) Band intensity ratio of XBP-1 spliced: XBP-1 unspliced was measured using Image J. (C). RNA was isolated from the lumbar spinal cords of EAE and control mice. cDNA was synthesized using random primers and specific ER gene transcripts were amplified using real time RT-PCR. Data are presented as mean ± SEM. This experiment was repeated three times. *$P<0.05$.

Figure 8 – Tunicamycin suppresses EAE disease profile. (A) EAE was induced in C57B/6 mice using MOG_{35-55} peptide. Tunicamycin was given to EAE mice daily for 5 days starting at disease induction (◊), or at the time of disease onset (□). (B) Mice were scored based on limb paralysis. The tail and each limb is given a score of 2 and 3 respectively if they are fully limp. Typically, EAE mice peak score is approximately 7 to 11 on a 14 point scale. (C) Maximum scores of each mouse were assessed and compared between groups (D) Scores from each mouse were added and represented as cumulative score. (B-D) Data are presented as mean ± SEM. This experiment was repeated once. *P<0.05. **P<0.01.

Figure 9 – The role of tunicamycin on ER stress and immune gene transcripts in the spinal cords of EAE mice *in vivo*. At 25 post EAE induction, lumbar spinal cords were isolated from EAE mice treated with PBS or tunicamycin at the 2 different time points: Tunicamycin at disease induction (EAE/late TM) or at the onset of EAE (EAE/late TM). RNA was isolated from lumbar spinal cords and a cDNA library was synthesized using random primers. Real time RT-PCR was performed using primers designed to specifically amplify (A) *CD3ε*, (B) *F4/80*, (C) *NOS2*, (D) *Arginase I*, (E) *XBP-1/S*, (G) *CHOP* and (H*) PERK*. (A-H) Data are presented as mean ± SEM. This experiment was not repeated. *P<0.05.

Figure 10 – EAE is CHOP independent. (A) CHOP +/+, +/- and -/- littermates were generated and EAE was subsequently induced. EAE clinical scores were assessed on a 14-point scale and followed from day 8 to day 25. (B) Average maximum clinical score. (C) Average cumulative clinical score. (A-C) Data are presented as mean ± SEM. This experiment was not repeated.

Figure 11 – The effect of crocin of ER stress and immune gene transcripts in EAE *in vivo*. EAE was induced in C57B/6 mice. Mice received PBS or crocin I.P. injections daily starting at day 7 post EAE induction until the end of the experiment. Spinal cords were isolated 25 day post EAE induction. RNA was isolated from the spinal cords and a cDNA library was synthesized using random primers. Real time RT-PCR was performed with primers designed to specifically amplify transcripts of (A) *CD3ε*, (B) *F4/80*, (C) *NOS2*, (D) *Arginase*, (E) *XBP-1/S*, (F) *BiP*, (G) *CHOP* and (H) *PERK*. (A-H) Data are presented as mean ± SEM. This experiment was not repeated. *P<0.05.

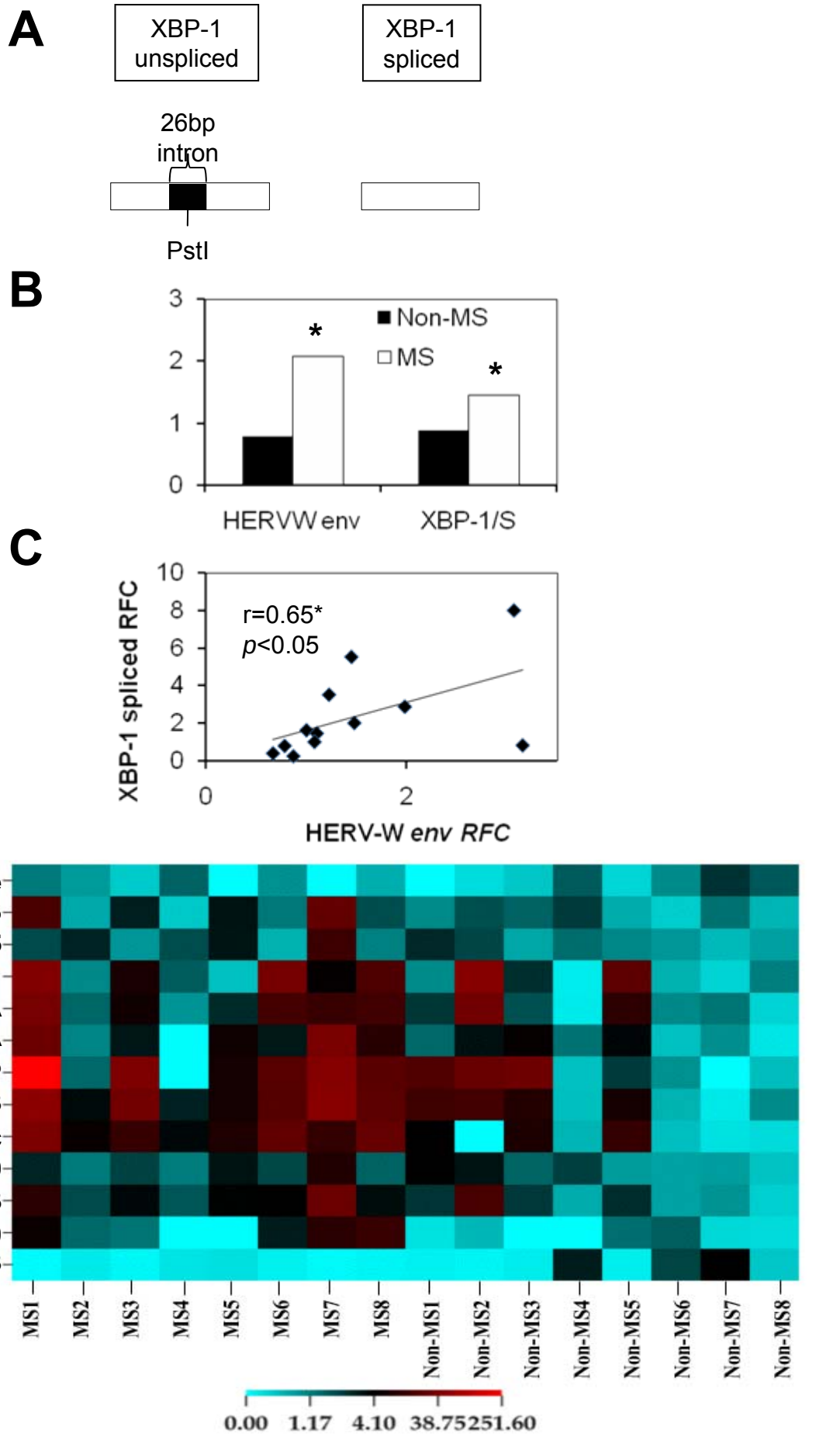
Figure 12 – The effect of crocin on the immunology of EAE disease pathology *in vivo*. Spinal cords were isolated 25 day post EAE induction from EAE mice treated with PBS or crocin, fixed with 4% formaldehyde and paraffin embedded. Sections 5-7 µm were cut with a microtome and immunostained for (1st row from the top) CD3 (T cells, **Arrows** indicate positive cells), (2nd row from the top) myelin basic protein (MBP) ('**M**' indicates myelin. '**D**' indicates demyelination, (3rd row from the top) silver stain (axons), (4th row from the top) ionized calcium binding adaptor molecule 1 (Iba-1) (macrophages/activated microglia). (Rows 1-3 from the top) Sections were viewed under light microscopy). (Bottom row) Sections were viewed with fluorescent microscopy.

Figure 13 - The effect of crocin on the ER stress of EAE disease pathology *in vivo*. Spinal cords were isolated 25 day post EAE induction from EAE mice treated with PBS or crocin, fixed with 4% formaldehyde and paraffin embedded. Sections 5-7 µm were cut with a microtome and stained for (top row, **arrows** indicate oligodendrocytes, **arrowheads** indicate astrocytes) BiP and (bottom row) CHOP. Arrows indicate positive cells.

Figure 14 – Crocin alters neurological disability during EAE. (A) EAE was induced on day 0 and crocin or PBS I.P. injections were given daily starting at day 7 post EAE induction until the end of the experiment (day 25). (B) Each day, starting at day 7 post EAE induction, clinical score was assessed on a 14 point scale based on limb paralysis. (C) Maximum EAE clinical scores from EAE and EAE animals treated with crocin. (D) Cumulative sum of EAE clinical scores during the entire course of EAE. All data are presented as mean ± SEM. This experiment was repeated once. *P<0.05. **P<0.01. ***P0.001.

Figure 15 – Disease mechanisms by which Syncytin-1-mediates ER stress-induced neuroinflammation and neurodegeneration. An environmental triggers the induction of Syncytin-1 in quiescent astrocytes. Astrocytes become activated and produce ROS and pro-inflammatory cytokines which cause demyelination, inhibits OPC maturation, inhibits remyelination and causes neurodegeneration. Crocin likely acts on a several points in this pathway including ROS production. The earliest effect of crocin appears to be on Syncytin-1 induced ER stress.

Figure 1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XBP-1 spliced
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PstI
B
Non-MS
MS
*
*
HERVW env
XBP-1/S
C
r=0.65*
p<0.05
XBP-1 spliced RFC
HERV-W env RFC
D
5α reductase
CD8β
IL12p35
HLA-DQA1
HLA-DRA
Granzyme A
GFAP
CD3
CD11c
IL23p19
TGF-β
IL-10
Foxp3/CD3
MS1
MS2
MS3
MS4
MS5
MS6
MS7
MS8
Non-MS1
Non-MS2
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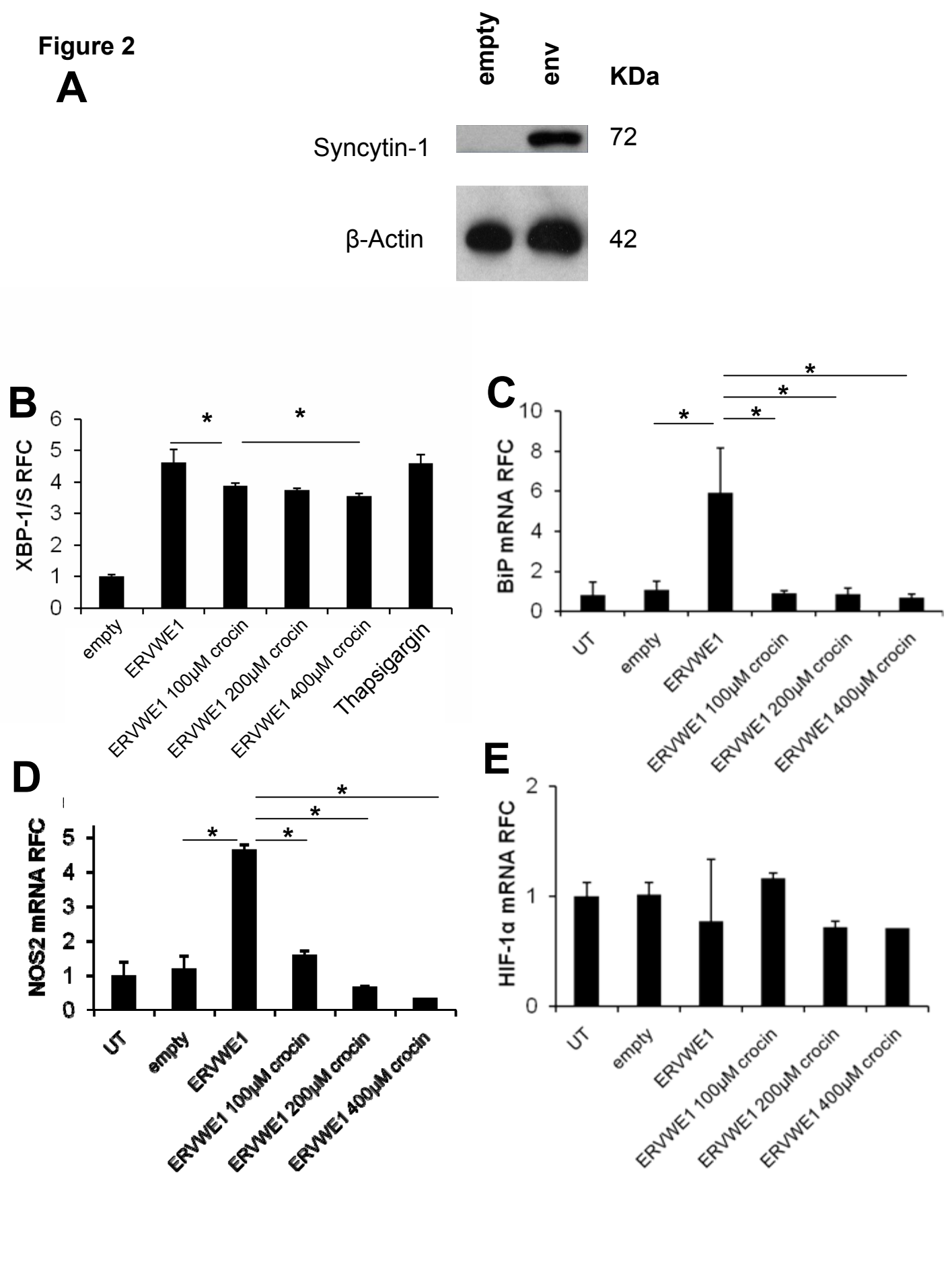
Figure 2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6
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ERVWE1
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ERVWE1 200μM crocin
ERVWE1 400μM crocin
Thapsigargin
C
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4
6
8
10
*
*
*
*
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ERVWE1
ERVWE1 100μM crocin
ERVWE1 200μM crocin
ERVWE1 400μM crocin
D
NOS2 mRNA RFC
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2
3
4
5
*
*
*
*
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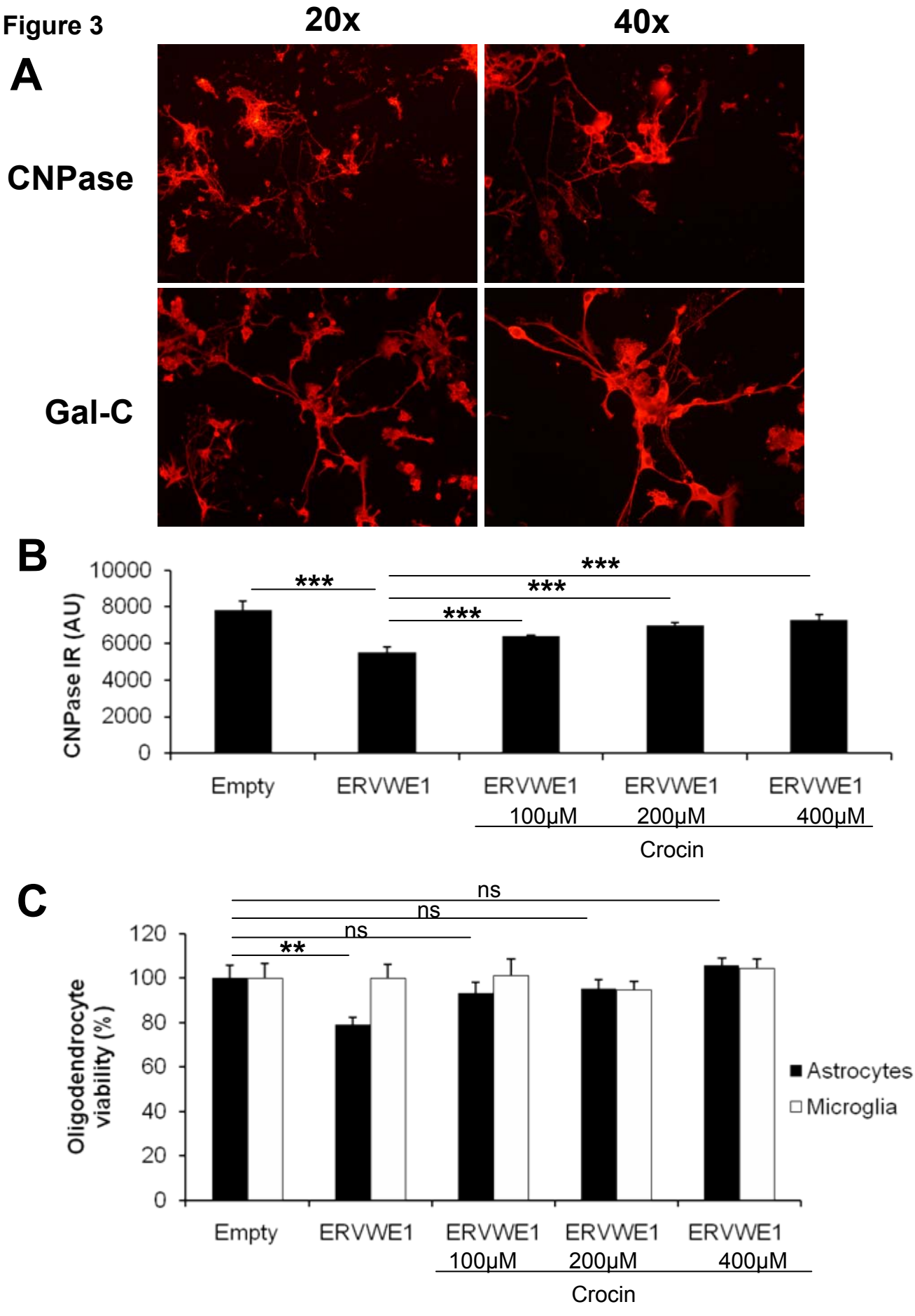
Figure 3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40x
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CNPase
Gal-C
B
CNPase IR (AU)
10000
8000
6000
4000
2000
0
***
***
***
***
Empty
ERVWE1
ERVWE1 100μM
ERVWE1 200μM
ERVWE1 400μM
Crocin
C
Oligodendrocyte viability (%)
120
100
80
60
40
20
0
ns
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Empty
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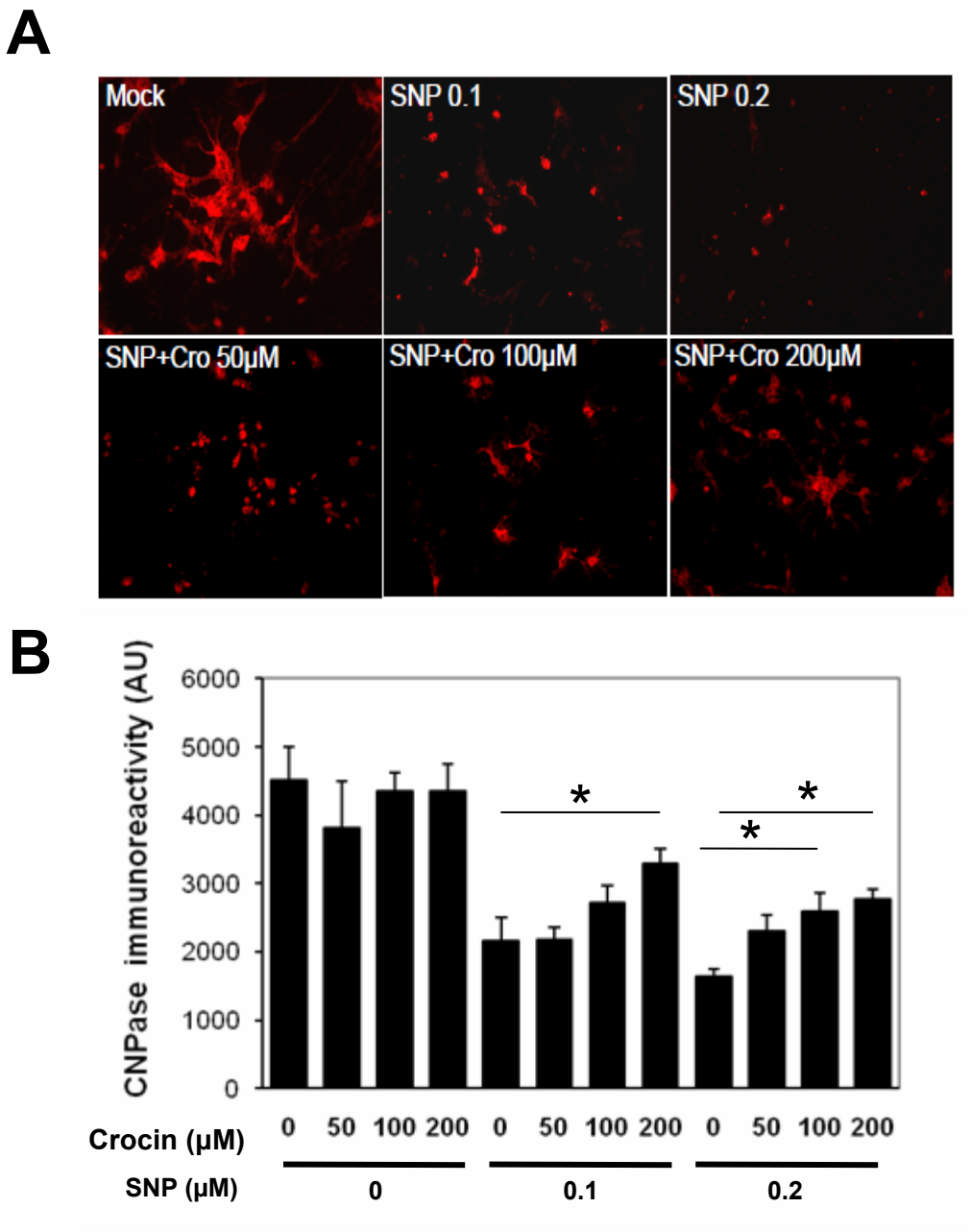
ERVWE1 100μM
ERVWE1 200μM
ERVWE1 400μM
Crocin
Astrocytes
Microglia

Figure 4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SNP 0.2
SNP+Cro 50μM
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CNPase immunoreactivity (AU)
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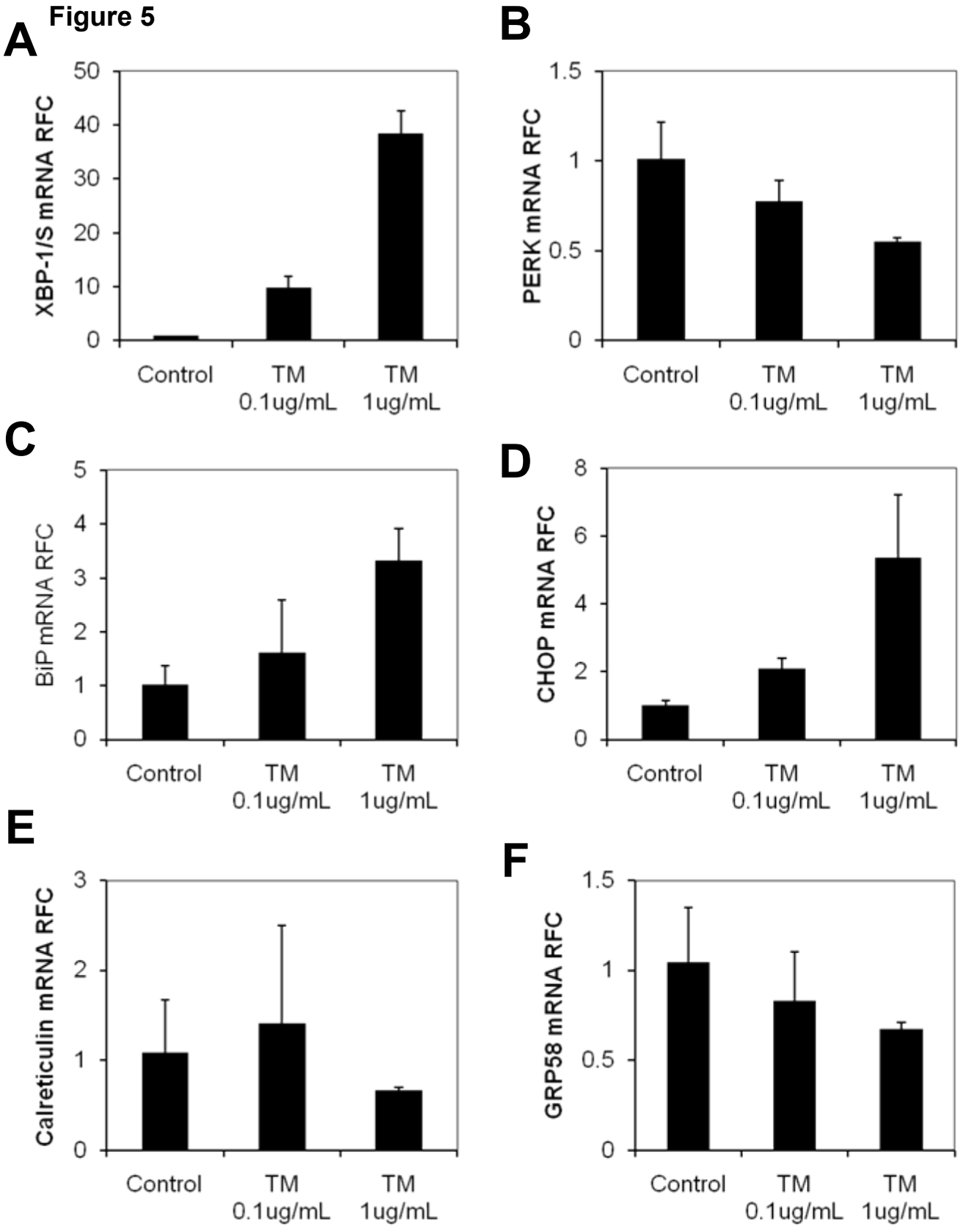
5000
4000
3000
2000
1000
0
*
*
*
Crocin (μM)
0 50 100 200 0 50 100 200 0 50 100 200
SNP (μM)
0
0.1
0.2

Figure 5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TM 1ug/mL
B
PERK mRNA RFC
1.5
1
0.5
0
Control
TM 0.1ug/mL
TM 1ug/mL
C
BiP mRNA RFC
5
4
3
2
1
0
Control
TM 0.1ug/mL
TM 1ug/mL
D
CHOP mRNA RFC
8
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4
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Control
TM 0.1ug/mL
TM 1ug/mL
E
Calreticulin mRNA RFC
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1
0
Control
TM 0.1ug/mL
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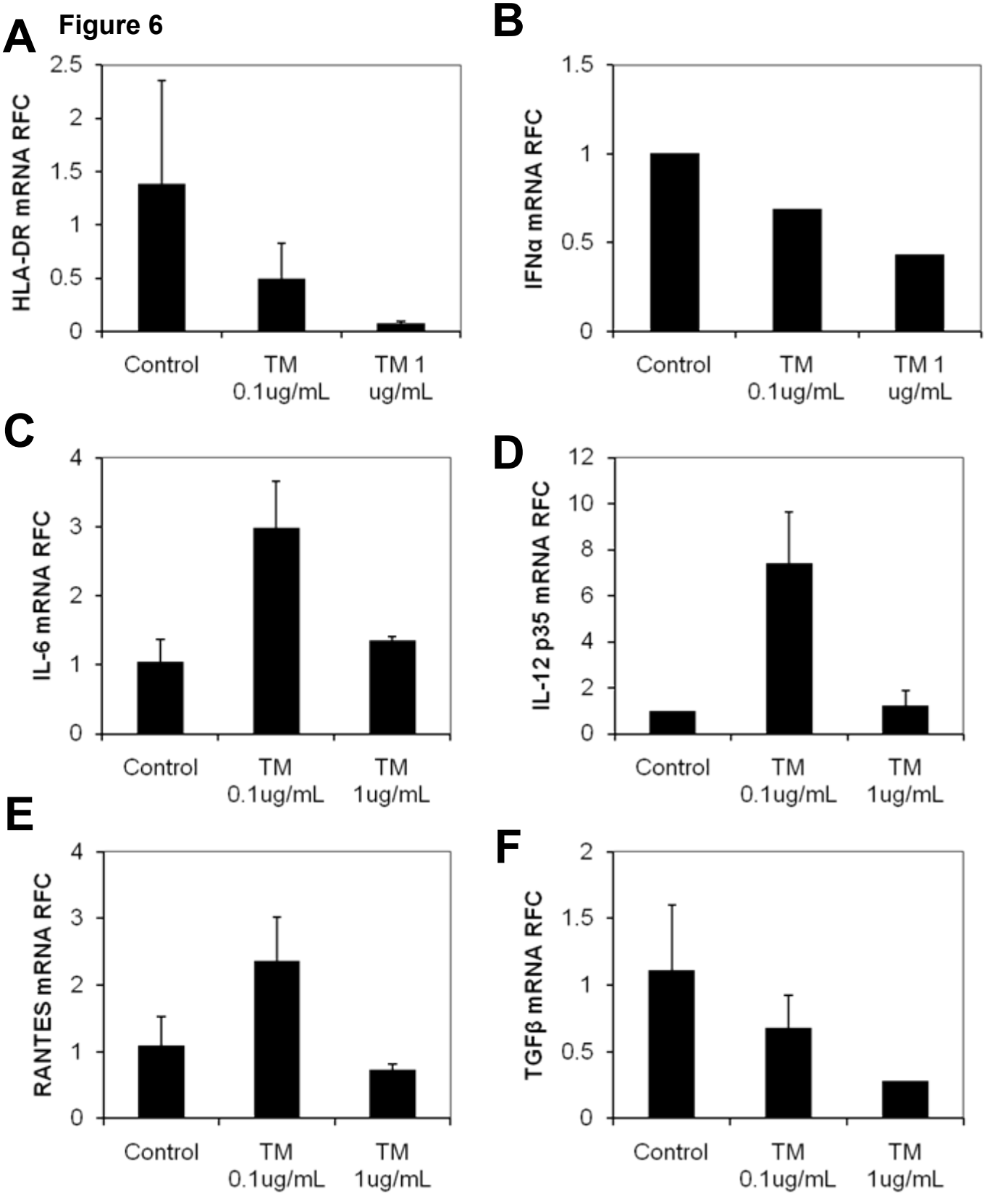
TM 1ug/mL

Figure 6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2
1.5
1
0.5
0
Control
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TM 1 ug/mL
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IFNα mRNA RFC
1.5
1
0.5
0
Control
TM 0.1ug/mL
TM 1 ug/mL
C
IL-6 mRNA RFC
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3
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1
0
Control
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TM 1ug/mL
D
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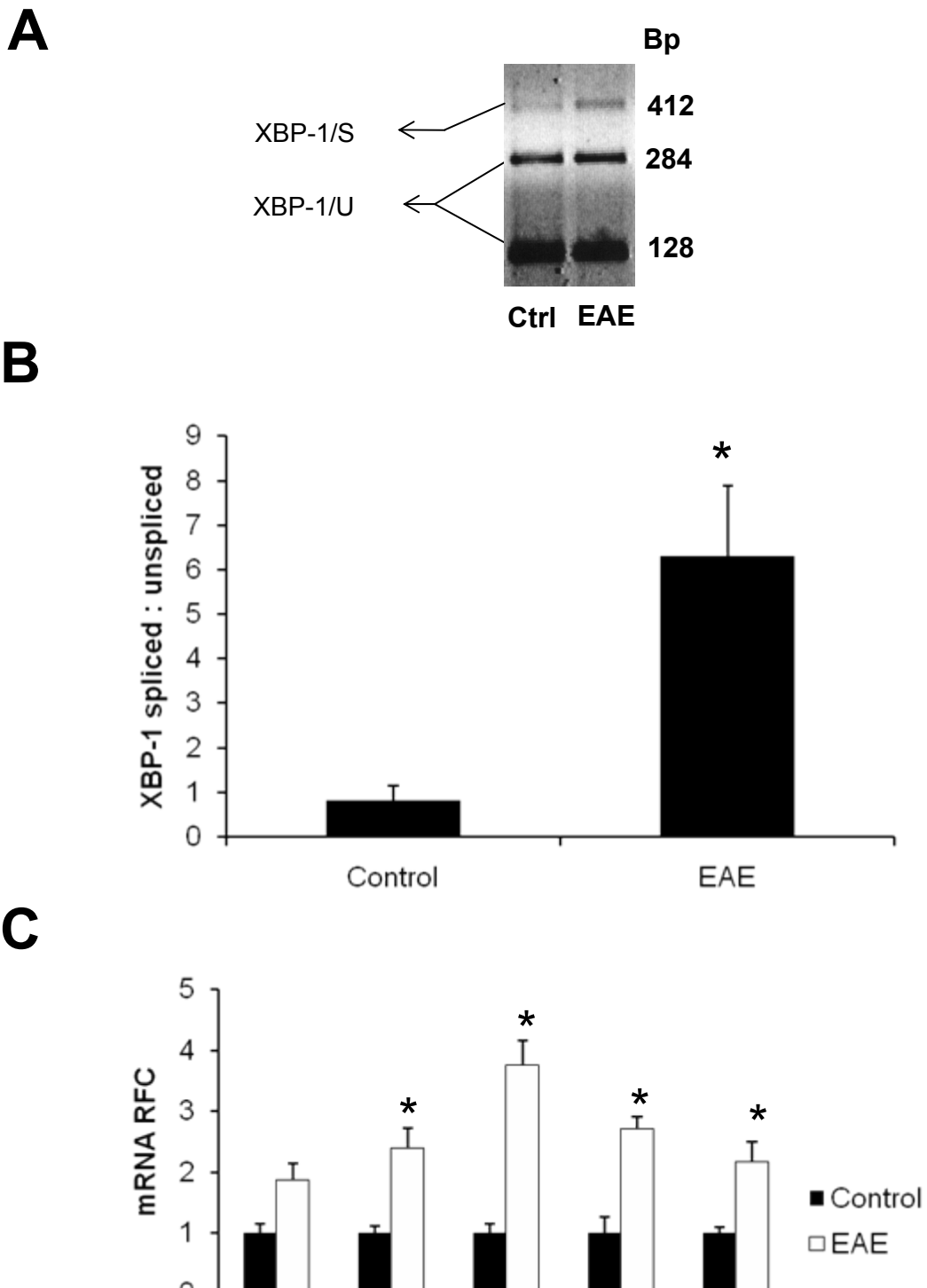
TM 1ug/mL

Figure 7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EAE
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2
3
4
5
6
7
8
9
*
Control
EAE
C
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PERK
BiP
GRP58
OASIS
CHOP
Control
EAE

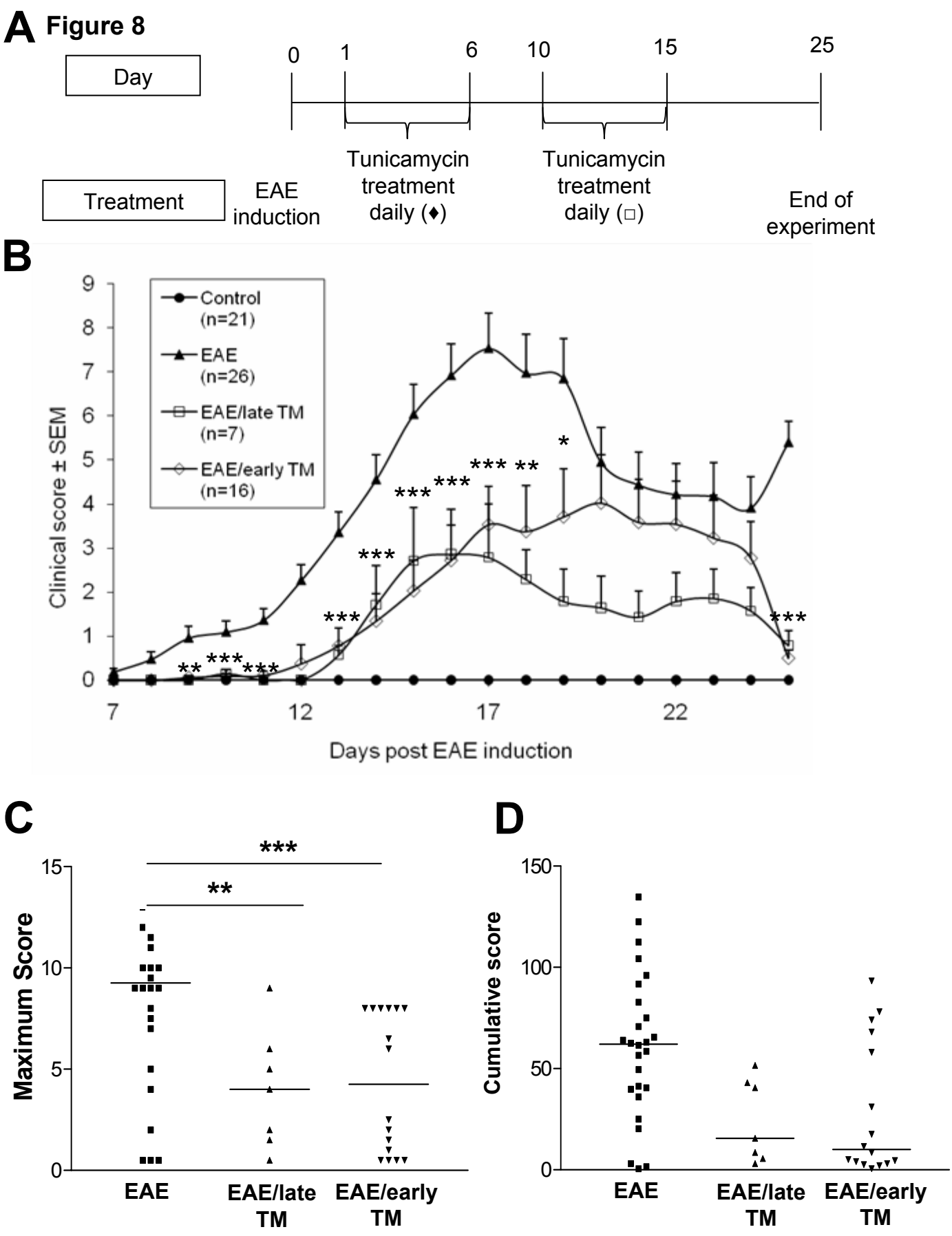
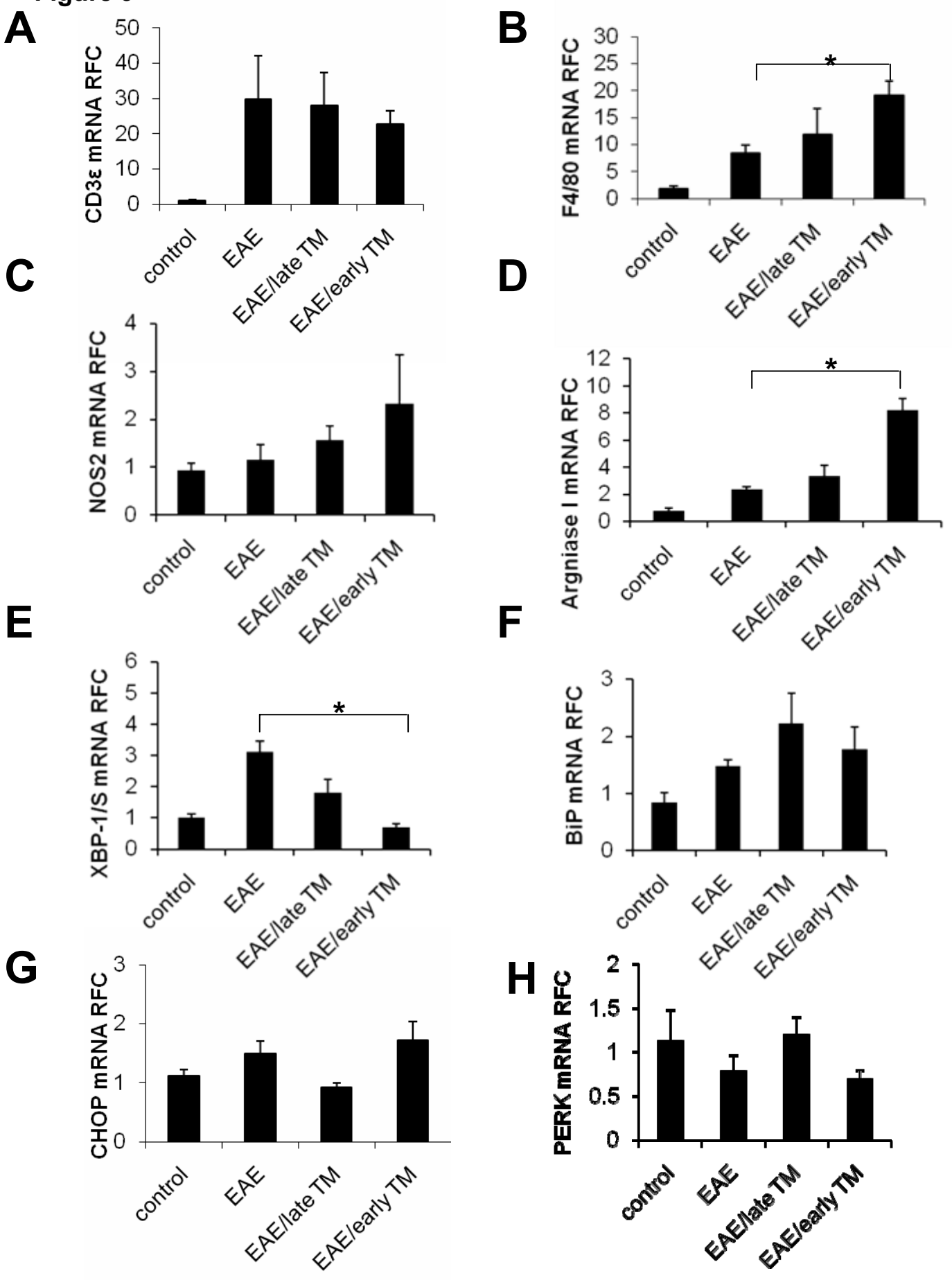

A
Figure 8
Day
0
1
6
10
15
25
Tunicamycin treatment daily (♦)
Tunicamycin treatment daily (□)
Treatment
EAE induction
End of experiment
B
Control (n=21)
EAE (n=26)
EAE/late TM (n=7)
EAE/early TM (n=16)
Clinical score ± SEM
Days post EAE induction
C
Maximum Score
EAE
EAE/late TM
EAE/early TM
D
Cumulative score
EAE
EAE/late TM
EAE/early TM

Figure 9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CD3ε mRNA RFC
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EAE/early TM
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F4/80 mRNA RFC
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control
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EAE/late TM
EAE/early TM
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EAE
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EAE
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EAE/early TM
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XBP-1/S mRNA RFC
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5
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3
2
1
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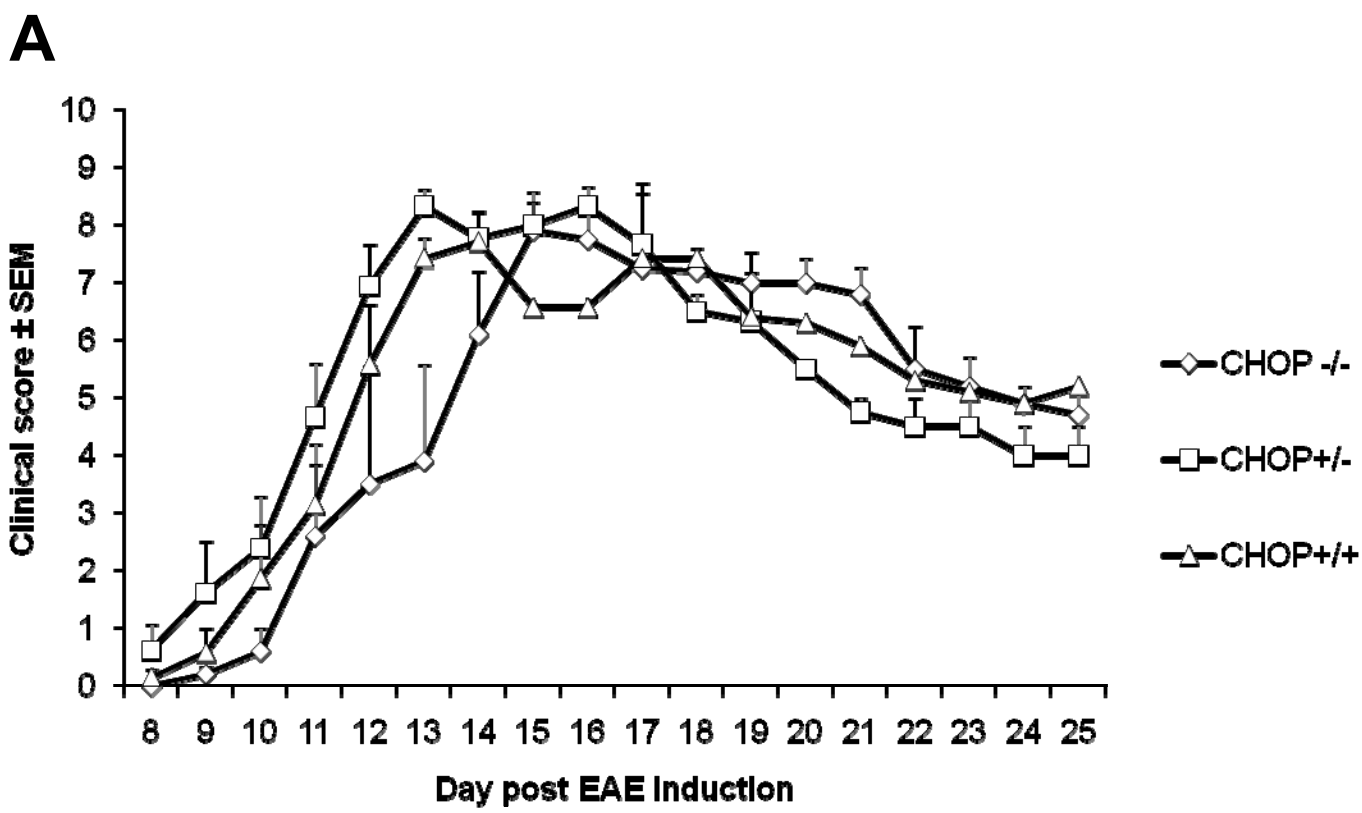
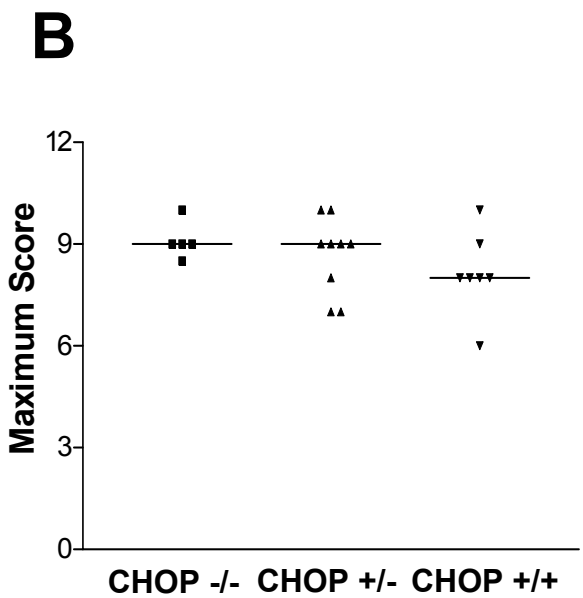
control
EAE
EAE/late TM
EAE/early TM
F
BiP mRNA RFC
3
2
1
0
control
EAE
EAE/late TM
EAE/early TM
G
CHOP mRNA RFC
3
2
1
0
control
EAE
EAE/late TM
EAE/early TM
H
PERK mRNA RFC
2
1.5
1
0.5
0
control
EAE
EAE/late TM
EAE/early TM

Figure 10


A
Clinical score ± SEM
10
9
8
7
6
5
4
3
2
1
0
8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Day post EAE Induction
CHOP -/-
CHOP+/-
CHOP+/+


B
Maximum Score
12
9
6
3
0
CHOP -/-
CHOP +/-
CHOP +/+

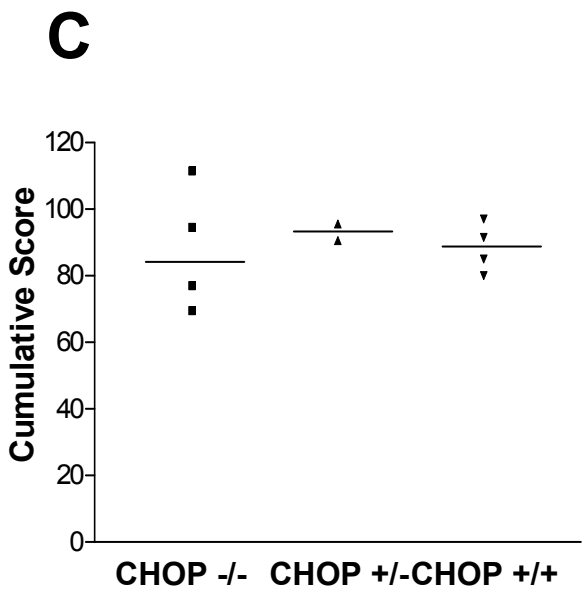

C
Cumulative Score
120
100
80
60
40
20
0
CHOP -/-
CHOP +/-
CHOP +/+

Figure 11

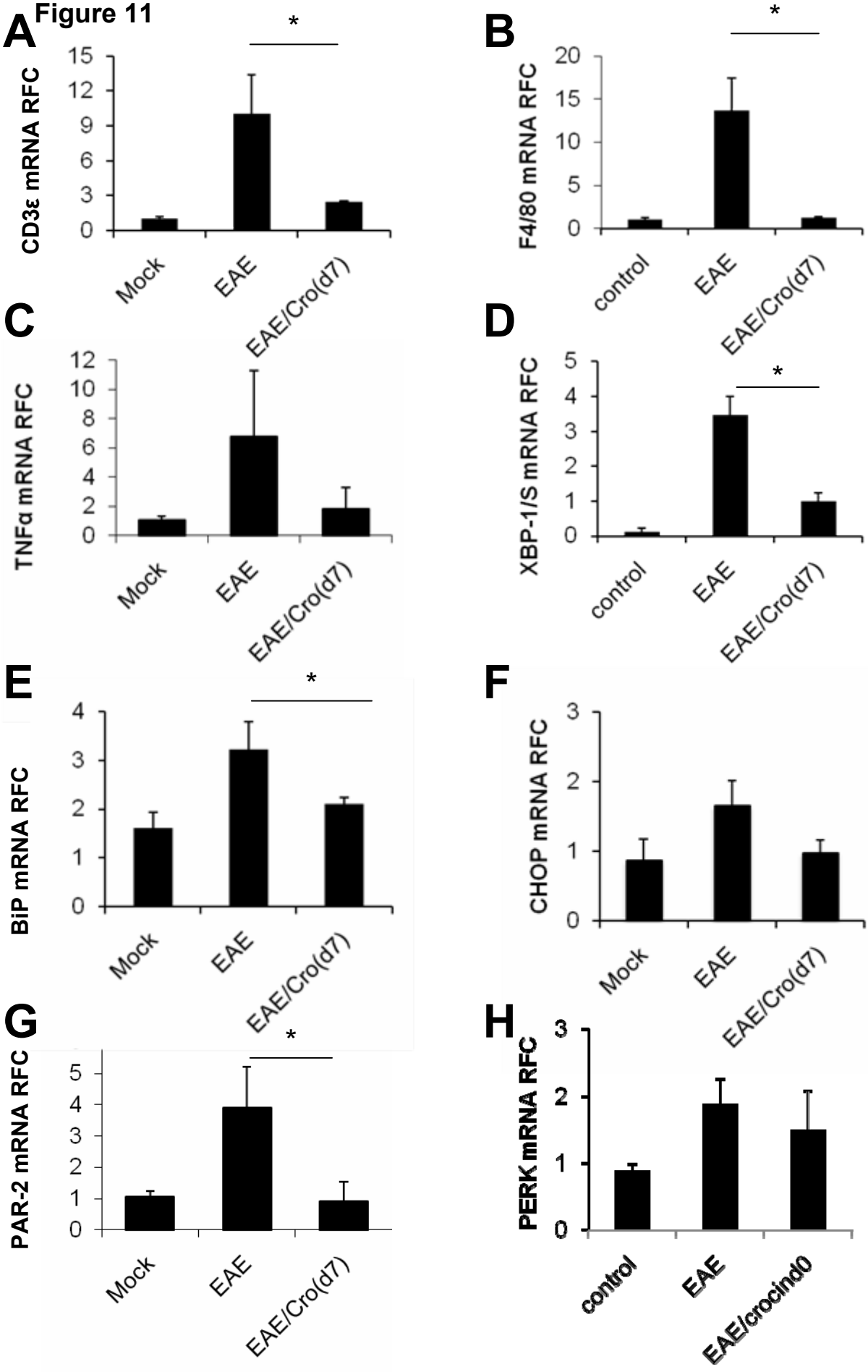

A
CD3ε mRNA RFC
15
12
9
6
3
0
*
Mock
EAE
EAE/Cro(d7)
B
F4/80 mRNA RFC
20
15
10
5
0
*
control
EAE
EAE/Cro(d7)
C
TNFα mRNA RFC
12
10
8
6
4
2
0
Mock
EAE
EAE/Cro(d7)
D
XBP-1/S mRNA RFC
5
4
3
2
1
0
*
control
EAE
EAE/Cro(d7)
E
BiP mRNA RFC
4
3
2
1
0
*
Mock
EAE
EAE/Cro(d7)
F
CHOP mRNA RFC
3
2
1
0
Mock
EAE
EAE/Cro(d7)
G
PAR-2 mRNA RFC
5
4
3
2
1
0
*
Mock
EAE
EAE/Cro(d7)
H
PERK mRNA RFC
3
2
1
0
control
EAE
EAE/crocind0

Figure 12

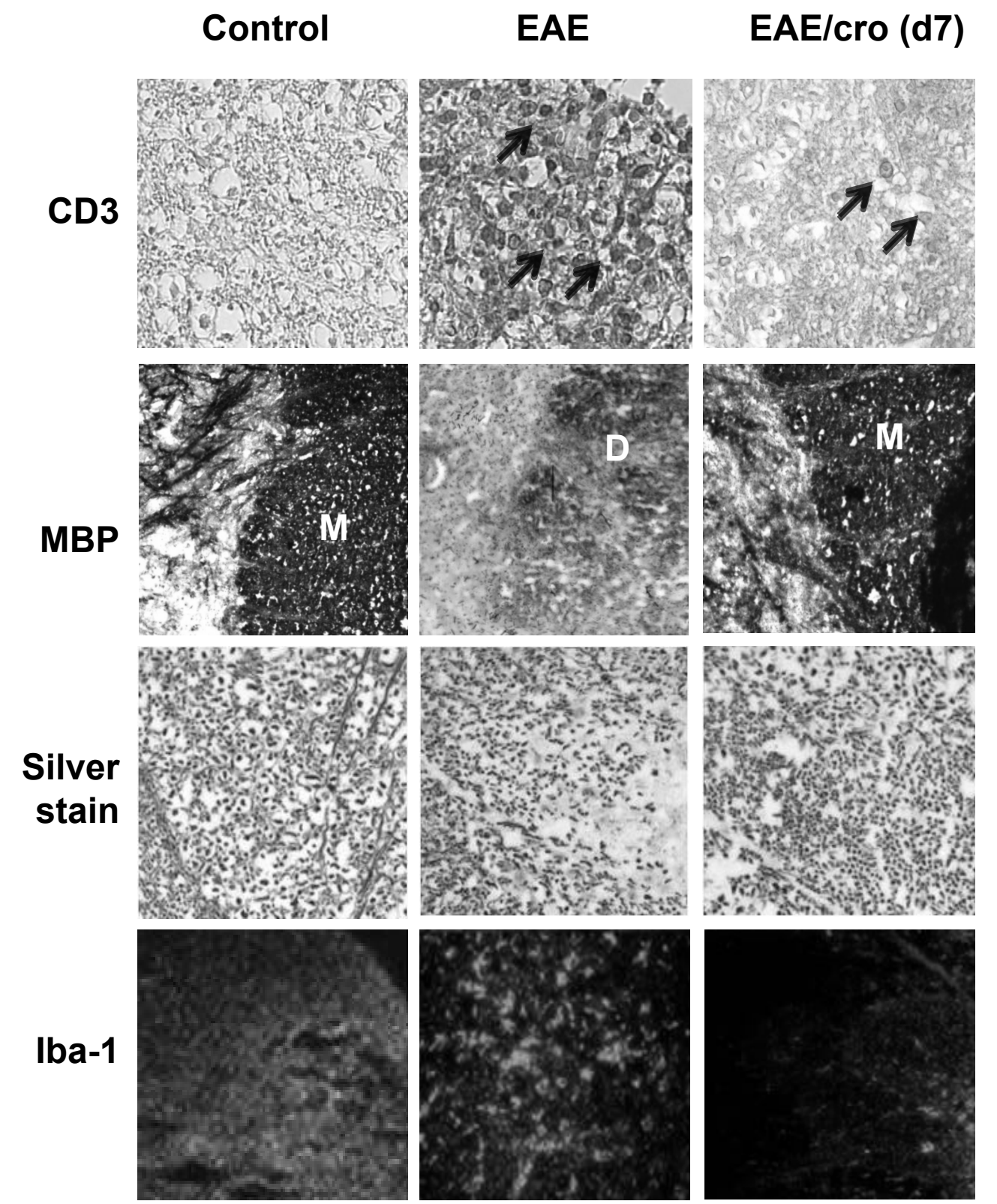


Figure 13

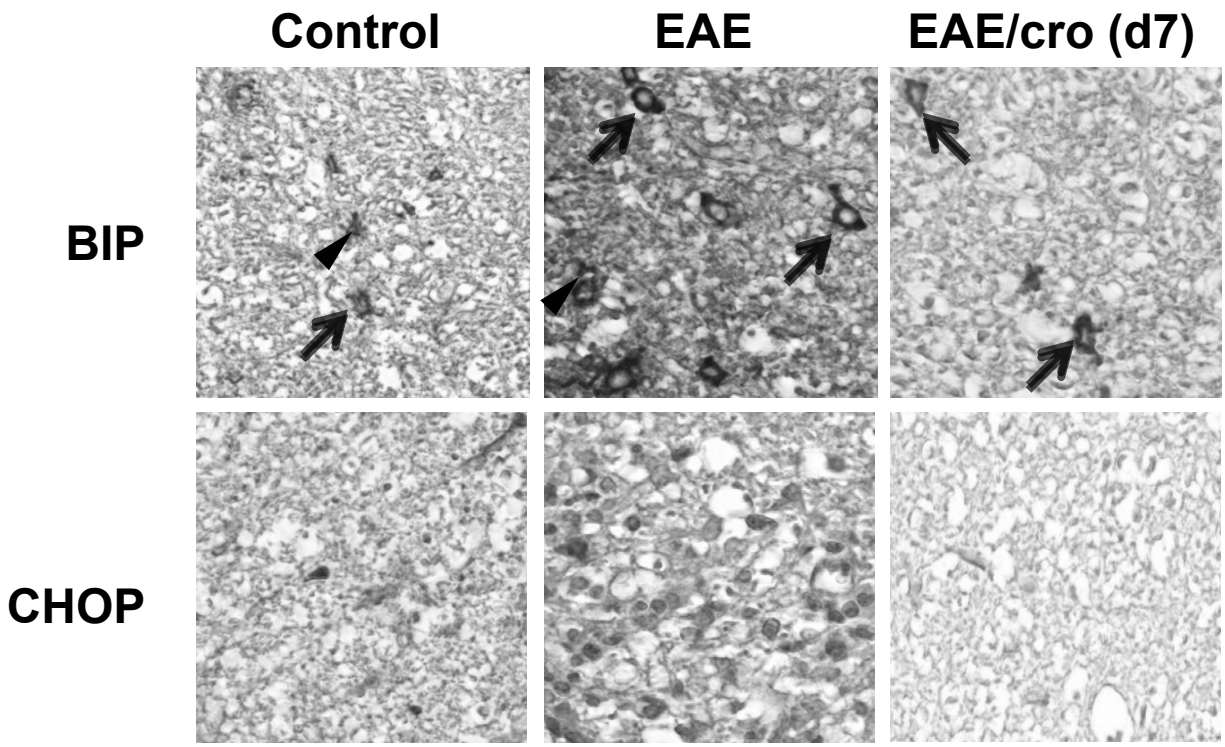
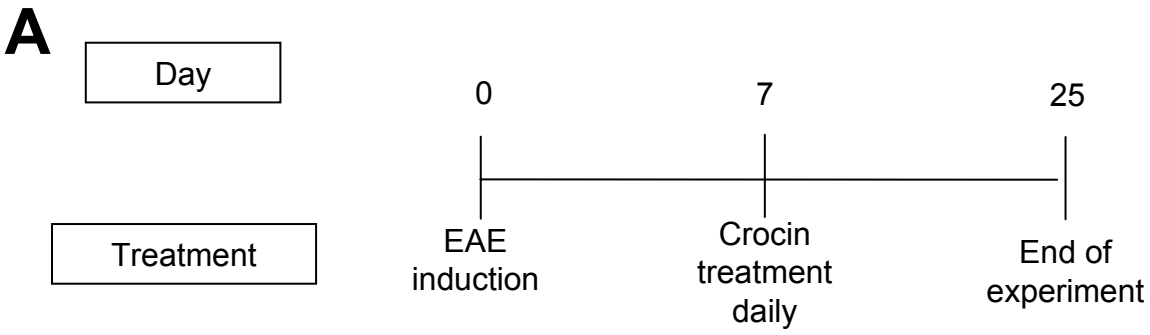
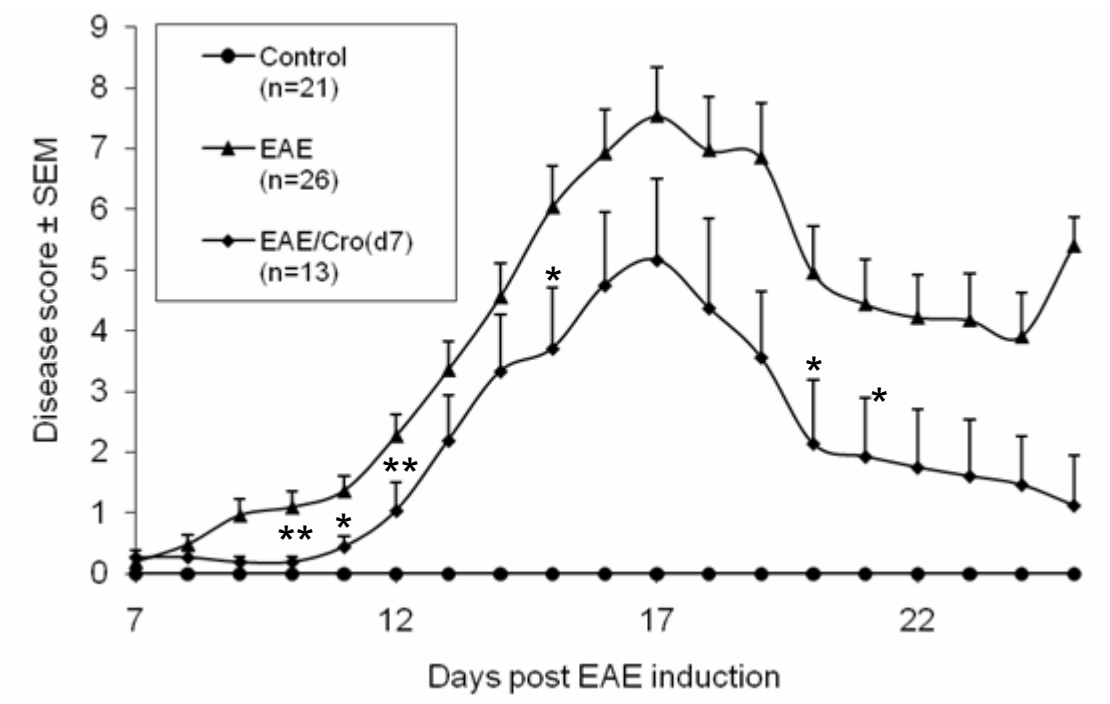
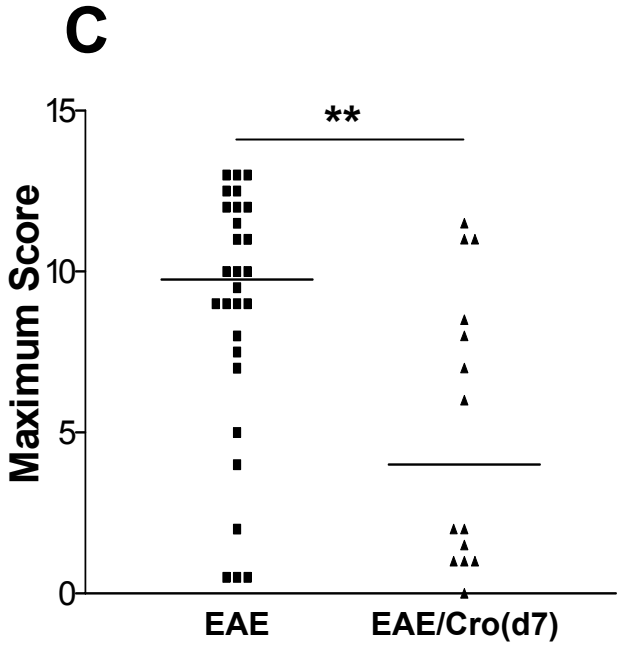


Figure 14


A
Day
0
7
25
Treatment
EAE induction
Crocin treatment daily
End of experiment


B
Control (n=21)
EAE (n=26)
EAE/Cro(d7) (n=13)
Disease score ± SEM
Days post EAE induction


C
Maximum Score
EAE
EAE/Cro(d7)

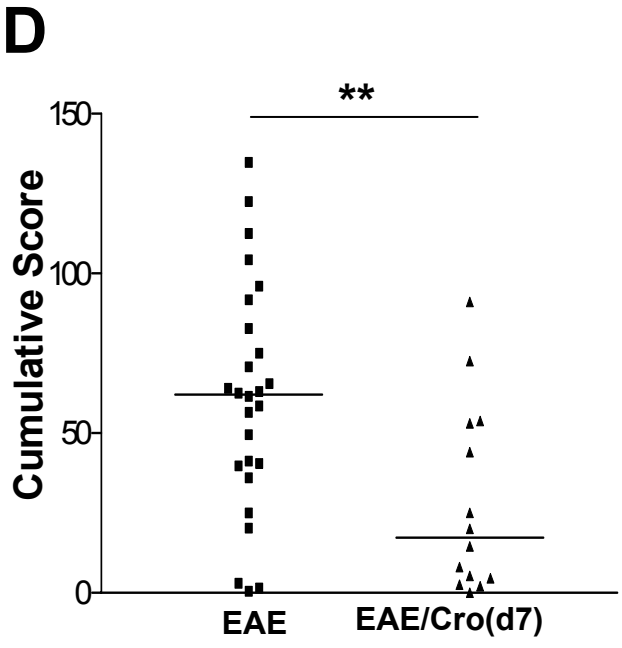
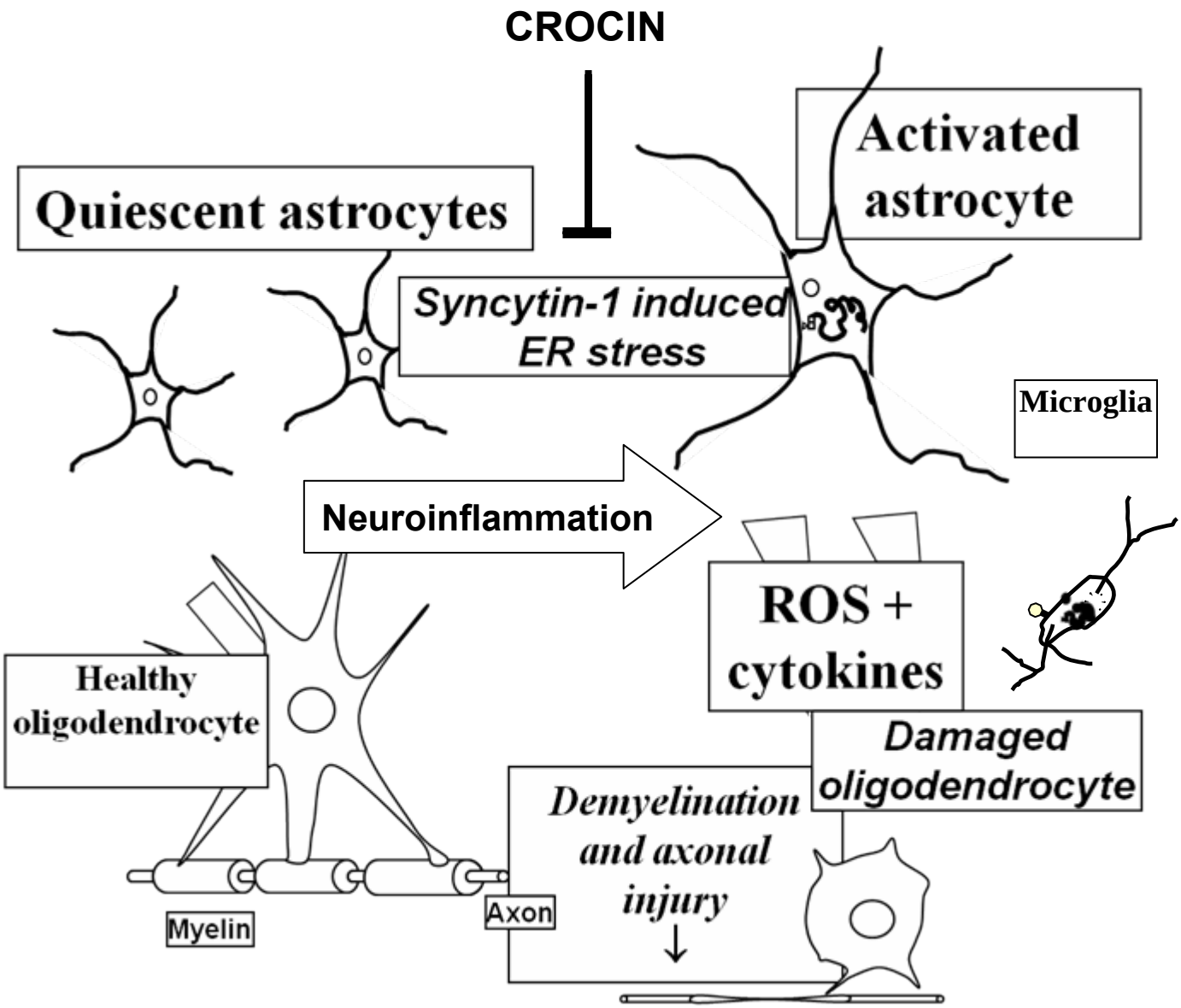

D
Cumulative Score
EAE
EAE/Cro(d7)

Figure 15


CROCIN
Quiescent astrocytes
Activated astrocyte
Syncytin-1 induced ER stress
Microglia
Neuroinflammation
ROS + cytokines
Healthy oligodendrocyte
Damaged oligodendrocyte
Demyelination and axonal injury
Myelin
Axon

Abbreviations

ALS – Amyotrophic Lateral Sclerosis

APC – Antigen Presenting Cell

ATF-6 – Activating Transcription Factor-6

BBB – Blood Brain Barrier

CHOP – C/EBP Homologous Protein

CNPase - Cyclic Nucleotide Peptidase

CNS – Central Nervous System

Cro – Crocin

CSF – Cerebrospinal Fluid

eIF-2α – Eukaryotic Translation Initiation Factor 2 alpha subunit

EAE – Experimental Autoimmune Encephalomyelitis

ER – Endoplasmic Reticulum

ERAD – Endoplasmic Reticulum Associated Degradation

ERK1/2 – Extracellular Signal-regulated Kinase ½

FGF-2 – Fibroblast Growth Factor-2

Gal-C – Galactocerebroside

GRK2 – G Protein Coupled Receptor Kinase 2

HERV – Human Endogenous Retrovirus

HIF-1 – Hypoxic Induced Factor-1

HLA – Human Leukocyte Antigen

IFN – Interferon

IL – Interleukin

IP – Intraperitonal

IRE-1 – Inositol Requirement Enzyme-1

JNK – c-JUN Kinase N-terminal Kinases

LFA-1 – Lymphocyte Function-associated Antigen 1

MBP – Myelin Basic Protein

MS – Multiple Sclerosis

MSRV – Multiple Sclerosis-associated Retrovirus

MHC II – Major histocompatibility Complex Class II

MMP – Matrix Metalloproteinase

MOG – Myelin Oligodendrocyte Glycoprotein

MRI – Magnetic Resonance Imaging

NAA – N-Acetylaspartate

NO – Nitric Oxide ODC – Oligodendrocyte

NOS2 – Nitric Oxide Synthase 2

OPC – Oligodendrocyte Progenitor Cell

NF-κB – Nuclear Factor Kappa-light-Chain-Enhancer of Activated B cells)

PAMP – Pathogen Associated Molecular Patterns

PAR-2 – Proteinase Activated Receptor-2

PDGF – Platelet Derived Growth Factor

PERK – PRKR-like Endoplasmic Reticulum Kinase

PRR – Pattern Recognition Receptors ROS – Reactive Oxygen Species

SC – Subcutaneous

SNP – Sodium Nitroprusside

SP – Serine Protease

TG - Thapsigargin

TGFβ – Transforming Growth Factor β

TLR – Toll-like Receptor

TM – Tunicamycin

TNF-α – Tumor Necrosis Factor alpha subunit

TRAF2 – Tumor Necrosis Associated Factor-2

UPR – Unfolded Protein Response

VLA-4 – Very Late Antigen 4

XBP-1 – X-box Binding Protein

Chapter 1 - Introduction

1.1 The unfolded protein response

1.1.1 The Endoplasmic Reticulum

The endoplasmic reticulum is a major organelle in eukaroyotic cells, which is uniquely responsible for protein synthesis, folding and modification. The endoplasmic reticulum has 3 basic functions: (1) protein synthesis, (2) protein folding and (3) calcium storage [1, 2]. When proteins are correctly folded, they are translocated outside the ER to the Golgi apparatus for protein trafficking [2]. However, when the levels of unfolded or misfolded proteins in the ER are too high for effective ER function, four main mechanisms can be activated [2]. Collectively these four mechanisms are termed the unfolded protein response (UPR) [2].

1.1.2 The unfolded protein response

The UPR is a homeostatic mechanism, which is utilized by cells to adapt to intra-cellular changes. The principal role of the UPR is to alleviate un- or mis-folded protein aggregation in the ER, ensuing intracellular homeostasis [3]. This homeostasis regulation is four-fold. The first mechanism of the UPR is *to increase the expression of ER chaperones* to increase the rate of correct protein folding in the ER and export them out of the ER [2]. If this mechanism fails, the second UPR mechanism is *translation attenuation*, which decreases the rate at which proteins are synthesized in the ER and hence decreases the rate at which misfolded and unfolded proteins accumulate in the ER [2]. If these

two mechanisms are not successful, the third UPR mechanism, *ER-associated degradation (ERAD)*, is activated [2]. During ERAD, proteins are tagged with ubiquitin and retrotranslocated out of the ER into the cytoplasm where they are degraded via the ubiquitin-proteosome pathway [4]. However, if ERAD fails, misfolded and unfolded proteins aggregate in the ER and this initiates the fourth and final UPR mechanism: *ER stress* [2]. ER stress can lead to many intra- and inter-cellular advantageous adaptations however if it is unregulated it can disrupt intra-cellular homeostasis by inducing growth arrest, DNA damage, oxidative stress, activation of ER chaperones, inflammation and apoptosis [2, 3, 5].

1.1.3 ER stress

ER stress is a highly conserved mechanism by which cells deal with aggregation of misfolded and unfolded proteins [18]. There are 3 main ER stress cascade initiators: PERK (PRKR-like endoplasmic reticulum kinase), IRE-1 (inositol requirement enzyme-1) and ATF-6 (activating transcription factor 6) [6, 7]. Another early central protein in the ER stress pathway is binding immunogenic protein (BiP). In the absence of ER stress, BiP is responsible for binding to the 3 main cascade initiators to prevent their respective cascade initiations. PERK is a protein kinase that is activated when there is ER stress; its role in ER stress is to phosphorylate eIF-2α and stop translation [1, 8]. IRE-1 has kinase and endoribonuclease activity however its cardinal function in ER

stress is to cleave an intron in pre-mature transcript of *XBP-1* (X-box binding protein) to yield a mature *XBP-1* transcript which has transcriptional regulation [5, 8]. The third ER stress sensor, ATF6, is a transcription factor that has a basic region and a leucine zipper motif [5, 6]. The principal role of ATF6 in ER stress is to enhance transcription of ER chaperones [5, 6]. In the absence of ER stress, the three ER stress sensors are bound to BiP and expressed at a very low level [7]. BiP, the main control for these three ER stress cascade initiators, is an ER glucose regulated chaperone that has a major function in ER stress [4-6]. Upon the initiation of ER stress, BIP dissociates from the three ER stress cascade initiators which activate their own unique sets of pathways [7].

Upon dissociation from BiP, PERK, will phosphorylate the 2α subunit of eukaryotic translation initiation factor (eIF-2α) to attenuate translation [5]. The eIF-2α subunit is involved in the initiation of protein translation in the ER [5]. Phosphorylated eIF-2α will enhance transcription of specific transcripts such as ATF4 or chaperones such as CCAT/enhancer binding C/EBP homologous protein (CHOP), which will activate the transcription of anti-oxidant genes and stimulate the expression of ERAD component genes [2, 5, 9]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a key transcriptional regulator and once it is in the nucleus it will activate transcription of several inflammatory genes[5]. Inhibitor of κBs (I-κB) is an inhibitor of NF-κB and has a shorter half life than NF-κB [5]. Therefore global attenuation

of translation by eIF-2α phosphorylation leads to NF-κB dissociation from Iκ-B [5]. NF-κB then becomes translocated in the nucleus and transcribed, resulting in increased expression of ROS-producing enzymes such as inducible nitric oxide synthase (iNOS) and an increase in pro-inflammatory cytokines in the central nervous system (CNS) will contribute to neuropathology of neuroinflammation [5, 10].

When *IRE-1*, the second main ER stress cascade initiator, dissociates from BiP*,* it will cleave an intron in the pre-mature *XBP-1* and yield a mature *XBP-1* splice variant transcript (*XBP-1/S)* [7]. XBP-1 is a transcriptional regulator in ER stress and also plays a role in major histocompatibility complex class II (MHC II) regulation [6, 11]. *XBP-1/S* will then translocate into the nucleus and activate the transcription of ER chaperones [2]. IRE-1 can also activate NF-κB when it forms a complex with tumor necrosis receptor-associated factor-2 (TRAF2) [12]. This will be discussed in the next section.

The third main ER-stress sensor, ATF-6, is a transcription factor that gets cleaved by serine proteases, SP1 and SP2, and then gets translocated into the nucleus to activate the transcription of ER chaperone genes [7].

1.1.4 ER stress and the nervous system

There is an emerging literature defining the role of the UPR in the nervous system, particularly in neurodegenerative and inflammatory disorders [3]. A common characteristic of many neurodegenerative

diseases is the accumulation of misfolded proteins, however it is unknown to what extent protein misfolding can be attributed to neurodegeneration [3]. The UPR is a homeostatic mechanism which is beneficial to the cell however; failure of induction or exploitation of the UPR can contribute to the pathogenesis of neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Parkinson's and MS [3]. Failure to induce ER stress, as the last resort of the UPR, can result in aberrant protein accumulations inside the ER which contribute to neurodegenerative disorders [13]. Similarly, over-expression of any ER stress cascade initiators: PERK, IRE or ATF6, can result in a range of potential consequences downstream of ER stress pathways including calcium signals, redox homeostasis and apoptosis [3, 5]. Moreover, there is now a strong consensus that ER stress is connected to inflammation by several factor molecules, particularly reactive oxygen species (ROS) and NF-κB [14].

1.1.5 ER stress and inflammation

ROS are highly reactive small molecules formed by the presence of unpaired electrons [15]. Protein folding is an energy demanding process that occurs in oxidizing conditions, particularly for the formation of disulphide bonds [14]. Because oxygen is the terminal electron recipient in the electron transport chain in formation of disulphide bonds, protein folding leads to the production of ROS [16]. Therefore, an increased

protein folding load in the ER produces accumulating ROS and potential inflammatory response [10].

Furthermore, NF-κB can be induced by the PERK or IRE-1 cascades to initiate inflammation[17]. NF-κB is a chief transcriptional regulator of induction of inflammation [18]. IκB is bound and inhibits NF-κB activity in the absence of inflammation [5]. As previously described, PERK leads to overall translation attenuation and since IκB has a shorter half-life than NF-κB, PERK induction will lead to the global activation of NF-κB [19]. Similarly, TRAF2 can form a complex with IRE-1α and also activate inflammatory genes through a phosphorylation cascade [20]. IRE-1α-TRAF2 complex can phosphorylate JNK, which phosphorylates transcription factor activator protein 1 (AP1) [21]. The precise mechanism how NF-κB initiates inflammation through ER stress however, experiments using calcium chelators and antioxidants indicate that ROS is also a signal for ER-associated NF-κB induction during ER stress [22]. Likewise metabolic factors, free radicals and calcium homeostasis are also factors which have the potential to link ER stress to inflammation [5].

While most neurodegenerative diseases involve inflammation, ER stress is important to avoid protein aggregation but controlled ER stress is also crucial to avoid inflammation [5]. Specifically, in the animal model of MS, experimental autoimmune encephalomyelitis (EAE), interferon-γ (IFN-γ) treatment has been shown to activate ER stress and cause apoptosis of oligodendrocytes [23]. Therefore, it is necessary to regulate

the balance between misfolded protein aggregates and inflammation to prevent the loss of ER homeostasis in neurological diseases. This concept supports the idea that ER stress might also play a crucial role in MS. Additionally; several studies of neurotropic murine retroviruses have reported the induction of inflammation and ER stress-related genes during viral infection [24]. Moreover, ER stress was specifically related to individual envelope protein expressed by the retrovirus HERV-W [24].

Similarly, several studies of neurotropic murine retroviruses have reported the induction of inflammation and ER stress-related genes during viral infection [24]. Specifically, ER stress is activated by a neurovirulent murine retrovirus, FrCas, which contains the envelope CasBrE [24]. It has been found that protein misfolding is restricted to the envelope protein Cas BrE [24].

1.1.6 The role of reactive oxygen species (ROS) in MS

While ER stress seems to play a role in MS pathogenesis, there is growing evidence that oxidative stress and ROS are also involved in the pathogenesis of MS [5, 25]. It has been demonstrated that redox reactants cause oligodendrocyte (ODC) death [25]. A study from the Power laboratory showed that antioxidants prevent Syncytin-1 induced ODC death [25]. Ferulic acid is an anti-inflammatory, anti-oxidant and ROS scavenging drug that was shown to inhibit Syncytin-1 induced neurological impairments in an animal [25]. Since ODCs are a chief target of injury in MS, ROS scavenging molecules, such as ferulic acid,

are critically important in the development of therapeutic agents for neurological disorders [25]. Equally, there is evidence indicating that ROS resulting from aberrant protein folding can cause ER stress and vice-versa [5]. For example, reduced glutathione is used to create disulfide bonds during correct protein folding [26]. With a depletion of reduced glutathione, proteins will be misfolded because disulfide bonds will not be able to form and this will affect protein function and generate ROS. *Crocus sativus* (crocin) is the active ingredient in saffron and is used in folk medicine [29, 30]. Crocin is also an anti-inflammatory agent, free-radical scavenger, inhibitor of oxidation reactions and suppresses ethanol-induced learning impairments [27, 28]. Crocin has also been reported to reduce TNF-α-induced death of neuronally differentiated PC-12 cells [29]. Since crocin is an ROS scavenger and abrogates neuron cytotoxicity caused by TNF-α, a pro-inflammatory cytokine, we decided to investigate its effect on ER stress and neuroinflammation in the context of MS and EAE.

1.2 Human Endogenous Retroviruses

1.2.1 Background

Human endogenous retroviruses (HERVs) are segments of proviral DNA, which were acquired as ancient infections 2 to 70 million years ago and become integrated into the human genome [30]. However, HERVs are no longer replication competent or infectious [31]. HERVs represent approximately 8% of the human genome [25].HERVs contain three

genes: *gag*, *pol* and *env* [32]. The *gag* (group antigen) gene encodes proteins in exogenous retroviruses, which comprise the capsid protein [32]. The *pol* gene encodes proteins for several enzymes, including reverse transcriptase, integrase and protease [32]. Lastly, the *env* gene encodes for subunit or transmembrane envelope proteins. The human genome contains approximately 70 *gag*, 100 *pol*, and 30 *env* HERV-W related regions [33]. Additionally, several retroviral elements are associated with retroviruses, e.g. long terminal repeats (LTRs) and long and short interspersed nuclear elements (LINEs, SINEs) [32]. These elements have the ability to modulate expression of HERVs [32].

1.2.2 Multiple sclerosis-associated retrovirus (MSRV)

MSRV was first discovered in the late 1980s when leptomeningeal cells were isolated from cerebrospinal fluid (CSF) of an MS patient [33]. Reverse transcription- polymerase chain reaction amplification of proviral DNA revealed a novel retroelement [33]. There is substantial evidence pointing to MSRV as a virulence factor in MS disease progression [33, 34]. Moreover, previous studies by Zawada *et al* indicated that MSRV *pol* sequences were increased in peripheral blood mononuclear cells (PBMCs) in MS patients [35]. While MSRV has been implicated in MS disease progression, it is also a member of the HERV-W family [32, 34]. Recent evidence by Antony *et al* showed that the *env* sequences of MSRV, HERV-W ERVWE1 on chromosome 7q, share 81% sequence identity [34].

1.2.3 Syncytin-1

Syncytin-1, the surface unit, encoded by HERV-W *env* (ERVWE1) [36]. It is expressed in the placenta and its primary role is to mediate syncytial fusion of the villous trophoplast in the placenta [36]. In addition to Syncytin-1's role in the placenta, data from our group indicated that it plays a role in neurodegeneration and neuroinflammation [25, 37]. ERVWE1/Syncytin-1 exhibit significantly increased transcript and protein envelope levels in the brains of MS patients compared to brains of non-MS controls, whereas there were very little changes in the transcript levels of *HERV-E, -K* and *–H* genes [25]. These observations suggest that HERV-W is specifically involved in the pathogenesis of MS. Syncytin-1, exerts neuroinflammatory effects and is over-expressed in the CNS of MS patients [37]. In a CNS inflammatory and neurodegenerative milieu of MS, Syncytin-1 was found to be more highly expressed in the glial cells of MS patients relative to patients without MS, specifically in astrocytes [25].

1.3 Neurocytology

1.3.3 Astrocytes

Astrocytes are the most abundant glial cell in the brain and have a wide range of functions [38]. Astrocytes are star-shaped glia that were long though to simply provide structural and support functions to the CNS [39]. However, astrocytes are now recognized to have numerous functions in both the healthy and diseased CNS [39]. More specifically,

astrocytes play an active role in shaping MS pathology [37, 39, 40]. There are two types of astrocytes: protoplasmic and fibrous [38]; protoplasmic astrocytes are located in gray matter whereas fibrous astrocytes are usually observed in the white matter [38]. The morphology of both types differs in that fibrous astrocytes exhibit longer processes than protoplasmic astrocytes. Functions of astrocytes in the healthy CNS include regulation of blood flow by interaction with blood vessels, fluid, ion, pH and transmitter homeostasis, synaptic transmission and CNS metabolism [38]. Also, recently astrocytes have been thought to maintain the integrity of the blood brain barrier (BBB) [38, 41]. It is widely understood that astrocytes can affect both anti- and pro-inflammatory responses [38]. When astrocytes are targeted in a neurodegenerative disease such as MS, they will alter multiple processes in the CNS [39]. For example during MS, astrocytes promote demyelination by activating autoreactive $CD4^+$ T cells via MHC class II, by secreting IL-12, IL-23 and IL-17 to polarize T cells to a T_H1 and T_H17 phenotype and by secreting chemokines to attract inflammatory cells such as T cells, infiltrating macrophages and activated microglia, which have detrimental roles in neuroinflammation and axonal damage [39]. Astrocytes can also hinder remyelination by inhibiting axonal regeneration, by preventing axonal growth because of astrogliosis (abnormal proliferation of astrocytes) and by preventing oligodendrocyte progenitor cell (OPC) maturation into oligodendrocytes [39]. The upregulation of glial fabrillary acidic protein

(GFAP), vimentin and nestin in the glial scar provides a physical barrier against remyelination [39]. Likewise, it has been shown that when human astrocytes are transfected with Syncytin-1, protein misfolding occurs [37].

1.3.2 Microglia

Microglia represent approximately 5 to 10% of adult CNS cells and are considered to be similar to tissue macrophages [42]. It is widely recognized that microglia act as the CNS's innate immune system and are essential for phagocytosis in the CNS [43]. In the past two decades, microglia have emerged as a pivotal players in mediating inflammatory responses in the CNS [42].

Like astrocytes, there are different types of microglia. They can be grouped into two main categories based on their location in the CNS[42]. Peri-neuronal microglia wrap around neuronal cell bodies and perivascular microglia which are in close proximity to blood vessels [42]. Microglia can exist in resting or active states [42]. The role for microglia in MS is not fully understood, however it is thought that perivascular microglia act as antigen presenting cells (APCs) for infiltrating T cells and initiate neuroinflammation by T_H1 cytokines, demyelinating agents and ROS species such as nitric oxide [42]. It is widely recognized that microglia are essential in the phagocytosis of myelin [44].However, similar to astrocytes, it has been shown that microglia can exert anti-inflammatory responses in MS as well [42]. When IL-4, an anti-inflammatory cytokine was injected into the CSF of EAE mice, an animal

model of MS, it was able to protect mice from neurological disease by activation of microglia [45]. Additionally, it has been demonstrated that microglia actively participate in the process of remyelination in remitting phase of relapsing-remitting MS patients [43].

1.3.3 Oligodendrocytes (ODCs)

Oligodendrocytes are the myelinating cells of the central nervous system [46]. Myelination of axons greatly increases the speed at which an action potential travels along the axon to the synapse [46]. Therefore, myelination is important for essential signal transduction in the central nervous system [46, 47]. In MS, where demyelination is a hallmark feature, loss of myelin contributes to impaired motor, cognitive and sensory functions by slowing signal transduction [47]. In healthy CNS, the number of oligodendrocytes are regulated by apoptosis, which is a controlled form of cell death [46]. However in a pathogenic milieu such as multiple sclerosis, oligodendrocytes are attacked by other glia and infiltrating T cells that have been activated by APCs which have recognized myelin antigens [47, 48]. It is clear that oligodendrocyte death induced by several different sources such as inflammatory cytokines, T cells, glia, however it is not well understood how these mechanisms are activated [48]. Since oligodendrocytes have a high energy and metabolic demand, it makes sense that they are so vulnerable in MS [48].

1.3.4 Neurons

Neurons are the principal cells in the CNS responsible for signal transduction. A neuron is composed of three anatomical parts: a cell body, an axon and synapse. The cell body is mononucleated, the axon is responsible for transmitting action potentials towards synapses where the action potential depolarizes dendrites, which are responsible for receiving action potentials, on a neighbouring neuron [49]. In multiple sclerosis, axonal transection and neuronal loss are cardinal neuropathological features and can be detected by Bielchowski silver staining in both MS patients and EAE mouse model [50].

1.3.5 Endothelial cells

Endothelial cells are important in transport of macronutrients and micronutrients, receptor-mediated signalling and leukocyte trafficking across the blood brain barrier [51]. The astrocyte-endothelial cell interaction increases endothelial cell tight junctions. Endothelial cells are critical in maintaining a healthy BBB to prevent selective permeability [52]. Pericytes are responsible for regulating endothelial cell proliferation, survival, migration and differentiation [52]. Neurons are metabolically demanding therefore require tight interaction with the vascular unit at the BBB [52].

1.3.6 Ependymal cells

The ependyma is the ciliated epithelium that lines the ventricular surfaces of the CNS [53]. Ependymal cells are involved in the production

of cerebrospinal fluid (CSF) and are important in trophic and metabolic support [53]. Ependymal cells share a tight association with the pluripotent stem cell [53]. Therefore, there is a role for ependymal cells as a modulator of stem cells. Ependymal cells also provide a trophic support role by producing growth factors, specifically fibroblast growth factors (FGF) [53, 54]. Well developed tight junctions are a crucial characteristic of ependymal cells [54]. In addition to tight junctions, ependymal cells also have gap junctions to regulate intracellular factors including ion homeostasis, volume control and adherent association between neighbouring cells [54]. Ion homeostasis and CSF flow are thought to be regulated by cilia, which beat in a manner consistent to the bulk of the CSF flow [54].

1.4 Multiple Sclerosis

1.4.1 History of Multiple Sclerosis

Jean-Martin Charcot is the most common name associated with framing the term Multiple Sclerosis (MS) in 1868 [47, 55]. It has been suggested that the first reported case of MS most likely occurred in the late fourteenth century [55]. The detailed documentation from the Vatican archives of Saint Ludwina of Schiedam from Holland, provides a potential description of MS. The first sign of weakness in Saint Ludwina was when she fell on her skates at the age of 16. The partial recovery from her fall was later accompanied by recurrent episodes of loss of balance, weakness and visual impairments. Subsequent similar cases occurred in

the next several centuries [55]. Charcot observed that many MS patients had a neurological disability such as cognitive and motor impairments [55]. With work by Edward Rindfleisch, who first described plaques around blood vessels in the brain, and Karl Fromman, who first described demyelination within lesions, Charcot was able assemble this information and to propose the term ‘multiple sclerosis’ [55].

Before 1960 there were over 100 therapies for MS including anticoagulants, antibiotics, histamine desensitisation, various diets, vaccines and anti-cancer agents, but none were effective [55]. With help of imaging techniques and advances in basic research, the last 50 years has seen the greatest progress in understanding the mechanisms of MS, pathology of MS and developing therapies to ameliorate disease the course [55].

1.4.2 MS clinical definition and types of MS

MS is an organ-specific autoimmune disease of the central nervous system (CNS): brain and spinal cord, characterized by neuroinflammation and neurodegeneration [41, 47]. The most common symptoms of MS include impairments of motor, sensory and neurocognitive functions [41]. MS is a heterogeneous disease and can have distinct symptoms in different individuals depending on which area of the brain is affected [41]. For example, optic neuritis represents inflammation in the optic nerve and can result in blindness. There are at least three types of MS patients: relapsing remitting (RR), primary

progressive (PP) and secondary progressive (SP) [47]. Different mechanisms leading to the different phenotypes are not well understood; approximately 75% of MS patients are RR-MS patients of which most will progress to become SP-MS [47]. RR-MS patients differ from PP- and SP-MS patients in that they will experience repeated relapses followed by resolution of neurological signs and symptoms, whereas the neurological disabilities of PP- and SP-MS patients will gradually progress.

1.4.3 MS epidemiology

It is widely understood that MS occurs more frequently in females [56]. Several reviews highlight the high female: male ratio in MS (3:1) however, the majority fail to explain the reason for these differences [56-58]. In the past 4 decades, the female: male ratio has increased steadily. The reason for this is uncertain however there are several factors which are suspected to play a role in the high female: male MS ratio including seasonal effects and hormonal fluctuations such as vitamin D levels and sex steroids [56]. However, these factors are still controversial because there are several factors such as ethnic diversity, family history of MS, longitudinal nature and genetics, which also contribute to MS risk[59]. It is difficult to solely isolate and study the gender risk of MS [59].

MS is most prevalent in countries distant from the equator, such as Canada, Scandinavia, and New Zealand. These countries received less sunlight and this might skew T cell phenotype or impair vitamin D biosynthesis[47]. It has been proposed that vitamin D insufficiency might

play a role in MS pathogenesis [47, 56]. However another hypothesis, the hygiene hypothesis explains why developed countries have higher prevalences in MS than third world or developing countries. This hypothesis states that individuals exposed to infectious agents at a young age have a decreased susceptibility to allergies and autoimmune diseases [60].

1.4.4 MS immunogenetics

Although MS is not purely a genetic disease, there are some genetic determinants, which make an individual more susceptible to MS. However, genetics alone do not explain MS and environmental triggers also contribute to disease induction [47]. Despite many studies and significant advances, the role for genetics in MS remains unclear [47]. There have been over 20 whole genome wide screens in different MS populations with 6,000 microsatellite markers [47]. Many T-cell mediated diseases confer genetic susceptibility in the HLA-DR and –DQ genes and MS is not an exception [47]. The highest susceptibility comes from two DR alleles that are in tight linkage disequilibrium [47, 61]. Linkage disequilibrium is non-random association of alleles at two or more loci [61]. For example, the effect from one allele in genetic susceptibility may not be caused by that allele but rather the allele it is associated with [61]. In MS it is thought that HLA-DR and -DQ confer the largest genetic susceptibility [47]. Although some MS patients showed increased frequencies in HLA-A7 and B3 alleles, MHC class I proteins, but these

association were traced back to linkage disequilibrium with the DB1*1501 allele [62]. It is thought that polymorphic residues of the T cell receptor (TCR)-exposed surfaces of the α-helical regions of DR-DQ-α and –β could be selected for autoimmune-prone T cells [47, 63].

However, a recent study by Baranzini *et. al.* showed evidence that genetics may not play a role in MS [64]. Here they demonstrated the first report of female twins with MS and individual genome sequences [64]. Baranzini *et. al*. found that there were no differences between twins in approximately 3.6 million single nucleotide polymorphisms or 0.2 insertion-deletion polymorphisms despite being discordant for MS occurrence [64]. Similarly, there were only 2 to 176 differences in the methylation of approximately 2 million CpG dinucleotides were detected between siblings of the three twin pairs [64]. These studies suggest that genetics and epigenetics do not exclusively dictate disease occurrence in MS. Despite the uncertainty of the involvement of genetics in MS, advances have been made and it is difficult to pin point an allele since MS is a variable and heterogeneous disease [47].

1.5 MS pathogenesis

1.5.1 Infections leading to MS

There are several bacterial and viral infections, which are thought to contribute indirectly to the pathogenesis of MS [47]. A list of viruses found to invade or be expressed in the CNS in association with MS include Epstein-Barr virus (EBV), human herpes simplex virus-6 (HSV-6),

human coronaviruses and human endogenous retroviruses (HERVs) [12]. Other than HERVs, herpes- viruses are candidate MS-triggering agents because of their neurotropism and tendency to produce latent and recurrent infections [47]. Although, anti-EBV antibodies are thought to be increased in MS patients, evidence supporting a direct role for EBV in MS is sparse and this idea remains controversial. [47].

1.5.2 Blood brain barrier

The blood brain barrier (BBB) is a complex multicellular structure composed of primarily endothelial cells, astrocytes, pericytes and tight junctions [52]. Primary functions of the blood brain barrier are to protect the CNS from toxic substances in the blood, supply nutrients and filter harmful substances in the brain to the bloodstream [52]. BBB integrity is important in keeping a homeostatic and healthy CNS [51]. BBB dysfunction is involved in a wide array of neurological diseases including neuroinflammatory and neurodegenerative disorders such as multiple sclerosis [51].

In multiple sclerosis, the BBB becomes more permeable to lymphocytes and macrophages, permitting them to migrate into the CNS [47, 51]. It is widely recognized that matrix metalloproteinases (MMPs), specifically MMP9, alter BBB permeability in MS lesions leading to BBB openings [51]. Similarly, occludin dephosphorylation alters BBB integrity [51].

1.5.3 Leukocyte transmigration

In multiple sclerosis, autoreactive T cells become activated and adhere to endothelial cells of the BBB via adhesion molecules such as lymphocyte function associated antigen-1 (LFA-1) and very late antigen (VLA-4) [47]. It is not completely understood how an inflammatory MS lesion occurs but it is assumed that proinflammatory cytokines (IFN-γ , IL-23, TNF-α, LT) and chemokines (RANTES, IP-10, IL-8) activate CNS resident cells and recruit immune cells [47]. It is not clear whether the BBB permeability is first altered by CNS cell activation or by cells in the periphery, but it is likely that both CNS and periphery cells contribute to increased BBB permeability. Activated immune cells traverse the BBB from the blood to the CSF or from the blood to parenchymal perivascular areas of the brain [65]. In multiple sclerosis, most leukocytes cross the BBB via the later mechanism [65]. Astrocytes can serve as intermediate signals to endothelial cells [65]. Similarly, perivascular macrophages and parenchymal microglia produce pro-inflammatory conditions to aid in leukocyte transmigration [65].

Leukocyte transmigration is a multi-step process. First , activated (autoreactive) leukocytes will roll along the endothelial surface and loosely tether their ligands to selectins on endothelial [65]. Activation of chemokine receptors initiates conformational changes in leukocytes leading to high-affinity interactions between leukocytes and endothelial cells [65]. After adhesion to the endothelial cell lining via integrins,

leukocytes will extend protrusions into the CNS through the BBB in search of guidance cues for extravasation [65].

1.5.4 Neuroinflammation and neurodegeneration

Once activated autoreactive leukocytes access the brain parenchyma they will cause neuroinflammation and neurodegeneration, which are part of the autoimmune disease process in MS [41]. During the neuroinflammation stage of MS, the (BBB) permeability is altered and allows the infiltration of T lymphocytes and other immune system mediators such as TNF-α and IFN-γ to traverse across the BBB into the CNS [41, 66]. This can lead to the apoptosis of oligodendocytes (ODCs), which heralds demyelination of axons [23, 67]. When myelin is destroyed, it is likely that additional myelin antigens are presented to T cells though epitope spreading and the bystander effect [47]. This causes additional recruitment of pro-inflammatory cytokines [47]. Neurodegeneration is also a key part of MS [68, 69]. Neuronal injury or axonal damage is what characterizes neurodegeneration [68]. It is thought that the neuroinflammation is directly responsible for neurodegeneration and widespread brain atrophy, however the interaction between neuroinflammation and neurodegeneration is not well understood [47]. ER stress represents an interlocking process in which both inflammation and cell death pathways are potential outcomes.

1.6 MS neuropathology

1.6.1 The active demyelinating lesion

The brain is composed of two structural components: the white and grey matter. The white matter consists primarily of myelinated axons. In multiple sclerosis the primary characteristic of the inflammatory demyeltinating lesions occurs in the white matter [70]. This is marked by activation of resident microglia and infiltration of leukocytes, primarily macrophages [70]. However, it cannot be disregarded that multiple sclerosis is primarily a T cell-mediated disease, mostly of the CD8+ subtype [47, 70]. It is unclear that macrophage and microglia directly attack myelin sheaths but rather it is more established they are directed towards T_H1 cells and cause the release of inflammatory and injury related molecules such as TNF-α and ROS [70]. There are four different patterns of demyelination as described in the following section,

1.6.2 Patterns of demyelination

Pattern I demyelination is mainly induced by macrophage and microglial injurious toxins, mainly proinflammatory molecules e.g. TNF-α, IFNγ, nitric oxide (NO) and ROS [70]. Pattern II demyelination involves the presence of antibodies against myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) [70]. This type of pattern shares some characteristics with the autoimmune disease Guillain Barré syndrome [47]. In Pattern III, oligodendrocytes become more vulnerable to destruction [47]. Additionally there is presence of hypoxia and anoxia

factors such as NOS2 [70]. Hypoxic induced factor-1 (HIF-1) has recently be recognized as pathogenic factor and might contribute to the mediation of Patern III demyelination [70]. Pattern IV is the last and rarest subtype of demyelination [70]. This patern involves non-apoptotic degeneration of oligodendrocytes, however this subtype is not well understood. MS has been primarily thought to occur in the white matter of the brain but recent literature suggests that pathological features of MS are also observed in the grey matter [61].

1.6.3 Grey matter

Grey matter consists over half the brain volume and is composed of neuronal cell bodies, neuropil (dendrites and unmyelinated axons), glial cells and capillaries [71]. Unlike white matter, most of the axons are unmyelinated. Additionally, white matter contains fewer neuronal cell bodies. Grey matter is situated mainly at the surface of the cerebral hemispheres, the cerebellum and deep inside the cerebrum (basal ganglia). The involvement of grey matter in MS pathology is becoming more evident [72]. At first MS was thought to occur in active demyelinating lesions in white matter of the brain [72]. However, in the past decade it has become clear that that the active MS lesions represents only a fraction of MS pathology [72]. Non-lesional white matter and grey matter are also damaged during the course of MS. It is evident that there is grey matter loss during the first clinical onset [73]. Adalsteinsson *et al* examined N-acetylaspartate (NAA) concentrations by

NMR (a measure of neuroaxonal integrity) in brain tissue appearing normal by MRI imaging in RR- and SP-MS patients [74]. There was no difference between the two subtypes of MS patients in white matter but a loss of NAA in the grey matter of SP-MS patients [74]. This suggests that grey matter pathology accentuates neurological impairment in SP-MS patients [74]. In the last decades, there is a increasing data suggesting that grey matter plays an important role in MS disease progression [72]. Grey matter and white matter pathology analysis in during MS progress has been facilitated by advances in magnetic resolution (MRI) technology [72].

1.6.4 Magnetic Resonance Imaging (MRI)

Magnetic Resonance Imaging (MRI) is a technique used to visualize internal structures in the body. In multiple sclerosis, MRI is a sensitive technique [75]. While MRI technique has provided a good insight into the pathology of MS it is limited as a technique [75]. Accuracy in MRIs are not definitive and this should be confirmed with biopsy tissue which is very limited [75]. Similarly, MRI largely captures active lesions and fails to recognize fully grey matter pathology [72]. Despite MRI limitations, its correlation with histopathology is giving some insight of MS lesions [75]. The strongest advantage of the MRI is that it is non-invasive

1.7 Experimental autoimmune encephalomyelitis (EAE): An animal model for MS

1.7.1 History of EAE

Experimental autoimmune encephalomyelitis is an animal model for multiple sclerosis. A myelin antigen and adjuvant emulsification injected into mice to initiates an immune response to the myelin sheath in the CNS. EAE was originally derived from a rabies vaccine; Thomas Rivers, as a senior virologist, saw a parallel between MS and neurological impairments associated with viral disease vaccines that could affect the CNS or rabies [76]. Additionally, Rivers recognized work done by Koritschoner *et al*. which indicated repeated inoculation of rabbits with normal human spinal cord occasionally resulted in paralysis [76]. It became apparent that brain-specific antibodies and T cells caused a demyelinating pathology driven by a specific immune response [76]. When brain emulsions were mixed with *Mycobacterium tuberculosis*, an adjuvant, and paraffin oil, animals became paralyzed, manifestining as EAE [76].

1.7.2 EAE methodology

To induce EAE, a myelin antigen, pertussis toxin and *Mycobacterium tuberculosis* are needed. Myelin oligodendrocyte protein (MOG), proteolipid protein (PLP) and myelin basic protein (MBP) are the principal myelin proteins from which myelin antigens are derived. An emulsification of the myelin antigen and *Mycobacterium tuberculosis* is

injected sub-cutaneously (sc) along with an intra-peritoneal (IP) injection of pertussis toxin. 48 hours later, a second pertussis toxin injection is given intraperitoneally (IP) This results in a CD4+ T cell mediated autoimmune reaction directed against myelin antigens and causes demyelination and pathology similar to human demyelinating diseases [47, 76, 77].

1.7.3 EAE pathogenesis and pathology

EAE immunopathology is characterized by focal demyelination, axonal loss and cellular infiltrations [77]. Myelin loss in EAE is most commonly found in the perivascualar region of the CNS [77]. The location of lesions in EAE differs with the antigen used but it predominates in the lumbar spinal cord when using MBP or MOG [77]. Occasionally, lesions and neuroinflammation are also located in the hind brain when using the MOG antigen [77]. In contrast to MS where CD8+ T cells are thought to be an important T cell infiltrate, CD4+ T cells predominantly infiltrated the CNS in the EAE model [77]. Macrophages and activated monocyte resident cells are also observed in mice with EAE [77].

1.7.4 Limitations of the EAE model

Although EAE provides a good model for MS, its pathology is not identical. As previously stated, CD8+ T cells predominate in MS, whereas, CD4+ T cells infiltrating the CNS are predominant in EAE [77]. Additionally, in MS demyelination is not limited to perivascular areas, but demyelination can occur anywhere in the CNS [77]. Also in EAE, the

cytokine profile is T_H1 biased in PLP and MBP models whereas in MS it is more broad and varies between patients [77].

1.8 Hypothesis and specific aims

I hypothesize that ER stress mediates neuroinflammation and neurodegeneration causing glia cell damage with ensuing exacerbation of multiple sclerosis. My specific aims include the following: (i) Define the differential *ex vivo* effects of Syncytin-1 on the induction of ER stress, (ii) Characterize the effects of crocin on glia cytotoxicity, (iii) investigate the effect of crocin on EAE neuropathology and behaviour and (iv) determine whether ER stress induction with tunicamycin (a classic ER stress inducer) during EAE exacerbates or ameliorates neurological impairment.

Chapter 2 – Methods and Materials

Herein, I provide a list of the pertinent methods and materials used within my thesis.

2.1 Animals

C57BL/6 female mice were purchased from The Jackson Laboratory and maintained in the Health Sciences Laboratory Animal Services facility of the University of Alberta under conventional housing conditions. All experiments were approved by the University of Alberta Animal Care Committee.

2.2 Cell cultures

Oligodendrocytes were isolated from adult Sprague-Dawley rat brains and plated in polyornithine-coated chamber slides (Nunc). Oligodendrocytes were maintained in MEM containing 10% FBS, 0.1% dextrose, 2 mM l-glutamine, and 1 mM sodium pyruvate, 1% Penicillin, 1% streptomycin, 240µL of 20µg/mL gentamycin and 1mL of 0.5µg/mL fungizone for 5 days. Human neuronal cultures were prepared from 15-19 week fetal brains obtained with consent (approved by the University of Alberta Ethics Committee), as previously described [78]. Briefly, fetal brain tissues were dissected, meninges were removed, and a single cell suspension was prepared by trituration through serological pipettes, followed by digestion for 30 min with 0.25% trypsin (Life Technologies, Burlington, ON, Canada) and 0.2 mg/ml DNase I (Roche Diagnostics, Mannheim, Germany) and passage through a 70-µm cell strainer (BD

Biosciences, Mississauga, ON, Canada). Cells were washed 2 times with fresh medium and plated in T-75 flasks coated with poly-L-ornithine (Sigma-Aldrich, Oakville, ON, Canada) at 6–8 X 10^7 cells/flask and medium for growing human fetal neuron (HFN), atrocyte and microglia cells was subsequently added (named HFN medium), which was MEM supplemented with 10% FBS (Life Technologies), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 1 X MEM nonessential amino acids (Life Technologies), 0.1% dextrose (Sigma-Aldrich), 100 U/ml Penicillin (Life Technologies), 100 µg/ml streptomycin (Life Technologies), 0.5 µg/ml amphotericin B (Life Technologies), and 20 µg/ml gentamicin (Life Technologies). Specifically, for neuronal cultures, 25 µM cytosine arabinoside (Sigma-Aldrich) were additionally supplemented to prevent astrocyte growth. Astrocyte cultures, without cytosine arabinoside, were passaged once a week and in 4-6 weeks the neurons were eliminated; the remaining astrocytes were ready for HIV transfection or infection. For microglial cell cultures, suspended microglial cells collected by centrifugation at 1200 rpm for 10 min at 1 week after cultures were established. The collected microglia cells were grow in a new plate with the above medium (without cytosine arabinoside) and ready for HIV transfection or infection in two days.

2.3 Human brain tissues

Brain samples from MS and non-MS patients were obtained from the Laboratory for Neurological Infection and Immunity Brain Bank,

University of Alberta(MS, *n* = 8; non-MS, *n* = 5). Normal-appearing frontal white matter was homogenized, lysed in TRIzol (Invitrogen), and used in real-time PCR analyses.

2.4 Induction and assessment of EAE

C57BL/6 female mice were obtained from The Jackson Laboratory and maintained in the Health Sciences Laboratory Animal Services facility of the University of Alberta under conventional housing conditions. Mice were injected subcutaneously with 50 μg MOG ($MOG_{35–55}$ peptide; prepared by the Peptide Synthesis Facility, University of Calgary) emulsified in 100 μL of complete Freund's adjuvant (Sigma-Aldrich) supplement with 5mg/mol heat killed mycobacteria H37 RA (Difco Laboratories). Animals received intraperitoneal injections of pertussis toxin (0.3 μg; List Biological Laboratories) at the same time as MOG immunization and repeated 48 h later. Control animals were only injected with complete Freund adjuvant and pertussis toxin. Animals were assessed daily for EAE severity for 25 d using a 0–14 rating scale. EAE mice treated with crocin received a 100mg/kg/day dose of crocin starting 7 days post induction (Sigma-Aldrich) while control EAE animals received daily PBS injections. All experiments were approved by the University of Alberta Animal Care Committee.

2.5 Western blotting and antibodies

Cells were lysed with Laemmli buffer and 0.1% β-mercaptoethanol. Proteins from whole cell lysates were separated using

polyacrylamide gel electrophoresis. Protein fractions were transferred to a nitrocellulose membrane overnight using electrophoresis. Membrane was blocked with 10% milk and labelled with anti-Syncytin-1 (house, 1:100) of polyclonal (primary) antibody overnight. The Syncytin-1 immunolabelled membrane was then probed with anti-rabbit secondary antibody (1:1000) for 2hrs. Membranes were developed with horseradish peroxidise and exposed to X-ray on film (Canon)

2.6 Histological analysis

Formalin-fixed spinal cords of EAE or control animals were embedded in paraffin before sectioning. 4-μm sections from lumbar spinal cords were stained with Bielschowsky's silver impregnation method [79]. White matter from the dorsal column of 4 different animals per group was scanned and photographed using a microscope (Axioskop2; Carl Zeiss MicroImaging, Inc.).

2.7 Immunohistochemistry and immunofluorescence

Formalin-fixed, paraffin-embedded sections of mouse lumbar spinal cord were deparaffinised and rehydrated using decreasing concentrations of ethanol. Antigen retrieval was performed by boiling the slides in 0.01 M trisodium citrate buffer, pH 6, for 10 min followed by incubation with 0.3% hydrogen peroxide to block endogenous peroxidases. Sections were then preincubated with 10% normal goat serum, 0.2% Triton X-100 overnight at 4°C to block nonspecific binding. Antibodies against Iba-1 (1:500; Wako), CD3 (1:100; Santa Cruz

Biotechnology, Inc.), and MBP (1:1,000; Sternberger Monoclonals) followed by appropriate secondary antibodies were used to detect macrophage/microglial, T lymphocyte, and myelin immunoreactivity respectively.

2.8 Infrared imaging

Cultured oligodendrocytes derived from adult rat brains were stained with O1 anti-galactocerebroside monoclonal antibody (provided by V.W. Yong, University of Calgary, Calgary, Canada) that recognizes mature oligodendrocytes, followed by a Cy3-conjugated goat anti–mouse antibody. Slides were imaged by infrared imaging with Licor program.

2. 9 Real-time RT PCR

Mouse tissues or cultured cells were homogenized in TRIzol (Invitrogen) and total RNA was purified from the aqueous phase using RNeasy mini columns (Qiagen). Following treatment of RNA with DNase I (Promega), cDNA was prepared by anchored oligo(dT) (d(T18)VN)-primed reverse transcription of equal quantities of RNA using either Superscript II or Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's recommended protocols. Semi-quantitative real-time PCR was performed using Bio-Rad iQ SYBR green supermix (Bio-Rad) on either an iQ5 or an iCycler (Bio-Rad) according to the manufacturer's recommended protocols. All PCR primers used herein are provided in the supplemental table (Table 1). All data were

normalized to *GAPDH* mRNA levels and expressed as relative fold change (RFC) compared with controls along with SEM.

2.10 Transfection and dual luciferase assay

HFA cells were pre-treated with 100, 200 or 400μM of crocin (Sigma) for 5 hours co-transfected with either pHCMV-env vector containing ERVWE1 or pHCMV-empty (ERVWE1 digested out with EcoRI) and a vector containing an internal control promoter constitutively driving the expression of Renilla *luciferase* and a promoter encoding firefly *luciferase*, which is only activated when XBP-1 is spliced. Lipofectamine LTX (Invitrogen) and PLUS reagent (Invitrogen) were used as transfection reagents. Luciferase activity was quantified using a luminometer and XBP-1 splicing was normalized to the internal constitutively expressed Renilla *luciferase*.

2.11 Crocin and tunicamycin dilutions

Crocin (Sigma) was diluted in PBS and stored at +4^{o}C in the dark. Each day the crocin solution was adjusted to physiological pH (pH 7.4) with hydrochloric acid and filter sterilized. Tunicamycin (Sigma-Aldrich) was diluted in basic water (pH 9), which was adjusted with sodium hydroxide. It was necessary to store tunicamycin in a basic solution because it is not stable in non-basic pH. Each day tunicamycin was further diluted and adjusted to physiological pH using hydrochloric acid. Prior to mouse injections, tunicamycin solution (pH 7.4) was filter sterilized as crocin was.

Chapter 3 – Syncytin-1 mediated ER stress in neuroinflammation

3.1 Correlation of Syncytin-1 with XBP-1 spliced transcript and inflammation markers

Anthony *et al* previously reported the presence of ER stress in the brains of MS patients compared to non-MS brains [37]. However, Anthony *et* al didn't examine the XBP-*1* spliced transcript variant, a hallmark for ER stress. Hence the *XBP-1* spliced transcript (XBP-1/S) levels were investigated by real time RT-PCR to confirm the increase in ER stress among MS brains compared to non-MS brains using a primers designed to amplify the *XBP-1* spliced transcript variant (Figure 1A). MS brains exhibited a 3-fold increase in median *XBP-1* spliced transcript levels (Figure 1B). This increase in XBP-1/S was accompanied an increase in median HERV-W *env* (*ERVWE1*) levels in MS brains (Figure 1B). A correlation analysis was performed disclosing that the XBP-1 spliced transcript variant was positively associated with *HERV-W env* transcript abundance (Figure 1C, r=0.65, p<0.05). Given that MS is a pro-inflammatory disease, which is mediated by T cells and involves induction of multiple immune molecules such as pro- and anti-inflammatory cytokines, chemokines and granzymes [47], a correlational analysis was performed to determine the association between the levels of *HERV-W env* transcripts and relevant immune genes in the brains of MS and non-MS patients (Table 2). Importantly, HERV-W *env* transcript levels were significantly correlated with TGF-β, CD3 and IL-12p35. These

data suggested that XBP-1 and Syncytin-1 were associated. Similarly a heat map confirmed the increase of MS associated genes (HLA-DQA1, granzyme A, GFAP, CD3 and CD11c) in that subset of MS patients (Figure 2D). These observations were verified by comparing the relative abundance of individual transcripts in MS and non-MS brains disclosing that the MS brains did indeed exhibit increased immune gene transcriptional activity (Figure 1D). These results prompted us to explore the impact of an anti-inflammatory compounds on ER stress induced by Syncytin-1 expression in astrocytes.

3.2 Crocin suppresses Syncytin-1-induced XBP-1 splicing in human astrocytes

Antony *et al* demonstrated that Syncytin-1 was most increased in the astrocytes within MS demyelinating lesions [34]. Given this finding and since there is a potential link between Syncytin-1 and XBP-1 splicing, Syncytin-1's effect on XBP-1 splicing as well as other ER stress-related genes was investigated in human astrocytes. HFAs were transfected with a ERVWE1/HERV-W *env* eukaryotic vector (phCMV env), which contained the HERV *env* region from the full length HERV-W provirus located on chromesome 7q21.1 (ERVWE1), to determine if XBP-1s was induced. Since crocin protects neuronally differentiated PC-12 cells against TNF-α induced cell death and is an ROS scavenger [29], it has the potential to affect ER stress such as XBP-1 splicing through its ROS scavenging properties. Similarly ferulic acid, another antioxidant, was

shown to abrogate Syncytin-1-induced neuroinflammation *in vivo* [25]. Hence, crocin had the potential to alter Syncytin-1-induced effects such as inflammation and ER stress.

Human astrocytes were transfected with the ERVWE1/Syncytin-1 encoding vector or a matched empty vector. Cells were lysed 24 hours later and probed for Syncytin-1 using a polyclonal antibody recognizing Syncytin-1 (Figure 2A). Human astrocytes transfected with ERVWE1 exhibited Syncytin-1 protein immunoreactivity, whereas those transfected with an empty vector did not exhibit Syncytin-1 immunoreactivity (Figure 2A). Additionally, crocin abrogated Syncytin-1-induced XBP-1 splicing and Syncytin-1 induced *BiP* and *NOS2* transcripts (Figure 2B-E). Thapsigargin is a prototypic ER stress inducing compound, hence it was used as a positive control to induce *XBP-1/S* levels (Figure 2B). Indeed, transfected astrocytes also exhibited increased *BiP* (Figure 2C), and *NOS2* (Figure 2D), all of which were suppressed by crocin in a concentration-dependent manner. Conversely, HIF-1α transcript levels were unaffected by Syncytin-1 expression with no ensuing effects mediated by crocin (Figure 2E). This suggests a plausible link between Syncytin-1, ER stress and selective ROS generators such as NOS2. These results also underscore Syncytin-1 capacity to induce ER stress-related genes, which were suppressed by the ROS scavenger, crocin, in a gene-specific manner.

3.3 Crocin diminishes oligodendrocyte cytotoxicity induced by supernatant from astrocytes over-expressing Syncytin-1

Astrocytes are the most abundant cell type in the brain and are responsible for a diversity of homeostatic functions [39]. Amongst these roles, oligodendrocyte maintenance and support are a pertinent function, particularly in multiple sclerosis. During MS, alterations in glia such as astrocytes are likely harmful to proximal cells, such as oligodendrocytes. Astrocytes exhibit perturbed function during times of stress or when a particular protein is knocked out or over-expressed. They can secrete different cytokine and chemokines, potentially altering oligodendrocyte function and viability. The actions of supernatants from human astrocyte over-expressing Syncytin-1 on oligodendrocytes were previously shown to be cytotoxic [80]. Hence, HFAs were transfected with matched empty or Syncytin-1 containing vectors; 6 hours post transfection fresh medium was added and 24 hours later supernatants were harvested and applied to rat oligodendrocytes, which had been differentiated for one week. Subsequently, oligodendrocytes were fixed with 4% formaldehyde and immunostained with antibodies detecting cyclic nucleotide peptidase (CNPase) and galactocerebroside (Gal-C), molecules found principally in oligodendrocytes to ensure differentiation (Figure 3A). Indeed Gal-C and CNPase immunostaining reveal extended processes (Figure 3A). Oligodendrocytes were cultured with astrocyte supernatants for 24 hours with or without crocin at varying concentrations of 100, 200 or 400μM

concentrations. Subsequently astrocytes were fixed with 4% formaldehyde and immunostained and immunostained with an antibody to CNPase. CNPase immunoreactivity was assessed semi-quantitatively by using infra-red imaging and normalized to healthy cells immunostained with secondary antibody (Figure 3B). Oligodendrocytes cultured with supernatants from astrocytes transfected with the Syncytin-1 vector exhibited significantly less viability than oligodendrocytes treated with supernatants from astrocytes transfected with empty vector (Figure 3B). When crocin was added to supernatant of astrocytes overexpressing syncytin-1, CNPase immunoreactivity was maintained in a concentration-dependant manner. These latter findings were recapitulated by DAPI staining of the oligodendrocyte cultures, also disclosing the cytoprotective effect of crocin (Figure 3C). These data suggested that astrocytes over-expressing syncytin-1 modulated oligodendrocyte viability. Moreover, crocin exerted a protective effect on oligodendrocytes following exposure to supernatants from astrocytes overexpressing Syncytin-1.

Microglial cells are the other cell type in which Syncytin-1 has been reported to be expressed, albeit to a lesser extent [37]. Since microglia are recognized to secrete pro-inflammatory cytokines such as TNF-α, which cause neurodegeneration, the effects of microglia overexpressing Syncytin-1 on oligodendrocyte viability were also examined. The experiment was conducted in the same manner as in HFAs, as described above. Oligodendrocytes exposed to supernatants

from microglia transfected with empty vector demonstrated greater cytotoxicity, as determined by a lower DAPI staining (Figure 3C). This finding was expected since transfection alone will cause a small population of cells to undergo cell death.

3.4 Crocin prevents nitric oxide-induced oligodendrocyte cytotoxicity

Since crocin abrogated Syncytin-1 induced XBP-1s levels in HFAs and prevented oligodendrocyte cytoxicity in the same experiments described above, crocin's effects on sodium nitroprusside (SNP)-induced oligodendrocyte cytotoxicity were investigated. Sodium nitroprusside is a potent NO donor [81]. Nitrogen reactive species are thought to be over-produced in the brains of MS patients and spinal cords of EAE animals as a result of inflammation [81]. NO is assumed to play a role in oligodendrocyte death and demyelination during MS [82]. Since SNP is a robust NO donor, it was relevant to study crocin's effect on SNP-induced oligodendocyte cytotoxicity. Oligodendrocytes were cultured with different concentrations of SNP and crocin for 24 hours with and without crocin treatment (Figure 4A). Following 24 incubation, oligodendrocytes were stain with CNPase and viewed by confocal microscopy (Figure 4A). Oligodendrocytes were less viable with increasing concentrations of SNP, as measured by CNPase staining. However, when crocin was added, CNPase was preserved, suggesting crocin was protective against SNP-induced cytotoxicity. CNPase immunoreactivity was measured

semi-quantitatively using infrared imaging technology (Figure 4B). As an ROS scavenger, it was possible that crocin was quenching ROS populations, particularly NO, which is released by SNP.

3.5 Examination of an ER stress-inducing agent, tunicamycin, on glial cells

Tunicamycin is antibiotic which is well recognized for its ability to induce ER stress in multiple cell types [83], although its actions on immune responses remain unknown. In fact, its effects in glial cells, particularly myeloid glia (microglia) were also uncertain but we suspected it might exert immunogenic properties because of the intimate relationship between ER stress and inflammation. Hence, we examined its effects on host responses at two concentrations (0.1 and 1.0 μg/ml) following a brief (6hr) exposure in cultured human microglia. These studies disclosed that tunicamycin predictably activated the ER stress-related genes, *XBP-1/S* (Figure 5A), *BiP* (Figure 5C), *CHOP* (Figure 5D) but not *PERK* (Figure 5B), especially at the higher concentration. In addition, the lower concentration of tunicamycin induced expression of several immune genes including *IL-6* (Figure 6C) and *IL-12p35* (Figure 5D) while it had inconsistent effects on *RANTES* (Figure 6E) *TGF-β* (Figure 6F). This prototypic ER stress inducer exerted both ER stress effects together with immunogenic actions. MS is a neuroinflammatory disease in which the generation of ROS occurs such as NO damaging oligodendrocytes [82]. Since crocin exerts anti-

inflammatory effects and was successful in protecting oligodendrocytes from SNP-induced cytotoxicity and from toxin secreted by astrocytes overexpressing Syncytin-1, we hypothesized crocin might have a therapeutic effect in a neuroinflammatory disease. Therefore, we decided to investigate the role of crocin *in vivo* in the animal model of MS, EAE. We also studied whether ER stress followed the same trend in EAE as in MS.

Chapter 4 – ER stress modulates EAE

4.1 ER stress is induced in the spinal cords of EAE mice

Since XBP-1 splicing was increased in the brains of MS patients compared to non-MS patients (Figure 1B), we next examined the transcript levels of XBP-1s and other ER stress genes in the spinal cords of mice with and without EAE mice at day 28 post-induction of EAE. A cDNA library was generated from RNA isolated from the lumbar spinal cords from EAE and healthy mice. The lumbar spinal cord was chosen because it is the anatomic region which exhibits the most abundant neuroinflammation during EAE. RT-PCR was used to specifically amplify both *XBP-1* spliced and unspliced transcript variants in the cDNA library. Resulting *XBP-1* amplicons were digested with PstI. Restriction digest products were separated using agarose gel electrophoresis, stained in ethidium bromide buffer and viewed under UV light. The PstI restriction site is excised when *XBP-1* unspliced is converted to *XBP-1* spliced

variant. Hence, the *XBP-1* unspliced transcript variant contains a *Pst*I restriction site and is susceptible to *Pst*I restriction digestion, whereas XBP-1s is not. Following amplification and digestion with *Pst*I, it would be expected for *XBP-1/S* to appear as a smaller amplicon compared with the XBP-1 unspliced transcript. Following RT-PCR with XBP-1 primers and digestion with PstI, the XBP-1s transcript was increased in the spinal cords of EAE mice compared to age-matched healthy mice (Figure 7A). Image analysis was used to quantify the DNA bands and compare *XBP-1/S:XBP-1* unspliced (*XBP-1/U*) ratio (Figure 7B). The mean ratio was 7-fold higher in the spinal cords of EAE mice compared to those in healthy animals (Figure 7B). Other ER stress transcript levels were also examined in the spinal cord of EAE mice (Figure 7C): *BiP*, an early ER stress marker, and *CHOP*, a late ER stress marker, were significantly upregulated in the spinal cords of EAE mice (Figure 7C). Similarly, *GRP58* and *OASIS* transcript levels were also increased in the spinal cords of EAE animals compared with healthy animals, however GRP58 is not a classic ER stress protein (Figure 7C). Together with increased XBP-1 splicing in the spinal cords of EAE mice, these data suggested ER stress was increased in the CNS during EAE, resembling findings in MS brains.

4.2 Tunicamycin treatment exerts neuroprotective effects in EAE but EAE is CHOP independent

4.2.1 Neurobehavioral consequences

Tunicamycin (TM) is a mixture of homologous nucleoside antibiotics produced by several species of *Streptomyces*. This antibiotic induces ER stress by blocking N-linked glycosylation on asparagines residues [83]. However it has also been found to alter BBB permeability [84]. Tunicamycin exposure to microglial cells resulted in the induction in ER stress genes together with concurrent activation of several innate immune genes including IL-12p35, -6 and IFNα at lower concentrations (Figure 6). Because of this diversity of effects including aberrant protein folding and immunogenic, we decided to investigate tumicamycin's actions in vivo in the MOG-EAE model. Tunicamycin was given intra-peritoneally (IP) at the time of EAE induction (day 0) or at the time of disease onset (approximately day 7) (Figure 8A). Surprisingly, tunicamycin exposure at the time of EAE induction significantly abrogated the subsequent severity of EAE while its exposure at the time of disease onset had no impact on the disease course (Figure 8B). Indeed, this same outcomes were also evident in terms of overall severity of EAE (Figure 8C) and cumulative disability (Figure 8D). These findings suggested that tunicamycin paradoxically reduced the severity of EAE depending on the timing of its delivery.

4.2.2 Neuroimmune actions of tunicamycin

To investigate the molecular underpinnings of the above neurobehavioral outcomes, we examined transcript levels in the spinal cord 25 days post-EAE induction, disclosing that XBP-1/S (Figure 9E), BiP (Figure 9F), PERK (Figure 9H) and CHOP (Figure 9G) levels did not differ significantly across experimental groups. Conversely, F4/80 transcript levels (Figure 9B) were significantly increased with tunicamycin exposure although CD3ε transcript levels were similar across the EAE groups (Figure 9A). Remarkably, NOS2 and Arginase 1 induction reflected changes in F4/80 levels, suggesting a role of alternative macrophage activation underlying the changes in disease phenotype (Figure 9B, D).

4.2.3 EAE is CHOP-independent

ER stress has many downstream effects of which inflammation and apoptosis are two of the main pathways. ER stress can activate CHOP, a protein responsible for ER stress-mediated apoptosis[85]. To determine if CHOP played a role in EAE we generated CHOP-/- and CHOP +/- animals. EAE was induced in CHOP -/-, +/- and +/+ animals to examine whether CHOP was needed for EAE pathogenesis. All three CHOP genotypes exhibited similar clinical scores (Figure 10A), maximum score (Figure 10B), and Cumulative Score (Figure 10C). These data suggested that CHOP is not necessary for EAE manifestation.

4.3 Crocin reduces neuroinflammation and ER stress in EAE spinal cords

4.3.1 Introduction

The role for crocin in abrogating Syncytin-1-induced XBP-1 splicing and glia cell death *in vitro* suggested protective actions against neuroinflammation. Hence, we examined if crocin could ameliorate EAE disease. EAE was induced in C57B/6 mice and treated with either PBS (placebo) or crocin daily at day 7 post immunization until the end of the experiment (day 25). Day 7 was chosen as the starting point of crocin treatment because that is usually the onset of EAE signs. The neurological impairment of control, EAE and EAE mice treated with crocin was followed for 25 days after disease onset.

4.3.2 Crocin reduces immune and ER stress gene expression during EAE

Given that crocin suppressed Syncytin-1 induced XPP-1 splicing in astrocytes, ER stress and immune gene expression was assessed herein. Real time RT-PCR was performed using primers to specifically amplify *CD3ε*, F4/80 *PAR-2*, *TNF-α*, *BiP*, *CHOP*, *PERK* and *XBP-1/S* (Figure 11). PAR-2 was examined because Noorbakhsh *et al* had previously demonstrated that PAR-2 KO mice are less susceptible to neurological impairment and disease pathology during EAE [69]. TNF-α was examined because it has been shown that crocin protects neuronally differentiated PC-12 cells against TNF- α induced cell death [29]. *BiP* and

CHOP were examined since they are early and late ER stress markers, respectively. *CD3ε* transcript levels were examined as a marker of T cells. Crocin diminished *PAR-2* and *TNF-α* transcript levels which indicated crocin suppressed the production of pro-inflammatory cytokines which are part of the EAE molecular profile. ER stress transcripts were also diminished, although to a lesser extent. As previously stated ER stress is beneficial if it is in homeostasis and not present in excess. Reduction of *BiP* and *CHOP* transcript levels may have shifted ER stress to a healthy level where it is no longer harmful to glia and neurons. While it is unlikely that crocin stops leukocyte transmigration, CD3ε was also diminished in crocin treated EAE mice. This is surprising however it is possible that crocin slowed the activation of more T cells in the periphery and their homing to the CNS. It would be worthwhile to examine leukocyte transcript levels in crocin treated EAE mice earlier in the EAE profile; for example, before the peak of neurological impairment. It would be expected that crocin would have little effect on leukocyte infiltration before peak of disease, even with the absence of neurological impairment because it is likely that leukocyte infiltration has already started before crocin administration.

4.3.3 Neuropathological features of EAE and crocin treatment

Spinal cords from EAE and control animals were isolated, formalin fixed and paraffin embedded. Neuropathological analyses showed $CD3^+$ positive T-cell numbers were lower in control and crocin-treated EAE

animals when compared to PBS-treated EAE animals (Figure 12, first row). Likewise, control and crocin-treated EAE mice exhibited reduced Iba-1 immunopositive macrophages/microglial cells numbers and size (Figure 12, fourth row). Myelin Basic Protein (MBP) immunoreactivity in control and crocin-treated EAE mice were increased compared with PBS-treated EAE mice (Figure 9, second row), suggesting minimal myelin disruption in control and EAE mice treated with crocin. Axons in spinal cord white matter tracts, indicated by silver staining were also reduced in PBS-treated EAE mice relative to control and crocin-treated EAE animals (Figure 12, third row). BiP immunoreactivity was increased in EAE animals, particularly in glial cells resembling oligodendrocytes and astrocytes, relative to controls and crocin-treated EAE animals (Figure 13, top row). Similarly, CHOP immunoreactivity was minimal in healthy controls and crocin-treated EAE animals but was very evident in glia within the white matter tracts of EAE animals (Figure 13, bottom row). Together these results reveal that crocin reduced neuroinflammation and limits axonal injury in the EAE disease context in conjunction with ER stress suppression.

4.3.4 Crocin is neuroprotective during EAE

As previously described, EAE was induced in C57B/6 mice with the MOG_{35-55} model on day 0 and mice were given daily crocin (EAE/cro d7) or PBS (EAE) injection daily starting on day 7 post EAE induction (Figure 14A). EAE mice treated with PBS show a typical MOG-induced

disease course with a disease onset at day 7 and peak neurological impairment at days 15 to 18 (Figure 14B) during the course of the disease. The majority of EAE mice had a fully paralyzed tails and hind limbs at the peak of disease, whereas those treated with crocin only had limp tails at the peak of disease (Figure 14B). EAE mice treated with crocin were 7 points lower on a 14 point scale during their maximum disease score compared to EAE mice treated with PBS (Figure 14C). Similarly the cumulative disease score of EAE mice treated with crocin was 5-fold lower than EAE mice treated with PBS (Figure 14D). Together, these data indicated that crocin diminished EAE neurological impairments during EAE.

Chapter 5 – Discussion

5.1 Syncytin-1-mediated ER stress in MS

The present study has extends the recognition of the relationship between Syncytin-1, neuroinflamamtion and ER stress, which have previously been described by Antony *et al* [34, 37]. These studies highlight a correlation between Syncytin-1 and XBP-1 splicing, a hallmark of ER stress. I have demonstrated a correlation between Syncytin-1 and many immune genes associated with multiple sclerosis (Figure 1). For example CD3, a marker for T cells, which are widely recognized to become activated by myelin antigens and migrate across the BBB into the CNS [86], is proportional to Syncytin-1 transcript levels. Similarly, IL12p35 transcripts were also correlated with Syncytin-1 (Figure 1 and Table 2). IL-12 is a proinflammatory cytokine produced by dendritic cells and macrophages [87]. Since there is a correlation between Syncytin-1, a HERV envelope protein which has been acquired through infections during evolution, and many MS- and proinflammatory-associated factors, it is plausible that Syncytin-1 is altering gene profiles in the brain when it is upregulated during MS. Another interpretation of this observation might be that neuroinflammation is inducing Syncytin-1 transcription, which further amplifies inflammatory gene expression and the ensuing development of MS. Interestingly, Syncytin-1 was found to be correlated with XBP-1 spliced transcript variant. Since Syncytin-1 and ER stress were found to be altered during MS, particularly in astrocytes, I examined

the differential effects of Syncytin-1 on the induction of ER stress by measuring XBP-1 splicing.

5.2 Crocin abrogates Syncytin-1-induced ER stress and glia cytotoxicity

Syncytin-1's contribution to placental syncytia formation during pregnancy is widely recognized, however Syncytin-1 role in health and disease has not been explored in depth. The present observations are the first evidence that Syncytin-1 exerts differential effects on a classic ER stress pathway, XBP-1 splicing. XBP-1 splicing can result in ROS production but most relevantly it can induce transcription of inflammatory genes [88]. XBP-1 has been implicated in neurodegenerative diseases, amyotrophic lateral sclerosis (ALS) and Alzheimer's disease [88]. Hetz *et al* have demonstrated that XBP-1 has a controlling function in autophagy and point out that the understanding mechanistic relationships between XBP-1 splicing and autophagy is critical to the development of a protection mechanism against neurodenegeration [88]. These findings were extended in that Syncytin-1, a cytotoxic HERV envelope glycoprotein, induces XBP-1 splicing in astrocytes (Figure 2B). Like ALS, MS is also an inflammatory neurodegenerative disease and therefore it is likely that XBP-1 plays a pivotal role in neurodegeneration. It is plausible that Syncytin-1 induces XBP-1 splicing and induces transcription activation of inflammatory genes in astrocytes which results in bystander activation of CD4+ T cells through glia such as microglia that release

TNF-α and IL-1 to initiate CD4+ T cell-mediated demyelination and neurodegeneration [89]. Bystander activation is the process by which self- and foreign-antigens share enough sequence similarity to activate autoreactive T cells [47]. In this case it is likely that Syncytin-1 is increasing activation of autoreactive T cells by the bystander activation of CD4+ T cells.

Over-expression of Syncytin-1 in MS, principally in astrocytes and macrophages, likely results in XBP-1 splicing as a consequence of the augmented intracellular protein burden which can initiate the UPR. I investigated the effects of crocin on Syncytin-1 induced XBP-1 splicing for several reasons. It has been shown that nitric oxide is increased during MS and contributes to demyelination [82]). Similarly, it has been reported that nitric oxide scavengers ameliorate EAE [90], and that ferulic acid, another antioxidant, prevented Syncytin-1 mediated-neuroinflammation during EAE [25]. There are several antioxidants available such as ferulic acid and vitamin E which could have been used to explore its effect on ER stress and glia cytoxicity in relevance to MS however crocin was chosen for reasons of convenience in public diet as well as its broad list of capabilities. As previously described crocin is the active ingredient in saffron, a natural spice commonly used in rice in north Indian and Persian cooking, Crocin has been shown to prevent ethanol-induced learning and memory impairments [91] and abrogate TNF-α-induced cell death of neuronally differentiated PC-12 cells [29].

Crocin also diminished oxidative and nitrative stress, marked by the presence of nitric oxide and malondialhyde, in the brain after cerebral ischemia [28]. Since crocin is easily accessible in our diet and protects against other CNS injuries as previously described, it was a prime candidate to investigate its effect on ER stress and MS. Indeed crocin diminished Syncytin-1 induced XBP-1 splicing in astrocytes in a concentration-dependent manner (Figure 2B). A direct transcriptional role for crocin is not clear and it is still unclear how it diminishes Syncytin-1-induced XBP-1 splicing.

Binding of pathogen associated molecular patterns (PAMP) leads to the initiation of intracellular cascades by binding to pattern-recognition receptors (PRP) which modulates transcription and a change in extra-cellular proteins or cellular secretion of host factors such as cytokines or chemokines. For example, lipopolysaccharide (LPS) is a PAMP that binds to toll-like receptor 4 (TLR4) to initiate a phosphorylation cascade to activation transcription of pro-inflammatory genes [92]. Oligodendrocytes are a central target cell in multiple sclerosis as they can undergo cell death, which results in demyelination. Microglia and astrocytes shape the CNS in healthy conditions but can also mediate disease mechanisms. For example microglial cells secrete pro-inflammatory molecules, which lead to neurodegeneration and demyelination [92]. In response to demyelinating injury, astrocytes secrete basic fibroblast growth factor 2 (FGF-2) and platelet derived

growth factor (PDGF), which assist in oligodendrocyte progenitor cell (OPC) proliferation and differentiation to restore myelination of axons [89]. It is conceivable that Syncytin-1 might induce ER stress and alter growth factor secretion, which results in impaired myelin homeostasis or remyelination post-injury. Therefore it is conceivable that astrocytes over-expressing Syncytin-1 secrete cytokines or growth factors which directly affect ODC viability.

Indeed, oligodendrocytes cultured with supernatants from astrocytes over-expressing Syncytin-1 were less viable as measured by CNPase immunoreactivity (Figure 3A). Similarly Syncytin-1 induced astrocyte *NOS2* expression, which has already been demonstrated in existing literature [34]. Antony *et* al had suggested a potential link between Syncytin-1 and oligodendrocyte damage, however the basis of this interaction was still unclear [34, 89]. My studies highlight oligodendrocyte damage caused by over expression of Syncytin-1 in astrocytes *in vitro*, involving a role of ER stress. It is well established that astrocytes prevent oligodendrocyte precursor cells' migration and maturation [93, 94], and that astrogliosis (increased astrocyte proliferation) is characteristic of chronic MS lesions [95]. It is possible that Syncytin-1 levels play a role in intra-cellular cascades in astrocytes which potentially alter the type of astrogliosis or astrocytes secretions. To further elucidate the role of Syncytin-1 in astrocyte, it would be worthwhile to examine how Syncytin-1 effects atrogliosis, remyelination

and astrocyte secretion *in vivo*. It is important to understand the mechanism by which Syncytin-1 induces ER stress.

Crocin was able to suppress Syncytin-1-induced XBP-1 splicing, oligodendrocyte cytotoxicity induced by astrocytes overexpressing Syncytin-1 and SNP-induced oligodendrocyte death. These findings suggest a potential link between crocin and Syncytin-1's actions. The mechanism by which crocin affects intra-cellular pathways is unclear; however, it has been found to inhibit translocation of G protein-coupled receptor kinase 2 (GRK2) from the cytosol to membrane while also diminishing extra-cellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation [28]. It is also apparent that crocin is neuroprotective by reducing ER stress and *NOS2* expression post injury (figure 2B, 3A, C), however it is crucial to study intracellular pathways involving signalling proteins to elucidate the origins of crocin's cytoprotective effect. Another study demonstrated crocin protects against hydrogen peroxide-induced endothelial injury by diminishing apoptosis [96]. Crocin's neuroprotective effects on TNF-α- [29], hydrogen peroxide-[96] , Syncytin-1- (figure 3A) and NO- (figure 4A,B) mediated cytotoxicity *in vitro* point to a potential therapeutic role for crocin in neurodegenerative and neuroinflammatory diseases.

5.3 Crocin diminishes EAE disease pathology

When crocin was administered to EAE mice 7 days post immunization, EAE disease pathology was reduced (Figure 12). As in

MS, ER stress transcripts was also increased in the spinal cords of EAE mice (Figure 7). It is unlikely that crocin impaired leukocyte infiltration initially because it is administered when leukocyte egress into the CNS has likely already begun. However, I propose that crocin might attenuate activation of T cells in the CNS. Additionally, with crocin's documented protective effect on hydrogen peroxide induced endothelial cells apoptosis and its anti-neuroinflammatory effect [29, 96], it is possible that crocin modulates BBB permeability possibly through suppression of inflammation-induced cell adhesion molecules on brain endothelia such as ICAM2, VCAM or one of the integrins. It will be important to investigate these topics further to elucidate the mechanisms by which crocin prevents axonal injury in the spinal cords of EAE mice.

These crocin-mediated findings were accompanied by a decrease in macrophage infiltration/microglia activation, as indicated by decreased Iba-1 staining with a concomitant preservation of MBP expression in crocin-treated EAE mice in comparison to PBS-treated EAE mice. It is well recognized that microglia activation is a detrimental pathogenic feature of neuroinflammatory diseases such as multiple sclerosis [97]. It appears that crocin alters the cytokine milieu produced by microglia and astrocytes during neuroinflammatory states. It would be worthwhile to examine the effect crocin has on pro- and anti-inflammatory cytokines, as well as on growth factors contributing to OPC proliferation and differentiation, in healthy and neuroinflammatory states. It is plausible

that crocin alters astrocyte and microglia cytokine and growth factor expression levels to favour a reduction in neuroinflammation and axonal transaction. Additionally, a reduction in XBP-1s and BiP expression in the crocin-treated EAE mice, in comparison with the PBS treated mice, consolidate a direct role for crocin in maintaining a homeostatic UPR.

In summary crocin suppresses EAE pathology by reducing macrophage infiltration/microglia activation concomitantly with reduced demyelination and diminished axonal loss. Despite the uncertainty in how crocin ameliorates EAE pathology, analysis of this study along with current literature suggest that crocin tailors ER stress mechanisms in astrocytes and microglia plays which shapes BBB permeability and cytokine profiles in the CNS during the EAE effector phase.

5.4 Crocin suppresses neurological disability in EAE mice

Herein we have demonstrated that crocin reduces EAE disease severity (Figure 13 B, C, D). Crocin ameliorated the EAE disease profile with a concurrent decrease in maximum and cumulative scores (Figure 14 B, C, D). Reduced behavioural defects in crocin-treated EAE mice fits in parallel with their diminished EAE pathology (Figure 12). EAE animals treated with crocin had fewer T cell infiltrates, fewer macrophages/activated microglia, and higher axonal numbers in the lumbar spinal cord compared to EAE mice. Indeed BiP and CHOP, a late and early ER stress marker respectively, were also reduced in the crocin treated EAE mice compared to EAE mice.

During EAE, leukocyte transmigration occurs prior to disease induction. Myelin antigen, in this case MOG_{35-55} peptide will be phagocytosed by APCs, processed and presented to T cells. These T cells will become activated in the periphery and begin to transmigrate into the CNS. Since crocin administration begins at the same time as disease induction, it is likely that crocin does not affect T cell activation/transmigration. Given that crocin has the ability to suppress Syncytin-1-mediated ER stress and glia cytotoxicty, it is likely that crocin tailors ER stress mechanisms to alter glia and immune cell cytokine profiles. Double immunostaining with GFAP and BiP would confirm that ER stress is principally occurring in astrocytes, as does in MS pathology. Additionally, perhaps there is a Syncytin-1 endogenous retroviral envelope ortholog in mice which regulates ER stress and glia intracellular pathways in a similar fashion to Syncytin-1. Despite the remaining questions regarding crocin's mechanism of action in neuroprotection during EAE, these studies highlight a potential therapeutic role for crocin in neuroinflammation, which was previously unrecognized.

5.5 Crocin is a potential therapeutic agent in MS and other neuroinflammatory disorders

Crocin has robust antioxidant and neuroprotective effects on a variety of mechanisms in the CNS. Additionally, since it is likely not immunosuppressive, this makes it a prime therapeutic candidate molecule for MS and other neuroinflammatory disorders. However, the

intracellular mechanisms by which crocin exerts its neuroprotective and antioxidant effects are unclear. Therefore it is essential to clarify several issues regarding crocin's role as a therapeutic agent. First, it is important to outline the cell types which are affected by crocin and similarly, what cytokines do crocin suppress/induce. Second, does crocin affect transcription, translation or post translational modifications of certain proteins? Thirdly, can crocin polarize a specific cell type; for example, does it polarize macrophages or T cells to protective M2 or Th2 phenotypes, respectively. It is also important to study the ER stress mechanisms, which are affected by crocin. Thus, crocin shows promise as a therapeutic agent but several issues require attention to extend the understanding of its mechanisms of action on neuroinflammation.

5.6 ER stress modulation ameliorates EAE

A recent study has reported a role for ER stress in MS [37]. Moreover, these studies show a direct ability for ER stress to alter EAE outcomes. Herein, treatment with tunicamycin, a prototypic ER stress inducer, at the time of induction of EAE abrogated neurological impairment in EAE, whereas tunicamcyin treatment at the onset of EAE disease had no effect (Figure 8B, C, D). Interestingly, *F4/80* and *arginase-1* transcript levels were significantly upregulated in EAE mice treated with tunicamycin at the same time as disease induction (EAE/early TM) compared to untreated EAE mice. An increase in F4/80, representative of macrophages was surprising since increased

macrophage infiltration in EAE is usually associated by a higher EAE disease score, not ameliorated neurological impairment, which was the case here. However, arginase is thought to be involved in polarizing macrophages to an anti-inflammatory (M2) phenotype [98]. Similarly mouse macrophage TGF-β1 inhibits NOS2 and induces arginase [98]. It is possible that tunicamcyin early treatment pre-conditions macrophages in the periphery to stimulate TGF-β1 production thus increasing arginase and polarizing macrophages to an M2 phenotype. It will be important to examine M2/M1 and TH2/Th1 ratios in the spinal cords of EAE mice in the context of tunicamycin treatment. It is likely that tunicamycin pre-conditioning of macrophages is a potential mechanism by which it ameliorates EAE clinical scores. An increase in macrophages, particularly anti-inflammatory macrophages in the CNS during EAE, might diminish neuroinflammation during EAE pathology.

Tunicamycin had little effect on ER stress transcripts, *BiP* and *CHOP.* However these transcripts were measured three weeks after tunicamycin treatment and after peak EAE symptoms. It might be expected that *BiP* and *CHOP* would be increased during tunicamcyin treatment, since tunicamycin is an ER stress inducer. However, since there was minimal neurological impairment in EAE mice treated with tunicamcyin at the time of disease induction, it is not surprising that this would be accompanied by a reduced ER stress burden.

The current studies with CHOP KO animals demonstrate that CHOP does not participate in EAE pathogenesis. CHOP has a variety of functions, but its chief function is to mediate apoptosis through ER stress. Since the EAE disease profiles, maximum and cumulative score were similar between CHOP-/-, CHOP+/- and CHOP+/+ animals, ER stress-mediated apoptosis is not required for EAE. During EAE, neuroinflammation may drive ER stress, which has previously been determined to predominate in astrocytes. However, during neuroinflammation astrocytes do not undergo apoptosis, they undergo astrogliosis. Therefore, it was expected that CHOP would not be required for EAE. The majority of the cells that undergo cell death are oligodendrocytes and neurons, in which axonal transection is most characteristic. However, apoptosis is not likely to be the only mechanism of cell death. It would be worthwhile to measure and compare early (BiP) and apoptosis related (CHOP) ER stress markers in the different cell types during EAE. All three ER stress cascade initiators, ATF6/OASIS, IRE1 and PERK, can all induce transcriptional activation of CHOP by binding to its promoter to mediate ER stress [85], however apoptosis is not the sole consequence of ER stress. ER stress can lead to inflammation; as previously described, ER stress is connected to inflammation through production of ROS, release of calcium from the ER, activation of NF-κB and JNK [5]. Since EAE in astrocytes and microglia is better characterized as a condition involving neuroinflammation rather

than apoptosis, which occurs in neurons and ODCs as a result of neuroinflammation and activation of innate immune pathways, it is likely CHOP is not important in EAE pathogenesis [99]. Similarly, different CHOP genotypes revealed no difference in EAE disease profile, which confirms CHOP is not necessary for EAE induction.

5.8 Conclusions

These studies provide compelling support for my hypothesis that Syncytin-1 and ER stress participate in the pathogenesis of MS and its animal model, EAE. Syncytin-1 exerted a vigorous effect on XBP-1 splicing in human astrocytes, which was partially abrogated by crocin. Additionally, crocin was neuroprotective *in vitro* against Syncytin-1- and NO- induced astrocyte and oligodendrocytotoxicity, respectively. Furthermore, crocin was able to diminish EAE pathology burden by reducing T cell and macrophage infiltrates, diminishing activation of microglia, abrogating axonal transaction, demyelination and reducing ER stress. This was associated with significantly less neurological impairment in EAE. Similarly early tunicamycin treatment was able to ameliorate EAE clinical scores, possibly by phenotype switching of macrophages and T cells to an M2 and Th2 phenotype, respectively. The proposed model here is that Syncytin-1 over-expression, as a result of the possession of specific alleles which render an individual susceptible to specific environmental cues such as viral infection, triggers ER stress in astrocytes and ensues (Figure 15). ER stress activates astrocytes to

release ROS and pro-inflammatory cytokines, which leads to oligodendrocyte cytotoxicity (Figure 15). Together with existing literature, it seems that crocin blocks several steps in this model, however the earliest is likely Syncytin-1 mediation of ER stress. These results underscore the role of Syncytin-1 and ER stress in MS and point to potential therapeutic targets for crocin in neuroinflammation.

5.9 Future Directions

The mechanisms by which Syncytin-1 mediates ER stress to activate astrocytes and mediate neuroinflammation and contribute to MS remain unclear. There are issues, both *in vitro* and *in vivo* that remain to be addressed. The processes by which Syncytin-1 mediates XBP-1 splicing are unknown. Thus, it would be worthwhile to transfect astrocytes with different amounts of Syncytin-1, measure XBP-1 splicing quantitatively and establish a standard curve to determine whether there is a minimum threshold for Syncytin-1 over-expression to induce XBP-1 splicing, or rather a threshold for Syncytin-1 expression. Similarly, double immunostaining with Syncytin-1 and intra-cellular component proteins would help determine where Syncytin-1 is located during overexpression in astrocytes. This would help elucidate whether Syncytin-1 directly interacts with the ER to modulate ER stress or does Syncytin-1 remain outside the ER and mediate a cascade to modulate ER stress indirectly.

Since astrocytes are the chief cells in which Syncytin-1 is over-expressed and ER stress is induced in the brains of MS patients, it is

essential to address several aims regarding astrocytes in the context of MS lesions. Does astrogliosis vary with the type of MS lesions. For example, it would be important to examine astrocyte numbers in chronic compared to acute lesions and in the periphery compared to the center of the lesion. Moreover, Syncytin-1 overexpression would need to be investigated in the context of type and spatial dimensions of lesions and correlated to astrogliosis. Another interesting facet would be to determine whether astrogliosis and Syncytin-1 over-expression is correlated with inflammatory cell infiltration. For example, does Syncytin-1 overexpression, precede or follow the creation of new lesions? Another intriguing aspect would be to determine if astrocytes over-expressing Syncytin-1 have altered functions in CNS homeostasis or in their interaction with immune cells, particularly inflammatory cells such as T cells. Previously studies have identified a potential antigen presentation function in astrocytes [100]. Although astrocytes do express have major histocomptibility class II complex (MHCII) proteins, they are inducible in *vitro* by IFNγ and modulated by TNFα [40]. Additionally there are studies indicating that mouse astrocytes expressing HLA alleles, can present myelin antigen to T cells [101]. Hence it is important to pursue whether Syncytin-1 changes astrocytes ability to present antigens, such as myelin antigens, in the CNS. Syncytin-1 has been found to induce XBP-1 splicing, a hallmark sign of ER stress, in astrocytes. However it remains

unclear which differential ER stress pathways are affected by Syncytin-1 over-expression.

Crocin exerts a variety of antioxidant and neuroprotective effects on several types of glia and other CNS cells. However it has also been shown to have a potent transcriptional repressor activity on genes activated by Syncytin-1. It would be interesting to investigate whether crocin affects transcription of Syncytin-1 and other inflammatory cells directly or is this mediated through intra-cellular pathways. Moreover, with which specific ER stress pathway(s) does crocin interact.

There is evidence pointing to the concept that tunicamycin alters EAE disease profile by polarizing immune cells to an anti-inflammatory phenotype; however, this is not certain. It would be prudent to continue to explore the tunicamcyin-mediated effect on the M1/M2 and Th1/Th2 macrophage and T helper cell paradigm, respectively. Similarly, understanding the mechanisms by which crocin reduces EAE pathology and disease burden is critical. Delineating EAE and control spinal cords in terms of immune and CNS cells with colocalization experiments would facilitate exploration of which cells are affected by ER stress, inflammation and ROS. Since CHOP, an ER stress mediator of apoptosis, was not required in EAE, it would be likely that ER stress-associated neuroinflammation is more likely a mechanism. Real time RT-PCR and immunostaining or immunoblotting of NF-κB, JNK and ROS

expression would extend the understanding of complex disease mechanisms.

References

1. Iwawaki, T., et al., *A transgenic mouse model for monitoring endoplasmic reticulum stress.* Nat Med, 2004. **10**(1): p. 98-102.
2. Yoshida, H., *ER stress and diseases.* FEBS J, 2007. **274**(3): p. 630-58.
3. Matus, S., et al., *The stress rheostat: an interplay between the unfolded protein response (UPR) and autophagy in neurodegeneration.* Curr Mol Med, 2008. **8**(3): p. 157-72.
4. Nishikawa, S., J.L. Brodsky, and K. Nakatsukasa, *Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD).* J Biochem, 2005. **137**(5): p. 551-5.
5. Zhang, K. and R.J. Kaufman, *From endoplasmic-reticulum stress to the inflammatory response.* Nature, 2008. **454**(7203): p. 455-62.
6. Kimata, Y., et al., *A role for BiP as an adjustor for the endoplasmic reticulum stress-sensing protein Ire1.* J Cell Biol, 2004. **167**(3): p. 445-56.
7. Kondo, S., et al., *OASIS, a CREB/ATF-family member, modulates UPR signalling in astrocytes.* Nat Cell Biol, 2005. **7**(2): p. 186-94.
8. Wolfson, J.J., et al., *Subtilase cytotoxin activates PERK, IRE1 and ATF6 endoplasmic reticulum stress-signalling pathways.* Cell Microbiol, 2008. **10**(9): p. 1775-86.
9. Marciniak, S.J., et al., *CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum.* Genes Dev, 2004. **18**(24): p. 3066-77.
10. Malhotra, J.D. and R.J. Kaufman, *Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword?* Antioxid Redox Signal, 2007. **9**(12): p. 2277-93.
11. Feldman, D.E., V. Chauhan, and A.C. Koong, *The unfolded protein response: a novel component of the hypoxic stress response in tumors.* Mol Cancer Res, 2005. **3**(11): p. 597-605.
12. Urano, F., et al., *Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1.* Science, 2000. **287**(5453): p. 664-6.
13. Nishitoh, H., et al., *ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1.* Genes Dev, 2008. **22**(11): p. 1451-64.

14. Tu, B.P. and J.S. Weissman, *The FAD- and O(2)-dependent reaction cycle of Ero1-mediated oxidative protein folding in the endoplasmic reticulum.* Mol Cell, 2002. **10**(5): p. 983-94.
15. Raha, S. and B.H. Robinson, *Mitochondria, oxygen free radicals, disease and ageing.* Trends Biochem Sci, 2000. **25**(10): p. 502-8.
16. Kobayashi, T., et al., *Respiratory chain is required to maintain oxidized states of the DsbA-DsbB disulfide bond formation system in aerobically growing Escherichia coli cells.* Proc Natl Acad Sci U S A, 1997. **94**(22): p. 11857-62.
17. Pahl, H.L. and P.A. Baeuerle, *Expression of influenza virus hemagglutinin activates transcription factor NF-kappa B.* J Virol, 1995. **69**(3): p. 1480-4.
18. Rius, J., et al., *NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha.* Nature, 2008. **453**(7196): p. 807-11.
19. Deng, J., et al., *Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2.* Mol Cell Biol, 2004. **24**(23): p. 10161-8.
20. Hu, P., et al., *Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression.* Mol Cell Biol, 2006. **26**(8): p. 3071-84.
21. Davis, R.J., *Signal transduction by the JNK group of MAP kinases.* Cell, 2000. **103**(2): p. 239-52.
22. Deniaud, A., et al., *Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis.* Oncogene, 2008. **27**(3): p. 285-99.
23. Lin, W., et al., *Interferon-gamma inhibits central nervous system remyelination through a process modulated by endoplasmic reticulum stress.* Brain, 2006. **129**(Pt 5): p. 1306-18.
24. Dimcheff, D.E., et al., *Endoplasmic reticulum (ER) stress induced by a neurovirulent mouse retrovirus is associated with prolonged BiP binding and retention of a viral protein in the ER.* J Biol Chem, 2004. **279**(32): p. 33782-90.
25. Antony, J.M., et al., *Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination.* Nat Neurosci, 2004. **7**(10): p. 1088-95.
26. Cuozzo, J.W. and C.A. Kaiser, *Competition between glutathione and protein thiols for disulphide-bond formation.* Nat Cell Biol, 1999. **1**(3): p. 130-5.
27. Sugiura, M., et al., *Crocin (crocetin di-gentiobiose ester) prevents the inhibitory effect of ethanol on long-term potentiation in the dentate gyrus in vivo.* J Pharmacol Exp Ther, 1994. **271**(2): p. 703-7.

28. Zheng, Y.Q., et al., *Effects of crocin on reperfusion-induced oxidative/nitrative injury to cerebral microvessels after global cerebral ischemia.* Brain Res, 2007. **1138**: p. 86-94.
29. Soeda, S., et al., *Crocin suppresses tumor necrosis factor-alpha-induced cell death of neuronally differentiated PC-12 cells.* Life Sci, 2001. **69**(24): p. 2887-98.
30. Ono, M., M. Kawakami, and T. Takezawa, *A novel human nonviral retroposon derived from an endogenous retrovirus.* Nucleic Acids Res, 1987. **15**(21): p. 8725-37.
31. Khodosevich, K., Y. Lebedev, and E. Sverdlov, *Endogenous retroviruses and human evolution.* Comp Funct Genomics, 2002. **3**(6): p. 494-8.
32. Colmegna, I. and R.F. Garry, *Role of endogenous retroviruses in autoimmune diseases.* Infect Dis Clin North Am, 2006. **20**(4): p. 913-29.
33. Dolei, A. and H. Perron, *The multiple sclerosis-associated retrovirus and its HERV-W endogenous family: a biological interface between virology, genetics, and immunology in human physiology and disease.* J Neurovirol, 2009. **15**(1): p. 4-13.
34. Antony, J.M., et al., *Comparative expression of human endogenous retrovirus-W genes in multiple sclerosis.* AIDS Res Hum Retroviruses, 2007. **23**(10): p. 1251-6.
35. Zawada, M., et al., *MSRV pol sequence copy number as a potential marker of multiple sclerosis.* Pol J Pharmacol, 2003. **55**(5): p. 869-75.
36. Potgens, A.J., et al., *Syncytin: the major regulator of trophoblast fusion? Recent developments and hypotheses on its action.* Hum Reprod Update, 2004. **10**(6): p. 487-96.
37. Antony, J.M., et al., *The human endogenous retrovirus envelope glycoprotein, syncytin-1, regulates neuroinflammation and its receptor expression in multiple sclerosis: a role for endoplasmic reticulum chaperones in astrocytes.* J Immunol, 2007. **179**(2): p. 1210-24.
38. Sofroniew, M.V. and H.V. Vinters, *Astrocytes: biology and pathology.* Acta Neuropathol. **119**(1): p. 7-35.
39. Nair, A., T.J. Frederick, and S.D. Miller, *Astrocytes in multiple sclerosis: a product of their environment.* Cell Mol Life Sci, 2008. **65**(17): p. 2702-20.
40. Williams, A., G. Piaton, and C. Lubetzki, *Astrocytes--friends or foes in multiple sclerosis?* Glia, 2007. **55**(13): p. 1300-12.
41. Miller, D.H., *Biomarkers and surrogate outcomes in neurodegenerative disease: lessons from multiple sclerosis.* NeuroRx, 2004. **1**(2): p. 284-94.
42. Muzio, L., G. Martino, and R. Furlan, *Multifaceted aspects of inflammation in multiple sclerosis: the role of microglia.* J Neuroimmunol, 2007. **191**(1-2): p. 39-44.

43. Graeber, M.B. and W.J. Streit, *Microglia: biology and pathology.* Acta Neuropathol. **119**(1): p. 89-105.
44. Rotshenker, S., *Microglia and macrophage activation and the regulation of complement-receptor-3 (CR3/MAC-1)-mediated myelin phagocytosis in injury and disease.* J Mol Neurosci, 2003. **21**(1): p. 65-72.
45. Furlan, R., et al., *Central nervous system gene therapy with interleukin-4 inhibits progression of ongoing relapsing-remitting autoimmune encephalomyelitis in Biozzi AB/H mice.* Gene Ther, 2001. **8**(1): p. 13-9.
46. Baumann, N. and D. Pham-Dinh, *Biology of oligodendrocyte and myelin in the mammalian central nervous system.* Physiol Rev, 2001. **81**(2): p. 871-927.
47. Sospedra, M. and R. Martin, *Immunology of multiple sclerosis.* Annu Rev Immunol, 2005. **23**: p. 683-747.
48. Zeis, T. and N. Schaeren-Wiemers, *Lame ducks or fierce creatures? The role of oligodendrocytes in multiple sclerosis.* J Mol Neurosci, 2008. **35**(1): p. 91-100.
49. Tombola, F., M.M. Pathak, and E.Y. Isacoff, *How does voltage open an ion channel?* Annu Rev Cell Dev Biol, 2006. **22**: p. 23-52.
50. Switzer, R.C., 3rd, *Application of silver degeneration stains for neurotoxicity testing.* Toxicol Pathol, 2000. **28**(1): p. 70-83.
51. Weiss, N., et al., *The blood-brain barrier in brain homeostasis and neurological diseases.* Biochim Biophys Acta, 2009. **1788**(4): p. 842-57.
52. Persidsky, Y., et al., *Blood-brain barrier: structural components and function under physiologic and pathologic conditions.* J Neuroimmune Pharmacol, 2006. **1**(3): p. 223-36.
53. Del Bigio, M.R., *Ependymal cells: biology and pathology.* Acta Neuropathol. **119**(1): p. 55-73.
54. Frautschy, S.A., P.A. Walicke, and A. Baird, *Localization of basic fibroblast growth factor and its mRNA after CNS injury.* Brain Res, 1991. **553**(2): p. 291-9.
55. Murray, T.J., *The history of multiple sclerosis: the changing frame of the disease over the centuries.* J Neurol Sci, 2009. **277 Suppl 1**: p. S3-8.
56. Eikelenboom, M.J., et al., *Gender differences in multiple sclerosis: cytokines and vitamin D.* J Neurol Sci, 2009. **286**(1-2): p. 40-2.
57. Koch-Henriksen, N. and P.S. Sorensen, *The changing demographic pattern of multiple sclerosis epidemiology.* Lancet Neurol. **9**(5): p. 520-32.
58. Kuhlmann, T., et al., *Gender differences in the histopathology of MS?* J Neurol Sci, 2009. **286**(1-2): p. 86-91.
59. Sadovnick, A.D., *European Charcot Foundation Lecture: the natural history of multiple sclerosis and gender.* J Neurol Sci, 2009. **286**(1-2): p. 1-5.

60. Okada, H., et al., *The 'hygiene hypothesis' for autoimmune and allergic diseases: an update.* Clin Exp Immunol, 2010. **160**(1): p. 1-9.
61. Barcellos, L.F., et al., *HLA-DR2 dose effect on susceptibility to multiple sclerosis and influence on disease course.* Am J Hum Genet, 2003. **72**(3): p. 710-6.
62. Friese, M.A., et al., *Opposing effects of HLA class I molecules in tuning autoreactive CD8+ T cells in multiple sclerosis.* Nat Med, 2008. **14**(11): p. 1227-35.
63. Merryman, P.F., et al., *Class II major histocompatibility complex gene sequences in rheumatoid arthritis. The third diversity regions of both DR beta 1 genes in two DR1, DRw10-positive individuals specify the same inferred amino acid sequence as the DR beta 1 and DR beta 2 genes of a DR4 (Dw14) haplotype.* Arthritis Rheum, 1989. **32**(3): p. 251-8.
64. Baranzini, S.E., et al., *Genome, epigenome and RNA sequences of monozygotic twins discordant for multiple sclerosis.* Nature. **464**(7293): p. 1351-6.
65. Man, S., E.E. Ubogu, and R.M. Ransohoff, *Inflammatory cell migration into the central nervous system: a few new twists on an old tale.* Brain Pathol, 2007. **17**(2): p. 243-50.
66. Lin, W., et al., *Endoplasmic reticulum stress modulates the response of myelinating oligodendrocytes to the immune cytokine interferon-gamma.* J Cell Biol, 2005. **169**(4): p. 603-12.
67. Rodriguez, M., *Effectors of demyelination and remyelination in the CNS: implications for multiple sclerosis.* Brain Pathol, 2007. **17**(2): p. 219-29.
68. Aktas, O., B. Kieseier, and H.P. Hartung, *Neuroprotection, regeneration and immunomodulation: broadening the therapeutic repertoire in multiple sclerosis.* Trends Neurosci, 2009.
69. Noorbakhsh, F., et al., *Proteinase-activated receptor 2 modulates neuroinflammation in experimental autoimmune encephalomyelitis and multiple sclerosis.* J Exp Med, 2006. **203**(2): p. 425-35.
70. Barnett, M.H., A.P. Henderson, and J.W. Prineas, *The macrophage in MS: just a scavenger after all? Pathology and pathogenesis of the acute MS lesion.* Mult Scler, 2006. **12**(2): p. 121-32.
71. Matthews, P.M., *An update on neuroimaging of multiple sclerosis.* Curr Opin Neurol, 2004. **17**(4): p. 453-8.
72. Chard, D. and D. Miller, *Grey matter pathology in clinically early multiple sclerosis: evidence from magnetic resonance imaging.* J Neurol Sci, 2009. **282**(1-2): p. 5-11.
73. Dalton, C.M., et al., *Early development of multiple sclerosis is associated with progressive grey matter atrophy in patients presenting with clinically isolated syndromes.* Brain, 2004. **127**(Pt 5): p. 1101-7.

74. Adalsteinsson, E., et al., *Gray matter N-acetyl aspartate deficits in secondary progressive but not relapsing-remitting multiple sclerosis.* AJNR Am J Neuroradiol, 2003. **24**(10): p. 1941-5.
75. Wayne Moore, G.R., *MRI-clinical correlations: more than inflammation alone-what can MRI contribute to improve the understanding of pathological processes in MS?* J Neurol Sci, 2003. **206**(2): p. 175-9.
76. Baxter, A.G., *The origin and application of experimental autoimmune encephalomyelitis.* Nat Rev Immunol, 2007. **7**(11): p. 904-12.
77. Sriram, S. and I. Steiner, *Experimental allergic encephalomyelitis: a misleading model of multiple sclerosis.* Ann Neurol, 2005. **58**(6): p. 939-45.
78. Zhu, Y., et al., *CXCR3 activation by lentivirus infection suppresses neuronal autophagy: neuroprotective effects of antiretroviral therapy.* FASEB J, 2009. **23**(9): p. 2928-41.
79. Tsutsui, S., et al., *RON-regulated innate immunity is protective in an animal model of multiple sclerosis.* Ann Neurol, 2005. **57**(6): p. 883-95.
80. Mizuno, T., et al., *Expression of cytokines during glial differentiation.* Brain Res, 1994. **656**(1): p. 141-46.
81. Boullerne, A.I., L. Nedelkoska, and J.A. Benjamins, *Role of calcium in nitric oxide-induced cytotoxicity: EGTA protects mouse oligodendrocytes.* J Neurosci Res, 2001. **63**(2): p. 124-35.
82. Bo, L., et al., *Induction of nitric oxide synthase in demyelinating regions of multiple sclerosis brains.* Ann Neurol, 1994. **36**(5): p. 778-86.
83. Steele, K.E., et al., *Tunicamycin enhances neuroinvasion and encephalitis in mice infected with Venezuelan equine encephalitis virus.* Vet Pathol, 2006. **43**(6): p. 904-13.
84. Finnie, J.W. and J.D. O'Shea, *Effect of tunicamycin on the blood-brain barrier and on endothelial cells in vitro.* J Comp Pathol, 1990. **102**(4): p. 363-74.
85. Oyadomari, S. and M. Mori, *Roles of CHOP/GADD153 in endoplasmic reticulum stress.* Cell Death Differ, 2004. **11**(4): p. 381-9.
86. Huseby, E.S., et al., *A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis.* J Exp Med, 2001. **194**(5): p. 669-76.
87. Kalinski, P., et al., *Dendritic cells, obtained from peripheral blood precursors in the presence of PGE2, promote Th2 responses.* Adv Exp Med Biol, 1997. **417**: p. 363-7.
88. Hetz, C., et al., *XBP-1 deficiency in the nervous system protects against amyotrophic lateral sclerosis by increasing autophagy.* Genes Dev, 2009. **23**(19): p. 2294-306.

89. Merson, T.D., M.D. Binder, and T.J. Kilpatrick, *Role of cytokines as mediators and regulators of microglial activity in inflammatory demyelination of the CNS.* Neuromolecular Med. **12**(2): p. 99-132.
90. Jolivalt, C.G., et al., *A novel nitric oxide scavenger in combination with cyclosporine A ameliorates experimental autoimmune encephalomyelitis progression in mice.* J Neuroimmunol, 2003. **138**(1-2): p. 56-64.
91. Abe, K. and H. Saito, *Effects of saffron extract and its constituent crocin on learning behaviour and long-term potentiation.* Phytother Res, 2000. **14**(3): p. 149-52.
92. De Nardo, D., et al., *Signaling crosstalk during sequential TLR4 and TLR9 activation amplifies the inflammatory response of mouse macrophages.* J Immunol, 2009. **183**(12): p. 8110-8.
93. Fok-Seang, J., et al., *An analysis of astrocytic cell lines with different abilities to promote axon growth.* Brain Res, 1995. **689**(2): p. 207-23.
94. Wilby, M.J., et al., *N-Cadherin inhibits Schwann cell migration on astrocytes.* Mol Cell Neurosci, 1999. **14**(1): p. 66-84.
95. Cannella, B. and C.S. Raine, *The adhesion molecule and cytokine profile of multiple sclerosis lesions.* Ann Neurol, 1995. **37**(4): p. 424-35.
96. Xu, G.L., et al., *Evidence of crocin against endothelial injury induced by hydrogen peroxide in vitro.* J Asian Nat Prod Res, 2006. **8**(1-2): p. 79-85.
97. Heppner, F.L., et al., *Experimental autoimmune encephalomyelitis repressed by microglial paralysis.* Nat Med, 2005. **11**(2): p. 146-52.
98. Mills, C.D., et al., *M-1/M-2 macrophages and the Th1/Th2 paradigm.* J Immunol, 2000. **164**(12): p. 6166-73.
99. Das, A., et al., *Activation of calpain and caspase pathways in demyelination and neurodegeneration in animal model of multiple sclerosis.* CNS Neurol Disord Drug Targets, 2008. **7**(3): p. 313-20.
100. Fontana, A., W. Fierz, and H. Wekerle, *Astrocytes present myelin basic protein to encephalitogenic T-cell lines.* Nature, 1984. **307**(5948): p. 273-6.
101. Soos, J.M., et al., *Astrocytes express elements of the class II endocytic pathway and process central nervous system autoantigen for presentation to encephalitogenic T cells.* J Immunol, 1998. **161**(11): p. 5959-66.