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Endoplasmic Reticulum stress in autoimmune central nervous system inflammation and demyelination

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS). MS is prevalent in Caucasians, where it affects about 0.05%-0.15% of the population. The cause of degeneration in MS remains largely enigmatic, but is generally considered to be the result of an autoimmune inflammatory reaction leading to demyelination, oligodendrocyte loss and axonal damage in the CNS. The disease is characterized by activated auto-reactive myelin-specific lymphocytes that home to the CNS where they initiate a vicious cycle of inflammation and tissue damage. The major targets in MS pathology are oligodendrocytes, the myelin-producing cells of the CNS, and neurons, and their loss is directly associated with clinical manifestations of the disease, including speech disturbances, sensation deficits and paralysis. Much knowledge concerning MS pathogenesis has resulted from studies on its animal model Experimental Autoimmune Encephalomyelitis (EAE).

Endoplasmic reticulum (ER) stress is likely to be a major pathway in the pathogenesis of MS. ER stress occurs upon the accumulation of unfolded or misfolded proteins in the ER initiating the **unfolded protein response (UPR).** The UPR has to deal with ER stress by increasing the folding capacity of the ER by reducing protein synthesis and promoting protein degradation though ER-associated degradation. Three different signalling cascades can be activated (Figure 1): the inositol-requiring transmembrane kinase/endonuclease 1 (IRE1) pathway, the pancreatic ER kinase (PERK) pathway and the activating transcription factor 6 (ATF6) pathway. Although all three branches are usually activated by any given ER stress event, the timing of activation can differ.

ER stress is part of normal cellular physiology, but can, however, become problematic in conditions of chronic, non-resolved stress, giving rise to inflammation and/or apoptosis. Recent observations suggest that the signalling pathways in the UPR and those controlling inflammation are interconnected and can activate each other through various mechanisms including the activation of NF-κB and MAP kinases (Zhang and Kaufman, 2008), suggesting that ER stress contributes to the pathology of many inflammatory diseases, including MS. Indeed, evidence is emerging that the UPR is involved in the disease pathogenesis of MS and EAE. Oligodendrocytes continuously produce large amounts of myelin to perform their function, making them prone to protein misfolding and ER stress. Expression of ER stress markers has been found to be upregulated in macrophages, microglia, astrocytes, and oligodendrocytes within demyelinated white matter lesions from MS patients. Moreover, elevated levels of phosphorylated-eIF2α, typical for PERK-dependent UPR signalling, have been observed in oligodendrocytes and infiltrating T-cells in the CNS during the course of EAE (Lin and Popko, 2009). Notably, IFN-γ exerts protective activities through the activation of the PERK-eIF2α pathway in oligodendrocytes (Lin et al., 2007). On the other hand, IFN-γ has suppressive activity on oligodendrocyte regeneration, inhibiting remyelination in MS and EAE demyelinated lesions (Lin et al., 2006). These data suggest that ER stress induction in fully myelinated mature oligodendrocytes promotes cell survival, but in actively (re)myelinating oligodendrocytes leads to cell death (Lin and Popko, 2009). Together, these observations clearly indicate the involvement of ER stress in MS and EAE pathology, suggesting that manipulation of the UPR may be beneficial in order to prevent disease. Since inflammation and ER stress may induce autophagy responses as a compensatory mechanism, also autophagy may be involved in MS (Adolph et al., 2013). **However, a better comprehension of the role and molecular mechanisms of UPR signaling and autophagy in MS/EAE is necessary.**

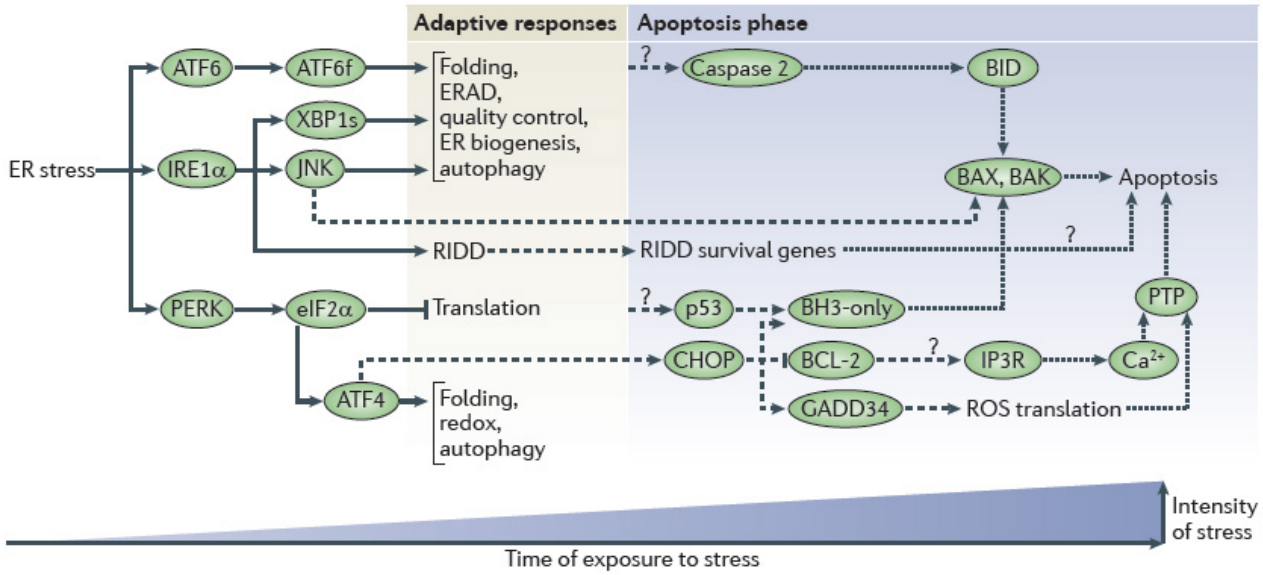

Figure 1. : ER stress cell fate decisions (Hetz, 2012). Different UPR-induced responses are observed over time in cells undergoing ER stress. Chronic ER stress induces inflammation and eventually apoptosis (through different poorly characterized mechanisms) in order to eliminate irreversibly damaged cells.

With this project we aim to better understand the contribution of autophagy and UPR signaling to the inflammatory processes associated with the development and progression of MS. The basic approach is to genetically manipulate genes coding for proteins essential for autophagy and UPR signaling in mice in specific neuronal populations, and to determine the effects of such mutation in neuronal development and MS pathogenesis. These studies will allow us to specify the role of UPR and autophagy signalling locally in the CNS, both in target cells (such as in neurons and oligodendrocytes) and in effector cells (such as in astrocytes and microglia) in the inflammatory and neurodegenerative processes happening during MS/EAE. Indeed, differences in UPR responses and sensitivity to ER stress may be expected depending on the cell type and the function they exert. It is thus critically important to determine how these various cell types at various stages of activation and disease development react to chronic inflammatory stress in order to better understanding the pathogenesis of MS (and other demyelinating diseases) which may have implications for the rational design of new therapeutics for the treatment of these pathologies.

1. Results 1st project year

1.1. Mouse conditional gene targeting

Conditional 'floxed' mice targeting NF-κB signaling (IKK2^{FL}), autophagy (Atg16l1^{FL}) and the three individual UPR signaling pathways (CHOP, XBP1^{FL}, IRE1α^{FL}, ATF6α^{FL}, PERK^{FL}) have been obtained or are in the process of being generated. Also the different Cre transgenic lines needed for CNS targeting have been introduced in the research group: Nestin-Cre transgenic mice for pan-CNS targeting, Thy1.2-Cre mice for neuron-specific targeting, MOGi-Cre mice for oligodendrocyte targeting, GFAP-Cre mice for astrocyte targeting, and Cx3Cr1-Ert2Cre mice for microglia targeting.

To study the CNS-specific role of ER stress and autophagy in the immunopathology of MS, we make use of the experimental MS model EAE, which can be induced by immunization of mice with myelin oligodendrocyte glycoprotein (MOG) or other encephalogenic agents. Next to EAE, brain-specific demyelination can also be induced by putting mice on a diet containing the neurotoxicant cuprizone. Advantage of this approach is that demyelination can afterwards be reversed by administration of normal food, allowing the study of brain remyelination and the involvement of ER stress signalling and

autophagy in this. Finally, protocols for *in vitro* studies on primary cells derived from the different tissue-specific knockout mice have been established, allowing biochemical studies complementary to the *in vivo* approaches.

1.2. CHOP-dependent neuronal apoptosis in EAE

ER stress has several downstream effects, of which inflammation and apoptosis are the two major outcomes. ER stress can activate CHOP, a protein responsible for ER stress-mediated apoptosis (Oyadomari and Mori, 2004). To determine whether CHOP plays a role in EAE pathogenesis, we induced EAE in wild-type ($CHOP^{+/+}$), heterozygous ($CHOP^{-/+}$) and CHOP deficient ($CHOP^{-/-}$) littermate mice.

Upon EAE induction, $CHOP^{-/-}$ mice did not show protection nor exacerbation of symptoms when compared to $CHOP^{-/+}$ and $CHOP^{+/+}$ mice (Figure 2 and table 1). Moreover, quantification of degree of demyelination through histology did not show any difference between the three groups of mice. Furthermore, inflammatory gene expression nor peripheral T cell activation did show any significant difference between the three genotypes (data not shown). From these observations, we can conclude that CHOP, and CHOP-dependent UPR signalling, does not crucially contribute to EAE onset and disease progression Since no difference in degree of demyelination could be observed in CHOP deficient conditions compared to controls, oligodendrocyte survival is not influenced by the absence of CHOP in conditions of EAE.

ER stress and CHOP expression can trigger apoptosis under physiological and pathophysiological conditions (Tabas and Ron, 2011). Indeed, using MEFs isolated from control and CHOP deficient mice, we could confirm the importance of CHOP in tunicamycin-induced cell death, since CHOP deficient MEFs are significantly protected from death induced by tunicamycin, a stimulus known to induce ER stress (data not shown). Although CHOP may possibly also contribute to death of oligodendrocytes in EAE, its effect may not be detectable in conditions of extensive inflammation where inflammatory cytokines such as TNF and Fas ligand may induce apoptosis independent of CHOP (Mc Guire et al., 2010).

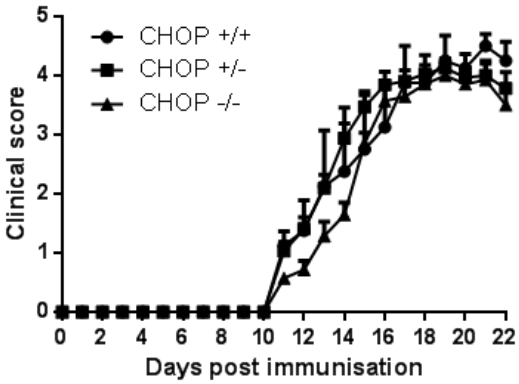

Figure 2. Clinical disease scores of $CHOP^{-/-}$ (n=7), $CHOP^{+/-}$(n=16) and $CHOP^{+/+}$ (n=4) mice after immunization with MOG peptide. Results are from three independent experiments and presented as mean values ± s.e.m.

Table 1. Clinical features of MOG_{35-55} induced EAE in CHOP mice

Genotype	Incidence	Mean day of disease onset	Maximum clinical score
$CHOP^{+/+}$	4 of 5 (80%)	14 ± 1.22	4.5 ± 0.2
$CHOP^{+/-}$	16 of 17 (94%)	13.12 ± 0.41	4.41 ± 0.13
$CHOP^{-/-}$	8 of 8 (100%)	14.38 ± 0.32	4.37 ± 0.12

Results are presented as mean values ± s.e.m.; combined data from three independent experiments.

1.3. XBP1-dependent UPR signaling in EAE

IRE1α is the most conserved transducer of UPR which acts through unconventional splicing of XBP1 mRNA. To investigate the importance of XBP1-dependent signalling in CNS inflammation, we generated CNS-specific XBP1 deficient mice and subjected them to EAE. $XBP1^{CNS\text{-}KO}$ mice, however, developed EAE pathology to the same extent as control littermates (Figure 3A). Also, on spinal cord histology nor on spinal cord gene expression, no significant differences could be observed (data not shown).

Since oligodendrocytes are particularly vulnerable to ER stress, we next investigated the response of oligodendrocyte-specific XBP1 knockout mice to EAE. However, although $XBP1^{ODC\text{-}KO}$ mice are behaving slightly better in EAE, especially at later time points, no significant differences with control mice could be observed (Figure 3B). In conclusion and opposite to what was expected, XBP1-dependent UPR signalling does not seem to be involved in autoimmune-mediated inflammation and demyelination.

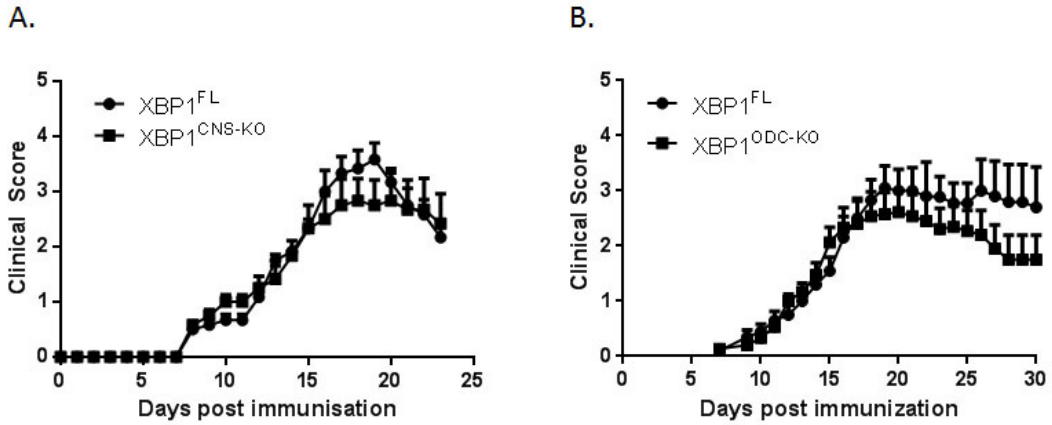

Figure 3. A. Clinical disease scores of XBP^{FL}(n=6) and $XBP^{CNS\text{-}KO}$ (n=6) littermate mice after immunization with MOG peptide. B. Clinical disease scores of XBP^{FL}(n=10) and $XBP^{ODC\text{-}KO}$ (n=15) littermate mice after immunization with MOG peptide. Results are presented as mean values ± s.e.m.

1.4. XBP1 in cuprizone-induced demyelination

Brain demyelination can be induced through the administration of cuprizone in chow diet. Although little is known of the mechanisms by which cuprizone induces CNS demyelination and inflammation, cuprizone administration induces activation of ER stress response genes (Figure 4).

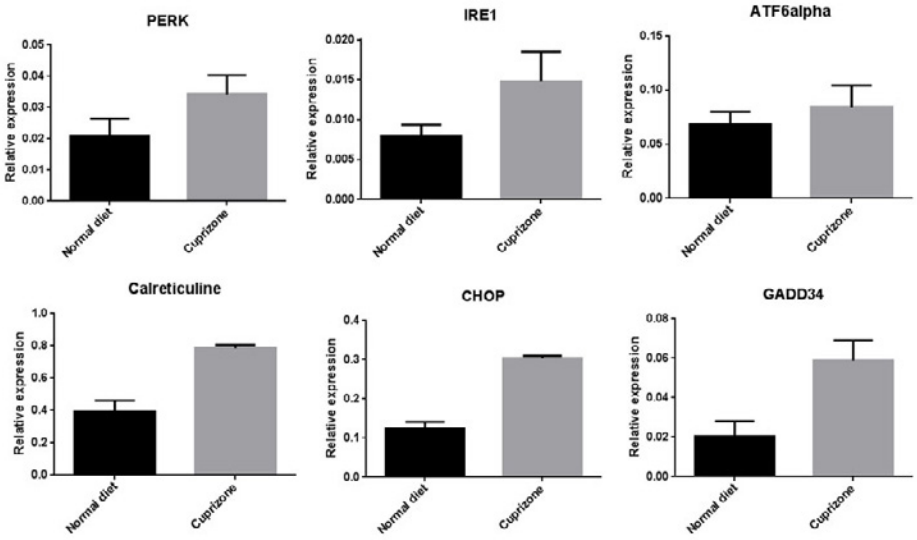

Figure 4. Cuprizone-induced expression of UPR genes. Expression of ER stress markers was assessed through qPCR on corpus callosum lysates isolated from wild-type C57BL/6 mice either or not treated with cuprizone for 5 weeks. Results are presented as mean values ± s.d.

Next, XBP1^{CNS-KO} mice and control littermates were given cuprizone for 5 weeks and degree of demyelination and inflammation was assessed in corpus callosum. Wild-type mice show a near complete demyelination of the corpus callosum, as expected, in contrast to XBP1^{CNS-KO} mice which were significantly protected from cuprizone-induced demyelination. On brain histology, XBP1^{CNS-KO} mice also showed a significant reduction in microgliosis and astrogliosis, and a higher number of mature oligodendrocytes (Figure 5). Since XBP1 deficiency was shown to be protective in a mouse model of ALS through enhanced clearance of protein aggregates by autophagy (Hetz et al., 2009), the protection of XBP1^{CNS-KO} mice in the model of cuprizone-induced demyelination may also depend on an increased autophagy of dysfunctional mitochondria protecting oligodendrocytes from cell death. XBP1 deficiency may also protect oligodendrocytes from apoptosis involving the pro-apoptotic proteins BAX, BAK, and ASK1-interacting protein 1 (AIP1) (Hetz et al., 2006). This hypothesis may also explain the mild protection of XBP1^{ODC-KO} mice in EAE. These studies are currently under investigation.

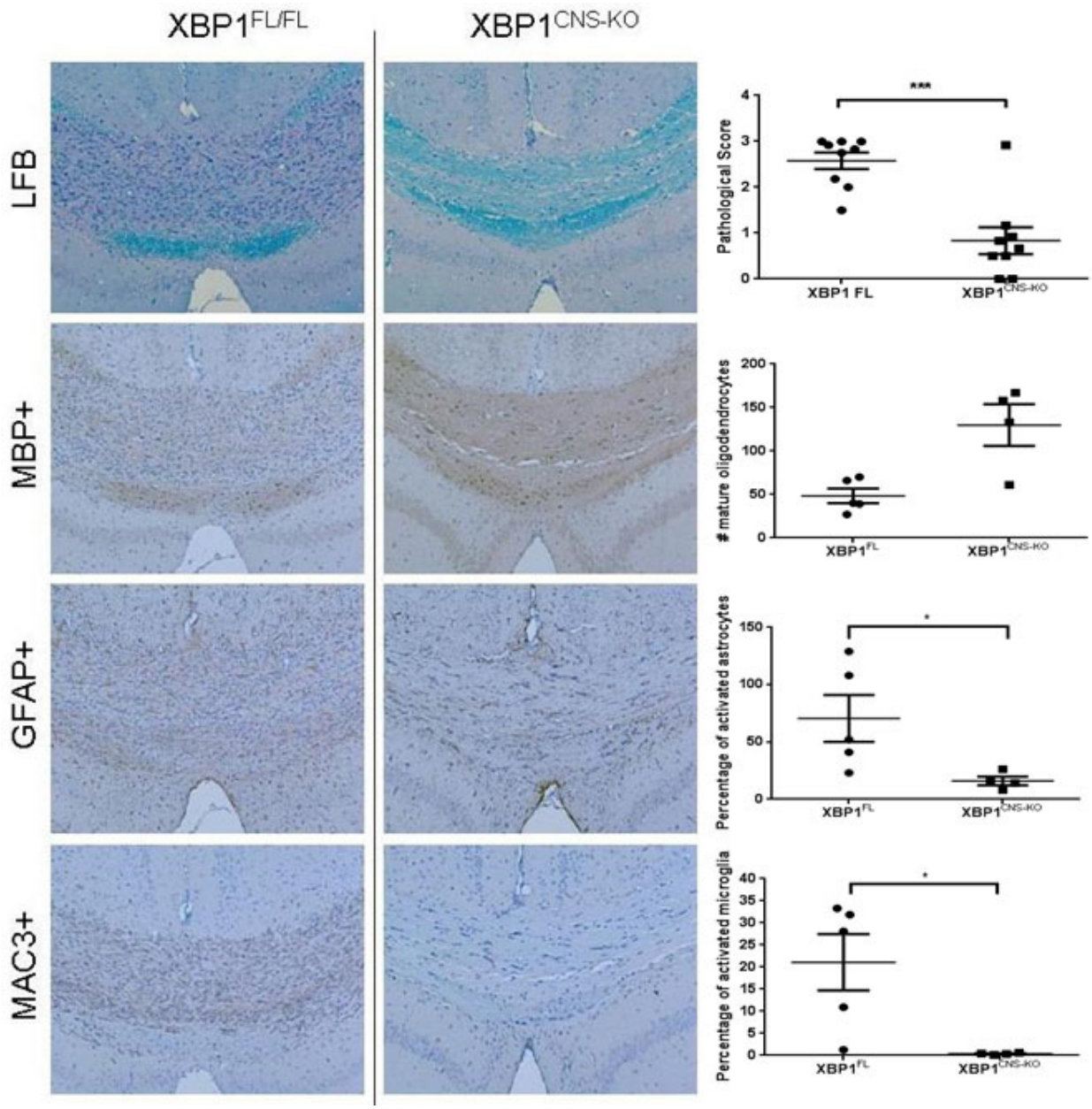

Figure 5. Cuprizone-induced demyelination is reduced in XBP1^{CNS-KO} mice. Representative pictures of coronal sections of the corpus callosum (left) and quantification (right) for degree of demyelination (LFB), oligodendrocyte numbers (MBP), astrogliosis (GFAP) and microgliosis (Mac3) in XBP1^{CNS-KO} mice and XBP1^{FL} littermates after 5 weeks of cuprizone treatment.

2. Future studies

During the second year of the project period, we will further elaborate on the phenotype of the CNS-specific XBP1 knockout mice in the model of cuprizone-induced inflammation and CNS demyelination and dissect the contribution of ER stress in the different CNS cell types involved. Next, new mouse lines with specific defects in IRE1α– and PERK-dependent UPR signalling and autophagy in CNS cell types have been generated allowing their study in both EAE and cuprizone models of MS. Also, *in vitro* biochemical studies using primary cultures isolated from the respective knockout mice will be used in order to establish the mechanisms by which ER stress and autophagy control inflammatory responses. Finally, our different brain-specific knockout mice will also be subjected to other models of CNS inflammation and pathology, such as to a model of cerebral ischemia. Although there is strong evidence that inflammation contributes to the pathology of cerebral ischemia, very little is known of the contribution of ER stress and autophagy to the inflammatory processes associated with the disease.

3. References

- Adolph TE, Tomczak MF, Niederreiter L, Ko HJ, Böck J, Martinez-Naves E, Glickman JN, Tschurtschenthaler M, Hartwig J, Hosomi S, Flak MB, Cusick JL, Kohno K, Iwawaki T, Billmann-Born S, Raine T, Bharti R, Lucius R, Kweon MN, Marciniak SJ, Choi A, Hagen SJ, Schreiber S, Rosenstiel P, **Kaser A**, Blumberg RS. (2013) *Nature* **503**, 272-276.
- Hetz, C., P. Bernasconi, J. Fisher, A. H. Lee, M. C. Bassik, B. Antonsson, G. S. Brandt, N. N. Iwakoshi, A. Schinzel, L. H. Glimcher and S. J. Korsmeyer (2006) *Science* **312**, 572-576.
- Hetz, C., A. H. Lee, D. Gonzalez-Romero, P. Thielen, J. Castilla, C. Soto and L. H. Glimcher (2008) *Proc Natl Acad Sci U S A* **105**, 757-762.
- Hetz, C. (2012) *Nat Rev Mol Cell Biol* **13**, 89-102
- Lin, W., and Popko, B. (2009) *Nat Neurosci* **12**, 379-385
- Lin, W., Bailey, S. L., Ho, H., Harding, H. P., Ron, D., Miller, S. D., and Popko, B. (2007) *J Clin Invest* **117**, 448-456
- Lin, W., Kemper, A., Dupree, J. L., Harding, H. P., Ron, D., and Popko, B. (2006) *Brain* **129**, 1306-1318
- Mc Guire, C., Volckaert, T., Wolke, U., Sze, M., de Rycke, R., Waisman, A., Prinz, M., Beyaert, R., Pasparakis, M., and van Loo, G. (2010) *J Immunol* **185**, 7646-7653
- Oyadomari, S., and Mori, M. (2004) *Cell Death Differ.* **11**, 381–389.
- Tabas, I. and D. Ron (2011) *Nat Cell Biol* **13**, 184-190
- Zhang, K., and Kaufman, R. J. (2008) *Nature* **454**, 455-462

4. Publications van Loo group 2012-2014 (acknowledging GSKE support)

- Vereecke, L., Vieira-Silva, S., Billiet, T., van Es, J.H., Mc Guire, C., Slowicka, K., Sze, M., van den Born, M., De Hertogh, G., Clevers, H., Raes, J., Rutgeerts, P., Vermeire, S., Beyaert, R. and **van Loo, G**. (2014) A20 controls intestinal homeostasis through cell-specific activities. Nat. Commun., 5, 5103.
- Takahashi, N., Vereecke, L., Bertrand, M.J.M., Duprez, L., Berger, S.B., Divert, T., Goncalves, A., Sze, M., Martens, S., Lefebvre, S., Gilbert, B., Gunther, C., Becker, C., Bertin, J., Gough, P.J., Declercq, W., **van Loo, G.** and Vandenabeele, P. (2014) RIPK1 ensures intestinal homeostasis by protecting the epithelium against apoptosis. Nature, 513, 95-99.
- Mc Guire, C., Elton, L., Wieghofer, P., Staal, J., Voet, S., Demeyer, A., Nagel, D., Krappmann, D., Prinz, M., Beyaert, R. and **van Loo, G**. (2014) Pharmacological inhibition of MALT1 protease activity by mepazine protects mice from experimental autoimmune encephalomyelitis. J. Neuroinflamm., 11, 124-135.
- Verhelst, K., **van Loo, G.** and Beyaert, R. (2014) A20: attractive without showing cleavage. EMBO Rep., 15 (7), 734-735.
- Vande Walle, L., Vanopdenbosch, N., Jacques, P., Fossoul, A., Verheugen, E., Vogel, P., Beyaert, R., Elewaut, D., Kanneganti, T.V., **van Loo, G.*** and Lamkanfi, M.* (2014) Negative regulation of the NLRP3 inflammasome by A20 protects against arthritis. Nature, in 512, 69-73 **(*equally contributed)**
- Catrysse, L., Vereecke, L., Beyaert, R. and **van Loo, G.** (2014) A20 in inflammation and autoimmunity. Trends Immunol., 35 (1), 22-31.
- Mc Guire, C., Prinz, M., Beyaert, R. and **van Loo, G.** (2013) Nuclear Factor-kB in multiple sclerosis pathology. Trends Mol. Med., 19 (10), 604-613.

– Mc Guire, C., Rahman, M., Schwaninger, M., Beyaert, R. and van Loo, G. (2013) The ubiquitin editing enzyme A20 (TNFAIP3) is upregulated during permanent middle cerebral artery occlusion but does not influence disease outcome. Cell Death Dis., 4:e531.
– Mc Guire, C., Wieghofer, P., Elton, L., Muyllaert, D., Prinz, M., Beyaert, R. and **van Loo, G.** (2013) Paracaspase MALT1 deficiency protects mice from autoimmune-mediated demyelination. J. Immunol., 190, 2896-2903.
– Maelfait, J., Roose, K., Bogaert, P., Sze, M., Saelens, X., Pasparakis, M., Carpentier, I., **van Loo, G.*** and Beyaert, R.* (2012) A20 (Tnfaip3) deficiency in myeloid cells protects against Influenza A virus infection. Plos. Pathog., 8 (3), e1002570. (*equally contributed)