CHAPTER 6

Summary and general discussion
In this chapter, the main findings of this doctoral dissertation are summarized and the results are discussed. In the end, research for future studies is proposed.

Summary

As resident immune cells of the central nervous system, microglia play a crucial role in the maintenance of brain homeostasis, synaptic plasticity, and network formation, defending the CNS against pathogens, and clearing apoptotic cells and debris. Microglia display a range of phenotypes which are shaped by the microenvironment or their activation history. In this thesis, we studied the (epi)genetic regulation of endotoxin tolerance and aging-associated priming in microglia. We have identified regulatory gene networks and evaluated the response of microglia to a range of pathogen-associated molecular patterns (PAMPs). We also investigated the consequences of microglia-specific deletion of the DNA repair enzyme Ercc1, associated with accelerated aging.

In chapter 2, the molecular basis of endotoxin tolerance in microglia was investigated. In microglia, endotoxin tolerance was induced by LPS challenges. The NF-κB signaling pathway, initiated by LPS-TLR4 interaction, induced the expression of pro-inflammatory genes in microglia, accompanied by the enrichment of histone modifications associated with increased gene expression, also referred to as “active” marks. After the inflammation resolved (after LPS treatment), the epigenome of the investigated genes was altered to a more “inactive” state and enriched for histone modifications associated with reduced gene expression. The transcription factor RelB was critically implicated in endotoxin tolerance induction in microglia.

In contrast to endotoxin tolerance, characterized by a reduced response of innate immune cells to inflammatory challenges, innate immune cells are also capable of developing an enhanced response when pathogens are re-encountered, a condition termed “trained immunity” (Netea et al., 2016). In chapter 3, the effects of β-glucan, a component from C. albicans and S. cerevisiae that induces trained immunity in innate immune cells, was studied on microglia. Like LPS, β-glucan tolerized microglia in vitro by activating the NF-κB signaling pathway. β-glucan first induced a metabolic shift in microglia in vitro to aerobic glycolysis and then to a state of both enhanced glycolysis and oxidative phosphorylation. The effects of β-glucan preconditioning in microglia were also addressed in vivo. β-glucan induced (transient) sensitization of microglia to
a second inflammatory challenge (LPS), characterized by enhanced expression of proinflammatory genes, which was accompanied by altered microglia morphology.

In chapter 4, the genome-wide (epi)genetic alterations underlying microglia endotoxin tolerance and priming were investigated. Transcriptional and epigenetic profiles of naïve and LPS pre-conditioned microglia, before and after a second LPS challenge were analyzed. Compared to LPS activation of naïve microglia, groups of genes were identified that display a different response to a second LPS challenge: tolerized (less induced by a 2nd LPS challenge) and responsive (equally induced by a 2nd LPS challenge). A co-regulated gene network was generated to delineate the gene network underlying endotoxin tolerance in microglia.

The (epi)genetic characteristics of hypersensitive, or primed, microglia and their exaggerated response to LPS were determined in accelerated aging, Ercc1 mutant mice. LPS hyper-responsive genes were also enriched for histone modifications associated with enhanced gene expression. The regulatory regions of genes with differential enrichment of histone modifications were enriched for Pu.1 binding motifs when comparing wild-type and aged microglia suggesting a role for Pu.1 in the microglia priming response.

In chapter 5, the Ercc1 gene was specifically deleted in microglia using Cx3cr1wt/creER transgenic mice. Microglia in Cx3cr1wt/creER:Ercc1ko/loxp mice after tamoxifen treatment exhibited similar phenotypical changes as microglia in generic Ercc1Δ/ko mice, in terms of altered morphology, increased cell proliferation, phagocytic activity, and increased expression of chemokines. In contrast to microglia from generic Ercc1Δ/ko mice, the expression of aging-associated genes was not significantly increased in Cx3cr1wt/creER:Ercc1ko/loxp microglia and no significantly exaggerated response to LPS stimulation was observed. These data strongly suggested that the microglia priming observed in generic Ercc1Δ/ko mice is primarily induced by their environment and not by intrinsic accumulation of DNA lesions. Microglia deficient for Ercc1 expressed genes related to aging rather than the typical priming-associated genes. Ercc1 deficient microglia were gradually replaced by other microglia, which was most likely due to apoptosis caused by excessive DNA damage.

**The biological relevance of endotoxin tolerance in microglia**

An excessive inflammatory response of microglia to peripheral inflammation or infection can result in impaired CNS performance and cognition. This notion is supported by the observation of long-term cognitive impairment after sepsis in both
rodents and humans (Iwashyna et al., 2010; Semmler et al., 2007; Semmler et al., 2013). Therefore, to prevent excessive CNS damage, the degree of microglial activation in response to peripheral inflammation might be tightly regulated (Foster et al., 2007). One of the mechanisms to limit excessive neuronal damage as a result of peripheral inflammation might be to limit microglial inflammatory activation. Here, we indeed found that a single inflammatory challenge permanently changed the epigenome and gene expression pattern of microglia in response to subsequent inflammatory challenge.

The term of endotoxin tolerance mainly refers to pro-inflammatory genes, however, the expression of anti-inflammatory genes, like \textit{Il10}, was highly enhanced in LPS pre-conditioned microglia. In addition, tolerized microglia are not immune paralyzed and are still capable to respond to inflammatory stimuli and mount an inflammatory response, e.g. the NF-\kappa B signaling pathway is still functional.

LPS preconditioned mice display impaired learning behavior, possibly due to change in microglia function, since the expression level of LPS target genes in microglia already returned to baseline levels when cognitive tests were performed. One may argue that tolerized microglia have altered synaptic pruning activity or produce less neuronal supportive factors, like BDNF (brain-derived neurotrophic factor).

Combining LPS-induced endotoxin tolerance of microglia with (transgenic) mouse models for neurodevelopmental or neurodegenerative diseases would be a good strategy to determine the functional ramifications of microglia endotoxin tolerance. As described in chapter 1, the pro-inflammatory activity of primed microglia in neurodegenerative diseases is considered to be harmful. Possibly peripheral inflammation-induced tolerance of microglia could be applied to attenuate this detrimental aspect would be a promising avenue of research to pursue.

\textbf{Transcriptome and epigenome analysis reveals the molecular regulatory networks of hypo- and hyper-responsiveness of microglia}

Mapping the epigenetic landscape of tolerized and primed microglia is important to understand how previous challenges and experiences are imprinted in the chromatin and determine cellular function and identity. Importantly, genome-wide analysis is a powerful tool that can provide functional insight into the cellular processes and regulatory gene networks that are implicated in microglia (dys)function in a variety of conditions and diseases (Galatro et al., 2017; Gosselin et al., 2014; Gosselin et al.,
2017; Lavin et al., 2014; Matcovitch-Natan et al., 2016). In addition, the epigenetic factors that confer these modifications could be possible targets for medical interventions and therapy.

During aging and in a variety of neurodegenerative diseases, microglia priming has been observed (Holtman et al., 2015; Norden et al., 2015; Perry and Holmes, 2014; Sierra et al., 2007). However, at present, the molecules and signals that induce this phenotype are not fully known and although a common gene signature was detected in primed microglia, also disease-specific gene signatures were present (Holtman et al., 2015). Additional experiments are required to determine the heterogeneity and stability of microglia priming.

In this thesis, we particularly investigated the transcriptional and epigenetic profiles of microglia tolerized by peripheral inflammatory challenges and microglia primed in accelerated aging mice. Multiple transcription factors were identified in the regulatory networks, which provided possible gene candidates to further investigate as potential drivers of these microglia phenotypes.

**β-glucan, a fungal component, sensitizes microglia**

In this study, we observed that an in vivo challenge with β-glucan sensitized microglia to a subsequent challenge with LPS. The morphological changes of hippocampal microglia in β-glucan pre-conditioned mice are quite pronounced. It is possible that these hypersensitive microglia could exacerbate the decline in cognition induced by peripheral inflammation, although behavioral and learning experiments were not performed. Furthermore, it would be interesting to assess the role of these sensitized microglia in mouse models of neurodevelopmental disorders or neurodegenerative diseases, e.g. 5xFAD or EAE mice, to evaluate to what extent sensitized microglia influence the development of pathology in these models.

It is unclear how β-glucan preconditioning induces microglia hypersensitivity and how comparable this effect is to disease-associated microglia priming and its underlying molecular mechanisms. The sensitization of microglia by β-glucan was relatively transient where microglia priming is long-lasting (e.g. microglia in generic Ercc1 mutant mice), therefore, investigating the epigenetic status of these cells might provide insight in these differences and responsible transcription factors and gene networks.

The protective effect of β-glucan preconditioning to a *C. albicans* infection that is lethal in naïve mice, was determined using a relatively short interval (4 days) between
the infection and β-glucan preconditioning (Cheng et al., 2014; Quintin et al., 2012). How long this protective effect of β-glucan persists is presently unknown. In another study, the training effect of β-glucan in vivo declined after 3 weeks indicated by serum cytokines levels (Garcia-Valtanen et al., 2017).

**Accumulation of DNA lesions leads to microglia aging instead of priming**

Microglia from generic hypomorphic Ercc1 mice are primed. Studies using a Camk2-cre driver that targeted Ercc1 deletion specifically to forebrain neurons (CamK2cre:Ercc1ko/flox) indicated that primed microglia were restricted to the region of forebrain neurons with Ercc1 deletion but not in the hindbrain (Raj et al., 2014). These data suggest that neuronal genotoxic stress is sufficient to induce priming in microglia. However, the effect of Ercc1 deficiency in microglia could also be a contributing factor to their primed phenotype. In this thesis, we determined if microglia-specific deletion of Ercc1 also induced priming in microglia. Microglia in Cx3cr1wt/creER:Ercc1ko/flox mice after tamoxifen treatment showed similar morphological changes as were observed for microglia in generic Ercc1Δ/ko mice. However, their transcriptome profiles are very distinct. Microglia from Cx3cr1wt/creER:Ercc1ko/flox mice, two months after tamoxifen treatment, exhibited properties of accelerated aging. Combined with previous studies from our lab, we propose that accumulation of DNA damage in microglia specifically leads to excessive microglial apoptosis and replacement, where a few cells escape and possibly become senescent. In generic Ercc1Δ/ko mice, the genotoxic stress in other CNS cells, likely neurons, resulted in microglia priming.
General discussion and future perspectives

Endotoxin tolerance in the hippocampus and at single cell resolution
In chapters 2, 3, and 4, the starting material of the in vivo experiments, i.e., RT-qPCR, ChIP-PCR, RNA sequencing, ATAC-sequencing, and ChIP-sequencing originated from sorted microglia from entire mouse brain. However, whether microglia from specific brain regions show similar gene expression patterns or epigenetic modifications is unclear but very unlikely (De Biase et al., 2017; Grabert et al., 2016; Mrdjen et al., 2018). A recent study showed that maternal inflammation attenuated the response of microglia to LPS in the offspring during adulthood, but that the microglia from the hippocampus were not tolerized, indicated by enhanced Il1b expression (Schaafsma et al., 2017). This heterogeneity of microglia, as described in chapter 1, needs to be considered in future studies. Data generated from bulk microglia may mask potential regional differences, in terms of gene expression and epigenetic signature. In addition, differences in microglia turnover rates between different CNS regions, which might have an effect on long-term epigenetic changes, will be lost in bulk samples. Nowadays, single-cell sequencing technology is increasingly applied in microglia research (Keren-Shaul et al., 2017; Matcovitch-Natan et al., 2016; Mathys et al., 2017). This will certainly improve our understanding of microglia heterogeneity. For instance, data from different studies show different conclusions in terms of determined turn-over rates (described in chapter 1), one could take advantage of single microglia sequencing to identify and segregate proliferating and apoptotic microglia based on the expression level of either cell cycle- (Han et al., 2018) or cell death-related genes (e.g. ki67 and caspase). Regarding endotoxin tolerance studies in this thesis, regional specific single-cell RNA sequencing would be a means to identify regional heterogeneity in tolerant microglia.

DNA methylation landscape in tolerized microglia, also more histone marks
In this thesis, six types of histone modifications and chromatin accessibility have been determined, and additional efforts on the involvement of other types of epigenetic modifications would complement these data. For instance, DNA methylation, which has been investigated in endotoxin-induced tolerance in a human macrophage cell line (El Gazzar et al., 2008) and has been used to mark enhancer regions in microglia (Gosselin et al., 2014; Gosselin et al., 2017). H4K20me3 is associated with regulation of the pro-inflammatory response in macrophages (Stender et al., 2012). Apart from
these epigenetic modifications, the responsible chromatin modifying enzymes need to be identified and investigated in different microglia phenotypes. We showed that H3K9me2 was involved in disrupting microglia inflammatory responses (chapter 2) but which histone modifier was responsible for this reduced LPS responsiveness remains unclear. To study microglia in mice deficient (either generic or conditional) for G9a or other related enzymes involved in H3K9 methylation would provide extra insight in the epigenetic regulation of microglia functions. Other candidates that might be involved in endotoxin tolerance in microglia are PHF2 and SMYD5 (catalyze H3K4me3 demethylation and methylation, respectively), because PHF2 is necessary to remove the H3K4me3, recruited by P65 (also known as RelA), in the promoter of pro-inflammatory genes, while SMYD5 is responsible to establish H3K4me3 and rewrite this modification after stimulation (Stender et al., 2012). NF-κB family members have been shown to recruit histone modifiers to specific regions, for instance, the genome-wide analysis of naïve and tolerized microglia indicated that RelA was one of the key transcription factors regulating the genes affected by LPS pre-conditioning (chapter 4), as was also indicated by a study in macrophages (Stender et al., 2012). One could also consider using inducible and microglia-specific RelA knockout mice to determine whether peripheral inflammation would still be capable to induce tolerance in RelA deficient microglia.

**Subtypes of microglia priming**

As described in Chapter 1, many terms have been used for an enhanced pro-inflammatory response of innate immune cells, e.g. training, priming, sensitization, hyper-/exaggerated/enhanced immune response, and hence it is necessary to clarify the definitions used in this thesis. In studying the process of LPS-induced IL-1β secretion, priming refers to the expression of pro-IL-1β, the first step of the pro-inflammatory response initiated by LPS, followed by the second step of secretion of an active IL-1β molecule (Lopez-Castejon and Brough, 2011). When priming is described in terms of epigenetics, e.g. epigenetic priming, it refers to a chromatin alteration prior to gene expression change. For instance, during development, primed enhancers are bound by related transcription factors and histone modifiers prior to recruitment of lineage inducing TFs to activate expression of these genes (Wang et al., 2015; Ziller et al., 2015).

Trained immunity refers to enhanced innate immunity, which, in most situations, means that monocyte-derived macrophages are in an altered state (regarding both...
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their epigenetic and metabolic state) after preconditioning with β-glucan, BCG, or oxLDL. These cells are capable to secrete higher levels of cytokines than naïve cells in response to a stimulus (described in chapter 1). Aged and disease-associated microglia are primed, and exhibit enhanced sensitivity to pro-inflammatory stimuli (which is comparable to trained monocytes), however, more importantly, these microglia also display enhanced phagocytic activity, at least in mice of physiological aging (24-month-old mice), accelerated aging (Ercc1 mutant), AD (APP/PS1 mutant), ALS (SOD1 mutant), or MS (chronic phase of experimental autoimmune encephalomyelitis, EAE) (Holtman et al., 2015; Keren-Shaul et al., 2017; Krasemann et al., 2017). Microglia priming is an adaptive state in response to neuronal damage and misfolded proteins or other factors. We postulate that priming of macrophages and microglia is different, as these cells are exposed to different triggers and are shaped by their tissue-specific microenvironment.

Raj et al. reported that microglia from third and fourth generation mTerc knockout mice (a mouse aging model based on accelerated telomere shortening) were hypersensitive to LPS stimulation but the expression of priming genes was unchanged (e.g. Cd11c, MHC II). Further experiments showed that the enhanced LPS responsiveness of microglia in mTERC KO mice was due to compromised blood-brain barrier integrity (Raj et al., 2015). In this case, mTerc deficient microglia are not considered to be primed. In addition, two days after β-glucan injection, microglia show an increased expression of Cd11c, Dectin 1, and CD68 (but not Axl, Mac2, and Apoe) as well as enhanced pro-inflammatory genes after peripheral inflammation. These microglia display part of priming features; however, this sensitization is relatively transient, and therefore different from priming observed during aging or in generic Ercc1 deficient mice. In summary, increased sensitivity of microglia to an inflammatory peripheral challenge is not restricted to aging-induced priming, but can also be caused by perturbed BBB integrity of exposure to PAMPs like β-Glucan.

Pathogen-dependent effects on microglia inflammatory responses and metabolism

The metabolism of microglia is a relatively unexplored research field. The metabolic alteration of immune cells largely depends on the type of pathogens and receptors involved (Lachmandas et al., 2016). For instance, Pam3CSK4 and LPS induce opposite changes in oxidative phosphorylation of macrophages. Therefore, it is necessary to study the metabolic alterations in microglia in response to different pathogens or
PAMPs. Besides, metabolism may also change over time, for example, LPS-induced metabolic reprogramming in macrophages could be partly restored by IL10 within 24 hours (Ip et al., 2017), and our results showed that the oxygen consumption rate first decreased after LPS stimulation but increased in LPS pre-conditioned microglia. Therefore, the dynamics of metabolic changes should be monitored to better understand microglia physiology.

Microglia were sensitized two days after a β-glucan challenge. Purified β-glucan can only partially mimic a fungal infection. It would be interesting to perform studies with C. albicans to investigate the effect on microglia phenotypical changes after exposure to entire pathogens.

**Conditional deletion of Ercc1 specifically in microglia**

After Cx3cr1 knockout mice being generated (Jung et al., 2000), the Cx3cr1 mice have been used to study microglia in respect to 1) microglia isolation, 2) identify the morphology, 3) track microglia migration based on fluorescence (when EGFP or EYFP reporter gene is inserted), 4) studying microglia phenotype after Cx3cr1 knockout (when the mutations are homozygous), and 5) driving conditional knockout of specific gene (when creER is driven by Cx3cr1 promoter). This method allows specific deletion of certain gene in certain time point, which has been used in several studies, for instance, Sall1 (Buttgereit et al., 2016), Dicer (Varol et al., 2017), Ifnar (Deczkowska et al., 2017), Mef2c (Deczkowska et al., 2017), Mecp2 (Cronk et al., 2015; Schafer et al., 2016) and Ercc1 (this thesis) knockout. However, due to the efficiency of tamoxifen-induced recombination and the persistent turnover of microglia, the heterogeneity of microglia (regarding successful cre-mediated recombination) needs to be considered, particularly when the targeted gene is (directly or indirectly) involved in cell cycle or apoptosis and possibly affects the turnover of microglia. Therefore, it is proposed, when using this system, to introduce a reporter gene to identify whether an individual cell is targeted or not. To investigate the effects of Ercc1 deletion in microglia using tamoxifen-treated Cx3cr1<sup>wt/creER;Ercc1<sup>ko/loxP;R26wt/tdTomato</sup> mice, it becomes then possible to segregate microglia with or without recombination by FACS sorting or to distinguish these two population by imaging. The efficiency of Ercc1 deletion could be determined much easier, the altered morphology and proliferation rate could be identified in targeted populations, and the gene expression profiles of only Ercc1 deficient microglia over time could be investigated. This is based on the assumption that the excision of the floxed Ercc1 and R26-tdTomato alleles is equally efficient.
In addition to its role in DNA damage repair, *Ercc1* is also implicated in gene transcription and cell cycle regulation (described in chapter 1). We have compared the gene expression profiles of microglia from *Cx3cr1*<sup>wt/creER:Ercc1<sup>ko/loxp</sup> and *Cx3cr1*<sup>wt/creER:Ercc1<sup>wt/loxp</sup> mice 5 days after tamoxifen gavage. At this time point, the accumulation of DNA lesions in *Ercc1* deficient microglia was assumed to still be limited. The results showed that no genes were significantly differentially expressed, 5 days after *Ercc1* deletion, which suggested a limited role of Ercc1 in (directly) regulating gene transcription in microglia. However, these results might be masked by the DNA damage induced by tamoxifen itself or the investigated time window. Alternatively, postnatal microglia from *Cx3cr1*<sup>wt/creER:Ercc1<sup>ko/loxp</sup> and *Cx3cr1*<sup>wt/creER:Ercc1<sup>wt/loxp</sup> pups could be cultured and *Ercc1* deficiency induced *in vitro*, allowing to monitor phenotypic changes in microglia following *Ercc1* deletion.

Whether *Ercc1* deficiency in microglia affects the CNS in general or neurons more specifically remains to be determined. Behavioral or cognitive tests could be performed 2 months after tamoxifen treatment when the majority of microglia in *Cx3cr1*<sup>wt/creER:Ercc1<sup>ko/loxp</sup> mice are *Ercc1* deficient. The sensitivity of hippocampal neurons to excitatory stress could be tested in organotypic brain slices prepared from these mice to further investigate the effect of *Ercc1* deletion-induced accelerated microglia aging in microglia-neuron interaction. However, transcriptomic comparison of human and mouse microglial aging profiles showed only few significantly overlapping genes, indicating that the aging-associated gene expression changes in microglia from mice and humans are quite distinct (Galatro et al., 2017). Therefore, it would be interesting to be able to determine the microglia phenotype in post-mortem brain samples from progeria syndromes (PS) patients with mutations in DNA repair genes and compare their phenotypes to microglia in corresponding mouse progeria models.
Conclusion

The data presented in this thesis show that peripheral inflammatory challenges can induce a permanent shift in microglia which is caused by stable epigenetic alterations. Accelerated aging of microglia by specific deletion of Ercc1 only partially recapitulated the phenotype of microglia in aged mice, pointing to the aging CNS as the key determinant of age-induced changes in microglia function.
References


