Long-term regulation of microglia
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Chapter 6

General discussion, summary and future perspectives
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The research topic of this doctoral dissertation concerns the acute and long-term consequences of microglia activation. Every person experiences episodes of inflammation in his or her life. Whether this is transient sickness, low-grade chronic inflammation or severe inflammatory events such as sepsis or traumatic injury, it impacts the adaptive and innate immune system, leaving behind a fingerprint within the epigenome of immune cells, possibly altering future responses. The results presented here show that microglia indeed retain imprints of previous experiences, which possibly permanently change their responsiveness and function.

In the last decade, the knowledge of microglia activation pathways has increased tremendously. However, the exact role of microglia in degenerative changes during aging and neurodegenerative diseases is still a subject of intense investigation and the picture is far from complete. The role of microglia in aging is primarily addressed in scenarios of old age and neurodegenerative disease models, where microglia are often described as being primed cells (Norden et al., 2014; Perry and Holmes, 2014). In contrast, the microglia dystrophy hypothesis of Alzheimer’s disease (AD) postulated by Streit (2004), states that ageing-induced microglia senescence impairs microglia neuroprotective mechanisms, thus facilitating neurodegenerative events (Streit, 2004; Streit and Xue, 2009). Most likely, both priming and loss of specialized functions of microglia during old age and neurodegenerative disease have detrimental effects on the brain. The microglia population is renewed at a very slow rate, around 28% of the microglia population per year and microglia are thus far one of the slowest dividing immune cells described and some microglia can potentially be decades old (Askew et al., 2017; Réu et al., 2017). In this sense, ageing of microglia, starts from the moment they derive from the yolk sac and colonize the CNS during embryonic development. It is here where they are exposed to maternal factors, and where imprinting and adaptation of the microglial phenotype starts, followed by all the challenges experienced during their lifetime.

Long-term effects of LPS on microglia phenotype

Remarkably, very little is known concerning the long-term effects of immune challenges on microglial responses and research mostly addressed acute activation effects over relatively short time periods. In chapter 2, the effect of LPS pretreatment on microglia activation followed by a subsequent LPS stimulus was evaluated in vitro on primary mouse microglia and in vivo in young adult mice. The main goal was to obtain insights in the duration of the tolerance inducing effect of LPS pretreatment; how persistently does LPS influence the secondary response of microglia and what molecular pathways are involved in these changes? In macrophages, pre-stimulation with LPS induces a transient state, which is termed endotoxin tolerance and is viewed/considered to serve as a protective mechanism of innate immune cells
to prevent a secondary exaggerated and possibly damaging inflammatory reaction after a previous inflammatory event (Biswas and Lopez-Collazo, 2009). Using LPS to mimic bacterial infection, the mechanism of sepsis has been studied in both mouse macrophages and human promonocytic THP cells and macrophages isolated from sepsis patients. To mimick sepsis, cells were pretreated with LPS followed by a secondary stimulus (El Gazzar et al., 2007). Although not completely unraveled, these research data have provided mechanistic insights into the molecular processes concerning the unresponsive state of immune cells. In tolerant THP cells RelB initiates recruitment of a repressive complex, resulting in histone H3 lysine 9 methyl-transferase G9a dependent H3K9 dimethylation and recruitment of DNA methyl-transferase DNMT3a/b resulting in heterochromatin formation and silencing of TNF-α and IL-1β (El Gazzar et al., 2009, 2008; Kondilis-Mangum and Wade, 2012). Whether or not microglia develop a similar tolerant phenotype using similar mechanisms until thus far was unknown. In chapter 2, we showed that, similar to what was reported in macrophages, microglia have a blunted inflammatory response to LPS after a previous LPS stimulation, both in vitro and in vivo. Macrophage and monocyte phenotypes are often described and characterized according to the M1/M2 classification, i.e. the classically activated and alternatively activated, polarization paradigm. This paradigm finds its origin in the response of M1 macrophages and M2 macrophages upon factors released from Th1 and Th2 cells, respectively. Where M1 macrophages are producing high amounts of proinflammatory cytokines and have microbicidal capacity, M2 macrophages are involved in resolving the inflammatory response preventing hyper inflammation and mediate tissue repair (Martinez and Gordon, 2014). The phenotype of endotoxin tolerant macrophages resembles M2 macrophages, showing decreased expression of proinflammatory cytokines and chemokines and upregulated genes involved in phagocytosis (Pena et al., 2011). However, the M1/M2 paradigm is currently under revision and is considered an oversimplification of both macrophage and microglia classification (Ransohoff, 2016). Macrophages and microglia polarize in response to microenvironmental cues, and can adopt phenotypic properties that cannot be categorized according to the M1/M2 classification (Heppner et al., 2015; Martinez and Gordon, 2014; Murray et al., 2014). Another pitfall is that M1/M2 classification of microglia in vitro cannot simply be extrapolated to the in vivo situation. Where the in vitro environment often is mediated by a single stimulus such as LPS, the in vivo surroundings are far more complex and will differ between pathological scenarios. With this in mind, in chapter 2, we showed that the blunted response of proinflammatory cytokine expression in LPS-tolerized microglia occurs both in vitro and in vivo. In vivo the effect of an LPS pretreatment by i.p. injection was still observed after 32 weeks, and possibly persists even longer. Since epigenetic modifications are highly involved in the regulation of the ET phenotype in monocytes (El Gazzar et al., 2007), we hypothesized that similar changes in microglia, which have long life span, will be able to induce long-term tolerance. In accordance with macrophages, we have shown that both in vitro and in vivo, epigenetic marks in tolerant microglia correlate with the blunted pro-inflammatory gene expression profile.
Where in tolerized microglia, activation marks AcH3 and H3K4me3 were reduced and the repressive mark H3K9me2 was increased at the IL-1β promotor. Interestingly, in chapter 2 we show that the tolerance in microglia only affects the expression of a small subset of proinflammatory genes in a heterogenous population of microglia collected from total brain. Due to the complex and regionally different environment and cues that microglia are exposed to in vivo, most likely specific subsets of microglia exist with their own transcriptome profile, within different brain regions (Grabert et al., 2016). In other words, it is possible that regional differences exist and not all microglia will express the same tolerant phenotype after a second LPS stimulation, as we have shown. With recent techniques, such as single cell transcriptomic profiling and single cell ATAC- and ChIP-seq a more detailed analysis of the phenotypes of microglia isolated from different region could be performed (Jaitin et al., 2014). The power of these techniques was recently shown, by Zeisel et al (2016), who identified 47 single cell subclasses in the mouse somatosensory cortex and hippocampal CA1 region by using large scale quantitative single cell RNA-sequencing. The resolution of this technique was shown to be remarkable, after clustering of cell types, ‘biclustering’ (where genes and cell types are simultaneously clustered) made it possible for example to identify and separate resident microglia from perivascular macrophages (Zeisel et al., 2015). With the use of single cell ChIP-seq, Rotem et al (2015) were able to identify three subpopulations among thousands of mouse ES cells, with three distinct epigenetic signatures correlating to more naïve ES cells with high pluripotency and cell that exhibited signs of early differentiation. Taking possible regional differences into account, in chapter 3, the response to prenatally administered LPS has been assessed in microglia from offspring that were isolated from total brain as well as from hippocampus. It was observed that microglia samples from total brain showed a tolerant phenotype, but microglia isolated from the hippocampus showed a more primed phenotype, with increased expression of IL-1β in response to a second LPS challenge.

**Biological relevance?**

One of the unresolved questions is: what is the biological relevance of microglia tolerance? And is this microglial endotoxin tolerance adaptive or maladaptive? It seems that the discussion whether or not the ET response is physiologically ‘good’ or ‘bad’ cannot be answered with a simple yes or no. In contrast to macrophages, which have a high turnover and where the ET phenotype is transient, microglia are long-lived cells in a self-renewing population, with low turnover (Askew et al., 2017; Prinz and Priller, 2014; Réu et al., 2017). This raises the question: ‘how long does ET last and what impact does it have on physiology?’. Although microglia are different to macrophages, ET is a mechanism that microglia as well can utilize in the CNS to prevent an exaggerated damaging inflammatory response to recurring stimuli. LPS-preconditioning has been reported to have both beneficial as detrimental effects in the CNS. In vivo, LPS-preconditioning of microglia has been associated with neuroprotection in animal
models of ischemia by prevention of an exacerbated inflammatory response during retinal (Halder et al., 2013) or cerebellar ischemia (Rosenzweig et al., 2004). Several other reports showed a long-term negative impact on learning and memory, and neuroanatomy of prenatal LPS exposure or preconditioning by i.p. LPS injection in adult mice (Graciarena et al., 2010; Lin et al., 2012; Semmler et al., 2007). These observations indicate that the biological effects of LPS-preconditioning depend on the time of preconditioning and type of tissue challenged. As is the case for macrophages, the biological relevance of microglia ET is difficult to define as good or bad, and probably ET will be a case of trade-off between protection/survival vs learning impairment. Furthermore, one could speculate that in an initial phase of inflammatory necessity, other macrophage populations, such as meningeal and choroid plexus macrophages (Prinz and Priller, 2014), can serve as an additional source of cytokines and chemokines. In this case microglia ET would be beneficial since their inflammatory response is not excessive but sufficient. In addition, it should be noted, as shown in chapter 2 and 3, that microglia prestimulated with LPS in vitro and in vivo still show expression of proinflammatory cytokines and are not completely immune suppressed. This means they are still able to respond or to respond in a different way, but that the gene activation program has altered. Genome-wide gene expression profiling of microglia (subsets) would provide more insight in the exact functional changes in LPS-preconditioned microglia. Another possibility is that microglia tolerance is a remnant of evolution, where humans in the past were subjected to a higher bacterial infectious pressure, where ET functioned to prevent the brain from being inflamed very frequently and where survival probably had a higher priority than learning and memory.

Microglial Endotoxin Tolerance: future “to do” list

The studies performed in chapters 2 and 3 were focused on characterizing the effect of pretreatment with LPS with regards to phenotype and molecular mechanisms involved in microglial ET. We have performed behavioral experiments, where young adult mice that were injected with LPS showed impaired learning in a T-maze up to 4 weeks after a single LPS treatment. Furthermore, offspring from LPS injected mothers showed reduced home cage activity, reduced anxiety in an elevated plus maze and impaired learning in a T-maze. These observed learning impairments are clearly the result of previous LPS treatment, but these results do not tell us whether the changed microglial phenotype underlies these learning impairments. However, in the hippocampus of prenatally LPS exposed offspring, stable downregulation of microglial BDNF was observed, whereas expression levels in total hippocampus tissue were only reduced after a second LPS injection. Behavior experiments were performed before the second LPS injection, and thus before any changes induced by it in total hippocampal BDNF expression. This led to the hypothesis that reduced expression of microglial BDNF impairs learning due to impaired synapse management by microglia, possibly perturbing synaptic pruning. This hypothesis is supported by observations made by Parkhurst et al (2013), who showed that microglia depletion in mice resulted in impaired learning,
whereas ‘genetic’ depletion of microglial BDNF had similar effects (Parkhurst et al., 2013). In order to clarify the exact role of microglia in the detrimental effects of LPS pre-exposure, microglial depletion experiments might prove useful. The tools for microglia depletion have improved considerably, and recently Elmore and colleagues (2014) reported an elegant way to deplete the microglia population by ~90% using an inhibitor of colony stimulating factor 1 receptor (CSF1R) (Elmore et al., 2014).

Depletion and subsequent replenishment of microglia in offspring that was prenatally treated with LPS could provide information about the microglia compartment involved in learning impairment induced by previous LPS exposure. Previously, pretreatment with LPS has been shown to induce a neuroprotective effect in retinal ischemia (Halder et al., 2013) and upon middle cerebral artery occlusion (Rosenzweig et al., 2004). To shed more light on the biological relevance of a tolerant microglia phenotype in the long run, it would be of interest to evaluate models for neurodegeneration in long-term LPS prestimulation paradigms as presented in chapter 2 and 3. In order to make more detailed predictions concerning advantages and disadvantages, analyzing genome-wide transcriptome and epigenome changes in tolerant microglia would be of great value. Using weighted gene correlated network analysis (WGCNA) of microglia expression data, Holtman et al (2015) identified the transcriptional signature of primed microglia (Holtman et al., 2015). This same method could be utilized to characterize key “hub” genes in tolerant microglia. Currently, RNA sequencing data of tolerant microglia have been generated in our group, more tolerized genes have been verified, and genome-wide ChIP-seq data are being analyzed. These data sets will provide more insight into the complete tolerant microglia regulatory gene networks and epigenetic regulation of transcription of these genes and will allow us to do more realistic functional predictions.

Importance of diet and microglial health

In chapter 4, the effect of diet on the CNS and in particular on microglia is highlighted. Evidence is accumulating that microglia priming occurs during aging or under chronic neurodegenerative conditions and that this phenotype might contribute to CNS deterioration or disease progression (Perry and Holmes, 2014). Diet can be used to influence extracellular environment and thus prevent/delay or induce such phenotypes in microglia. Recently, In the mouse aging ERCC1Δc mutant mouse model, caloric restriction tripled the mean lifespan of these mice. Mice under caloric restriction maintained motor control and their neuronal population was significantly protected. It was shown that caloric restriction does so by alleviating accumulating DNA damage (Vermeij et al., 2016). Previously, it was shown that specifically targeting neurons with this mutation, primes microglia in their environment (Raj et al., 2014). By alleviating this damage, caloric restriction may help to prevent microglia priming. In chapter 4, we studied the effects of high fat diet in combination with aging on microglia phenotype. In this study, we also investigated the putative intervening effects of
caloric restriction and physical exercise, which both are implicated in contributing to neuroprotective mechanisms during aging and neurodegenerative diseases (Heilbronn and Ravussin, 2003; Ma et al., 2017). We did not detect increased transcription of microglia activation markers induced by HFD, as described in literature where the hypothalamus is described as a site of microglia inflammation (Milanski et al., 2009; Posey et al., 2009; Thaler et al., 2012; Zhang et al., 2008). This is possibly attributable to the differences in age at which time experiments were conducted. Indeed, Baufeld et al (2016) showed that HFD elicited an acute response in the directly after the start of HFD, but a shift towards a more anti-inflammatory profile was observed after 8 weeks (Baufeld et al., 2016). With increasing age at 24 months we observed an increased activation of microglia in important white matter bundles, fimbria of the hippocampus and internal capsule, and this was aggravated by HFD. Decreasing white matter integrity has been correlated with increasing cognitive deficits in aging (Bennett and Madden, 2014) and increased inflammation and demyelination during aging are most prominent in white matter during ageing (FM et al., 2009). In chapter 4, we showed that caloric restriction was able to prevent aging-induced activation of microglia in white matter only in LFD mice. This suggests that the neuroprotective actions of caloric restriction are inhibited by HFD. One proposed effect of caloric restriction is the upregulation of BDNF (Stranahan et al., 2009). In chapter 3, along with another report (Parkhurst et al., 2013), it has been discussed that microglia-derived BDNF can have very important roles in cognitive processes. It would be interesting in future experiments to subject aged HFD and LFD mice to caloric restriction to determine whether or not caloric restriction improves cognitive performance. Also, it will be of importance to determine the gene expression- and epigenetic profile of microglia isolated specifically from the white matter of these mice. Another point of interest would be to assess how primed microglia respond to subsequent LPS injections at old age and whether or not primed microglia can adopt a tolerant phenotype as we observed in chapter 2 and 3 or that somehow this ability is lost during the process of aging.

**Intervention by dietary additives**

In chapter 3 we showed that prenatal treatment with LPS induced a more primed microglia phenotype, in the hippocampus. This has been associated previously with impaired learning and memory (Williamson et al., 2011). Diet has been suggested to be an interesting factor to reduce chronic microglial activation. Indeed, it has been suggested that dietary compounds such as flavonoids, n-3 PUFA’s and bioactive natural molecules such as resveratrol can reverse age-induced microglia priming (Johnson, 2015). In **chapter 5**, using a lentiviral in-vitro microglia reporter model (NF-κB- reporter), we screened several substances that are naturally occurring in certain foods or are well known food additives. We identified that especially magnesium sulfate has anti-inflammatory properties. To verify that magnesium supplementation, or at least keeping magnesium levels sufficient, is beneficial for microglia and a healthy brain, in vivo experiments should be performed. In this respect, mice maintained
on a Mg\textsuperscript{2+} deficient diet for 10-21 days showed impaired fear conditioning compared to animals on a control diet (Bardgett et al., 2005). The data underscore the importance of maintaining Mg\textsuperscript{2+} levels in the body. [Mg\textsuperscript{2+}] in cerebrospinal fluid/brain is high compared to plasma levels and is tightly regulated and limited by active transport. This makes the Mg\textsuperscript{2+} level in the brain difficult to increase by oral supplementation (Slutsky et al., 2010). To study the effects of magnesium in vivo, a magnesium depletion study would be the preferred method to use. In addition to microglia priming, preliminary experiments using our generated BV2 NF-κB -reporter cell line, we showed that pretreatment with magnesium sulfate was able to prevent endotoxin tolerance of BV2 cells in sense of NF-κB.

**Understanding microglia physiology key to healthy ageing?**

In this dissertation, several aspects of the regulation of microglia activity have been discussed. The role of microglia in a range of neuroinflammatory and neurodegenerative conditions has become increasingly clear. A key aspect of understanding how microglia are involved in these processes is to determine how microglia are affected by previous experiences and in recent years significant advances have been made on this topic, including research described in this dissertation. Data presented in this dissertation show that microglia in mice have a long-term ‘memory’ that determines their phenotype, which is regulated by epigenetic mechanisms that are possibly retained in a self-renewing microglia population. Modulating the inflammatory state and response of microglia continuously by a healthy diet and lifestyle might be an important key to healthy ageing.
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