Imbalanced immunity in multiple sclerosis

Koning, N.

Publication date
2009

Summarizing discussion

7

The studies described in this thesis aimed to provide a better understanding of the brain's intrinsic immune suppressive systems with respect to a severe neuroinflammatory disease: multiple sclerosis (MS). Although the results are separately discussed in each chapter, I will here summarize the findings, highlight their importance as a whole, and give some suggestions for future research.

Immune suppressive systems in a balanced immune system

As Matsue in 2005 aptly expressed: 'the immune system must accomplish two opposing tasks: inducing efficient immunological protection against potentially harmful pathogens and, at the same time, preventing excessive and prolonged immunological responses that may lead to tissue destruction'.[248] In other words, proper functioning of the immune system requires a well regulated immunological balance maintained by both activating and inhibitory signals. Defects in either one of these signals consequently results in imbalanced immunity. Because the majority of studies regarding inflammatory diseases have focused on immune activators, the contribution of intrinsic immune suppressors has been underestimated, but is clearly equally important. Examples of potent immune suppressive systems required for immunological balance are cytotoxic T lymphocyte antigen (CTLA)-4 in T cells, and TREM2, CD47-SIRPα and CD200-CD200R in myeloid cells. For the latter pair of molecules, this concept was recently illustrated in the lung, using influenza as a model.[181] Alveolar macrophages are immune suppressed by high CD200R expression and during influenza infection CD200R levels further increased, which is suggested to diminish inflammation. Blocking CD200R signaling using $CD200^{-/-}$ mice resulted in increased viral clearance due to enhanced immune reaction, but coincided with increased mortality as inflammation was excessive. Triggering CD200R with agonistic reagents in turn dampened inflammation. This study demonstrates that the role of inhibitory mechanisms such as CD200-CD200R interaction relies in setting a threshold for immune activation and, when this is exceeded and inflammation has established, further provides a site-specific mechanism to control the immune response and prevent disproportionate tissue damage.

Physiological implications of immune suppressive systems

Immune regulation in the CNS

Special care for overzealous immune reactions should be taken in the CNS. Whereas most other organs are able to repair inflammatory damage, neurons hardly regenerate upon injury, and therefore need to be protected in several ways. The

extremely high expression of CD200 (and CD47) in the brain likely increases the threshold for inflammation and maintains an immune suppressed environment (**chapters 2** and **3**).[111,120] In MS patients however, myelin breakdown, axonal damage and neuronal loss ensue from seemingly uncontrolled inflammation. Apparently, the immunological balance is lost, which may not only be due to enhanced immune activating factors, but may also be ascribed to decreased immune inhibitory input through CD200 and CD47 as shown in **chapter 2**.[111] The consequence is a diminished threshold which facilitates immune activation, and may thus underlie uncontrolled inflammation. However, it seems that the brain has additional mechanisms to restore homeostasis during pathology, by enhancing CD200-CD200R interaction, by induction of CD200 expression on astrocytes in MS lesions (**chapter 3**). This might be similar to the situation studied in the lung.[181] Nevertheless, astrocytes cannot sufficiently compensate the loss of CD200 as overall expression of CD200 in MS lesions is decreased, which might be due to loss of expression of this molecule on neurons and oligodendrocytes, as these are the two main cell types expressing CD200 and are most heavily affected by the disease.

CD200-CD200R interaction is not the only system with immune modulatory functions in the brain. Other examples are glucocorticoids (GCs) produced from hypothalamo-pituitary-adrenal (HPA)-axis activation and sex hormones as estrogen and testosterone. Both types of hormones have multiple actions including anti-inflammatory and neuroprotective effects.[249-251] Upon treatment, glucocorticoids and estrogens are potentially beneficial for MS patients as they can reduce the volume and number of gadolinium enhancing lesions.[46,152] In many MS patients, endogenous cortisol production is increased although clinical effectiveness is yet to be determined.[252-254] This indeed may form a mechanism to control the immune system, as demonstrated by the finding that suppression of the HPA-axis by active hypothalamic MS lesions correlated with a worsened disease course.[255] Protective effects of endogenous estrogen are suggested by studies showing that elevated levels of estrogen during the third trimester of pregnancy are associated with a decrease in MS symptoms.[256] Its immune regulatory capacity is demonstrated by the observation that symptoms rapidly exacerbate post-partum, when estrogen levels drop.[256] The expression analysis of several genes in MS lesions, described in **chapter 2** of this thesis, shows amongst others an increased amount of ERα in MS lesions, possibly reflecting an endogenous mechanism to modulate local inflammation. Interestingly, in neurons of the human paraventricular nucleus, ERα colocalizes with CRH, the driving force of the HPA-axis.[257] Since estrogen response elements are present in the promoter region of CRH,[258] one of the mechanisms by which estrogen exerts its beneficial effects may be the activation of the HPA-axis

and thus the enhancement of glucocorticoid levels.

The systems mentioned above are hypothesized to act in response to inflammation in order to restore the immunological balance by inducing CD200 expression in astrocytes, activating the HPA-axis to release cortisol and to increase the expression of estrogen receptors. Interestingly, inflammation in MS lesions at some point resolves, but the mechanisms behind this ceased activity are currently unknown. It might be speculated that the systems mentioned above and described in this thesis are involved in halting inflammation in an established lesion. In contrast to fluctuating hormone levels, CD200 and CD200R are constitutively expressed in the brain. The sustained neuron-glia and glia-glia interactions (**chapter 3**) would thus incessantly control the immune response, suggesting that this system may therefore be more effective in continuously balancing immunity compared to systems that are induced upon inflammation. The decreased expression of CD200 would therefore significantly contribute to the imbalanced immunity in MS. Accordingly, these systems are excellent targets for therapy, as will be discussed below.

Shaping of immune responses by CD200-CD200R

CD200-CD200R interaction may not only be involved in suppression of the innate immune system, but potentially also in modeling its response. The data presented in **chapter 4** shows that IL-4 induced alternative activation of macrophages (M2 cells) triggers enhanced CD200R expression on these cells. M2 cells display an anti-inflammatory phenotype and are known to mediate tissue remodeling and repair as well as to induce Th2 responses by expressing anti-inflammatory cytokines.[22,23] As the effects of CD200R triggering, i.e. decreased production of IL-6 and TNF,[124] are in line with those seen in M2 cells, it is likely that CD200R signaling is responsible for many M2 cell activities. In MS, infiltrating and activated macrophages have a pro-inflammatory phenotype, but macrophages that have ingested myelin switch to an anti-inflammatory phenotype consistent with M2 cells.[29] In concordance, **chapter 3** of this thesis shows that foamy macrophages express CD200R. Notably, this is after myelin ingestion, whereas an early shift to an anti-inflammatory phenotype would possibly prevent inflammation and lesion development. Several current therapies that appear beneficial in MS treatment can shift a type I to a type II immune response (**chapter 1**). Proper activation of CD200R by a therapeutic agonist could therefore be highly beneficial in MS, not only to suppress immune activity, but also to shift towards a type II immune response with a more favorable outcome.

To achieve a switch in the immune response in MS lesions, CD200R signaling in the CNS should be increased, for example by enhancing its ligand, CD200. However, the regulation of CD200 is still obscure. Although a single group claimed that

a remarkably high dose of IL-4 enhances neuronal CD200 expression,[193,259] the experiments with physiological levels of IL-4, described in **chapter 5** of this thesis, did not confirm this. Indeed, IL-4 may play a role in the regulation of macrophage activation, but rather by inducing CD200R expression on polarized macrophages as discussed above (**chapter 4**). In **chapter 4** and **5**, we show that CD200R is also expressed on microglia. Interestingly, CD200R expression on these cells is not enhanced by IL-4, indicating that microglia either do not polarize, have already polarized, or are influenced by other factors than IL-4. Nevertheless, regarding MS it is important to note that the expression of CD200R in lesions was unchanged,[111] indicating that it is available as therapeutic target. In absence of knowledge on how to enhance endogenous CD200 expression, administration of soluble CD200 or a CD200R agonistic antibody may provide a good therapeutic approach to diminish and shape the inflammatory response and regain homeostasis in the brain.

Peripheral immune regulation by GITR-GITRL

Apart from studies concerning immune suppression in the brain, we also studied a mechanism of peripheral immune homeostasis. **Chapter 6** shows that interaction of glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) and its ligand (GITRL) is involved in balancing the adaptive immune system by increasing both regulatory and effector T cell populations. Functional consequences of controlling the numbers of both regulatory and effector T cells by GITR stimulation appeared protective in experimental autoimmune encephalomyelitis (EAE), as it significantly delayed disease onset. In MS patients, regulatory T cells were thought to have reduced suppressive activities,[260,261] but this was recently revised when non-regulatory $CD127^{hi}$ expressing T cells were removed from the population of $CD4^{+}CD25^{hi}$ regulatory T cells that subsequently had similar suppressive capacities when compared to healthy individuals.[262] Regulatory T cells are highly important in controlling potentially harmful responses for example to self-antigens.[263] Although regulatory T cell function apparently is not altered in MS, the $CD127^{hi}$ T cell population was highly activated in patients. These results indicate that the imbalance in immune regulation is also displayed in the T cell compartment. Hence, GITR-GITRL signaling could be an interesting mechanism to rebalance responses of the peripheral adaptive immune system.

Clinical implications of immune suppressive systems in the CNS

As mentioned above, immune suppressive systems are essential for a proper functioning and balanced immune system. Especially in the CNS where they provide

in concert with other mechanisms such as the blood-brain barrier a constitutive immune suppressed environment. In this way the immunological survey in the CNS, predominantly carried out by microglia, is still occurring, but will not elicit substantial inflammatory responses. This is highly important, because protection of neurons and other CNS cell types from inflammation-mediated degeneration is paramount, as neuronal damage will hardly be repaired. In several conditions however, strong microglia activation is observed. MS is an extreme example, where activated microglia, but also infiltrating macrophages are thought to be effector cells driving disease development. Microglia activation is also evident in stroke, brain trauma, Alzheimer's disease, Parkinson's disease and in ageing, where they are considered to contribute to neurodegeneration.[264-268] Evidence is growing on the relation of microglia activation with CD200 levels in these conditions. During ageing in rats, microglia activation showed a strong correlation with reduced amounts of CD200.[269] In humans, decreased immune suppression via CD200-CD200R has first been implicated in MS (this thesis), and recently a study in brains of patients with Alzheimer's disease elegantly showed a significant reduction in mRNA and protein expression levels of CD200 but also CD200R in the hippocampus of these patients.[132] Although direct evidence is still lacking, CD200 expression is furthermore suggested to be linked to microglia activation in Parkinson disease.[270] Enhancing CD200-CD200R interaction could therefore be beneficial in many neurodegenerative conditions where activated microglia may cause or enhance neuropathology.

A prerequisite for a potential therapeutic target such as the CD200-CD200R interaction is its sufficient capacity to effectively dampen immune activation. The spontaneous activation of microglia in absence of CD200 already indicates the power of this immune suppressive system, but its strength has been further demonstrated in studies concerning tumor immunology and anti-viral responses. For example, CD200 expression is enhanced on several tumors and is subsequently associated with a decreased anti-tumor immune response and worsened survival prognosis.[131,271-273] Also several viruses express CD200-like molecules that have been shown to bind to CD200R and indeed are able to suppress myeloid immune responses.[274-277] Although elevated levels of CD200 in these examples are undesired, they clearly demonstrate the effectiveness of CD200-CD200R interaction in dampening immune responses. *Wld*^{S} mice have inherently elevated levels of CD200 in the CNS.[129] Importantly for MS, this enhanced CD200 expression appeared responsible for suppression of inflammation and protection against EAE compared to wild type littermates. Conclusively, these studies indicate that CD200-CD200R interaction can be expected a powerful tool for treatment of MS.

To enhance immune suppression as MS therapeutic, it is important that the drug is able to enter the brain, where the imbalance in the immune system is most evident. Whereas most compounds cannot cross the blood-brain barrier and thus cannot enter the brain in healthy individuals, this might be relatively easy in MS, since damage of the blood-brain barrier is a well known feature in active MS lesions. Hence, systemically administered small molecule CD200R agonists like CD200 fusion proteins or agonistic CD200R antibodies, likely enter the brain easily and could thus act locally to suppress inflammation and restore the immune suppressed environment. Maximizing the stability and increasing the affinity of CD200R agonists would furthermore be important in the development of such a therapeutic.

Future directions

Many questions still need to be answered and necessitate further research focusing on immune suppressive systems in MS. As discussed before, CD200 expression can be induced in astrocytes in MS. This indicates that CD200 expression is regulated, but it remains unclear what factor is responsible. Similarly, the intensity of CD200 expression varies among different tissues, with the highest expression found in the CNS.[120] So far, the influence of cytokines on CD200 has been studied, but other soluble or membrane-bound proteins should also be studied. For example, we showed that cytokines could not affect CD200 expression in cultured neuroblastoma cells, but these cells seemed to express higher levels of CD200 protein when clusters were formed, as seen by immunohistochemistry (data not shown). Although this could not yet be confirmed quantitatively on mRNA or protein level, this observation argues for involvement of cell adhesion molecules. In addition, the role of different CNS cell types in influencing CD200 expression could also be studied.

An important question is what the cause is for the decreased amount of CD200 and CD47 in MS lesions. It could be a simple reflection of damaged neurons and oligodendrocytes. However, CD47 was also decreased in white matter directly adjacent to MS lesions (**chapter 2**) and recent preliminary data suggest a reduction in expression of both CD200 and CD47 in normal appearing white matter that does not directly surround MS lesions as tissue without MS lesions was used and gene expression data were consistent within this tissue as well as in the different donors (J. Melief, pers. comm.). Since activation of microglia, as demonstrated by up-regulation of MHC class II molecules, is not yet detectable in these areas, these findings corroborate the concept that immune suppression in the brains of MS patients is impaired, leading to a decreased immunological threshold that hence facilitates

the development of inflammatory foci. Indeed, this may be indicative of an event prior to neuronal and oligodendrocyte injury, but does not exclude the possibility that affected neurons or oligodendrocytes are an initial key factor in MS lesion ontogeny since decreased expression of neurofilament and myelin basic protein (MBP) can also be found in white matter not directly surrounding MS lesions (J. Melief). Future research should therefore focus on the mechanisms of the reduced expression of the immune suppressive molecules in the CNS of MS patients and whether this correlates to microglia activation, which is linked to the earliest stages of lesion development,[6,33,34] in order to understand the cause and consequence of this hampered immune balance.

In the gene encoding CD200, 196 single nucleotide polymorphisms (SNPs) have been identified, of which 7 result in an amino acid change (www.genecards.org). In the CD200R gene, 199 SNPs are known including 7 changes in amino acid sequence. However, it is at present unknown whether these alterations have functional consequences. SNP analysis should therefore be performed on post-mortem brain tissue in order to study the associations and implications of these polymorphisms with MS or any other neurodegenerative disease.

CD200 in the brain most obviously binds CD200R on perivascular macrophages and microglia. The biology of human microglia in MS is a largely unexplored field, with the most important reason that these cells are hardly accessible and methods to isolate them from post mortem tissue in a pure form are currently limited. Based on previous work on microglia isolations from rodent brains described by Sedgwick and colleagues,[162,184,185] **chapter 5** of this thesis shows a reliable and rapid method of isolating and culturing resident microglia from human post-mortem tissue. This opens the door to study the dynamics of these cells from control subjects and patients with different neurodegenerative diseases, including MS. Since macrophage activation is known to be diverse, depending on the stimulus, it would be highly interesting to know whether microglia also show this diversity upon activation and if this would correlate with neurotoxic or neuroprotective effects that have been ascribed to activated microglia.[31] To obtain information on the activation and polarization status of human macrophages and microglia, one could perform a differential screening of gene and protein expression for example directly after isolation of the cells or after stimulation with pro- and anti-inflammatory compounds. In addition, cells can also be compared for example between relatively young donors and older donors, or between donors with no disease of the central nervous system and MS patients. Especially in older donors and in MS patients, microglia are expected to show a higher activation status compared to microglia derived from younger or control donors. These screenings can also be

performed on macrophages and microglia derived from mice as the higher availability of tissues compared to human donors is an important advantage. However, macrophage polarization in mice may have different underlying mechanisms compared to those in human (**chapter 4**), indicating that data from mice macrophages and microglia can not unquestioningly be extrapolated to humans. Finally, microglia and macrophages that have ingested myelin in MS lesions, display an alternatively activated phenotype[29] and express CD200R (**chapter 3**). In following studies, it should be tested whether inducing high CD200R expression on non-myelin laden macrophages prevents inflammation-mediated phagocytosis such as seen in MS. In line with this, it should also be studied if CD200 could mediate polarization of macrophages, once classically activated, towards alternative activation and how this affects phagocytosis and inflammation.

Additional mouse studies could provide important information on the role of macrophage regulation in the disease model for MS. For instance, depleting macrophages from CD200$^{-/-}$ mice would identify the role of peripheral macrophages in EAE in the absence of CD200. Conditional knockouts of CD200R on macrophages, by using the Lysozyme M(Cre)[280] or F4/80(Cre) mice, is expected to increase the development and severity of EAE because of a lack of inhibition of these cells. In addition, bone marrow chimaeras of WT and CD200R$^{-/-}$ mice are considered valuable in dissecting the role of blood-derived macrophages versus resident microglia.

Other mechanisms that control the activation of microglia, such as CD47-SIRPα interaction should be further studied as well. For example, their broad expression pattern is comparable to that of CD200, but the cell types positive for these molecules have not been identified in detail, neither are factors that can regulate their expression. CD47-SIRPα interactions play a role in phagocytosis of erythrocytes, but it is currently unknown whether it affects myelin phagocytosis. The effects of CD47-SIRPα and of CD200-CD200R on phagocytic capacities of macrophages and microglia should be elucidated in future studies. Unfortunately, double knockout mice for CD200 and CD47 will not be helpful as the mice appear not viable (R.M. Hoek, pers. comm.). TREM2 which in the brain is mainly expressed by microglia, has been demonstrated to have immune suppressive activities in EAE.[278,279] However, its ligand is still unknown and limited information is available on the mechanisms by which TREM2 exerts its anti-inflammatory functions. More research is therefore needed to elucidate the immune suppressive properties of TREM2.

Finally, the most important and relevant question to be answered is whether CD200-CD200R interaction alone, or in combination with the other intrinsic immune suppressive mechanisms mentioned above, can indeed restore balanced immunity in MS and can finally stop the progression of this very disabling disease.