Mediators of central immune response and peripheral immunosuppression in human and experimental stroke

Mediatoren der zentralen Immunantwort und der peripheren Immunsuppression im humanen und experimentellen Schlaganfall

I n a u g u r a l d i s s e r t a t i o n

zur

Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Ernst-Moritz-Arndt-Universität Greifswald

vorgelegt von
Juliane Schulze
geboren am 13.02.1985
in Luckau

Greifswald, 24.11.2016

Dekan: Prof. Dr. Werner Weitschies

1. Gutachter : Prof. Dr. Barbara Bröker

2. Gutachter: Prof. Dr. Dr. Johannes Boltze

Tag der Promotion: 29.05.2017

Content

1 Summary

1.1 Introduction and Results

1.1.1 Disease

In 2013, 10.3 million new strokes occurred globally; 67% of these strokes were ischemic and 6.5 million persons died from their stroke (Feigin et al., 2015; Hankey, 2013). Stroke is the 2nd leading cause of death worldwide (WHO, 2014) and the leading cause of long-term disability with 113 million disability adjusted life years (DALYs) in 2013 (Feigin et al., 2015; Mozaffarian et al., 2015). As our population ages, more attention must be paid to diseases of the elderly, including stroke. Given the prevalence of stroke and the devastating effects it has on its victim, it is important to identify new strategies for stroke prevention and acute stroke therapy.

The causes of stroke are heterogeneous. Risk factors include non-modifiable factors like age, sex, ethnicity and family history as well as modifiable risk factors including obesity/nutrition, smoking, hypertension, and physical inactivity (Romero, Morris, & Pikula, 2008). Ischemic stroke is the most common type of stroke and generally occurs when a thrombus or embolus occludes a blood vessel supplying a part of the brain. A hemorrhagic stroke is caused when a blood vessel ruptures and there is bleeding in (intracerebral hemorrhage) or around (subarachnoid hemorrhage) the brain.

Modelling stroke

To better understand stroke and in order to study potential therapeutic interventions, experimental models have been established in different species of animals. Such models are necessary as access to patient material is largely limited to blood samples, and the systemic effects of stroke cannot be investigated *in vitro*. And since ischemic stroke is the most frequent type of stroke, the middle cerebral artery occlusion (MCAO) has become the most widely used experimental model of human stroke. Most stroke research is done in small animals, like mice, rats and rabbits. There are models in which cerebral ischemia can be induced without the need for craniotomy (embolic, intraluminal filament MCAO, photothrombosis, endothelin-1-induced) and those that require craniotomy like electrocoagulation of the distal middle cerebral artery. Among these experimental stroke models, MCAO with an intraluminal filament has many

advantages with a less traumatic surgical approach than those models that require craniotomy, the ability to achieve reperfusion which mimics the clinical situation of thrombolysis, good reproducibility in comparison with other models and short surgery time allowing for more efficiency and the minimization of anesthesia effects. Our experimental stroke studies in rats and mice were therefore performed using the intraluminal filament MCAO. The advantages and disadvantages of different experimental stroke models have been reviewed (Fluri, Schuhmann, & Kleinschnitz, 2015). The Stroke Therapy Academic Industry Roundtable (STAIR) recommends that positive results should be confirmed in another species and should also be reproducible in a different stroke model.

1.1.2 Immunology of Stroke

Under normal physiological conditions, an anti-inflammatory milieu is maintained within the central nervous system (CNS). The picture below depicts the immunologic alterations occurring after (ischemic) stroke. Details are given in our review articles (Schulze, Vogelgesang, & Dressel, 2014; Vogelgesang, Becker, & Dressel, 2014). In short, due to the brain injury that is caused by the ischemic stroke a local inflammation in the brain occurs.

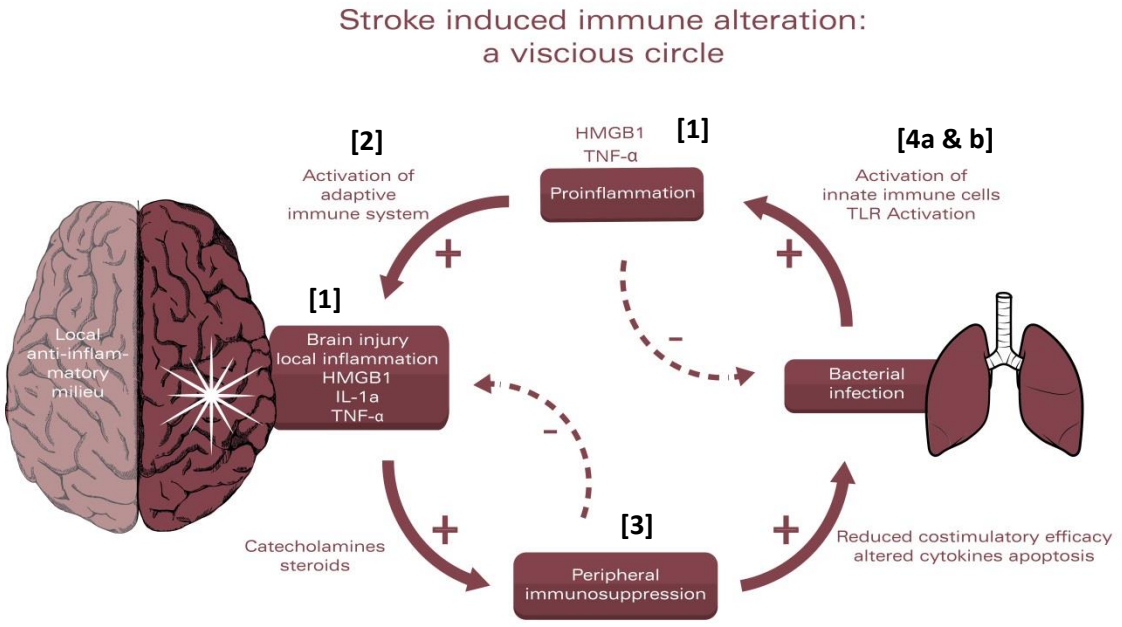


Fig.1: Modified from (Vogelgesang et al., 2014). The numbers in brackets in this picture indicate the different approaches with which the immunologic changes following stroke where investigated in this dissertation.

In the following, a brief overview of the different aspects of the vicious circle of stroke-induced immune alterations will be provided. These will then be explained in more detail, and the different approaches that have been taken in this study to shed light on the immune alterations in stroke [1-4, see Fig.1] will be described.

Pro-inflammatory mediators are released from the site of injury and act locally, but these same mediators gain access to the systemic circulation and induce an inflammation within the periphery. This pro-inflammatory process was investigated in approach 1. On the other hand, stroke induces the production and release of catecholamines and steroids that lead to a peripheral immunosuppression. This immunosuppression is thought to dampen the immune "attack" on the injured brain, but it also increases the risk of bacterial infections as the ability to combat pathogens is impaired. As part of the immune system's suppressive cellular mediators, regulatory T cells (Treg) were investigated in approach 3. Infections, on the other hand, lead to activation of the innate and adaptive immune responses that cause secondary inflammatory damage to the brain. This was investigated with approach 2. In approach 4a and 4b we furthermore investigated the impact of genetic variations on the risk of infection and the patient outcome.

1.1.2.1 Response to the ischemic injury

In ischemic stroke, the disruption of blood supply to an area of the brain leads to abrogation of oxygen and glucose transport. The resulting energy failure immediately causes change in ionic homeostasis including increased intracellular concentrations of Na+ und Ca2+, glutamate induced excitotoxicity, generation of reactive oxygen species and eventually neuronal cell death in the ischemic core (Lai, Zhang, & Wang, 2014). Cytoplasmic ionic accumulation also contributes to swelling and further vascular compression preventing a reperfusion of the affected area. At the periphery of the ischemic region, called penumbra, damage develops more slowly and neuronal cell death might be still prevented. This acute phase lasts for minutes to a few hours. In the subacute phase, hours to days after stroke, apoptosis, necrosis and neuroinflammation occur as a result of the damage caused in the acute phase. The endogenous inflammation following the initial injury leads to a secondary brain damage. During the chronic phase, which occurs days and weeks after the ischemic insult, there is repair and regeneration of brain tissue.

Normally the blood brain barrier (BBB) shields the CNS from the peripheral circulation and the tight junctions of the endothelium regulate the diffusion between the different compartments. The BBB, consisting of endothelial cells, pericytes and astrocytes, is damaged after stroke.

Approach 1: Do DAMPs released due to brain damage signal an inflammatory response and can they help to predict the patients' outcome?

"Damage/danger associated molecular patterns" (DAMPs) are released from necrotic cells and can activate the innate immune response through pattern recognition receptors like the toll-like receptors (TLR). In the brain, DAMPs released from tissue injured by ischemia activate microglia and trigger the local production of pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α, chemokines and adhesion molecules. Disruption of the BBB also allows for these DAMPS to enter the systemic circulation. Examples of DAMPs include uric acid, nucleic acids, nucleotides like ATP & UTP, lipids and heat shock proteins. Together with pro-inflammatory cytokines they are likely to induce and affect an endogenous inflammatory response in the periphery (Gelderblom, Sobey, Kleinschnitz, & Magnus, 2015; Shichita, Ito, & Yoshimura, 2014).

High mobility group box protein 1 (HMGB1) is a nuclear histone-binding protein that can be passively released upon cell damage or actively secreted upon activation of cells and functions as a pro-inflammatory mediator (Lotze & Tracey, 2005; Scaffidi, Mistelli, & Bianchi, 2002). HMGB1 is a classic DAMP that binds to TLR2, TLR4 and the receptor for advanced glycation end products (RAGE) to activate NF-κB (Bierhaus et al., 2005; Yu et al., 2006). In experimental studies of stroke, neutralizing HMGB1 or abrogating HMGB1-induced RAGE signaling decreases infarct size and attenuates the inflammatory response in the infarct area (Liu et al., 2007; Muhammad et al., 2008)

In this study detailed in chapter 2.2, we questioned if early released endogenous DAMPs like HMGB1 signal the post-stroke inflammatory response and if their presence may predict outcome from ischemic stroke.

Patients, Materials and Methods

For this study 110 acute stroke patients were recruited. Plasma was obtained at multiple time points after stroke. HMGB1 plasma concentrations were correlated to stroke severity and biomarkers of inflammation, stroke outcome and autoimmune responses to brain antigens.

Results

Acute ischemic stroke resulted in an increase of the HMGB1 plasma concentration that was measurable up to 30 days after stroke and was highest immediately after stroke. Plasma HMGB1 correlated with the number of circulating leukocytes but not with the infarct volume. It was not independently predictive of stroke outcome or of the development of an autoimmune response to brain antigens.

Conclusion

The activated leukocytes seem to be the major source of plasma HMGB1 post stroke rather than the brain injury itself as HMGB1 concentrations are correlated with the number of leukocytes but not the infarct volume.

1.1.2.2 Local immune response of the ischemic brain

The CNS-immune system interactions lead to an activation of immune cells in the periphery through pro-inflammatory mediators like TNF-α, IL-6, IL-1α, IL-1β and a subsequent infiltration of activated immune cells like macrophages, granulocytes, T and B cells into the damaged brain (An et al., 2014). On the one hand this immune response causes a secondary inflammation-mediated injury of brain tissue but on the other hand it is necessary for phagocytosis of damaged neuronal cells and repair mechanisms.

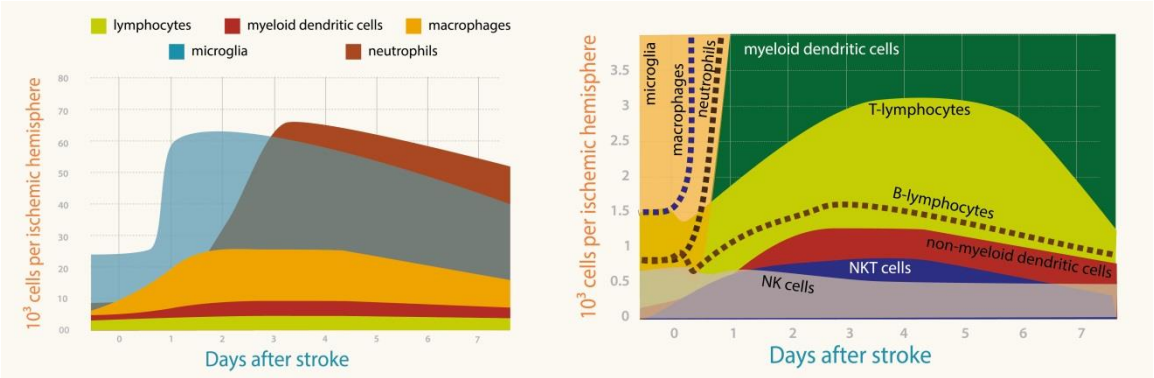


Fig.2a & b (graphs modified from (Gelderblom et al., 2009)). A: The figure depicts the time course and amount of immune cells infiltrating into the ischemic hemisphere after stroke. B: Shows the time course and amount of infiltration of lymphocyte subsets (only summarized as lymphocytes in Fig.2a) into the ischemic hemisphere post stroke.

Macrophages and neutrophils were shown to be first to infiltrate the brain after ischemic damage within hours. Later during the post-stroke inflammation, lymphocytes are recruited into the ischemic brain within 2-3 days after stroke onset (Gelderblom et al., 2009). In an experimental study, T and B cell lymphocyte deficient SCID (severe combined immunodeficiency) mice developed smaller stroke lesions and less inflammation than C57Bl/6 immunologically intact controls (Hurn et al., 2007). Kleinschnitz et al. showed in a study with T and B lymphocyte deficient Rag mice and a reconstitution of T and B cells, that B cells seem to have a neuroprotective role, whereas T cells seem to contribute to the endogenous inflammation (Kleinschnitz et al., 2010). Furthermore, the disrupted BBB also allows brain antigens that are normally shielded to enter the periphery and gives the possibility of activation of autoreactive T cells (Kyra J. Becker, Kindrick, Lester, Shea, & Ye, 2005; Planas et al., 2012). The role of neural antigens and their presentation has recently been reviewed (Miro-Mur, Urra, Gallizioli, Chamorro, & Planas, 2016).

As T cells seem to contribute to the secondary inflammatory damage to the brain it is important to identify different T cell subsets as they resemble different qualities of immune response. T cells develop either into naïve CD4 T cells (also called T helper or Th cells), into naïve CD8 T cells (also named cytotoxic T cells or CTL) or into natural Treg (nTreg). Naïve Th cells can be skewed into different effector cell subpopulations depending on the cytokine environment during activation, of which the most important ones are: i) pro-inflammatory primed Th1 cells that produce IFN-γ, ii) (mostly) anti-inflammatory primed Th2 cells that produce IL-4, iii) pro-inflammatory Th17 cells producing IL-17 (associated with chronic inflammatory diseases), iv) suppressive induced Treg (iTreg) producing IL-10 and TGF-β (Raphael, Nalawade, Eagar, & Forsthuber, 2015). Due to their pro-inflammatory potential, Th1 and Th17 cells are of interest as mediators of a secondary damage upon infiltration to the brain.

Approach 2: Do autoreactive pro-inflammatory T lymphocytes influence the outcome after experimental stroke?

As detailed in chapter 2.3 “The immunologic profile of adoptively transferred lymphocytes influences stroke outcome in recipients” in this study we investigated if autoreactive T lymphocyte responses modulate or worsen outcome after experimental stroke.

Animals, Materials and Methods

In donor rats a severe stroke was induced by 3h MCAO. The T cell response was forced towards pro-inflammation by the administration of lipopolysaccharide (LPS). When LPS is administered at the time of stroke to model infection with Gram-negative bacteria, the proportion of lymphocytes with a Th1-type immune response towards the brain antigen myelin basic protein (MBP) is increased after stroke (Kyra J. Becker et al., 2005).

Donor lymphocytes were obtained from the spleen of donor rats 1 month after experimental stroke. The MBP specific effector function of donor cells was determined prior to adoptive transfer using an *ELIspot* assay for IFN-γ, IL-17 and TGF-β1 secretion in response to MBP. A Th1 immune response was defined by the ratio of the relative increase in the number of MBP specific IFN-γ to the relative increase in the number of MBP specific TGF-β1 secreting cells and a Th17 immune response as the ratio of the relative increase in the number of MBP specific IL-17 to the relative increase in the number of MBP specific TGF-β1 secreting cells. The donor cells were labelled with the cell permeable dye Carboxyfluorescein succinimidyl ester (CFSE) and injected into recipient animals at reperfusion 2 hours after MCAO. The functional and clinical outcome of recipient animals was determined with functional tests and a neurological score. Brain sections of recipient animals were stained for fluorescein (CFSE+ cells), IL-17, IFN-γ and CD8 by immunofluorescent staining.

Results

Animals that received MBP specific donor cells with either an MBP specific Th1 or Th17 dominated immune response at the time of stroke experienced a worse clinical outcome than animals that received donor cells without a Th1 or Th17 dominated immune response. The more robust the Th1 and Th17 response in donor cells the worse the functional performance (Rotarod) in recipient animals was. Furthermore, robust Th17 responses to MBP were associated with worse neurological scores in recipient animals. In animals receiving Th1+ donor cells, there were more infiltrated donor cells (CFSE+ cells) in the infarcted hemisphere 1 day after MCAO compared to animals that received Th1- donor cells. In animals receiving Th17+ donor cells, there were more IFN-γ+ and more CD8+ cells in the infarcted hemisphere 3 days after MCAO compared to animals that received Th17- donor cells.

Conclusion

The quality of the lymphocyte response to self-antigen (MBP) influences stroke outcome as inflammatory primed MBP specific T cells led to a worse outcome. As LPS, mimicking an infection with Gram-negative bacteria, increases the number of inflammatory primed MBP specific lymphocyte responses, it shows a possible mechanism how infections could negatively influence patient outcome.

1.1.2.3 Peripheral immune alterations

Besides the activation and infiltration of immune cells to the brain, the CNS-immune interaction also involves the suppression of immune responses in the periphery - most likely to prevent activation and infiltration of autoreactive immune cells (as investigated with approach 2) thus limiting secondary brain injury. Together, activation of the hypothalamic-pituitary-adrenal (HPA)-axis after stroke leading to a release of glucocorticoids from the adrenal gland and the activation of the sympathetic nervous system (SNS) with direct innervation of lymphoid organs and the release of epinephrine and norepinephrine are thought to lead to changes in peripheral innate and adaptive immune system. In our review article, we provided an overview on how glucocorticoid and sympathetic responses affect immune cells after stroke. For instance, many immune cells express both glucocorticoid and adrenergic receptors, thus providing a mechanism by which activation of the SNS and the HPA-axis following stroke can directly affect the peripheral immune system (Schulze et al., 2014). These issues are further detailed in chapter 2.7. Functional impairments affect mechanisms of the innate and adaptive immune system.

In human stroke patients numerous signs of immune suppression can be observed. Already on admission to the hospital and on the following days, stroke patients suffer from a T lymphopenia compared to age-matched controls. A long lasting lymphopenia was also reported in experimental stroke models, for example by Prass et al (Prass et al., 2003). Furthermore the HLA-DR on monocytes which monocytes need in order to present antigens and successfully induce an immune response is reduced in stroke patients (Vogelgesang et al., 2008). The neutrophils, as part of the first line defense against pathogens, are functionally impaired. It could be shown that the oxidative burst and *NETosis* which both are important mechanisms to kill bacteria are reduced and defective in stroke patients. Stress hormones like epinephrine and

norepinephrine can induce these deficits in neutrophils *in vitro* (Ruhnau et al., 2014). SIIS is also documented in experimental stroke studies. A shift from Th1 cytokines like IFN-γ to Th2 cytokines like IL-4 was found in experimental stroke (Prass et al., 2003). Stroke-induced effects can also be detected in the spleen after experimental stroke as the spleen becomes atrophic mostly due to apoptosis of lymphocytes (Mracsko et al., 2014; Offner et al., 2006).

The described alterations in both the innate and adaptive immune response may explain the well documented high susceptibility to infections in stroke patients. Most often pneumonia and urinary tract infections occur. Reviewed by Emsley and Hopkins, rates of these so called stroke-associated infections (SAI) vary depending on the study from 1-33% for respiratory tract infections and 2-27% for urinary tract infections (Emsley & Hopkins, 2008). The problem of post-stroke infections is that they worsen outcome and increase 30-day mortality to 27% in acute stroke patients with pneumonia in comparison with 4% in patients without pneumonia (Katzan, Cebul, Husak, Dawson, & Baker, 2003).

The blockade of the SNS and HPA axis with propranolol and RU486 could prevent the stroke-induced impairments in experimental stroke studies (Mracsko et al., 2014; Prass et al., 2003; Yan & Zhang, 2014). A recent experimental stroke study by Römer et al. has shown that inhibiting the stroke-induced immunosuppression (SIIS) by blockade of the SNS with propranolol and the HPA axis with mifepristone increases autoreactive T cell responses but the blockade does not worsen the long term outcome (Romer et al., 2015). There is delicate “balance” between dampening the secondary inflammation-mediated brain injury and the increased mortality due to stroke-associated infections.

Approach 3: Do Tregs influence the post-stroke immunosuppression, the secondary brain damage and outcome after stroke?

Due to their immunosuppressive capacity Tregs are of interest as a potential mediator of post-stroke immunosuppression. This CD4+ T cell subpopulation can inhibit effector cells directly by cell contact and secretion of immunosuppressive cytokines such as IL-10 and TGF-β or indirectly by inhibition of stimulatory capacity of antigen-presenting cells (APCs) e.g. via CTLA-4. The role of Tregs in the stroke-induced immunosuppression as well as in the prevention of post-stroke autoimmune responses is still controversial. Studies in stroke patients and experimental stroke yielded contradictory results. Possible explanations are discussed in chapter 1.2.

A lack of Tregs leads to autoimmune diseases, immunopathology and allergy (Sakaguchi, Yamaguchi, Nomura, & Ono, 2008). So with Tregs actively suppressing autoimmune reactivity,

they were thought to prevent inflammatory secondary brain damage. Adenosine triphosphate (ATP), which is released after ischemic damage of the brain, functions as a pro-inflammatory signal. The ectonucleotidase CD39 expressed by Tregs cleaves the pro-inflammatory extracellular ATP to inhibitory antiproliferative AMP (adenosine monophosphate) and thus eliminates a pro-inflammatory stimulus. This links the CD39 expression on the surface of Tregs to their active suppressive function.

Patients, Animals, Materials and Methods

In this study, a 45min transient intraluminal Middle Cerebral Artery Occlusion (MCAO) was performed in young adult and aged Dereg (Depletion of regulatory T cells) mice. Dereg mice allow for *in vivo* depletion of Foxp3+ Tregs as well as identification of Tregs by their Foxp3-GFP expression. Dereg mice carry a diphtheria toxin receptor-eGFP under the control of an additional Foxp3 promoter that enables a specific depletion of Treg by administration of diphtheria toxin. T2-weighted MRI was performed for lesion volume assessment. CD39 expression on Tregs in the blood and spleen of young adult and aged naïve as well as young adult and aged Dereg mice following MCAO was determined. Treg frequencies and CD39 expression on Tregs were also determined in stroke patients at multiple time points after stroke and in age-matched healthy controls.

Results

In a comparison of specific markers on the surface of Tregs indicating their suppressive capacity, we found an increase of CD39 in the blood of naïve Dereg mice with ageing. This could also be confirmed in human healthy donors of different ages. Following experimental stroke we found a decrease of CD39 on Tregs in the blood of aged mice indicating a reduction of Treg function in aged mice after stroke. This reduction of CD39 on the surface of Tregs was also observed in human stroke patients compared to healthy age-matched controls. Furthermore Treg frequencies in human peripheral blood were reduced after stroke compared to age-matched healthy controls.

Conclusion

In the context of stroke-induced immunosuppression and a prevention of autoimmunity against the brain, Tregs might just play a minor role. They seem to be impaired in their suppressive capacity after stroke as indicated by the reduced frequency of Tregs in stroke patients as well as the reduction of CD39 expression on Tregs in the blood of human stroke patients as well as in experimental stroke. Furthermore, our data on the CD39 expression on Tregs point out similar

age-related differences in Treg activity in humans and mice. As other immune effector functions might be affected by ageing as well, this should be considered in experimental stroke studies.

1.1.3 Translation of results from bench to bedside

Limited therapeutic options

Limited therapeutic options are a serious problem in ischemic stroke. So far there are only two effective options for reperfusion of the occluded vessel in ischemic stroke. One therapeutic approach is intravenous administration of the thrombolytic recombinant tissue plasminogen activator (rtPA) within 3-4.5 hours after ischemic stroke onset. Patients who receive rtPA are more likely to have minimal or no disability at 3 months (Gumbinger et al., 2014; Hacke et al., 2008; NINDS, 1995). Due to the relatively short time window for administration and potential contraindications, thrombolysis is only applicable in up to 5% of ischemic stroke patients (Fonarow et al., 2011). The only other option are endovascular procedures with a slightly wider timeframe of 8 hours (European Stroke Organisation Executive & Committee, 2008). While rtPA and recanalization are interventions aiming at the restoration of blood supply to the brain, there are no approved therapeutic interventions targeting the local inflammatory response in the brain or the immune alterations in the periphery. A summary of clinical trials aiming at immunomodulation in ischemic stroke has recently been reviewed by Veltkamp and Gill (Veltkamp & Gill, 2016).

Stroke-induced immunosuppression (SIIS) and SAI are challenging problems in stroke care since they increase post-stroke mortality and worsen the patient outcome (Emsley & Hopkins, 2008). As detailed in chapter 1.1.2.2, already on admission to the hospital profound signs of a suppressed immune system for example lymphopenia are apparent. Although in experimental studies treatment with antibiotics in order to prevent SAI and improve outcome was successful, translation from “bench to bedside” failed. In different studies (ESPIAS, PANTHERIS, STROKE-INF, PASS) preventive antibiotic therapy in stroke did not improve the patients’ outcome (Chamorro et al., 2005; Kalra et al., 2015; Klehmet et al., 2009; Westendorp et al., 2015).

Identification of predictive markers

In order to meet the challenges of effective stroke treatment and care, the search for “biomarkers” that help to predict the risk of stroke, the clinical outcome of patients as well as

the individual risk of post-stroke infection as early as possible are of great importance (Jickling & Sharp, 2015). The identification of markers that classify patients at risk of infection or that predict their clinical outcome could not only improve stroke care but also help to create new approaches in experimental studies. Genetic variations of the individual patient could have an impact on the extent of the local inflammatory response as well as peripheral immune alterations.

Approach 4a & b: Can we identify SNPs in patients that serve as predictive markers for a patients' risk of infection and outcome?

Single base pair mutations, known as single nucleotide polymorphisms (SNPs) are the most common genetic variation in the human genome. Non-synonymous SNPs result in a change of the amino acid sequence of the corresponding protein. These changes in protein can affect function (loss or gain) as well as interactions. The majority of SNPs have two alleles. Based on the observed frequency one distinguishes between a "major" and a "minor" allele. An individual can be homozygous for either the major or minor allele or be heterozygous (Crawford & Nickerson, 2005).

Both IL-1 and TLR4 are part of the initiation of the endogenous inflammatory process due to the primary brain damage as well as part of the first line of defense against bacteria when stroke-associated infections actually occur. Interleukin-1 receptor antagonist (IL-1ra) binds competitively to the IL-1 receptor (IL-1R) without inducing the downstream signaling and thus inhibits IL-1 mediated pro-inflammatory effects. The *IL1RN* gene encodes for the IL-1ra protein. Rafiq et al. reported that the minor allele at rs4251961 is associated with lower IL-1ra plasma concentrations and increased IL-1β (Rafiq et al., 2007). For TLR4 it has been reported that the TLR4 *Asp299* allele in comparison to *Gly299* is associated with increased TLR4 activation as well as higher levels of inflammatory cytokines, acute-phase reactants & soluble adhesion molecules and a higher risk of atherosclerosis (Steinhardt et al., 2010).

In the following studies we investigated the influence of an *IL1RN* SNP and two *TLR4* SNPs on the risk of infection after stroke and the patients' outcome.

Approach 4a: IL-1ra

As detailed in chapter 2.5 we investigated if a SNP in the *IL1RN* gene influences IL-1ra concentrations and the risk of infection after stroke as well as the patients' outcome.

Patients, Materials and Methods

113 stroke patients were included in the study. Plasma IL-1ra concentrations at multiple time points and clinical outcome were determined at 1, 3, 6 and 12 months after stroke. Infection status was documented during hospitalization. SNP genotyping of *IL1RN* rs4251961 was performed.

Results

There is a rapid and sustained elevation of IL-1ra with the highest IL-1ra concentrations on day 7 after acute ischemic stroke. Patients with the most severe strokes had the highest levels of IL-1ra and higher plasma IL-1ra was associated with increased risk of infection other than pneumonia. However in stroke patients the plasma IL-1ra concentrations were not influenced by the *IL1RN* genotypes. The presence of the C allele was associated with a decreased risk of infections other than pneumonia. Although the initial plasma IL1-ra was not predictive of good long-term outcome carriers of the minor C allele were more likely to have a good long-term outcome.

Conclusion

In contrast to previous reports, we did not detect differences in plasma IL-1ra between subjects with different alleles of the rs4251961 SNP in this study. IL-1ra and the *IL1RN* gene may influence the risk of infection after stroke but for our data this seems to be limited to infections other than pneumonia.

Approach 4b: TLR4

Similar to the *IL1RN* SNP study, as detailed in chapter 2.6, we investigated the effect of two *TLR4* SNPs on neurological outcome, infection, and inflammatory markers like C-reactive protein (CRP) in acute ischemic stroke. The two investigated SNPS are located in the coding region of the *TLR4*

gene in exon 3 and result in amino acid changes. It has been shown that these SNPs change the extracellular TLR4 domain and lead to hyporesponsiveness to LPS (Arbour et al., 2000).

Patients, Material and Methods

113 stroke patients were included in this study. Plasma samples were taken at multiple time points after ischemic stroke, neurologic outcome was assessed at 1, 3, 6 and 12 months and infection status was documented while the patients were hospitalized. In this study SNP genotyping of two *TLR4* SNPs (1063 A/G [Asp299Gly] and 1363 C/T [Thr399Ile]) was performed using two different SNP genotyping kits (rs4986790 and rs4986791). For SNP rs4986790 the G allele is the minor allele and for SNP rs4986791 the T allele is the minor allele.

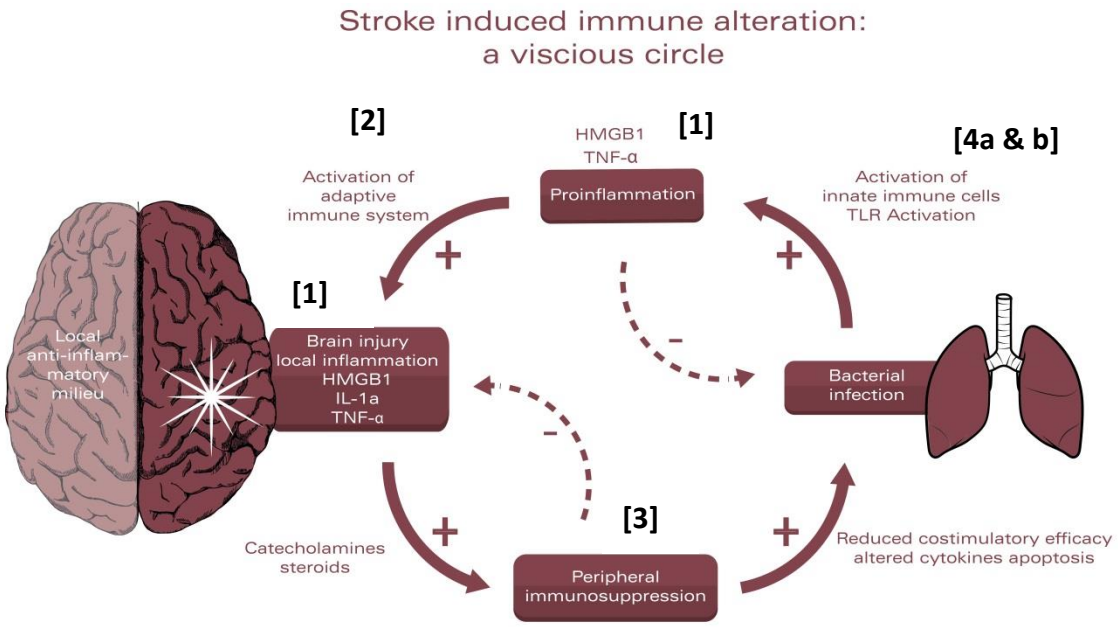
Results

At 3 months after stroke, the group of patients with either one or both of the *TLR4* SNPs minor alleles had significantly worse neurological outcomes in adjusted analyses. The patients carrying one or both *TLR4* minor alleles also exhibited transiently increased leukocyte counts and plasma levels of CRP and IL-1ra. Increased leukocyte counts and elevated plasma CRP were independently associated with poor outcome after stroke. Associations of the *TLR4* minor alleles with IL-1ra, the leukocyte count and CRP persisted after adjusting for infection.

Conclusion

Certain functionally significant genetic variants in the *TLR4* gene are associated with a worse neurological outcome and alterations in systemic markers of inflammation following acute ischemic stroke. Our finding that variant *TLR4* SNPs are associated with transient increases in both leukocyte count and CRP could explain the *TLR4* SNP effect on outcome. Similar to *TLR4*, SNPs in other genes related to the first line immune response might play a role in the individual's risk of post-stroke infection and the patient's outcome.

1.2 Discussion



In this dissertation aspects of post-stroke CNS-immune interactions have been investigated to gain a better understanding of stroke-induced immune alterations and thereby improve the success in translation of results from bench to bedside. The inflammatory response to the ischemic injury and the stroke-induced immunosuppression in the periphery are closely interlinked. A fine balance is necessary when searching for new therapeutic approaches as interventions aiming at one aspect of the post-stroke immune alterations might impact the other as well with the danger of causing adverse effects on the patients' outcome.

Passively released HMGB1 mediates initiation of systemic inflammatory response [1]

HMGB1, released from dying brain tissue, links the central brain injury with a systemic inflammatory response. In our study, HMGB1 was elevated following stroke but its levels were not independently predictive of patient outcome. Plasma HMGB1 was better correlated with leukocyte numbers than with infarct volume suggesting that circulating HMGB1 secreted by leukocytes may overwhelm the release by injured brain tissue after stroke. Of note, there are different isoforms of HMGB1- a fully reduced isoform in a hypoacetylated state indicating a passive release from necrotic cells and a matured cytokine-inducing isoform. In a study by Liesz and colleagues from 2015, they found that the HMGB1 isoform released early after stroke serves

as a chemoattractant but at 24 hours after stroke the prevalent isoform in the serum of patients was the cytokine-inducing isoform of HMGB1. Taken together, our study and the further characterization of the isoforms by Liesz et al. show the importance of HMGB1 in initiating the inflammatory response after stroke. In an experimental model of stroke, blockade of HMGB1 before stroke induction ameliorated the ischemic brain damage and RAGE-deficiency reduced the infarct size (Muhammad et al., 2008). In another study blockade of HMGB1 prior to stroke induction did not improve outcome but mice that were deficient in RAGE had a reduction in splenic lymphocyte apoptosis and in the loss of circulating T cells after experimental stroke (Liesz, Dalpke, et al., 2015). Two experimental stroke studies in rats showed a reduction of infarct size, the inhibition of microglial activation and inflammatory mediators as well as an increased clearance of serum HMGB1 with an anti-HMGB1 treatment (Liu et al., 2007; Zhang et al., 2011). It was also suggested that the HMGB1-mediated ongoing inflammation leads to an exhaustion of immune cells and thus contributes to the post-stroke immune dysfunction (Liesz, Dalpke, et al., 2015). These observations show that even though a mediator acts in a predominantly pro-inflammatory manner it could also have immunosuppressive effects depending on the setting. Any immunomodulating therapeutic approach has to take adverse effects into account as well. As HMGB1 is elevated for days, anti-HMGB1 treatment after stroke could give some further insights on the role of HMGB1 in post-stroke inflammation.

Inflammatory priming of Th1 cells mediates worse outcome [2]

Once an inflammatory response to the ischemic damage is initiated, activation and infiltration of immune cells occur and are likely to contribute to a secondary brain damage (Iadecola & Anrather, 2011). Experimental stroke studies with transgenic mice showed a detrimental effect of T but not B lymphocytes. In stroke patients as well as in experimental stroke, it could be shown that Th1 type immune responses to MBP are associated with worse outcome (K. J. Becker et al., 2011). Our study for the first time showed a direct correlation between the robustness of a Th1 and Th17 response to MBP and the functional outcome of T cell transfer into animals with stroke. This shows that inflammatory priming of infiltrating immune cells actually mediates the outcome. Furthermore the fact that LPS, mimicking a Gram-negative infection, at the time point of stroke skews the T cell response to a Th1 phenotype implicates the consequences of a post-stroke infection. An actual infection with generation of IFN-γ could increase the likelihood of a Th1 dominated immune response and thus worsen the outcome. As mentioned before, in different studies (ESPIAS, PANTHERIS, STROKE-INF, PASS) preventive antibiotic therapy in stroke

did not improve the patients' outcome (Chamorro et al., 2005; Kalra et al., 2015; Klehmet et al., 2009; Westendorp et al., 2015). Once the inflammatory anti-infective immune response has been initiated, elimination of the invasive pathogens by antibiotic treatment may come too late to mitigate the adverse effects of inflammation on the brain.

In chapter 2.3 we could show that the priming of infiltrating lymphocytes influences the outcome. Although autoreactive immune cells seem to worsen the outcome after stroke by increasing the secondary damage there is no development of a general autoimmune disease against the brain like in multiple sclerosis (MS). After stroke brain antigens are present in the cerebrospinal fluid, the serum and lymphoid tissue (Jauch et al., 2006; Planas et al., 2012). An explanation for this could be that in stroke an endogenous inflammation and the antigen leakage due to the damaged blood brain barrier might trigger an autoimmune response but are just temporary. Once the brain tissue damage and the blood brain barrier are repaired brain antigens are shielded again. On the other hand, in MS a viral/exogenous trigger of the autoimmune response is suspected. Viral antigens that are similar to brain antigens like myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) are thought to start the immune activation. Due to the antigen similarity between the viral antigens and the autoantigen they might also attack MBP- or MOG-expressing brain cells. Although there is still no clear etiology for MS, in this disease the antigen triggering the misguided (auto-)immune response might be present in the periphery, for example as still discussed the Epstein-Barr-Virus or Human Herpes-6-Virus (Broccolo, Fusetti, & Ceccherini-Nelli, 2013; Lucas et al., 2011). Whereas in stroke the brain antigens only enter the circulation as long as the BBB is damaged and thus the autoimmune response might only be temporary. This might explain why there is no development of a CNS-directed autoimmune disease after an ischemic insult. Furthermore the SIIS also contributes to the regulation of immune activation caused by the ischemic damage. Not only is this the case in ischemic stroke but similar events can be observed in myocardial infarction where there is a release of autoantigens following myocardial damage and an endogenous inflammation (Hofmann et al., 2012). As we reviewed in chapter 2.7, there are similar mechanisms of immunosuppression in acute ischemic stroke and acute myocardial infarctions.

Tregs – role in SIIS? [3]

While the brain tissue necrosis causes a local and systemic endogenous inflammation, the CNS dampens it via activating the HPA axis and the sympathetic nervous system in order to protect itself from overwhelming inflammatory infiltration. The impact of Tregs, as a suppressive CD4 T

cell subpopulation, in SIIS has been under investigation. The contradicting results of these studies have been reviewed by Liesz et al. and Hu et al. (Liesz, Hu, Kleinschnitz, & Offner, 2015; Xu, Li, & Jiang, 2013) and have shown how important publications of contradicting, neutral and negative results are. In our study we could show that there is a significant reduction of Tregs in the blood of stroke patients following stroke. Furthermore the frequency of suppressive active Tregs (CD39 expressing Tregs) is reduced in the blood of stroke patients and aged mice following MCAO. Thus it seems unlikely that Tregs play a major role in SIIS. They might still be a valuable therapeutic target as Tregs also enter the brain following stroke (Stubbe et al., 2013). But also boosting of Treg function or adoptive transfer of Tregs has led to inconclusive results in different studies (Liesz, Hu, et al., 2015). Similar to studies by Ren and Stubbe, our unpublished experimental results also show that Treg depletion has no influence on stroke volume or outcome in young adult mice (Ren, Akiyoshi, Vandenbark, Hurn, & Offner, 2011; Stubbe et al., 2013). There are several possible explanations of the contradicting results in the studies on the role of regulatory T cells. As different models for experimental stroke induction (transient and permanent), different Treg depletion strategies (conditional knock-out versus antibody-mediated depletion) and different occlusion times were used, it might not be surprising that there is no clear picture yet. Furthermore, it has been published that Tregs also exert non-immunologic effects that might have an impact (Kleinschnitz, Kraft, & Dreykluft, 2013).

Similar to the role of regulatory T cells the effect of an anti-CD49d treatment is one example among many of contradicting results in experimental stroke models yielded in individual laboratories. The infiltration of leukocytes to the injured brain depends on molecules like the very late antigen-4 (VLA-4). VLA-4 is an integrin heterodimer that consist of integrin α4 (CD49d) and integrin β1 (CD29). This integrin heterodimer interacts with the vascular cell adhesion molecule-1 (VCAM-1) for transmigration of the cells. Liesz et al. showed that treatment with anti-CD49d antibodies improved the outcome in experimental stroke models without further impairing the peripheral immune response (Liesz et al., 2011). In contrast to this, Langhauser et al. later published that anti-CD49d treatment reduced T lymphocyte infiltration but did not improve outcome (Langhauser et al., 2014). In 2009 Bath et al. suggested multicenter trials for experimental stroke in order to improve translation from experimental stroke models into clinical trials. This was done for the anti-CD49d treatment by 6 independent research centers in a preclinical randomized controlled multicenter trial (pRCT) in Europe and published in 2015 by Llovera et al. (Llovera et al., 2015). The pRCT on the effect of an anti-CD49d monoclonal antibody treatment showed a reduction of leukocyte invasion and infarct volumes in models inducing small cortical infarction but failed to show this effect in transient proximal MCAO

inducing larger lesions. This showed that already the use of a different model of stroke induction could lead to contradicting results. A similar pRCT approach could give some concluding insights into the role of Tregs and might in general improve the translation from bench to bedside.

Consideration of age and risk factors in stroke models [3]

Furthermore there is also a need to refine experimental stroke models. Stroke risk factors and stroke outcomes are heterogeneous. The majority of ischemic strokes occur in older humans with a broad spectrum of comorbidities and risk factors. When modelling ischemic stroke, the age equivalent in the animal model and risk factors like over-nutrition or atherosclerosis should be taken into account as well. One important finding of our study on Tregs was that there is evidence of similarities in immunosenescence of humans and mice. There was a similar increase of CD39 expression on Tregs with age in healthy humans and naïve mice. But in most experimental stroke studies so far young mice are used for several reasons such as housing limitations and higher costs of old animals. Another challenge when considering aged mice for experimental studies is their higher mortality in various experimental disease models (Schütze et al., 2014; Starr et al., 2014).

Identification of new predictors for improved targeting of therapeutic interventions [4a & b]

The failure to translate positive results with promising agents/compounds and therapeutic approaches from experimental models to clinic studies has been a major problem of stroke research over the past decades. One approach to overcome the translational roadblock might be the mentioned pRCTs to improve the reliability of the pre-clinical studies. As we reviewed, there is similar post-injury immunosuppression in stroke and myocardial infarction. Hence, transferring interventions that have proved successful in acute diseases with shared disease mechanism or settings of similar injury-related immune responses to stroke therapy may be a promising approach. For example, tolerization with injury-specific (auto)antigens showed beneficial effects in both stroke and myocardial infarct (Frenkel et al., 2009; Gee, Kalil, Thullbery, & Becker, 2008; Hofmann & Frantz, 2015).

Moreover, the dose translation from the animal model to humans, also known as allometry, is a very important issue. Several experimental studies with granulocyte colony stimulating factor (G-CSF) showed neuroprotective and neuroregenerative effects in rodents but failed to meet

primary and secondary end points in a clinical trial (Ringelstein et al., 2013). Moreover, the G-CSF had immunologic side effects. In the clinical study a higher dose of G-CSF has led to more pronounced immunomodulating effects of the treatment that might have overwhelmed the CNS-directed effects. Probably this was due to a non-allometric dose translation as a retranslation into a rodent model showed (Wagner et al., 2014).

Stroke patients are a very heterogeneous cohort, which limits the power of clinical studies. Stratification of stroke patients would be very helpful for targeting specific interventions to those patients most likely to benefit from them. Therefore, the scientific community is searching for biomarkers and predictors of disease risk and/or outcome in stroke. To be able to identify such predictors and biomarkers in stroke, studies in large cohorts are needed. The limitation of our studies of SNPs is the relatively small sample size. It is possible that changes in IL-1ra production induced by the acute stroke may have overwhelmed any potential effect of the IL-1ra SNPs. Still, genetic studies like our SNP studies in much larger cohorts like the Study of Health in Pomerania (SHIP) with 8000 participants to search for other predictive SNP markers would be of great interest (Jurgens, Volzke, & Tost, 2014). Also, in such a large cohort a prospective approach could give some insights on genetic risk factors predisposing for stroke occurrence or even predicting SAI or outcome. Furthermore, in a much larger cohort subanalyses could be interesting for example to predict not only the risk of infection but also the kind of infection or to detect gender-related differences. That way stroke care could be much more directed.

SNPs affecting stress hormone expression or functionality of their receptors would also be of great interest considering the stress hormone-induced immunosuppression after stroke and the associated infections are an unsolved challenge in stroke treatment. For example, the catechol-O-methyltransferase (COMT) metabolizing catecholaminergic neurotransmitters such as norepinephrine, epinephrine and dopamine could be an interesting candidate. The A allele of SNP rs165774 of the *COMT* gene is associated with a lower COMT activity (Meloto et al., 2015). This means that stress hormones are metabolized more slowly in carriers of the minor A allele compared with the major G allele. The dopamine-beta-hydroxylase (DBH) plays an essential role in the catecholamine synthesis by converting dopamine to norepinephrine. The major C allele of the common DBH C-970T variant is associated with an increased DBH plasma activity and increased epinephrine excretion compared to the minor T allele (Chen et al., 2010). As a lot of different mediators play a role in post-stroke immune alterations, there are many more SNP candidates that would be of great interest.

1.3 Literature

An, C., Shi, Y., Li, P., Hu, X., Gan, Y., Stetler, R. A., . . . Chen, J. (2014). Molecular dialogs between the ischemic brain and the peripheral immune system: dualistic roles in injury and repair. *Prog Neurobiol, 115*, 6-24. doi: 10.1016/j.pneurobio.2013.12.002

Arbour, N. C., Lorenz, E., Schutte, B. C., Zabner, J., Kline, J. N., Jones, M., . . . Schwartz, D. A. (2000). *TLR4* mutations are associated with endotoxin hyporesponsiveness in humans. *Nature Genetics, 25*, 5.

Becker, K. J., Kalil, A. J., Tanzi, P., Zierath, D. K., Savos, A. V., Gee, J. M., . . . Cain, K. C. (2011). Autoimmune responses to the brain after stroke are associated with worse outcome. *Stroke, 42*(10), 2763-2769. doi: 10.1161/STROKEAHA.111.619593

Becker, K. J., Kindrick, D. L., Lester, M. P., Shea, C., & Ye, Z.-C. (2005). Sensitization to brain antigens after stroke is augmented by lipopolysaccharide. *Journal of Cerebral Blood Flow & Metabolism, 25*(12), 1634-1644. doi: 10.1038/sj.jcbfm.9600160

Bierhaus, A., Humpert, P. M., Morcos, M., Wendt, T., Chavakis, T., Arnold, B., . . . Nawroth, P. P. (2005). Understanding RAGE, the receptor for advanced glycation end products. *J Mol Med (Berl), 83*(11), 876-886. doi: 10.1007/s00109-005-0688-7

Broccolo, F., Fusetti, L., & Ceccherini-Nelli, L. (2013). Possible role of human herpesvirus 6 as a trigger of autoimmune disease. *ScientificWorldJournal, 2013*, 867389. doi: 10.1155/2013/867389

Chamorro, A., Horcajada, J. P., Obach, V., Vargas, M., Revilla, M., Torres, F., . . . Mensa, J. (2005). The Early Systemic Prophylaxis of Infection After Stroke study: a randomized clinical trial. *Stroke, 36*(7), 1495-1500. doi: 10.1161/01.STR.0000170644.15504.49

Chen, Y., Wen, G., Rao, F., Zhang, K., Wang, L., Rodriguez-Flores, J. L., . . . O'Connor, D. T. (2010). Human dopamine beta-hydroxylase (DBH) regulatory polymorphism that influences enzymatic activity, autonomic function, and blood pressure. *J Hypertens, 28*(1), 76-86. doi: 10.1097/HJH.0b013e328332bc87

Crawford, D. C., & Nickerson, D. A. (2005). Definition and clinical importance of haplotypes. *Annu Rev Med, 56*, 303-320. doi: 10.1146/annurev.med.56.082103.104540

Emsley, H. C. A., & Hopkins, S. J. (2008). Acute ischaemic stroke and infection: recent and emerging concepts. *The Lancet Neurology, 7*(4), 341-353. doi: 10.1016/s1474-4422(08)70061-9

European Stroke Organisation Executive, C., & Committee, E. S. O. W. (2008). Guidelines for management of ischaemic stroke and transient ischaemic attack 2008. *Cerebrovasc Dis, 25*(5), 457-507. doi: 10.1159/000131083

Feigin, V. L., Krishnamurthi, R. V., Parmar, P., Norrving, B., Mensah, G. A., Bennett, D. A., . . . Group, G. B. D. S. P. E. (2015). Update on the Global Burden of Ischemic and Hemorrhagic Stroke in 1990-2013: The GBD 2013 Study. *Neuroepidemiology, 45*(3), 161-176. doi: 10.1159/000441085

Fluri, F., Schuhmann, M. K., & Kleinschnitz, C. (2015). Animal models of ischemic stroke and their application in clinical research. *Drug Des Devel Ther, 9*, 3445-3454. doi: 10.2147/DDDT.S56071

Fonarow, G. C., Smith, E. E., Saver, J. L., Reeves, M. J., Bhatt, D. L., Grau-Sepulveda, M. V., . . . Schwamm, L. H. (2011). Timeliness of tissue-type plasminogen activator therapy in acute ischemic stroke: patient characteristics, hospital factors, and outcomes associated with door-to-needle times within 60 minutes. *Circulation, 123*(7), 750-758. doi: 10.1161/CIRCULATIONAHA.110.974675

Frenkel, D., Pachori, A. S., Zhang, L., Dembinsky-Vaknin, A., Farfara, D., Petrovic-Stojkovic, S., . . . Weiner, H. L. (2009). Nasal vaccination with troponin reduces troponin specific T-cell

responses and improves heart function in myocardial ischemia-reperfusion injury. *Int Immunol, 21*(7), 817-829. doi: 10.1093/intimm/dxp051

Gee, J. M., Kalil, A., Thullbery, M., & Becker, K. J. (2008). Induction of immunologic tolerance to myelin basic protein prevents central nervous system autoimmunity and improves outcome after stroke. *Stroke, 39*(5), 1575-1582. doi: 10.1161/STROKEAHA.107.501486

Gelderblom, M., Leypoldt, F., Steinbach, K., Behrens, D., Choe, C. U., Siler, D. A., . . . Magnus, T. (2009). Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. *Stroke, 40*(5), 1849-1857. doi: 10.1161/STROKEAHA.108.534503

Gelderblom, M., Sobey, C. G., Kleinschnitz, C., & Magnus, T. (2015). Danger signals in stroke. *Ageing Res Rev, 24*(Pt A), 77-82. doi: 10.1016/j.arr.2015.07.004

Gumbinger, C., Reuter, B., Stock, C., Sauer, T., Wietholter, H., Bruder, I., . . . Schlaganfall, A. G. (2014). Time to treatment with recombinant tissue plasminogen activator and outcome of stroke in clinical practice: retrospective analysis of hospital quality assurance data with comparison with results from randomised clinical trials. *BMJ, 348*, g3429. doi: 10.1136/bmj.g3429

Hacke, W., Kaste, M., Bluhmki, E., Brozman, M., Dávalos, A., Guidetti, D., . . . investigators, f. t. E. (2008). Thrombolysis with Alteplase 3 to 4.5 Hours after Acute Ischemic Stroke. *The New England Journal of Medicine, 359*(13), 13.

Hankey, G. J. (2013). The global and regional burden of stroke. *The Lancet Global Health, 1*(5), e239-e240. doi: 10.1016/s2214-109x(13)70095-0

Hofmann, U., Beyersdorf, N., Weirather, J., Podolskaya, A., Bauersachs, J., Ertl, G., . . . Frantz, S. (2012). Activation of CD4+ T lymphocytes improves wound healing and survival after experimental myocardial infarction in mice. *Circulation, 125*(13), 1652-1663. doi: 10.1161/CIRCULATIONAHA.111.044164

Hofmann, U., & Frantz, S. (2015). Role of lymphocytes in myocardial injury, healing, and remodeling after myocardial infarction. *Circ Res, 116*(2), 354-367. doi: 10.1161/CIRCRESAHA.116.304072

Hurn, P. D., Subramanian, S., Parker, S. M., Afentoulis, M. E., Kaler, L. J., Vandenbark, A. A., & Offner, H. (2007). T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. *J Cereb Blood Flow Metab, 27*(11), 1798-1805. doi: 10.1038/sj.jcbfm.9600482

Iadecola, C., & Anrather, J. (2011). The immunology of stroke: from mechanisms to translation. *Nat Med, 17*(7), 796-808. doi: 10.1038/nm.2399

Jauch, E. C., Lindsell, C., Broderick, J., Fagan, S. C., Tilley, B. C., Levine, S. R., & Group, N. r.-P. S. S. (2006). Association of serial biochemical markers with acute ischemic stroke: the National Institute of Neurological Disorders and Stroke recombinant tissue plasminogen activator Stroke Study. *Stroke, 37*(10), 2508-2513. doi: 10.1161/01.STR.0000242290.01174.9e

Jickling, G. C., & Sharp, F. R. (2015). Biomarker panels in ischemic stroke. *Stroke, 46*(3), 915-920. doi: 10.1161/STROKEAHA.114.005604

Jurgens, C., Volzke, H., & Tost, F. (2014). [Study of health in Pomerania (SHIP-Trend): : Important aspects for healthcare research in ophthalmology]. *Ophthalmologe, 111*(5), 443-447. doi: 10.1007/s00347-013-2924-9

Kalra, L., Irshad, S., Hodsoll, J., Simpson, M., Gulliford, M., Smithard, D., . . . Rebollo-Mesa, I. (2015). Prophylactic antibiotics after acute stroke for reducing pneumonia in patients with dysphagia (STROKE-INF): a prospective, cluster-randomised, open-label, masked endpoint, controlled clinical trial. *The Lancet, 386*(10006), 1835-1844. doi: 10.1016/s0140-6736(15)00126-9

Katzan, I. L., Cebul, R. D., Husak, S. H., Dawson, N. V., & Baker, D. W. (2003). The effect of pneumonia on mortality among patients hospitalized for acute stroke. *Neurology, 60*(4), 620-625. doi: 10.1212/01.wnl.0000046586.38284.60

Klehmet, J., Harms, H., Richter, M., Prass, K., Volk, H. D., Dirnagl, U., . . . Meisel, C. (2009). Stroke-induced immunodepression and post-stroke infections: lessons from the preventive antibacterial therapy in stroke trial. *Neuroscience, 158*(3), 1184-1193. doi: 10.1016/j.neuroscience.2008.07.044

Kleinschnitz, C., Kraft, P., & Dreykluft, A. (2013). Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature. *Blood*. doi: 10.1182/blood-201204-426734

Kleinschnitz, C., Schwab, N., Kraft, P., Hagedorn, I., Dreykluft, A., Schwarz, T., . . . Stoll, G. (2010). Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood, 115*(18), 3835-3842. doi: 10.1182/blood-2009-10-249078

Lai, T. W., Zhang, S., & Wang, Y. T. (2014). Excitotoxicity and stroke: identifying novel targets for neuroprotection. *Prog Neurobiol, 115*, 157-188. doi: 10.1016/j.pneurobio.2013.11.006

Langhauser, F., Kraft, P., Gob, E., Leinweber, J., Schuhmann, M. K., Lorenz, K., . . . Kleinschnitz, C. (2014). Blocking of alpha4 integrin does not protect from acute ischemic stroke in mice. *Stroke, 45*(6), 1799-1806. doi: 10.1161/STROKEAHA.114.005000

Liesz, A., Dalpke, A., Mracsko, E., Antoine, D. J., Roth, S., Zhou, W., . . . Veltkamp, R. (2015). DAMP signaling is a key pathway inducing immune modulation after brain injury. *J Neurosci, 35*(2), 583-598. doi: 10.1523/JNEUROSCI.2439-14.2015

Liesz, A., Hu, X., Kleinschnitz, C., & Offner, H. (2015). Functional role of regulatory lymphocytes in stroke: facts and controversies. *Stroke, 46*(5), 1422-1430. doi: 10.1161/STROKEAHA.114.008608

Liesz, A., Zhou, W., Mracsko, E., Karcher, S., Bauer, H., Schwarting, S., . . . Veltkamp, R. (2011). Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke. *Brain, 134*(Pt 3), 704-720. doi: 10.1093/brain/awr008

Liu, K., Mori, S., Takahashi, H. K., Tomono, Y., Wake, H., Kanke, T., . . . Nishibori, M. (2007). Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. *FASEB J, 21*(14), 3904-3916. doi: 10.1096/fj.07-8770com

Llovera, G., Hofmann, K., Roth, S., Salas-Perdomo, A., Ferrer-Ferrer, M., Perego, C., . . . Liesz, A. (2015). Results of a preclinical randomized controlled multicenter trial (pRCT): Anti-CD49d treatment for acute brain ischemia. *Sci Transl Med, 7*(299), 299ra121. doi: 10.1126/scitranslmed.aaa9853

Lotze, M. T., & Tracey, K. J. (2005). High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol, 5*(4), 331-342. doi: 10.1038/nri1594

Lucas, R. M., Hughes, A. M., Lay, M. L., Ponsonby, A. L., Dwyer, D. E., Taylor, B. V., & Pender, M. P. (2011). Epstein-Barr virus and multiple sclerosis. *J Neurol Neurosurg Psychiatry, 82*(10), 1142-1148. doi: 10.1136/jnnp-2011-300174

Meloto, C. B., Segall, S. K., Smith, S., Parisien, M., Shabalina, S. A., Rizzatti-Barbosa, C. M., . . . Diatchenko, L. (2015). COMT gene locus: new functional variants. *Pain, 156*(10), 2072-2083. doi: 10.1097/j.pain.0000000000000273

Miro-Mur, F., Urra, X., Gallizioli, M., Chamorro, A., & Planas, A. M. (2016). Antigen Presentation After Stroke. *Neurotherapeutics, 13*(4), 719-728. doi: 10.1007/s13311-016-0469-8

Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., Cushman, M., . . . Stroke Statistics, S. (2015). Heart disease and stroke statistics--2015 update: a report from the American Heart Association. *Circulation, 131*(4), e29-322. doi: 10.1161/CIR.0000000000000152

Mracsko, E., Liesz, A., Karcher, S., Zorn, M., Bari, F., & Veltkamp, R. (2014). Differential effects of sympathetic nervous system and hypothalamic-pituitary-adrenal axis on systemic immune cells after severe experimental stroke. *Brain Behav Immun, 41*, 200-209. doi: 10.1016/j.bbi.2014.05.015

Muhammad, S., Barakat, W., Stoyanov, S., Murikinati, S., Yang, H., Tracey, K. J., . . . Schwaninger, M. (2008). The HMGB1 receptor RAGE mediates ischemic brain damage. *J Neurosci, 28*(46), 12023-12031. doi: 10.1523/JNEUROSCI.2435-08.2008

NINDS. (1995). Tissue Plasminogen Activator for Acute Ischemic Stroke. *The New England Journal of Medicine, 333*.

Offner, H., Subramanian, S., Parker, S. M., Wang, C., Afentoulis, M. E., Lewis, A., . . . Hurn, P. D. (2006). Splenic Atrophy in Experimental Stroke Is Accompanied by Increased Regulatory T Cells and Circulating Macrophages. *The Journal of Immunology, 176*(11), 6523-6531. doi: 10.4049/jimmunol.176.11.6523

Planas, A. M., Gomez-Choco, M., Urra, X., Gorina, R., Caballero, M., & Chamorro, A. (2012). Brain-derived antigens in lymphoid tissue of patients with acute stroke. *J Immunol, 188*(5), 2156-2163. doi: 10.4049/jimmunol.1102289

Prass, K., Meisel, C., Hoflich, C., Braun, J., Halle, E., Wolf, T., . . . Meisel, A. (2003). Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. *J Exp Med, 198*(5), 725-736. doi: 10.1084/jem.20021098

Rafiq, S., Stevens, K., Hurst, A. J., Murray, A., Henley, W., Weedon, M. N., . . . Frayling, T. M. (2007). Common genetic variation in the gene encoding interleukin-1-receptor antagonist (IL-1RA) is associated with altered circulating IL-1RA levels. *Genes Immun, 8*(4), 344-351.

Raphael, I., Nalawade, S., Eagar, T. N., & Forsthuber, T. G. (2015). T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine, 74*(1), 5-17. doi: 10.1016/j.cyto.2014.09.011

Ren, X., Akiyoshi, K., Vandenbark, A. A., Hurn, P. D., & Offner, H. (2011). CD4+FoxP3+ regulatory T-cells in cerebral ischemic stroke. *Metab Brain Dis, 26*(1), 87-90. doi: 10.1007/s11011-010-9226-6

Ringelstein, E. B., Thijs, V., Norrving, B., Chamorro, A., Aichner, F., Grond, M., . . . Investigators, A. (2013). Granulocyte colony-stimulating factor in patients with acute ischemic stroke: results of the AX200 for Ischemic Stroke trial. *Stroke, 44*(10), 2681-2687. doi: 10.1161/STROKEAHA.113.001531

Romer, C., Engel, O., Winek, K., Hochmeister, S., Zhang, T., Royl, G., . . . Meisel, A. (2015). Blocking stroke-induced immunodeficiency increases CNS antigen-specific autoreactivity but does not worsen functional outcome after experimental stroke. *J Neurosci, 35*(20), 7777-7794. doi: 10.1523/JNEUROSCI.1532-14.2015

Romero, J. R., Morris, J., & Pikula, A. (2008). Stroke prevention: modifying risk factors. *Ther Adv Cardiovasc Dis, 2*(4), 287-303. doi: 10.1177/1753944708093847

Ruhnau, J., Schulze, K., Gaida, B., Langner, S., Kessler, C., Broker, B., . . . Vogelgesang, A. (2014). Stroke alters respiratory burst in neutrophils and monocytes. *Stroke, 45*(3), 794-800. doi: 10.1161/STROKEAHA.113.003342

Sakaguchi, S., Yamaguchi, T., Nomura, T., & Ono, M. (2008). Regulatory T cells and immune tolerance. *Cell, 133*(5), 775-787. doi: 10.1016/j.cell.2008.05.009

Scaffidi, P., Mistelli, T., & Bianchi, M. E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature, 418*, 6. doi: 10.1038/nature00858

Schulze, J., Vogelgesang, A., & Dressel, A. (2014). Catecholamines, steroids and immune alterations in ischemic stroke and other acute diseases. *Aging Dis, 5*(5), 327-339. doi: 10.14336/AD.2014.0500327

Schütze, S., Ribes, S., Kaufmann, A., Manig, A., Scheffel, J., Redlich, S., . . . Nau, R. (2014). Higher mortality and impaired elimination of bacteria in aged mice after intracerebral infection with E. coli are associated with an age-related decline of microglia and macrophage functions. *Oncotarget, 5*(24), 20.

Shichita, T., Ito, M., & Yoshimura, A. (2014). Post-ischemic inflammation regulates neural damage and protection. *Front Cell Neurosci, 8*, 319. doi: 10.3389/fncel.2014.00319

Starr, M., Takahashi, H., Okamura, D., Zwischenberger, B., Mrazek, A., Ueda, J., . . . Saito, H. (2014). Increased coagulation and suppressed generation of activated protein C in aged mice during intra-abdominal sepsis. *Am J Physiol Heart Circ Physiol, 308*, 9. doi: 10.1152/ajpheart.00289.2014.-Sepsis

Steinhardt, A. P., Aranguren, F., Tellechea, M. L., Gomez Rosso, L. A., Brites, F. D., Martinez-Larrad, M. T., . . . Taverna, M. J. (2010). A functional nonsynonymous toll-like receptor 4 gene polymorphism is associated with metabolic syndrome, surrogates of insulin resistance, and syndromes of lipid accumulation. *Metabolism, 59*(5), 711-717. doi: 10.1016/j.metabol.2009.09.015

Stubbe, T., Ebner, F., Richter, D., Engel, O., Klehmet, J., Royl, G., . . . Brandt, C. (2013). Regulatory T cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO. *J Cereb Blood Flow Metab, 33*(1), 37-47. doi: 10.1038/jcbfm.2012.128

Veltkamp, R., & Gill, D. (2016). Clinical Trials of Immunomodulation in Ischemic Stroke. *Neurotherapeutics, 13*(4), 791-800. doi: 10.1007/s13311-016-0458-y

Vogelgesang, A., Becker, K. J., & Dressel, A. (2014). Immunological consequences of ischemic stroke. *Acta Neurol Scand, 129*(1), 1-12. doi: 10.1111/ane.12165

Vogelgesang, A., Grunwald, U., Langner, S., Jack, R., Broker, B. M., Kessler, C., & Dressel, A. (2008). Analysis of lymphocyte subsets in patients with stroke and their influence on infection after stroke. *Stroke, 39*(1), 237-241. doi: 10.1161/STROKEAHA.107.493635

Wagner, D. C., Posel, C., Schulz, I., Schicht, G., Boltze, J., Lange, F., . . . Weise, G. (2014). Allometric dose retranslation unveiled substantial immunological side effects of granulocyte colony-stimulating factor after stroke. *Stroke, 45*(2), 623-626. doi: 10.1161/STROKEAHA.113.003812

Westendorp, W. F., Vermeij, J.-D., Zock, E., Hooijenga, I. J., Kruyt, N. D., Bosboom, H. J. L. W., . . . Nederkoorn, P. J. (2015). The Preventive Antibiotics in Stroke Study (PASS): a pragmatic randomised open-label masked endpoint clinical trial. *The Lancet, 385*(9977), 1519-1526. doi: 10.1016/s0140-6736(14)62456-9

WHO. (2014). WHO Factsheet No.310. *World Health Organization*.

Xu, X., Li, M., & Jiang, Y. (2013). The paradox role of regulatory T cells in ischemic stroke. *ScientificWorldJournal, 2013*, 174373. doi: 10.1155/2013/174373

Yan, F. L., & Zhang, J. H. (2014). Role of the sympathetic nervous system and spleen in experimental stroke-induced immunodepression. *Med Sci Monit, 20*, 2489-2496. doi: 10.12659/MSM.890844

Yu, M., Wang, H., Ding, A., Golenbock, D. T., Latz, E., Czura, C. J., . . . Yang, H. (2006). HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock, 26*(2), 174-179. doi: 10.1097/01.shk.0000225404.51320.82

Zhang, J., Takahashi, H. K., Liu, K., Wake, H., Liu, R., Maruo, T., . . . Nishibori, M. (2011). Anti-high mobility group box-1 monoclonal antibody protects the blood-brain barrier from ischemia-induced disruption in rats. *Stroke, 42*(5), 1420-1428. doi: 10.1161/STROKEAHA.110.598334

2 Publications

2.1 Overview and contributions

2.1.1 Overview

The studies presented in this dissertation aimed at the investigation of mediators that participate in the local immune response as well as in peripheral immune alterations following ischemic stroke.

In chapter 2.2 we focused on HMGB1 as a potential initiator of the inflammatory response in the brain as well as a mediator of the ischemic damage into the periphery.

In chapter 2.3 we investigated whether the quality of the inflammatory response of infiltrating T cells determines the outcome of stroke. Therefore we determined the response of donor splenocytes to autoantigens prior to adoptive transfer in rats undergoing experimental stroke.

In chapter 2.4 we focused on peripheral immunosuppression and questioned whether Treg as an immunosuppressive T cell subset are part of this immunosuppression.

In chapters 2.5 and 2.6 studies on single nucleotide polymorphisms are detailed to investigate the influence of SNPs on stroke outcome and their predictive value for stroke.

In chapter 2.7 we reviewed the catecholamine- and steroid-induced changes of the immune system in stroke and also made comparison to other acute diseases like traumatic brain injury or myocardial infarction.

2.1.2 Contributions

1: Severe Stroke induces long-lasting alterations of high-mobility group box 1.

Schulze J, Zierath D, Tanzi P, Cain K, Shibata D, Dressel A, Becker K. Stroke. 2013 Jan; 44(1):246-8. doi: 10.1161/STROKEAHA.112.676072. Epub 2012 Nov 29. PMID: 23204053

Schulze J: performed HMGB1 ELISAs, data evaluation, drafted manuscript; Zierath D: data collection, critical revision of article; Tanzi P: data collection, critical revision of article; Cain K: data collection, critical revision of article; Shibata D: data collection, critical revision of article; Dressel A: critical revision of article; Becker K: conceived of the research, obtained funding, designed study, drafted manuscript.

2: The immunologic profile of adoptively transferred lymphocytes influences stroke outcome of recipients.

Zierath D, **Schulze J**, Kunze A, Drogomiretskiy O, Nhan D, Jaspers B, Dressel A, Becker K. J Neuroimmunol. 2013 Oct 15; 263(1-2):28-34. doi: 10.1016/j.jneuroim.2013.07.014. Epub 2013 Jul 29.PMID: 23948692

Zierath D: performed MCAO, data collection, critical revision of article; Schulze J: performed isolation and culture of transferred cells, behavioral testing on rats, CFSE staining of donor cells, performed ELIspot assays of donor cells, tissue sectioning, established immunohistochemical staining of the brain sections, critical revision of article; Kunze A: data collection; Drogomiretskiy O: data collection; Nhan D: data collection; Jaspers B: data collection ; Dressel A: critical revision of article; Becker KJ: conceived of the research, obtained funding, designed study, drafted manuscript.

3: Reduced numbers and impaired function of regulatory T cells in peripheral blood of ischemic stroke patients

Ruhnau J*, **Schulze J***, von Sarnowski B, Heinrich M, Langner S, Pötschke C, Wilden A, Kessler C, Bröker BM, Vogelgesang A, Dressel A. Mediators Inflamm. 2016; 2016: 2974605. doi: 10.1155/2016/2974605. Epub 2016 Mar 17. PMID: 27073295

Ruhnau J: human Treg data, drafted manuscript; Schulze J: mouse Treg data, drafted manuscript, supervised A. Wilden; von Sarnowski B: patient recruitment, critical revision of article; Heinrich M: established Treg FACS staining; Langner S: infarct volume assessment of stroke patients; Pötschke C: supervised FACS data collection; Wilden A: data collection; Kessler C: obtained funding, critical revision of article; Bröker BM: provided Dereg mice, critical revision of article. Vogelgesang A: drafted manuscript, data evaluation, designed study. Dressel A: conceived of the research, designed study, drafted manuscript.

* Equal contribution

4: Stroke, IL-1ra, IL1RN, infection and outcome.

Becker KJ, Dankwa D, Lee R, **Schulze J**, Zierath D, Tanzi P, Cain K, Dressel A, Shibata D, Weinstein J. Neurocrit Care. 2014 Aug; 21(1):140-6. PMID: 24233813

Becker KJ: conceived of the research, obtained funding, designed study, drafted manuscript; Dankwa D: data collection; Lee R: data collection; Schulze J: performed *IL1RN* SNP genotyping, data analysis with software, critical revision of article; Zierath D: data collection, critical revision of article; Tanzi P: data collection, critical revision of article; Cain K: data collection, critical revision of article; Dressel A: critical revision of article; Shibata D: data collection, critical revision of article; Weinstein J: data collection, critical revision of article.

5: Functional polymorphisms in toll-like receptor 4 are associated with worse outcome in acute ischemic stroke patients.

Weinstein JR, **Schulze J**, Lee RV, Phillips H, Zierath D, Tanzi P, Shibata D, Cain KC, Becker KJ. Neuroreport. 2014 May 28; 25(8):580-4. doi:10.1097/WNR.0000000000000140. PMID: 24784586

Weinstein JR: conceived of the research, designed study, drafted manuscript; Schulze J: performed *TLR4* SNP genotyping, data collection, critical revision of article; Lee RV: data collection; Philips H: data collection; Zierath D: data collection, critical revision of article; Tanzi P: data collection, critical revision of article; Shibata D: data collection, critical revision of article; Cain KC: data collection, critical revision of article ; Becker KJ: obtained funding, designed study, data collection, critical revision of article

6: Catecholamines, steroids and immune alterations in ischemic stroke and other acute diseases

Schulze J*, Vogelgesang A* and Dressel A. Aging Dis. 2014 Oct 1; 5(5):327-39. doi: 10.14336/AD.2014.0500327. eCollection 2014 Oct. Review. PMID: 25276491

Schulze J & Vogelgesang A: drafting and revision of manuscript. Dressel A: conceived of the article, critical revision of article.

* Equal contribution

2.2 Severe stroke induces long-lasting alterations of HMGB1

Severe Stroke Induces Long-Lasting Alterations of High-Mobility Group Box 1

Juliane Schulze, BS; Dannielle Zierath, BS; Patricia Tanzi, BSN, RN, CCRC; Kevin Cain, PhD; Dean Shibata, MD; Alexander Dressel, MD; Kyra Becker, MD

Background and Purpose—The signals that initiate the poststroke inflammatory response are unknown. High-mobility group box (HMGB) 1 protein is a nuclear protein that is passively released from necrotic tissue and is able to activate leukocytes, which in turn secrete HMGB1. HMGB1 is also able to activate antigen-presenting cells and therefore stands at the crossroads of innate and adaptive immunity.

Methods—Plasma HMGB1 concentrations were determined at multiple time points after ischemic stroke (N=110) and correlated to stroke severity and biomarkers of inflammation. The relationships between HMGB1, stroke outcome, and autoimmune responses to brain antigens were also assessed.

Results—Stroke resulted in an increase in HMGB1 that persisted for 30 days. Plasma HMGB1 was correlated with the number of circulating leukocytes but was not predictive of either stroke outcome or the development of autoimmune responses to brain antigens. Patients with a $T_H1(+)$ response to myelin basic protein at 90 days after stroke, however, had higher plasma HMGB1.

Conclusions—HMGB1 appears to be involved in the postischemic inflammatory response, but it remains unclear whether HMGB1 initiates this response or merely reflects activation of leukocytes by another signal. **(*Stroke*. 2013;44:246-248.)**

Key Words: alarmin ■ DAMP ■ HMGB1 ■ inflammation ■ monocytes ■ stroke

Stroke induces complex changes in the immune response, leading to systemic inflammation as well as impaired host defense.[1–4] Both the degree of inflammation and the degree of host response impairment are related to stroke severity and infarct volume.[1,2,4] The dysfunction in host defense is mediated by the sympathetic nervous system; the signals that initiate systemic inflammation are unknown.[3]

The poststroke systemic inflammatory response is not directed in an antigen-specific fashion. Systemic infections that activate the innate immune response, however, increase the likelihood of T_H1-type immune responses to brain antigens in patients with stroke.[5] The link between infection and the development of central nervous system autoimmunity may be mediated by danger-associated molecular patterns, which are derived from pathogens or released from host cells.[6] High-mobility group box (HMGB) 1 is a ubiquitous nuclear protein released from necrotic cells and secreted by activated leukocytes.[7] Once released, HMGB1 functions as a danger-associated molecular pattern by activating antigen-presenting cells through toll-like receptors, and the receptor for advanced glycation end-products.[6]

Neutralizing HMGB1 improves outcome in experimental stroke.[8,9] The relationship between plasma HMGB1 and clinical stroke outcome is unknown. In this study we investigate whether plasma HMGB1 concentrations in patients (1) reflect infarct size, (2) promote $T_H1(+)$ responses to brain antigens, or (3) are predictive of stroke outcome.

Materials and Methods

Research Subjects

The patient population for this study is described elsewhere.[5] Patients with acute ischemic stroke were enrolled as soon as possible after stroke onset. Blood was drawn at 24 hours (±6 hours; N=38), 72 hours (±12 hours; N=98), 7 days (±1 day; N=94), 30 days (±5 days; N=89), 90 days (±5 days; N=72), 180 days (±5 days; N=70), and 365 days (±5 days; N=24) after stroke. The study was approved by the Institutional Review Board. Patients or their surrogates provided informed consent.

Clinical Data

Stroke severity was determined by the National Institutes of Health Stroke Scale, and outcome by the modified Rankin Scale. Infarct volume on initial diffusion-weighted magnetic resonance imaging was calculated by the ABC/2 method.[10]

Laboratory Studies

Leukocyte counts and concentrations of C-reactive protein were determined by hospital clinical laboratories. Additional plasma was immediately frozen at −80°, and HMGB1 concentrations determined by enzyme-linked immunoassay (IBL International); the sensitivity of the assay was 0.20 ng/mL. Isolated lymphocytes were isolated and frozen in liquid nitrogen until use.

Received September 5, 2012; final revision received October 2, 2012; accepted October 4, 2012.

From the University of Greifswald (J.S., A.D.) Greifswald, Germany; and Harborview Medical Center, University of Washington School of Medicine, Seattle, WA (D.Z., P.T., K.C., D.S., K.B.).

Correspondence to Kyra Becker, MD, Harborview Medical Center, University of Washington School of Medicine, Box 359775 HMC, 325 9th Ave, Seattle, WA 98104. E-mail kjb@uw.edu

***Stroke* is available at http://stroke.ahajournals.org** **DOI: 10.1161/STROKEAHA.112.676072**

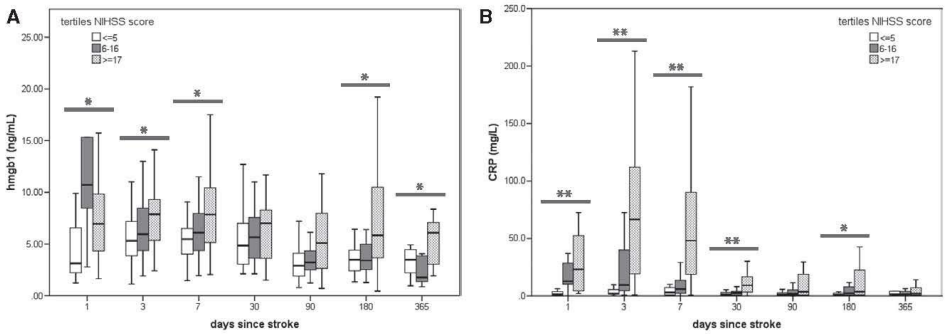


Figure. Changes in plasma high-mobility group box (HMGB) 1 and CRP over time after stroke. Both plasma HMGB1 (**A**) and C-reactive protein (CRP) (**B**) are higher among patients with severe stroke; these differences persist for months after stroke onset. Tertiles of stroke severity differ from each other at **P*<0.05 or ***P*<0.01 level (Kruskal-Wallis H test).

$T_H1(+)$ responses to lymphocytes were determined, as described elsewhere.[5]

Statistics

Descriptive data are presented as median and interquartile range; group comparisons were performed using the Kruskal-Wallis H test or the Mann–Whitney U test. Correlations are presented as either Pearson *r* or Spearman ρ. Logistic regression was used to assess the contribution of HMGB1, C-reactive protein, and leukocyte subsets to poor outcome (modified Rankin Scale >3) at 90 days after stroke, and to the risk of developing a $T_H1(+)$ response to myelin basic protein (MBP). Significance was set at $P<0.05$.

Results

A total of 114 patients were enrolled in the parent study; baseline characteristics are described elsewhere.[5] Plasma HMGB1 was available for 110 of these patients, who are the subject of this report. At day 3 after stroke, there were weak correlations between HMGB1, infarct volume ($r=0.217$, $P=0.024$), and stroke severity ($\rho=0.230$, $P=0.015$). Plasma HMGB1 and CRP were highest in patients with severe strokes (National Institutes of Health Stroke Scale ≥17), and remained elevated for months (Figure).

Neither the number of leukocytes nor the plasma concentrations of HMGB1 early after stroke were independently predictive of stroke outcome at 90 days (Table 1). Higher concentrations of CRP early after stroke, however, were associated with worse 90 day outcomes. The number of leukocytes was highly correlated (independent of infarct volume) to plasma HMGB1 throughout the study period: $r=0.415$, $P=0.415$ at day 1; $r=0.312$, $P=0.002$ at day 3; $r=0.0297$, $P=0.004$ at week 1; $r=0.374$, $P<0.001$ at month 1; $r=0.475$, $P<0.001$ at month 3; and $r=0.539$, $P=0.010$ at year 1. The relationship between CRP and HMGB1 was more variable.

Among patients with a $T_H1(+)$ response to MBP at 90 days,[5] plasma HMGB1 and CRP were also elevated at that time point (Table 2). There was, however, no relationship between HMGB1 concentrations early after stroke onset and the propensity to develop a $T_H1(+)$ response to MBP at 90 days.

Discussion

A systemic inflammatory response is common after stroke. Alarmins like HMGB1 are candidate molecules that could initiate the innate immune response following tissue damage.[11,12]

Table 1. Predictive Value of Early (Day 3) Markers of Inflammation on Poor Outcome (mRS>3) at 90 Days After Stroke

	Unadjusted		Adjusted for NIHSS		Adjusted for NIHSS and Age	
90 days (N=102)	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
Leukocytes (per thou/μL)	1.183 (1.047–1.336)	0.007	1.035 (0.884–1.212)	NS	1.033 (0.879–1.213)	NS
Neutrophils (per thou/μL)	1.367 (1.138–1.641)	0.001	1.120 (0.884–1.418)	NS	1.133 (0.893–1.438)	NS
Lymphocytes* (per thou/μL)	0.181 (0.052–0.638)	0.008	0.518 (0.132–2.031)	NS	0.575 (0.144–2.294)	NS
Monocytes (per thou/μL)	41.40 (5.481–312.8)	<0.001	7.490 (0.527–106.5)	0.137	6.832 (0.427–109.3)	0.174
HMGB1 (per ng)	0.998 (0.939–1.061)	NS	0.960 (0.882–1.044)	NS	0.974 (0.896–1.059)	NS
CRP (per 10 mg/L)	1.311 (1.160–1.482)	<0.001	1.166 (1.026–1.325)	0.019	1.197 (1.038–1.380)	0.013

CI indicates confidence interval; CRP, C-reactive protein; HMGB, high-mobility group box; mRS indicates modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; NS, not significant; OR, odds ratio; and thou/μL, thousand per μL.

*All values represent the highest recorded value within the first 3 days except for lymphocytes, where the lowest recorded value was used.

Table 2. Differences in Inflammatory Markers Between Patients With a Th1(+) Response to MBP at 90 Days and Those Without

	Th1(+) Response to MBP		
90 days	Yes (N=19)	No (N=47)	*P Value*
Leukocytes (thou/μL)	7.73 (5.94, 9.41)	6.94 (5.39, 7.99)	0.111
Neutrophils (thou/μL)	4.44 (3.36, 6.51)	4.01 (3.09, 5.29)	NS
Lymphocytes (thou/μL)	1.75 (1.37, 2.14)	1.73 (1.33, 2.14)	NS
Monocytes (thou/μL)	0.57 (0.44, 0.66)	0.50 (0.41, 0.66)	NS
HMGB1 (ng/mL)	5.70 (2.51, 8.11)	3.27 (2.12, 4.49)	0.030
CRP (mg/L)	8.10 (0.80, 21.1)	1.90 (0.60, 3.92)	0.036

T_H1(+) response to MBP is a response greater than that seen in 75% of the control population.[5]

CRP indicates C-reactive protein; HMGB, high-mobility group box; MBP indicates myelin basic protein; and NS, not significant.

Given that HMGB1 is released from necrotic cells, we hypothesized that HMGB1 concentrations would reflect the degree of tissue injury. Similar to a previous study, however, plasma HMGB1 was only weakly associated with infarct volume.[13] Activated leukocytes are also a source of HMGB,[7] and the robust association between HMGB1 and the leukocyte numbers suggest that immune cells might be the primary source of plasma HMGB1 following stroke.

Given its ability to promote inflammation and activate antigen-presenting cells through toll-like receptors and advanced glycation end products, we expected that high concentrations of plasma HMGB1 early after stroke onset would be predictive of poor outcome and predispose to autoimmune responses to brain antigens, yet plasma HMGB1 was predictive of neither. The lack of an association between HMGB1, infarct size, and autoimmune responses to brain antigens suggests that HMGB1 is not the single factor initiating inflammation or activating antigen-presenting cells after stroke. At day 90 after stroke, however, those patients with a T_H1(+) response to MBP had increased plasma HMGB1; the source of this HMGB1 is unknown.

In summary, plasma HMGB1 is elevated following ischemic stroke; patients with severe stroke have higher HMGB1, and these elevations last for months. The correlation between plasma HMGB1 and leukocyte numbers is more robust than that between plasma HMGB1 and infarct volume, suggesting that plasma HMGB1 reflects secretion by leukocytes. Finally, HMGB1 did not predict stroke outcome or development of autoimmune responses to MBP. Further studies are needed to define the role of HMGB1 in poststroke inflammation.

Sources of Funding

This work was funded by National Institute of Neurological Disorders and Stroke R01NS049197.

Disclosure

None.

References

1. Buck BH, Liebeskind DS, Saver JL, Bang OY, Yun SW, Starkman S, et al. Early neutrophilia is associated with volume of ischemic tissue in acute stroke. *Stroke*. 2008;39:355–360.
2. Audebert HJ, Rott MM, Eck T, Haberl RL. Systemic inflammatory response depends on initial stroke severity but is attenuated by successful thrombolysis. *Stroke*. 2004;35:2128–2133.
3. Prass K, Meisel C, Höflich C, Braun J, Halle E, Wolf T, et al. Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. *J Exp Med*. 2003;198:725–736.
4. Hug A, Dalpke A, Wieczorek N, Giese T, Lorenz A, Auffarth G, et al. Infarct volume is a major determiner of post-stroke immune cell function and susceptibility to infection. *Stroke*. 2009;40:3226–3232.
5. Becker KJ, Kalil AJ, Tanzi P, Zierath DK, Savos AV, Gee JM, et al. Autoimmune responses to the brain after stroke are associated with worse outcome. *Stroke*. 2011;42:2763–2769.
6. Oppenheim JJ, Tewary P, de la Rosa G, Yang D. Alarmins initiate host defense. *Adv Exp Med Biol*. 2007;601:185–194.
7. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol*. 2005;5:331–342.
8. Liu K, Mori S, Takahashi HK, Tomono Y, Wake H, Kanke T, et al. Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. *FASEB J*. 2007;21:3904–3916.
9. Muhammad S, Barakat W, Stoyanov S, Murikinati S, Yang H, Tracey KJ, et al. The HMGB1 receptor RAGE mediates ischemic brain damage. *J Neurosci*. 2008;28:12023–12031.
10. Sims JR, Gharai LR, Schaefer PW, Vangel M, Rosenthal ES, Lev MH, et al. ABC/2 for rapid clinical estimate of infarct, perfusion, and mismatch volumes. *Neurology*. 2009;72:2104–2110.
11. Qiu J, Nishimura M, Wang Y, Sims JR, Qiu S, Savitz SI, et al. Early release of HMGB-1 from neurons after the onset of brain ischemia. *J Cereb Blood Flow Metab*. 2008;28:927–938.
12. Kim JB, Sig Choi J, Yu YM, Nam K, Piao CS, Kim SW, et al. HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. *J Neurosci*. 2006;26:6413–6421.
13. Vogelgesang A, May VE, Grunwald U, Bakkeboe M, Langner S, Wallaschofski H, et al. Functional status of peripheral blood T-cells in ischemic stroke patients. *PLoS ONE*. 2010;5:e8718.

2.3 The immunologic profile of adoptively transferred lymphocytes influences stroke outcome of recipients

Journal of Neuroimmunology 263 (2013) 28–34

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

The immunologic profile of adoptively transferred lymphocytes influences stroke outcome of recipients☆

Dannielle Zierath [a], Juliane Schulze [b], Allison Kunze [a], Olga Drogomiretskiy [a], Derek Nhan [a], Brett Jaspers [a], Alexander Dressel [b], Kyra Becker [a,*]

[a] University of Washington School of Medicine, Seattle, WA, USA
[b] University of Greifswald, Greifswald, Germany

ARTICLE INFO

Article history:
Received 28 September 2012
Received in revised form 9 July 2013
Accepted 17 July 2013

Keywords:
Stroke
Immune response
Th1
Th17
Lymphocyte

ABSTRACT

Animals that have myelin basic protein (MBP) specific lymphocytes with a Th1(+) phenotype have worse stroke outcome than those that do not. Whether these MBP specific cells contribute to worsened outcome or are merely a consequence of worse outcome is unclear. In these experiments, lymphocytes were obtained from donor animals one month after stroke and transferred to naïve recipient animals at the time of cerebral ischemia. The MBP specific phenotype of donor cells was determined prior to transfer. Animals that received either MBP specific Th1(+) or Th17(+) cells experienced worse neurological outcome, and the degree of impairment correlated with the robustness of MBP specific Th1(+) and Th17(+) responses. These data demonstrate that the immunologic phenotype of antigen specific lymphocytes influences stroke outcome.

1. Introduction

Following stroke there is a transient breakdown in the blood–brain barrier (BBB) that allows cells of the immune system to gain access to the brain (Jander et al., 1995). In addition, dying neurons and glia release proteins into the systemic circulation allowing cells of the immune system to come into contact with CNS specific proteins in the periphery (Jauch et al., 2006). The possibility for developing an immune response to brain antigens thus exists, and the nature of that response depends upon the microenvironment at the site of antigen presentation. In animal models, we showed that Th1 type immune responses to antigens such as myelin basic protein (MBP) were uncommon after stroke, but that Th1 responses could be enhanced by an inflammatory insult (systemic injection of lipopolysaccharide [LPS]) during the period of BBB compromise (Becker et al., 2005). In these experiments, a Th1(+) response to MBP was associated with worse stroke outcome (Becker et al., 2005; Gee et al., 2008, 2009). Whether the Th1 response to brain antigens contributed to the poor outcome or was merely a marker of poor outcome has not yet been adequately addressed. In the current study, the influence of lymphocytes primed to MBP by stroke (and injection of LPS) on stroke outcome was assessed by adoptive transfer of these cells to naïve animals. Lymphocytes were harvested from donor animals one month after stroke and injected into recipient animals at the time of middle cerebral artery occlusion (MCAO). The immunologic phenotype of the transferred cells (MBP specific Th1[+] or Th17[+]) was determined and their effect on the outcome of recipients assessed.

2. Methods

2.1. Animals

All protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Donor animals (male Lewis rats, 325–375 g, Charles River) underwent 3 h middle MCAO and received either an intraperitoneal (IP) injection of lipopolysaccharide (LPS; 1 mg/kg) or normal saline (1 mL/kg) at the time of reperfusion. The prolonged period of ischemia (3 h) and LPS was used to skew the immune response towards MBP to that of a Th1(+) response (Becker et al., 2005). Sham-operated animals underwent ligation of the common carotid artery without MCAO and were similarly treated with either LPS or saline. Donor animals were sacrificed at 1 month after MCAO — the optimal time point to detect the Th1 immune response to MBP based on our prior studies (Becker et al., 2005). Recipient animals (male Lewis rats, 325–375 g, Charles River) underwent MCAO (2 h) and at the time of reperfusion received an IP injection of splenocytes (1×10^8 cells in 1 mL normal saline) from donor animals. Recipient animals received cells from only 1 donor animal and were sacrificed at 1 day, 3 days or 1 week after MCAO (Fig. 1). Temperature was maintained at normothermia during MCAO, but animals were allowed to spontaneously thermoregulate thereafter. A shorter period of ischemia (2 h) was used in recipient

☆ Funding was provided by NINDS 5R01NS056457.
* Corresponding author at: Box 359775 HMC, 325 9th Ave, Seattle, WA 98104-2499, USA. Tel.: +1 206 744 3251; fax: +1 206 744 8787.
E-mail address: kjb@uw.edu (K. Becker).

0165-5728/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jneuroim.2013.07.014

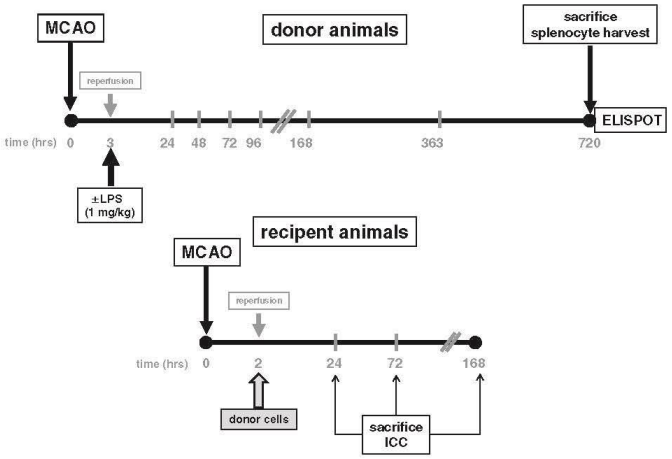


Fig. 1. *Experimental protocol.* Donor animals underwent 3 h of MCAO (or sham surgery) and received either LPS or saline at the time of reperfusion. Animals were sacrificed at 720 h (1 month), splenocytes harvested and cells cultured with MBP for 48 h. A subset of cells was subjected to ELISPOT assay to determine the TH1 and TH17 responses to MBP. Recipient animals underwent 2 h MCAO and donor cells were injected into recipient animals at the time of reperfusion. Neurologic function was assessed at 24 h (1 day), 72 h (3 days) and 168 h (1 week) after MCAO. Subsets of animals were sacrificed at each time point for histological analysis.

animals so that a detrimental effect of the adoptively transferred cells could be detected.

2.2. Donor cell preparation

Spleens were removed from donor animals at the time of sacrifice and processed into single cell suspensions. Cells (5×10^6/mL) were cultured in media supplemented with bovine MBP (2 μg/mL, Sigma Aldrich) for 48 h to expand the population of MBP reactive cells. Cells were washed extensively prior to injection. Splenocytes from a subset of animals were labeled with carboxy-fluorescein diacetate succinimidyl ester (CFDA SE) according to manufacturer's recommendations (Invitrogen) to track their migration after transfer. One donor animal generally provided enough cells for transfer to up to two different recipient animals.

2.3. ELISPOT assays

At the time of sacrifice, ELISPOT assays were done on isolated donor lymphocytes to detect the MBP specific secretion of IFN-γ, IL-17 and TGF-β1 (R&D Systems). Briefly, cells were cultured in media alone or in media supplemented with human MBP (50 μg/mL, Sigma Aldrich) for 48 h in 96 well plates (Multiscreen®-IP, Millipore). Plates were developed using standard protocols (R&D Systems). After plate development, spots were counted with the aid of a semi-automated system (Metamorph®) and expressed as the ratio of the relative increase in the number of MBP specific IFN-γ to the relative increase in the number of MBP specific TGF-β1 secreting cells (TH1 response) or as the ratio of the relative increase in the number of MBP specific IL-17 to the relative increase in the number of MBP specific TGF-β1 secreting cells (TH17 response).

$$\text{TH1 response} = \frac{\text{number of IFN}-\gamma\text{ spots with MBP} \div \text{number of IFN}-\gamma\text{ spots in media alone}}{\text{number of TGF}-\beta\text{ spots with MBP} \div \text{number of TGF}-\beta\text{ spots in media alone}}$$

$$\text{TH17 response} = \frac{\text{number of IL}-17\text{ spots with MBP} \div \text{number of IL}-17\text{ spots in media alone}}{\text{number of TGF}-\beta1\text{ spots with MBP} \div \text{number of TGF}-\beta1\text{ spots in media alone}}.$$

The ratios of the number of MBP specific IFN-γ and IL-17 secreting cells to that of MBP specific TGF-β1 secreting cells (TH1 and TH17 responses, respectively) were used to better reflect the overall immunologic phenotype of the adoptively transferred cells. The most robust TH1 response seen in any naïve/sham-operated animal was 1.16 and the most robust TH17 response seen in any naïve/sham-operated animal was 1.15. Animals were thus considered to be TH1(+) or TH17(+) if they had responses that exceeded these values (1.16 and 1.15, respectively).

2.4. Behavioral outcomes

The neurological score of recipient animals was determined at 3 h, 1 day, 3 days and 1 week after MCAO (Bederson et al., 1986). Recipient animals were trained on the rotarod for 5 sessions over 5 days prior to MCAO. After MCAO, rotarod performance was assessed at 1 day, 3 days and 1 week and expressed as a percent of pre-stroke baseline. Performance of the foot fault test was also assessed at these time points and the results expressed as a percentage of foot faults per total number of steps taken (Lubics et al., 2005).

2.5. Histology

At the time of sacrifice, recipient animals were perfused with saline followed by 4% paraformaldehyde. Brains were removed, post-fixed in 4% paraformaldehyde, cryoprotected in 25% sucrose and frozen at −80 °C until sectioning. Sections (20 μm) were stained for IFN-γ and IL-17 (Abcam). Additional sections were stained for lymphocytes using a CD3 antibody (Abcam) detected with a rhodamine tagged secondary antibody (Jackson labs); CFDA SE labeled lymphocytes were identified with an anti-fluorescein antibody (Invitrogen) and detected with a fluorescein tagged secondary antibody (Abcam). CD8+ cells were detected using a mouse anti-rat CD8 antibody (Abcam). Cells were counted in coronal brain sections correlating to bregma 2.70 mm and −0.26 mm. The number of cells within 6 high power fields

(100×) in each of the 8 different brain regions outlined in Fig. 3*a* was determined.

2.6. Statistics

Non-parametric data are displayed as the mean and (interquartile range [IQR]) and compared using the Mann–Whitney *U* test. Parametric data are displayed as the mean and standard deviation (unless otherwise indicated) and compared by *t*-test. Categorical data are compared using the χ^2-test statistic. Correlations are performed using Pearson's r for parametric data and Spearman's rho (ρ) for non-parametric data. Significance was set at $P < 0.05$.

3. Results

The absolute change in the numbers of cells secreting IFN-γ, TGF-β1 and IL-17 upon culture with MBP (in comparison to culture in media alone) after stroke is depicted in Fig. 2. The fact that there was a decrease in the number of cells secreting cytokines after culture with MBP in some animals suggests that MBP downregulated secretion of those cytokines. The horizontal line in each panel represents the highest value seen in sham-operated animals. Stroke was associated with an increase in the proportion of animals that had an MBP dependent upregulation in the secretion of IFN-γ and IL-17. The effect of LPS administration at stroke onset was notably associated with an increase in the MBP specific IFN-γ response (Fig. 2*a*).

To better reflect the immunologic status of this pool of splenocytes, the ratio of the relative increase in the number of MBP specific cells secreting IFN-γ to that of the relative increase in the number of MBP specific cells secreting TGF-β1 was calculated (as a marker of the TH1 response) and the ratio of the relative increase in the number of MPB specific cells secreting IL-17 to that of the relative increase in the number of MBP specific cells secreting TGF-β1 was calculated (as a marker of the TH17 response). The immunologic phenotype of donor cells, TH1(+) *versus* TH17(+), is displayed in Table 1. The proportion of animals with a TH1(+) response was increased by the administration of LPS at the time of MCAO, while TH17(+) responses were associated with MCAO alone.

Neither the stroke nor the LPS treatment status of the donor animals affected the outcome of recipient animals (data not shown). Animals that received TH17(+) cells at the time of MCAO, however, had higher temperatures in the post-stroke period and higher (worse) neurological scores 1 week after MCAO (Table 2). Animals that received cells with either a TH1(+) or TH17(+) phenotype also performed less well on the rotarod after stroke (Table 2). To further test the relationship between the immunologic phenotype of the donor cells and their effect of the outcome of recipient animals, the robustness of the TH1 and TH17 response was correlated to each outcome measure. Table 3 shows that the more robust the TH1 and TH17 response in donor cells the worse the performance on the rotarod in recipient animals. Additionally, more robust TH17 responses to MBP were associated with worse (higher) neurological scores in recipient animals.

Table 1
TH1 and TH17 responses of donor animals based on treatment status.

	Number of animals with TH1(+) and TH17(+) responses		
	Naïve/sham	MCAO; LPS(−)	*P*
TH1(+)	0/9	2/8 (25%)	0.11
TH17(+)	**0/9**	**5/8 (62%)**	**<0.01**
	Naïve/sham	MCAO; LPS(+)	*P*
TH1(+)	**0/9**	**10/18 (56%)**	**<0.01**
TH17(+)	**0/9**	**9/18 (50%)**	**<0.01**
	MCAO; LPS(−)	MCAO; LPS(+)	*P*
TH1(+)	2/8 (25%)	10/18 (56%)	0.15
TH17(+)	5/8 (62%)	9/18 (50%)	>0.20

TH1(+) indicates a TH1 response > 1.16, TH17(+) response indicates a TH17(+) response >1.15 as defined in the methods section. MCAO = middle cerebral artery occlusion, LPS = lipopolysaccharide, LPS(+) = treated with LPS at the time of MCAO, LPS(−) = treated with saline at the time of MCAO. Statistics are by χ^2.
Bold indicates significant results (P < 0.05).

Immunocytochemistry was done for IFN-γ, IL-17, fluorescein (to identify CFDA SE labeled cells) and CD8 at the time of sacrifice (1, 3, or 7 days after MCAO). Fig. 3 shows compares the number of these cells in the brain based on the phenotype (Th1[+] or Th17[+]) of the cells. There were more fluorescein$^+$ cells in the infarcted hemisphere 1 day after MCAO in animals receiving TH1(+) donor cells (Fig. 3*d*). Among animals receiving TH17(+) donor cells, there were more IFN-γ$^+$ and more CD8$^+$ cells in the infarcted hemisphere 3 days after MCAO (Fig. 3*f* and *i*). No fluorescein labeled cells were found in the brains of sham-operated animals or in the non-infarcted hemisphere in animals undergoing MCAO. At 3 days after MCAO/adoptive transfer, there was a strong correlation between the number of fluorescein labeled cells and the number of IFN-γ$^+$ ($r = 0.76$, $P = 0.001$), IL-17$^+$ ($r = 0.54$, $P = 0.04$), and CD8$^+$ ($r = 0.70$, $P = 0.007$) cells. The correlation between the number of fluorescein labeled

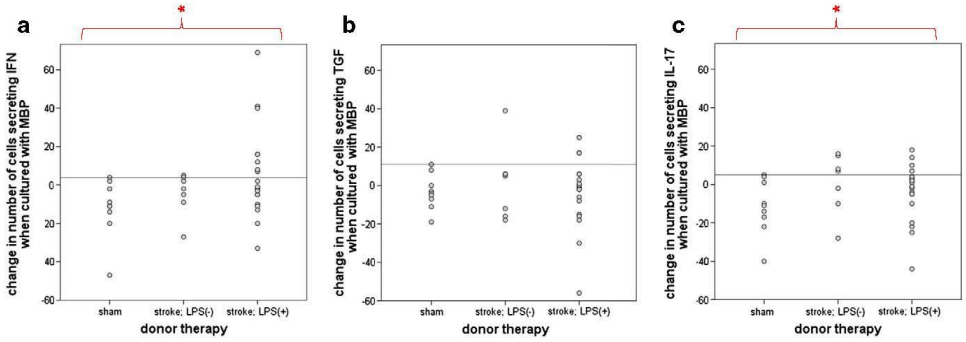


Fig. 2. Change in numbers of cells secreting IFN-γ (a), TGF-β1 (b) and IL-17 (c) after culture with MBP (in comparison to culture in media alone). The Y-axis shows the increase or decrease in cell number (per 100,000 cells) following culture with MBP and the X-axis shows the treatment of the donor animals. The horizontal line indicates the highest number of MBP specific cells seen in sham-operated animals. The proportion of animals with stroke induced increases in MBP specific IFN-γ and IL-17 responses (in comparison to highest number seen in sham-operated animals) is displayed in panels a and c. LPS administration to donor animals was also associated with an increase in the absolute numbers of MBP specific IFN-γ secreting cells (a; $P = 0.03$ by *ANOVA*). Stroke (with or without LPS administration) did not result in a significant change in the number of MBP specific TGF-β1 secreting cells (b). * indicates that the treatment groups differ by $P < 0.05$ (χ^2).

Table 2
Differences in neurological outcomes of recipient animals based on the phenotype of donor cells.

Donor status:	TH1(+)	TH1(−)	P	TH17(+)	TH17(−)	P
Temperature						
Baseline	36.3 (36.1, 36.6) N = 18	36.4 (36.1, 36.6) N = 25	>0.20	36.4 (36.0, 36.6) N = 21	36.4 (36.1, 36.6) N = 22	>0.20
3 h	38.4 (38.2, 39.0) N = 18	38.8 (38.4, 39.2) N = 25	>0.20	39.0 (38.4, 39.3) N = 21	38.5 (38.1, 39.0) N = 22	0.09
1 day	38.0 (37.8, 38.3) N = 18	38.0 (37.6, 38.5) N = 25	>0.20	38.2 (38.0, 38.5) N = 21	37.7 (37.2, 38.1) N = 22	<0.01
3 days	37.7 (37.4, 37.8) N = 12	37.6 (37.3, 37.7) N = 17	>0.20	37.7 (37.5, 37.9) N = 14	37.4 (37.2, 37.7) N = 15	0.03
1 week	37.3 (37.0, 37.7) N = 5	37.6 (37.1, 37.7) N = 8	>0.20	37.2 (36.7, 37.6) N = 6	37.6 (37.1, 37.7) N = 7	>0.20
Neuroscore						
Baseline	–	–	–	–	–	–
3 h	3 (2, 3) N = 18	3 (2, 3) N = 25	>0.20	3 (2, 3) N = 21	3 (2, 3) N = 22	0.16
1 day	3 (3, 4) N = 18	3 (3, 4) N = 25	>0.20	3 (3, 4) N = 21	3 (2, 4) N = 22	>0.20
3 days	3 (2, 3) N = 12	3 (2, 3) N = 17	>0.20	3 (2, 3) N = 14	3 (2, 3) N = 15	>0.20
1 week	2 (2, 2) N = 5	2 (0, 3) N = 8	>0.20	2 (2, 3) N = 6	2 (0, 2) N = 7	<0.05
Foot fault (% of total steps)						
Baseline	0 (0, 2) N = 18	0 (0, 1) N = 25	>0.20	0 (0, 2) N = 21	0 (0, 0) N = 22	0.17
3 h	–	–	–	–	–	–
1 day	87 (73, 96) N = 18	80 (58, 90) N = 25	0.14	88 (72, 94) N = 21	80 (59–89) N = 22	0.15
3 days	86 (61, 96) N = 12	71 (46, 85) N = 17	0.08	82 (62, 94) N = 14	71 (44, 84) N = 15	0.05
1 week	25 (18, 48) N = 5	32 (11, 51) N = 8	>0.20	31 (19, 45) N = 6	25 (10, 53) N = 7	>0.20
Rotarod performance (% of baseline)						
Baseline	–	–	–	–	–	–
3 h	–	–	–	–	–	–
1 day	9.7 (5.9, 19.1) N = 18	10.8 (7.2, 60.7) N = 25	0.18	10.3 (5.9, 21.4) N = 21	9.8 (6.9, 78.7) N = 22	>0.20
3 days	5.8 (5.0, 7.6) N = 12	6.6 (5.9, 80.0) N = 17	0.18	5.6 (4.8, 7.0) N = 14	10.0 (6.0, 100) N = 15	0.01
1 week	6.5 (6.0, 48.8) N = 5	23.6 (11.0, 100) N = 8	0.04	7.5 (6.0, 35.9) N = 6	27.9 (8.6, 100) N = 7	0.04

TH1(+) represents a value greater than sham-operated (>1.16), TH17(+) represents a value greater than all sham-operated animals (>1.15). Statistics are by Mann–Whitney *U* test.

cells and the number of $CD8^+$ cells persisted to 7 days ($r = 0.64$, $P = 0.04$). Fig. 4 shows the infiltration of donor ($fluorescein^+$) cells 1 day after MCAO in recipients of MBP specific TH1(+) cells.

4. Discussion

There is increasing evidence that lymphocytes contribute to ischemic brain injury (Iadecola and Anrather, 2011). There is also ample evidence demonstrating the occurrence of autoimmune responses to brain antigens after stroke, but the pathologic consequences of these responses are unknown (Rocklin et al., 1971; Youngchaiyud et al., 1974; Kallen et al., 1977; Wang et al., 1992). We previously showed that a TH1 type immune response to MBP is associated with worse outcome in experimental models of stroke (Becker et al., 2005; Gee et al., 2008; Gee et al., 2009) and that TH1 responses to MBP are associated with worse clinical outcome in a cohort of stroke patients 3 months after stroke onset (Becker et al., 2011). The association of the TH1 response to MBP and worse outcome, however, does not prove that the lymphocytes with the TH1 phenotype mediate the worse outcome. The purpose of this study was to more definitively address the role of MBP specific lymphocytes in modulating outcome from stroke. The contribution of MBP specific lymphocytes was evaluated by adoptive transfer into recipient animals. For clinical relevance, the donor lymphocytes were generated in animals undergoing severe stroke (3 h MCAO) and skewed towards a TH1(+) response by the administration of LPS. Animals that received donor cells with either an MBP specific TH1(+) or TH17(+) phenotype at the time of stroke experienced worse clinical outcome than animals that received donor cells without a TH1(+) or TH17(+) phenotype. Further, there was a direct correlation between the robustness of both the TH1 and TH17 responses to MBP and functional outcome at multiple time points after stroke. These observations suggest that adoptively transferred cells mediate the worsened outcome.

Most animals undergoing MCAO do not develop a TH1(+) response to MBP (Becker et al., 2005). Systemic administration of LPS, however,

Table 3
Correlation between the robustness of the TH1 and TH17 responses of donor cells and the neurological outcome of recipients.

Correlations	1 day		3 days		1 week	
	TH1	TH17	TH1	TH17	TH1	TH17
Temperature[a]	−0.22	0.15	0.07	0.13	−0.18	−0.32
	0.12	>0.20	>0.20	>0.20	>0.20	>0.20
Neurological score[b]	−0.02	−0.01	0.17	**0.42**	0.23	0.51
	>0.20	>0.20	>0.20	**0.01**	>0.20	P = 0.05
Foot fault test[b]	0.12	0.15	0.30	0.33	0.04	0.28
	>0.20	>0.20	0.09	0.06	>0.20	>0.20
Rotarod[b]	−0.10	−0.07	**−0.36**	−0.32	**−0.65**	**−0.52**
	>0.20	>0.20	**0.04**	0.07	**<0.01**	**<0.05**

[a] Pearson's r.
[b] Spearman's rho.

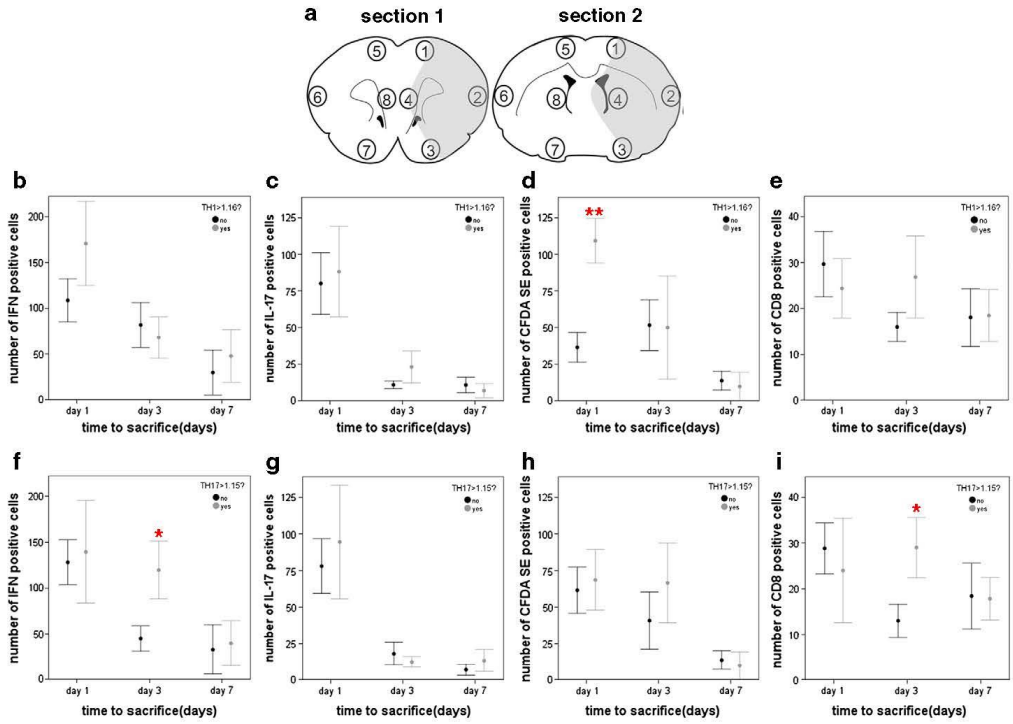


Fig. 3. The numbers of cells staining for IFN-γ, IL-17, fluorescein and CD8 were determined in 4 regions within the infarcted hemisphere and in 4 regions within the non-infarcted hemisphere in two different coronal brain sections (a). Cells were counted in 6 adjacent high power fields (100×) within each of the 4 brain regions in the infarcted and non-infarcted hemispheres. The graphs show the total cell counts for regions 1 through 4 in both coronal sections (as there were virtually no identifiable cells in non-infarcted hemisphere). Animals receiving cells from a TH1(+) donor had more CFDA SE positive (or fluorescein$^+$) cells in the infarcted hemisphere at day 1 after MCAO (d), but there were no differences in the number of IFN-γ$^+$ (b), IL-17$^+$ (c) or CD8$^+$ (d) cells. Animals receiving cells from a TH17(+) donor had more IFN-γ$^+$ and CD8$^+$ cells at day 3 after MCAO (*f* and *i*). Data are presented as the mean and SEM. $^*P < 0.05$ and $^{**}P < 0.01$ by *t*-test.

skews the immune response towards that of a TH1(+) response in animals undergoing severe stroke (3 h MCAO) (Becker et al., 2005). In accord with our prior studies, we observed an increase in the TH1 response to MBP among animals treated with LPS. LPS, however, did not appear to increase the MBP specific TH17 response after stroke. The requisites for generation of TH1 and TH17 cells differ; the former is dependent on IFN-γ in the environment of antigen presentation and the latter upon the presence of both TGF-β1 and IL-6 (Zhou et al., 2009). Few studies address stroke induced changes in plasma IFN-γ, and those that do show no appreciable changes (Urra et al., 2009) or even a decrease in IFN-γ (Vogelgesang et al., 2010) in the days after stroke. Further, IFN-γ does not appear to be reliably upregulated in the ischemic brain (Lambertsen et al., 2004). LPS, however, is known to induce the production of IFN-γ (Pulendran et al., 2001). Infections, especially those caused by Gram-negative organisms, are associated with an increase IFN-γ (Kohler et al., 1993; Lainee et al., 2005; Paats et al., 2013). These data argue that the usual scenario after stroke would not favor the development of TH1 responses, but that post-stroke infection (with generation of IFN-γ) could increase this likelihood. Unlike IFN-γ, both IL-6 and TGF-β1 are known to be markedly upregulated following ischemic stroke. Systemic IL-6 increases within hours after stroke onset (Beamer et al., 1995; Waje-Andreassen et al., 2005). There is also rapid upregulation of IL-6 and TGF-β1 expression in ischemic brain (Krupinski et al., 1996; Suzuki et al., 1999; Legos et al., 2000; Ali et al., 2001). The cytokines necessary for the development of a TH17 response are thus usually present after stroke. These observations might explain why LPS was needed to provoke the development of TH1(+) responses while stroke itself was associated with a TH17(+) response in at least 50% of animals.

In the animal model used in our experiments, LPS is used as a proxy for systemic infection. Infections, especially pneumonia and urinary tract infections, are very common following stroke (Westendorp et al., 2011). As might be expected, patients who develop infection are more likely to develop TH1 responses to MBP, and these TH1 responses are associated with worse clinical outcome (Becker et al., 2011). We have not previously evaluated the association between MBP specific TH17 responses and stroke outcome. The current study, however, suggests that both MBP specific TH1 and TH17 immune responses may not only be associated with worse stroke outcome, but may mediate this worse outcome. Importantly, the absolute number of MBP specific cells secreting IFN-γ and IL-17 in this study is similar to that seen for PLP in models of EAE (Targoni et al., 2001). Further, the numbers of MBP specific cells are similar to those seen in the circulation of patients with multiple sclerosis (Jansson et al., 2003).

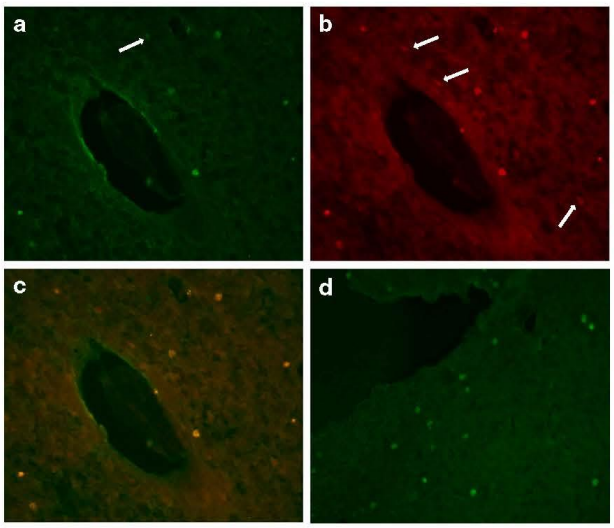


Fig. 4. Immunocytochemistry for CFDA SE (fluorescein) (a), CD3 (b), and both (c) at 20×. The donor cells for this animal were TH1(+) but not TH17(+); the recipient animal was sacrificed 1 day after MCAO. The arrow in panel a shows a fluorescein$^+$ cell that is not CD3$^+$, and the arrows in panel b show CD3$^+$ cells that are not fluorescein$^+$. Panel d shows fluorescein$^+$ (donor) cells in a different animal that received MBP specific TH1(+) but not TH17(+) cells and was sacrificed 1 day after MCAO.

CFDA SE(+) or fluorescein$^+$ cells were found in the infarcted hemispheres of recipient animals, demonstrating that these cells traffic into ischemic brain; the infiltration of these fluorescein$^+$ cells was most robust at 1 day after MCAO in animals receiving TH1(+) cells. Interestingly, there were more IFN-γ$^+$ (not IL-17$^+$) cells in the brains of animals receiving TH17(+) donor cells at day 3 after MCAO, but there appears to be a population of TH17(+) cells that also secrete IFN-γ (Suryani and Sutton, 2007; Murphy et al., 2010). We did not see a significant difference in the number of IFN-γ$^+$ or IL-17$^+$ cells among animals receiving TH1(+) or TH17(+) donor cells at other time points. More CD8$^+$ cells were seen among recipients of TH17(+) cells 3 days after MCAO, suggesting these animals experienced a more robust inflammatory response, which may help to explain the worse functional outcome in recipients of TH17(+) cells. To better address the relative numbers of IFN-γ$^+$, IL-17$^+$, fluorescein$^+$ and CD8$^+$ cells, flow cytometry with intracellular cytokine staining of lymphocytes isolated from the ischemic brain could be done in future studies. In addition, these studies might address the contribution of inflammatory cells other than lymphocytes to the CNS inflammatory response in animals receiving MBP specific TH1(+) and TH17(+) cells.

In concert, our data argue strongly that lymphocytes are able to modulate outcome from stroke and that the phenotype of the lymphocytes is important in determining the nature of this modulation. MBP specific TH1(+) and TH17(+) cells both worsen outcome from stroke while MBP specific cells with a TREG phenotype improve outcome from stroke (Becker et al., 2003). Whether TH1(+) and TH17(+) cells differentially effect outcome was not directly evaluated in this study, but in animal models of experimental autoimmune encephalomyelitis (EAE), TH17 type cells are inducing more severe disease than TH1 type cells (Jager et al., 2009). After stroke, endogenously developing immune responses towards brain antigens may thus be either detrimental (TH1, TH17) or beneficial (TREG). This observation suggests that manipulation of the post-ischemic immune response is thus a potential therapeutic strategy for the treatment of stroke.

References

Ali, C., Docagne, F., Nicole, O., Lesne, S., Toutain, J., Young, A., et al., 2001. Increased expression of transforming growth factor-beta after cerebral ischemia in the baboon: an endogenous marker of neuronal stress? J. Cereb. Blood Flow Metab. 21, 820–827.

Beamer, N.B., Coull, B.M., Clark, W.M., Hazel, J.S., Silberger, J.R., 1995. Interleukin-6 and interleukin-1 receptor antagonist in acute stroke. Ann. Neurol. 37, 800–805.

Becker, K., Kindrick, D., McCarron, R., Hallenbeck, J., Winn, R., 2003. Adoptive transfer of myelin basic protein-tolerized splenocytes to naive animals reduces infarct size: a role for lymphocytes in ischemic brain injury? Stroke 34, 1809–1815.

Becker, K.J., Kindrick, D.L., Lester, M.P., Shea, C., Ye, Z.C., 2005. Sensitization to brain antigens after stroke is augmented by lipopolysaccharide. J. Cereb. Blood Flow Metab. 25, 1634–1644.

Becker, K.J., Kalil, A.J., Tanzi, P., Zierath, D.K., Savos, A.V., Gee, J.M., et al., 2011. Autoimmune responses to the brain after stroke are associated with worse outcome. Stroke 42, 2763–2769.

Bederson, J.B., Pitts, L.H., Tsuji, M., Nishimura, M.C., Davis, R.L., Bartkowski, H., 1986. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke 17, 472–476.

Gee, J.M., Kalil, A., Thullbery, M., Becker, K.J., 2008. Induction of immunologic tolerance to myelin basic protein prevents central nervous system autoimmunity and improves outcome after stroke. Stroke 39, 1575–1582.

Gee, J.M., Zierath, D., Hadwin, J., Savos, A., Kalil, A., Thullbery, M., et al., 2009. Long term immunologic consequences of experimental stroke and mucosal tolerance. Exp. Transl. Stroke Med. 1, 3.

Iadecola, C., Anrather, J., 2011. The immunology of stroke: from mechanisms to translation. Nat. Med. 17, 796–808.

Jager, A., Dardalhon, V., Sobel, R.A., Bettelli, E., Kuchroo, V.K., 2009. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. J. Immunol. 183, 7169–7177.

Jander, S., Kraemer, M., Schroeter, M., Witte, O.W., Stoll, G., 1995. Lymphocytic infiltration and expression of intercellular adhesion molecule-1 in photochemically induced ischemia of the rat cortex. J. Cereb. Blood Flow Metab. 15, 42–51.

Jansson, A., Emerudh, J., Kvarnstrom, M., Ekerfelt, C., Vrethem, M., 2003. Elispot assay detection of cytokine secretion in multiple sclerosis patients treated with interferon-beta1a or glatiramer acetate compared with untreated patients. Mult. Scler. 9, 440–445.

Jauch, E.C., Lindsell, C., Broderick, J., Fagan, S.C., Tilley, B.C., Levine, S.R., 2006. Association of serial biochemical markers with acute ischemic stroke: the National Institute of Neurological Disorders and Stroke recombinant tissue plasminogen activator stroke study. Stroke 37, 2508–2513.

Kallen, B., Nilsson, O., Thelin, C., 1977. Effect of encephalitogenic protein on migration in agarose of leukocytes from patients with multiple sclerosis. A longitudinal study of patients with relapsing multiple sclerosis or with cerebral infarction. Acta Neurol. Scand. 55, 47–56.

Kohler, J., Heumann, D., Garotta, G., LeRoy, D., Bailat, S., Barras, C., et al., 1993. IFN-gamma involvement in the severity of gram-negative infections in mice. J. Immunol. 151, 916–921.
Krupinski, J., Kumar, P., Kumar, S., Kaluza, J., 1996. Increased expression of TGF-beta 1 in brain tissue after ischemic stroke in humans. Stroke 27, 852–857.
Lainee, P., Efron, P., Tschoeke, S.K., Elies, L., De Winter, H., Lorre, K., et al., 2005. Delayed neutralization of interferon-gamma prevents lethality in primate Gram-negative bacteremic shock. Crit. Care Med. 33, 797–805.
Lambertsen, K.L., Gregersen, R., Meldgaard, M., Clausen, B.H., Heibol, E.K., Ladeby, R., et al., 2004. A role for interferon-gamma in focal cerebral ischemia in mice. J. Neuropathol. Exp. Neurol. 63, 942–955.
Legos, J.J., Whitmore, R.G., Erhardt, J.A., Parsons, A.A., Tuma, R.F., Barone, F.C., 2000. Quantitative changes in interleukin proteins following focal stroke in the rat. Neurosci. Lett. 282, 189–192.
Lubics, A., Reglodi, D., Tamas, A., Kiss, P., Szalai, M., Szalontay, L., et al., 2005. Neurological reflexes and early motor behavior in rats subjected to neonatal hypoxic-ischemic injury. Behav. Brain Res. 157, 157–165.
Murphy, A.C., Lalor, S.J., Lynch, M.A., Mills, K.H., 2010. Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. Brain Behav. Immun. 24, 641–651.
Paats, M.S., Bergen, I.M., Hanselaar, W.E., Groeninx van Zoelen, E.C., Hoogsteden, H.C., Hendriks, R.W., et al., 2013. Local and systemic cytokine profiles in nonsevere and severe community-acquired pneumonia. Eur. Respir. J. 41, 1378–1385.
Pulendran, B., Kumar, P., Cutler, C.W., Mohamadzadeh, M., Van Dyke, T., Banchereau, J., 2001. Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. J. Immunol. 167, 5067–5076.
Rocklin, R.E., Sheremata, W.A., Feldman, R.G., Kies, M.W., David, J.R., 1971. The Guillain–Barre syndrome and multiple sclerosis. In vitro cellular responses to nervous-tissue antigens. N. Engl. J. Med. 284, 803–808.
Suryani, S., Sutton, I., 2007. An interferon-gamma-producing Th1 subset is the major source of IL-17 in experimental autoimmune encephalitis. J. Neuroimmunol. 183, 96–103.
Suzuki, S., Tanaka, K., Nogawa, S., Nagata, E., Ito, D., Dembo, T., et al., 1999. Temporal profile and cellular localization of interleukin-6 protein after focal cerebral ischemia in rats. J. Cereb. Blood Flow Metab. 19, 1256–1262.
Targoni, O.S., Baus, J., Hofstetter, H.H., Hesse, M.D., Karulin, A.Y., Boehm, B.O., et al., 2001. Frequencies of neuroantigen-specific T cells in the central nervous system versus the immune periphery during the course of experimental allergic encephalomyelitis. J. Immunol. 166, 4757–4764.
Urra, X., Cervera, A., Obach, V., Climent, N., Planas, A.M., Chamorro, A., 2009. Monocytes are major players in the prognosis and risk of infection after acute stroke. Stroke 40, 1262–1268.
Vogelgesang, A., May, V.E., Grunwald, U., Bakkeboe, M., Langner, S., Wallaschofski, H., et al., 2010. Functional status of peripheral blood T-cells in ischemic stroke patients. PLoS One 5, e8718.
Waje-Andreassen, U., Krakenes, J., Ulvestad, E., Thomassen, L., Myhr, K.M., Aarseth, J., et al., 2005. IL-6: an early marker for outcome in acute ischemic stroke. Acta Neurol. Scand. 111, 360–365.
Wang, W.Z., Olsson, T., Kostulas, V., Hojeberg, B., Ekre, H.P., Link, H., 1992. Myelin antigen reactive T cells in cerebrovascular diseases. Clin. Exp. Immunol. 88, 157–162.
Westendorp, W.F., Nederkoorn, P.J., Vermeij, J.D., Dijkgraaf, M.G., van de Beek, D., 2011. Post-stroke infection: a systematic review and meta-analysis. BMC Neurol. 11, 110.
Youngchaiyud, U., Coates, A.S., Whittingham, S., Mackay, I.R., 1974. Cellular-immune response to myelin protein: absence in multiple sclerosis and presence in cerebrovascular accidents. Aust. N. Z. J. Med. 4, 535–538.
Zhou, L., Chong, M.M., Littman, D.R., 2009. Plasticity of CD4+ T cell lineage differentiation. Immunity 30, 646–655.

2.4 Reduced numbers and impaired function of regulatory T cells in peripheral blood of ischemic stroke patients

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 2974605, 9 pages
http://dx.doi.org/10.1155/2016/2974605

Research Article

Reduced Numbers and Impaired Function of Regulatory T Cells in Peripheral Blood of Ischemic Stroke Patients

Johanna Ruhnau,[1] Juliane Schulze,[1] Bettina von Sarnowski,[1] Marie Heinrich,[1] Sönke Langner,[2] Christian Pötschke,[3] Anika Wilden,[1] Christof Kessler,[1] Barbara M. Bröker,[3] Antje Vogelgesang,[1] and Alexander Dressel[1]

[1]*Department of Neurology, University Medicine Greifswald, 17475 Greifswald, Germany*
[2]*Department of Diagnostic Radiology and Neuroradiology, University Medicine Greifswald, 17475 Greifswald, Germany*
[3]*Department of Immunology, University Medicine Greifswald, 17475 Greifswald, Germany*

Correspondence should be addressed to Alexander Dressel; adressel@uni-greifswald.de

Received 11 December 2015; Accepted 8 February 2016

Academic Editor: Jens Geginat

Background and Purpose. Regulatory T cells (Tregs) have been suggested to modulate stroke-induced immune responses. However, analyses of Tregs in patients and in experimental stroke have yielded contradictory findings. We performed the current study to assess the regulation and function of Tregs in peripheral blood of stroke patients. Age dependent expression of CD39 on Tregs was quantified in mice and men. *Methods.* Total FoxP3$^+$ Tregs and CD39$^+$FoxP3$^+$ Tregs were quantified by flow cytometry in controls and stroke patients on admission and on days 1, 3, 5, and 7 thereafter. Treg function was assessed by quantifying the inhibition of activation-induced expression of CD69 and CD154 on T effector cells (Teffs). *Results.* Total Tregs accounted for 5.0% of CD4$^+$ T cells in controls and <2.8% in stroke patients on admission. They remained below control values until day 7. CD39$^+$ Tregs were most strongly reduced in stroke patients. On day 3 the Treg-mediated inhibition of CD154 upregulation on CD4$^+$ Teff was impaired in stroke patients. CD39 expression on Treg increased with age in peripheral blood of mice and men. *Conclusion.* We demonstrate a loss of active FoxP3$^+$CD39$^+$ Tregs from stroke patient's peripheral blood. The suppressive Treg function of remaining Tregs is impaired after stroke.

1. Introduction

Ischemic stroke-induced immune alterations (SIIA) are thought to affect the outcome of stroke patients, in part by enhancing their susceptibility to bacterial infection. This has been shown in experimental stroke models and in patients [1–3]. The alterations in the immune system are thought to be mediated by rapid activation of the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. In stroke patients, plasma levels of stress hormones correlate with the extent of immune alterations [4, 5].

In addition to the systemic immune-suppressive alterations observed in the peripheral blood, a local inflammatory immune response also develops. Within 24 h, leukocytes accumulate in the ischemic brain region [6, 7]. Recent observations suggest that lymphocytes but not granulocytes can trespass the blood brain barrier and infiltrate the ischemic lesion and the penumbra [8–10]. To date, the mechanisms that regulate this local inflammatory response and the role of the leukocyte subtypes involved are only partially understood. In experimental stroke models, T cells sensitized to CNS antigens have been transferred into mice lacking B and T cells. These autoreactive T cells were found in the stroke lesion, and their transfer enhanced the severity of stroke [11]. Furthermore, immunological tolerization of T cells to CNS autoantigens has beneficial effects on experimental stroke outcome [12]. Together, these data suggest that local inflammation following stroke has some autoimmune properties.

Regulatory T cells (Tregs) are modulators of adaptive immune responses and play an important role in maintaining

tolerance to self-antigens. Depletion of murine $CD25^+CD4^+$ Tregs or abrogation of their function exacerbates various autoimmune diseases, including autoimmune gastritis, thyroiditis, and type 1 diabetes [13]. Thus, it has been hypothesized that, in stroke, Tregs may dampen the immunological cascades that result in secondary brain damage, which is not directly caused by ischemia. This concept is supported by the findings of Liesz et al., who used antibody-mediated depletion of $CD25^+$ cells to eliminate Tregs and reported worse outcome in an experimental stroke model [14]. In contrast, Kleinschnitz et al. have reported that depleting $FoxP3^+$ Tregs with an inducible diphtheria toxin receptor construct under the control of the FoxP3 promoter decreased brain damage [15]. Additional conflicting data have been reported, wherein Treg depletion using a similar mouse model in a different experimental stroke model did not alter the infarct volume within the initial 4 days after stroke [16]. More recently, controversial data continue to be reported from experimental stroke models. Boosting Treg function with a superagonistic anti-CD28 antibody (CD28SA) 3 h after middle cerebral artery occlusion (MCAO) reduced brain damage and improved outcome, while pretreatment with the same CD28SA antibody worsened clinical outcome and treatment immediately after MCAO had no effect on the ischemic brain volume in another study [17, 18]. In humans, Treg enumeration and function in peripheral blood have also yielded contradictory results. While Hug et al. were unable to detect changes in Treg function in patients with ischemic stroke [5], Yan et al. reported impaired function, but an increased percentage of Tregs [19].

In part, these contradictory findings could result from the fact that even $FoxP3^+$ Tregs are not a homogenous population; instead, they can be divided into several subgroups that differ in function and can be distinguished by surface antigen expression [20]. CD45RA is expressed on naive $FoxP3^+$ cells. Even in adulthood, some naive $FoxP3^+$ cells can be found in the circulation. Upon antigen stimulation, $CD45RA^+$ Tregs lose their CD45RA expression, start to proliferate, and differentiate to a more-suppressive Treg phenotype [13, 20]. CD39 is a rate-limiting ectonucleotidase that cleaves the proinflammatory extracellular adenosine triphosphate (ATP) [21, 22] to inhibitory and antiproliferative adenosine monophosphate [23]. Indeed, expression of CD39 has been shown to identify functionally active, suppressive Tregs in rodents and humans [24].

We performed the current study to determine the regulation of Treg subsets and Treg function in the peripheral blood of human stroke patients.

2. Methods

2.1. Human Studies

2.1.1. Patients and Controls. Patients (age: >18 y) with acute middle cerebral artery infarction were eligible for the study within 12 h of disease onset. They were recruited at the Department of Neurology of the University Medicine Greifswald if their National Institutes of Health Stroke Scale (NIHSS) score was ≥6, they had no signs of infection on admission, and their plasma levels of C-reactive protein (CRP) were ≤50 mg/L and of procalcitonin (PCT) ≤0.5 ng/mL. Patients were excluded if they took immunesuppressive drugs, suffered from known malignancies, or had an NIHSS score of <6. Treatment complied with best medical care standards and took place in a dedicated stroke unit. Recombinant tissue plasminogen activator administration and thrombectomy took place as clinically indicated. Control subjects were either healthy or recruited from the Ophthalmology Clinics at the University Medicine Greifswald. Control subjects were of similar age and had no known neurological or immunological disorders and fulfilled the same criteria for CRP and PCT as stroke patients. Patient and control characteristics are listed in Table 1(a). In addition a cohort of 32 younger healthy controls (age 21–79 years) was recruited to analyze age dependency of CD39 expression on Treg.

Patients were allocated into the stroke associated infection (SAI+) cohort if they had developed (a) clinical signs of infection (pneumonia, urinary tract infections, and fever of unknown origin); (b) serum concentrations of CRP >50 mg/L; and (c) PCT serum concentrations >0.5 ng/mL. Only patients that matched none of the criteria throughout the whole study period were considered free of infection (SAI−). Patients who fulfilled some criteria but not all were excluded from the comparison of stroke patients with and without SAI. These criteria were designed to identify two distinct populations of patients as published previously [3]. Details on SAI+ and SAI− patients are given in Table 1(b).

2.1.2. Ethics Statement. The study protocol was approved by the ethics committee of the Medical Faculty, University of Greifswald (No. III UV 30/01). All patients gave written informed consent directly or through a surrogate where appropriate.

2.1.3. CT Imaging. Routine cerebral CT images (sequential cCT native, 4.5 mm slice thickness, and supra- and infratentorial; mAs = 50; kV = 120) were acquired on a 16-row multislice CT scanner (Somatom 16; Siemens Medical Systems, Erlangen, Germany). To calculate lesion size, images were analyzed with OSIRIX 5.6. Regions of interest were defined manually, and the lesion volume was calculated semiautomatically.

2.1.4. Blood Sampling. Blood samples were obtained immediately upon admission and then between 6:00 a.m. and 8:00 a.m. on days 1, 3, 5, and 7. Investigators were not blinded for control and stroke patient samples, but they were unaware of stroke severity.

2.1.5. Phenotyping of Human Tregs. Aliquots of 200 μL of whole blood, anticoagulated with ethylenediaminetetraacetic acid (EDTA), were incubated with appropriate combinations of fluorescence-conjugated monoclonal antibodies to stain surface molecules. After lysing of erythrocytes (Buffer A; Human FoxP3 Buffer Set, BD Biosciences, Heidelberg, Germany), cells were washed twice and prepared for intracellular staining without further stimulation. After 30 min incubation

Table 1: (a) Subjects characteristics, Treg phenotyping. (b) Participants characteristics of patients (SAI+) suffering from an infection and patients (SAI−) who remained uninfected after stroke.

(a)

	Total number	Age†	NIHSS‡	Lesion volume (mm^3)§	Localization of infarction	Thrombolysis/ thrombectomy	Male	Female
Control subjects	26	69,5 (51–88)	NA	NA	NA	NA	12	14
Stroke patients	48	77 (55–93)	13 (23–6)	71,5 (4,99–1022)	45 MCA 3 MCA + anterior	17	16	32

(b)

	Total number	Age†	NIHSS‡	Lesion volume (mm^3)§	Localization of infarction	Thrombolysis/ thrombectomy	Male	Female
Noninfected cohort (SAI−)	15	77 (62–93)	8 (6–23)	44,05 (4,99–1022)	15 MCA 1 MCA + anterior	4	5	10
Infected cohort (SAI+)	7	74 (55–87)	17,5 (13–19)	174,24 (18,52–318,27)	7 MCA	2	4	3

†Mean (range); ‡median (range); §median (range); NA: not applicable; MCA: middle cerebral artery; SAI: stroke associated infection.

with anti-FoxP3-antibodies coupled with Alexa Fluor 647 (BioLegend, San Diego, CA, USA) and an additional washing step, cells were measured on a BD Canto II or BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA).

The monoclonal antibodies used to determine expression of cell surface molecules were CD25-PE-Cy7, CD49d-FITC, and CD4-V500 (BD Biosciences, Heidelberg, Germany) and CD45RA-PerCP-Cy5.5 and CD39-PE (BioLegend, San Diego, CA, USA). Isotype control antibodies coupled to PE, PerCP-Cy5.5, and Alexa Fluor 647 were from BioLegend and those coupled to FITC, PE-Cy7, and V500 were from BD Biosciences.

For each stroke patient or control sample six appropriate fluorescence minus one (FMO) controls were prepared to identify positive events by flow cytometry. To account for any nonspecific binding of the epitope-specific antibody the appropriate isotype control was added to each FMO control.

Tregs were quantified as $CD4^+CD49d^-FoxP3^+$ cells. Human proinflammatory effector cells can be transiently $FoxP3^+$ but bear CD49d. Therefore, $CD49^+$ cells were excluded from the $FoxP3^+$ population [27]. Within this population of Tregs, expression of CD45RA was used to identify naive Tregs, while CD39 surface expression was used to detect activated Tregs. There were no $CD39^+CD45RA^+$ cells; however, there was a consistent population of $CD39^+CD45RA^-$ Tregs. Flow cytometry results were evaluated by FlowJo software 7.6.5 (Tree Star Inc., Ashland, OR, USA).

2.1.6. Isolation of Human Tregs. Tregs were isolated with the $CD4^+CD25^{+high}CD127^{-/dim}$ Regulatory T Cell Isolation Kit II (human) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In brief, peripheral blood mononuclear cells (PBMCs) were isolated with a Ficoll gradient (Biochrom AG, Berlin, Germany). The $CD4^+CD25^+CD127^-$ T Cell Biotin-Antibody Cocktail II was used to negatively enrich for $CD4^+CD127^-$ cells followed by a positive selection of $CD25^+$ cells.

To assess the purity of these $CD4^+CD25^{+high}CD127^{-/dim}$ cells, samples and PBMCs were tested by flow cytometry. Hence, cells were incubated with the appropriate amount of extracellular antibody and prepared for intracellular staining with FoxP3-Alexa Fluor 647 (BioLegend) using the Human FoxP3 Buffer Set (BD Biosciences). For extracellular staining, CD25-PE-Cy7, CD4-FITC (both BD Biosciences), and CD127-Pacific Blue (BioLegend) were used. In all experiments, the purity of $CD4^+CD25^{+high}CD127^{-/dim}FoxP3^+$ cells was ≥95%.

2.1.7. Suppression Assay. Once the Tregs were isolated, they were incubated with 100 000/well PBMCs in different ratios (Treg : PBMC: 1 : 1, 1 : 2, and 1 : 4) in a flat-bottomed plate. In accordance with the instructions of the BD FastImmune Human Regulatory T Cell Function Kit (BD Biosciences), cells were activated with an appropriate amount of CD3/CD28 beads (Dynabeads Human T Activator CD3/CD28; Invitrogen, Carlsbad, CA, USA) and incubated with CD154-APC antibodies. After 7 h of incubation at 37°C and 5–7% CO_2, cells were additionally stained with CD69-PE-Cy7, CD4-FITC, CD25-PE, and CD3-PerCP-Cy5.5 (BD FastImmune Human Regulatory T Cell Function Kit; BD Biosciences). Hence, CD25 served to discriminate Tregs from Teffs in this assay, since the molecule is constitutively expressed on the former but not yet induced within the 7 h activation period in the latter.

Samples were measured by flow cytometry on a BD LSR II (BD Biosciences) in accordance with the company's advice. T cell activation was evaluated on two T effector cell (Teff) populations, on $CD25^-CD3^+CD4^+$ T helper cells and on $CD25^-CD3^+CD4^-$ cells, which are mainly $CD8^+$ T cells and are therefore referred to as cytotoxic T cells within this paper. The suppressive capacity of the added $CD4^+CD25^{+high}CD127^{-/dim}$ Tregs was determined as inhibition of CD69 and CD154 expression on the Teff populations.

2.2. Animal Studies

2.2.1. Animals. All animal experiments were approved by the local government authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF), Mecklenburg-Vorpommern). DEREG (Depletion of regulatory T cells) mice were bred in our animal facility (Zentrale Service- und Forschungseinrichtung für Versuchstiere (ZSFV), Greifswald). DEREG mice carry a DTR-eGFP (DTR: diphtheria toxin receptor, GFP: green fluorescent protein) transgene under the control of an additional FoxP3 promoter allowing Treg depletion by low dose diphtheria toxin injection [25]. Here, we only took advantage of the eGFP expression of Treg for the identification of the subset and did not deplete Treg cells.

2.2.2. Ischemia Model. Male undepleted DEREG mice of different ages (8–46 weeks) underwent left MCAO using the filament model. Anesthesia was induced at 2.5% isoflurane with 70% N_2O/30%O_2 and maintained at 2% isoflurane with 70% N_2O/30%O_2 during surgery. Body temperature was measured with a rectal probe and maintained using a feedback-controlled heating pad for a body temperature of 37°C ± 0.5°C. Briefly, the common carotid artery and the external carotid artery were dissected and ligated. A silicon-coated filament (Doccol Corporation, MA, USA) was introduced into the common carotid artery and advanced into the internal carotid artery until the origin of the middle cerebral artery. The surgery time for ischemia induction did not exceed 15 minutes. The filament was withdrawn after 45 min occlusion time. Body weight and body temperature were measured according to protocol.

2.2.3. Mouse MRI. For 7T-animal MRI (ClinScan, Bruker Biospin, Ettlingen, Germany) mice were anesthetized with 1-2% isoflurane and 1 L/min oxygen. During brain scans respiration was monitored and animals were kept warm using an external water bath. For brain scans at day 1 after MCAO a 3D-T2 weighted imaging (mouse brain coil, TR = 2000 ms, TE = 37 ms, FoV 19 × 25 mm, thickness 0.45 mm) and additional diffusion weighted imaging for visualization of the acute infarct were performed. For evaluation of T2 lesion volume of the brain MRI data were analyzed by two independent investigators with respect to lesion location and size. Regions of interest were selected manually and the volume was calculated semiautomatically using OsiriX software.

2.2.4. Flow Cytometry of CD39 Expression on Murine Treg. CD39 expression was determined on FoxP3 expressing Treg of naive (8–48 weeks old) and stroked undepleted DEREG mice. Transient MCAO was described before. Similar to naive mice, on d3 following stroke blood was withdrawn directly from the heart and anticoagulated with EDTA. Spleens were then collected after transcardial perfusion with ice-cold 0.9% saline in deeply anesthetized mice. After homogenization and hypotonic lysis of red blood cells, the single cell suspension was used for flow cytometric analysis of CD39 expression on $CD4^+$ $FoxP3^+$ Treg. Unwanted Fc receptor staining was blocked by initial incubation of cell suspensions with TruStainFcX13 (anti-mouse CD16/32, BioLegend, San Diego, CA, USA). Tregs were identified by anti-CD4-Brilliant Violet 605 antibody (BioLegend, San Diego, CA, USA) and the transgenic expression of eGFP under control of the FoxP3 promotor. CD39 surface expression was determined on $CD4^+FoxP3^+$ lymphocytes by staining of CD39 with an anti-CD39-PE antibody (BioLegend) and its isotype control IgG2a PE (BioLegend, San Diego, CA, USA). Flow cytometry was performed on a Becton Dickinson LSRII. Data were analyzed using FlowJo (Tree Star Inc., OR, USA).

2.3. Statistical Analysis. All datasets were tested for deviations from Gaussian distribution with the Kolmogorov-Smirnov test. Data that passed the test were analyzed by repeated-measures analysis of variance (ANOVA), with Bonferroni's multiple-comparison test as a posttest. Since some of the data in each *in vitro* experiment failed the normality test, we used nonparametric testing throughout. The Kruskal-Wallis test, with Dunn's multiple-comparison test as a posttest, was used as appropriate. Posttests were only performed if the initial testing revealed significant differences between the groups. Correlations were determined by Pearson correlation analysis. Tests were performed with 95% confidence intervals (two tailed). All analyses were carried out with the software GraphPad-PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA). A p value of <0.05 was regarded as significant.

3. Results

3.1. CD39 Expression on Tregs Increases with Age. Since it is not known how age affects the CD39 subset of Tregs we determined the age dependent expression of CD39 on $CD49^-FoxP3^+$ Tregs. In our population the percentage of Tregs of $CD4^+$ T cells did not change significantly, while the percentage of CD45RA expressing naive Tregs declined with age ($r = -0.8422$; $p < 0.0001$) (Supplemental Figure S1) (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/2974605). CD39 expression increased with age ($r = 0.6612$; $p < 0.0001$) (Figure 1(a)).

3.2. Tregs Are Reduced in the Peripheral Blood of Stroke Patients. Flow cytometric analysis confirmed the well-described loss of lymphocytes in the peripheral blood of stroke patients, which was highly significant on all days ($p = 0.0035$). The proportion of $CD4^+$ T cells to total lymphocytes was not significantly altered in this stroke patient cohort, indicating that $CD4^+$ T cells are lost in a similar quantity from peripheral blood as $CD4^-$ lymphocytes (Figure 2). Total Tregs, which accounted for 5.0% (median) (range 1.3–10.2%) of $CD4^+$ T cells in healthy controls, were reduced in stroke patients to 2.8% (median) (range 0.03–8.1%) on admission and remained below control values until day 7 ($p = 0.0095$). This reduction was due to a loss of $CD39^+$ activated Tregs ($p = 0.0335$), which reached a minimum of 1.2% (median) (range 0.2–7.1%) of all $CD4^+$ T cells on day 5. Naive Tregs, which express CD45RA, remained largely unchanged (Figure 2(c)). In controls, 92.1% median (range

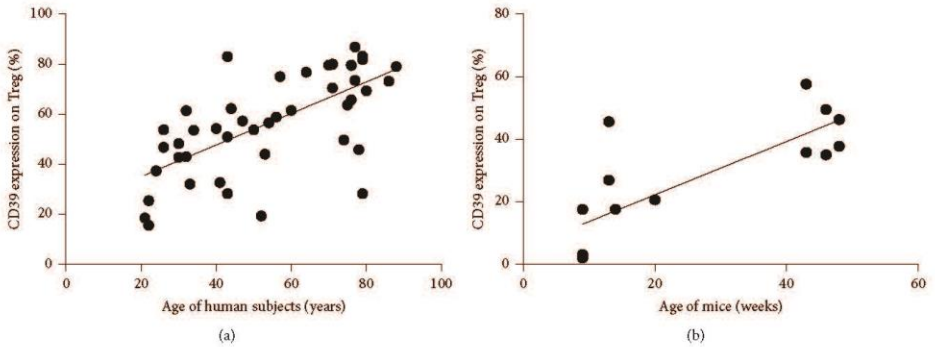


FIGURE 1: CD39 expression on Treg correlates positively with age in man and mice. (a) Human, peripheral blood: the percentage of CD39 expression was determined on $CD4^+CD25^+CD49d^-FoxP3^+$ Treg and correlated to age for a total of 45 healthy controls (age from 21 to 88 years). Pearson $r = 0.6612$, $p < 0.0001$, and R squared = 0.4372. (b) Mice, blood: the percentage of CD39 expression was determined on $CD4^+CD25^+CD49d^-FoxP3^+$ Treg and correlated to age for a total of 15 naive animals (age from 8 to 48 weeks). Pearson $r = 0.7895$, $p < 0.0005$, and R squared = 0.6233.

71.0–98.3%) of Tregs expressed the activation marker CD25. In stroke patients, CD25 expression was not significantly altered ($p = 0.0600$) (Supplemental Figure S2A).

We also assessed the percentage of $CD4^+CD25^+$ cells in stroke patients, as this has been used previously as a marker for Tregs; however, activated T cells also upregulate CD25 on their cell surface. Stroke patients had 30.4% (median) (range 4.2–71.6%) $CD4^+CD25^+$ cells on the day of admission, which did not differ significantly from control values 41.2% (median) (range 5.1–73.7%) ($p = 0.7886$) (Supplemental Figure S2B).

No differences were seen when we compared the Treg populations between sexes. There was no robust correlation between percentage of Tregs and their subpopulations with respect to the neurological deficit or stroke size.

3.3. Age Dependent Regulation of $CD39^+$ Treg in Mice. Using nondepleted DEREG animals the GFP expression of $FoxP3^+$ Treg was used to quantify peripheral blood Treg. In aged mice the proportion of CD39 expressing Treg was increased compared to young adult mice (Figure 1(b)). Induction of cerebral ischemia by transient filament MCAO resulted in similar infarct sizes in young and aged mice (Figure 3(a)). Nevertheless CD39 expression on Treg in SIIA differed between young and aged animals. While there was no effect on CD39 expressing Treg in young mice the age related increase in CD39 expression in peripheral blood was reversed by MCAO in aged mice (Figure 3(b)).

3.4. Impaired Treg Function in Stroke Patients. Treg function was quantified by inhibition of the activation-induced upregulation of CD69 and CD154 on the surface of Teffs. We therefore compared this activation marker expression on T helper cells and cytotoxic T cells derived from stroke patients and controls. Directly *ex vivo* T helper cells from stroke patients expressed more CD69 on their surface ($p = 0.0243$) compared to controls, while CD154 expression did not differ between the groups ($p = 0.4140$) (data not shown). There was no difference in the expression of CD69 or CD154 on cytotoxic T cells from control versus stroke patients (CD69, $p = 0.2238$, and CD154, $p = 0.1449$). Upon CD3/CD28 activation *in vitro*, no differences in CD69 or CD154 expression on the cell surface of either $CD4^+$ T cells or $CD8^+$ T cells were seen between stroke patients and controls (data not shown).

Treg-mediated inhibition of T helper cell activation was impaired in stroke patients. At a ratio of 1:2 and 1:1 Tregs : PBMCs, the inhibition of CD154 expression on $CD4^+$ effector cells was reduced in stroke patients compared to healthy controls ($p < 0.0001$) (Figure 4(b)). Inhibition of the early-activation marker CD69 on T helper cells remained unaltered (Figure 4(a)). The effect of Tregs on cytotoxic T cells was not altered in stroke patients (Figures 4(c) and 4(d)).

Since Yan et al. detected no changes in Treg in a cohort of stroke patients that remained free of infection throughout the hospital stay, we reanalyzed our data according to infection status [19, 26]. As shown in Figure 5 the effect of stroke on total Treg and subsets was more pronounced in those patients that went on to develop SAI during their hospitalization.

4. Discussion

In this study, we found decreased numbers and impaired function of Tregs in the peripheral blood of stroke patients. The decrease in Tregs following stroke was detectable upon admission and remained statistically significant through day 5. This reduction in Tregs was not evenly distributed among all $FoxP3^+$ Treg subsets but was most pronounced in the active $CD39^+$ Treg population.

The reduced efficacy of Treg-mediated Teff suppression in stroke patients is in agreement with reports by Yan et al.,

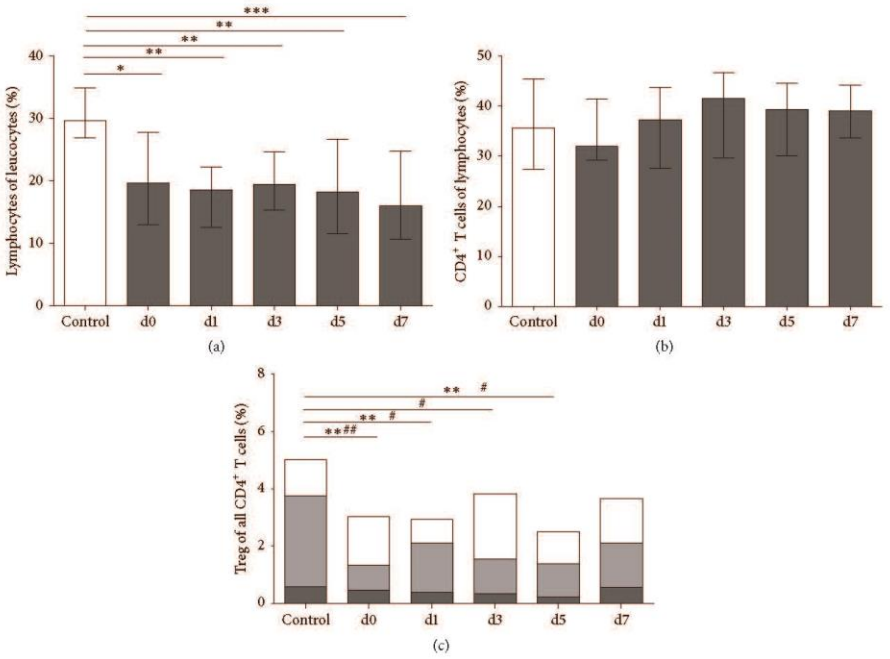


FIGURE 2: T helper cells and Tregs and their subpopulations. Cytometric analysis of nonstroke controls (white bars) and stroke patients (dark grey bars) was performed for (a) lymphocytes ($n_{control,d0,1,3,5,7}$ = 15, 35, 35, 35, 32, 30, resp.), shown as the percentage of leukocytes. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. (b) $CD4^{+}$ T helper cells ($n_{control,d0,1,3,5,7}$ = 15, 35, 35, 35, 32, 29, resp.), shown as the percentage of lymphocytes. (c) Total Tregs ($n_{control,d0,1,3,5,7}$ = 15, 35, 35, 34, 32, 27, resp.) combined with their subpopulations. Naive Tregs ($n_{control,d0,1,3,5,7}$ = 15, 34, 35, 33, 31, 27, resp.) are shown by dark grey bars and activated Tregs ($n_{control,d0,1,3,5,7}$ = 14, 35, 34, 33, 32, 27, resp.) by light grey bars. Patient samples were obtained on admission (d0), the next morning (d1), and on days 3, 5, and 7 (d3, d5, and d7, resp.). $^{**}p < 0.01$ for Tregs in stroke patients versus controls; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ for activated Tregs in patients versus controls. Medians and interquartile ranges (a, b) are provided.

who found a similar suppression using proliferation as a read-out [19, 26]. However, a study by Hug et al. was unable to detect altered Treg function in stroke patients [5]. In this previous study, no dose-response curve was obtained and the suppression measurable using Tregs and Teffs of control individuals was only 10%. Therefore, the conditions chosen by Hug et al. for the assay may not have been sufficiently sensitive to detect impaired Treg function. We applied an assay that is based on activation marker expression on T responder cells rather than proliferation. The validity of inhibition of CD154 expression on Teff as a marker for Treg suppressive activity has been demonstrated [27]. This approach enabled us to distinguish between the effect of Treg on $CD4^{+}$ and $CD4^{-}$ Teff.

Since all three studies used autologous Teff cells as responder cells, an enhanced resistance of Teff to Treg-mediated inhibition cannot be excluded. We observed a predominant loss of CD39 expressing Tregs, which represents the functionally active Treg subset, with a concomitant shift in Treg composition toward the naive subset. It is therefore plausible that Treg function rather than Teff susceptibility is impaired in stroke patients.

Our data corroborate recent observations by Li et al., who detected a reduced percentage and suppressive activity of Tregs in stroke patients, and extend their findings by demonstrating that functionally active $CD39^{+}$ Tregs are predominantly reduced following stroke [28]. Furthermore, to our knowledge, this is the first study to address the inhibition of activation of cytotoxic Teffs in stroke patients. In contrast to the impaired inhibition of T helper cells, reduction of cytotoxic Teff activation was not altered in stroke patients.

Our quantitative findings, however, are in contrast to two reports by Yan et al., who detected an increase in $FoxP3^{+}$ Tregs following stroke [19, 26]. These differences may be related to the stroke population recruited; in our study all patients had MCA ischemia and an NIHSS ≥6, whereas Yan et al. recruited all ischemic stroke patients, including those who were less severely affected. More importantly, Yan et

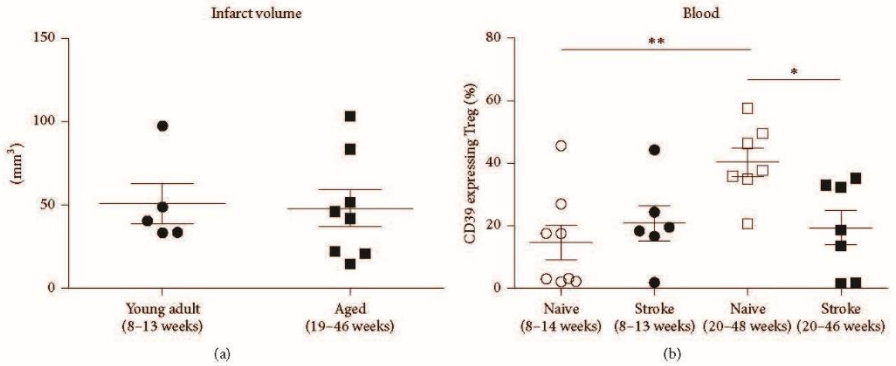


FIGURE 3: Age dependency of infarct volume and CD39 expression on Treg after experimental stroke in mice. (a) The infarct volume as assessed by MRI in young adult and aged mice was compared on day 1 after transient middle cerebral artery occlusion. (b) The CD39 expression on $CD4^+CD25^+CD49d^-FoxP3^+$ Treg in blood was compared between naive and stroked young adult and naive and stroked aged mice on day 3 after transient middle cerebral artery occlusion. $^*p < 0.05$, $^{**}p < 0.01$. Means and SEM are provided.

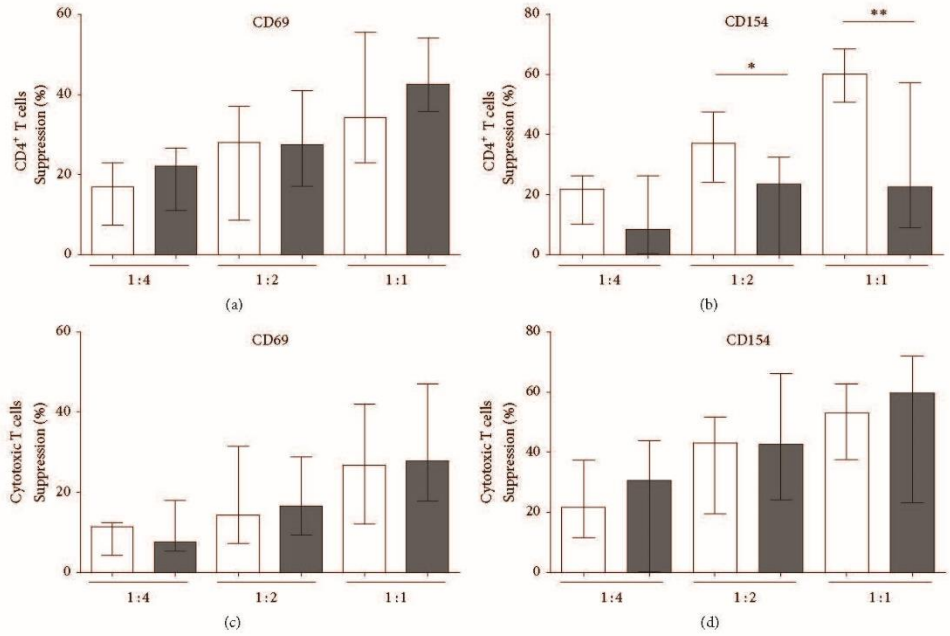


FIGURE 4: Suppressive activity of Tregs. Treg function was evaluated by measuring the $CD4^+CD25^+CD127^{dim/-}$ Treg-mediated inhibition of CD69 (a, c) and CD154 (b, d) induction on T effector cells (Teff) after anti-CD3/anti-CD28 stimulation. Suppression is shown for T helper cells (a, b) and cytotoxic T cells (c, d) in control subjects (white bars) versus stroke patients (dark grey bars) on day 3 with different Treg : PBMC concentrations (Treg : PBMC = 1 : 4; 1 : 2; and 1 : 1). $n_{\text{control,stroke on day 3}} = 11$ and 10, respectively. $^*p < 0.05$ and $^{**}p < 0.01$ for stroke patients versus controls. Medians and interquartile ranges are provided.

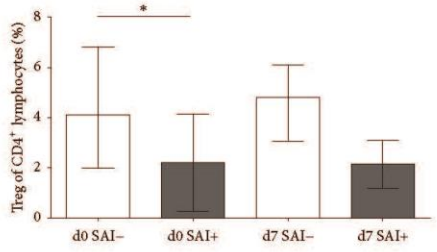


FIGURE 5: Comparison of SAI− and SAI+ patients. On the day of admission (d0) and day 7 after stroke (d7) the percentage of Treg of $CD4^+$ T helper cells was compared between patients without poststroke infections SAI− (white bars; $n_{d0,d7} = 14, 12$, resp.) and patients with poststroke infections SAI+ (grey bars; $n_{d0,d7} = 7, 7$, resp.). $^*p < 0.05$. Means and SD are provided.

al. excluded patients with "acute infections after stroke." Since SAIs are more likely to occur in patients with stroke-induced immunosuppression [2], this approach is likely to exclude patients with the most severe stroke-induced immune alterations. In our study, we also excluded patients with signs of infection on admission; however, patients with subsequent SAIs were not excluded. To evaluate whether this could account for the apparent contradiction, we performed a subanalysis comparing patients with and without subsequent infections. While failing to reach statistical significance, Tregs appeared to be more strongly reduced in patients with SAI compared to those without SAI. This supports our hypothesis that the differences in patient populations may account for the seemingly contradictory findings between our findings and the data reported by Yan et al.

The major limitation of the study is the fact that our analysis is restricted to the peripheral blood as other immune compartments are not readily accessible in patients. Therefore we cannot exclude that the active Tregs have migrated into the tissues. Our animal data indicate that aged mice could provide a suitable model to determine the fate of Treg subsets following stroke. Immunosenescence has been shown to alter the clinical course of diseases and also affect Treg subsets [29–31]. We extend this knowledge by demonstrating that the active Treg population circulating in the peripheral blood increases with age. The role of Treg in regulating SIIA remains disputed and differs between the experimental models [14–18]. Our data suggest that Tregs are regulated differently during immunosenescence, an aspect rarely reflected in current experimental stroke models.

5. Conclusion

Our data demonstrate that $CD4^+CD49^-Foxp3^+$ Tregs are reduced in the peripheral blood of stroke patients. Among those the active $CD39^+$ Treg subset is the most affected, a finding mirrored in functional studies demonstrating impaired suppressive activity of stroke patient derived Treg *in vitro*. Whether these alterations contribute to secondary immune-mediated brain damage or whether reduced Treg function is beneficial in stroke remains to be investigated. The observation that the proportion of Treg expressing CD39 increases with age and is differentially regulated in young adult and aged mice highlights the importance to consider immunosenescence in the design of experimental stroke models.

Disclosure

The funders had no role in the study design, data collection and the analysis, decision to publish, or preparation of the paper.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Johanna Ruhnau and Juliane Schulze have contributed equally. Antje Vogelgesang and Alexander Dressel have contributed equally.

Acknowledgments

Antje Vogelgesang and Alexander Dressel received funding from the EU grant EnVision (FP7-REGPOT-2010, Grant no. 264143). Johanna Ruhnau and Marie Heinrich were funded by the Gerhard Domagk program of the University Medicine Greifswald. Juliane Schulze was funded by the DFG Graduate School GRK840/1-03. The authors thank Dr. Stefan Clemens, Department of Ophthalmology, University Medicine Greifswald, for providing access to patients scheduled to receive cataract surgery. They also thank Dr. Jens Kühn, Department of Experimental Radiology, University Medicine Greifswald, for support in the animal MRI facility. DEREG mice breeding pairs were kindly provided by Jochen Hühn, Department of Experimental Immunology, Helmholtz Centre for Infection Research, Braunschweig, and Tim Sparwasser, Institute of Infection Immunology, TWINCORE, Centre for Experimental and Clinical Infection Research, a joint venture between the Medical School Hannover and the Helmholtz Centre for Infection Research, Hannover.

References

[1] K. Prass, C. Meisel, C. Höflich et al., "Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation," *The Journal of Experimental Medicine*, vol. 198, no. 5, pp. 725–736, 2003.

[2] A. Vogelgesang and A. Dressel, "Immunological consequences of ischemic stroke: immunosuppression and autoimmunity," *Journal of Neuroimmunology*, vol. 231, no. 1-2, pp. 105–110, 2011.

[3] A. Vogelgesang, U. Grunwald, S. Langner et al., "Analysis of lymphocyte subsets in patients with stroke and their influence on infection after stroke," *Stroke*, vol. 39, no. 1, pp. 237–241, 2007.

[4] A. Chamorro, S. Amaro, M. Vargas et al., "Catecholamines, infection, and death in acute ischemic stroke," *Journal of the Neurological Sciences*, vol. 252, no. 1, pp. 29–35, 2007.

[5] A. Hug, A. Liesz, B. Muerle et al., "Reduced efficacy of circulating costimulatory cells after focal cerebral ischemia," *Stroke*, vol. 42, no. 12, pp. 3580–3586, 2011.

[6] T. Stubbe, F. Ebner, D. Richter et al., "Regulatory T cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO," *Journal of Cerebral Blood Flow and Metabolism*, vol. 33, no. 1, pp. 37–47, 2013.

[7] G. Yilmaz and D. N. Granger, "Leukocyte Recruitment and Ischemic Brain Injury," *NeuroMolecular Medicine*, vol. 12, no. 2, pp. 193–204, 2010.

[8] G. Enzmann, C. Mysiorek, R. Gorina et al., "The neurovascular unit as a selective barrier to polymorphonuclear granulocyte (PMN) infiltration into the brain after ischemic injury," *Acta Neuropathologica*, vol. 125, no. 3, pp. 395–412, 2013.

[9] S. Fumagalli, J. A. Coles, P. Ejlerskov et al., "In vivo real-time multiphoton imaging of T lymphocytes in the mouse brain after experimental stroke," *Stroke*, vol. 42, no. 5, pp. 1429–1436, 2011.

[10] F. Ortolano, P. Maffia, G. Dever et al., "Advances in imaging of new targets for pharmacological intervention in stroke: real-time tracking of T-cells in the ischaemic brain," *British Journal of Pharmacology*, vol. 159, no. 4, pp. 808–811, 2010.

[11] K. J. Becker, D. L. Kindrick, M. P. Lester, C. Shea, and Z. C. Ye, "Sensitization to brain antigens after stroke is augmented by lipopolysaccharide," *Journal of Cerebral Blood Flow and Metabolism*, vol. 25, no. 12, pp. 1634–1644, 2005.

[12] J. M. Gee, A. Kalil, M. Thullbery, and K. J. Becker, "Induction of immunologic tolerance to myelin basic protein prevents central nervous system autoimmunity and improves outcome after stroke," *Stroke*, vol. 39, no. 5, pp. 1575–1582, 2008.

[13] S. Sakaguchi, M. Miyara, C. M. Costantino, and D. A. Hafler, "FOXP3+ regulatory T cells in the human immune system," *Nature Reviews Immunology*, vol. 10, no. 7, pp. 490–500, 2010.

[14] A. Liesz, E. Suri-Payer, C. Veltkamp et al., "Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke," *Nature Medicine*, vol. 15, no. 2, pp. 192–199, 2009.

[15] C. Kleinschnitz, P. Kraft, A. Dreykluft et al., "Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature," *Blood*, vol. 121, no. 4, pp. 679–691, 2013.

[16] X. Ren, K. Akiyoshi, A. A. Vandenbark, P. D. Hurn, and H. Offner, "$CD4^+FoxP3^+$ regulatory T-cells in cerebral ischemic stroke," *Metabolic Brain Disease*, vol. 26, no. 1, pp. 87–90, 2011.

[17] A. Liesz, W. Zhou, S.-Y. Na et al., "Boosting regulatory T cells limits neuroinflammation in permanent cortical stroke," *The Journal of Neuroscience*, vol. 33, no. 44, pp. 17350–17362, 2013.

[18] M. K. Schuhmann, P. Kraft, G. Stoll et al., "CD28 superagonist-mediated boost of regulatory T cells increases thrombo-inflammation and ischemic neurodegeneration during the acute phase of experimental stroke," *Journal of Cerebral Blood Flow and Metabolism*, vol. 35, no. 1, pp. 6–10, 2015.

[19] J. Yan, S. J. Read, R. D. Henderson et al., "Frequency and function of regulatory T cells after ischaemic stroke in humans," *Journal of Neuroimmunology*, vol. 243, no. 1-2, pp. 89–94, 2012.

[20] M. Miyara and S. Sakaguchi, "Human FoxP3+ CD4+ regulatory T cells: their knowns and unknowns," *Immunology and Cell Biology*, vol. 89, no. 3, pp. 346–351, 2011.

[21] S. Gallucci, M. Lolkema, and P. Matzinger, "Natural adjuvants: endogenous activators of dendritic cells," *Nature Medicine*, vol. 5, no. 11, pp. 1249–1255, 1999.

[22] P. Matzinger, "The danger model: a renewed sense of self," *Science*, vol. 296, no. 5566, pp. 301–305, 2002.

[23] G. Borsellino, M. Kleinewietfeld, D. Di Mitri et al., "Expression of ectonucleotidase CD39 by $Foxp3^+$ Treg cells: hydrolysis of extracellular ATP and immune suppression," *Blood*, vol. 110, no. 4, pp. 1225–1232, 2007.

[24] M. Mandapathil, S. Lang, E. Gorelik, and T. L. Whiteside, "Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression," *Journal of Immunological Methods*, vol. 346, no. 1-2, pp. 55–63, 2009.

[25] K. Lahl, C. Loddenkemper, C. Drouin et al., "Selective depletion of $Foxp3^+$ regulatory T cells induces a scurfy-like disease," *Journal of Experimental Medicine*, vol. 204, no. 1, pp. 57–63, 2007.

[26] J. Yan, J. M. Greer, K. Etherington et al., "Immune activation in the peripheral blood of patients with acute ischemic stroke," *Journal of Neuroimmunology*, vol. 206, no. 1-2, pp. 112–117, 2009.

[27] D. Hill, N. Eastaff-Leung, S. Bresatz-Atkins et al., "Inhibition of activation induced CD154 on $CD4^+$ $CD25^-$ cells: a valid surrogate for human Treg suppressor function," *Immunology and Cell Biology*, vol. 90, no. 8, pp. 812–821, 2012.

[28] Q. Li, Y. Wang, F. Yu et al., "Peripheral Th17/Treg imbalance in patients with atherosclerotic cerebral infarction," *International Journal of Clinical and Experimental Pathology*, vol. 6, no. 6, pp. 1015–1027, 2013.

[29] A. Jagger, Y. Shimojima, J. J. Goronzy, and C. M. Weyand, "Regulatory T cells and the immune aging process: a mini-review," *Gerontology*, vol. 60, no. 2, pp. 130–137, 2014.

[30] M. A. Murray and S. H. Chotirmall, "The impact of immunosenescence on pulmonary disease," *Mediators of Inflammation*, vol. 2015, Article ID 692546, 10 pages, 2015.

[31] A. Pera, C. Campos, N. López et al., "Immunosenescence: implications for response to infection and vaccination in older people," *Maturitas*, vol. 82, no. 1, pp. 50–55, 2015.

2.5 Stroke, IL-1ra, *IL1RN*, Infection and Outcome

neurocritical care society Neurocrit Care (2014) 21:140–146
DOI 10.1007/s12028-013-9899-x

ORIGINAL ARTICLE

Stroke, IL-1ra, *IL1RN*, Infection and Outcome

Kyra J. Becker · Dorender Dankwa · Richard Lee · Juliane Schulze · Dannielle Zierath · Patricia Tanzi · Kevin Cain · Alexander Dressel · Dean Shibata · Jonathan Weinstein

Published online: 14 November 2013

Abstract
Background Infection is a common phenomenon following stroke, and adversely affects outcome. Previous studies suggest that interleukin-1 receptor antagonist (IL-1ra) and single nucleotide polymorphisms (SNPs) in the *IL1RN* gene might influence the risk of post-stroke infection and outcome. In this study, we addressed the effects of the rs4251961 SNP in *IL1RN* on infection risk and outcome.
Methods Subjects with acute ischemic stroke were enrolled within 72 h of symptom onset and followed up to 1 year. Plasma IL-1ra was measured at multiple time points and outcome assessed at 1, 3, 6, and 12 months. Active surveillance for infection occurred while subjects were hospitalized. Subjects were genotyped for the *IL1RN* rs4251961 polymorphism.
Results In the population of 113 subjects for this study, those with the minor C allele of rs4251961 polymorphism in *IL1RN* were more likely to be Caucasian, hypertensive, and to be afflicted with coronary heart disease. Higher plasma IL-1ra was associated with an increased risk of infection (other than pneumonia), and the minor C allele of rs4251961 was independently associated with a decreased risk of infection (other than pneumonia). Initial plasma IL-1ra was not predictive of long-term outcome, but patients with the minor C allele of rs4251961 were more likely to experience good (modified Rankin Score <2) long-term outcome.
Conclusions These data indicate that IL-1ra and *IL1RN* may influence the risk of infection after stroke, but this influence seems limited to infections other than pneumonia. Further studies are needed to better understand the complexities of immune regulation on infection and outcome after stroke.

Keywords IL-1ra · IL1RN · Stroke · Infection · Outcome

K. J. Becker (✉) · D. Dankwa · R. Lee · D. Zierath · P. Tanzi · J. Weinstein
Department of Neurology, University of Washington School of Medicine, HMC 325 9th Ave, Box 359775, Seattle, WA 98104, USA
e-mail: kjb@u.washington.edu

J. Schulze · A. Dressel
Department of Neurology, University of Greifswald, Greifswald, Germany

K. Cain
Department of Biostatistics, University of Washington School of Medicine, Seattle, WA, USA

D. Shibata
Department of Radiology, University of Washington School of Medicine, Seattle, WA, USA

Introduction

Interleukin-1 receptor antagonist (IL-1ra) is an endogenous immunomodulatory cytokine encoded by *IL1RN* on chromosome two that inhibits the actions of IL-1α and IL-1β [1, 2]. IL-1ra is secreted by cells of the immune system as well as a variety of other cell types; it is also considered to be an acute phase reactant and is secreted by the liver [3]. In animal studies, exogenous administration of IL-1ra or overexpression of IL-1ra improves outcome from experimental stroke [4]. In a clinical study, we found that elevated plasma IL-1ra early after stroke independently predicted infection within the first 15 days after stroke [5]. Importantly, infection is an independent predictor of poor

outcome after stroke [6, 7]. Polymorphisms in *IL1RN* that affect the production of IL-1ra thus have the potential to influence infection risk and stroke outcome. The minor *IL1RN**2* allele of the variable number tandem repeat (VNTR) of *IL1RN*, for instance, is reported to be associated with increased IL-1ra production in most studies [8–11]. There are some studies, however, which have noted that *IL1RN**2* is associated with decreased IL-1ra production, increased inflammation and inflammatory diseases, as well as risk of cancer [12–17]. These observations suggest that the genetic regulation of IL-1ra and inflammation is thus complex and likely context dependent.

In a study of 391patients with ischemic stroke, those who were homozygous for *IL1RN**2* had better neurological outcomes, but the allele was associated with an increased risk of early death [18]. The authors hypothesized that *IL1RN**2* was associated with increased IL-1ra (although IL-1ra was not assessed), and IL-1ra is neuroprotective experimental stroke. Further, the increase in early death was attributed to infection, although no infections were explicitly tracked. The minor C allele of the single nucleotide polymorphism (SNP) rs4251961 SNP in *IL1RN* is associated with lower concentrations of plasma IL-1ra, and increased IL-1β and C-reactive protein (CRP) [19, 20]. In this study, we sought to determine whether the plasma IL-1ra concentrations after stroke were predictive of outcome and whether the rs4251961 polymorphism in *IL1RN* influenced the risk of post-stroke infection risk or stroke outcome.

Materials and Methods

Research Subjects

Patients with ischemic stroke admitted to Harborview Medical Center from 9/2005 to 5/2009 who were at least 18 years of age were enrolled within 72 h of symptom onset. Individuals with ongoing therapy for malignancy, known history of HIV, hepatitis B or C, history of brain tumor, anemia (hematocrit <35 on admission), and those taking immunomodulatory medications were excluded. Blood was drawn as soon as possible after stroke onset and at 3, 7, 30, 90, 180 and 365 days. Blood was also drawn from 40 volunteers to determine normative data for IL-1ra. All aspects of this study were approved by the University of Washington Institutional Review Board; subjects with stroke or their surrogates as well as control subjects provided informed consent.

Clinical Data

Clinical and demographic data were collected on all subjects with stroke. Stroke severity was determined by the National Institutes of Health Stroke Scale (NIHSS) score and outcome by the modified Rankin Scale (mRS). There was active ascertainment of infection in patients. Infection was defined as clinical symptoms of an infection (including fever as well as pyuria for urinary tract infection [UTI], productive cough, and radiographic evidence of consolidation

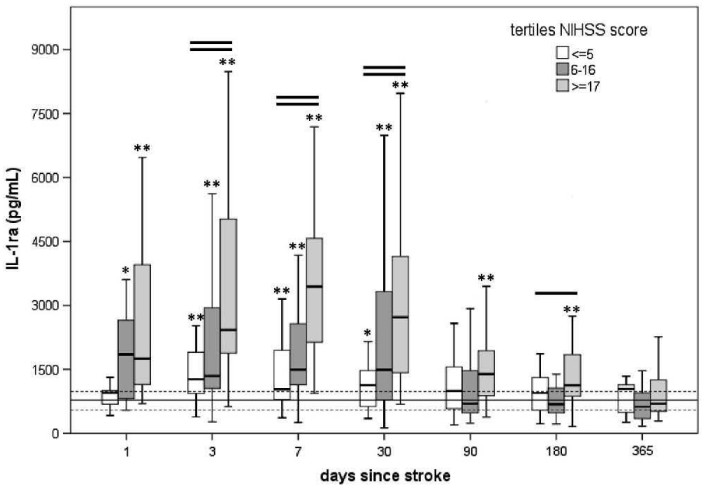


Fig. 1 Changes in plasma IL-1ra over time as a function of stroke severity. Values are depicted as the median and IQA. The *solid horizontal line* represents the median value for the control population; the *dashed lines* represent the IQA. One *horizontal line* over the *box plots* at a given time point indicates that the groups differ from each other at $P < 0.05$; *two horizontal lines* indicate that the groups differ from each other at $P < 0.01$ (Kruskal–Wallis H test). An *asterisk* (*) indicates that the group differs from controls at $P < 0.05$; ** indicates that the group differs from controls at $P < 0.01$ (Mann–Whitney U test)

for pneumonia [PNA]) and positive culture data). The date of infection onset was considered to be the date of symptom onset. Antibiotic therapy (ABX) was used as appropriate to treat infections (prophylactic ABX therapy was not used). MRI was done as part of usual clinical care (generally within 24 h of admission). Total infarct volume on diffusion weighted MRI imaging was calculated by the ABC/2 method [21]. Outcome was assessed in person at 1, 3, 6, and 12 months after stroke.

Laboratory Studies

Plasma was frozen at −80 °C within an hour after blood draw; the concentration of circulating IL-1ra was measured using a cytometric bead-based system (Fluorokine MAP®; R&D Systems). The sensitivity of the assay was 2.23 pg/mL.

IL1RN SNP Genotyping

DNA was extracted from blood plasma samples using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) per manufacturer's protocols. Genotyping for the *IL1RN* rs4251961 was carried out using TaqMan SNP Genotyping Assay Sets and Master Mix (Applied Biosystems, Carlsbad, CA). In brief, 2 ng of sample DNA was genotyped per manufacturer's protocols on StepOnePlus™ Real-Time PCR System (Applied Biosystems) under the following cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. An allelic discrimination plot was then generated using StepOne Software v2.0 (Applied Biosystems). All samples were processed in triplicate. The reproducibility of the plasma-based PCR genotyping method was confirmed by carrying out identical PCR-based genotyping on DNA extracted from isolated leukocytes in a subset ($N = 42$) of subjects. In these 42 subjects there was 100 % concordance between the plasma-based and leukocyte-based samples.

Statistics

Descriptive data for continuous variables are presented as mean and standard deviation (SD) or median and interquartile range (IQR). Data for categorical variables are presented as percentages. Group comparisons are performed using analysis of variance (ANOVA) and the t-tests for parametric data, and the Kruskal–Wallis H test or Mann–Whitney U test for nonparametric data. Categorical data are compared using the χ^2 test statistic. Logistic regression was used to estimate the odds ratio (OR), and 95 % confidence interval (CI) for the effect of plasma IL-1ra and the rs4251961 SNP in *IL1RN* on infection risk and

Table 1 Characteristics of patients with and without the minor C allele of the rs4251961 SNP in *IL1RN*

	TT 55/113 49 %	TC 41/113 36 %	CC 17/113 15 %	*P*	TT 55/113 49 %	TC or CC 58/113 51 %	*P*
Patient characteristics							
Age	55 (57, 67)	54 (45, 65)	65 (52, 71)	NS	55 (57, 67)	56 (46, 66)	NS
Caucasian	46/55 (84 %)	39/41 (95 %)	17/17 (100 %)	0.06	46/55 (84 %)	56/58 (97 %)	0.02
Gender (female)	16/55 (29 %)	15/41 (37 %)	7/17 (41 %)	NS	16/55 (29 %)	22/58 (38 %)	NS
Prior stroke	18/55 (27 %)	15/41 (37 %)	7/17 (41 %)	NS	18/55 (27 %)	22/58 (38 %)	NS
Stroke characteristics							
NIHSS	10 (3, 18)	12 (4, 20)	10 (4, 22)	NS	10 (3, 18)	12 (5, 20)	NS
Infarct volume (cc)	6.1 (0.5, 64.2)	19.8 (4.3, 147.9)	14.6 (2.5, 47.7)	NS	6.1 (0.5, 64.2)	18.6 (3.5, 129.3)	0.11
Stroke risk factors							
AF	9/55 (16 %)	4/41 (10 %)	3/17 (18 %)	NS	9/55 (16 %)	7/58 (12 %)	NS
CHD	8/55 (14 %)	12/41 (29 %)	7/17 (41 %)	0.05	8/55 (14 %)	19/58 (33 %)	0.02
DM	11/55 (20 %)	8/41 (20 %)	8/17 (47 %)	0.05	11/55 (20 %)	16/58 (28 %)	NS
HTN	23/55 (42 %)	24/41 (58 %)	13/17 (76 %)	0.03	23/55 (42 %)	37/58 (64 %)	0.02
Smoker	23/55 (42 %)	15/41 (37 %)	4/17 (24 %)	NS	23/55 (42 %)	19/58 (33 %)	NS
Infection by day 15[a]							
Any	14/55 (25 %)	11/40 (28 %)	3/16 (19 %)	NS	14/55 (25 %)	14/56 (25 %)	NS
PNA	2/55 (4 %)	5/40 (12 %)	2/16 (12 %)	NS	2/55 (4 %)	7/56 (12 %)	0.09
Any but PNA	12/55 (22 %)	7/40 (18 %)	1/16 (6 %)	NS	12/55 (22 %)	8/56 (14 %)	NS

NIHSS National Institutes of Health Stroke Scale, *AF* atrial fibrillation, *CHD* coronary heart disease, *DM* diabetes mellitus, *HTN* hypertension, *PNA* pneumonia

[a] Some patients had more than one infection, which is the reason that the number of PNAs and other infections exceeds any infection; *NS* not significant and indicates $P \geq 0.200$

Table 2 Risk of infection by day 15 based on plasma IL-1ra concentration at 72 h after stroke onset

Infection by day 15	Any infection		PNA		Any infection but PNA	
	OR (95 % CI)	*P*	OR (95 % CI)	*P*	OR (95 % CI)	*P*
All patients	*N* = 28		*N* = 9		*N* = 20	
IL-1ra (per 1,000 pg/mL)	1.25 (1.06–1.48)	0.01	1.07 (0.92–1.24)	NS	1.21 (1.05–1.39)	0.01
IL-1ra (per 1,000 pg/mL) + NIHSS	1.18 (1.02–1.38)	0.03	0.97 (0.79–1.19)	NS	1.17 (1.02–1.34)	0.03
Excluding patients with infection to day 1	*N* = 27		*N* = 9		*N* = 19	
IL-1ra (per 1,000 pg/mL)	1.22 (1.03–1.45)	0.02	1.08 (0.93–1.25)	NS	1.18 (1.03–1.36)	0.02
IL-1ra (per 1,000 pg/mL) + NIHSS	1.17 (1.00–1.36)	0.04	0.98 (0.80–1.22)	NS	1.15 (1.00–1.32)	0.05
Excluding patients with infection to day 2	*N* = 23		*N* = 9		*N* = 15	
IL-1ra (per 1,000 pg/mL)	1.26 (1.05–1.51)	0.01	1.08 (0.93–1.25)	NS	1.23 (1.05–1.41)	0.01
IL-1ra (per 1,000 pg/mL) + NIHSS	1.22 (1.04–1.45)	0.02	0.98 (0.79–1.21)	NS	1.19 (1.02–1.39)	0.02
Excluding patients with infection to day 3	*N* = 22		*N* = 8		*N* = 14	
IL-1ra (per 1,000 pg/mL)	1.25 (1.05–1.49)	0.01	1.07 (0.92–1.25)	NS	1.21 (1.05–1.40)	0.01
IL-1ra (per 1,000 pg/mL) + NIHSS	1.22 (1.04–1.44)	0.02	0.97 (0.78–1.21)	NS	1.19 (1.02–1.38)	0.02
Excluding patients with infection to day 4	*N* = 20		*N* = 6		*N* = 14	
IL-1ra (per 1,000 pg/mL)	1.22 (1.03–1.43)	0.02	1.03 (0.84–1.26)	NS	1.22 (1.05–1.42)	0.01
IL-1ra (per 1,000 pg/mL) + NIHSS	1.20 (1.02–1.42)	0.02	0.89 (0.68–1.18)	NS	1.20 (1.03–1.41)	0.02
Excluding patients with infection to day 5	*N* = 16		*N* = 2		*N* = 14	
IL-1ra (per 1,000 pg/mL)	1.22 (1.04–1.44)	0.01	1.06 (0.80–1.40)	NS	1.22 (1.05–1.42)	0.01
IL-1ra (per 1,000 pg/mL) + NIHSS	1.21 (1.03–1.43)	0.02	0.91 (0.64–1.30)	NS	1.20 (1.02–1.41)	0.02
Excluding patients with infection to day 6	*N* = 16		*N* = 2		*N* = 14	
IL-1ra (per 1,000 pg/mL)	1.22 (1.04–1.44)	0.01	1.06 (0.80–1.40)	NS	1.22 (1.05–1.42)	0.01
IL-1ra (per 1,000 pg/mL) + NIHSS	1.21 (1.03–1.43)	0.02	0.91 (0.64–1.30)	NS	1.20 (1.02–1.41)	0.02
Excluding patients with infection to day 7	*N* = 15		*N* = 2		*N* = 13	
IL-1ra (per 1,000 pg/mL)	1.24 (1.05–1.46)	0.01	1.06 (0.80–1.40)	NS	1.23 (1.05–1.44)	0.01
IL-1ra (per 1,000 pg/mL) + NIHSS	1.25 (1.05–1.49)	0.01	0.91 (0.64–1.30)	NS	1.23 (1.04–1.45)	0.02
Excluding patients with infection to day 8	*N* = 14		*N* = 1		*N* = 13	
IL-1ra (per 1,000 pg/mL)	1.23 (1.05–1.45)	0.01	1.04 (0.68–1.60)	NS	1.23 (1.05–1.44)	0.01
IL-1ra (per 1,000 pg/mL) + NIHSS	1.24 (1.04–1.48)	0.02	NC	NC	1.23 (1.04–1.46)	0.02

Patients are excluded stepwise based on their time to infection to avoid confounding by infection-related increases in IL-1ra. Two patients were excluded from analyses—1 was infected at the time of stroke onset and the other died before day 15

OR odds ratio, *CI* confidence interval, *IL-1ra* interleukin-1 receptor antagonist, *PNA* pneumonia, *NIHSS* National Institutes of Health Stroke Scale, *NS* not significant ($P \geq 0.200$), *NC* not calculable

Table 3 Risk of infection by day 15 after stroke for each C allele of the rs4251961 SNP in *IL1RN*

Infection by day 15	Any infection *N* = 28		PNA *N* = 9		Infection (not PNA) *N* = 20	
	OR (95 % CI)	*P*	OR (95 % CI)	*P*	OR (95 % CI)	*P*
C allele	0.90 (0.49–1.64)	NS	1.72 (0.78–3.81)	0.18	0.54 (0.24–1.24)	0.15
C allele + NIHSS	0.76 (0.36–1.60)	NS	2.12 (0.70–6.45)	0.19	0.46 (0.19–1.14)	0.09
C allele + NIHSS + race	0.72 (0.34–1.53)	NS	2.02 (0.66–6.18)	NS	0.48 (0.19–1.19)	0.11
C allele + NIHSS + race + CHD	0.57 (0.25–1.31)	0.19	1.87 (0.57–6.08)	NS	0.38 (0.14–1.00)	0.05
C allele + NIHSS + race + CHD + HTN	0.59 (0.25–1.37)	NS	1.83 (0.56–5.97)	NS	0.39 (0.14–1.06)	0.06
C allele + NIHSS + race + CHD + HTN + IL-1ra[a]	0.50 (0.21–1.22)	0.13	1.60 (0.49–5.22)	NS	0.32 (0.11–0.97)	0.04

Two patients were excluded from analyses—1 was infected at the time of stroke onset and the other died before day 15

SNP single nucleotide polymorphism, *OR* odds ratio, *CI* confidence interval, *IL-1ra* interleukin-1 receptor antagonist, *NIHSS* National Institutes of Health Stroke Scale, *CHD* coronary heart disease, *HTN* hypertension, *NS* not significant ($P \geq 0.200$)

[a] Indicates the plasma concentration of IL-1ra at 72 h after stroke onset

outcome. Good outcome was defined as mRS < 2. Significance was set at $P < 0.05$.

Results

Genotyping was possible in 113 of the 114 subjects enrolled in this study. Among these 113 subjects there was a rapid and sustained elevation of IL-1ra after stroke (Fig. 1). Subjects with the most severe strokes (NIHSS $\geq$17) had the highest plasma IL-1ra. IL-1ra concentrations were the highest on day 7 after stroke, but remained elevated in comparison to the control population until at least 180 days after stroke. The concentrations of IL-1ra in plasma were more highly correlated to clinical stroke severity (NIHSS score) than infarct volume at all-time points; at 3 days after stroke onset, the correlation between IL-1ra and NIHSS score was $\rho = 0.399$, $P < 0.001$, and the correlation between IL-1ra and infarct volume was $\rho = 0.265$, $P < 0.010$. Subsequent models were thus corrected for the NIHSS score.

The *IL1RN* allele frequencies for rs4251961 did not differ from the Hardy–Weinberg equilibrium among our subject population: TT (49 %), TC (36 %), CC (15 %). The characteristics of these subjects are detailed in Table 1. Subjects with the minor C allele of the rs4251961 SNP in *IL1RN* were more likely to be Caucasian, having coronary heart disease (CHD) and hypertension (HTN). The data suggest increased rates of PNA in subjects with the minor C allele, but this increase was not statistically significant. There were no differences in the concentrations of plasma IL-1ra at any time point among subjects with different genotypes (data not shown).

We previously showed that an early elevation in IL-1ra was an independent risk factor for infection in the first 15 days after stroke [5]. The median time to PNA was 5 days; the mean time to any infection was 8 days. The effect of IL-1ra on infection risk was true only for infections other than PNA (Table 2). Despite the lack of difference in IL-1ra concentrations among subjects with different genotypes, we built logistic regression models to test the association between infection and the minor C allele of rs4251961 SNP. The model was controlled for stroke severity (using the NIHSS) as well as those characteristics that differed ($P < 0.100$) among subjects with the different genotypes at baseline (Table 1). The presence of a C allele did not affect the overall risk of infection, but was associated with a decrease in the risk of infections other than PNA (Table 3).

No effect of early plasma IL-1ra on stroke outcome at any time point was seen using a logistic regression model controlled for known predictors of stroke outcome (age, stroke severity and infection) as well as for characteristics that differed between subjects with different *IL1RN* genotypes (race, CHD, HTN). The effect of the *IL1RN* genotype on outcome after stroke was also explored controlling for these same variables (Table 4). At 1 year after stroke onset, those individuals with a C allele were nearly 8 times more likely (when controlled for initial NIHSS) to experience a good outcome (mRS <2).

Conclusions

In the cohort of 113 subjects for this study with ischemic stroke, we found that those with the minor C allele of the

Table 4 The predictive value of the minor C allele of the *IL1RN* SNP rs4251961 on good outcome (mRS < 2) after stroke

Outcome at	30 days $N = 22$ (104)		90 days $N = 32$ (103)		180 days $N = 34$ (92)		365 days $N = 36$ (81)	
	OR (95 % CI)	*P*	OR (95 % CI)	*P*	OR (95 % CI)	*P*	OR (95 % CI)	*P*
C allele	1.48 (0.57–3.85)	NS	1.13 (0.49–2.62)	NS	1.05 (0.45–2.45)	NS	1.22 (0.51–2.97)	NS
C allele + NIHSS	2.72 (0.85–8.68)	0.09	2.28 (0.71–7.29)	0.16	2.13 (0.63–7.25)	NS	3.59 (0.88–14.74)	0.08
C allele + NIHSS + age	2.78 (0.86–8.99)	0.09	2.31 (0.72–7.45)	0.16	2.14 (0.63–7.27)	NS	3.62 (0.88–14.83)	0.07
C allele + NIHSS + age + race	2.70 (0.82–8.82)	0.10	2.46 (0.74–8.13)	0.14	2.19 (0.63–7.61)	NS	3.14 (0.73–13.46)	0.12
C allele + NIHSS + age + race + CHD	2.16 (0.62–7.46)	NS	2.12 (0.62–7.23)	NS	1.66 (0.45–6.04)	NS	3.00 (0.67–13.37)	0.15
C allele + NIHSS + age + race + CHD + HTN	2.49 (0.69–9.02)	0.17	2.50 (0.70–9.00)	0.16	1.86 (0.48–7.13)	NS	6.52 (1.07–39.83)	0.04
C allele + NIHSS + age + race + CHD + HTN + infection[a]	2.33 (0.63–8.63)	NS	2.34 (0.62–8.83)	NS	1.68 (0.43–6.61)	NS	7.67 (1.02–57.46)	<0.05

N = the number of patients with a good outcome (total number of patients available for assessment at that time point)

SNP single nucleotide polymorphism, *mRS* modified Rankin Scale, *OR* odds ratio, *CI* confidence interval, *NIHSS* National Institutes of Health Stroke Scale, *CHD* coronary heart disease, *HTN* hypertension, *NS* not significant ($P \geq 0.200$)

[a] Denotes infection by day 15 after stroke

rs4251961 SNP in *IL1RN* were more likely to be Caucasian and more likely to have CHD and HTN. This observation needs to be confirmed in a larger population, but it is intriguing to speculate that the minor allele of this SNP could contribute to vascular risk factors and vascular damage. In fact, mice that lack *IL1RN* develop arterial inflammation [22]. The C allele of rs4251961 is associated with decreased ex vivo cellular production of IL-1ra, decreased plasma IL-1ra, and increased plasma IL-1β and CRP in healthy individuals [20, 23]. These observations suggest that the C allele of the rs4251961 SNP is associated with chronic inflammation and may predispose to vascular disease. The fact that we did not detect differences in plasma IL-1ra among subjects with different alleles of the rs4251961 SNP in this study may be related to the relatively small sample size, the possibility that changes in IL-1ra production induced by the acute stroke may have overwhelmed any potential effect of the SNPs, or that genetic regulation of IL-1ra is complex and context dependent.

We previously showed that plasma IL-1ra was an independent predictor of post-stroke infection. We now extend those analyses and show that the increased risk applies only to infections other than PNA. Based on prior reports showing a decrease in IL-1ra in subjects with the C allele of rs4251961, we anticipated that subjects with this allele would experience a decreased risk of infection. The data, however, show no association between the C allele and overall infection risk. Interestingly, similar to the data for IL-1ra, there appears to be a differential effect of the C allele on infection risk depending on the type of infection in question; the C allele was associated with a decrease in the risk of infections other than PNA. Since the risk of PNA is so closely linked to stroke severity and the ability to protect the airway (including the level of consciousness and the degree of dysphagia/risk for aspiration), these observations suggest that the effect of cytokines/genes on infection risk (*i.e.*, PNA) might be overpowered by other factors (*i.e.*, dysphagia and the risk of aspiration).

There are strong preclinical data suggesting a neuroprotective role for IL-1ra in cerebral ischemia; both overexpressions of IL-1ra and exogenous administration of IL-1ra are associated with improved outcome from experimental stroke [4]. Our data show a marked increase in endogenous levels of IL-1ra after stroke onset with an associate increase in infection independent of stroke severity. Exogenous administration of IL-1ra and medications that increase IL-1ra production also appear to be associated with an increased risk of infection [24–26].Gromadzka and colleagues [18], in their study, suggest that the *IL1RN**2 allele of the VNTR polymorphism is associated with increased IL-1ra production and suggest that this allele may be associated with better long-term outcome because of the neuroprotective properties of IL-1ra. They further suggest that the early increase in mortality associated with the *IL1RN**2 allele of the VNTR polymorphism may have been related to infection, although no data about infections were provided. While most publications suggest that the *IL1RN**2 allele of the VNTR polymorphism is associated with increased IL-1ra [8–11], there is no universal agreement on this point. The regulation of IL-1ra is likely dependent on a complement of genes as well as environmental context; since IL-1ra was not assessed in this study, the actual mechanism by which *IL1RN**2 affected outcome is unclear. We hypothesized that the C allele of rs4251961, which is associated with decreased ex vivo cellular IL-1ra production and decreased plasma IL-1ra in healthy individuals [19, 20], would be associated with worse stroke outcome. On the contrary, subjects with this allele were more likely to experience good outcomes after stroke, although the results were not significant at each time point.

To our knowledge, this is the first study to examine the effects of the rs4251961 SNP in *IL1RN* on post-stroke infection and stroke outcome. The complexities of understanding how the immune response following stroke modulates the risk of infection and stroke outcome are highlighted. Previously, it was shown that endogenous increases in IL-1ra were associated with the increased risk of infection and that SNPs in *IL1RN* which lead to increased IL-1ra production, are associated with better outcome in those who remain infection free [5, 18]. In the current study, we showed that the C allele of rs4251961, which leads to decreased IL-1ra production, was associated with decreased risk of infection (other than PNA) as predicted, but counter to what was predicted, this SNP was also associated with good outcome. Because the study is relatively small ($N = 113$) and the number of variables entered into the logistic regression models is relatively large, the findings will need to be replicated in a larger population. The preliminary data presented here, however, suggest that the genetic profile of individuals may affect the risk of post-stroke infection and influence stroke outcome.

Acknowledgments We thank all of the individuals who volunteered for participation in this study.

Conflict of Interests There are no competing interests to disclose.

Funding This research was funded by NINDS 5R01NS049197.

References

1. Arend WP. Interleukin 1 receptor antagonist. A new member of the interleukin 1 family. J Clin Invest. 1991;88:1445–51.
2. Steinkasserer A, Spurr NK, Cox S, Jeggo P, Sim RB. The human IL-1 receptor antagonist gene (*IL1RN*) maps to chromosome 2q14-q21, in the region of the IL-1 alpha and IL-1 beta loci. Genomics. 1992;13:654–7.

3. Arend WP, Palmer G, Gabay C. IL-1, IL-18, and IL-33 families of cytokines. Immunol Rev. 2008;223:20–38.
4. Banwell V, Sena ES, Macleod MR. Systematic review and stratified meta-analysis of the efficacy of interleukin-1 receptor antagonist in animal models of stroke. J Stroke Cerebrovasc Dis. 2009;18:269–76.
5. Tanzi P, Cain K, Kalil A, et al. Post-stroke infection: a role for IL-1ra? Neurocrit Care. 2011;14:244–52.
6. Westendorp WF, Nederkoorn PJ, Vermeij JD, Dijkgraaf MG, van de Beek D. Post-stroke infection: a systematic review and meta-analysis. BMC Neurol. 2011;11:110.
7. Aslanyan S, Weir CJ, Diener HC, Kaste M, Lees KR. Pneumonia and urinary tract infection after acute ischemic stroke: a tertiary analysis of the GAIN International trial. Eur J Neurol. 2004;11:49–53.
8. Danis VA, Millington M, Hyland VJ, Grennan D. Cytokine production by normal human monocytes: inter-subject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism. Clin Exp Immunol. 1995;99:303–10.
9. Hurme M, Santtila S. IL-1 receptor antagonist (IL-1Ra) plasma levels are co-ordinately regulated by both IL-1Ra and IL-1beta genes. Eur J Immunol. 1998;28:2598–602.
10. Santtila S, Savinainen K, Hurme M. Presence of the IL-1RA allele 2 (*IL1RN**2) is associated with enhanced IL-1beta production in vitro. Scand J Immunol. 1998;47:195–8.
11. Candiotti KA, Yang Z, Morris R, et al. Polymorphism in the interleukin-1 receptor antagonist gene is associated with serum interleukin-1 receptor antagonist concentrations and postoperative opioid consumption. Anesthesiology. 2011;114:1162–8.
12. Tountas NA, Casini-Raggi V, Yang H, et al. Functional and ethnic association of allele 2 of the interleukin-1 receptor antagonist gene in ulcerative colitis. Gastroenterology. 1999;117:806–13.
13. Rocha AM, De Souza C, Rocha GA, et al. *IL1RN* VNTR and IL2-330 polymorphic genes are independently associated with chronic immune thrombocytopenia. Br J Haematol. 2010;150:679–84.
14. Smolonska J, Wijmenga C, Postma DS, Boezen HM. Meta-analyses on suspected chronic obstructive pulmonary disease genes: a summary of 20 years' research. Am J Respir Crit Care Med. 2009;180:618–31.
15. Queiroz DM, Oliveira AG, Saraiva IE, et al. Immune response and gene polymorphism profiles in Crohn's disease and ulcerative colitis. Inflamm Bowel Dis. 2009;15:353–8.
16. Vencovsky J, Jarosova K, Ruzickova S, et al. Higher frequency of allele 2 of the interleukin-1 receptor antagonist gene in patients with juvenile idiopathic arthritis. Arthritis Rheum. 2001;44:2387–91.
17. Xue H, Lin B, Ni P, Xu H, Huang G. Interleukin-1B and interleukin-1 RN polymorphisms and gastric carcinoma risk: a meta-analysis. J Gastroenterol Hepatol. 2010;25:1604–17.
18. Gromadzka G, Sarzynska-Dlugosz I, Czlonkowska A. *IL1RN* intron 2 polymorphism caused by variable number tandem repeats is associated with 1 year outcome in patients with ischemic stroke. J Neurol Neurosurg Psychiatry. 2007;78:183–6.
19. Rafiq S, Stevens K, Hurst AJ, et al. Common genetic variation in the gene encoding interleukin-1-receptor antagonist (IL-1RA) is associated with altered circulating IL-1RA levels. Genes Immun. 2007;8:344–51.
20. Reiner AP, Wurfel MM, Lange LA, et al. Polymorphisms of the IL1-receptor antagonist gene (*IL1RN*) are associated with multiple markers of systemic inflammation. Arterioscler Thromb Vasc Biol. 2008;28:1407–12.
21. Sims JR, Gharai LR, Schaefer PW, et al. ABC/2 for rapid clinical estimate of infarct, perfusion, and mismatch volumes. Neurology. 2009;72:2104–10.
22. Nicklin MJ, Hughes DE, Barton JL, Ure JM, Duff GW. Arterial inflammation in mice lacking the interleukin 1 receptor antagonist gene. J Exp Med. 2000;191:303–12.
23. Rafiq S, Melzer D, Weedon MN, et al. Gene variants influencing measures of inflammation or predisposing to autoimmune and inflammatory diseases are not associated with the risk of type 2 diabetes. Diabetologia. 2008;51:2205–13.
24. Becker K, Tanzi P, Kalil A, Shibata D, Cain K. Early statin use is associated with increased risk of infection after stroke. J Stroke Cerebrovasc Dis. 2013;22:66–71.
25. Montaner J, Chacon P, Krupinski J, et al. Simvastatin in the acute phase of ischemic stroke: a safety and efficacy pilot trial. Eur J Neurol. 2008;15:82–90.
26. Emsley HC, Smith CJ, Georgiou RF, et al. A randomised phase II study of interleukin-1 receptor antagonist in acute stroke patients. J Neurol Neurosurg Psychiatry. 2005;76:1366–72.

2.6 Functional polymorphisms in toll-like receptor 4 are associated with worse outcome in acute ischemic stroke patients

Functional polymorphisms in toll-like receptor 4 are associated with worse outcome in acute ischemic stroke patients

Jonathan R. Weinstein[a], Juliane Schulze[a], Richard V. Lee[a], Hunter Phillips[a], Dannielle Zierath[a], Patricia Tanzi[a], Dean Shibata[b], Kevin C. Cain[c] and Kyra J. Becker[a]

Toll-like receptor-4 (TLR4) is important in neuroinflammation. Single nucleotide polymorphisms (SNPs) in *TLR4*, including 1063 A/G [Asp299Gly] and 1363 C/T [Thr399Ile], are associated with altered immune responses but their effect on acute ischemic stroke (AIS) outcome is unknown. We collected demographic, clinical, laboratory, radiologic, and genotype data on 113 AIS patients and performed multivariate analyses to assess associations between *TLR4* SNP haplotype and either neurological outcome, infection, or inflammatory markers. In adjusted analyses, *TLR4* SNPs were associated with worse outcome as well as increases in circulating leukocytes, C-reactive protein, and interleukin-1 receptor antagonist. In AIS, variations in *TLR4* may influence neurological outcome (for video abstract, please see Supplemental digital content 1 file,

http://links.lww.com/WNR/A274). NeuroReport **25:580–584**

NeuroReport 2014, **25**:580–584

Keywords: infection, outcome, single nucleotide polymorphisms, stroke, toll-like receptor 4

Departments of [a]Neurology, [b]Radiology and [c]Biostatistics, University of Washington, Seattle, Washington, USA

Correspondence to Jonathan R. Weinstein, MD, PhD, Department of Neurology, University of Washington, Box 356465, 1959 NE Pacific Street, Seattle, WA 98195, USA
Tel: +1 206 221 5362; fax: +1 206 685 8100;
e-mail: jweinste@u.washington.edu

Present address: Juliane Schulze: Department of Neurology, University of Greifswald, Greifswald, Germany.

Received 30 December 2013 **accepted** 3 February 2014

Introduction

Acute ischemic stroke (AIS) induces profound alterations in both systemic and central nervous system immune responses [1]. In the periphery, these changes include robust increases in plasma concentrations of proinflammatory markers such as C-reactive protein (CRP) and interleukin (IL)-6 [1]. In brain, this response includes production of proinflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) from astrocytes and microglia [2] as well as a marked influx of leukocytes into the ischemic hemisphere [2].

Toll-like receptors (TLRs) are a family of pattern recognition receptors involved in identification of, and response to, foreign pathogens [3]. In brain, TLRs are expressed by microglia and astrocytes [2] and are critical in initiation of innate immune response to injury [3]. Thirteen TLRs have been identified and each recognizes different pathogen-associated molecular patterns including bacterial cell wall/membrane components such as lipotechoic acid (TLR2) and lipopolysaccharide (endotoxin) (TLR4) [3]. Activation of TLRs by endogenous ligands (also known as danger-associated molecular patterns or DAMPs) released from ischemia-injured cerebral vasculature and parenchyma is a possible mechanism for initiation of both inflammatory and immunomodulatory responses in AIS [2,4]. A number of TLR4-activating DAMPs have been identified in brain including heat shock proteins, high-mobility group box 1, and peroxiredoxins [2,4]. In animal models, TLR4 signaling has been implicated in poststroke neuroinflammation and injury [5,6]. TLR4 is also critical in the robustly neuroprotective ischemic preconditioning phenomenon [7].

Single nucleotide polymorphisms (SNPs) in genes encoding proteins involved in the immune response can influence clinical outcomes following AIS [8]. There are ~20 known SNPs in *TLR4* [9]. Two of these *TLR4* SNPs, 1063 A/G (Asp299Gly) and 1363 C/T (Thr399Ile), occur at significant frequencies (> 5%) in populations across the globe [10]. In Whites, these two *TLR4* SNPs cosegregate; thus the double SNP *299/399* haplotype occurs more frequently than either of the individual SNP haplotypes (*299/wt* or *wt/399*) [9,10]. Functionally, SNPs at either or both loci result in TLR4 proteins with altered ligand binding domains [11] and the *299/399* haplotype is associated with hyporesponsiveness to lipopolysaccharide in some [9,12], but not all [13], prior studies. In addition, the *299/399* haplotype is associated with increased risk of systemic infection [10,14].

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (*www.neuroreport.com*).

0959-4965 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins

DOI: 10.1097/WNR.0000000000000140

The effect of these *TLR4* SNPs on poststroke infection and clinical outcome is unknown.

Methods

Research subjects

The patient population for this study was described elsewhere [15,16]. The University of Washington's Institutional Review Board approved this study. Patients or their surrogates provided informed consent.

Data collection

Demographic, clinical, and radiological data were collected on all patients. Stroke severity at time of presentation was quantified using the National Institutes of Health Stroke Scale score. Total infarct volume on initial diffusion weighted MRI was calculated by the ABC/2 method [17]. Stroke subtypes were classified as described [18]. Stroke outcome was determined at 3 months by the modified Rankin Scale (mRS); poor outcome was defined as mRS greater than 2. Infections included in our analyses were in-hospital urinary tract infections and pneumonia. Clinical criteria for these infections were as previously defined [15]. All patients with infection were treated with antibiotics.

Laboratory studies

Leukocyte counts and plasma CRP levels were determined by the hospital laboratory. Concentrations of circulating cytokines [IL-6, IL-10, TNFα, and IL-1 receptor antagonist (IL-1ra)] were measured with a cytometric bead-based system (Fluorokine MAP; R&D Systems, Minneapolis, Minnesota, USA). Values below the specific limit of detection for each cytokine were assigned a value equivalent to the lower level of detection.

TLR4 SNP genotyping

Prior studies examining the 1063 A/G (Asp299Gly) and 1363 C/T (Thr399Ile) *TLR4* loci have used different groupings of the variant haplotypes (*299/wt*, *wt/399*, and *299/399*) to demonstrate significant associations with clinical parameters. Some have looked at the *299/399* haplotype alone [13,14], whereas others have looked at associations with any of the three variant haplotypes combined (*299/wt*, *wt/399*, or *299/399*) [9,10]. The two approaches have complementary scientific merit [9] and we chose to do both throughout this study although the data presented in the tables show only results with the combined haplotypes (*299/wt*, *wt/399*, or *299/399*). DNA extraction from blood plasma samples, quantitative reverse transcription-PCR, and allelic discrimination analyses were performed as described [19]. Target SNP reference identification numbers were rs4986790 and rs4986791. All samples were processed in triplicate. Reproducibility of the genotyping method was confirmed as described [19].

Statistical analyses

Descriptive data are presented as median and interquartile range for continuous variables and percentages for categorical variables. Group comparisons were performed using the Mann–Whitney *U*-test or the χ^2-test statistic as appropriate. Logistic regression was used to estimate the odds ratio (OR) and 95% confidence interval (CI) for the effect of the *TLR4* SNPs on infection by day 15 and on neurologic outcome at 3 months. With a relatively small sample as in this study, there is only good statistical power for detecting a large effect. For example, for the association of genotype with clinical outcome (Table 2) the power is 32, 59, and 73% for detecting an OR of 5, 10, or 15, respectively. For the association of genotype with infection (Table 3) the power is 51, 79, and 90% for detecting an OR of 5, 10, or 15, respectively. For Table 4, there is 85% power for detecting a difference of 1.0 SD between groups.

Results

Of the 113 patients in the study, 10 (8.8%) were heterozygous for a haplotype that included either one or both of the indicated *TLR4* SNPs: 8 (7.1%) had the double SNP (*299/399* haplotype) and 2 (1.8%) had the single SNP (*wt/399* haplotype). The remaining 103 (91.1%) patients had neither SNP (*wt/wt* haplotype). The observed genotype frequencies for both SNPs were in Hardy–Weinberg equilibrium (*http://www.oege.org/*).

There were no significant differences in baseline characteristics or medical history of patients on the basis of whether or not they had a defined *TLR4* SNP (i.e. *wt/wt* vs. *299/wt*, *wt/399*, or *299/399*) (Table 1). Neither initial stroke severity nor infarct volume differed between patients with a *TLR4* SNP and those without (Table 1). Patients with a *TLR4* SNP haplotype, however, were more likely to have lacunar stroke and less likely to have cardioembolic stroke than patients with the *wt/wt* haplotype (Table 1). Rates of infection were similar among all patients irrespective of *TLR4* haplotype (Table 1). Table 1 shows only results for patients who had any of the three *TLR4* SNP-positive haplotypes investigated, although results were not significantly different when only patients with the cosegregating dual SNP *299/399* alone, were included in the analysis.

Table 2 displays the association between *TLR4* SNP haplotype and poor neurological outcome (mRS > 2) at 3 months. Results are shown both unadjusted and adjusted for variables known to affect stroke outcome. Table 2 shows that there was no association between the presence of a *TLR4* SNP and poor outcome in the unadjusted analysis. However, after adjusting for initial stroke severity and age, the presence of a *TLR4* SNP was associated with poor outcome. Neither further adjustment for stroke etiology nor the presence of infection significantly altered this association. Results for the *299/399* SNP haplotype alone were largely similar to

Table 1 Baseline characteristics and other clinical and radiological parameters for patients without or with a TLR4 SNP haplotype

	TLR4 haplotype		
Parameters	*wt/wt* (*N*=103)	*299/wt*, *wt/399*, or *299/399 SNP* (*N*=10)	*P*
Baseline characteristics			
Age	57 (46–67)	46 (42–64)	0.31
Sex (female) [*N* (%)]	33 (32)	5 (50)	0.25
Medical history [*N* (%)]			
AF	16 (16)	0 (0)	0.18
CHD	26 (25)	1 (10)	0.28
DM	23 (22)	4 (40)	0.21
HTN	53 (51)	7 (70)	0.26
Smoker	40 (39)	2 (20)	0.24
HLD	74 (72)	7 (70)	0.90
Clinical/radiological data on patients' stroke			
Initial NIHSS score	11 (4–20)	9 (3–18)	0.46
Initial infarct volume (cc)	12 (2–90)	12 (0.1–117)	0.46
Stroke etiology [*N* (%)]			
Lacunar	8 (8)	3 (30)	**0.02**
Cardioembolic	30 (29)	0 (0)	**0.046**
Large artery atherosclerosis	16 (16)	1 (10)	0.64
Dissection	6 (6)	1 (10)	0.60
Other[a]	21 (20)	2 (20)	0.76
Unknown	22 (21)	3 (30)	0.53
Infections [*N* (%)]			
All infections	26 (25)	3 (30)	0.76
PNA	12 (12)	0 (0)	0.33

AF, atrial fibrillation; CHD, coronary heart disease; DM, diabetes mellitus; HLD, hyperlipidemia; HTN, hypertension; NIHSS, National Institutes of Health Stroke Scale; PNA, pneumonia; SNP, single nucleotide polymorphism; TLR4, Toll-like receptor-4; wt, wild type.
Significant *P* values are shown in bold.
[a]Other etiology category included one iatrogenic, three paradoxical emboli, and 19 with either multiple competing etiologies or uncertain etiology.

Table 2 Association between TLR4 SNP (1063 A/G [Asp299Gly] and/or 1363 C/T [Thr399Ile]) and poor outcome (modified Rankin Scale score>2) at 3 months post-AIS

Presence of TLR4 SNP predicts poor outcome		
Model adjusted for	OR (95% CI)	*P*
TLR4 SNP[a]	2.21 (0.58–8.38)	0.24
TLR4 SNP + NIHSS	9.22 (1.43–59.5)	**0.02**
TLR4 SNP + NIHSS + age	12.92 (1.78–93.90)	**0.01**
TLR4 SNP + NIHSS + age + stroke etiology	15.46 (2.04–117.04)	**0.008**
TLR4 SNP + NIHSS + age + stroke etiology + infection	14.16 (1.75–114.81)	**0.01**

AIS, acute ischemic stroke; CI, confidence interval; NIHSS, National Institutes of Health Stroke Scale; OR, odds ratio; SNP, single nucleotide polymorphism; TLR4, Toll-like receptor-4.
Significant *P* values are shown in bold.
[a]Numerical breakdown of unadjusted data: 99/113 (88%) of patients in study had outcome data available at this time point; 42/99 (42%) of patients with available outcome data at this time point had poor outcome; 6/10 (60%) of patients with available outcome data at this time point and a TLR4 SNP had a poor outcome; 36/89 (40%) of patients with available outcome data at this time point without a TLR4 SNP had a poor outcome.

Table 3 Association between TLR4 SNP (1063 A/G [Asp299Gly] and/or 1363 C/T [Thr399Ile]) and infection at 15 days post-AIS

Presence of TLR4 SNP predicts infection		
Model adjusted for	OR (95% CI)	*P*
TLR4 SNP	1.25 (0.30–5.23)	0.76
TLR4 SNP + NIHSS	2.08 (0.41–10.52)	0.38
TLR4 SNP + NIHSS + age	2.22 (0.44–11.20)	0.34

AIS, acute ischemic stroke; CI, confidence interval; NIHSS, National Institutes of Health Stroke Scale; OR, odds ratio; SNP, single nucleotide polymorphism; TLR4, Toll-like receptor-4.

those for the three variant *TLR4* SNP haplotypes combined: unadjusted, OR±95% CI = 1.47 (0.35–6.27), *P* = NS; adjusted for initial stroke severity and age, 10.79 (1.32–88.4), *P* = 0.027; adjusted further for stroke etiology and infection, 12.61 (1.42–111.9), *P* = 0.023.

Overall, 29/113 (25.7%) of patients in this cohort had an infection by day 15 after AIS (Table 1). Having one or both *TLR4* SNPs was not associated with an increased likelihood of infection in the unadjusted analysis [OR±95% CI = 1.25 (0.30–5.23), *P* = NS] (Table 3). After controlling for initial stroke severity and age there was still no significant association found between the presence of a *TLR4* SNP-containing haplotype and the risk of infection, 2.22 (0.44–11.20), *P* = NS (Table 3). Similarly, no significant association was found between the *299/399* haplotype alone and risk of infection, 2.06 (0.30–14.33), *P* = NS, post adjustment for stroke severity and age.

Patients with a haplotype containing either one or both *TLR4* SNPs had higher white blood cell (WBC) counts at 3 days and 1 week post-AIS (Table 4); this elevation was independent of stroke severity. A similar pattern of WBC elevation was seen for the *299/399* haplotype alone (data not shown). The increase in WBC counts was driven by an increase in polymorphonuclear cell counts (Table 4). No significant changes in either lymphocyte or monocyte counts were found with any of the variant *TLR4* haplotypes examined. Plasma CRP was also elevated in the SNP-positive patient group at 3 months after stroke onset. This increase in CRP at 3 months was also seen in

Table 4 Markers of inflammation from AIS onset to 3 months in patients who either did not have, or did have, one of the variant TLR4 SNP haplotypes under investigation here (*299/wt, wt/399*, or *299/399*): indicated in table as '+ SNP'

	72h			1 week			1 month			3 months		
Time	wt/wt	+ SNP	*P*	wt/wt	+ SNP	*P*	wt/wt	+ SNP	*P*	wt/wt	+ SNP	*P*
WBCs ($\times 10^3/\mu l$)	7.44 (5.99, 10.00)	8.97 (7.68, 12.42)	**0.025**	7.66 (6.02, 9.78)	10.70 (7.46, 13.00)	**0.003**	6.53 (5.34, 8.54)	8.43 (6.82, 11.15)	0.074	6.88 (5.42, 8.12)	7.99 (7.41, 10.10)	0.139
PMNs ($\times 10^3/\mu l$)	5.04 (3.44, 6.87)	6.08 (5.06, 8.06)	**0.023**	4.80 (3.36–6.44)	5.49 (3.95, 8.26)	0.104	4.12 (3.00, 5.43)	5.18 (4.22–7.33)	0.087	3.89 (3.00, 5.43)	5.48 (4.10, 7.30)	0.059
Lymphs ($\times 10^3/\mu l$)	1.47 (1.22, 1.82)	1.71 (1.42, 1.94)	0.755	1.80 (1.40–2.25)	1.80 (1.69, 2.13)	0.918	1.78 (1.27–2.04)	1.96 (1.63, 2.45)	0.175	1.73 (1.34, 2.14)	1.83 (1.41, 2.59)	0.468
Monos ($\times 10^3/\mu l$)	0.64 (0.46, 0.86)	0.61 (0.46, 0.82)	0.742	0.66 (0.50, 0.89)	0.68 (0.58, 1.04)	0.363	0.52 (0.42, 0.68)	0.50 (0.47, 0.60)	0.727	0.50 (0.41, 0.65)	0.59 (0.52, 0.67)	0.127
CRP (mg/l)	9.5 (2.6, 48.1)	8.8 (1.9, 87.1)	0.208	7.20 (2.20, 25.95)	8.20 (3.85, 48.65)	0.811	2.70 (0.95, 9.15)	4.00 (0.95–10.92)	0.900	1.90 (0.60, 5.90)	2.50 (0.90, 12.00)	**0.002**
IL-6 (pg/ml)	1.94 (ND, 7.23)	1.66 (ND, 15.16)	0.941	1.26 (ND, 4.39)	2.87 (1.47, 4.83)	0.999	ND (ND, 2.12)	ND (ND, 2.52)	0.817	ND (ND, 1.62)	1.41 (ND, 1.69)	0.611
IL-10 (pg/ml)	ND (ND, 0.93)	ND (ND, ND)	0.182	ND (ND, 0.76)	ND (ND, 0.56)	0.519	ND (ND, 0.70)	ND (ND, ND)	0.301	ND (ND, 0.70)	ND (ND, ND)	0.369
TNFα (pg/ml)	1.87 (ND, 2.96)	ND (ND, 4.05)	0.669	2.12 (ND, 3.92)	ND (ND, 4.72)	0.069	1.88 (ND, 3.89)	2.18 (ND, 4.30)	0.519	1.98 (ND, 3.26)	1.76 (ND, 4.77)	0.827
IL-1ra (pg/ml)	1645 (998, 2946)	2401 (765, 4137)	0.094	1777 (981, 3189)	2086 (1458, 8401)	**0.009**	1400 (812–3214)	2029 (1448, 3150)	0.272	1009 (579, 1745)	1238 (606, 2579)	0.982

Data values shown are medians (interquartile range).
AIS, acute ischemic stroke; CRP, high-sensitivity C-reactive protein; IL, interleukin; IL-1ra, IL-1 receptor antagonist; ND, not detected; PMNs, polymorphonuclear cells; monos, monocytes; lymphs, lymphocytes; SNP, single nucleotide polymorphism; TLR4, Toll-like receptor-4; TNF, tumor necrosis factor; WBCs, white blood cells; wt, wild type.
Significant *P* values are shown in bold.
P=*P* value (adjusted for initial stroke severity) for comparisons between *wt/wt* haplotype versus either the *299/wt, wt/399*, or *299/399* haplotypes.

the *299/399* haplotype alone (data not shown). Of the cytokines assayed (IL-6, IL-10, TNFα, and IL-1ra), the only significant difference between SNP-positive and *wt/wt* patients was for IL-1ra, which was higher in the SNP-positive patient group at the 1-week time point (Table 4).

Discussion

This study demonstrates that two functionally significant *TLR4* SNPs were associated with poor neurological outcome following AIS. At 3 months after stroke, the group of patients with either one or both of the *TLR4* SNPs (*299/wt*, *wt/399*, or *299/399* haplotypes) had significantly worse clinical outcomes. This association persisted when limiting the analysis to patients with the *299/399* haplotype alone and also in multivariate models controlling for age, stroke severity, stroke etiology and infection. The *TLR4* SNP patient group also exhibited transiently increased WBC counts and plasma levels of CRP and IL-1ra.

The two *TLR4* SNPs investigated are in strong linkage disequilibrium in Whites [9,14]. Our results are consistent with this in that eight of the 10 *TLR4* SNP-positive patients were heterozygous for the *299/399* dual SNP haplotype. The *299/399* haplotype is associated with increased rates of systemic infection [10,14], however we did not find an association with infection in our cohort. Because the rates of poststroke infection are generally high, the association between these SNPs and infection risk may have been overwhelmed by stroke-related factors. Given the limited statistical power of this study, it is also possible that the lack of a demonstrated association between genotype and infection could be due to a type II (false negative) error.

Despite the absence of a demonstrated association between *TLR4* SNP haplotype and infection in our cohort, the possibility that poor outcomes in *TLR4* SNP patients could be related to infection was considered. Poststroke infection is an independent risk factor for poor outcome [1] and worse outcome was seen among patients with poststroke infection in our cohort [16]. Our data, however, showed that adjustment for infection had little impact on the association between *TLR4* SNP haplotype and clinical outcome (Table 2). Thus, poststroke infection is unlikely to explain this association. It is important to note that our modest sample size and multiple comparisons mandate caution in the interpretation of these results. However, the primary association between *TLR4* SNP haplotype and clinical outcome here was a singular prespecified a-priori hypothesis. Thus, the possibility of a type 1 (false positive) error is reduced [20]. Nevertheless, our findings will need to be replicated in an independent cohort of AIS patients.

Given the findings in Table 2, a mechanism to account for the effect of *TLR4* SNP haplotype on outcome, independent of infection, should be proposed. Our findings

seem to contrast with those in rodent studies where spontaneous mutations in [6] or complete absence of [21] the *TLR4* gene results in reduced infarct volumes and improved neurobehavioral outcomes. The *TLR4* SNPs investigated here, however, result in structural alterations in human TLR4 that are different than in mice [12]. The responses of these SNP-altered TLR4 proteins to endogenous ligands (or DAMPs) released from ischemic brain tissue are unknown. The extent of injury and recovery in the brain following stroke may be modulated by the TLR4-response of resident microglia and/or infiltrating macrophages to DAMPs [2,4]. Alternatively, modification of TLR4 function in the immune cells of SNP-positive patients could result in an attenuation of baseline TLR4-dependent 'preconditioning' [7].

Experimental data support a beneficial role for the anti-inflammatory cytokine IL-1ra in stroke [22]. In a recent study characterizing the same patient cohort as described here, elevated plasma IL-1ra was independently associated with an increased risk of poststroke infection but not clinical outcome [16]. Our data indicate that patients with a *TLR4* SNP haplotype have transiently increased IL-1ra plasma levels. This association persisted after controlling for stroke severity (Table 4) and was not significantly altered by adjustment for infection (data not shown). The biological explanation for this association, independent of infection, is uncertain. However, the *TLR4* SNPs studied here alter both plasma levels of cytokines [23] and immune cell release of cytokines following stimulation with TLR4 agonists [10,12]. Thus, the IL-1ra finding here is consistent with previous literature.

Increased WBC counts and elevated plasma CRP are independently associated with poor outcome after stroke [1]. Our finding that variant *TLR4* SNPs are associated with transient increases in both WBC count and CRP is interesting and could help explain the *TLR4* SNP effect on outcome. As with IL-1ra, the associations with both WBC count and CRP persisted after adjusting for infection (data not shown). The biological explanations for these associations are also uncertain. However, our findings here are again consistent with prior work [24,25].

Conclusion

Functionally significant variations in *TLR4* are associated with: (i) worse neurological outcome and (ii) alterations in systemic markers of inflammation following AIS. These data are the first to suggest a direct connection between TLR4 function and stroke pathophysiology in humans.

Acknowledgements

NIH/NINDS grants NS065008 (J.R.W.) and NS049197 (K.J.B.).

Conflicts of interest

There are no conflicts of interest.

References

1 Becker KJ. Modulation of the postischemic immune response to improve stroke outcome. *Stroke* 2010; **41 (Suppl)**:S75–S78.
2 Weinstein JR, Koerner IP, Moller T. Microglia in ischemic brain injury. *Future Neurol* 2010; **5**:227–246.
3 Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 2010; **11**:373–384.
4 Shichita T, Ago T, Kamouchi M, Kitazono T, Yoshimura A, Ooboshi H. Novel therapeutic strategies targeting innate immune responses and early inflammation after stroke. *J Neurochem* 2012; **123 (Suppl 2)**:29–38.
5 Brea D, Blanco M, Ramos-Cabrer P, Moldes O, Arias S, Perez-Mato M, *et al.* Toll-like receptors 2 and 4 in ischemic stroke: outcome and therapeutic values. *J Cereb Blood Flow Metabo* 2011; **31**:1424–1431.
6 Caso JR, Pradillo JM, Hurtado O, Lorenzo P, Moro MA, Lizasoain I. Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. *Circulation* 2007; **115**:1599–1608.
7 Pradillo JM, Fernandez-Lopez D, Garcia-Yebenes I, Sobrado M, Hurtado O, Moro MA, *et al.* Toll-like receptor 4 is involved in neuroprotection afforded by ischemic preconditioning. *J Neurochem* 2009; **109**:287–294.
8 Marousi S, Ellul J, Antonacopoulou A, Gogos C, Papathanasopoulos P, Karakantza M. Functional polymorphisms of interleukin 4 and interleukin 10 may predict evolution and functional outcome of an ischaemic stroke. *Eur J Neurol* 2010; **18**:637–643.
9 Ferwerda B, McCall MB, Verheijen K, Kullberg BJ, van der Ven AJ, Van der Meer JW, *et al.* Functional consequences of toll-like receptor 4 polymorphisms. *Mol Med* 2008; **14 (5–6)**:346–352.
10 Ferwerda B, McCall MB, Alonso S, Giamarellos-Bourboulis EJ, Mouktaroudi M, Izagirre N, *et al.* TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. *Proc Natl Acad Sci USA* 2007; **104**:16645–16650.
11 Schroder NW, Schumann RR. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. *Lancet Infect Dis* 2005; **5**:156–164.
12 Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, *et al.* TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000; **25**:187–191.
13 Douville RN, Lissitsyn Y, Hirschfeld AF, Becker AB, Kozyrskyj AL, Liem J, *et al.* TLR4 Asp299Gly and Thr399Ile polymorphisms: no impact on human immune responsiveness to LPS or respiratory syncytial virus. *PloS One* 2010; **5**:e12087.
14 Bochud PY, Chien JW, Marr KA, Leisenring WM, Upton A, Janer M, *et al.* Toll-like receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. *N Engl J Med* 2008; **359**:1766–1777.
15 Becker KJ, Kalil AJ, Tanzi P, Zierath DK, Savos AV, Gee JM, *et al.* Autoimmune responses to the brain after stroke are associated with worse outcome. *Stroke* 2011; **42**:2763–2769.
16 Tanzi P, Cain K, Kalil A, Zierath D, Savos A, Gee JM, *et al.* Post-stroke infection: a role for IL-1ra? *Neurocrit Care* 2010; **14**:244–252.
17 Sims JR, Gharai LR, Schaefer PW, Vangel M, Rosenthal ES, Lev MH, *et al.* ABC/2 for rapid clinical estimate of infarct, perfusion, and mismatch volumes. *Neurology* 2009; **72**:2104–2110.
18 Adams HP, Woolson RF, Helgason C, Karanjia PN, Gordon DL. a.t.T. Investigators. Low molecular weight heparinoid, ORG 10172 (danaparoid), and outcome after acute ischemic stroke: a randomized controlled trial. The Publications Committee for the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) Investigators. *JAMA* 1998; **279**:1265–1272.
19 Becker KJ, Dankwa D, Lee R, Schulze J, Zierath D, Tanzi P, *et al.* Stroke, IL-1ra, IL1RN, infection and outcome. *Neurocrit Care* 2013 (in press).
20 Austin PC, Mamdani MM, Juurlink DN, Hux JE. Testing multiple statistical hypotheses resulted in spurious associations: a study of astrological signs and health. *J Clin Epidemiol* 2006; **59**:964–969.
21 Yang QW, Lu FL, Zhou Y, Wang L, Zhong Q, Lin S, *et al.* HMBG1 mediates ischemia-reperfusion injury by TRIF-adaptor independent Toll-like receptor 4 signaling. *J Cereb Blood Flow Metab* 2011; **31**:593–605.
22 Banwell V, Sena ES, Macleod MR. Systematic review and stratified meta-analysis of the efficacy of interleukin-1 receptor antagonist in animal models of stroke. *J Stroke Cerebrovasc Dis* 2009; **18**:269–276.
23 Koch A, Hamann L, Schott M, Boehm O, Grotemeyer D, Kurt M, *et al.* Genetic variation of TLR4 influences immunoendocrine stress response: an observational study in cardiac surgical patients. *Crit Care* 2011; **15**:R109.
24 Pace E, Giarratano A, Ferraro M, Bruno A, Siena L, Mangione S, *et al.* TLR4 upregulation underpins airway neutrophilia in smokers with chronic obstructive pulmonary disease and acute respiratory failure. *Hum Immunol* 2011; **72**:54–62.
25 Steinhardt AP, Aranguren F, Tellechea ML, Gomez Rosso LA, Brites FD, Martinez-Larrad MT, *et al.* A functional nonsynonymous toll-like receptor 4 gene polymorphism is associated with metabolic syndrome, surrogates of insulin resistance, and syndromes of lipid accumulation. *Metabolism* 2010; **59**:711–717.

2.7 Catecholamines, steroids and immune alterations in ischemic stroke and other acute diseases

Aging and Disease
www.aginganddisease.org
Volume 5, Number 5; 327-339, October 2014
http://dx.doi.org/10.14336/AD.2014.0500327

Review Article

Catecholamines, Steroids and Immune Alterations in Ischemic Stroke and Other Acute Diseases

Juliane Schulze[#], Antje Vogelgesang[#] and Alexander Dressel*

Section of Neuroimmunology, Department of Neurology, University Medicine Greifswald, Germany

[Received December 21, 2013; Revised May 9, 2014; Accepted May 27, 2014]

ABSTRACT: The outcome of stroke patients is not only determined by the extent and localization of the ischemic lesion, but also by stroke-associated infections. Stroke-induced immune alterations, which are related to stroke-associated infections, have been described over the last decade. Here we review the evidence that catecholamines and steroids induced by stroke result in stroke-induced immune alterations. In addition, we compare the immune alterations observed in other acute diseases such as myocardial infarction, brain trauma, and surgical trauma with the changes seen in stroke-induced immune alterations.

Key words: ischemic stroke, immune, aging, catecholamines, steroids

Cerebrovascular diseases, including ischemic stroke, are the second most common cause of death worldwide after ischemic heart disease; stroke is the third leading cause of death in the USA [1, 2]. Stroke-related disability ranks third when disability-adjusted life-years are assessed [3]. The clinical outcome of stroke patients is not solely determined by the infarct size and localization, but is also altered by subsequent infections. Stroke-associated infections (SAIs), of which pneumonia is the most common, impair outcome and increase mortality [4, 5]. The frequency of SAIs ranges from 15%-42% depending on the inclusion criteria of the respective study [4, 5]. In recent years it has become evident that increased susceptibility to infections is related to stroke-induced alterations in the immune system. Loss of lymphocytes, lymphocyte dysfunction, and monocyte deactivation following stroke have been observed in both experimental stroke models and in stroke patients [6-8].

If the mechanisms by which stroke induces these immune alterations became known, these pathways could be targeted in future therapeutic trials. The central nervous system and the immune system communicate with each other via several pathways: the sympathetic nervous system, the parasympathic nervous system, and the hypothalamic-pituitary-adrenal (HPA) axis [9]. These pathways can be triggered through the nervous system by the brain itself or as a response to afferent vagus stimulation. In addition, the immune system can induce a stress response through inflammatory cytokines, which may be locally produced or may reach the central nervous system via the blood stream. Ischemic brain injury has been shown to affect several of these pathways [9].

Catecholamine effects and receptors on immune cells

To alter immune responses, catecholamines must be present in the microenvironment of the leukocytes, and the cells must express a receptor to detect these hormones [10]. Direct sympathetic innervation is found in both primary and secondary lymphoid organs, where norepinephrine and epinephrine are released from the sympathetic nerve endings and immune cells express α- and β-adrenoreceptors that transduce the signal into the cell (Table 1) [10]. Expression levels differ due to epigenetic regulation by histones and DNA methylation [11-13].

***Correspondence should be addressed to:** Alexander Dressel, Section of Neuroimmunology, Dept. of Neurology, University Medicine Greifswald, 17475 Greifswald, Germany. Email: adressel@uni-greifswald.de.[#]contributed equally
ISSN: 2152-5250

Table 1. Expression of adrenoreceptors on immune cells.

Most innate immune cells	Both αAR and βAR families
Bone marrow-derived dendritic cells	α1AR α2AR β1AR β2AR
Monocytes/macrophages	β2AR α1AR α2AR
Natural killer cells Resting and activated B cells Naïve T cells and Th1 cells, but not Th2 cells	β2AR
Regulatory T cells and Th17 cells	No data available

Reviewed in [14]. AR, adrenoreceptor.

Since lymphocytes are primed in lymphoid organs, and catecholamine concentrations within the spleen and lymph nodes are likely to exceed plasma concentrations due to direct sympathetic innervations, sympathetic activation can effectively alter immune responses [15]. Furthermore, catecholamines are not only released from nerve terminals and the adrenal medulla, but can also be actively produced, stored, and secreted by immune cells themselves [16]. Catecholamines released from immune cells serve as auto/paracrine regulators of lymphocyte activity, for example through the suppression of lymphocyte proliferation, cytokine production, and the induction of apoptosis [17, 18]. The biological relevance of immune cell-derived catecholamines versus the catecholamines released by the sympathetic response is not known [19].

Catecholamine release leads to a quick two-phased mobilization: initial lymphocytosis is succeeded by granulocytosis and reduced lymphocytes in the peripheral blood. Lymphocyte recruitment seems to mainly be mediated by β2-adrenoreceptors (β2AR) and originates from the marginal pool and the spleen, whereas granulocytes are predominantly recruited from the marginal pool and the lung via α-adrenoreceptor stimulation [20]. While this biphasic response of lymphocytes to β2AR engagement is well described the underlying mechanisms are only partly resolved. The effect depends on the time of receptor engagement in relation to the activation and differentiation state of the cell, the involved molecular signaling pathway, and the cytokine microenvironment (Table 2) (for reviews see [21-23]). In addition, high concentrations of catecholamines are known to induce lymphocyte apoptosis [17].

Glucocorticoid effects and receptors on immune cells

Another effective pathway for the interaction of the central nervous system and the immune system is the HPA axis. Activation of the HPA axis starts with the release of corticotropin-releasing hormone from the hypothalamus, which induces the secretion of adrenocorticotropic hormone, which leads to the secretion of glucocorticoids from the adrenal gland [10]. Glucocorticoids have long been known to exert anti-inflammatory and immunosuppressive effects, and are broadly used as an anti-inflammatory treatment. In the absence of ligands, the glucocorticoid receptor (GCR) resides in the cytoplasm in a complex with heat shock proteins and immunophilins [24]. The GCR is constitutively expressed in virtually all cell types, but different tissue-specific expression patterns lead to tissue-specific outcomes in different diseases [25].

When binding its ligand in the cytoplasm, the GCR can interact with signaling pathways of the T-cell receptor signaling complex and thus modulate pro-inflammatory gene expression [26]. The primary actions of the GCR are evident in the nucleus. Upon ligand binding, chaperone complex remodeling exposes nuclear localization sequences on the GCR, leading to its nuclear translocation [27]. The GCR has two ways of modulating gene expression. As a dimer, GCR binds the glucocorticoid response element, when undimerized, GCR binds pro-inflammatory transcription factors such as AP-1, NF-kB, IRF-3, STAT, CREB, NFAT, T-bet, and GATA-3, leading to their inhibition [27]. Further details are reviewed in [28]. Cellular effects of stress hormones are summarized in Table 2.

Table 2. Cellular effects of stress hormones in immune cells

	Glucorticoids	Catecholamines
DC	• induce apoptosis in immature DCs [29] • reduction of MHCII, costimulatory molecules and cytokine expression [30, 31] • inhibition of migration in vivo and in vitro [32]; [33] by downregulation of CCR7 [34] • induction of a tolerogenic DC phenotype that induces T cell anergy, suppression of T cells and generates Tregs [35]	• enhanced surface expression of MHCII, CD80 and CD86 [36] • control cell migration via α1bAR [37] and induction of an anti-inflammatory cytokine profile [38] • enhancement of IL-33 production thus promoting Th2 responses [39] inhibited the lipopolysaccharide (LPS)-stimulated production of interleukin (IL)-23, IL-12 p40, tumor necrosis factor (TNF)-alpha and IL-6 [40]
Macrophages/ monocytes	• suppressed activation by IL-10 induction and inhibition of upregulation of pro-inflammatory CD163 [41] • development of myeloid suppressor cell like phenotype [42]	• cAMP/PKA dependent stimulation of IL-10 promoter/enhancer [43] • upregulate L-selectin in vitro [44] • inhibit IL-6 secretion via αAR [45] but • induce secretion of IL-6 (in the presence of GC) via βAR [46]
Neutrophils	• suppression of adhesion molecule expression inhibits rolling, adhesion and transmigration [47] • increase of BM-derived neutrophils in blood [48] • promotes necrosis [49]	• increase the total circulating neutrophil pool for a few hours [50] • increase expression and release of Hsp72 [51] • suppression of CD11b and inhibition of suppression of CD62L (L-selectin) [52] • decreased phagocytosis of zymosan in vitro [53]
B cells	• reduction of splenic and LN B cell numbers • inhibit B cell progenitor proliferation • enhance IgE, suppressed IgG production [54, 55]	• state of B cell activation decisive about effect of CA: - enhanced IgG1 production and IgE on NE exposure during antigen processing or after Th2 coculture [56] - increase in costimulatory capacity (CD86 upregulation) [57] - β2AR engagement in presence of IL-4 enhances IgE [58]
T cells	• affect thymocyte maturation by inducing apoptosis in thymocytes; more sensitive than Teff than Treg cells [59, 60] • physiological doses: shift from Th1 response to Th2 [61] • pharmacological doses induce anti-inflammation: - reduce RORγt in Th17 cells [62] - inhibit TH1 function by direct inhibition of STAT4 and T-bet [63, 64] - suppress STAT6 function in Th2 by interfering with GATA [65, 66]	• β2AR engagement enhances IFNγ production in TH1 cells the presence of IL-12 in pre activated TH1 cells [67] • inhibit IFN-γ in resting TH1 cells [13]
NK cells	• impair NK cell function via histone deacetylation and transrepression [68]	• inhibit NK cytotoxic functions by: - reduced TNF-α, IFN-γ, and GM-CSF - impaired, target binding [69]

DC dendritic cells; CCR C-C chemokine receptor; NK natural killer cells;
Treg regulatory T cells; Teff effector T cells; BM bone marrow; LN Lymph node; NE norepinephrine; CA Catecholamine.

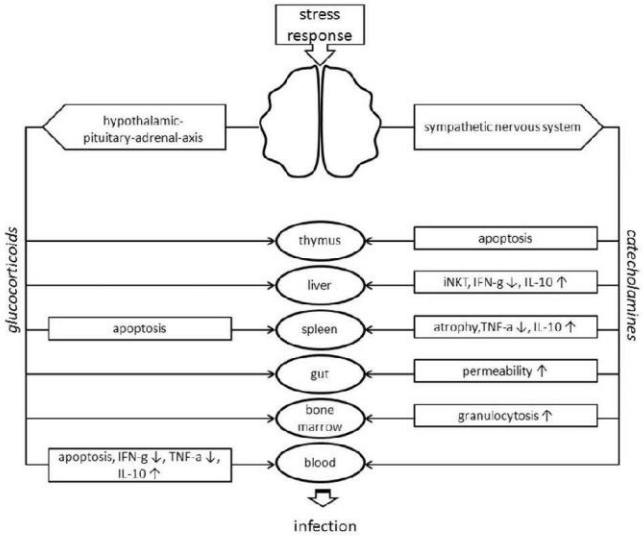


Figure 1. Systemic effects of the stress response in ischemic stroke. The scheme depicts those organ specific immune alterations that occur in stroke and have been experimentally linked to the activation of either the HPA axis or the sympathetic nervous system. As a result the stress response reduces the ability to fight bacteria and increases the risk of subsequent infection. (HPA, hypothalamic-pituitary gland-adrenal; iNKT, invariant natural killer T cells; IFN-g, Interferon-gamma; IL-10, Interleukin-10; TNF-a, tumor necrosis factor-alpha; ↑, increase, ↓, decrease)

Stress responses have been described in stroke; the pathways delineated above have been implicated in stroke-induce immune alterations (SIIAs). In this article, we review the clinical and experimental evidence that stress hormones are indeed the mediators linking ischemic brain injury with SIIA. Other acute diseases such as myocardial infarction (MI), surgical trauma, and trauma-related injury also induce a stress response. We will summarize what is known with respect to immunological changes in patients with these diseases (section 4.1), and discuss whether changes observed in SIIA that enhance the risk for SAIs are unique to ischemic brain injury or extend to other diseases. This review will not address the role of the immune system and SIIAs in secondary immune-mediated organ damage, which has been described in experimental stroke and traumatic brain injury (TBI) [70-73].

Stress hormones in stroke

Alterations induced by catecholamines and steroids in specific organs are summarized in Figure 1. Here we will focus on cell type specific alterations and clinical consequences.

Stroke: catecholamines and clinical outcome

Several studies of stroke patients have investigated whether epinephrine, norepinephrine, or its metabolites metanephrine and normetanephrine can be used as markers for the extent of SIIA and whether these molecules correlate with patient outcome and the occurrence of SAIs. Metanephrine and normetanephrine, which lack biological activity but are relatively stable, can be determined in urinary samples [74]. Data across different stroke patient populations and research groups consistently indicate that catecholamines are associated with an increased risk of post-stroke infections. Higher levels of metanephrine and normetanephrine on admission and on day 1 enhance the risk of developing SAIs [4, 75, 76]. In agreement with these findings, mortality at 3 months was associated with higher levels of

normetanephrine on admission and day 1, at least in one study [75].

Furthermore, the cellular changes observed in SIIA, including lymphocytopenia and reduced monocytic HLA-DR expression that leads to impaired immune function, have been linked to catecholamine levels in stroke patients in the ESPIAS and PANTHERIS studies [4, 75]. This observation may be due to the biphasic effect of catecholamines on immune cells, including the autocrine, apoptosis-inducing regulation that was described earlier. However, the observation that lymphocytes remaining in the circulation of stroke patients are primed towards proinflammation [76] is not in line with the earlier report that Th2 cells lack the β2AR [13], and therefore T cells with an anti-inflammatory phenotype should escape catecholamine-induced apoptosis. This scenario suggests that additional mediators are involved in regulating T-cell responses in stroke patients.

The phenotypic findings in human stroke-induced SIIA are very similar to the observations made in experimental stroke; lymphocyte apoptosis in the spleen and thymus, lymphocytopenia in the periphery, and a defective interferon (IFN)-γ response in monocytes predispose patients to post-stroke infections, with bacteremia and spontaneous pneumonia in experimental stroke [77]. Thus, the animal model appears well suited to investigate the underlying pathophysiology of SIIA.

Selective inhibition of the effects of sympathetic nervous-system activation at an early time point, but not the blockage of the effects of HPA axis activation, prevented systemic infections and improved survival in stroked mice [77, 79]. These findings suggest that catecholamines, but not glucocorticoids, are causally related to SAI. Moreover, adoptive transfer of splenocytes, especially T and natural killer cells from control mice into stroked animals, restored the recipient's defense against pathogens [77]. Both the loss of lymphocytes due to apoptosis and the functional impairment of lymphocytes can be explained by the known β2AR-mediated effects of catecholamines. Additionally, adrenoreceptor antagonism has been demonstrated to inhibit splenic atrophy, reduce the infarct volume, and modulate cytokine expression in the spleen following experimental stroke, but did not affect a specific lymphocyte population within the splenocyte fraction [80]. Thus converging evidence from animal models and human studies support the role of β-adrenergic stimulation in SIIA.

Stroke: glucocorticoids and clinical outcome

The stress hormone cortisol is also transiently elevated following stroke [81-83]. However, the data for the kinetics of cortisol in plasma concentrations as well as the relationship of cortisol with SAI remain contradictory. The increase of plasma cortisol levels has been reported to persist through day 5 post-stroke [81] , while others observed elevated plasma levels on admission that normalized within 24 h [84, 85].

The correlation of cortisol levels with stroke severity, infarct volumes, unfavorable outcome, and even higher mortality has been repeatedly demonstrated [86-90]. However, the association of cortisol levels with markers of SIIA and post-stroke infections remains poorly understood. Whereas our own data support an association of plasma cortisol levels on day 1 with SAI [76] , other studies detected no association of plasma cortisol levels with SAI or with monocyte function [4, 84]. Another study linked interleukin (IL)-6 levels to cortisol levels; in stroke patients, IL-6 levels correlated significantly with cortisol levels, and morning serum IL-6 levels independently predicted evening/night cortisol levels, which has been interpreted as evidence for cytokine-induced HPA axis activation following stroke [91].

In experimental stroke, a three-fold increase in serum corticosterone levels (the primary glucocorticoid in rodents) compared to naïve animals was observed at 4 h after stroke; only 24 h after sham/permanent middle cerebral artery occlusion surgery, corticosterone levels in stroked animals returned nearly to the levels of naïve animals [92].

While several studies have addressed the role of glucocorticoids in brain-lesion development in stroke, there is a surprising paucity of data investigating the effects of glucocorticoids on immune function and infection in experimental stroke. The seminal study by Prass et al. has long been the only investigation of GCR antagonism with respect to SIIA and SAI. They reported that glucocorticoid inhibition reduced apoptosis of splenocytes and lymphopenia following stroke [77]. However, in contrast to β-adrenergic inhibition, GCR antagonism did not prevent pneumonia. A very recent study now reported similar findings demonstrating in experimental stroke that the inhibition of glucocorticoid effects reversed lymphocytopenia while inhibition of β2AR restored interferon release in lymphocytes [78].

Do stress hormones mimic SIIA in vitro?

As reviewed in the preceding sections, clinical data and animal models suggest that catecholamines are the major mediator of SIIA. However, it is difficult to prove causality due to the complex pathways activated in whole animals and in patients. In vitro studies could provide complementary evidence if the effects observed in vivo could be replicated in vitro.

Table 3. Immune alterations immediately after disease onset.

	Ischemic stroke (IS)		Traumatic brain injury (TBI)		Myocardial infarction (MI)		(Surgical) trauma (ST/T)	
	animal	human	animal	human	animal	human	animal	human
innate immune system								
White blood cell		↑[98, 100]		↑[101]	↑[102]	↑[98, 103]		
monocytic HLA-DR		↓[98, 100, 104]				↓[98]	↓[105, 106]	↓[106, 107]
monocytic LPS activatability		↓[98, 104]				↓[98]		↓[108]
cytokines								
IL-10		↑[104, 109]	↑[110]	↑[94]		↑[43]		↑[111]
TNF-α	↓[77]	↓[100]	↑[110]			↓[98]	↑[112]	
IL-6		↑[98, 109]	↑[110]	↑[101, 113]	↑[98, 102]		↑[112]	↑[111]
HMGB-1		↑[76, 99]		↑[114]		↑[99]		↑[95, 115]
adaptive immune system								
circulatory lymphocyte number	↓[77]	↓[7, 100, 109, 116, 117]		↓[113]		↓[98]	↓[105, 106]	↓[107]
T lymphocyte activation		↓[116] ↑[76]				↑[118]		
IgG, IgM		=[117]		↓[119, 120]				
T-cell proliferation to mitogen		↓[117] =[76]		↓[113]				
hormones								
catecholamines	↑[77]	↑[4, 75, 76, 109]		↑[94]		↑[96, 97]		↑[95]
cortisol/corticosterone	↑[77]	↑[100, 104]				↑[98]	↑[106]	

LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.

In peripheral blood mononuclear cells activated through the T-cell receptor in vitro, non-toxic concentrations of norepinephrine induce pro- and anti-inflammatory cytokine expression [93], while the synthetic glucocorticoid dexamethasone reduces cellular activation, lowering the number of cytokine-producing lymphocytes and inhibiting both Th1- and Th2-type cytokines [93]. In this setting, which included the combined in vitro application of dexamethasone and norepinephrine, the dexamethasone-induced alteration dominated [93]. Exposure to norepinephrine or terbutaline, a β2AR agonist, before T-cell receptor stimulation inhibits IFNγ production, as evident in the defective IFNγ response after stroke [12].

However, when peripheral blood mononuclear cells are not activated via the T-cell receptor but through the Toll-like receptors, application of dexamethasone and norepinephrine in vitro did not alter cytokine secretion (A. Kasprik, A. Dressel unpublished observations). These findings suggest that stress hormones may alter immune responses in a manner that is dependent on the activation pathway. We have observed that the in vitro application of catecholamines results in defunct upregulation of CTLA-4 expression on CD4+ T cells upon activation, mimicking observations made in stroke patients [76]. Whether this impaired regulation of CTLA-4 is functionally relevant in stroke patients is not known.

Mechanisms of immunosuppression in various diseases

The preceding sections have summarized the currently available evidence on stress hormones and SIIA. Taken together, these data suggest that catecholamines and glucocorticoids are key factors inducing immunosuppression in cerebral ischemia, enhancing susceptibility to SIA. If this emerging pathophysiological concept is correct, the immunological alterations seen in SIIA may not be unique to ischemic stroke, but may extend to other diseases that also induce an acute release of catecholamines and glucocorticoids. A rapid increase in the plasma levels of catecholamines and steroids has long been described in TBI, MI, and surgical trauma and trauma (ST/T), among others [94-97]. Furthermore, a relationship between clinical outcome and initial catecholamine levels has been described for ST/T, similar to the relationship detected in stroke [4, 75, 76, 95]. To the best of our knowledge only two studies have compared

immune alterations in stroke and MI [98, 99]. We therefore reviewed publications addressing immunosuppressive mechanisms in TBI, MI, or ST/T. Cross-study comparisons have limited validity, as patient characteristics, time points of sample acquisition, assays used to determine the activation status, and measured cytokines differ between studies. Despite these limitations, there is a striking consistency in the pattern of immune alterations observed within 48 h after disease onset. These patterns are summarized in Table 3 and detailed in section 4.1 and 4.3.

Immune response and cytokines

ST/T, MI, and TBI induce increased white blood cell counts and reduced monocytic HLA-DR, similar to findings in cerebral ischemia [7, 94, 95, 98, 101, 103, 106]. Monocytic TNF-α production upon lipopolysaccharide challenge is diminished in ischemic stroke as well as in TBI. The proinflammatory cytokine IL-6 is upregulated in ischemic stroke, TBI, and MI, as is the antiinflammatory cytokine IL-10 [98, 101, 110, 111]. Furthermore HMGB1 which has been shown to be a strong proinflammatory mediator is also elevated in TBI, ST/T, and ischemic stroke [76, 95, 115, 121, 122].

The reduced number of circulatory T lymphocytes (CD4+, CD8+) is an overall phenomenon observed in ischemic stroke, TBI, MI, and ST/T. While proliferation in response to mitogenic stimuli is impaired in TBI [113] and surgery [123], it is indistinguishable from controls in ischemic stroke, where T cells are prone to proinflammation [76] or reported to be also impaired [117]. In TBI, even B cells are unable to mount normal IgM or IgG responses [119, 120].

Gut barrier

TBI was reported to lead to secondary infections in up to 75% of affected comatose patients [120]. One possible shared source of bacterial infections is endogenous bacteria, Escherichia coli translocated from the patient's own gut. Increased permeability was reported in experimental ischemic stroke, in TBI [124], in intracerebral hemorrhage [125], and in patients who underwent surgical trauma [126-128]. Vagal nerve stimulation was found to inhibit bacterial translocation in TBI [129].

Hormones

Increased catecholamine or cortisol/corticosterone levels are present very early after disease onset in all of the reviewed diseases (ischemic stroke, TBI, MI, ST/T) [4, 75, 76, 94-98, 100, 104, 106, 109]. The blockade of catecholamine effects through the administration of propranolol was beneficial in ischemic stroke and in TBI [77, 94]. For TBI, beta blockade therapy is also suggested to be beneficial [130]. However, the effect of beta-blockade premedication in ischemic stroke patients is still not well enough investigated to draw conclusions. The levels of catecholamines 1 h post trauma (controls 0.3 ng/mL vs. TBI 3.27 ng/mL) correlate with mortality [95], while the results for TBI are contradictory [131, 132].

Summary and conclusion

Stress hormones are important regulators of immune cell function via adreneoreceptors and GCR. Lymphoid organs are directly innervated and nerve terminals release catecholamines. Moreover immune cells can actively secret hormones. There is strong evidence that the increase of stress hormones early after stroke is a marker for the extent of SIIA that can be expected to develop. Surprisingly, the experimental evidence suggests that catecholamine-triggered pathways, but not well-known immunosuppressive glucocorticoid-induced alterations, are causally related to SAI. However, which immune alterations induced by catecholamines mediate the enhanced susceptibility to infection in stroke patients remains to be determined. A recent observation from our laboratory provides evidence that catecholamine induced immune alterations are not limited to lymphocytes and monocytes but may extend to granulocyte function. The ability of monocytes and granulocytes to generate oxygen radicals (oxidative burst) was impaired in stroke patients compared to healthy controls. In vitro data suggest that catecholamines and glucocorticoids can both reduce oxidative burst [133].

In stroke and TBI, the brain injury itself is thought to trigger rapid activation of the stress pathways. MI patients suffer from thoracic pain and possibly respiratory distress, triggering a stress response in the absence of brain damage; similarly, patients with trauma also experience acute stress due to the trauma and to the resulting injury. Cross-study comparisons of ischemic stroke and TBI with ST/T and MI suggest that these diseases lead to similar induction of immunosuppression and subsequent infection through common activation of the stress response independently of direct central nervous system involvement (Figure 1).

Due to the similarities observed in alterations to the immune system in these very different diseases, we propose that SIIAs constitute a common response of the immune system to acute stress. SIIAs, which currently refer to "stroke-induced immune alterations", may therefore be better termed as "stress-induced immune alterations." Direct comparisons of the stress-induced immune alterations in various diseases are required to test

this hypothesis, which would offer a common therapeutic target to prevent infection and improve patient outcome across a wide spectrum of acute diseases.

Competing Interests

The authors declare that no competing interests exist.

Acknowledgement

We thank the German Research Foundation, Research Training Group 840 and the EU grant EnVision (FP7-REGPOT-2010, Grant-No 264143) for supporting our research.

The funders had no role in the decision to publish or the preparation of the review.

References

[1] Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ (2006). Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet, 367: 1747-1757

[2] Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, et al. (2010). Executive summary: heart disease and stroke statistics--2010 update: a report from the American Heart Association. Circulation, 121: 948-954

[3] Murray CJ, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C, et al. (2012). Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet, 380: 2197-2223

[4] Harms H, Reimnitz P, Bohner G, Werich T, Klingebiel R, Meisel C, et al. (2011). Influence of stroke localization on autonomic activation, immunodepression, and post-stroke infection. Cerebrovasc Dis, 32: 552-560

[5] Vermeij FH, Scholte op Reimer WJ, de Man P, van Oostenbrugge RJ, Franke CL, de Jong G, et al. (2009). Stroke-associated infection is an independent risk factor for poor outcome after acute ischemic stroke: data from the Netherlands Stroke Survey. Cerebrovasc Dis, 27: 465-471

[6] Dirnagl U, Klehmet J, Braun JS, Harms H, Meisel C, Ziemssen T, et al. (2007). Stroke-induced immunodepression: experimental evidence and clinical relevance. Stroke, 38: 770-773

[7] Vogelgesang A, Grunwald U, Langner S, Jack R, Broker BM, Kessler C, et al. (2008). Analysis of lymphocyte subsets in patients with stroke and their influence on infection after stroke. Stroke, 39: 237-241

[8] Urra X, Obach V, Chamorro A (2009). Stroke induced immunodepression syndrome: from bench to bedside. Curr Mol Med, 9: 195-202

[9] Meisel C, Schwab JM, Prass K, Meisel A, Dirnagl U (2005). Central nervous system injury-induced immune deficiency syndrome. Nat Rev Neurosci, 6: 775-786

[10] Straub RH, Westermann J, Scholmerich J, Falk W (1998). Dialogue between the CNS and the immune system in lymphoid organs. Immunol Today, 19: 409-413

[11] Kohm AP, Sanders VM (1999). Suppression of antigen-specific Th2 cell-dependent IgM and IgG1 production following norepinephrine depletion in vivo. J Immunol, 162: 5299-5308

[12] Sanders VM, Baker RA, Ramer-Quinn DS, Kasprowicz DJ, Fuchs BA, Street NE (1997). Differential expression of the beta2-adrenergic receptor by Th1 and Th2 clones: implications for cytokine production and B cell help. J Immunol, 158: 4200-4210

[13] Ramer-Quinn DS, Baker RA, Sanders VM (1997). Activated T helper 1 and T helper 2 cells differentially express the beta-2-adrenergic receptor: a mechanism for selective modulation of T helper 1 cell cytokine production. J Immunol, 159: 4857-4867

[14] Charlene A. McQueen, editor. Comprehensive Toxicology, Kidlington: Elsevier Science & Technology; 2010

[15] Dunn AJ (1995). Interactions Between the Nervous System and the Immune System: Implications for Psychopharmacology. In: Bloom FE, Kupfer DJ, editors. Psychopharmacology: The Fourth Generation of Progress, Lippincott Williams and Wilkins.

[16] Musso NR, Brenci S, Setti M, Indiveri F, Lotti G (1996). Catecholamine content and in vitro catecholamine synthesis in peripheral human lymphocytes. J Clin Endocrinol Metab, 81: 3553-3557

[17] Josefsson E, Bergquist J, Ekman R, Tarkowski A (1996). Catecholamines are synthesized by mouse lymphocytes and regulate function of these cells by induction of apoptosis. Immunology, 88: 140-146

[18] Bergquist J, Tarkowski A, Ekman R, Ewing A (1994). Discovery of endogenous catecholamines in lymphocytes and evidence for catecholamine regulation of lymphocyte function via an autocrine loop. Proc Natl Acad Sci U S A, 91: 12912-12916

[19] Flierl MA, Rittirsch D, Huber-Lang M, Sarma JV, Ward PA (2008). Catecholamines-crafty weapons in the inflammatory arsenal of immune/inflammatory cells or opening pandora's box? Mol Med, 14: 195-204

[20] Benschop RJ, Rodriguez-Feuerhahn M, Schedlowski M (1996). Catecholamine-induced leukocytosis: early observations, current research, and future directions. Brain, behavior, and immunity, 10: 77-91

[21] Sanders VM (2012). The beta2-adrenergic receptor on T and B lymphocytes: do we understand it yet? Brain, behavior, and immunity, 26: 195-200
[22] Kin NW, Sanders VM (2006). It takes nerve to tell T and B cells what to do. Journal of leukocyte biology, 79: 1093-1104
[23] Straub RH (2004). Complexity of the bi-directional neuroimmune junction in the spleen. Trends Pharmacol Sci, 25: 640-646
[24] Pratt WB, Toft DO (2003). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med (Maywood), 228: 111-133
[25] Zhou J, Cidlowski JA (2005). The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. Steroids, 70: 407-417
[26] Lowenberg M, Stahn C, Hommes DW, Buttgereit F (2008). Novel insights into mechanisms of glucocorticoid action and the development of new glucocorticoid receptor ligands. Steroids, 73: 1025-1029
[27] De Bosscher K, Haegeman G (2009). Minireview: latest perspectives on antiinflammatory actions of glucocorticoids. Mol Endocrinol, 23: 281-291
[28] Baschant U, Tuckermann J (2010). The role of the glucocorticoid receptor in inflammation and immunity. J Steroid Biochem Mol Biol, 120: 69-75
[29] Moser M, De Smedt T, Sornasse T, Tielemans F, Chentoufi AA, Muraille E, et al. (1995). Glucocorticoids down-regulate dendritic cell function in vitro and in vivo. European journal of immunology, 25: 2818-2824
[30] Kitajima T, Ariizumi K, Bergstresser PR, Takashima A (1996). A novel mechanism of glucocorticoid-induced immune suppression: the inhibiton of T cell-mediated terminal maturation of a murine dendritic cell line. The Journal of clinical investigation, 98: 142-147
[31] Matyszak MK, Citterio S, Rescigno M, Ricciardi-Castagnoli P (2000). Differential effects of corticosteroids during different stages of dendritic cell maturation. European journal of immunology, 30: 1233-1242
[32] Tuckermann JP, Kleiman A, Moriggl R, Spanbroek R, Neumann A, Illing A, et al. (2007). Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy. The Journal of clinical investigation, 117: 1381-1390
[33] Cumberbatch M, Dearman RJ, Kimber I (1999). Inhibition by dexamethasone of Langerhans cell migration: influence of epidermal cytokine signals. Immunopharmacology, 41: 235-243
[34] Vizzardelli C, Pavelka N, Luchini A, Zanoni I, Bendickson L, Pelizzola M, et al. (2006). Effects of dexamethazone on LPS-induced activationand migration of mouse dendritic cells revealed by a genome-wide transcriptional analysis. European journal of immunology, 36: 1504-1515
[35] Rutella S, Danese S, Leone G (2006). Tolerogenic dendritic cells: cytokine modulation comes of age. Blood, 108: 1435-1440
[36] Kim BJ, Jones HP (2010). Epinephrine-primed murine bone marrow-derived dendritic cells facilitate production of IL-17A and IL-4 but not IFN-gamma by CD4+ T cells. Brain, behavior, and immunity, 24: 1126-1136
[37] Maestroni GJ (2000). Dendritic cell migration controlled by alpha 1b-adrenergic receptors. J Immunol, 165: 6743-6747
[38] Maestroni GJ (2002). Short exposure of maturing, bone marrow-derived dendritic cells to norepinephrine: impact on kinetics of cytokine production and Th development. Journal of neuroimmunology, 129: 106-114
[39] Yanagawa Y, Matsumoto M, Togashi H (2011). Adrenoceptor-mediated enhancement of interleukin-33 production by dendritic cells. Brain, behavior, and immunity, 25: 1427-1433
[40] Goyarts E, Matsui M, Mammone T, Bender AM, Wagner JA, Maes D, et al. (2008). Norepinephrine modulates human dendritic cell activation by altering cytokine release. Experimental dermatology, 17: 188-196
[41] Ehrchen J, Steinmuller L, Barczyk K, Tenbrock K, Nacken W, Eisenacher M, et al. (2007). Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes. Blood, 109: 1265-1274
[42] Varga G, Ehrchen J, Tsianakas A, Tenbrock K, Rattenholl A, Seeliger S, et al. (2008). Glucocorticoids induce an activated, anti-inflammatory monocyte subset in mice that resembles myeloid-derived suppressor cells. Journal of leukocyte biology, 84: 644-650
[43] Platzer C, Docke W, Volk H, Prosch S (2000). Catecholamines trigger IL-10 release in acute systemic stress reaction by direct stimulation of its promoter/enhancer activity in monocytic cells. Journal of neuroimmunology, 105: 31-38
[44] Rainer TH, Lam N, Cocks RA (1999). Adrenaline upregulates monocyte L-selectin in vitro. Resuscitation, 43: 47-55
[45] Straub RH, Herrmann M, Berkmiller G, Frauenholz T, Lang B, Scholmerich J, et al. (1997). Neuronal regulation of interleukin 6 secretion in murine spleen: adrenergic and opioidergic control. Journal of neurochemistry, 68: 1633-1639
[46] Straub RH, Dorner M, Riedel J, Kubitza M, Van Rooijen N, Lang B, et al. (1998). Tonic neurogenic inhibition of interleukin-6 secretion from murine spleen caused by

opioidergic transmission. The American journal of physiology, 274: R997-1003
[47] Pitzalis C, Pipitone N, Perretti M (2002). Regulation of leukocyte-endothelial interactions by glucocorticoids. Annals of the New York Academy of Sciences, 966: 108-118
[48] Stroncek DF, Yau YY, Oblitas J, Leitman SF (2001). Administration of G--CSF plus dexamethasone produces greater granulocyte concentrate yields while causing no more donor toxicity than G--CSF alone. Transfusion, 41: 1037-1044
[49] Heasman SJ, Giles KM, Ward C, Rossi AG, Haslett C, Dransfield I (2003). Glucocorticoid-mediated regulation of granulocyte apoptosis and macrophage phagocytosis of apoptotic cells: implications for the resolution of inflammation. The Journal of endocrinology, 178: 29-36
[50] Parks KR, Davis JM (2012). Epinephrine, cortisol, endotoxin, nutrition, and the neutrophil. Surgical infections, 13: 300-306
[51] Giraldo E, Multhoff G, Ortega E (2010). Noradrenaline increases the expression and release of Hsp72 by human neutrophils. Brain, behavior, and immunity, 24: 672-677
[52] Trabold B, Gruber M, Frohlich D (2007). Functional and phenotypic changes in polymorphonuclear neutrophils induced by catecholamines. Scandinavian cardiovascular journal : SCJ, 41: 59-64
[53] Kaufmann I, Eisner C, Richter P, Huge V, Beyer A, Chouker A, et al. (2007). Psychoneuroendocrine stress response may impair neutrophil function in complex regional pain syndrome. Clin Immunol, 125: 103-111
[54] Cupps TR, Edgar LC, Thomas CA, Fauci AS (1984). Multiple mechanisms of B cell immunoregulation in man after administration of in vivo corticosteroids. J Immunol, 132: 170-175
[55] Cupps TR, Gerrard TL, Falkoff RJ, Whalen G, Fauci AS (1985). Effects of in vitro corticosteroids on B cell activation, proliferation, and differentiation. The Journal of clinical investigation, 75: 754-761
[56] Kasprowicz DJ, Kohm AP, Berton MT, Chruscinski AJ, Sharpe A, Sanders VM (2000). Stimulation of the B cell receptor, CD86 (B7-2), and the beta 2-adrenergic receptor intrinsically modulates the level of IgG1 and IgE produced per B cell. J Immunol, 165: 680-690
[57] Kohm AP, Mozaffarian A, Sanders VM (2002). B cell receptor- and beta 2-adrenergic receptor-induced regulation of B7-2 (CD86) expression in B cells. J Immunol, 168: 6314-6322
[58] Pongratz G, McAlees JW, Conrad DH, Erbe RS, Haas KM, Sanders VM (2006). The level of IgE produced by a B cell is regulated by norepinephrine in a p38 MAPK- and CD23-dependent manner. J Immunol, 177: 2926-2938
[59] Wang D, Muller N, McPherson KG, Reichardt HM (2006). Glucocorticoids engage different signal transduction pathways to induce apoptosis in thymocytes and mature T cells. J Immunol, 176: 1695-1702
[60] Chen X, Murakami T, Oppenheim JJ, Howard OM (2004). Differential response of murine CD4+CD25+ and CD4+CD25- T cells to dexamethasone-induced cell death. European journal of immunology, 34: 859-869
[61] Ramirez F, Fowell DJ, Puklavec M, Simmonds S, Mason D (1996). Glucocorticoids promote a TH2 cytokine response by CD4+ T cells in vitro. J Immunol, 156: 2406-2412
[62] Momcilovic M, Miljkovic Z, Popadic D, Markovic M, Savic E, Ramic Z, et al. (2008). Methylprednisolone inhibits interleukin-17 and interferon-gamma expression by both naive and primed T cells. BMC immunology, 9: 47
[63] Franchimont D, Galon J, Gadina M, Visconti R, Zhou Y, Aringer M, et al. (2000). Inhibition of Th1 immune response by glucocorticoids: dexamethasone selectively inhibits IL-12-induced Stat4 phosphorylation in T lymphocytes. J Immunol, 164: 1768-1774
[64] Liberman AC, Druker J, Refojo D, Holsboer F, Arzt E (2009). Glucocorticoids inhibit GATA-3 phosphorylation and activity in T cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 23: 1558-1571
[65] Maneechotesuwan K, Yao X, Ito K, Jazrawi E, Usmani OS, Adcock IM, et al. (2009). Suppression of GATA-3 nuclear import and phosphorylation: a novel mechanism of corticosteroid action in allergic disease. PLoS medicine, 6: e1000076
[66] Biola A, Andreau K, David M, Sturm M, Haake M, Bertoglio J, et al. (2000). The glucocorticoid receptor and STAT6 physically and functionally interact in T-lymphocytes. FEBS letters, 487: 229-233
[67] Swanson MA, Lee WT, Sanders VM (2001). IFN-gamma production by Th1 cells generated from naive CD4+ T cells exposed to norepinephrine. J Immunol, 166: 232-240
[68] Bush KA, Krukowski K, Eddy JL, Janusek LW, Mathews HL (2012). Glucocorticoid receptor mediated suppression of natural killer cell activity: identification of associated deacetylase and corepressor molecules. Cellular immunology, 275: 80-89
[69] Gan X, Zhang L, Solomon GF, Bonavida B (2002). Mechanism of norepinephrine-mediated inhibition of human NK cytotoxic functions: inhibition of cytokine secretion, target binding, and programming for cytotoxicity. Brain, behavior, and immunity, 16: 227-246
[70] Brait VH, Arumugam TV, Drummond GR, Sobey CG (2012). Importance of T lymphocytes in brain injury, immunodeficiency, and recovery after cerebral ischemia. J Cereb Blood Flow Metab, 32: 598-611

[71] Morganti-Kossmann MC, Satgunaseelan L, Bye N, Kossmann T (2007). Modulation of immune response by head injury. Injury, 38: 1392-1400
[72] Morganti-Kossmann MC, Rancan M, Stahel PF, Kossmann T (2002). Inflammatory response in acute traumatic brain injury: a double-edged sword. Curr Opin Crit Care, 8: 101-105
[73] Kamel H, Iadecola C (2012). Brain-immune interactions and ischemic stroke: clinical implications. Arch Neurol, 69: 576-581
[74] Whiting MJ, Doogue MP (2009). Advances in biochemical screening for phaeochromocytoma using biogenic amines. Clin Biochem Rev, 30: 3-17
[75] Chamorro A, Amaro S, Vargas M, Obach V, Cervera A, Gomez-Choco M, et al. (2007). Catecholamines, infection, and death in acute ischemic stroke. J Neurol Sci, 252: 29-35
[76] Vogelgesang A, May VE, Grunwald U, Bakkeboe M, Langner S, Wallaschofski H, et al. (2010). Functional status of peripheral blood T-cells in ischemic stroke patients. PLoS One, 5: e8718
[77] Prass K, Meisel C, Hoflich C, Braun J, Halle E, Wolf T, et al. (2003). Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. J Exp Med, 198: 725-736
[78] Mracsko E, Liesz A, Karcher S, Zorn M, Bari F, Veltkamp R (2014). Differential effects of sympathetic nervous system and hypothalamic-pituitary-adrenal axis on systemic immune cells after severe experimental stroke. Brain, behavior, and immunity,
[79] Wong CH, Jenne CN, Lee WY, Leger C, Kubes P (2011). Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. Science, 334: 101-105
[80] Ajmo CT, Jr., Collier LA, Leonardo CC, Hall AA, Green SM, Womble TA, et al. (2009). Blockade of adrenoreceptors inhibits the splenic response to stroke. Exp Neurol, 218: 47-55
[81] Fassbender K, Schmidt R, Mossner R, Daffertshofer M, Hennerici M (1994). Pattern of activation of the hypothalamic-pituitary-adrenal axis in acute stroke. Relation to acute confusional state, extent of brain damage, and clinical outcome. Stroke, 25: 1105-1108
[82] Franceschini R, Gandolfo C, Cataldi A, Del Sette M, Cianciosi P, Finocchi C, et al. (1994). Twenty-four-hour beta-endorphin secretory pattern in stroke patients. Stroke, 25: 2142-2145
[83] Olsson T (1990). Urinary free cortisol excretion shortly after ischaemic stroke. J Intern Med, 228: 177-181
[84] Emsley H (2003). An early and sustained peripheral inflammatory response in acute ischaemic stroke: relationships with infection and atherosclerosis. Journal of neuroimmunology, 139: 93-101
[85] Marklund N, Peltonen M, Nilsson TK, Olsson T (2004). Low and high circulating cortisol levels predict mortality and cognitive dysfunction early after stroke. J Intern Med, 256: 15-21
[86] Neidert S, Katan M, Schuetz P, Fluri F, Ernst A, Bingisser R, et al. (2011). Anterior pituitary axis hormones and outcome in acute ischaemic stroke. J Intern Med, 269: 420-432
[87] Christensen H, Boysen G, Johannesen HH (2004). Serum-cortisol reflects severity and mortality in acute stroke. J Neurol Sci, 217: 175-180
[88] O'Neill PA, Davies I, Fullerton KJ, Bennett D (1991). Stress hormone and blood glucose response following acute stroke in the elderly. Stroke, 22: 842-847
[89] Murros K, Fogelholm R, Kettunen S, Vuorela AL (1993). Serum cortisol and outcome of ischemic brain infarction. J Neurol Sci, 116: 12-17
[90] Anne M, Juha K, Timo M, Mikko T, Olli V, Kyosti S, et al. (2007). Neurohormonal activation in ischemic stroke: effects of acute phase disturbances on long-term mortality. Curr Neurovasc Res, 4: 170-175
[91] Szczudlik A, Dziedzic T, Bartus S, Slowik A, Kieltyka A (2004). Serum interleukin-6 predicts cortisol release in acute stroke patients. J Endocrinol Invest, 27: 37-41
[92] Weidenfeld J, Leker RR, Gai N, Teichner A, Bener D, Ovadia H (2011). The function of the adrenocortical axis in permanent middle cerebral artery occlusion: effect of glucocorticoids on the neurological outcome. Brain Res, 1407: 90-96
[93] Torres KC, Antonelli LR, Souza AL, Teixeira MM, Dutra WO, Gollob KJ (2005). Norepinephrine, dopamine and dexamethasone modulate discrete leukocyte subpopulations and cytokine profiles from human PBMC. Journal of neuroimmunology, 166: 144-157
[94] Woiciechowsky C, Asadullah K, Nestler D, Eberhardt B, Platzer C, Schoning B, et al. (1998). Sympathetic activation triggers systemic interleukin-10 release in immunodepression induced by brain injury. Nat Med, 4: 808-813
[95] Johansson PI, Stensballe J, Rasmussen LS, Ostrowski SR (2012). High circulating adrenaline levels at admission predict increased mortality after trauma. The journal of trauma and acute care surgery, 72: 428-436
[96] Ostrowski SR, Pedersen SH, Jensen JS, Mogelvang R, Johansson PI (2013). Acute myocardial infarction is associated with endothelial glycocalyx and cell damage and a parallel increase in circulating catecholamines. Crit Care, 17: R32
[97] Christensen NJ, Videbaek J (1974). Plasma catecholamines and carbohydrate metabolism in patients

with acute myocardial infarction. The Journal of clinical investigation, 54: 278-286
[98] Haeusler KG, Schmidt WU, Foehring F, Meisel C, Guenther C, Brunecker P, et al. (2012). Immune responses after acute ischemic stroke or myocardial infarction. Int J Cardiol, 155: 372-377
[99] Goldstein RS, Gallowitsch-Puerta M, Yang L, Rosas-Ballina M, Huston JM, Czura CJ, et al. (2006). Elevated high-mobility group box 1 levels in patients with cerebral and myocardial ischemia. Shock, 25: 571-574
[100] Haeusler KG, Schmidt WU, Fohring F, Meisel C, Helms T, Jungehulsing GJ, et al. (2008). Cellular immunodepression preceding infectious complications after acute ischemic stroke in humans. Cerebrovasc Dis, 25: 50-58
[101] Kossmann T, Hans VH, Imhof HG, Stocker R, Grob P, Trentz O, et al. (1995). Intrathecal and serum interleukin-6 and the acute-phase response in patients with severe traumatic brain injuries. Shock, 4: 311-317
[102] Ruparelia N, Digby JE, Jefferson A, Medway DJ, Neubauer S, Lygate CA, et al. (2013). Myocardial infarction causes inflammation and leukocyte recruitment at remote sites in the myocardium and in the renal glomerulus. Inflammation research: official journal of the European Histamine Research Society ... [et al.], 62: 515-525
[103] Fukui D, Yasukawa H, Sugi Y, Oba T, Nagata T, Kyogoku S, et al. (2012). Transient reduction and activation of circulating dendritic cells in patients with acute myocardial infarction. Int J Cardiol, 160: 216-219
[104] Urra X, Cervera A, Obach V, Climent N, Planas AM, Chamorro A (2009). Monocytes are major players in the prognosis and risk of infection after acute stroke. Stroke, 40: 1262-1268
[105] Riegger T, Conrad S, Liu K, Schluesener HJ, Adibzahdeh M, Schwab JM (2007). Spinal cord injury-induced immune depression syndrome (SCI-IDS). The European journal of neuroscience, 25: 1743-1747
[106] Menges P, Kessler W, Kloecker C, Feuerherd M, Gaubert S, Diedrich S, et al. (2012). Surgical trauma and postoperative immune dysfunction. Eur Surg Res, 48: 180-186
[107] Riegger T, Conrad S, Schluesener HJ, Kaps HP, Badke A, Baron C, et al. (2009). Immune depression syndrome following human spinal cord injury (SCI): a pilot study. Neuroscience, 158: 1194-1199
[108] Kirchhoff C, Biberthaler P, Mutschler WE, Faist E, Jochum M, Zedler S (2009). Early down-regulation of the pro-inflammatory potential of monocytes is correlated to organ dysfunction in patients after severe multiple injury: a cohort study. Crit Care, 13: R88
[109] Klehmet J, Harms H, Richter M, Prass K, Volk HD, Dirnagl U, et al. (2009). Stroke-induced immunodepression and post-stroke infections: lessons from the preventive antibacterial therapy in stroke trial. Neuroscience, 158: 1184-1193
[110] Li M, Li F, Luo C, Shan Y, Zhang L, Qian Z, et al. (2011). Immediate splenectomy decreases mortality and improves cognitive function of rats after severe traumatic brain injury. J Trauma, 71: 141-147
[111] Silveira FP, Nicoluzzi JE, Saucedo Junior NS, Silveira F, Nicollelli GM, Maranhao BS (2012). Evaluation of serum levels of interleukin-6 and interleukin-10 in patients undergoing laparoscopic versus conventional cholecystectomy. Rev Col Bras Cir, 39: 33-40
[112] Chu W, Li M, Li F, Hu R, Chen Z, Lin J, et al. (2013). Immediate splenectomy down-regulates the MAPK-NF-kappaB signaling pathway in rat brain after severe traumatic brain injury. The journal of trauma and acute care surgery, 74: 1446-1453
[113] Dziedzic T, Slowik A, Szczudlik A (2004). Nosocomial infections and immunity: lesson from brain-injured patients. Crit Care, 8: 266-270
[114] Wang KY, Yu GF, Zhang ZY, Huang Q, Dong XQ (2012). Plasma high-mobility group box 1 levels and prediction of outcome in patients with traumatic brain injury. Clinica chimica acta; international journal of clinical chemistry, 413: 1737-1741
[115] Peltz ED, Moore EE, Eckels PC, Damle SS, Tsuruta Y, Johnson JL, et al. (2009). HMGB1 is markedly elevated within 6 hours of mechanical trauma in humans. Shock, 32: 17-22
[116] Urra X, Cervera A, Villamor N, Planas AM, Chamorro A (2009). Harms and benefits of lymphocyte subpopulations in patients with acute stroke. Neuroscience, 158: 1174-1183
[117] Czlonkowska A, Korlak J (1979). [Humoral immune response in patients with stroke]. Pol Tyg Lek, 34: 485-487
[118] Cheng X, Liao YH, Ge H, Li B, Zhang J, Yuan J, et al. (2005). TH1/TH2 functional imbalance after acute myocardial infarction: coronary arterial inflammation or myocardial inflammation. Journal of clinical immunology, 25: 246-253
[119] Griffin GD (2011). The injured brain: TBI, mTBI, the immune system, and infection: connecting the dots. Mil Med, 176: 364-368
[120] Wolach B, Sazbon L, Gavrieli R, Broda A, Schlesinger M (2001). Early immunological defects in comatose patients after acute brain injury. J Neurosurg, 94: 706-711
[121] Schulze J, Zierath D, Tanzi P, Cain K, Shibata D, Dressel A, et al. (2013). Severe stroke induces long-lasting alterations of high-mobility group box 1. Stroke, 44: 246-248
[122] Kawabata H, Setoguchi T, Yone K, Souda M, Yoshida H, Kawahara K, et al. (2010). High mobility group box 1 is upregulated after spinal cord injury and is associated

with neuronal cell apoptosis. Spine (Phila Pa 1976), 35: 1109-1115

[123] Angele MK, Chaudry IH (2005). Surgical trauma and immunosuppression: pathophysiology and potential immunomodulatory approaches. Langenbecks Arch Surg, 390: 333-341

[124] Castillo L, Velasco N, Wainstein C, Bugedo G, Hernandez G, Klaassen J, et al. (1999). [Transcranial cytokine gradient and intestinal permeability in acute severe brain injury]. Rev Med Chil, 127: 639-646

[125] Hernandez G, Hasbun P, Velasco N, Wainstein C, Bugedo G, Bruhn A, et al. (2007). Splanchnic ischemia and gut permeability after acute brain injury secondary to intracranial hemorrhage. Neurocrit Care, 7: 40-44

[126] Jiang XH, Li N, Li JS (2003). Intestinal permeability in patients after surgical trauma and effect of enteral nutrition versus parenteral nutrition. World J Gastroenterol, 9: 1878-1880

[127] O'Boyle CJ, MacFie J, Mitchell CJ, Johnstone D, Sagar PM, Sedman PC (1998). Microbiology of bacterial translocation in humans. Gut, 42: 29-35

[128] MacFie J (1997). Bacterial translocation in surgical patients. Ann R Coll Surg Engl, 79: 183-189

[129] Bansal V, Costantini T, Ryu SY, Peterson C, Loomis W, Putnam J, et al. (2010). Stimulating the central nervous system to prevent intestinal dysfunction after traumatic brain injury. J Trauma, 68: 1059-1064

[130] Heffernan DS, Inaba K, Arbabi S, Cotton BA (2010). Sympathetic hyperactivity after traumatic brain injury and the role of beta-blocker therapy. J Trauma, 69: 1602-1609

[131] Salehpoor F, Bazzazi AM, Estakhri R, Zaheri M, Asghari B (2010). Correlation between catecholamine levels and outcome in patients with severe head trauma. Pak J Biol Sci, 13: 738-742

[132] Woolf PD, Hamill RW, Lee LA, Cox C, McDonald JV (1987). The predictive value of catecholamines in assessing outcome in traumatic brain injury. J Neurosurg, 66: 875-882

[133] Ruhnau J, Schulze K, Gaida B, Langner S, Kessler C, Broker B, et al. (2014). Stroke alters respiratory burst in neutrophils and monocytes. Stroke, 45: 794-800

3 Appendix

3.1 Short Summary

Ischemic stroke is the second leading cause of death worldwide and a disease with a variety of risk factors including hypotension, nutrition/obesity, and smoking but also increased age. In an ageing society stroke is a great challenge and leaves the survivors with disabilities. The aim of this dissertation was to investigate the immunologic changes post ischemic stroke, in order to use a better understanding for new therapeutic approaches as well as for improvement of translation of results from bench to bedside. Ischemic stroke leads to a local and peripheral immune activation. On the other side an immune dysfunction/suppression occurs, that leads to a higher risk of stroke-associated infections. In this dissertation, a long-lasting elevation of HMGB1 after stroke and a correlation with blood leukocyte numbers could be shown. HMGB1 seems to be an important mediator of an endogenous inflammation and an interesting target for post-stroke immunomodulation. In a further study we showed that the quality of the immune response of infiltrating T cells has an impact on the neurologic outcome and functional recovery after experimental stroke. Importantly, a mechanism of how infections, mimicked by LPS injections, could worsen the outcome of stroke patients was revealed. In the context of stroke-induced immunosuppression regulatory T cells as an immunosuppressive T cells subset seem to not play a role as their suppressive capacity is reduced after stroke. Interestingly, the CD39 expression on Tregs is similarly increasing with age in humans and mice. This shows the importance of an age equivalent in experimental studies. In search of predictors for the outcome after stroke as well as the risk of infections, we performed single nucleotide polymorphism genotyping in the *IL-1RN* and *TLR4* gene of stroke patients. Functional significant variants in the *IL-1RN* and *TLR4* genes may have an impact on outcome and systemic markers of inflammation post stroke but these findings need to be replicated in studies with much larger cohorts.

3.2 Kurzzusammenfassung

Der Schlaganfall ist die zweithäufigste Todesursache weltweit und eine Erkrankung mit vielfältigen Risikofaktoren, unter anderem Bluthochdruck, Ernährung/Adipositas, Rauchen aber auch hohem Alter. In einer alternden Gesellschaft ist der Schlaganfall eine große Herausforderung in der Behandlung und hinterlässt die Überlebenden häufig mit Behinderungen. Ziel dieser Arbeit war es, die immunologischen Veränderungen in der Folge des ischämischen Schlaganfalls zu untersuchen, um durch das bessere Verständnis neue Therapieansätze generieren und die Translation experimenteller Studien in die Klinik zu verbessern. In der Folge einen ischämischen Schlaganfalls kommt es lokal sowie peripher zu einer Immunaktivierung. Auf der anderen Seite tritt aber auch eine Immundysfunktion/-suppression auf, die zu einem erhöhten Risiko für Schlaganfall-assoziierte Infektionen führt. In dieser Arbeit wurde gezeigt, dass HMGB1 nach einem Schlaganfall langanhaltend im Plasma erhöht ist und mit der Leukozytenzahl im Blut korreliert. HMGB1 scheint ein wichtiger Mediator der endogenen Inflammation zu sein und wäre interessant für eine immunmodulatorische Intervention. In einer weiteren Studie dieser Arbeit wurde gezeigt, dass die Qualität der Immunantwort von infiltrierenden T-Zellen einen Einfluss auf das neurologische Outcome sowie die funktionelle Erholung nach Schlaganfall hat. Außerdem konnte stellvertretend durch LPS gezeigt werden, wie Infektionen zu einem verschlechterten Outcome bei Schlaganfall-Patienten führen könnten. In einer Studie über regulatorische T-Zellen wurde festgestellt, dass diese vermutlich keinen Einfluss auf die Schlaganfall-induzierte Immunsuppression haben, da ihre suppressive Aktivität nach Schlaganfall reduziert ist. Die Expression von CD39 auf der Oberfläche von Tregs zeigt bei Menschen und Mäusen einen ähnlichen Anstieg mit zunehmendem Alter. Dies liefert wichtige Hinweise für eine Notwendigkeit von gealterten Mäusen in tierexperimentellen Schlaganfallstudien. Auf der Suche nach Prädiktoren für das Outcome nach Schlaganfall sowie das Risiko für Infektionen wurden Studien zum Auftreten von verschiedenen Einzelnucleotidpolymorphismen im *IL-1RN* und *TLR4* Gen durchgeführt. Funktionell signifikante Varianten in den *IL-1RN* und *TLR4* Genen können zum Outcome und zu Veränderungen von systemischen Entzündungsmarkern nach akutem ischämischem Schlaganfall beitragen. Die Ergebnisse sollten jedoch noch in einer größer angelegten Studie repliziert werden.

3.3 Abbreviations & Figures

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BBB	Blood brain barrier
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CRP	C-reactive protein
CSF	cerebrospinal fluid
CTL	cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte antigen
DAMP	Danger-associated molecular pattern
DALY	Disability adjusted life years
DBH	Dopamine beta hydroxylase
Dereg	Depletion of regulatory T cells
G-CSF	granulocyte colony stimulating factor
GFP	green fluorescent protein
HLA-DR	Human leukocyte antigen-
HMGB	High mobility group box protein
HPA axis	Hypothalamic-pituitary-adrenal axis
IFN	Interferon
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
IS	Immune system
LPS	Lipopolysaccharide
MBP	Myelin basic protein
MCAO	Middle cerebral artery occlusion
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
pRCT	preclinical randomized controlled multicenter trial

Rag	Recombination-activating genes
RAGE	Receptor for advanced glycation end products
rtPA	Recombinant tissue plasminogen activator
SAI	Stroke-associated infection
SCID	Severe combined immunodeficiency
SHIP	Study of Health In Pomerania
SIIS	Stroke induced immunosuppression
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
STAIR	Stroke Treatment Academic Industry Roundtable
TGF	tumor growth factor
TNF	tumor necrosis factor
Th cell	T helper cell
TLR	toll-like receptor
Treg	regulatory T cell
UTP	uridine triphosphate
VLA	very late antigen
VCAM	vascular cell adhesion molecule
WBC	white blood cell
WHO	World Health Organization

Figures

Figure 1	Figure modified from (Vogelgesang et al., 2014). The numbers in the picture indicate the different approaches with which the immunologic changes following stroke where investigated in this dissertation.
Figure 2a	Graph modified from (Gelderblom et al., 2009). The figure depicts the time course and amount of immune cells infiltrating into the ischemic hemisphere after stroke.
Figure 2b	Graph modified from (Gelderblom et al., 2009). The figure shows the time course and amount of infiltration of lymphocyte subsets (only summarized as lymphocytes in Fig.2a) into the ischemic hemisphere post stroke.

3.4 Publications

Original Papers

Ruhnau J, **Schulze J**, von Sarnowski B, Heinrich M, Langner S, Pötschke C, Wilden A, Kessler C, Bröker BM, Vogelgesang A, Dressel A. Reduced Numbers and Impaired Function of Regulatory T Cells in Peripheral Blood of Ischemic Stroke Patients. Mediators Inflamm. 2016; 2016:2974605. doi: 10.1155/2016/2974605. Epub 2016 Mar 17.

Weinstein JR, **Schulze J**, Lee RV, Phillips H, Zierath D, Tanzi P, Shibata D, Cain KC, Becker KJ. Functional polymorphisms in toll-like receptor 4 are associated with worse outcome in acute ischemic stroke patients. Neuroreport. 2014 May 28; 25(8):580-4. doi: 10.1097/WNR.0000000000000140. PMID: 24784586

Becker KJ, Dankwa D, Lee R, **Schulze J**, Zierath D, Tanzi P, Cain K, Dressel A, Shibata D, Weinstein J. Stroke, IL-1ra, IL1RN, infection and outcome. Neurocrit Care. 2014 Aug; 21(1):140-6. PMID: 24233813

Zierath D, **Schulze J**, Kunze A, Drogomiretskiy O, Nhan D, Jaspers B, Dressel A, Becker K. The immunologic profile of adoptively transferred lymphocytes influences stroke outcome of recipients. J Neuroimmunol. 2013 Oct 15;263(1-2):28-34. doi: 10.1016/j.jneuroim.2013.07.014. Epub 2013 Jul 29.PMID: 23948692

Schulze J, Zierath D, Tanzi P, Cain K, Shibata D, Dressel A, Becker K. Severe stroke induces long-lasting alterations of high-mobility group box 1.Stroke. 2013 Jan;44(1):246-8. doi: 10.1161/STROKEAHA.112.676072. Epub 2012 Nov 29. PMID:23204053

Reviews

Schulze J, Vogelgesang A, Dressel A. Catecholamines, steroids and immune alterations in ischemic stroke and other acute diseases. Aging Dis. 2014 Oct 1;5(5):327-39. doi: 10.14336/AD.2014.0500327. eCollection 2014 Oct. Review. PMID: 25276491

Abstracts

Schulze J, Ruhnau J, von Sarnowski B, Heinrich M, Langner S, Pötschke C, Wilden A, Kühn J, Kessler C, Bröker BM, Dressel A, Vogelgesang, A. Age dependent effects of Treg depletion on brain lesion development in experimental stroke. 9th International Symposium on Neuroprotection and Neurorepair, Leipzig 2016

Schulze J, Ruhnau J, Dressel A, Vogelgesang A. Impaired Regulatory Tcell Function in Stroke Patients and Experimental Stroke in Aged Mice, European Stroke Organisation Conference 2015, Glasgow

Schulze J, Henck V, Langner S, Bröker BM, Dressel A, Vogelgesang A. Depletion regulatorischer T-Zellen (Treg) im experimentellen Schlaganfallmodell. 73. Jahrestagung der Gesellschaft für Nervenheilkunde Mecklenburg-Vorpommern, Neubrandenburg, 2014

Schulze J, Ziebart J, Henck V, Langner S, Bröker BM, Vogelgesang A, Dressel A. Experimental Stroke: serial 7T MRI study of Treg-depleted mice. 8th International Symposium on Neuroprotection and Neurorepair, Magdeburg, 2014

Baguhl R, Joseph C, Tsakmakides T, **Schulze J**, Dressel A. Impact of caloric restriction on aged stroked rats: evidence from neurogenesis and infl ammation in the brain. 2nd International Workshop “Molecular Imaging in Medical Research”, EnVision, Greifswald, 2013

Sander C, **Schulze J**, Vogelgesang A, Ziebart J, Dressel A. Germinal centers and Tregs following experimental stroke - Imaging the spleen of DBH and Dereg mice. 2nd International Workshop “Molecular Imaging in Medical Research”, EnVision, Greifswald, 2013

Schulze J, Zierath D, Tanzi P, Shibata D, Dressel A, Becker KJ**.** HMGB1 levels following stroke-predictor of long term outcome? International Stroke Conference, New Orleans, USA 2012 (ranked among the top 10 percent of the AHA’s specialty conferences 2012)

Weinstein JR, **Schulze J**, Lee RV, Zierath D, Tanzi P, Shibata D, Becker KJ. Functional Polymorphisms in Toll-like Receptor 4 Predict Worse Outcome in Acute Ischemic Stroke Patients, International Stroke Conference, New Orleans, USA 2012

Zierath D, **Schulze J**, Kunze A, Becker KJ. Autoimmune Responses to Brain following Stroke: an Epiphenomenon with Consequences, Stroke Meeting, Antalya, Turkey 2011