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Translational aspects of postischaemic inflammation

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Content

1 Aim of the study

Within only a few minutes acute ischaemic stroke leads to deprivation of oxygen and glucose supply as well as subsequent necrotic cell death in the brain parenchyma. These events trigger the release of mediators and danger signals, which in turn promote the evolvement of a severe postischaemic inflammatory response. Research in the mouse model has shown that the innate as well as the adaptive immunity contribute to these processes. Among others, T cells and neutrophils are key players in the pathophysiology of postischaemic inflammation. Recently, the roles of the inflammatory cytokine IL-17 and the innate-like γδ T cells have been highlighted in the mouse model. In humans, there is also evidence for an involvement of neutrophils, conventional T cells and IL-17. The role of γδ T cells remains unclear so far.

This study was designed to gain further insight into the pathophysiology of stroke in humans and to find evidence for the transferability of the mice findings to humans. We analysed the absolute numbers and frequencies of different leukocyte subsets in the peripheral blood of stroke patients. The main focus was laid on the different T cell subsets and we raised the question if there are observable differences in the distribution of T cell subsets after stroke. We also analysed these T cell subsets in the cerebrospinal fluid and investigated the cytokine profile of CSF γδ T cells. Additionally, we performed *in vitro* experiments with γδ T cells from healthy donors to further characterise the phenotype and functional properties of these cells. In order to find a link between IL-17 and neutrophil infiltration, we assessed the effect of IL-17 on the production of neutrophil chemoattractants by brain endothelium *in vitro*. By analysing the activation status of neutrophils in the peripheral blood we tried to provide further evidence for an involvement of these cells in human postischaemic inflammation.

2 Zielsetzung

In der Pathophysiologie des akuten Schlaganfalls steht anfänglich der Mangel von essentiellen Stoffwechselprodukten wie Sauerstoff und Glukose im Vordergrund, welcher innerhalb von wenigen Minuten zu dem Untergang von Hirngewebe führt. Infolge der Freisetzung von Botenstoffen und Warnsignalen im Rahmen des Gewebeunterganges entwickelt sich die sogenannte postischämische Entzündungsreaktion, die eine Zunahme der Gewebezerstörung bewirkt. Forschungsarbeiten an der Maus haben gezeigt, dass sowohl die unspezifische als auch die adaptive Immunität an diesen Abläufen beteiligt sind. T-Zellen und neutrophile Granulozyten scheinen, neben anderen, eine entscheidende Rolle in der Pathophysiologie der postischämischen Entzündung zu spielen. Kürzlich wurde in diesem Zusammenhang hervorgehoben, dass das inflammatorische Zytokin IL-17 und die γδ T-Zellen, die den Zellen der angeborenen Immunantwort ähneln, eine entscheidende Rolle spielen. Klinische Studien am Menschen konnten genauso Beweise für die Beteiligung von konventionellen T-Zellen und neutrophilen Granulozyten liefern. Die Funktion der γδ T-Zellen ist derzeit noch nicht geklärt.

Das Hauptziel der vorliegenden Arbeit war es, Einsicht in die pathophysiologischen Vorgänge nach einem ischämischen Schlaganfall zu erlangen und die Anwendbarkeit der Kenntnisse aus dem Tiermodel auf den Menschen zu überprüfen. Zu diesem Zweck untersuchten wir Blut und Liquor von Patienten, die einen Schlaganfall erlitten hatten. Den Schwerpunkt der Studie legten wir auf die Analyse der verschieden T-Zell Populationen. Wir betrachteten, inwiefern die Verteilung der verschiedenen Suptypen im Blut von einem Schlaganfall beeinflusst wird. Weiterhin untersuchten wir die Verteilung dieser Zellen im Liquor und analysierten die Zytokinproduktion der dort zu findenden γδ T-Zellen. Um mehr Kenntnisse bezüglich der humanen γδ T-Zellen zu gewinnen, betrachteten wir Phenotyp und funktionelle Eigenschaften dieser Zellen aus gesunden Spendern *in vitro*. Zusätzlich untersuchten wir den Effekt von IL-17 auf die Chemokinproduktion von humanen Endothelzellen, mit der Absicht, einen Zusammenhang zwischen der Gewebsinfiltration durch Neutrophile und diesem Zytokin herzustellen. Diesem Ansatz weiter folgend, suchten wir in Seren von Schlaganfallpatienten nach Parametern, die eine Aktivierung der Neutrophilen vermuten ließen.

3 Introduction

3.1 Ischaemic stroke

The World Health Organisation has classified a stroke as a "neurological deficit of cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours". Stroke represents the third leading cause of death in the western world and is the most common cause of disability in adults. The incidence is estimated to be around 130-340/100.000 (Khaw and Kessler 2006). One of the major risk factors is hypertension, followed by increasing age, diabetes mellitus and heart conditions, such as atrial fibrillations. Smoking, obesity and hyperlipidaemia also lead to an elevated risk. Moreover, differences among family, race and gender have been observed.

Strokes can be divided into the two major categories of haemorrhagic and ischaemic, with the latter one being the more frequent subtype in 75-80% (Khaw and Kessler 2006) of the cases. An ischaemic stroke is caused by the loss of cerebral blood-flow in a localised area. In its classic manifestation, a thromboembolism, originating from the surface of ulcerous, arteriosclerotic plaques, or an embolus, generated in the heart, provokes the stroke. Alternatively, a stroke can be caused by vasculitis, arterial dissections and haemodynamic alterations.

The restriction of blood supply leads to permanent damage of neuronal cells and therefore loss of function in the affected part of the brain. This can cause severe neurological deficits. Immediately post-stroke excitotoxic mechanisms are responsible for the lethal damage of the neuronal tissue. However, within hours, a severe inflammatory response emerges. This sterile inflammation involves a massive influx of various immune cells and the release of many inflammatory mediators, deteriorating the tissue damage.

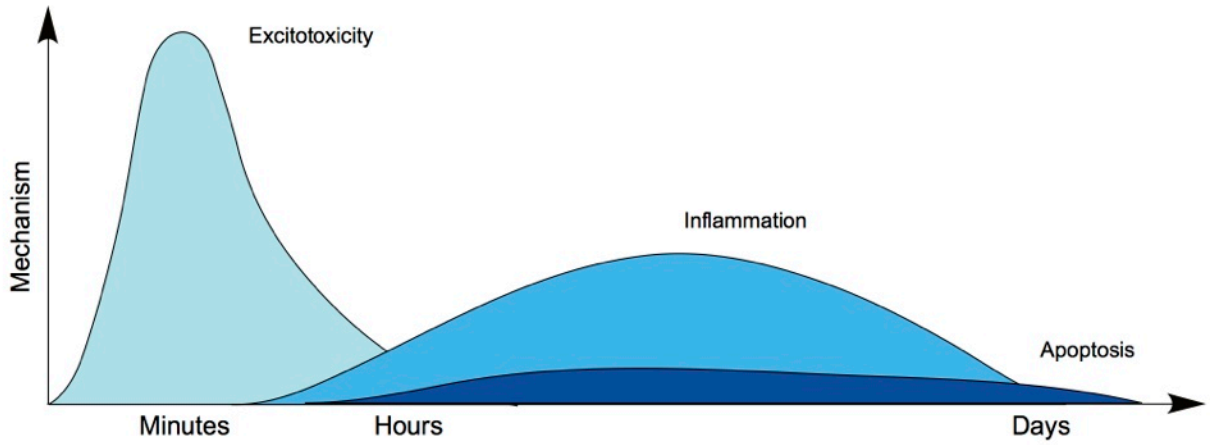

Figure 1: Early excitotoxic mechanisms contribute to the tissue damage in the ischaemic core. However, within hours an inflammatory reaction aggravates the injury and conduces further lethal damage of cells in the penumbra area (adapted from Dirnagl, Iadecola et. al., 1997)

3.2 The immune system and its key players in postischaemic inflammation

The immune system consists of a variety of effector cells and molecules that protect the body against external and internal threats. Two arms can be recognised: The innate and the adaptive immune system.

3.2.1 Innate immunity

The innate immune system predates the evolution of the adaptive immunity. It recognises pathogens in a non-specific manner and acts as a first-line defence. The innate immune system becomes activated after the recognition of certain patterns that are conserved among extensive groups of pathogens, but can also sense alarm signals from injured and stressed cells. It consists of humoral components like the complement system, and cellular ones, such as granulocytes, macrophages, dendritic and natural killer cells. Important mechanisms are the removal of foreign substances through phagocytosis and production of cytokines to recruit immune cells to the site of inflammation. Other crucial tasks are the processing of antigen in order to activate the adaptive immune system as well as the provision of costimulatory signals.

3.2.1.1 Neutrophil granulocytes

Neutrophils constitute an essential part of the innate immune system as the very first line of defence. They rise from stem cells in the bone marrow, mature there completely during granulocytopoesis and eventually start circulating the bloodstream. The name granulocyte is derived from the cytoplasmatic granules, which contain specific enzymes and peptides directed against microbial and other pathogens. They are the most abundant leukocyte population in the human blood, but have an estimated halftime of only 90 hours (Pillay, den Braber et al. 2010). Circulating neutrophils either undergo apoptosis and are degraded in spleen and liver by macrophages or migrate to injured or infected tissue. They die there while expelling their cytotoxic contents in order to protect the body against danger and pathogens.
To enter the extravascular space they depend on interaction with the endothelium. Upon activation with stimuli such as TNFα (tumour necrosis factor α), IL-1β, (interleukin 1β) or IL-17 (interleukin 17), endothelial cells upregulate cell adhesion molecules like P- and E-selectins and establish the initial contact. Firmer adhesion is mediated by the interaction of ICAM-1 (intracellular adhesion molecule 1) and ICAM-2 on the endothelium with the β_2-integrin LFA-1 (lymphocyte function-associated antigen 1) on the neutrophils, followed by transcellular or paracellular migration through the endothelium. Furthermore, the activated endothelium provides chemokines like IL-8 and CXCL2 (Chemokine C-X-C motif ligand 2), which operate in a chemotactic as well as an activating way for the neutrophils (Borregaard

2010). Recruitment to the site of inflammation in the tissue is driven by a gradient of local chemokines. Subsequently, neutrophils produce cytokines themselves to activate other immune cells and amplify the inflammatory response.
To eliminate pathogens, neutrophils can either phagocytose opsonised microorganisms and digest them in the phagosomes, or release their granules with antimicrobial peptides and enzymes. Recently, a third mechanism has been described in which neutrophils release fibres of DNA (desoxyribonucleic acid) together with embedded granular contents (Brinkmann, Reichard et al. 2004). These formations are called neutrophil extracellular traps (NETs) and can trap and kill bacteria extracellularly. NETs also seem to harbour activation properties in different diseases (Gupta, Hasler et al. 2007; Kessenbrock, Krumbholz et al. 2009).

3.2.1.2 Macrophages

Monocytes develop into macrophages in the tissue under the influence of distinct cytokines. Attracted through chemotaxis these macrophages migrate to inflamed tissue and contribute to acute and chronic inflammation. Their main function is the phagocytosis of pathogens and debris to prevent damage to the body (Murphy 2008). Moreover, they have an important secretory function: They produce anti-inflammatory cytokines such as IL-10 (interleukin 10) and TGF-β (transforming growth factor β), proinflammatory cytokines like IL-1β, TNFα and IL-6 (interleukin 6), as well as reactive oxygen species. They are also able to process antigen, present it by MHC (major histocompatibility complex) class II-molecules and activate the adaptive immune system. Organ resident macrophages are a special subtype, which are specific for a particular part of the body and have distinct morphology and phenotypes (Gordon and Martinez 2010). They are constitutively present, fulfil basic trophic and homeostatic function and are among others responsible for tissue remodelling and the secretion of various cytokines, growth factors and metabolites.

3.2.1.3 Microglia

Microglia are the resident immune cells of the brain and the only cells in the healthy central nervous system parenchyma with a myeloid origin. They constitute about 10% of the cells in the adult human brain (Ransohoff and Cardona 2010). Microglial cells are mononuclear phagocytes, originating from monocytes that populate the embryonic brain early in development and then differentiate into microglia. They are self-renewing and do not develop from infiltrating monocytes throughout life (Ajami, Bennett et al. 2011).
The microglia of the healthy brain has been termed ‚resting' and has a ramified morphology. This phenotype maintains homeostasis in the brain, cleans up cellular debris and constantly surveils the neuronal tissue (Ransohoff and Cardona 2010). Triggered by pro-inflammatory

cytokines, changes in the extracellular potassium or cell necrosis, microglia enlarge their soma, upregulate myeloid markers and adopt a more ameboid shape. Activated microglia can produce a large variety of mediators, including inflammatory cytokines like IL-1β and TNFα. Furthermore, they provide chemokines, which direct immune cells to the site of inflammation, and reactive oxygen species, contributing to acute and chronic inflammation. Microglia can also phagocytose, process antigen and activate T cells.

3.2.2 Adaptive immunity

In contrast to the innate immunity, which is non-specific and operates quickly, the adaptive immunity requires several days after an antigen challenge to gain functionality. It is, however, able to mount a specific and much stronger response. During the early development of B and T cells, which represent the cellular components of the adaptive immunity, a vast number of different antigen-receptors is created, which is unique to each individual lymphocyte. Cells that recognise self-antigens are eliminated quickly; the others migrate to the periphery and potentially encounter their antigen. Upon activation through a specific antigen one cell can expand clonally and mount the specific response. The interaction with cells of the innate immune system, which provide important costimulatory signals, is essential for the clonal expansion. Activated B cells produce soluble antibodies which constitute the humoral component of the adaptive immunity. The two different effector types of T cells either show high cytotoxic activity ($CD8^+$) or supportive function ($CD4^+$), amplifying the cellular and humoral responses of the immune system. Another important feature of the adaptive immune system is the ability to create an immunological memory, which ensures a much faster response upon activation by an antigen the body has encountered before.

3.2.2.1 $CD4^+$ cells

T helper cells (T_h) originate from the bone marrow and populate the thymus, where they undergo several selection steps to acquire self-reference and avoid autoreactivity. In contrast to most other immune cells they are not directly involved in killing pathogens, but rather play an important role in providing signals for the immune system. After thymic development they enter the blood stream and migrate to the lymphoid organs. When a T cell encounters its antigen, the T cell receptor (TCR) recognises a specific domain presented by MHC class II-molecules on a professional antigen presenting cell (APC). Subsequently, the activation cascade is turned on when the costimulatory molecules CD80 and CD86 interact with the CD28 on the T cell. The cell starts to proliferate and upregulates the IL-2 receptor in addition to secreting IL-2, which is an essential cytokine for growth and survival of T cells.

After activation they can develop into one of the different lineages Th1, Th2, Th17 or inducible T regulatory cells. The differentiation into Th1 cells requires the presence of IL-12 (interleukin 12), and these cells then produce cytokines like IFNγ (interferon γ), promoting macrophage activation in order to eliminate intracellular pathogens. Th2 cells develop under the influence of IL-4 (interleukin 4) and drive the humoral response by stimulating B cells to produce soluble antibodies and antibody class switch. Th 17 cells need TGFβ and IL-6 (interleukin 6) to evolve, mainly produce IL17-A and F, and play a major role in fighting pathogens like *candida* and *staphylococcus*. Both Th1 and Th17 are also involved in autoimmunity and tissue inflammation (Veldhoen 2009). The fourth subset are the inducible T regulatory cells (iTreg), identified by the transcription factor forkhead box protein 3 (FOXP3) (Curotto de Lafaille and Lafaille 2009). They evolve in the periphery under the influence of TGFβ and the absence of IL-6 and are responsible for maintaining peripheral tolerance. Furthermore other subsets like the Th9 (Veldhoen, Uyttenhove et al. 2008) or follicular T helper cells (Vinuesa, Tangye et al. 2005) have been proposed and the enigma about the plasticity among the $CD4^+$ lineages is yet to be solved (Murphy and Stockinger 2010).

3.2.2.2 Naturally occurring regulatory T cells

Just like other T cell subpopulations naturally occurring regulatory T cells (nTreg) become committed to their lineage in the thymus. Probably correlating with the purpose of maintaining peripheral tolerance, they have some limited affinity to self-peptides. They play an essential role in preventing autoimmune disease and limiting chronic inflammatory processes (Vignali, Collison et al. 2008).

Human regulatory T cells are identified by the expression of the transcription factor FOXP3 (Hori, Nomura et al. 2003) and the surface markers $CD4^+CD25^{high}CD127^{low}$ (Seddiki, Santner-Nanan et al. 2006). They employ various suppression mechanisms such as the secretion of the inhibitory cytokines IL-10 and TGF-β, metabolic disruption of other T cells due to IL-2 deprivation, or the inhibition of maturation and functioning of dendritic cells (Vignali, Collison et al. 2008). Imbalance and malfunction of T regulatory cells can lead to severe immune dysfunction (Kim, Rasmussen et al. 2007).

3.2.2.3 γδ T cells

γδ T cells represent a small subset of circulating T cells. Unlike conventional αβ T cells, where the TCR consists of an α and a β chain, these cells use a γ and a δ chain. Similar to conventional T cells they originate from stem cells in the bone marrow and populate the thymus. Whereas αβ T cells leave the thymus continuously, γδ T cells, however, emigrate in

waves, which also takes place earlier in development (Dunon, Courtois et al. 1997; Murphy 2008). Probably, they are the first T cells to develop in all species (Hayday 2000) and the first having a mature character (De Rosa, Andrus et al. 2004). Thus, γδ T cells potentially play a role in the immunity of early life, compensating for the not yet fully developed adaptive immune system (Moens, Brouwer et al. 2011). This is supported by the fact that they are critical in neonatal control of infection (Cairo, Mancino et al. 2008; Vermijlen, Brouwer et al. 2010). The γδ T cell repertoire changes throughout life. This can not only be seen in decreasing numbers (Giachino, Granziero et al. 1994), but also in the tendency to less variety, suggesting that the adult cells are only some remaining representatives (Morita, Parker et al. 1994).

In contrast to conventional T cells their TCR repertoire has limited diversity; the majority does not express the coreceptors CD4 or CD8 and is not MHC class I or II restricted. Only rarely do γδ T cells recognise antigens in the form of peptides. They can, nevertheless, identify unconventional antigens like phosphorylated microbial metabolites and lipid antigens (Beetz, Wesch et al. 2008) through MHC class I-like molecules as well as probably other yet unknown receptors. It has been reported that they can be directly activated via toll-like receptors (TLR) (Pietschmann, Beetz et al. 2009). Another uncommon feature is the ability to efficiently process antigen, leading to αβ T cell proliferation and differentiation, with the γδ T cells even providing the necessary costimulatory signals (Brandes, Willimann et al. 2005). Although belonging to the T cell lineage, γδ T cells share many properties with cells of the innate immune system. This makes them a unique cell type playing a substantial role at the cross-roads of innate and adaptive immunity. On the one hand they participate in the first-line defence, react rapidly upon stimulation, and can activate adaptive immune cells; on the other hand they also express a clonally variable antigen receptor and harbour immunological memory.

The relative quantity of γδ T cells varies to a great degree among species and tissue distribution in the body. In adult humans they account for 2-6 % of the T cells in the peripheral blood, but are particularly enriched in intraepithelial sites, where they can account for as much as 40% of the T cells (Meresse and Cerf-Bensussan 2009). The classification of human γδ T cells is based on the expression of the different Vγ and Vδ chains, leading to at least two major subtypes. Cells expressing the Vδ1 chain are residing the tissue and represent high proportions of lymphocytes in the skin, lung, intestine, female reproductive tract and tongue (Zheng 2002). The Vδ1 chain can pair with any of the Vγ chains, which in mice often shows an allocation to a specific tissue. This subset displays a more regulatory phenotype. They also express higher levels of genes like CCR7 (chemokine C-C motif receptor 7) and CD62-L (CD62-ligand), resulting in homing to lymphoid and non-inflamed tissue (Kress, Hedges et al. 2006), where they fulfil basic homeostatic functions. Cells bearing the Vδ2

chain in turn express more genes involved in inflammation (Kress, Hedges et al. 2006) and preferably pair with the Vγ9 chain. In adults they constitute between 50-90% of all γδ T cells in the peripheral blood (Schondelmaier, Wesch et al. 1993). The Vδ2 subset is activated by various microorganisms like *listeria* or *plasmodium* and plays an important role in tumour clearance (Nedellec, Bonneville et al. 2010). Phosphoantigens, which are endogenous metabolites of the cholesterol pathway, are the physiological stimuli in this context. They exclusively stimulate the Vδ2γ9 subset (Tanaka, Morita et al. 1995). Most microbial organisms use the non-mevalonate pathway to produce isoprenoids, and the byproduct 1-hydroxy-2-methyl-2-buten-4-yl-4-diphosphate (HDMAPP) is able to stimulate Vδ2 cells already at nano-molar concentrations. In contrast, eukaryotic cells use the mevalonate pathway. The byproduct dimethylallyl-phosphate (DMAPP) and its isomer isopentenyl-pyrophosphate (IPP) are also able to sufficiently stimulate Vδ2 cells, but in this context much higher concentrations are needed. These concentration depended activation ensures an efficient response against microorganisms. In this way the body‘s own cells are protected with the exception of tumour cells, which produce very large amounts of IPP and DMAPP.

Almost all γδ T cells can rapidly produce IFNγ upon stimulation (Beetz, Wesch et al. 2008) and are prominent sources of IL-17 in animal models of various diseases. IL-17 producing Vδ2 cells also exist in humans and the Vδ2γ9 subset can be driven into an $IL17^{+}IFN\gamma^{+}$ phenotype through the stimulation with HDMAPP, IL-1β, IL-23 and TGFβ (Ness-Schwickerath, Jin et al. 2010). These cells have been reported to express CCR6 as well as CD161 (Caccamo, La Mendola et al. 2011). CD161 is a marker expressed by all IL-17 producing human T cell subsets (Maggi, Santarlasci et al. 2010). Th17 cells, which show many parallels to IL-17 producing γδ T cells (Martin, Hirota et al. 2009), also express CCR6 and CD161 (Annunziato, Cosmi et al. 2008). Interestingly, the vast majority of adult Vδ2 cells show a non-naive phenotype (De Rosa, Andrus et al. 2004). This suggests that they have already been primed to produce their specific cytokine early in life. The polarisation of neonatal IL-17 producing Vδ2 cells requires different conditions (Ness-Schwickerath, Jin et al. 2010) and they seem to produce IL-17 only (Moens, Brouwer et al. 2011). Studies elucidating the properties of IL-17 producing γδ T cells have mostly focused on mice. There is no murine equivalent to the human Vδ2 cells and little is known about the phenotypic and functional characteristics of these cells.

3.3 Pathophysiology of postischaemic inflammation

Brain tissue is almost exclusively dependent on the supply of oxygen and glucose. The deprivation of these essential sources leads to an ischaemic cascade, causing failure of processes crucial for the viability of the cells. Neurones are most vulnerable to hypoxia (Lipton 1999), and cease to function after already 60-90 seconds under hypoxic conditions. The production of high energy phosphates cannot be maintained, leading to a disruption of the ionic membrane gradient. This causes an extensive increase of extracellular glutamate. The following activation of different glutamate receptors contributes to the accumulation of intracellular Ca^{2+}. This initiates cellular events that cause tissue damage, such as triggering necrosis as well as apoptosis and the production of free oxygen radicals (Dirnagl, Iadecola et al. 1999). Cells in the ischaemic core, where the supply is reduced the most, are irreversibly damaged and quickly undergo necrosis. The tissue in the periphery (penumbra) is still viable, but dysfunctional and extremely vulnerable to any incident in its surroundings. After the initial restriction of blood supply the situation deteriorates in the case of restored blood flow. Reperfusion and reoxygenation lead to an aggravation of tissue damage through the induction of a severe inflammatory, albeit sterile immune response (Eltzschig and Eckle 2011).

3.3.1 Sterile inflammation in the brain

Ischaemia affects neurones as well as glial cells and the endothelium. Even if the blood flow is restored again, cell clotting, pericyte contractions (Yemisci, Gursoy-Ozdemir et al. 2009) and imbalance of nitric oxide (NO) and reactive oxygen species (ROS) production in favour of the ROS (Grisham, Granger et al. 1998) impair the microvessel circulation. Furthermore, the endothelium, which is very vulnerable to reoxygenation (Carden and Granger 2000), reacts with the upregulation of transcription factors like NFκb (nuclear factor-κb) (Cummins, Berra et al. 2006) and HIF-1 (hypoxia inducible factor 1) (Ruscher, Isaev et al. 1998) through hypoxia-dependent mechanisms. Subsequently, the permeability of the endothelium increases (Ogawa, Gerlach et al. 1990; Ogawa, Koga et al. 1992), leading to a blood-brain barrier breakdown. Leukocyte adhesion molecules like ICAM-1 as well as P- and E-selectins are upregulated and promote leukocyte infiltration and inflammation. This exacerbates the postischaemic inflammation, a determining coefficient of the outcome of stroke.

The acute inflammatory response after ischaemia is partially physiological and occurs in any damaged tissue, also in the absence of microorganisms. It has an integral role in wound healing and tissue remodelling, removing cellular debris and hazardous agents to heal sustained damage (Chen and Nunez 2010). In the context of brain ischaemia, however, the response exaggerates and deteriorates the mechanism crucial for regeneration of tissue in the penumbra area. Many of the processes involved in sterile inflammation are similar to the

mechanisms observed during microbial infection. Pattern recognition receptors (PPRs) are not only important in sensing conserved microbial antigens, but also recognise dangerous non-infectious material and endogenous danger-associated molecular patterns (DAMPs), which are released following tissue injury. The increase of extracellular ATP (adenosine triphosphate), which is released by depolarised neuronal and glial cells (Melani, Turchi et al. 2005), is an early neuronal danger signal. This activates microglia via P2X7 (P2X purinoreceptor 7) (Cavaliere, Dinkel et al. 2005) as well as macrophages in the ROS dependent oxidative stress response (Cruz, Rinna et al. 2007). Subsequently, when cells start to suffer from necrosis, which is very immunostimulatory, more DAMPs are released. The results are immune cell infiltration and cytokine production (Basu, Binder et al. 2000; Scaffidi, Misteli et al. 2002). Double-stranded DNA and nucleotides, which are released by dying cells, act as ‚eat-and-find-me' signals for phagocytic cells (Elliott, Chekeni et al. 2009). They also activate inflammasomes, which results in IL-1β secretion (Burckstummer, Baumann et al. 2009; Hornung, Ablasser et al. 2009).

In addition, there is evidence that TLR engagement can induce a sterile inflammatory response (Chen and Nunez 2010) and that DAMP induced activation plays an important role in cerebral ischaemia (Brea, Blanco et al. 2011). HSP60 (heat shock protein 60) and HMGB1 (high-mobility group protein B1) can activate TLR 2 and 4 (Vabulas, Ahmad-Nejad et al. 2001; Yu, Wang et al. 2006) and the activation of TLR 4 is enhanced by oxidative stress (Powers, Szaszi et al. 2006). The stimulation of TLR receptors promotes the recruitment of several adaptive proteins to activate NF-κb. That in turn, increases the expression of signalling TLR 2 and 6 (Kuhlicke, Frick et al. 2007), which triggers the production of inflammation mediators like TNFa and IL-1β. Just recently, peroxiredoxins have been shown to induce the production of inflammatory cytokines like IL-23 in macrophages via TLR 2 and 4 (Shichita, Hasegawa et al. 2012). All these processes contribute to the inflammatory reaction and infiltration of immune cells.

3.3.2 Role of inflammatory cells

Research in the MCAO (middle cerebral artery occlusion) model in rodents has shown that infiltrating inflammatory cells play a crucial role in postischaemic inflammation. The cellular infiltrate of the ischaemic lesion is dominated by macrophages, microglia and neutrophils (Gelderblom, Leypoldt et al. 2009). The first two of those are increased already in the first 12 hours after stroke, whereas neutrophils reach their maximum around day three (Gelderblom, Leypoldt et al. 2009).

Although lymphocytes only constitute a small fraction of infiltrating cells, they play a prominent role in the evolvement of postischaemic inflammation. Recombination activating

gene-2 deficient mice that lack functional B and T cells (Shinkai, Rathbun et al. 1992) have much smaller infarct volumes for example. Further investigations attributed this effect to the absence of T cells (Yilmaz, Arumugam et al. 2006).

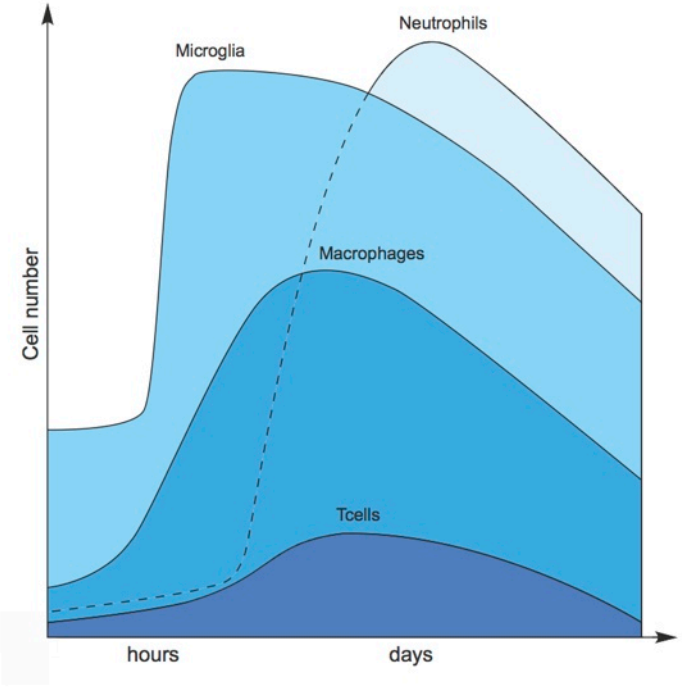

Figure 2: Time course of immune cells important in postischaemic inflammation (adapted from Gelderblom et al., 2009).

3.3.2.1 Innate immunity

Microglia are thought to be the first immune cells to be activated (Schilling, Besselmann et al. 2003) and produce inflammatory mediators like TNFα, IL-1β and IL-6 (Barone, Arvin et al. 1997; Rothwell, Allan et al. 1997). Their production of ROS might have further impact on the blood-brain-barrier breakdown (Yenari, Xu et al. 2006), which facilitates the migration of non-resident immune cells to the ischaemic lesion. Macrophages contribute to postischaemic inflammation in a similar way, but are recruited at a later point of time (Schilling, Besselmann et al. 2003). IL-1β, which is mainly released by the macrophages, induces an upregulation of adhesion molecules on endothelial cells for further recruitment of monocytes and neutrophils (Wang, Feuerstein et al. 1995; Gabay, Lamacchia et al. 2010).

Neutrophils in turn, account for a substantial number of infiltrating cells, which correlates with the severity of the brain tissue damage. They produce large amounts of ROS and pro-inflammatory cytokines, especially in response to necrotic cells. Although neutrophil-depleted mice have been shown to have smaller lesion sizes (Matsuo, Onodera et al. 1994; Hudome, Palmer et al. 1997), the specific role in contributing to ischaemia related tissue injury still needs to be elucidated.

3.3.2.2 Adaptive immunity

The role of adaptive immunity in general is controversial, as the temporal profile of involvement in brain damage is not consistent with established concepts of the adaptive immunity (Yilmaz, Arumugam et al. 2006). Although immunisation of T cells with myelin antigens decreases the lesion size (Becker, McCarron et al. 1997), it seems unlikely that an autoimmune response resulting from presentation of beforehand hidden CNS (central nervous system) antigens is the major pathophysiological mechanism. Adaptive immune cells emerge already 24 to 48 hours after stroke. Mice that lack co-stimulatory signals for T cell activation like CD28 or B7 molecules do not have smaller infarct volumes (Kleinschnitz, Schwab et al. 2010). Nevertheless, T cells are important in the pathophysiology of postischaemic inflammation and mice depleted of $CD4^+$ or $CD8^+$ cells show a reduction of lesion size (Yilmaz, Arumugam et al. 2006). $CD4^+$ T cells are important sources of IFNγ already early after stroke (Yilmaz, Arumugam et al. 2006; Shichita, Sugiyama et al. 2009). Although IFNγ-deficient mice have no smaller infarct volume (Shichita, Sugiyama et al. 2009) elevated IFNγ mRNA (messenger ribonucleic acid) levels are found in the ischaemic hemisphere (Li, Kostulas et al. 2001).

Shichita et al. recently highlighted the role of γδ T cells and IL-17, an important inflammatory cytokine that induces and mediates various inflammatory responses in the central nervous system. They noticed that these cells account for a major proportion of infiltrating T lymphocytes, although they only represent a small subset in the periphery, and were the predominant source of IL-17. IL-17-deficient mice, in turn, showed reduced infarct volume and lower mRNA levels of IL-1β and TNFα. Mice deficient in IL-23, a cytokine necessary to drive IL-17 production by T cells, also showed reduced infarct volumes.

But not all CNS-infiltrating T cell subsets are harmful. T regulatory cells for example, might be protective in brain ischaemia. Their cell number increases after stroke (Offner, Subramanian et al. 2006). IL-10 administration, with IL-10 being one of the major cytokines produced by these cells, results in a reduction of infarct size (Spera, Ellison et al. 1998) and lowers the cerebral expression of other pro-inflammatory cytokines (Liesz, Suri-Payer et al. 2009). The mechanisms and interactions are not fully understood yet. *In vivo* depletion of T regulatory cells through the administration of anti-CD25 enlarges the infarct size and the cytokines TNFα, INFγ and IL-1β are substantially upregulated. (Liesz, Suri-Payer et al. 2009). Still, it needs to be considered that the anti-CD25 could not only affect the T regulatory cells, but also other regulatory and protective pathways. The injection of diphtheria toxin in the DEREG mouse model for example, a method to exclusively deplete T regulatory cells *in vivo*, did not affect the infarct size (Ren, Akiyoshi et al. 2011). Therefore, further research investigating the role of T regulatory cells is necessary.

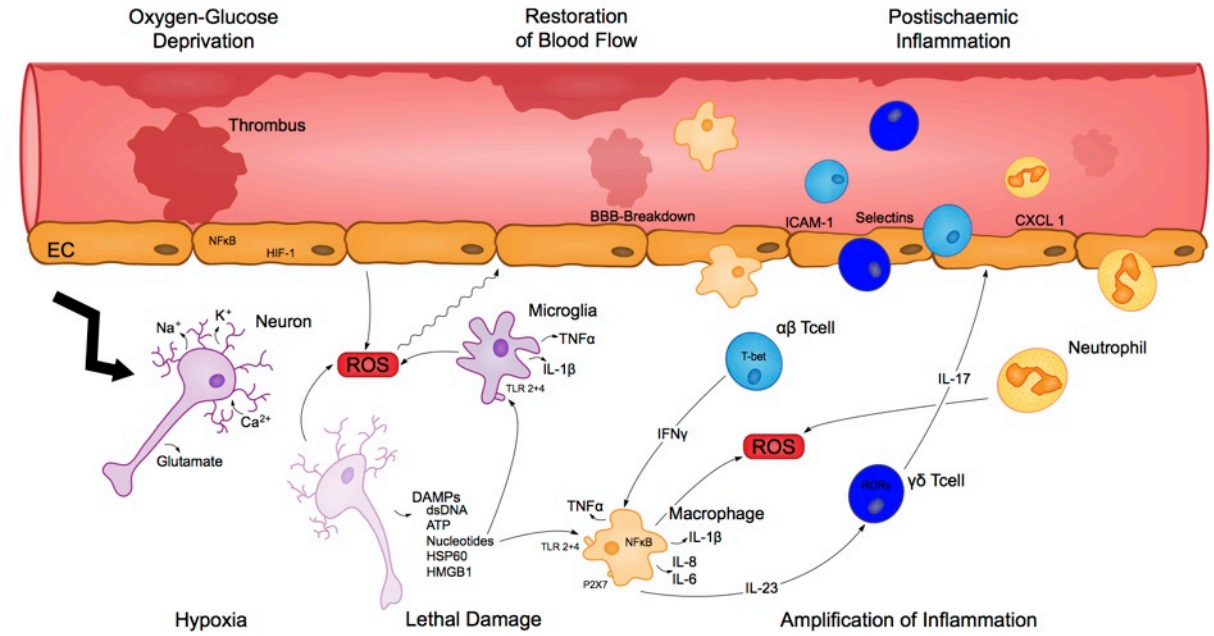

Figure 3: Evolvement and amplification of postischaemic inflammation.

3.3.3 Supporting evidence in humans for the existence of a similar pathophysiology

Given the difficult access to human brain tissue most studies on human stroke depend on indirect observations, histopathological and radiological findings. A study, which was designed to explore mechanisms of mountain sickness, showed the connection of hypoxia and inflammation in humans. Study subjects in high altitudes had elevated levels of IL-6 and C-reactive protein (CRP) (Hartmann, Tschop et al. 2000). MRI (magnetic resonance imaging) studies performed in stroke patients also strengthen the proposed pathophysiology: Repeated imaging of the brain showed an enlargement of the ischaemic lesion over time by more than 20% in selected patients (Baird, Benfield et al. 1997). This is a known feature of the MCAO model in rodents (Shichita, Sugiyama et al. 2009). The enlargement of the lesion volume was dependent on the initial diffusion-perfusion mismatch (Beaulieu, de Crespigny et al. 1999), which represents the penumbra area in MRI imaging. A diffusion-weighted MRI scan was highly predictive for the size of the infarct lesion in the chronic stage (Ritzl, Meisel et al. 2004). By observing the migration patterns, SPECT (single photon emission computed tomography) and other MRI studies focused on the role of infiltrating cells. For the SPECT studies peripheral leukocytes were first isolated from patients shortly after stroke onset, then labelled with radioactive substances to make them detectable in the CT, and later re-injected. Within the first 24 hours polymorphonuclear leukocytes in the ischaemic hemisphere showed a large increase, which returned to normal levels after one month (Akopov, Simonian et al. 1996). Furthermore, an accumulation of circulating neutrophils (Price, Menon et al. 2004) and mononuclear phagocytes (Jander, Schroeter et al. 2007), which were most likely macrophages, was found.

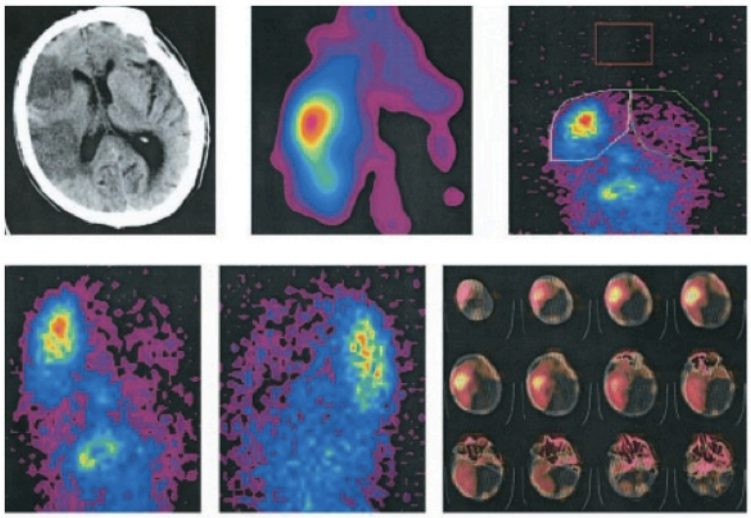

Figure 4: Accumulation of autologous indium-111 labelled neutrophils in the in ischaemic area 42 hours after the clinical onset shown by Price, Menon et al. 2004.

Leukocyte accumulation could also be confirmed in brain autopsies. Neutrophils infiltrated the ischaemic hemisphere already in the acute phase (1-2 days after symptom onset). Infiltrating macrophages were found in the subacute phase (3-30 days after symptom onset) (Mena, Cadavid et al. 2004; Price, Menon et al. 2004), as well as T cells and dendritic cells (DCs) (Yilmaz, Fuchs et al. 2010). Microglia were activated in the penumbra area (Arsene, Vasilescu et al. 2011) and an upregulation of the inflammatory molecules NF-κb and CXCL2 (Wu, Zhang et al. 2010) was observed. Importantly, a massive increase of IL-17 positive cells was found in the ischaemic hemisphere (Li, Zhong et al. 2005), supporting the pivotal role of this cytokine in the evolvement of postischaemic inflammation. Elevated IL-17 mRNA levels were also found in human peripheral blood mononuclear cells of stroke patients (Kostulas, Pelidou et al. 1999).

3.4 Systemic impact of a stroke on the immune system

The clinical course of stroke patients is often complicated through pneumonia, urinary tract and other infections. This is attributed to the CNS-mediated immunosuppression, a common phenomenon after acute CNS injury (Woiciechowsky, Asadullah et al. 1998). The immune system and the brain are linked (Elenkov, Wilder et al. 2000) and cytokines including IL-1β, TNFα and IL-6 can stimulate specialised cells of the hypothalamus in order to synthesise corticotropin releasing hormone (Turnbull and Rivier 1999). This in turn induces the secretion of glucocorticoids in the adrenal gland via the adrenocorticotropin hormone, which suppresses the production of pro-inflammatory mediators as well as promoting the release of IL-4, IL-10 and TGFβ. These cytokines subsequently mediate antiproliferative and apoptotic effects on immune cells. The stress-mediated release of catecholamines contributes to immuno-suppression as well (Vogelgesang, May et al. 2010).

On a cellular level, a rapid $CD4^+$-lymphopenia with long lasting diminished IFNγ secretion is observed in stroke patients (Klehmet, Harms et al. 2009). Fever that is often associated with these infections worsens the outcome and was found to correlate with a larger infarct volume (Reith, Jorgensen et al. 1996). Post-stroke infections contribute crucially to the outcome and are the most common cause of death in the late phase of stroke.

3.5 Treatment

Currently the prevention of further tissue damage through ischaemia focuses on the early restoration of blood flow in the infarcted area to recreate functionality. This remains the treatment of choice so far. Due to the limited time window of a maximum of six hours, only few patients can benefit from this. Therefore, new treatment options with a less restricted time window are needed. Since the inflammatory response after stroke seems to contribute crucially to further tissue damage, the use of immunomodulatory drugs that specifically block pathways relevant in postischaemic inflammation could be an alternative strategy.

Fingolimod is a sphingosin 1-phosphate receptor modulator that inhibits the emigration of lymphocytes from the lymph nodes and therefore migration to the site of inflammation. Its application showed a reduced infarct size and improved the outcome of stroke in the rodent model (Wei, Yemisci et al. 2011). Also treatment with recombinant TCR ligands that inhibit autoreactive T cells could successfully decrease lesion size in the mouse (Akiyoshi, Dziennis et al. 2011).

However, it is difficult to transfer the findings from mice to humans, and clinical trials have failed to show a protective effect so far. One of the clinical trials in fact worsened the outcome of stroke, although it was successfully tested in the rodent model (Sherman, Bes et al. 2001). There, a monoclonal antibody against ICAM-1 was tested, which inhibits leucocyte adhesion to vascular endothelium. However, the reduction of inflammation after stroke seems to be a promising option. Especially IL-17 might be a possible target for a novel therapeutic approach, considering the pivotal role of this cytokine in the mouse model and its presence in human autoptic brain tissue.

4 Materials and methods

4.1 Materials

4.1.1 Media and supplements

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	PAA
Accutase	PAA
Bovine serum albumin (BSA)	PAA
$Ca^{2+}M^{+}$ free Hank‘s balanced salt solution (HBSS)	PAA
Dextran-500	Sigma
Dimethylsulfoxid (DMSO)	Applichem
Dulbecco‘s Phosphate Buffered Saline (PBS)	PAA
Endothelial basal medium (EBM-2)	Lonza
Ethylenediaminetetraacetic acid (EDTA), 0.5M	Fluka
Ethylenediaminetetraacetic acid-Trypsin	Gibco
Gentamicin Sulfate	Lonza
Hydrocortisone	Sigma
L-Glutamine, 200mM	Gibco
Lymphocyte Seperation Medium	PAA
Penicillin/Streptomycin,100x	PAA
RPMI 1640	Gibco
Fetal calf serum (FCS)	Biochrom AG
X-VIVO 15, serum free medium	Lonza

4.1.2 Complete media and solutions

T cell medium	10% FCS 1% Penicillin-Streptomycin L-Glutamine 2mmM in RPMI
γδ Medium	10 % FCS 1% Penicillin-Streptomycin 2mM L-Glutamine 1mM Sodium Pyruvate in RPMI
Endothelial Cell Medium	5% FCS 1% Penicillin-Streptomycin HEPES 10mM bFGF 1ng/ml Hydrocortisone 1,4 µM in EBM-2

Freezing Medium	65 % RPMI 25 % FCS 10 % DMSO
FACS Buffer	0,1% BSA 0.02 % sodium azide in 1x PBS

4.1.3 Cell stimulation

1-hydroxy-2-methyl-2-buten-4-yl-4-diphosphate (HDMAPP)	Echelon
anti-CD3 (clone OKT 3)	Bioxell
Brefeldin A	eBioscience
Ionomycin	Sigma Aldrich
Live/dead fixable dead cell stain kit	Invitrogen
Phytohemagglutinin (PHA)	Sigma Aldrich
Phorbol myristate acetate (PMA)	Sigma Aldrich
Toll-like receptor agonist set	InVivoGen

4.1.4 Cytokines and growth factors

bFGF	R&D
hr IL-1β	R&D
hr IL-2	Hoffmann-Roche
hr IL-4	PeproTech
hr IL-6	R&D
hr IL-17	R&D
hr IL-23	R&D
GM-CSF	PeproTech
TGF-β	eBioscience

4.1.5 Reagents for cell culture

2-beta mercaptoethanol	Invitrogen
Sodium pyruvate	Gibco
Vybrant CFDA-SE (Carboxyfluorescein diacetat succinimidyl ester) cell tracer kit	Invitrogen

4.1.6 Reagents for flow cytometry

FACS Flow	BD Bioscience
Fixation buffer	eBioscience
Lysing Solution	BD Bioscience
Permeabilisation buffer	eBioscience

4.1.7 Antibodies for flow cytometry

mouse α human CD3 IgG1 PE-Cy5.5, UCHT1	Biozol
mouse α human CD4 IgG1κ AF488, RPA-T4	BD Bioscience
mouse α human CD4 IgG1κ V500, RPA-T4	BD Bioscience
mouse α human CD4 IgG1κ PE-Cy7, RPA-T4	BD Bioscience
mouse α human CD8 IgG1κ V450, RPA-T8	BD Bioscience
mouse α human CD14 IgG2bκ V450, MΦP9	BD Bioscience
mouse α human CD16 IgG1κ APC-Cy7, 3G8	BD Bioscience
mouse α human CD19 IgG1κ PE-Cy7, HIB19	BD Bioscience
mouse α human CD25 IgG1κ PE, M-A251	BD Bioscience
mouse α human CD27 IgG1κ APC-H7, M-T271	BD Bioscience
mouse α human CD28 IgG1κ PE-Cy7, CD28.2	BD Bioscience
mouse α human CD39 IgG1 PE-Cy7, eBioA1	eBioschience
mouse α human CD45 IgG1κ V500, HI30	BD Bioscience
mouse α human CD45RA IgG2bκ PE-Cy7, HI100	BD Bioscience
mouse α human CD56 IgG1 APC, N901	Beckman Coulter
mouse α human CD69 IgG1κ APC-Cy7, FN50	BD Bioscience
mouse α human CD127 IgG1κ PerCP-Cy5.5, HIL-7R-M21	BD Bioscience
mouse α human CD161 IgG1κ APC, HP-3G10	Biolegend
mouse α human CD196, IgG1 PE, R6H1	BD Bioscience
mouse α human Vδ2 IgG1 FITC, IMMU389	Beckmann Coulter
mouse α human TCRγδ IgG1κ PE, 11F2	BD Bioscience
mouse α human TCRγδ IgG1κ FITC, 11F2	BD Bioscience
mouse α human IL-23R IgG2b PE, 218213	R&D
mouse α human IL-17A IgG1κ AF647, eBio64DEC17	eBioscience
mouse α human IFNγ IgG1κ FITC, 4S.B3	Biolegend

4.1.8 NET-ELISA

mouse α human myeloperoxidase, IgG2b, 4A4	ABD Serotec
Commercial cell death detection ELISA kit	Roche
ELISA-Plate	Corning Incorporated

4.1.9 RNA Isolation and RT-PCR

Chloroform	SIGMA
Isopropyl	SIGMA
Ethanol	Merck
TRIZOL	Invitrogen
Maxima First Standard cDNA synthesis kit	Fermentas
dNTP Mix	Fermentas
DNAse	Quiagen

β-Actin primers and probe set	Applied Bioscience
CCL2 primers and probe set	Applied Bioscience
CCL6 primers and probe set	Applied Bioscience
IL-8 primers and probe set	Applied Bioscience

4.1.10 Cell lines

Human brain capillary endothelial cells: hCMEC/D3	(Weksler, Subileau et al. 2005)

4.1.11 Equipment

Benchtop refrigerated centrifuges	Eppendorf, Beckman Coulter
ELISA-Reader, VICTOR^3	Perkin Elmer
FACS Aria cell sorter	BD Bioscience
FACS CantoII	BD Bioscience
Freezers	Liebherr
Fridges	Liebherr
Incubator, Incu Safe Thermo Scientific	Sanyo
Microscope	Zeiss
NanoDrop 2000	Thermo Scientific
Neubauer chamber	Marienfeld
Pipets	Eppendorf/Gilson
Racks	Roth
rt-PCR-system, StepOnePlus	Applied Bioscience
Sterile bank, class II standard	Thermo Scientific
Thermal cycler, Mastercycler gradient	Eppendorf
Vortex-Genie 2, class II standard	Thermo Scientific
Waterbath	Eppendorf, GFL

4.1.12 Consumables

Cryo tubes	Greiner
Eppendorf tubes 0.5ml, 1.5ml	Eppendorf
FACS tubes	BD Bioscience
EDTA vaccutainer	Sarstedt
Parafilm ‚M‘	Pechiney
Pipette tips	Sarstedt
Serological pipets	Falcon
Tissue culture flasks 15ml, 75ml	Sarstedt
Tissue culture plates 12 wells, 48 wells, 96 wells	Sarstedt
Tubes 15ml, 50ml	Greiner

4.1.13 Software

Adobe Illustrator CS5	Adobe Systems Incorporated
BD FACSDiva™ 6.0	BD Bioscience
Endnote X3	Thomson Reuters
FlowJo 9.4	Celeza GmbH
GraphPad Prism 5.0	Graph Pad Software
IBM SPSS Statistics 20.0	IBM

4.2 Methods

4.2.1. Donors

Control patients and stroke patients were recruited from the Neurology Department of the University Medical Centre Hamburg-Eppendorf. For each patient we were able to collect one serum tube, between one and three EDTA-tubes and one tube of cerebrospinal fluid, in those cases where lumbar puncture was performed. Our study is approved by the local ethics committee (PV3392, Postischämische Inflammation des Gehirn - Studie zur Phänotypisierung und biologischen Marken, Applicants: Prof. Dr.med. Christian Gerloff and PD Dr. med Tim Magnus, approved on April 24 2010) and informed consent was obtained from all study subjects. We included patients with MRI or CT (computed tomography) proof of an ischaemic stroke with known onset of symptoms within the last seven days. The minimum age was 18 and patients needed to be legally able to give informed consent. Exclusion criteria were hypercytosis (>50/3 cells) in the CSF, signs of sepsis (CRP > 200) and lymphopenia or leukopenia of unknown origin. We obtained samples from 42 patients between the ages of 31 and 92.

patient ID	age	sex	area	type	CSF	CRP	leukocytes	other diagnosis
S10012DO	79	f	media	C	yes	<5	<11	
S10016CH	72	f	media	C	yes	17	14	no focus found
S10018FR	46	m	media	SC	yes	<5	<11	
S10024ON	31	f	media	SC	yes	<5	<11	
S10028CL	57	m	media	SC	yes	<5	<11	
S10035DE	37	m	media	SC	yes	<5	<11	
S10039LO	43	m	posterior	SC	yes	<5	<11	
B10038MA	72	f	media	C	yes	132	<11	unspecific after surgery
B10040ER	83	f	media	C	yes	40	<11	UTI
S10042WO	62	m	media	SC	yes	8	<11	no focus found
S10044KI	44	f	media	C	yes	7	<11	no focus found
S10047EB	80	m	media	SC	yes	<5	<11	
S10048TA	45	f	posterior	C	yes	<5	<11	
S10054SI	57	f	media	SC	yes	<5	<11	
S10061IN	72	f	media	SC	yes	<5	<11	
S10063HO	47	m	posterior	SC	yes	<5	<11	
B10064AN	83	f	media	C	no	<5	<11	
B10065DO	89	f	media	C	no	13	<11	pneumonia
B10066BA	60	f	media	SC	no	116	<11	unspecific after surgery
S10068SO	43	f	media	SC	yes	15	<11	UTI
B10069HO	70	m	media	C	no	58	<11	UTI, pneumonia
B10070AN	67	f	media	SC	no	<5	<11	
B10073ED	88	f	media	C	no	19	<11	UTI
B10074GE	79	m	media	SC	no	8	<11	no focus found
B10075WE	82	m	media	C	no	99	<11	pneumonia

patient ID	age	sex	area	type	CSF	CRP	leukocytes	other diagnosis
B10076AD	56	m	media	C	no	32	<11	no focus found
B10077HE	81	f	media	SC	no	76	<11	no focus found
B10079WA	92	m	media	C	no	<5	<11	
B10080EV	76	f	media	C	no	19	<11	UTI
B10081GE	52	m	posterior	SC	no	<5	<11	
B10082FR	76	m	media	SC	no	13	<11	no focus found
B10083HE	77	m	anterior	C	no	<5	<11	
S10084HO	54	m	media	SC	yes	<5	13,5	
B10085AN	45	f	media	SC	no	<5	<11	
B10086EV	84	f	posterior	SC	no	83	<11	pneumonia
B10087EL	90	f	media	SC	no	<5	<11	
B10088HE	82	m	media	SC	no	<5	<11	
B10089ER	89	f	media	C	no	<5	<11	
B10092ER	80	m	media	C	no	12	15	pneumonia
S10093NU	25	f	posterior	SC	no	<5	<11	
S10094GE	65	m	media	SC	no	8	<11	no focus found
S10097CA	48	m	media	C	yes	<5	<11	

f:female m:male

Table 1: List of included stroke patients with specification of age, sex, infarct localisation, CSF acquisition, level of C-reactive protein (CRP), leukocyte count and additional diagnosis.

The control population consisted of 30 age-matched patients. Twenty-eight were diagnosed with a non-inflammatory neurological disease (OND) and two with other inflammatory neurological disease (OIND).

patient ID	age	sex	condition	CSF	diagnosis
C10011CH	47	f	OND	yes	first-time epileptic fit
C10013KA	39	m	OND	yes	temporary hypaesthesia
C10015MA	53	f	OND	yes	wernicke‘s encephalopathy
C10019MA	59	f	OND	yes	occult inflammatory adducens palsy, arthrosis
C10023RA	50	m	OND	yes	ideopathic parkinson‘s syndrome, alcohol dependency
C10030EB	54	m	OND	yes	segmental primary dystonia
C10033BA	62	f	OND	yes	suspicion of transient ischaemic attack, leukoencephalopathy
C10034YA	49	f	OND	yes	amaurosis fugax, rheumatoid arthritis
C10041UT	66	f	OND	yes	chronic pain syndrome
C10043SE	41	m	OND	yes	ideopathic parkinson‘s syndrome
C10045MA	64	f	OND	yes	multiple myeloma
C10049HE	79	f	OND	yes	s/p breast cancer, s/p bladder cancer
C10053MA	46	m	OIND	yes	neuroborreliosis
C10056BA	28	m	OIND	yes	meningoencephalitis of unknown origin
C10059PE	58	m	OND	yes	persistent headache after dissection of the cortid artery
C10060DI	63	m	OND	yes	brainstem ischaemia
C10098IN	74	f	OND	no	chronic pain syndrome, depression
C10099MA	70	f	OND	no	polyneuropathy
C10100CH	81	f	OND	no	parkinson‘s disease
C10101BR	56	f	OND	no	chronic headache
C10102AL	69	m	OND	no	Ca^{2+} deficiency, associated myelopathy
C10103KA	68	f	OND	no	normal pressure hydrocephalus
C10105HO	72	m	OND	no	dementia
C10108UR	76	f	OND	no	parkinson‘s disease
C10109IL	71	f	OND	no	headache of unknown origin
C10110SI	83	f	OND	no	SAE DD cervical myelopathy
C10111CO	58	f	OND	no	legparesis of unknown origin
C10112UT	73	f	OND	no	parkinson‘s disease
C10113FR	70	m	OND	no	parkinson‘s disease, trimethoprim-treatment
C10114CA	75	f	OND	no	epilepsy, plasmozytoma, microangiopathy

f:female, m:male

Table 2: Control patients with specification of age, sex, condition, CSF acquisition and diagnosis.

4.2.2 Isolation of peripheral blood mononuclear cells (PBMCs)

Human PBMCs were isolated by density gradient centrifugation. This technique allows the separation of cells, organelles and macromolecules based on their size, shape and density. The gradient is created by layering a cell solution on a polysucrose of high molecular weight (400kD). During centrifugation the various components migrate differentially through the gradient and form distinct layers. The mononuclear cells are found at the interphase between plasma and the polysucrose. In order to isolate PBMCs, human peripheral blood was diluted 1:2 in PBS at room temperature (RT). Thirty ml of the diluted blood were carefully layered on 20 ml lymphocyte-separation-medium in 50 ml falcon tubes and centrifuged for 30 minutes at 400x g and 20° C without brakes. After centrifugation, PBMCs appeared as a white layer in the interphase of plasma and lymphocyte separation medium. They were collected by aspiration and washed three times in cold PBS. The number of cells was determined in a Neubauer counting chamber and cells were either frozen or directly used for further experiments.

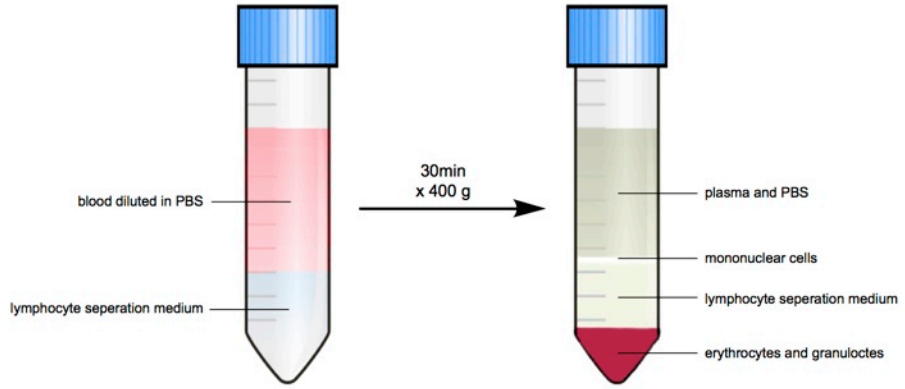

Figure 5: Principle of density gradient centrifugation.

4.2.3 Isolation of mononuclear cells from cerebrospinal fluid

Samples were collected during diagnostic procedures and processed immediately. Cells were collected after ten minutes of centrifugation at 1500 rpm and either stained for FACS analysis or expanded. Additionally the CSF fluid was collected, frozen and stored at -80°C.

4.2.4 Flow cytometry

Flow cytometry uses the principle of light scattering and characteristics of fluorochrome molecules to generate specific multi-parameter data from cells. A single-wavelength beam of light, most commonly a laser, is directed onto a hydro-dynamically focused stream of fluid, which contains the fluorochrome-stained cells. The cells intercept with the light source and the forward-scattered light (FSC) correlates with the cell volume. The side-scattered light (SSC), however, is proportional to the granularity of the cells. The fluorochrome molecules are excited to a higher state of energy. This energy is released in form of a photon light with

specific spectral properties, unique to each of the fluorochromes. Optical long and band pass filters direct the light to different optical detectors, where it is converted into electrical impulses. These impulses are amplified and processed before being transformed into digital information, which is graphically plotted. Depending on the number of laser and detection filters, different fluorochromes can be measured in parallel. The fluorochromes are bound to specific antibodies, which are directed against diverse cell molecules. Together with the FSC and SSC a detailed and precise phenotypical characterisation of individual cells can be performed. All data was acquired on a FACS CantoII with the BD FACSDiva™ 6.0 software. For analysis FlowJo 9.4 was used.

4.2.5 Surface staining of whole blood and CSF cells

Whole blood or PBMCs were incubated with fluorochrome labelled antibodies for 30 minutes at room temperature. Subsequently, samples containing whole blood were incubated for 10 minutes with lysing solution, centrifuged for five minutes at 1500 rpm, washed with 3ml FACS-buffer and then resuspended in 300µl FACS-buffer. Tubes containing the CSF were washed once with 1ml FACS-buffer after staining and then immediately resuspended in 300µl FACS-buffer.

4.2.6 Expansion of CSF cells

Isolated mononuclear cells from the CSF were counted and immediately transferred to γδ-medium. Over the course of two weeks stimulation was performed with repetitive administration of 10nM/ml HDMAPP every five to six days. 200 U/ml hrIL-2 were added every three to four days. Selected samples were additionally stimulated with 2.5µg/ml PHA (Phytohemagglutinin) and 200.000 irradiated (70Gy) feeder cells on day zero.

Patient ID	HDMAPP	hrIL-2	PHA	feeder cells
C10007GU	-	+	+	+
C10045MA	+	+	+	+
C10049HE	+	+	+	+
C10056BA	+	+	-	+
C10059PE	+	+	-	+
C10060DI	+	+	-	-
S10068SO	+	+	-	-
S10094GE	+	+	-	-
S10097CA	+	+	-	-

Table 3: Stimuli used for CSF-cell expansion.

4.2.7 Intracellular cytokine staining of PBMCs and expanded CSF cells

To assess the production of cytokines, cells were transferred to a serum-free culture medium (X-VIVO) and activated with 50 ng/ml PMA and 1 µg/ml ionomycin for five hours at 37°C, 5% CO_2. In order to facilitate the accumulation of intracellular cytokines protein transport

was inhibited with 10µg/ml brefeldin A after one hour of incubation with PMA and ionomycin. Subsequently, cells were washed, blocked with hIgG and incubated with live/ dead-staining on ice for 25 minutes. Afterwards, cells were stained with surface antibodies for 30 minutes at room temperature and washed once again before being fixed for 20 minutes. To allow the cytokine-antibodies to enter the intracellular space, cells were permeabilised by two washing cycles with permeabilisation buffer. Incubation with the cytokine-antibodies was performed for 30 minutes at room temperature, followed by another washing step with permeabilisation buffer. Finally, cells were resuspended in 300µl permeabilisation buffer and analysed by flow cytometry.

4.2.8 Isolation and expansion of peripheral blood γδ T cells

γδ T cells were isolated by fluorescent-activated cell sorting. Therefore, whole PBMCs were labelled with anti-TCR γδ antibody using a concentration of 2µl/1million cells. The labelled PBMCs were sorted in a flow cytometer that can not only measure the expression of markers, but can also separate a selected population. An appliance inside this cytometer can create vibrations that break the fluid stream of cells into droplets. Each droplet only contains one cell which after measuring the fluorescent characteristics can be sorted in a separate container. For further experiments, isolated γδ T cells or whole PBMCs were cultured in γδ T cell medium and stimulated with 100U/ml hrIL-2 and 10nM HDMAPP. Restimulation was performed every five to six days.

4.2.9 Proliferation assay of γδ T cells

CFDA-SE is a cell permeant dye that binds to cytoplasmatic structures and remains inside the cell. Upon cell division, the amount of dye spreads equally between the daughter cells and can then by measured by flowcytometry. To assess T cell proliferation PBMCs were labelled with 2µM CFDA-SE dilution at 37°C for 15 minutes. After quenching in medium for 30 minutes, cells were washed with medium twice. Subsequently, cells were stimulated with 100 U/ml IL-2, 2.5 µg/ml PHA or 10 nM/ml HDMAPP. One week after stimulation the proliferation of cells expressing the γδ TCR was assessed by a flowcytometric surface staining.

4.2.10 Isolation of peripheral blood neutrophils

Neutrophils were isolated by density gradient centrifugation. Peripheral blood was diluted 3:1 in 3% dextran solution in a 15 ml falcon tube, mixed carefully and let to rest. After 30 minutes two phases could be distinguished. The lower one contained the majority of erythrocytes, whereas lymphocytes, monocytes and granulocytes were located in the upper phase. The leucocyte containing phase was collected, carefully layered on lymphocyte separation

medium and centrifuged for 30 minutes at 400x g. The granulocytes appeared as a dense layer at the bottom of the tube. They were collected and washed with HBSS. To eliminate the remaining erythrocytes, cells were resuspended in H_2O for 30 seconds, and tonicity was restored by adding HBSS. Cells were washed and used for further experiments.

4.2.11 In vitro generation of monocyte derived macrophages and DCs

Monocytes and DCs were isolated from freshly isolated PBMCs by adherence. Cells were diluted in RPMI to a concentration of $5x10^6$ cells/ml and sowed at a density of 1.5 x 10^6 cells/cm^2. They were incubated for two hours at 37° C, and non-adherent cells were removed by extensive washing with PBS. In order to obtain macrophages, adherent monocytes were incubated for two weeks without additional growth factors. For DC differentiation adherent monocytes were incubated for seven days in complete medium which was supplemented with 0.1µg/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 0.04µg/ml IL-4.

4.2.12 Inducing IL-17 production in Vδ2γ9 cells

In order to induce IL-17 production in Vδ2γ9 cells, PBMCs were stimulated with 0.3µM/ml HDMAPP in the presence or absence of 1ng/ml IL-1β, 1ng/ml IL-6, 50ng/ml IL-23 and 1ng/ml TGFβ for a maximum of 20 days. IL-2 was added on day three, 10, and 17. To assess the cytokine production, cells were stained on day seven, 13 or 20.

4.2.13 Impact of an infectious environment on the IL-17 production of γδ T cells

An infectious environment was mimicked by a dendritic cell-conditioned medium (DCCM) and IL-6, which is secreted by immune cells during infections. Therefore, neutrophils were isolated from the peripheral blood. In order to induce apoptosis, neutrophils were exposed to UV-irradiation for 30 minutes. Apoptotic cells were added to dendritic cell cultures and incubated for 18 hours at 37°C degrees to obtain the DCCM. Whole PBMCs were cultured in the DCCM in either the absence or presence of 1ng/ml IL-6, 0.1µg/ml OKT3 and 50ng/ml IL-23 for three days and a cytokine staining for IL-17 and IFNγ was performed.

4.2.14 TLR stimulation of γδ T cells

γδ T cells were sorted by flow cytometry and stimulated with TLR agonists in either the presence or absence of 0.1µg/ml OKT3, 10nM HDMAPP and 50ng/ml IL-23. The same experiment design was used for whole PBMCs. Following TLR receptor agonists were used for stimulation: 1µg/ml Pam3CSK4 (synthetic bacterial triacylated lipoprotein) for TLR 1 and 2; 10^8 HKLM (heat killed listeria monocytogenes)/ml for TLR 2; 5µg/ml Poly (I:C) HMW (polyinosinic-polycytidylic acid with a high molecular weight) for TLR 3; 1µg/ml LPS

(lipopolysaccharid) for TLR 4; 1ng/ml Flagellin for TLR 5; 1µg/ml FSL-1 (synthetic mycoplasmal triacylated lipoprotein) for TLR 6; 1µg/ml Iminiquod for TLR 7; 1µg/ml ssRNA for TLR 8 and 1µM ODN2006 (unmethylated cytosin-phosphatidyl-guanin oligonucleotide) for TLR 9. Cytokine production of γδ T cells was assessed after three or five days.

4.2.15 Cocultures of macrophages and γδ T cells

In order to observe the effect of TLR-activated antigen presenting cells (APCs) on the cytokine production of γδ T cells, macrophages were stimulated with TLR ligands and co-cultured with PBMCs. Initially, macrophages were stimulated with 1µg/ml Pam3CSK4, 10^8 HKLM/ml or 1µg/ml LPS in a 96-well plate. After two days PBMCs were cocultured with the stimulated macrophages and the macrophage conditioned medium (MCM), the macrophages and fresh medium (FM), the MCM only or medium containing nothing but the TLR ligands (M). HDMAPP and 100U/ml IL-2 were present in all conditions. An intracellular cytokine staining of the γδ T cells was performed after five days of culture.

4.2.16 Quantitative rt-PCR of mRNA from stimulated hCMEC/D3

The effect of IL-17 and TNFα on the production of neutrophil chemoattractants by brain endothelial cells was assessed by quantitative rt-PCR (real-time polymerase chain reaction). Monolayers of a brain endothelial cell line (hCMED/D3) were stimulated with 0.1ng/ml, 1ng/ml or 10ng/ml TNFα in either the absence or presence of 20ng/ml IL-17. After 24 hours RNA was homogenised using 500µl TRIZOL reagent (1ml/10cm^2). 100µl chloroform (0.2ml/1ml TRIZOL-reagent) were added, mixed carefully and samples were centrifuged at 12000 x g for 15 minutes at 4°C. The upper aqueous phase was collected and RNA was precipitated by adding 250µl isopropyl alcohol. The RNA obtained, was quantified in a spectrophotometer and diluted in H_20 to a concentration of 1400ng/ml in a volume of 14µl.
For cDNA (complementary desoxyribonucleic acid) transcription 2µl reverse transcriptase and 10µl dNTPs were added, and transcription was performed in a thermal cycler for 10 minutes at 25° C, 30 minutes at 50° C and five minutes at 85° C. Real-time PCR was used to quantify the gene expression of IL-8, CCL2 (CC chemokine ligand 2) and CXCL6. This method monitors the amplification of a target gene in real time using fluorescent technology. 1µl of IL-8, CCL2, CXCL6 or β-actin primers, 10µl of dNTP Mix with DNA-polymerase were added to 9 µl of obtained cDNA and run in a rt-PCR-system for 45 cycles. Each cycle consisted of 15 seconds at 95°C to denaturate the dsDNA (double stranded DNA), 20 seconds at 60°C to allow the primer to anneal to the ssDNA (single stranded DNA) and 60 seconds at 80°C for synthesising the complementary strain.

Relative quantification was calculated using the ΔΔCt method, which compares the fluorescent signal from the target gene with the signal of constitutively expressed genes that subserve as an internal reference. The Ct value corresponds to the numbers of cycles needed to exceed a constantly defined fluorescence level and the difference of the Ct value of the target gene and the constitutively expressed gene is calculated (ΔCt). After this normalisation, the ΔCt from a control sample is subtracted from the ΔCt of the treated sample, resulting in the ΔΔCt. Applying the formula $2^{-\Delta\Delta Ct}$, the relative difference of gene expression levels is calculated. Samples were run in duplicates and normalised to the expression of β-actin.

4.2.17 NET-ELISA

A sandwich ELISA was used to measure NET formation in the sera of stroke and control patients (Kessenbrock, Krumbholz et al. 2009). Since myeloperoxidase (MPO) and DNA fibres are prominent components of the NETs, anti-MPO was used as a capturing antibody and anti-DNA as a detection antibody. An ELISA plate was coated with 75µl/well anti-MPO antibody at a concentration of 5µg/ml overnight, and washed with PBS three times the next day. Afterwards the wells were blocked with 1% BSA for one hour, washed three times with PBS and incubated with 40µl patient serum and 80µl peroxidase labelled anti-DNA antibody. After two hours of incubation at room temperature the plate was washed three times with PBS, and 100µl of 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid-substrate (ABTS) were added. The plate was incubated for 40 minutes at 37°C and subsequently measured with an ELISA reader at 405nm.

4.2.18 Statistical analysis

GraphPad Prism 5.0 and IBM SPSS Statistics 20.0 software were used for statistical evaluation of the data. Unpaired student‘s t-test was utilised to compare the average values of the frequencies and subtypes of the different cell populations in the peripheral blood as well as NET formation in sera of patients and healthy donors. Deviations are shown as the standard error. We used paired student‘s t-test to analyse correlations between CSF and peripheral blood. For the graphical presentation we utilised whisker-plots, which show the median and all quartiles. In all experiments regarding the IL-17 production of γδ T cells unpaired student‘s t-test was performed. Error bars represent the standard deviation. Results were considered significant if $p \leq 0.05$.

5 Results

5.1 Phenotype of leukocyte subsets in peripheral blood and CSF

We designed five different antibody cocktails to identify the different leukocyte subsets and investigate their activation status. Peripheral blood was stained with all cocktails, and if available, cerebrospinal fluid was stained with cocktail ‘subsets II‘ only, due to the low number of cells available.

Subsets I	Subsets II	T reg	T effector	T activation
CD14 V450	CD14 V450	CD8 V450	CD8 V450	CD8 V450
CD45 V500	CD45 V500	CD4 V500	CD4 V500	CD4 V500
CD4 Al488	Vδ2 FITC	CD25 PE	Vδ2 FITC	Vδ2 FITC
γδ PE	γδ PE	CD127 PE-Cy5.5	γδ PE	γδ PE
CD3 PE-Cy5.5	CD3 PE-Cy5.5	CD39 PE-Cy7	CD3 PE-Cy5.5	CD3 PE-Cy5.5
CD19 PE-Cy7	CD4 PE-Cy7		CD45RA PE-Cy7	CD28 PE-Cy7
CD56 APC	CD56 APC		CD56 APC	CD56 APC
CD16 APC-Cy7	CD69 APC-Cy7		CD27 APC H7	CD69 APC-Cy7

Table 4: Antibody cocktails used for identification of the phenotype of peripheral blood and CSF leukocytes.

5.1.1 Gating strategy

Leukocytes were identified and separated from cell debris by the expression of CD45. We selected lymphocytes ($CD16^{-}CD14^{-}$), neutrophils ($CD16^{+}CD14^{-}$), eosinophils ($CD16^{-}CD14^{low}$) and monocytes ($CD14^{high}$) from the leukocyte population (Fig. 6).

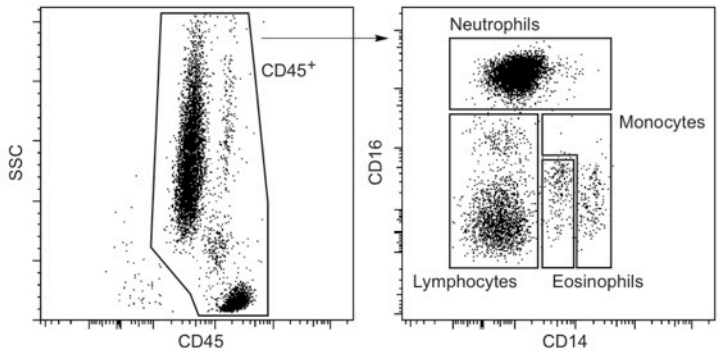

Figure 6: Gating strategy for the selection of leukocytes and subsequent selection of lymphocytes, neutrophil granulocytes, eosinophil granulocytes and monocytes.

We separated B cells from the other lymphocytes according to the expression of CD19. Subsequently, NK cells (natural killer cells) ($CD56^{+}CD3^{-}$) and T cells ($CD3^{+}$) were selected from the non-B cell lymphocyte population. We further divided NK cells into NK $CD56^{bright}CD16^{-}$ with the ability to produce more regulatory operating cytokines, and NK $CD56^{dim}CD16^{+}$, which have higher cytotoxic capacities (Poli, Michel et al. 2009) (Fig. 7).

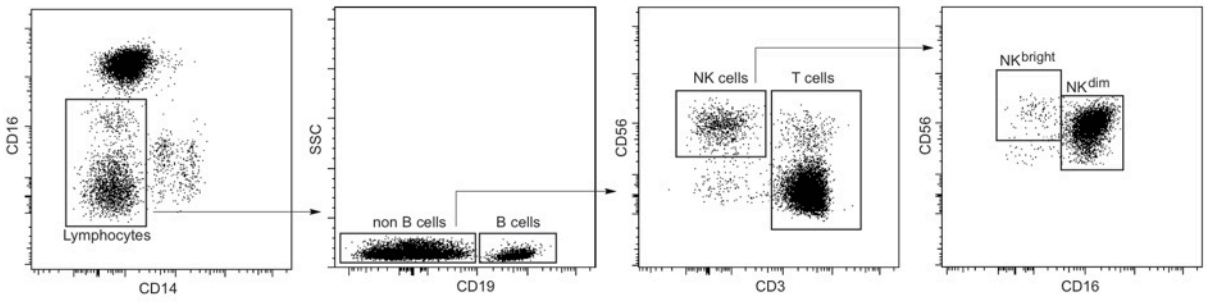

Figure 7: Selection of B cells, T cells and NK cells from the lymphocyte population.

We further subdivided the T cell population into conventional and unconventional T cells. γδ T cells were identified through the expression of γδ TCR and the Vδ2 subset was considered separately. We selected NKT cells (natural killer like T cells) ($CD56^{+}CD3^{+}$) from the non-γδ-T cell population. The remaining cells were assumed to be conventional αβ T cells (Fig. 8).

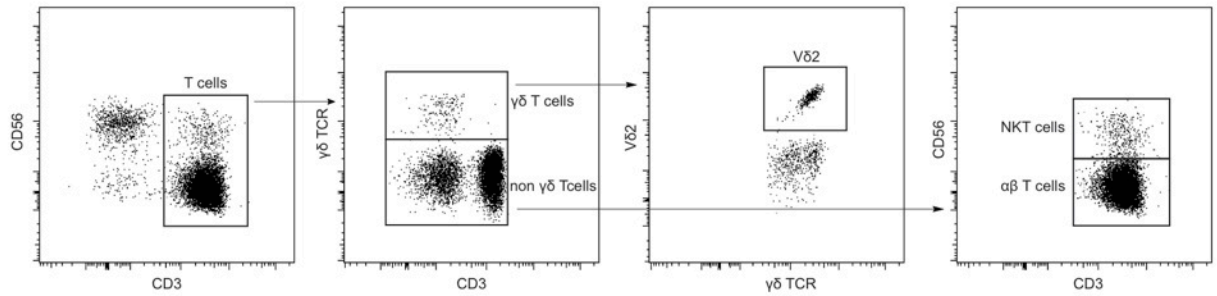

Figure 8: Identification of γδ T cells, the Vδ2 subset, NKT cells and αβ T cells.

For detailed analysis of T cells, we selected T helper, T cytotoxic and T regulatory cells from the lymphocytes, which were identified by their appearance in the forward and side scatter plot. We identified T helper and T cytotoxic cells according to the expression of CD4 or CD8, respectively. Regulatory T cells were defined as $CD4^{+}$ $CD25^{high}CD127^{low}$ lymphocytes. In this population we further assessed the expression of CD39, an ATP-degrading ecto-enzyme. CD39 marks a subset of T regulatory cells, which possesses an important anti-inflammatory potential (Borsellino, Kleinewietfeld et al. 2007) (Fig. 9).

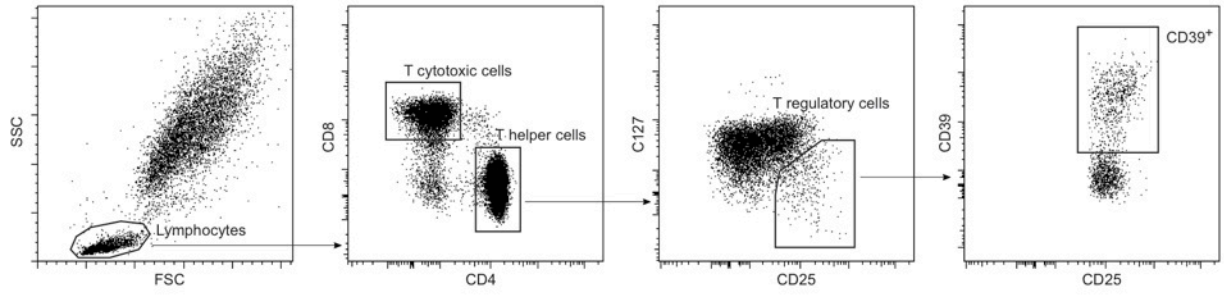

Figure 9 : Selection of T helper, T cytotoxic and T regulatory cells.

Moreover, we defined the maturational status of T helper, T cytotoxic and γδ T cells based on the expression of CD45RA and CD27. Naive T cells were characterised as $CD45RA^{+}CD27^{+}$. The $CD45RA^{-}CD27^{+}$ population was defined as central memory cells, whereas the $CD45RA^{-}CD27^{-}$ cells were characterised as effector memory cells (Appay and Rowland-Jones 2004). We assessed the expression levels of CD69 and CD25, the alpha chain of the IL-2 receptor, to determine the activation status. Finally, the frequency of $CD28^{-}$ cells was taken into consideration. The decrease of CD28 has been proposed to be a sign of immune senescence (Vallejo, Weyand et al. 2004), but it is also related to persistent immune activation (Vallejo, Brandes et al. 1999) as well as auto-reactive T helper cells (Vallejo, Mugge et al. 2000) (Fig. 10).

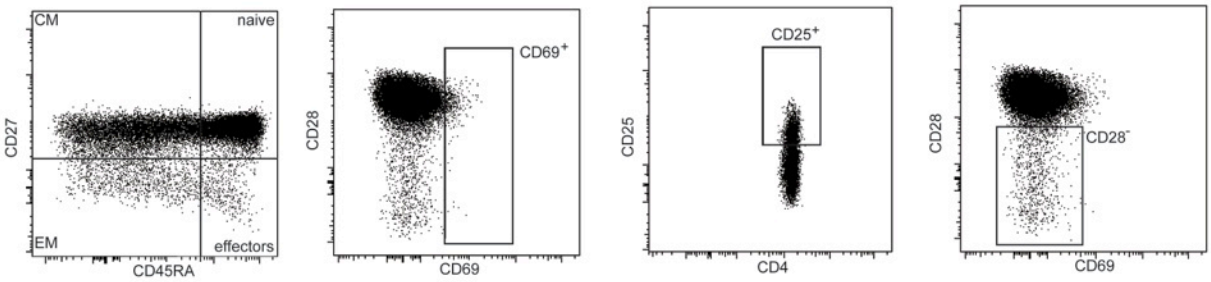

Figure 10: Assessment of the maturation and activation status, shown on the example of T helper cells.

5.1.2 Analysis of leukocytes in the peripheral blood of stroke and control patients

We compared the average values of the different leukocyte populations in control and stroke patients to investigate the effect of stroke on these cells. The present study included ten control patients with other neurological disease (OND) and 14 stroke patients (Stroke). Out of the stroke patients eight had small subcortical strokes (Sco) and six had larger cortical strokes (Co). Absolute numbers and frequencies are listed in table 5.

5.1.2.1 Leukocyte subsets

Transient neutrophilia and lymphopenia are known consequences of stroke. In our study we observed higher absolute numbers of total leukocytes in stroke patients compared to the controls. This difference was significant between the cortical group and control, while the subcortical group was similar to the control. The increase of total leukocyte counts in the cortical group was due to an elevated number of neutrophils, which was not detectable in the subcortical group. Lymphocytes were decreased in stroke patients. The cortical group had significantly lower numbers of lymphocytes compared to the control patients, and the subcortical group showed a trend towards reduced lymphocyte numbers as well. We observed no major difference in the monocyte population between stroke and control patients (Fig. 11).

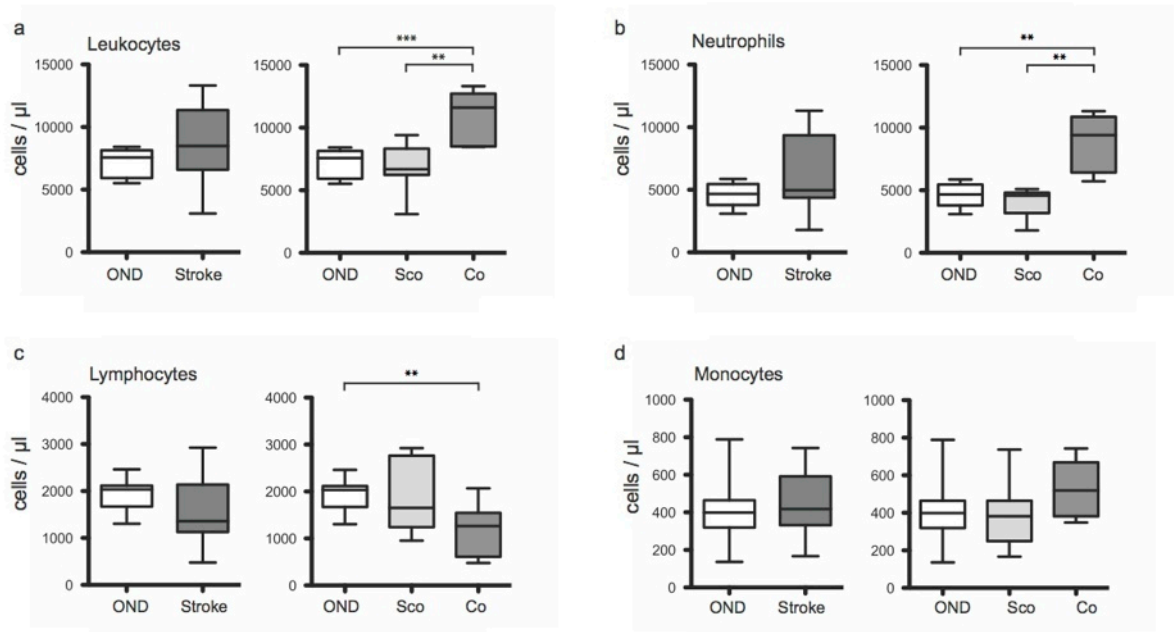

*Figure 11: Median values of absolute numbers (solid bar) of leukocytes (**a**), neutrophils (**b**), lymphocytes (**c**) and monocytes (**d**) with interquartile range (bar height) and minimum/maximum value (whiskers) across the quartile; ** $p < 0.01$ and *** $p < 0.001$.*

NK cells were decreased after stroke, especially in patients with a cortical stroke. The subcortical group showed diminished numbers in the same way, although there was no statistically significance. No differences were found regarding the proportion of the NK $CD56^{dim}CD16^{+}$ population, but the frequency of the regulatory NK $CD56^{bright}CD16^{-}$ cells was significantly elevated in stroke patients. Both the cortical and subcortical group showed the same trend concerning the NK $CD56^{bright}CD16^{-}$ population. We observed a slight decrease of NKT cells in stroke patients, which was more pronounced in severely affected patients (Fig. 12).

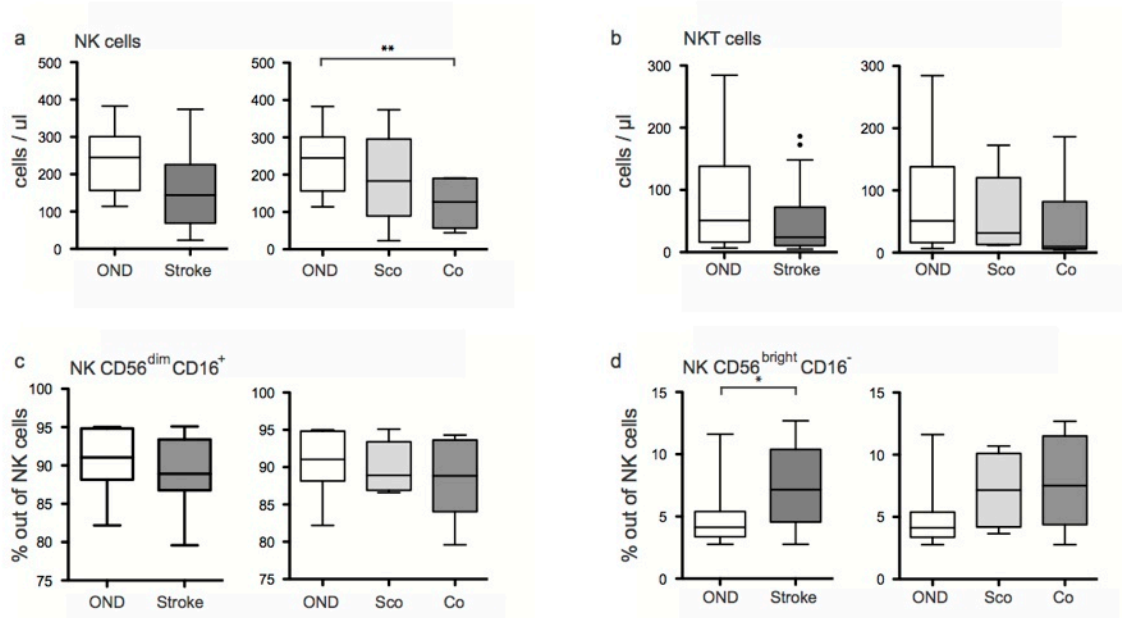

*Figure 12: Median values of absolute numbers (solid bar) of NK cells (**a**) and NKT cells (**b**) as well as the frequencies (solid bar) of NK dim (**c**) and NK bright (**d**) out of the NK cells with interquartile range (bar height) and minimum/maximum value (whiskers) across the quartile; * $p < 0.05$ and ** $p < 0.01$.*

We did not find a difference in the absolute numbers of B cells. αβ T cells were diminished in stroke patients and we observed a significant difference between the cortical group and OND. γδ T cell numbers did not vary between stroke and controls patients, as well as the frequency of the Vδ2 cells (Fig. 13).

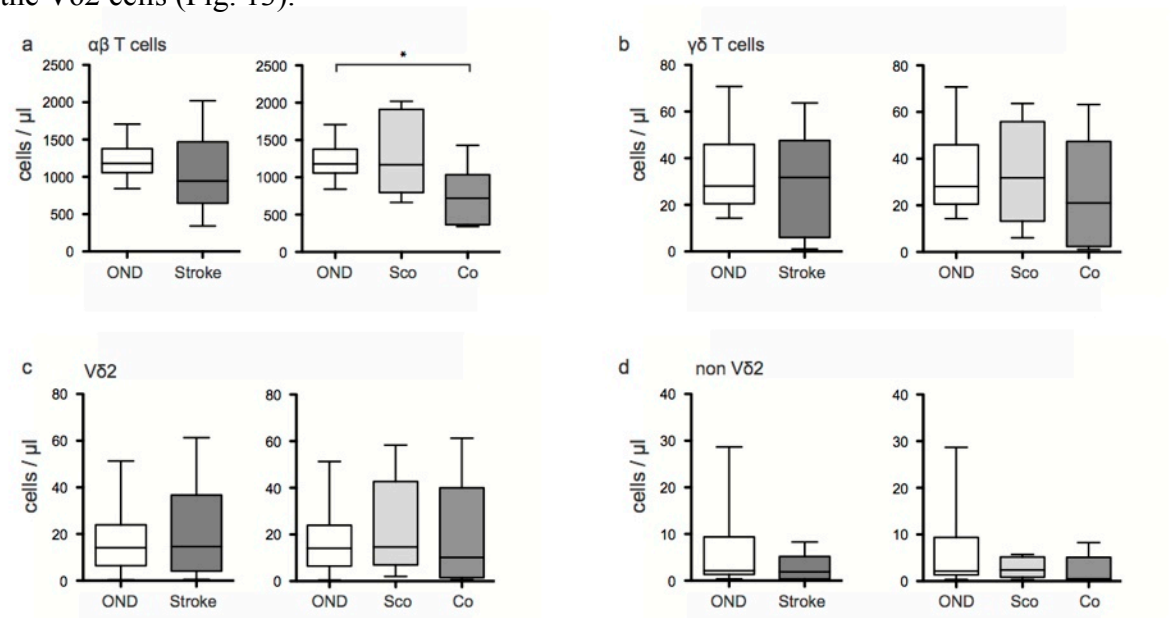

*Figure 13: Median values of absolute numbers (solid bar) of αβ T cells (**a**), γδ T cells (**b**) Vδ2 (**c**) and non Vδ2(**d**) with interquartile range (bar height) and minimum/maximum value (whiskers) across the quartile; * $p < 0.05$.*

Within the T cell,s it were the T helper cells, which were decreased after stroke. A significant difference between the cortical group and OND was found, whereas the subcortical group did not differ from OND. Likewise, the number of T cytotoxic cells was diminished, with the difference only being present in the cortical group. The number of T regulatory cells was not affected and neither was the percentage of $CD39^+$ T regulatory cells (Fig.14).

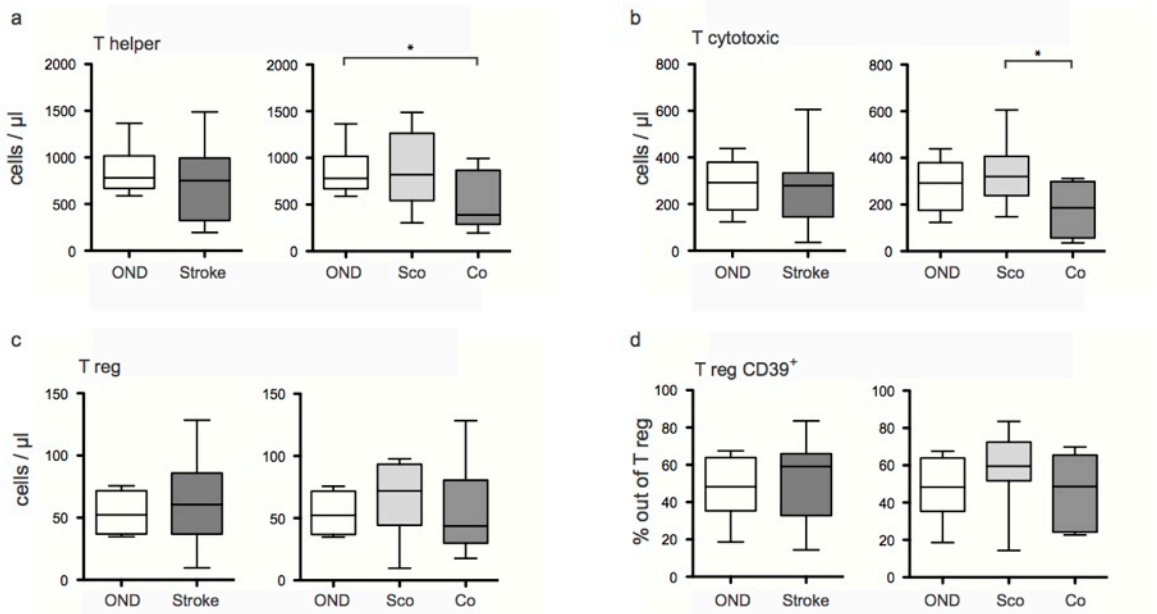

*Figure 14: Median values of absolute numbers (solid bar) of T helper cells (**a**), T cytotoxic cells (**b**) and T regulatory cells (**c**) as well as frequency (solid bar) of $CD39^+$ T regulatory cells (**d**) with interquartile range (height) and minimum/maximum value (whiskers) across the quartile; * $p < 0.05$.*

In summary, we observed that neutrophils account for the increase of total leukocytes, whereas lymphocytes decrease in patients immediately after stroke. Yet, only the severely affected patients showed differences in neutrophil numbers, while lymphocytes were affected in all stroke patients. The observed lymphopenia was due to a decrease of αβ T cells; T helper as well as T cytotoxic cells accounted for this deficit. This effect was more pronounced in severely affected patients. Also the absolute number of NK cells was decreased in stroke patients, with a higher percentage of the more regulatory NK^{bright} population. We observed no changes within the γδ T cell population and T regulatory cells.

5.1.2.2 Activation and differentiation status of T helper, T cytotoxic and γδ T cells

The loss of the costimulatory molecule CD28 on CD4 cells is considered to be a sign of chronic activation. It is also related to persistent immune activation, and some of those cells show high cytotoxic activity. $CD28^-$ T helper cells were significantly diminished in stroke patients. The cortical group had the lowest number of $CD28^-$ T helper cells. Also the subcortical group showed a strong trend towards fewer $CD28^-$ cells. T cytotoxic cells showed a significant decrease of $CD28^-$ cells, but we observed no major differences between the cortical and subcortical group. The role of CD28 in γδ T cells is not clear, but a down-regulation of CD28 after activation has been reported. We did not observe substantial alterations between stroke and control patients. Especially in the stroke cohort, frequencies of $CD28^-$ γδ T cells and Vδ2 cells varied to a great degree in each patient (Fig. 15).

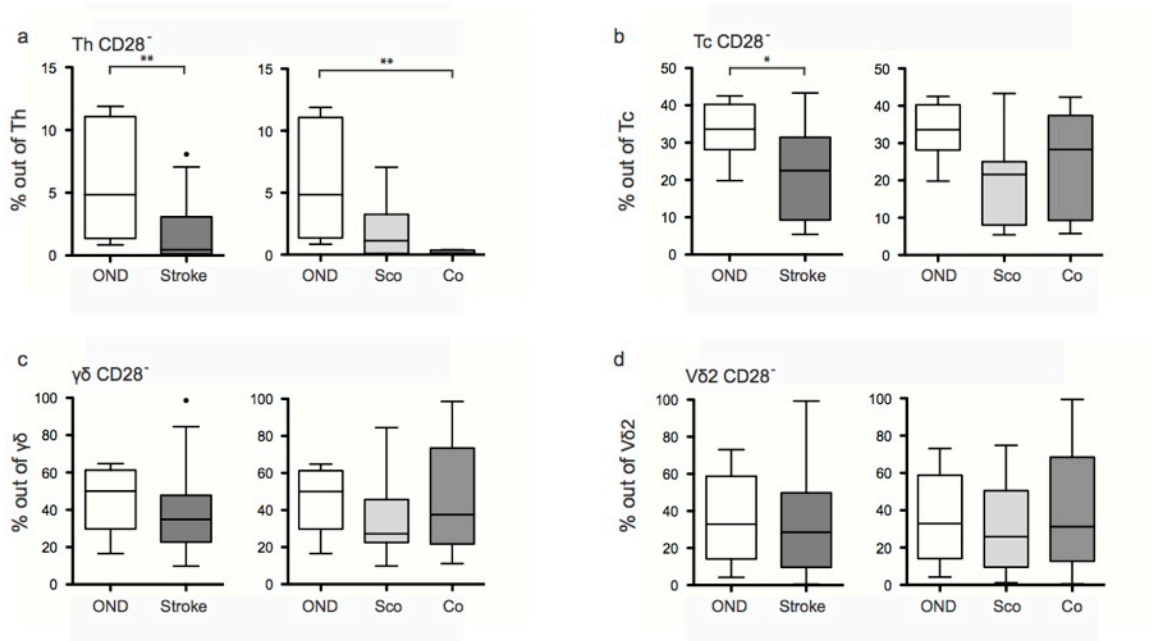

*Figure 15: Median values of frequencies (solid bar) of $CD28^-$ T helper cells (**a**), $CD28^-$ T helper cells (**b**), $CD28^-$ γδ T cells (**c**) and $CD28^-$ Vδ2 cells (**d**) with interquartile range (bar height) and minimum/maximum value (whiskers) across the quartile; * $p < 0.05$ and ** < 0.01.*

T cells were activated after stroke. We analysed the expression of the activation markers CD25 and CD69 on T helper cells, T cytotoxic cells, γδ T cells and the Vδ2 subset of the γδ T cells. The expression of CD25, which is upregulated after 24 hours upon activation, fluctuated strongly between zero and almost 50 % on T helper and T cytotoxic cells. Yet, the patients of the cortical group showed an increase of this marker, both on T helper and T cytotoxic cells. CD69 as a marker of early activation was clearly upregulated on the T helper cells of severely affected stroke patients. The upregulation of CD69 was even more pronounced on T cytotoxic cells. Similar to the observations on the T helper and T cytotoxic cells, γδ T cells and the Vδ2 subset had a higher expression of CD69, which was significant for the Vδ2 cells of the cortical group (Fig. 16).

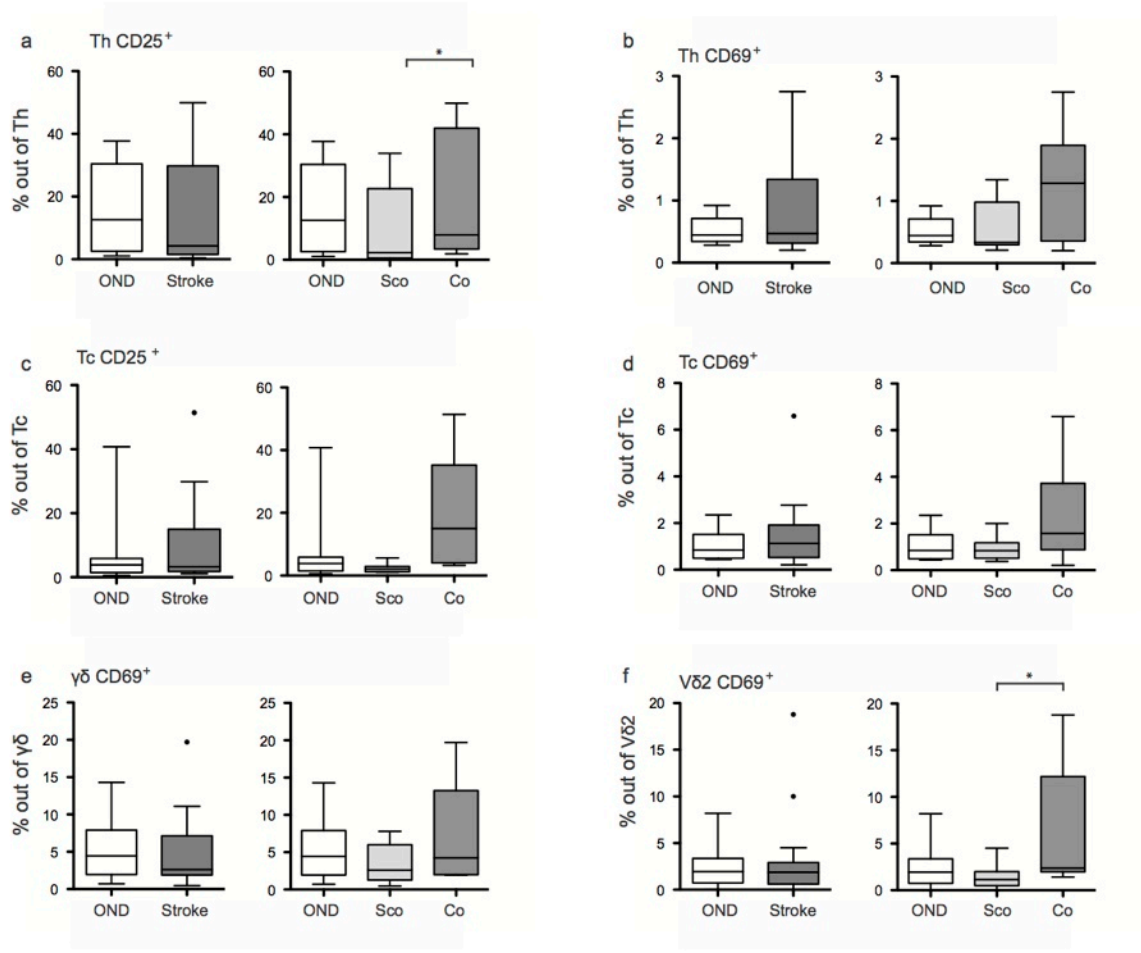

*Figure 16: Median values of frequencies (solid bar) of CD25⁺ T helper cells (**a**), CD69⁺ T helper cells (**b**), CD25⁺ T cytotoxic cells (**c**) CD69⁺ T cytotoxic cells (**d**), CD69⁺ γδ T cells (**e**) and CD69⁺ Vδ2 cells (**f**) with interquartile range (bar height) and minimum/maximum value (whiskers) across the quartile; * $p < 0.05$*

To recapitulate, the expression of CD28 was diminished in T helper and T cytotoxic cells of stroke patients, but remained unaltered in γδ T cells. Furthermore, all T cells expressed higher levels of CD69 in severely affected patients, which was particularly pronounced in the Vδ2 cells.

	OND	Stroke	Sco	Co
Leukocytes/µl	**7216 ± 342**	**8587 ± 769**	**6777 ± 670**	**11000 ± 846**
Neutrophils/µl	**4585 ± 298**	**6121 ± 789**	**4058 ± 402**	**8872 ± 911**
Lymphocytes/µl	**1938 ± 106**	**1574 ± 201**	**1866 ± 271**	**1183 ± 232**
Monocytes/µl	447 ± 46	413 ± 53	387 ± 62	527 ± 62
NK cells	**243 ± 26**	**162 ± 27**	**191 ± 41**	**123 ± 25**
% of NK^{dim}	91 ± 1	89 ± 1	90 ± 1	88 ± 2
% of NK^{bright}	**4.9 ± 0.8**	**7.4 ± 0.9**	**7.1 ± 1.0**	**7.8 ± 1.5**
NKT cells	86 ± 29	51 ± 17	58 ± 23	44 ± 29
B cells	180 ± 28	173 ± 39	191 ± 55	149 ± 57
αβ T cells	**1217 ± 78**	**1060 ± 145**	**1293 ± 188**	**748 ± 166**
T helper cells	**857 ± 80**	**719 ± 106**	**870 ± 142**	**519 ± 130**
% of $CD25^+$	16.6 ± 4.4	13.2 ± 4.54	2.4 ± 0.6	18.5 ± 8.4
% of $CD28^-$	**5.8 ± 1.9**	**1.3 ± 0.6**	**2.1 ± 0.9**	**0.19 ± 0.08**
% of $CD69^+$	0.52 ± 0.22	0.86 ± 0.20	0.56 ± 0.15	1.25 ± 0.37
T cytotoxic cell	**285 ± 35**	**268 ± 40**	**335 ± 49**	**179 ± 48**
% of $CD25^+$	**7.3 ± 3.8**	**10.4 ± 4.1**	**2.4 ± 0.6**	**19.8 ± 7.6**
% of $CD28^-$	**33.3 ± 3.3**	**22.4 ± 3.7**	**21.9 ± 4.7**	**24.3 ± 6.6**
% of $CD69^+$	1.06 ± 0.20	1.52 ± 0.43	0.93 ± 0.19	2.30 ± 0.93
T regulatory cells	54 ± 5	61 ± 9	66 ± 11	55 ± 16
% of $CD39^+$	47 ± 5	53 ± 5	58 ± 7	46 ± 9
γδ T cells	33.6 ± 5.7	30.2 ± 6.1	34 ± 7.5	25 ± 11
% of $CD28^-$	67 ± 10	45 ± 9	45 ± 11	46 ± 15
% of $CD69^+$	5.20 ± 1.28	4.98 ± 1.40	3.32 ± 0.94	7.21 ± 2.90
Vδ2 cells	16.6 ± 4.6	21.5 ± 6.1	23 ± 7.2	19 ± 9.7
% of $CD28^-$	61 ± 11	39.5 ± 9	40.0 ± 12.3	38.7 ± 16.5
% of $CD69^+$	**2.41 ± 0.74**	**3.48 ± 1.35**	**1.44 ± 0.51**	**6.19 ± 2.83**

Table 5: Absolute numbers or frequencies and the standard error of the different leukocyte subpopulations as well as the percentages of the expression of activation and differentiation markers in stroke and control patients; statistically significant differences are highlight in bold font.

5.1.3 Frequencies of leukocyte subsets in the cerebrospinal fluid

The cerebrospinal fluid is examined for diagnosis of a variety of neurological diseases. In stroke, CSF is of diagnostic value if rare causes like vasculitis or other autoimmune diseases are suspected. It has been shown that an identical clone can be found both in the CSF as well as in the brain parenchyma in patients diagnosed with multiple sclerosis (Skulina, Schmidt et al. 2004). In the present study, we examined paired CSF samples of 10 control and 12 stroke patients. Out of these 12, eight patients had small subcortical strokes and four suffered from larger cortical strokes. In all cases lymphocytes were the most abundant population in the CSF. We did not observe any differences in the frequency of lymphocytes in the CSF or the

CSF-blood ratio between stroke and control patients. The percentage of NK and NKT cells was decreased in the CSF and we found a slight trend towards reduced numbers of NK and NKT cells in the CSF of stroke patients (Fig. 17).

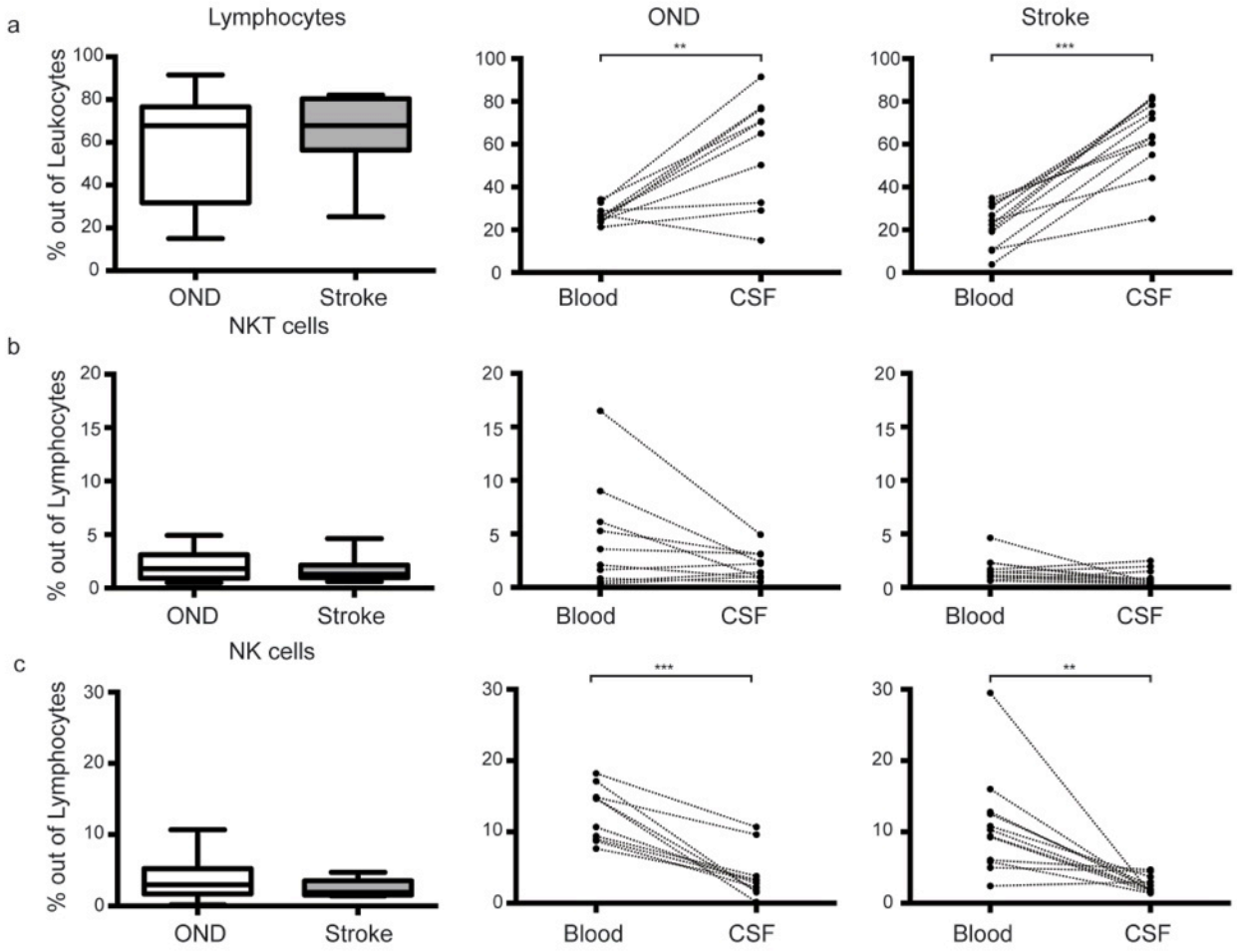

*Figure 17: Median values of frequencies (solid bar) of lymphocytes (**a**) out of the leukocytes as wells as NKT cells (**b**) and NK cells(**c**) out of the CSF lymphocytes with interquartile range (bar width) and minimum/ maximum value (whiskers) across the quartile as well as a comparison of the frequencies of these cells in blood and CSF (middle and right panel); **p < 0.01 and *** p < 0.001.*

T cells account for the majority of lymphocytes in the CSF. Stroke patients showed a significant increase of αβ T cells in the cerebrospinal fluid. We also observed a higher proportion of T helper cells in the CSF compared to the peripheral blood in these patients. However, we could not detect a difference in the frequency of the CSF T helper cells between stroke and control. We observed a slight trend towards reduced numbers of γδ T cells in the CSF of stroke patients. The γδ T cell proportion of overall T cells was even lower in this compartment. This effect was more pronounced in stroke patients. The Vδ2 subset did not show any difference between stroke and control patients, and the frequency in the CSF was similar to the one found in the peripheral blood (Fig. 18).

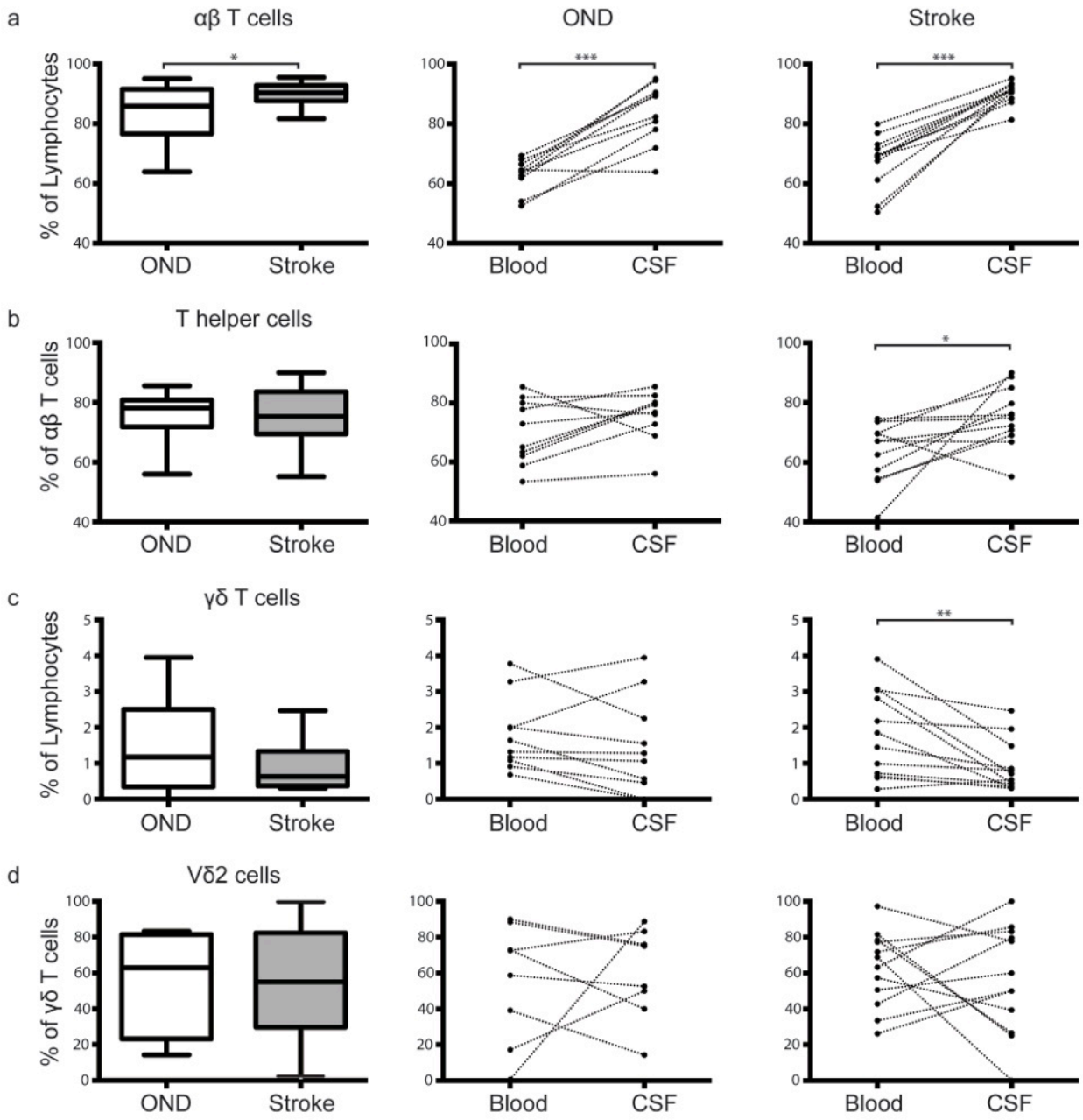

*Figure 18: Median values of frequencies (solid bar) of αβ T cells out of the lymphocytes (**a**), T helper cells as well as (**b**) γδ T cells out of the T cells(**c**) and Vδ2 cells out of the γδ T cells (**d**) with interquartile range (bar height), minimum/maximum value (whiskers) across the quartile and a comparison of the frequencies of these cells in blood and CSF (middle and right panel); * $p < 0.05$, **$p < 0.01$ and *** $p < 0.001$.*

	Peripheral Blood		CSF	
	OND	Stroke	OND	Stroke
Lymphocytes	27.0 ± 1.3 %	20.4 ± 2.7 %	57.8 ± 7.9 %	65.1 ± 5.0 %
NK cells	12.5 ± 1.2 %	10.8 ± 1.8 %	3.90 ± 1.09 %	2.50 ± 0.34 %
NKT cells	4.59 ± 1.60 %	3.47 ± 1.15 %	2.05 ± 0.43 %	1.60 ± 0.34 %
αβ T cells	**62.8 ± 1.8 %**	**66.7 ± 2.3 %**	**83.7 ± 3.2 %**	**90.6 ± 1.0 %**
T helper cells	70.1 ± 3.5 %	67.3 ± 3.5 %	75.8 ± 2.7 %	75.3 ± 2.8%
γδ T cells	1.79 ± 0.32 %	1.89 ± 0.38 %	1.44 ± 0.43 %	0.89 ± 0.20 %
Vδ2 cells	54.1 ± 9.2 %	64.1 ± 5.8 %	60.0 ± 9.0 %	56.4 ± 8.7%

Table 6: Frequencies and standard error of the different leukocyte subpopulations in the CSF and peripheral blood from stroke and control patients.

5.1.4 IFNγ and IL-17 production by T cells in the cerebrospinal fluid

γδ T cells are important sources of IL-17 in the mouse model of stroke. The present study analysed the cytokine profile of CSF T cells, mainly focusing on the γδ T cells. Considering the limited amount of cerebrospinal fluid and the low cell number it was essential to expand the cells prior to the staining.

5.1.4.1 Expansion of CSF T cells

γδ T cells are of relatively low quantity in the CSF. We tested different stimuli to increase the number of these cells (see Table 3, 4.2.6). Stimulation with PHA (phytohaemagglutinin), IL-2 and irradiated feeder cells showed an expansion of T cells in general (Fig. 19a). All T cell subsets proliferated well, but we could not obtain enough γδ T cells to perform a representative staining. Simultaneous stimulation with PHA and the phosphoantigen HDMAPP (1-hydroxy-2-methyl-2-buten-4-yl-4-diphosphate) (Fig. 19b), a specific stimulus for the Vδ2 cells, could not reach sufficient expansion of γδ T cells. This result was most likely a consequence of a superior growth of αβ T cells due to competition for IL-2. The stimulation with HDMAPP only, which is not recognised by αβ T cells, led to a substantial growth of γδ T cells (Fig. 19c). The presence of feeder cells was not required any longer, since the non-peptide molecule HDMAPP does not promote proliferation in a classic fashion. No costimulatory signals for conventional T cells were needed under this condition.

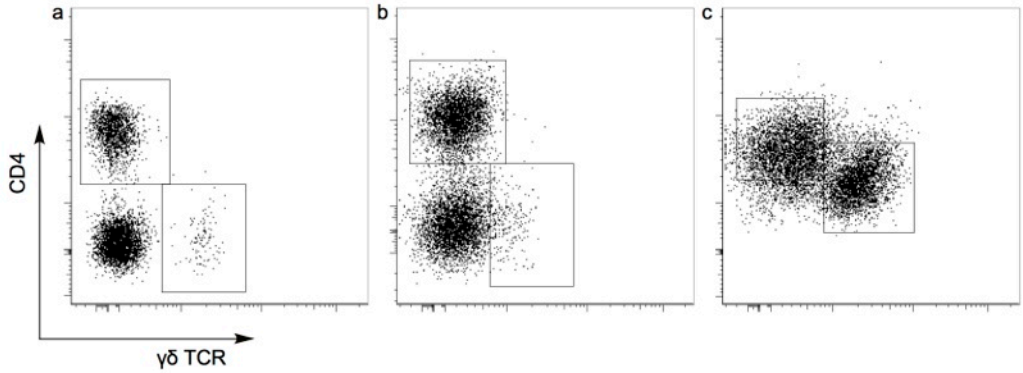

*Figure 19 : Proliferation of CD3⁺ CSF cells after two weeks of expansion with PHA (**a**), PHA and HDMAPP (**b**) and HDMAPP only (**c**).*

5.1.4.2 Cytokine profile of expanded Vδ2γ9 CSF cells

The cytokine profile of expanded CSF γδ T cells was independent of the used stimulus (data not shown). The majority of γδ T cells produced IFNγ, but we could never detect significant IL-17 production by these cells (Fig. 20a). Additionally, the cytokine production of the CD4⁺ T cells was taken into consideration for control purposes. These cells produced less IFNγ and IL-17 in small amounts (Fig. 20b). We observed no difference in the cytokine profile among stroke and control patients (Fig. 20c). The attempt to detect IL-17 in the CSF via ELISA was unsuccessful (data not shown), because the test was not sensitive enough to detect values at the physiological level around 15pg/ml (Rentzos, Rombos et al. 2010).

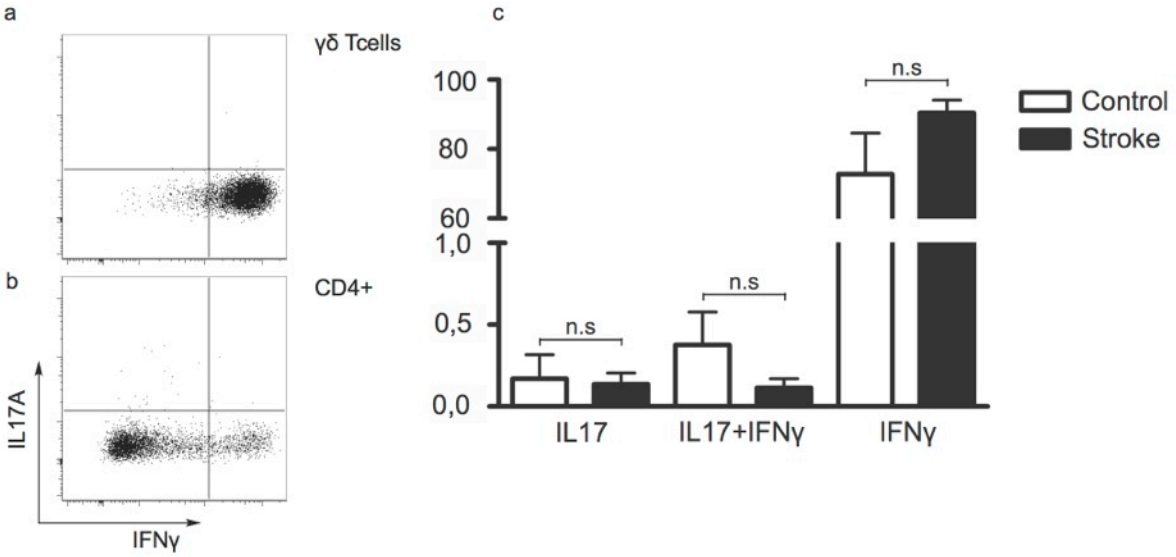

*Figure 20 : Production of cytokines by CSF γδ T cells expanded for two weeks with HDMAPP. Representative FACS plot of IFNγ and IL-17 production of expanded γδ T cells (**a**) and CD4⁺ cells. Comparison of the IL-17, IFNγ or IL-17 and IFNγ producing CSF γδ T cells from stroke and control patients.*

5.2 Differentiation and expansion of IL-17 producing Vδ2γ9 cells from healthy donors

In the mouse model of stroke it was found that IL-17 producing γδ T cells contribute crucially to the inflammatory processes after stroke (Shichita, Sugiyama et al. 2009). However, when analysing γδ T cell populations in the blood and CSF of stroke patients, we did not find

significant differences regarding this population. It is known that γδ T cells of mice and humans show major differences, so we addressed the question if human γδ T cells could produce inflammatory cytokines that are relevant in the mouse model of stroke. Therefore, we examined phenotype and functional properties of these cells in healthy donors.

5.2.1 Phenotype of *ex vivo* peripheral Vδ2γ9 cells

γδ T cells are known to produce IL-17 in response to various microorganisms, as well as rapidly reacting with IFNγ production upon stimulation. To determine the cytokine production of *ex vivo* γδ T cells of healthy individuals, PBMCs from three donors were taken and an intracellular cytokine staining for IL-17 and IFNγ was performed. We found that 45% of the γδ T cells produced IFNγ, but no substantial IL-17 production was observed (Fig. 21 a,b). Since it is known that a subset of IL-17 producing Vδ2γ9 cells expresses CCR6 and CD161, we performed a surface staining for these markers from fresh blood of three different donors. Ten percent of the γδ T cells were double positive for CCR6 and CD161, mainly the Vδ2 subtype (Fig. 21c). A cytokine staining conducted in parallel from the same blood did not show IL-17 producing γδ T cells (data not shown). The staining for the expression of the IL-23 receptor (IL-23R), whose ligand enhances the IL-17 production and is a marker for Th17 cells, revealed 1% IL-23R$^+$ γδ T cells (Fig. 21d). This data indicates that *ex vivo* γδ T cells from healthy individuals clearly produce IFNγ and express markers associated with IL-17 production, but do not produce IL-17 in this context.

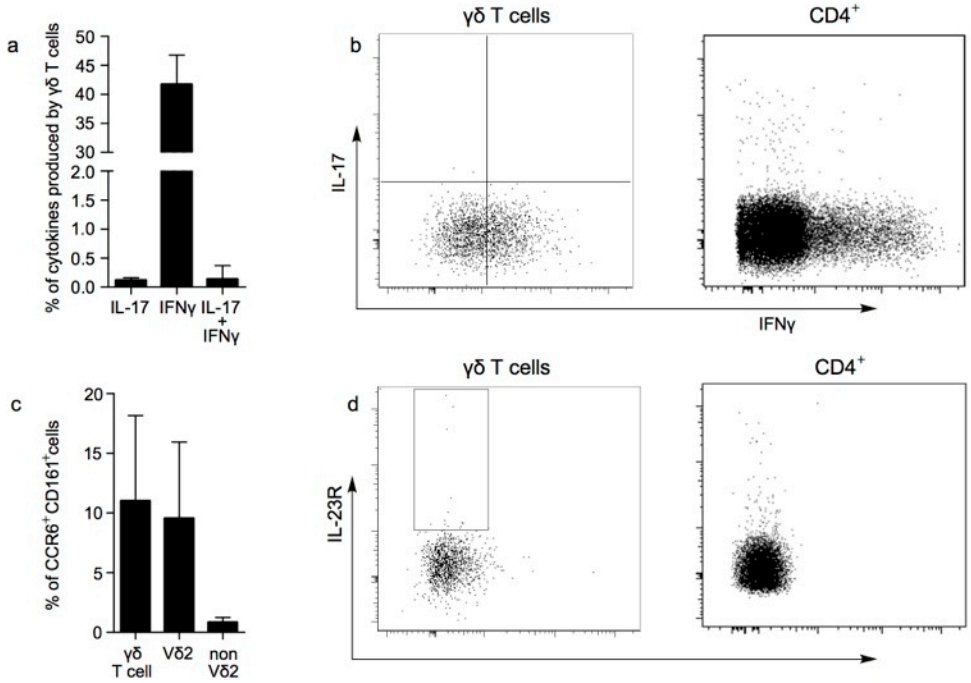

*Figure 21: Cytokine production and phenotype of ex vivo Vδ2 cells from healthy donors. Diagram of the IL-17, IFNγ as well as IL-17 and IFNγ double producing Vδ2 cells (**a**). Representative FACS plot of the IFNγ and IL-17 cytokine production of ex vivo Vδ2 cells (**b**). CCR6 and CD161 double positive ex vivo γδ T cells, Vδ2 and non Vδ2 (**c**) Expression of the IL-23 receptor by γδ T cells and CD4$^+$ cells (**d**).*

5.2.2 Expansion of γδ T cells with different stimuli

Phosphoantigens like HDMAPP (1-hydroxy-2-methyl-2-buten-4-yl-4-diphosphate) are known to sufficiently expand γδ T cells. We compared the proliferative capacity of whole PMBCs in response to a specific stimulus (HDMAPP) for the γδ T cells and a polyclonal TCR activator (OKT3) after one week. The FACS analysis showed that almost all γδ T cells proliferated upon stimulation with PHA. HDMAPP stimulation did not induce proliferation in all γδ T cells, since it only exclusively expands the Vδ2γ9 subset. However, only considering the proliferating cells, HDMAPP stimulation resulted in much stronger proliferation of the γδ T cells (Fig. 22a).

Furthermore, we investigated the effect of HDMAPP stimulation on the cytokine profile of cells. We expanded Vδ2 cells for two weeks (Fig. 22b) and observed a strong upregulation of IFNγ production up to 95%, but no substantial IL-17 production (Fig. 22c). Thus, Vδ2γ9 expand sufficiently in response to HDMAPP and change their cytokine profile towards a high IFNγ production, but do not make IL-17.

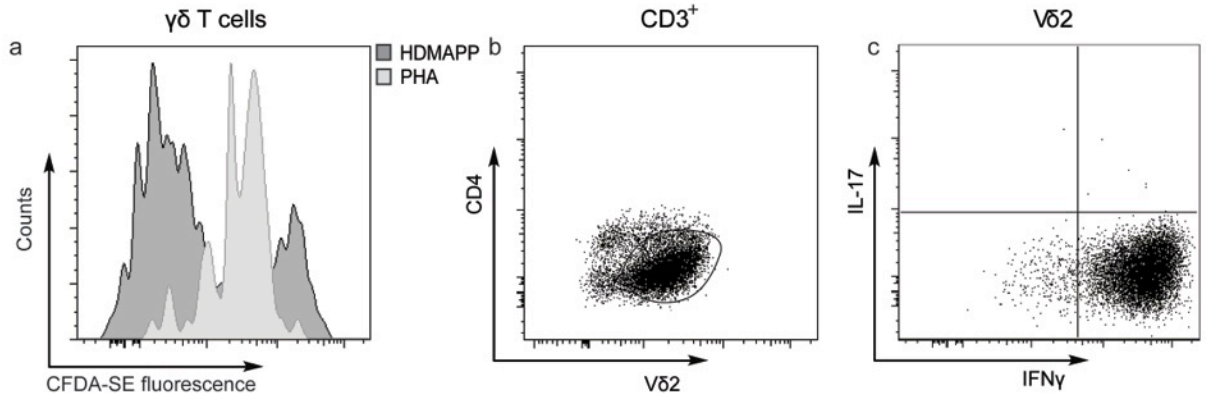

*Figure 22: Proliferation and cytokine production of γδ T cells in response to HDMAPP. Histogramm of CFSE-labelled γδ T cells after stimulation with HDMAPP or PHA (**a**). Phenotype of HDMAPP stimulated T cells after one week (**b**) and IL-17 and IFNγ production of these Vδ2 cells (**c**).*

5.2.3 Stimulation of Vδ2γ9 cells in the presence of IL-23, IL-1β and TGFβ

The *in vitro* requirements for generating IL-17A-producing Vδ2γ9 cells have not been clearly identified yet, but it has been published that the stimulation with HDMAPP, IL-23, IL-1β and TGFβ is sufficient to induce IL-17 production in Vδ2γ9 cells (Ness-Schwickerath, Jin et al. 2010). We stimulated PBMCs from healthy donors with different combinations of these cytokines and HDMAPP for either seven, 13 or 20 days (Fig. 23a). Stimulation with PMA and ionomycin was performed for five hours or overnight to further verify the condition. Surprisingly, all attempts to generate IL-17A producing Vδ2γ9 cells remained unsuccessful. $CD4^+$ cells, however, which were taken as control, showed an increase of IL-17A production upon stimulation with IL-23, IL-1β, IL-6 and TGFβ (Fig. 23a). These results indicate that, in our settings, the stimulation of Vδ2γ9 cells with IL-23, IL-1β and TGFβ does not induce IL-17 production.

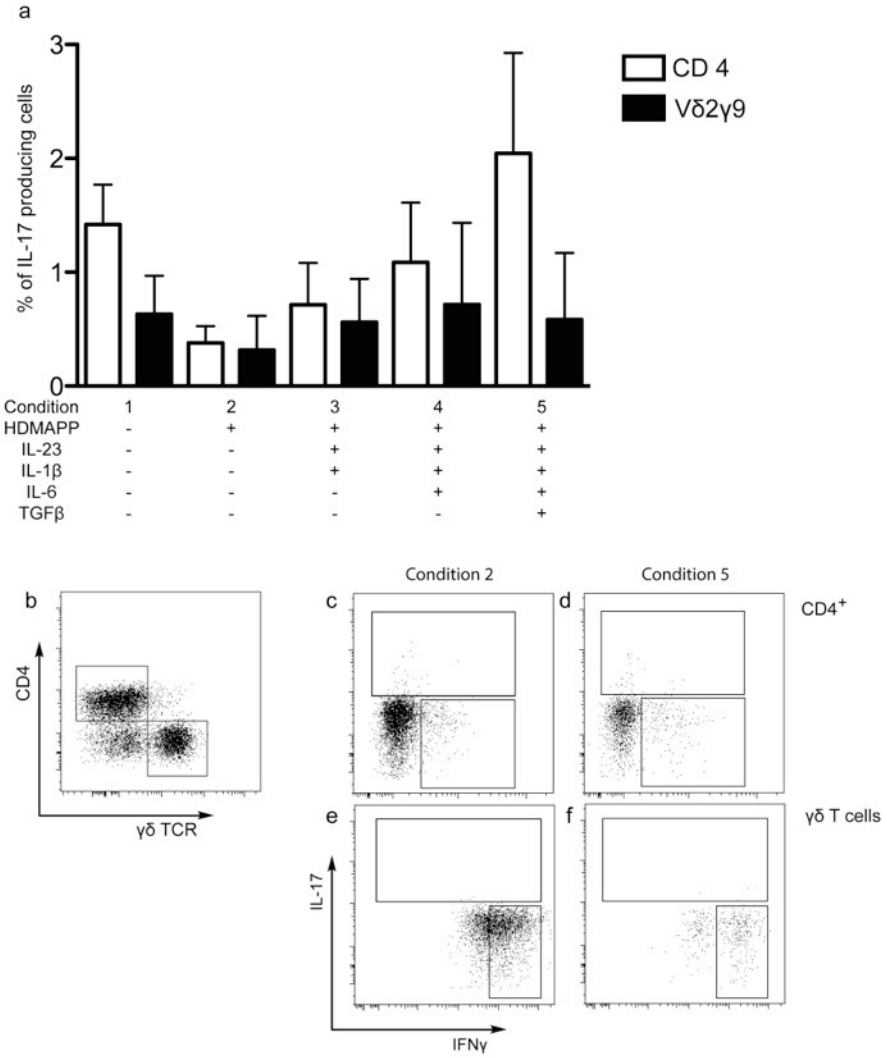

*Figure 23: IL-17 production of CD4⁺ and Vδ2γ9 cells after polarisation with HDMAPP, IL-23, IL-1β, IL-6 and TGFβ. Diagram of IL-17 production by CD4⁺ and Vδ2γ9 (**a**). Separation of polarised Vδ2γ9 cells and CD4⁺ cells (**b**). Representative FACS plot of the IFNγ and IL-17 production by CD4⁺ (**c,d**) and Vδ2γ9 (**e,f**) exemplary on condition 2 and 5.*

5.2.4 Cytokine production by γδ T cells in the context of an infectious environment

Phagocytosis of infected apoptotic cells is a relevant physiological stimulus for αβ T cells to produce IL-17 in mice (Torchinsky, Garaude et al. 2009). We examined if this is also applicable to human γδ T cells. Therefore, we first cocultured DCs with apoptotic neutrophils to obtain a dendritic cell conditioned medium (DCCM). Then, PBMCs were cultured in this DCCM in either the absence or presence of OKT3, IL-6 and IL-23. A cytokine staining was performed after three days. The conditioned medium with the addition of the cytokine IL-6 (condition 4), mimicking the infectious environment, was expected to be sufficient to prime IL-17 production. More than 85% of the γδ T cells produced IFNγ (Fig. 24c) upon stimulation with the DCCM and IL-6, but no substantial IL-17 production was observed (Fig. 24c). The addition of IL-23 did not change the cytokine profile. Approximately 15 % of the CD4⁺ cells produced IFNγ, but did not show any upregulation of IL-17 either, although condition 4 had

been shown to be optimal in mice. Furthermore, we observed a decrease of IL-17 production when both OKT3 and IL-23 where present. Thus, imitation of an infectious environment does neither induce IL-17 production in human γδ T cells, nor in human $CD4^+$ cells.

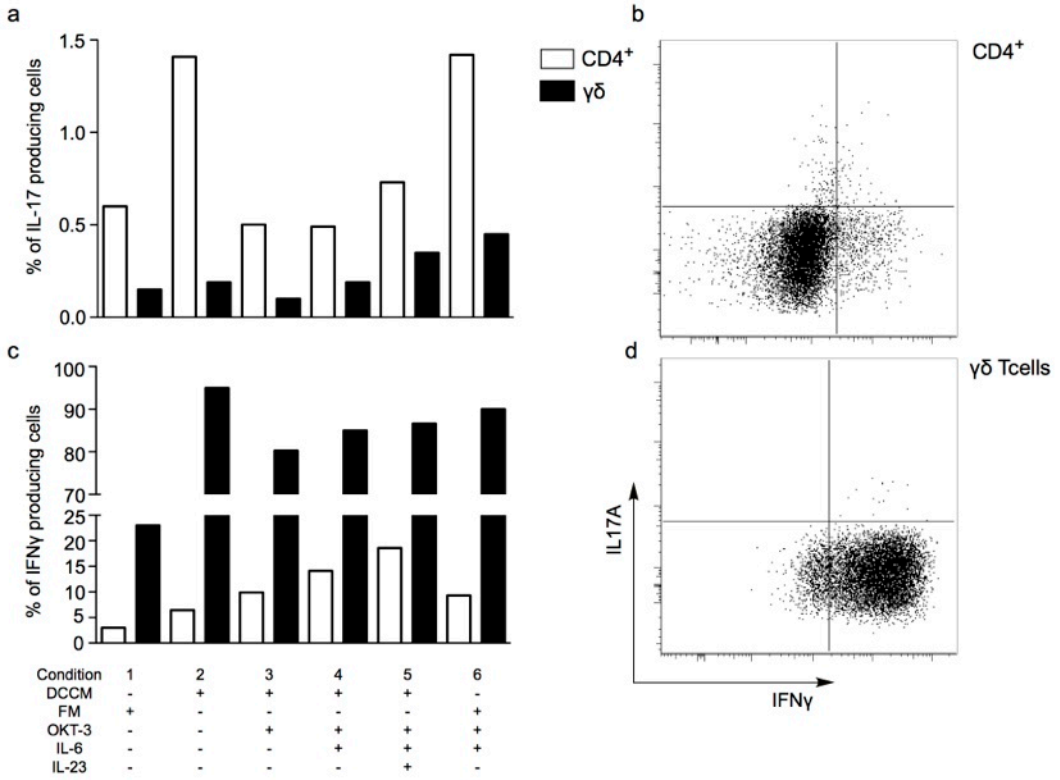

*Figure 24: Production of cytokines by γδ T cells and $CD4^+$ in an infectious environment. The diagram shows the production of IL-17 (**a**) and IFNγ (**c**) by the T cell subtypes after stimulation. Representative FACS plot of a $CD4^+$ (**b**) and γδ T cell (**d**) intracellular staining as an example on condition 4.*

5.2.5 Effect of direct TLR stimulation on the cytokine production by γδ T cells

Engagement of TLR 1 and 2 enhances the proportion of IL-17 producing γδ T cells in mice (Martin, Hirota et al. 2009). Also human γδ T cells express functional TLRs. To investigate if human γδ T cells can also respond to TLR agonists with IL-17 production, PBMCs were stimulated with ligands for TLR agonists in the presence of HDMAPP and IL-2. We assessed the cytokine production on day three. Although we found a slight upregulation of IL-17 producing γδ T cells upon stimulation with ligands for TLR 1, 2 and 4, we did not consider the results to be representative, due to the small number of γδ T cells (Fig. 25a). To verify the findings of the previous experiment and increase the cell number, we sorted γδ T cells by flow cytometry, and subsequently stimulated them with agonists for TLR 1 and 2, 2 and 4 in the presence or absence of IL-23 and OKT-3. We took $CD4^+$ cells as control and performed the cytokine staining on day five. Ninety percent of the γδ T cells produced IFNγ, but we did not detect substantial IL-17 production (Fig. 24c). Furthermore, we found no changes between the different conditions. $CD4^+$ cells instead, produced between 6.3% and 10.9% IFNγ and we

observed a strong increase in IL-17 production (17,1 %) upon stimulation with the TLR 2 ligand, OKT3 and IL-23 (Fig. 25b). In addition, we took freshly heat killed listeria monocytogenes (TLR 2 agonist) to confirm the results of the experiments with the commercially available TLR ligands. HDMAPP or OKT3 were added to provide a sufficient activation stimulus as well as the cytokine IL-23, and cells were stimulated for five days. Consistent with the other experiments we found a high IFNγ, but no IL-17 production by γδ T cells (data not shown). According to these observations, direct TLR engagement does not have an inducing effect on the IL-17 production of γδ T cells.

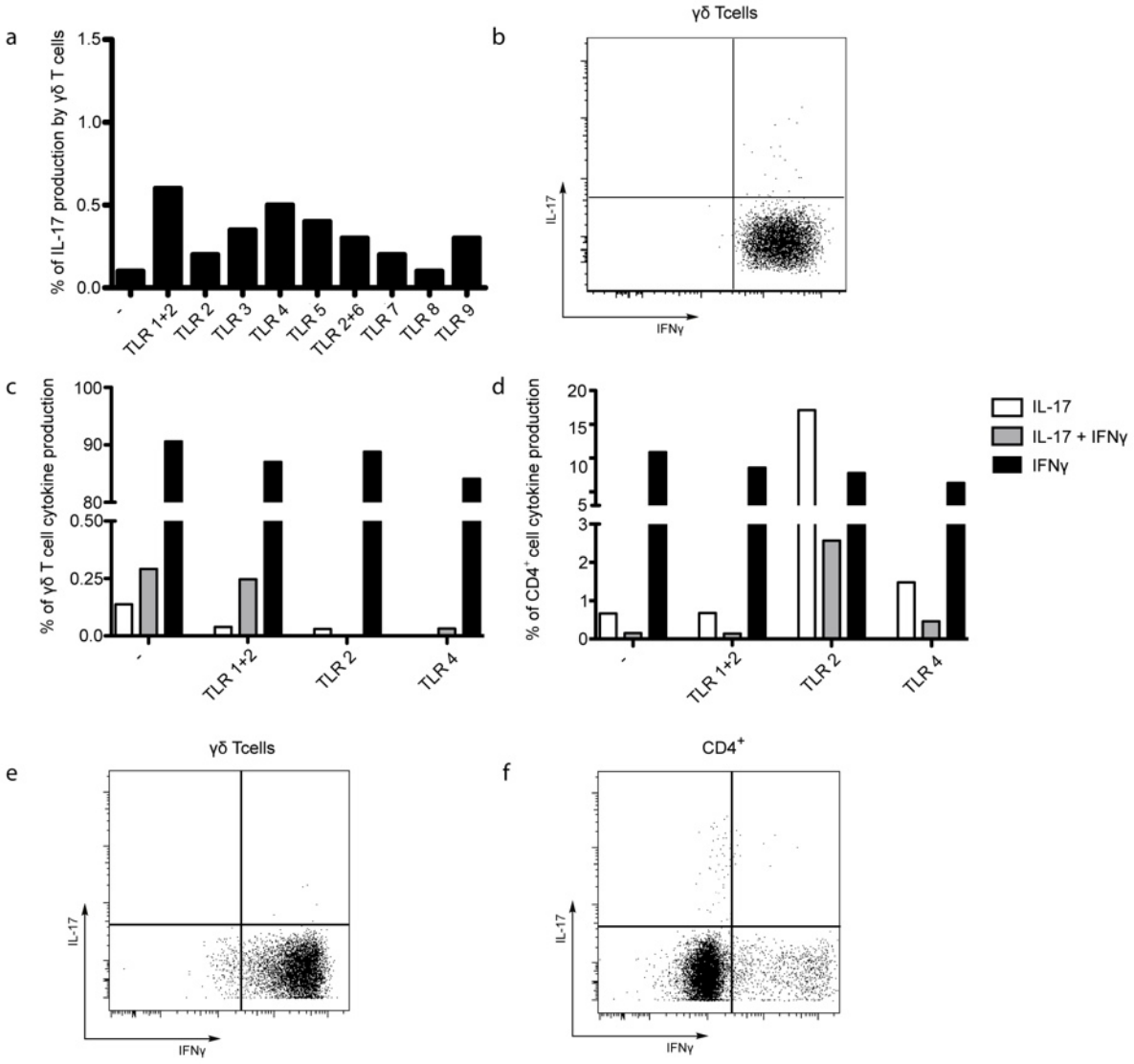

*Figure 25: IL-17 and IFNγ production by γδ T cells after different stimulation strategies with TLR ligands. IL-17 production after stimulation with ligands for TLR 1-9 in the presence of HDMAPP and IL-2 (**a**) and representative FACS plot of γδ T cells stimulated with an agonist for TLR 1+2 (**b**). IFNγ and IL-17 production of sorted γδ T cells and CD4+ cells after the stimulation with TLR1+2, 2 and 4 agonists (**c**,**d**) and a representative FACS plot of γδ T cells and CD4+ cells stimulated with TLR1+2 (**e**,**f**).*

	OKT3	IL-23	CD4+ IL17	CD4+ IL17 IFNγ	CD4+ IFNγ	γδ T cells IL17	γδ T cells IL17 IFNγ	γδ T cells IFNγ
-	-	-	0,6	0,1	7,7	0,0	0,1	70,2
-	-	+	0,7	0,2	10,3	0,0	0,1	86,2
-	+	-	1,0	0,2	9,9	0,0	0,0	91,0
-	+	+	**0,7**	**0,2**	**10,9**	**0,1**	**0,3**	**90,6**
TLR 1+2	-	-	0,7	0,2	9,3	0,0	0,0	92,7
TLR 1+2	-	+	0,7	0,3	10,4	0,0	0,1	88,5
TLR 1+2	+	-	0,6	0,1	8,0	0,0	0,0	89,8
TLR 1+2	+	+	**0,7**	**0,1**	**8,6**	**0,0**	**0,2**	**87,0**
TLR2	-	-	0,6	0,2	9,2	0,0	0,0	94,1
TLR2	-	+	0,8	0,2	8,9	0,0	0,1	90,3
TLR2	+	-	0,4	0,0	6,3	0,1	0,1	86,4
TLR2	+	+	**17,1**	**2,6**	**7,8**	**0,0**	**0,0**	**84,1**
TLR4	-	-	0,7	0,1	8,6	0,1	0,0	91,5
TLR4	-	+	0,7	0,3	9,1	0,0	0,1	94,6
TLR4	+	-	0,4	0,1	8,8	0,0	0,0	88,9
TLR4	+	+	**1,5**	**0,5**	**6,3**	**0,0**	**0,0**	**88,8**

Table 7: IFNγ and IL-17 production of the sorted γδ T cells and CD4+ cells; results marked in bolt were taken for figure 25 c and d.

5.2.6 Effect of TLR-stimulated macrophages on the cytokine production of γδ T cells

To investigate the effect of TLR-activated antigen presenting cells (APC) on the IL-17 production of γδ T cells, PBMCs were cocultured with TLR-pre-activated macrophages (MΦ) in the presence of HDMAPP and IL-2. After five days a cytokine staining was performed. None of the TLR agonists could sufficiently stimulate macrophages to produce substances inducing IL-17 production in γδ T cells. IFNγ positive cells remained at a relatively low level between 10% to 40% (Fig. 26a,c,d), although HDMAPP was present. $CD4^+$ cells, which were used for control purposes, produced between 1.5% to 5% IFNγ. We could not recognise a pattern that explains the alterations of the cytokine production in response to the different TLR ligands. The IL-17 production by conventional T cells, however, was enhanced for all tested TLRs in the presence of macrophages and macrophage conditioned medium (MCM) (Fig. 26b,d,f). These results indicate that TLR stimulated APCs have no effect on the IL-17 production of γδ T cells, but that their presence and the mediators produced might have an influence on Th17 cells.

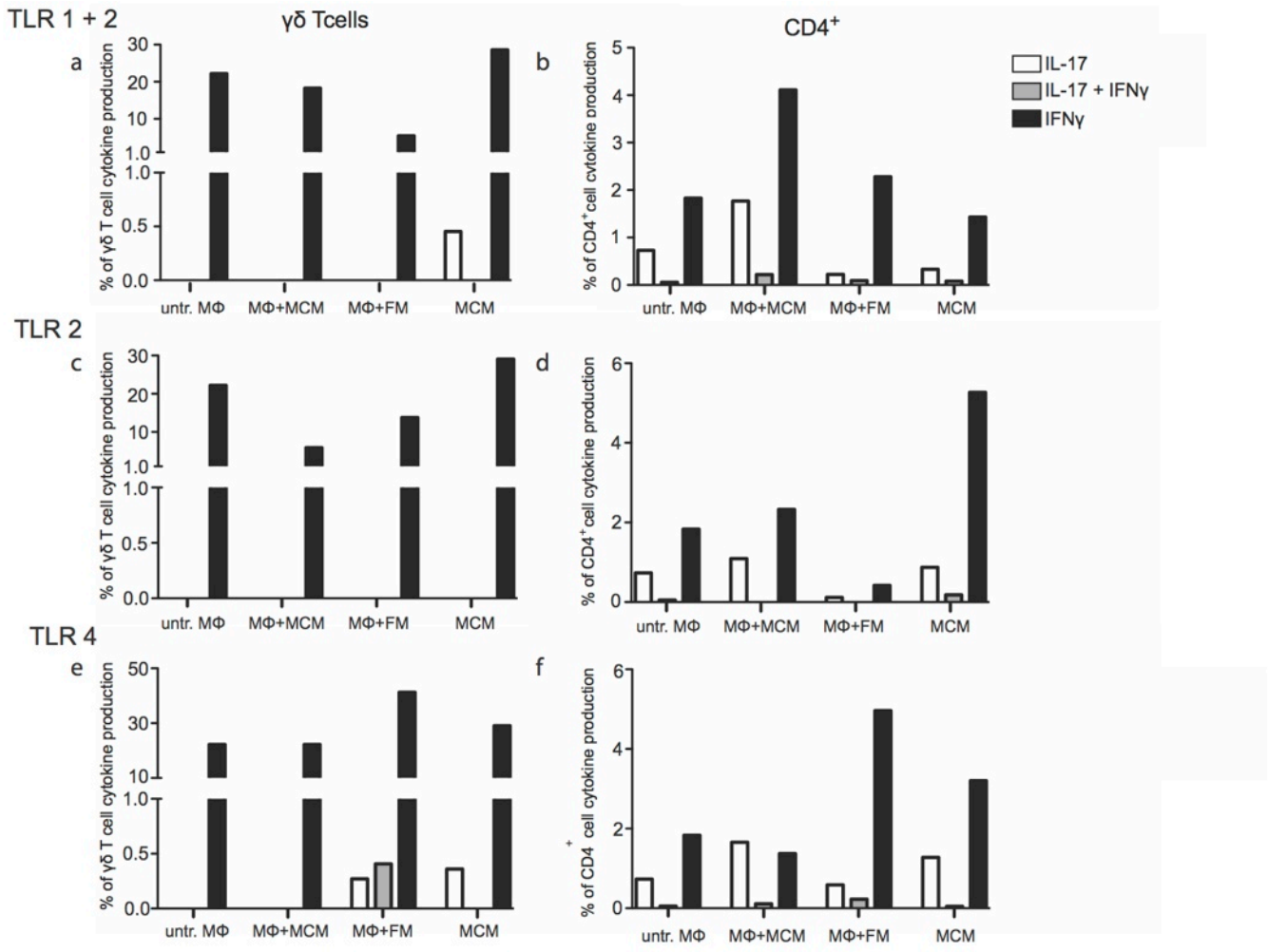

*Figure 26 :IL-17 and IFNγ production of T cell subsets after indirect TLR stimulation. Cytokine production of γδ T cells and CD4⁺ with TLR1+2 (**a**,**b**), TLR 2 (**c**,**d**) or TLR4 (**e**,**f**) stimulated macrophages and MCM, stimulated macrophages and fresh medium (FM) or MCM only.*

5.3 Impact of IL-17 on the production of neutrophil chemoattractants

IL-17 mediates the recruitment of neutrophils to the site of inflammation by stromal cells. To observe if this is also applicable for brain endothelial cells, we investigated the effect of IL-17 on the human immortalised brain capillary endothelial cell line hCMEC/D3. The stimulation with TNFα alone already lead to an increased mRNA expression of IL-8, CCL2 and CXCL6. The increase correlated positively with rising concentration of the TNFα. The addition of IL-17 enhanced the production of the neutrophil chemoattractant IL-8 (Fig. 27a) and CXCL6 (Fig. 27c) and we observed a synergistic effect of IL-17 and TNFα. This was most prominent for IL-8, using a suboptimal concentration of TNFα. For control purposes, we also assessed the effect of IL-17 and TNFα on the production of the monocyte chemoattractant CCL-2 and we could not detect a synergistic effect (Fig. 27b). Thus, IL-17 acts on brain endothelial cells and possibly other stromal cells expressing the IL-17 receptor by enhancing the production of neutrophil chemoattractants.

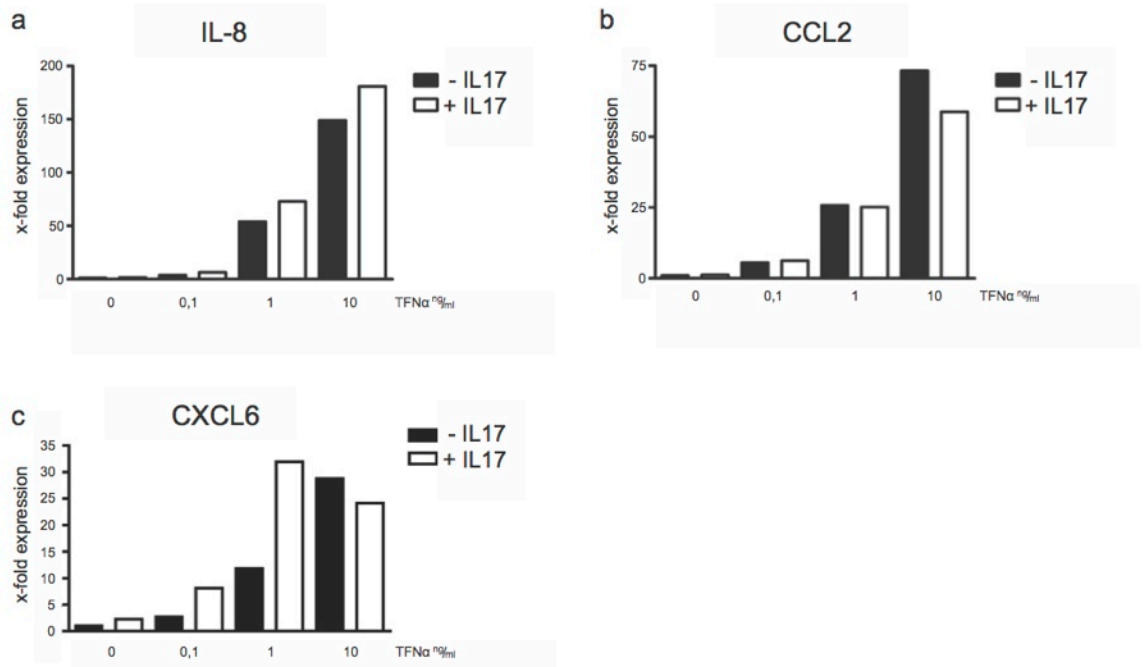

*Figure 27: IL-17 synergises with TNFα for the production of neutrophil chemoattractants. Relative gene expression of IL-8 (**a**), CCL2 (**b**) and CXCL6 (**c**) by brain endothelial cells after stimulation with increasing concentrations of TNFα in the absence or presence of IL-17.*

5.4 NET formations in the sera of stroke and control patients

The absolute number of neutrophils in the peripheral blood of humans correlates positively with the infarct size after stroke (Buck, Liebeskind et al. 2008). To find signs of neutrophil activation in the sera of stroke patients, we analysed the sera for the formation of neutrophil extracellular traps (NETs). We observed a significant increase of NETs in the sera of stroke patients compared to the age-matched controls (Fig. 28). To exclude the effect of post-stroke infections like pneumonia and urinary tract infections that are frequent in patients after stroke, the patients with a CRP higher than five were eliminated. Comparison of controls and non-infected stroke patients still showed a trend towards increased levels of NET formation, although the results where not significant. Hence, there is evidence for increased neutrophil activation in the periphery after stroke.

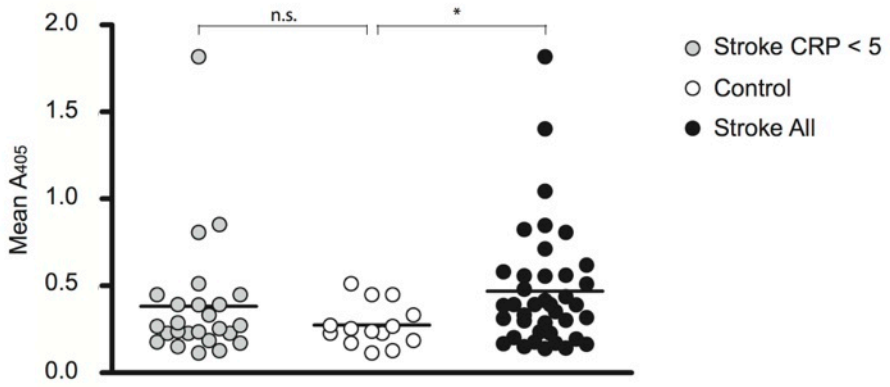

*Figure 28: Relative quantification of MPO-DNA complexes in serum samples of stroke patients and control patients measured by ELISA (n controls = 14, n stroke all= 39, n stroke CRP < 5 =25) ; * p < 0.05.*

6 Discussion

Postischaemic inflammation is considered a cause rather than a consequence of brain injury. Resident as well as invading immune cells contribute to neuronal tissue damage. This study investigates the role and interaction of invading leukocyte subsets in humans, with the focus on γδ T cells, IL-17 and neutrophils.

6.1 Changes of leukocyte subsets in the peripheral blood

Analysis of the peripheral blood leukocytes from stroke patients reflects both the inflammatory immune reaction in the brain as well as the following systemic immunosuppression. First of all, we observed higher numbers of total leukocytes in stroke than in healthy patients, which is caused by a relative and absolute increase of neutrophils. This increase was only detectable in severely affected patients with large infarcted areas, suggesting that the increase of neutrophils correlates with the size of the damaged area. MRI imaging performed in humans after stroke could even show that the accumulation of neutrophils in the peripheral blood is proportional to the lesion volume (Buck, Liebeskind et al. 2008), highlighting them as a marker of stroke size and also connecting neutrophils to the severity of the inflammatory response. Masked by the increase of absolute leukocyte numbers, stroke patients showed a decrease in lymphocytes. This is mainly caused by a decrease of αβ T cells, which is due to the systemic immunosuppression. We confirmed that both T helper as well as T cytotoxic cells account for this loss (Vogelgesang, Grunwald et al. 2008; Hug, Dalpke et al. 2009; Klehmet, Harms et al. 2009; Urra, Cervera et al. 2009).

Despite the decline in numbers, our results showed that T cells display an activated phenotype in severely affected patients (Vogelgesang, Grunwald et al. 2008; Klehmet, Harms et al. 2009; Urra, Cervera et al. 2009). The activation markers CD69 and CD25 were upregulated on T helper and T cytotoxic cells. γδ T cells upregulated these activation molecules as well and the upregulation of CD69 was even more pronounced on Vδ2 cells. Although, there where no detectable changes in γδ T cell numbers in the peripheral blood, the activated status of the γδ T cells suggests an involvement in the processes of postischaemic inflammation. Whether they are important directly in the brain or in the periphery, to potentially compensate for the impaired function of conventional T cells, can not be determined though. Regarding the phenotype of the conventional T cells, we observed a drastic decrease of $CD28^-$ T helper cells in stroke patients. This decrease is in contrast to published data where an increase after stroke is described that could even be considered as a risk factor (Nowik, Nowacki et al. 2007). In that study, however, only patients with a first-ever arteriosclerosis-related ischaemic stroke were included and compared to individuals having arteriosclerosis but without history of stroke. Since $CD28^-$ T helper cells are elevated and important in arteriosclerotic plaque

destabilisation, causing vascular events in other diseases (Dumitriu, Araguás et al. 2009), this could explain the opposing results. In our study the factor arteriosclerosis was not considered, and therefore, the decrease of $CD28^-$ cells could be related to another phenomenon. The upregulation of CD28 on T cells due to the immune activation occurring after stroke and shift towards an effector phenotype might be a possible explanation.

T regulatory cells can suppress the exaggerated inflammatory response in the brain (Liesz, Suri-Payer et al. 2009). We were not able to detect an effect of stroke on the frequency of regulatory T cells, although this has been described already. One study found decreased levels in the peripheral blood at day two (Urra, Cervera et al. 2009), whereas another study observed elevated numbers of T regulatory cells in patients after stroke (Yan, Greer et al. 2009) at day seven. Assembling the results of these two studies it seems possible that the number of T regulatory cells follows a distinct time course after stroke. First, there could be an early decrease due to stress mediated release of catecholamines that leads to a rapid decline of all $CD4^+$ cells (Klehmet, Harms et al. 2009). This in turn, could be followed by recovery and even subsequent proliferation of regulatory T cells to encounter the acute inflammation induced by ischaemic stroke. Our samples were taken between day three and day five, when cell numbers possibly return to baseline after the early decline, but before proliferation.

The analysis of NK cell numbers showed a decline after stroke. Yet, other studies did not observe changes regarding these cells (Vogelgesang, Grunwald et al. 2008; Peterfalvi, Molnar et al. 2009; Urra, Cervera et al. 2009). Since our study population was relatively small, differing results are probably due to a statistic effect. Furthermore, we observed an increase of the more regulatory NK^{bright} population, indicating that they might be involved in immunosurveillance processes after stroke. Considering the limited data about the role of NK cells in stroke it is difficult to clarify whether this is only a side-effect or an influencing factor.

6.2 Distribution and cytokine profile of leukocytes in the CSF

Analysis of the peripheral blood is not suitable to directly draw conclusions about processes in the brain, since the peripheral blood also reflects the systemic immunosuppression found in patients after acute stroke. Therefore, it is not always possible to state the origin of the observed changes. To get a more specific insight into the events directly taking place in the ischaemic area, we analysed the cerebrospinal fluid of stroke patients compared to patient with other non-inflammatory diseases. CSF is a suitable compartment to look for changes in the brain and in some CNS diseases identical clones can be found both in the CSF and the brain parenchyma (Skulina, Schmidt et al. 2004). Still, it needs to be considered that the entry of immune cells into the brain is strongly regulated by the blood-brain barrier, which separates the brain from the systemic circulation. Successful diapedesis requires appropriate

interaction with the activated endothelium to first enter the perivascular space, which is not continuosly connected to the subarachnoidal space that contains the CSF. Continued migration to the brain parenchyma is allowed after interaction with glia cells that build a highly structured wall around the vessels and produce further positive migratory signals (Wilson, Weninger et al. 2010). Immune cells that are relevant in the ischaemic lesion first need to enter the brain parenchyma, before they can possibly be found in the CSF, which could be facilitated by the leakage of the blood-brain-barrier after stroke.

Compared to the controls, our analysis of the CSF shows an increase of the frequency of αβ T cells after stroke, which indicates an involvement in postischaemic inflammation. The number of γδ T cells, however, is not altered, although an important role in this context has been proposed in the mouse model (Shichita, Sugiyama et al. 2009; Gelderblom, Weymar et al. 2012). Likewise, the level of IL-17 and IFNγ, produced by the *in vitro* expanded γδ T cells from the CSF, does not differ between stroke patients and controls. These results could falsify the importance of γδ T cells, but it is more likely that the cerebrospinal fluid is not the optimal compartment to look for postischaemic inflammation-related changes. In progressive multi-focal leucoencephalopathy, for example, pathogenetically relevant T cells can only be found in the brain parenchyma itself, but not in the CSF (Aly, Yousef et al. 2011). However, this approach provides reliable results for some of the CNS associated diseases like multiple sclerosis, where an increase of IL-17 producing T helper cells is observed in the CSF (Brucklacher-Waldert, Stuerner et al. 2009). Meningitis is another disease where IL-17 producing γδ T cells are present in the CSF (Caccamo, La Mendola et al. 2011), the pathology though, takes places in the subarachnoidal space. Since meningitis patients have elevated cell numbers in the CSF, this group could perform the cytokine staining directly after the lumbar puncture. The number of CSF cells in stroke patients, however, is very low and we had to expand the cells for two weeks with HDMAPP before assessing the cytokine production. It is not clear which kind of influence the expansion with HDMAPP has for the IL-17 production. The IFNγ production of γδ T cells from healthy donors increases after two weeks and it is likely that there is a general impact on the cytokine profile and therefore the IL-17 production as well. Furthermore, it is possible that the γδ T cells only produce IL-17 in a distinct time window, and that we obtained the CSF outside this window.

6.3 Potential role of γδ T cells

In mice, γδ T cells are the main source of IL-17 in brain inflammation, and both γδ T cells and IL-17 worsen the outcome of stroke (Shichita, Sugiyama et al. 2009; Gelderblom, Weymar et al. 2012). IL-17 producing γδ T cells have also been found in a mouse model of myocardial ischaemia (Liao, Xia et al. 2012), further relating these cells to ischaemia related tissue injury.

In models of acute inflammation, γδ T cells are a major producer of the IL-17 (Martin, Hirota et al. 2009) mediating the recruitment of neutrophils, and IL-17 production by γδ T cells is essential for the migration of neutrophils to the infarcted area in stroke (Gelderblom, Weymar et al. 2012).

Considering these findings, it is surprising that we did not observe any changes of the γδ T cell population in the peripheral blood and CSF in patients after stroke. However, γδ T cells are a minor subpopulation in the peripheral blood, and even drastic changes would only have a minor impact within the distribution of all leukocytes. The cell counts in the peripheral blood are very variable (stroke: mean 30/µl; max. 64/µl; min. 1/µl; OND: mean 34/µl; max. 70/µl; min 14/µl) and the absolute number of γδ T cells decreases with age (Michishita, Hirokawa et al. 2011). Therefore a very homogenous study population considering the factor age would be needed. In the present study the age of the patients ranged from 31 to 84 years and it was unexpectedly difficult to recruit patients with severe strokes, since they had to be able to sign the informed consent. Furthermore, human and murine γδ T cells show major differences (Korn and Petermann 2012). Mice have different Vγ chains than humans, and the Vγ chains are often related to an allocation to a specific tissue. The Vδ2γ9 subset, which is the only one that can be activated by the phosphoantigens, is unique to humans and is the most abundant population in the peripheral blood of adults. This is the subset we focused on and it is not known if the human Vδ2γ9 cells reflect the IL-17 producing γδ T cells in mice. Another important feature of the human γδ T cells, which does not apply to the population in mice, is the ability to recognise antigens presented by CD1 molecules (Dutronc and Porcelli 2002). Although, human γδ T cells have been shown to produce IL-17 in various contexts (Fenoglio, Poggi et al. 2009; Poggi, Catellani et al. 2009; Caccamo, La Mendola et al. 2011; Cai, Shen et al. 2011), the physiological requirements are not defined yet.

In the present study we examined the properties of γδ T cells from healthy donors and tried to induce IL-17 production in various conditions. In line with current literature *ex vivo* γδ T cells from healthy individuals clearly produce IFNγ and express markers associated with IL-17 production, but we were not able to observe spontaneous IL-17 production. We did not succeed to reproduce published data in our laboratory (Ness-Schwickerath, Jin et al. 2010), stimulating Vδ2γ9 cells with HDMAPP, IL-23, IL-1β and TGFβ in order to induce IL-17 production. A very stringent gating strategy for the IL-17$^+$ γδ T cells as well as subtle differences in media, cytokines or serum obtained from other companies could be an explanation for the opposing results. The fact that we could neither induce IL-17 production in human γδ T cells nor CD4$^+$ cells by mimicking an infectious environment, as it has been shown for mice Th17 cells (Torchinsky, Garaude et al. 2009), might be related to the differences of these cells in between the species human and mice. Similarly, it has been

reported for mice that direct TLR engagement does have an inducing effect on the IL-17 production of γδ T cells (Shibata, Yamada et al. 2007; Martin, Hirota et al. 2009). Yet, this is not applicable for humans. These findings are consistent with published data, stating that engagement of TLR 2 and TLR 4 only has an indirect effect on human γδ T cells (Wesch, Peters et al. 2011). The indirect stimulation with TLR activated APCs, as it has been shown for Vδ1 cells in human lyme arthritis (Collins, Shi et al. 2008), shows also no effect on the IL-17 production of Vδ2γ9 cells, underlining the differences between the γδ T cell subpopulations.

Hence, human γδ T cells are not only different from mice γδ T cells, but also more resistant to IL-17 polarisation applying the standard protocols that have successfully been used for Th17 cells. So far, our results do not provide enough evidence in favour of an important role of γδ T cells in human postischaemic inflammation. To investigate if and how these cells are involved in this context, further characterisation and analysis of the requirements for activation, differentiation and cytokine production for human γδ T cells is required.

6.4 Neutrophils and IL-17 in stroke

Neutrophils have been implicated in ischaemia-related tissue injury in a wide range of pathologies including acute kidney injury, myocardial infarction and liver injury (Caldwell, Okaya et al. 2005; Chen, Sato et al. 2006; Awad, Rouse et al. 2009; Liao, Xia et al. 2012). IL-17 contributes crucially to the infiltration of neutrophils, and studies in mice models of hepatic (Kono, Fujii et al. 2011) and myocardial (Liao, Xia et al. 2012) ischaemia reperfusion injury support this hypothesis. IL-17 activates stromal cells and mediates the production of chemoattractants like IL-8 in order to promote neutrophil migration (Roussel, Houle et al. 2010). However, it can not act on neutrophils directly since they do not express a functional IL-17 receptor (Pelletier, Maggi et al. 2010). In the mice model of stroke, γδ T cells produce the IL-17 essential for the migration of neutrophils to the infarcted area (Gelderblom, Weymar et al. 2012).

We found that IL-17 enhances the production of neutrophil chemoattractants also in human brain endothelial cells. There was a mild synergistic effect of TNFα, if used in suboptimal concentrations, and IL-17 on the production of IL-8 and CXCL-6 mRNA. Furthermore, the sera taken from stroke and control patients showed evidence for an activated status of neutrophils in stroke patients. We observed elevated levels of NETs in the sera of stroke patients and these NETs can be induced *in vitro* through stimulation with IL-8 (Brinkmann, Reichard et al. 2004). NET formation is also induced by placenta-derived IL-8 in preeclampsia (Gupta, Hasler et al. 2005) and IL-8 produced by activated endothelial cells (Gupta, Joshi et al. 2010). Current literature describes higher levels of circulating DNA and

histones in stroke patients (Tsai, Lin et al. 2011) as well as increased levels of IL-8 (Kostulas, Pelidou et al. 1999; Al-Bahrani, Taha et al. 2007; Ormstad, Aass et al. 2011). The increase of DNA and histones reflects the severity of cerebral damage (Tsai, Lin et al. 2011) and might specifically relate to NET formation, since both DNA and histones are essential components of the NETs. Thus, our results support the important role of IL-17 on the infiltration of neutrophils mediated by the endothelium, although it might not be produced by γδ T cells in humans.

6.5 Transferability of the mice findings to human stroke

Most of our current knowledge about the pathophysiology and cells that contribute to postischaemic inflammation has been obtained through research in the MCAO mouse model. First of all it needs to be considered that there are significant differences in the brain morphology of humans and mice. Neuronal and glial densities are quantitatively different and the brain of mice is not gyrated. The glucose and oxygen metabolism in rodents is three times higher than in humans, and is has been proposed that the human penumbra is smaller and exists for a shorter period of time compared to mice (Dirnagl, Iadecola et al. 1999). Also the collaterals of the brain arteries vary to a great degree among different mouse strains. Some strains have been shown to have a less pronounced circulus arteriosus willisi (Barone, Knudsen et al. 1993) that ensures collateral blood flow in the case of an occlusion. The C57/BL6 strain that is often used in the MCAO model, lacks functional collaterals between the internal and external carotid artery (Hecht 2008), which are commonly important in humans. Furthermore, the MCAO model in rodents generally results in a large infarcted area, whereas minor strokes are also commonly observed in humans. Hence, the MCAO model only reflects one of the subtypes of human stroke and it is not clear if the same pathological mechanisms also contribute to postischaemic inflammation in minor strokes. Another major difference between humans and mice can be observed in the composition of the immune system. In humans, neutrophils are the predominant leukocyte population in the blood (≈ 70%), whereas in mice, neutrophils only account for 20% of the blood leukocytes and lymphocytes represent the numerically superior population (Doeing, Borowicz et al. 2003). Yet, it is not clear if this difference causes any functional consequences (Mestas and Hughes 2004). In multiple sclerosis research, however, it was shown in a clinical trial that IFNγ, which successfully suppressed the disease in mice, in fact exacerbated multiple sclerosis in humans (Panitch, Hirsch et al. 1987). The murine immune system resembles the human system remarkably well though, and only 300 genes are unique to each species (Mestas and Hughes 2004). Thus, much of the knowledge obtained from the mice model is probably applicable to humans.

IL-17 has been found in the ischaemic hemisphere of humans (Li, Zhong et al. 2005) and our study shows a positive effect of IL-17 on the production of neutrophil adhesion molecules by human brain endothelial cells. Furthermore, neutrophils have been shown to infiltrate the ischaemic lesion in humans (Mena, Cadavid et al. 2004; Price, Menon et al. 2004), which leaves little doubt about the pathogenic role of IL-17 and neutrophils in humans. Only the source of the IL-17 remains unclear.

We were not able to support the potential role of γδ T cells in humans. Several options should be taken into consideration. It is possible that we did not include the relevant subtype with the current reagents, considering the major differences of human and mice γδ T cells as well as the uniqueness of the Vδ2γ9 cells to humans. Cells expressing the Vδ1 chain could also account for the IL-17 production. It also seems possible that another leukocyte population is responsible for the IL-17 production. To identify this cell type it should be considered that maximum levels of IL-17 are found between day three to five in human stroke lesions (Li, Zhong et al. 2005). It is therefore quite likely that another cell with innate immune properties accounts for the IL-17 production. Both mucosa associated invariant T cells (MAIT cells) and human invariant NKT cells can produce IL-17 (Rachitskaya, Hansen et al. 2008; Dusseaux, Martin et al. 2011) upon stimulation with PMA or with anti-CD3 and IL-23, respectively. IL-17 producing neutrophils have also been found in a number of pathological immune processes in humans. The majority of IL-17 producing cells in the synovial fluid of patients with active inflammatory arthritis were identified as neutrophils by the expression of CD15 (Moran, Heydrich et al. 2011). In mycosis fungoides, which is the most common form of cutaneous T cell lymphoma, skin biopsies revealed IL-17 positive neutrophils (Fontao, Brembilla et al. 2011). After acute rejection of renal allografts (Yapici, Kers et al. 2011) and in ANCA(anti-neutrophil cytoplasmic antibody)-associated glomerulonephritis (Velden, Paust et al. 2012) neutrophils were the main IL-17 producers. It is remarkable that neutrophils were also found to be the main source of IL-17 in a mouse model of renal ischaemia reperfusion injury (Li, Huang et al. 2010). There is an abundance of neutrophils in ischaemic stroke lesions, and it seems possible that they amplify their own infiltration as they actually produce IL-17 themselves. Interestingly, even astrocytes are able to produce IL-17 in significant amounts (Tzartos, Friese et al, 2008), suggesting that there is a large variety of potential IL-17 producers.

However, IL-17 should not be the only focus; other mechanisms could contribute to the postischaemic inflammation as well. Conventional T cells are decreased in absolute numbers after stroke and have a long lasting impaired IFNγ production (Klehmet, Harms et al. 2009), which is due to the central nervous system mediated immunosuppression. T cells still show an activated phenotype, indicating a role in the evolvement of postischaemic inflammation. Comparative analyses of serum samples from stroke patients revealed increased levels of glial

fibrillary acidic protein (GFAP) (Herrmann, Vos et al. 2000), myelin basic protein (MBP) and neurone-specific enolase (NSE) as well as autoantibodies against MBP and NSE (Kamchatov, Ruleva et al. 2009). Another study showed an increased number of MBP-positive cells in the palatine tonsil and increased immunoreactivity to MBP was associated with larger infarct volume and poorer outcome (Planas, Gomez-Choco et al. 2012). Considering the activated status of the T cells it would be conceivable that molecules like MBP, GFAP or NSE could activate T cells, either in the periphery or even directly in the brain, and cause some kind of autoimmune-like response. Although studies in the mouse model have shown that the early detrimental effect of T cells can not be contributed to these adaptive autoimmune processes (Kleinschnitz, Schwab et al. 2010), a delayed response seems possible. T cells could easily enter the brain trough the damaged blood-brain-barrier, which is a product of the preceding inflammatory and hypoxic events. The administration of a recombinant TCR ligand against oligodendrocyte glycoprotein (MOG)-reactive T cells, for example, significantly reduced the infarct size in mice (Akiyoshi, Dziennis et al. 2011; Dziennis, Mader et al. 2011). This would fit the concept of adaptive immunity and could be an explanation for the activated status of conventional T cells.

To summarise, there is abundant evidence for the involvement of IL-17 and neutrophils, as well as conventional and unconventional T cells in the pathophysiology of postischaemic inflammation. IL-17 is found in the ischaemic lesion and in the periphery; neutrophils infiltrate the lesion and show an activated phenotype. Similarly, T cells are activated. With current knowledge it cannot be determined whether this is crucial for the processes mediating the neutrophil infiltration via IL-17 or if this is a parallel pathway. Other possibilities include a presentation of CNS-antigens or even other yet unknown mechanisms independent of the the IL-17-neutrophil axis. Still, further research is needed to directly identify the IL-17 producing cell as this cytokine is one of the key players in stroke pathophysiology.

7 Summary

This study was designed to gain further insight into the pathophysiologic events following ischaemic stroke in humans and to find evidence for the transferability of the mice findings to humans. We analysed peripheral blood and CSF leukocytes as well as the sera of patients suffering from acute ischaemic stroke. We furthermore aimed to characterise phenotypical and functional properties of γδ T cells from healthy donors and performed *in vitro* stimulation of brain endothelial cells with IL-17.

We found that the peripheral blood of stroke patients reflected both the inflammatory immune reaction in the brain and the following systemic immunosuppression. We observed an increase of neutrophils and a decline of T cytotoxic as well as T helper cells, but the number of γδ T cells remained unaltered. Despite the decline in numbers, T cells displayed an activated phenotype, which was most pronounced in the Vδ2 cells. Additionally, we successfully expanded the CSF γδ T cells of some stroke patients, but could not detect significant IL-17 production by these cells. Further efforts to explore the requirements for the induction of IL-17 producing γδ T cells from healthy donors remained unsuccessful. We were not able to induce IL-17 production in Vδ2γ9 cells. Still, we were able to detect markers associated with IL-17 production. Concerning the neutrophils, we found an increase of NET production, which is associated with neutrophil activation. We also showed that stimulation with IL-17 increased the production of neutrophil-chemoattractants by brain endothelial cells.

Considering our results and current literature there is abundant evidence for the involvement of IL-17 and neutrophils in the pathophysiology of postischaemic inflammation. The source of the IL-17 in this context, however, is not known yet. We could neither find enough evidence for nor against the pivotal role of γδ T cells, like it has been proposed in the mouse model. It is crucial to first identify the physiological requirements for IL-17 production by γδ T cells in humans, before further exploring their role in the pathophysiology of postischaemic inflammation.

8 Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid
ANCA	Anti-neutrophil cytoplasmic antibody
APC	Antigen presenting cell
APC	Allophycocyanin
AF	Alexa fluor
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CCL	CC chemokine ligand
CCR	Chemokine C-C motif receptor
CD	Cluster of differentiation
cDNA	Complementary desoxyribonucleic acid
Co	Patients with cortical strokes
dsDNA	double stranded desoxyribonucleic acid
ssDNA	single stranded desoxyribonucleic acid
CFDA-SE	Carboxyfluorescein diacetat succinimidyl ester
CFS	Cerebrospinal Fluid
CNS	Central nervous system
CRP	C-reactive protein
CT	Computed Tomography
CXCL	Chemokine C-X-C motif ligand
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DCCM	Dendritic cell conditioned medium
DMAPP	Dimethylallyl-phosphate
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
dNTP	Desoxynucleotid
EBM	Endothelial basal medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FM	Fresh medium with conditioned macrophages
FOXP3	Forkhead Box Protein 3
FSC	Forward-scattered light
FSL-1	Synthetic mycoplasmal triacylated lipoprotein
GFAP	Glial fibrillary acidic protein
HBSS	$Ca^{2+}M^{+}$ free Hank‘s balanced salt solution
HDMAPP	1-hydroxy-2-methyl-2-buten-4-yl-4-diphosphate
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1	Hypoxia inducible factor 1
HKLM	Heat killed listeria monocytogenes
HMGB1	High-mobility group protein B1
HMW	High molecular weight
hr	Human recombinant
HSP60	Heat shock protein 60
ICAM-1	Intercellular adhesion molecule 1

ICAM-2	Intercellular adhesion molecule 2
IFNγ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IPP	Isopentenyl-pyrophosphate
L	Ligand
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharid
M	Medium with TLR ligands
MBP	Myelin basic protein
MCAO	Middle cerebral artery occlusion
MCM	Macrophage conditioned medium
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MΦ	Macrophages
mRNA	Messenger ribonucleic acid
NET	Neutrophil extracellular trap
NK cells	Natural killer cells
NFκb	Nuclear factor-κb
NKT cells	Natural killer like T cells
NO	Nitric oxide
NSE	Neurone-specific enolase
ODN2006	Unmethylated cytosin-phosphatidyl-guanin oligonucleotide
OND	Other non-inflammatory neurological disease
OIND	Other inflammatory neurological disease
P2X7	P2X purinoreceptor 7
Pam3CSK4	Synthetic bacterial triacylated lipoprotein
PBS	Phosphate buffered saline
PBMCs	Peripheral blood mononuclear cells
PE	Phycoerythrin
PHA	Phytohemagglutinin
PMA	Phorbol myristate acetate
Poly (I:C)	Polyinosinic-polycytidylic acid
PPR	Pattern recognition receptor
ROS	Reactive oxygen species
RT	Room temperature
rt-PCR	Real time polymerase chain reaction
Sco	Patients with small subcortical strokes
SPECT	Single photon emission computed tomography
SSC	Side-scattered light
Tc	T cytotoxic cell
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper cell
TLR	Toll-like receptors
TNFα	Tumour necrosis factor α
Treg	T regulatory cell
UTI	Urinary tract infection

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12 Eidesstattliche Erklärung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

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