IL-13 AND ITS BENEFICIAL EFFECTS ON ISCHEMIC STROKE

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Tutkimuksen tarkoituksena oli selvittää interleukiini-13 -hoidon vaikutuksia aivoinfarktin hoidossa. Vaikutuksia mallinnettiin terveillä uroshiirillä, joille aiheutettiin kirurgisesti aivohalvaus, minkä jälkeen hiiriä hoidettiin joko interleukiinivalmisteella tai lumehoidolla. Interleukiinihoidon saaneita (N = 8) verrattiin lumehoitoa saaneisiin (N = 7) hiiriin kolme päivää aivoinfarktin jälkeen hyödyntämällä värjättyjä aivoleikkeitä ja magneettikuvia sekä määrittämällä muiden sytokiinien pitoisuuksia plasmasta. Tulokset analysoitiin vertaamalla ryhmiä kaksisuuntaisessa T-testissä.

Kontrolliryhmään verratessa havaittiin, että interleukiinilla hoidetuilla hiirillä aivoinfarktin aiheuttama vaurioalue oli pienempi ja mikrogliasolujen ja makrofagien aktivaatio painottui kohti hermosoluja suojaavaa toimintaa. Tutkimuksessa havaittiin myös viitteitä astrosyyttien aktiivisuuden vähenemisestä hoitoa saaneilla hiirillä, mutta tulos ei saavuttanut tilastollista merkitsevyyttä. Hoidolla ei ollut vaikutusta ohjelmoituun solukuolemaan, aktivoituneiden mikrogliasolujen ja makrofagien kokonaismäärään eikä muiden sytokiinien pitoisuuksiin. Hoito ei myöskään vaikuttanut mikrogliasolujen tai makrofagien ilmiasua säätelevän reseptorin aktiivisuuteen.

Tutkimuksen tuloksena havaittiin interleukiini-13:n edullinen vaikutus aivoinfarktin hoidossa. Kyseessä oli kuitenkin ensimmäinen tutkimus tästä aiheesta, minkä keskeisinä jatkotutkimusaiheina ovat esimerkiksi hoidon vaikutukset eri ajankohtina, tarkat vaikutusmekanismit sekä sukupuolen ja liitännäissairauksien vaikutukset hoidon tehoon. Tutkimuksen tulokset antavat kuitenkin viitteitä siitä, että aivoinfarktiin on jonain päivänä mahdollista soveltaa toisenlaisia, madollisesti turvallisempia, hoitomuotoja kuin liuotushoito.

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LAINE, MIKA L.: IL-13 and its beneficial effects on ischemic stroke

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The aim of the study was to look at the effects of interleukin-13 treatment on ischemic stroke. The effects were modelled with healthy male mice, on which ischemic stroke was surgically induced, after which the mice were treated either with interleukin preparation or with placebo. Interleukin-treated ones (N = 8) were compared to placebo-treated ones (N = 7) 3-day post ischemia, utilizing stained brain sections and magnetic resonance images, and determing the concentrations of other cytokines from plasma. The results were analyzed by comparing the groups in two-way T-test.

When comparing to the control group, it was observed that the mice treated with interleukin had smaller lesion areas, caused by ischemic stroke, and the activation of microglia and macrophages shifted towards neuroprotective activity. On the study, indications of decreased astrocytic activity among treated mice was also observed, however, the result did not reach statistical significance. Treatment had no effect on apoptosis, the total amount of microglia and macrophages, or the concentrations of other cytokines. The treatment had also no effect on receptors regulating the expression of microglia and macrophages.

As the outcome of the study, the confirmation of interleukin-13 having a beneficial effect on ischemic stroke was observed. This was the first study regarding this topic, on which treatment effects on different time points, specific mechanisms of action, and the role of gender and co-morbidities on the effects of the treatment, are essential topics for following studies. The results of the study, however, indicate that, perhaps one day, it is possible to apply different, perhaps safer, treatment methods for ischemic stroke, other than thrombolysis.

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1. Introduction

Ischemic stroke has been estimated to have caused the death of 6,7 million people in 2015, and cardiovascular diseases kills more people annually than any other disease or insult (WHO 2016). Ischemic stroke is nowadays treated with tissue plasminogen activators (tPA) which is effective only when given within a few hours after the incident and the treatment has an increased risk for hemorrhages (Schellinger et al 2008). It has been observed that inflammation plays a role in ischemic stroke where the responses are mediated by various molecules, such as different cytokines, nitric oxide (NO) and reactive oxygen species (ROS) (Kim et al 2016). Several studies have shown (Korhonen et al 2015, Park et al 2014, Frenkel et al 2005) the neuroprotective effects of immunomodulators on ischemic stroke models, and these protective effects have been observed to be mediated through increased expression of anti-inflammatory cytokines. Since interleukin (IL) 13 is an anti-inflammatory cytokine (Kim et al 2016), IL-13 treatment on ischemic stroke could hypothetically have neuroprotective effects, thus this study is conducted.

The effects of IL-13 are studied using male mice and the ischemic stroke is induced by using a permanent surgical method (see table 1, p. 15), and the neuroprotective effects are compared to the control group at 3 days post ischemia (dpi). Treatment effects are analyzed with magnetic resonance imaging (MRI), and immunohistochemistry is used to analyze the effects on the more neuroprotective, M2-type microglia/macrophages, on the total number of microglia/macrophages on ischemic brain, on induced apoptosis, and on astrocytic activation. Cytometric bead array (CBA) was also conducted to observe the treatment's effects on expression of other cytokines. For IL-13 treatment to be neuroprotective, should it at least reduce lesion size and induce M2-type cell polarization, comparing to the control group.

The main goal of this study is to determine whether IL-13 treatment is neuroprotective in ischemic stroke. Based on these results, it is possible to carry out more specific studies about the mechanism of action, treatment adaptation on different phenotypes, such as obese or diabetic, and perhaps eventually suitability for humans experiencing ischemic stroke.

2. Background

2.1. General information

Ischemic stroke is a life-threatening insult usually caused by blood clots or atherosclerosis, and which killed approximately 6.7 million people worldwide in 2015. The major risk factors for the ischemic stroke are increased blood pressure, unhealthy diet and obesity (WHO 2016). The symptoms vary depending on location of the stroke, usually being (80 – 90 %) in the branching arteries in carotid area, especially in the middle cerebral artery (MCA). Main symptoms are hemiplegia/hemiparesthesia, dizziness, aphasia and disturbances of vision or eye movements (Roine 2016). The state is treated with tissue plasminogen activators (tPA) by causing thrombolysis, but the therapy is beneficial only a few hours after the beginning of ischemia and not suitable for everyone because of hemorrhage risk (Schellinger et al 2008). Anticoagulants are often used after the event to prevent ischemia from reappearing (Roine 2016) but they also have a lot adverse effects (PubChem 2017). These treatment issues force us to find a new possible treatment for ischemic stroke (Korhonen et al 2015).

2.1.1. Risk factors

Atherosclerosis is a major risk factor for stroke since the third of all strokes in Finland are caused by atherosclerosis or embolism, which can be caused, for example, by atrial fibrillation (Roine 2016). In atherosclerosis, several molecules and cells, such as lipoprotein particles and monocytes, infiltrate the sub-endothelial space and begin to secrete chemokines and create plagues. The plague has a lipid- and cholesterol rich center with calcification and various inflammatory cells on the edge. As the plague grows, it becomes more unstable and can eventually be ruptured, causing a atherothrombotic occlusion. Although, even without breaking apart, the plague can cause focal ischemia and thrombus formation if it is large enough to significantly reduce the blood flow to the tissue (Gimbrone & García-Cardeña 2016). Increased plasma cholesterol can result in blockage of arteries and it has been even suggested to decrease angiogenesis in ApoE deficient mice (Zecharian et al 2013) and thus contribute to atherosclerosis in many ways.

People with diabetes have a hazard ratio of 2.27 for ischemic stroke, compared to non-diabetic people (Emerging Risk Factors Collaboration 2010). The risk for ischemic stroke is especially higher amongst people over 55-65 years with type 2 diabetes. Diabetes will disturb normal endothelial function, stiffen blood vessels, maintain inflammatory state and it often interferes with diastolic filling of the left ventricle. It can also interfere with normal vasodilatation by impairing NO synthesis or its response. Having diabetes will increase the risk for other co-morbidities, such as atherosclerosis and arterial stiffness, thus increasing the risk of ischemic stroke even more (Figure 1) (Chen et al 2016). Higher glucose concentrations at the beginning of ischemia are known to increase lesion size (Quast et al 1997), decrease blood circulation by aggregation of platelets (Weir et al 1997) and worsen the clinical outcome after thrombolytic treatment by increasing hemorrhagic risk and delaying reperfusion (Bruno et al 2002). Intensive diabetes therapy (INT) has been proven effective for decreasing the risk of diabetic people for cardiovascular diseases and ischemic stroke. The positive changes can also be mediated by, for example, a decreased blood pressure (Chen et al 2016).

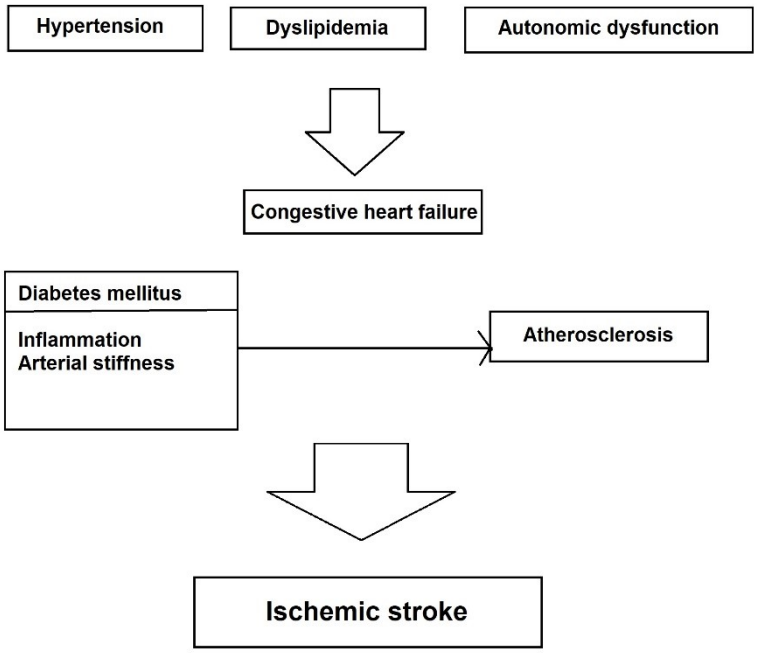

Figure 1: Risk factors, co-morbidities and their relationship on the pathophysiology of ischemic stroke. Modified from Chen et al (2016).

Hypertension is one of the most important risk factors for ischemic stroke since it can increase the risk for stroke by 4 times (Arboix 2015). Increase of systolic pressure alone can increase the risk for recurrent ischemic stroke, however, the increase of diastolic pressure will also increase the risk (Arima et al 2006). On the other hand, also a low blood pressure can worsen the outcome after ischemic stroke (McManus & Liebeskind 2016). Hypertension will stress the endothelium and can increase BBB permeability, promoting the progression of atherosclerosis. It will also interfere with autoregulation, thus increasing the risk for either

ischemic or hemorrhagic stroke (Dubow & Fink 2011, Johansson 1999). Even a small reduction of blood pressure can significantly reduce the risk for both the first and recurrent ischemic stroke (Progress Collaborative Group 2001).

Hyperlipidemia has been observed to increase the amount of inflammatory sensitive proteins in blood and the overall risk of cardiovascular diseases (Engström et al 2002). High LDL- and triglyceride levels in blood increase the risk of ischemic stroke, whereas high levels of HDL will decrease the risk (Dhungana 2014). However, total serum cholesterol is inversely associated with the risk of intracranial hemorrhage (Yano et al 1989).

2.2. Mechanism of cell death on ischemic stroke

When brain ischemia occurs, it significantly reduces the blood flow and thus the oxygen and glucose supply in a certain area. As the flow falls, the vital functions of brain cells, such as protein synthesis and glucose metabolism, are disrupted. At rate 25 ml/100g/min the neurons can only utilize anaerobic glycolysis which leads to ATP deprivation, lactate accumulation and descending pH of the brain cells. If the blood flow falls below 16 – 18 ml/100g/min, neurons lose their electrical function and eventually the ion pumps at the cell membrane cease. This leads to a massive efflux of K^+ and influx of Ca^{2+}, Na^+, and Cl^-. Water is drawn into the brain cells because of increased osmotic pressure (Markus 2004). Excessive amounts of intracellular Ca^{2+} will open a mitochondrial permeability transition pore (MPTP) which allows small molecules, such as water and Ca^{2+}, to enter and disrupt mitochondrial ATP synthesis and increase the production of reactive oxygen species (ROS) (Karch & Molketin 2015). Eventually this leads to necrosis, which includes changes in the nucleus, loss of structure and cell rupture, spilling the contents to the extracellular space (Majno & Joris 1995).

Intracellular Ca^{2+} is a major proapoptotic substance in excessive amounts, increased by reduction of blood flow (see above) or by glutamate receptor activation. Since astrocytes are not able to remove glutamate from extracellular space during severe ischemia, activation of NMDA and AMPA receptors and a massive Ca^{2+} influx occur (Barreto et al 2011, Dugan et al 1995). Ca^{2+} can directly activate proteases, which can disrupt microtubule system of the cell (Barone & Feuerstein 1999) Increased intracellular Ca^{2+} can also activate MPTP. It will

allow small molecules to enter the mitochondria and disrupt its function by hydrolyzing ATP or by swelling caused by water and also increase the ROS production. This event usually leads eventually to necrosis (Karch & Molketin 2015). Mitochondria have several Blc-2 family substances which regulate opening of the pore: Blc-2 and Blc-xL inhibit whereas Bax and Bak promote this process (Arun et al 2016). Mitochondrial outer membrane permeabilization (MOMP) is also a BAX/BAK driven process where the pore is created for cytochrome c to exit the mitochondria. The major difference is that mitochondria can maintain oxidative phosphorylation after MOMP, and thus produce ATP (Chipuk et al 2006).

Caspases are cell proteases that become activated during ischemic stroke and are known to promote cell apoptosis (Sims & Muyderman 2010). Activation of these enzymes takes place when they are dimerized. They can be classified to apoptotic (2, 3, 6, 7, 8, 9, 10) and anti-apoptotic (1, 4, 5, 14), and further to initiator (1, 2, 4, 5, 8, 9, 10) and effector (3, 6, 7) caspases (Pop & Salvesen 2009). Some anti-apoptotic caspases have been observed to promote pyroptosis, an inflammatory cell mediated cell death (Labbé & Saleh 2008). Initiator caspases are activated via intrinsic or extrinsic pathway (Figure 2). On internal activation, increased intracellular Ca^{2+} and ROS makes it possible for cytochrome C to exit mitochondria through MOMP and activate apoptotic protease activating factor 1 (Apaf-1) oligomerization which then activates caspase-9 (Riedl & Salvesen 2007). External activation is caused by a ligand, for example FasL, to form a complex, which activates caspase-8. These both pathways eventually activate caspase-3 leading to apoptosis by DNA fragmentation (LeBlanc & Ashkenazi 2003). However, caspases require ATP for activation thus in major ATP deficiency the cells die through necrosis instead of apoptosis (Karch & Molketin 2015).

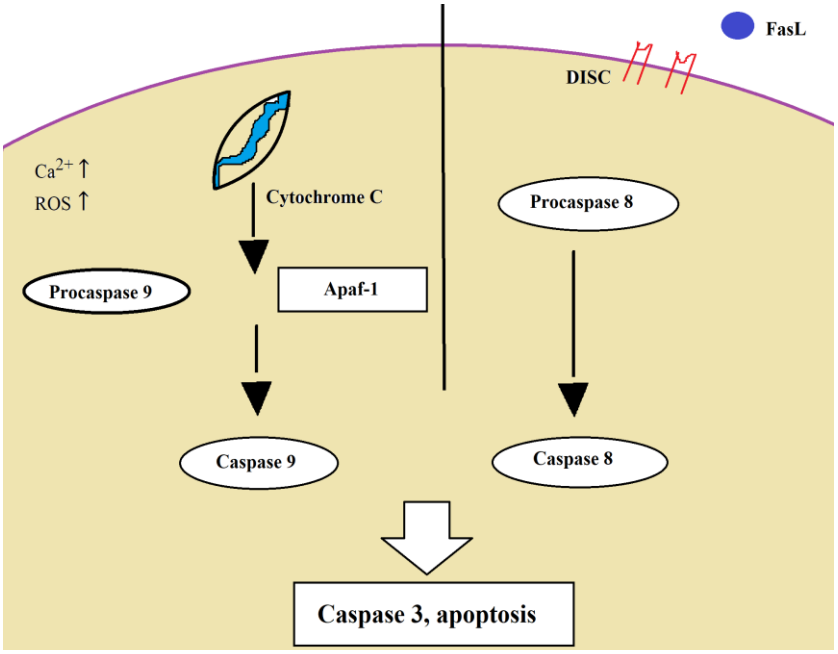

Figure 2: Intrinsic, shown left, and extrinsic pathway, shown right, of apoptosis. Figure modified from Dhungana (2014).

The cells in the core area of ischemia usually go through necrosis, whereas apoptosis is mainly induced in the peripheral area. The blood flow in the penumbra is strong enough to keep the cells potentially viable and thus it is possible to save these neurons (Markus 2004). Necroptotic pathway is induced by caspase inhibition, where cell death is mediated by death receptors and receptor interacting protein kinases (Karch & Molketin 2015). Also, reperfusion can cause necrosis, since respiratory restoration by oxygen can enhance mitochondrial calcium uptake and induce ROS generation. If calcium is also released from sarcoplasmic reticulum, it can open MPTP, causing ATP deprivation and cell death (Bhosale et al 2015).

2.3. Inflammation in ischemic stroke

2.3.1. Cytokines

Cytokines are proteins that are a major part of immune system, since they induce proper responses to antigens regulating both innate and adaptive immune response. They also maintain homeostasis during infection and inflammatory states. Cytokines can also over-activate immune system, for example in allergic reactions or autoimmune diseases (Holloway et al 2002) and contribute towards many non-communicable diseases, such as stroke, atherosclerosis and cancer (Brough & Denes 2015). The main cells that produce cytokines in periphery are macrophages, monocytes, platelets, endothelial cells, T-cells and NK-cells, whereas in the central nervous system (CNS) cytokines are also secreted by glial cells and neurons (Barone & Feuerstein 1999). It has been observed that on ischemic stroke the expression of proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, is increased and is linked to worsened clinical outcome (Kim et al 2016).

To increase the expression of cytokines, immune cells must recognize damage-associated molecular patterns (DAMP), such as substances released from necrotic cells, with their pattern recognition receptors (PRR). Produced, usually proinflammatory, cytokines then bind to their receptor and further increase cytokine gene expression by activating NF-κB, p38 and other signaling pathways (Brough & Denes 2015). Some proinflammatory cytokines, such as TNF-α, can directly induce apoptosis by forming a death domain when binding to TNFR1 and have anti-inflammatory effects (Wang et al 2007). However, TNF-α is involved in ischemic tolerance, since reduction in TNFR1 expression inhibited the protective effects if

ischemic preconditioning in rats (Pradillo et al 2005). Anti-inflammatory cytokines, such as TGF-β, IL-10 and IL-1 receptor antagonist, usually act by inhibiting proinflammatory cytokine activation, thus reducing the inflammation (Wang et al 2007). In ischemic stroke, IL-1β mRNA expression increases 15-30 minutes after ischemia and TNF-α expression is increased within 1-3 hours. Both of these cytokines have a biphasic expression within 6-36 hours after ischemia (Wang et al 2007). IL-1α is observed to activate even earlier than IL-1β (Brough & Denes 2015). It should be noted that these timelines of cytokine signaling events are model-dependent.

IL-13 is an anti-inflammatory cytokine that results in anti-inflammatory responses and might thus have beneficial effects in ischemic conditions. Interestingly, Tanshinone I (TS1), a pigment isolated from the herbal medicine *Salvia miltiorrhiza Bunge* displaying cytotoxicity against human macrophages and IFN-γ production in certain lymph node cells, has been proven to inhibit PGE_2 production and decrease the activity of COX_2, thus reducing the inflammation after inducing the inflammation with lipopolysaccharide (LPS) treatment (Kim et al 2002). In gerbils, TS1 treatment resulted in maintenance of increased levels of anti-inflammatory cytokines IL-4 and IL-13 for 4 days after ischemia-reperfusion. However, the immunohistochemistry after 4 days of reperfusion showed that treatment with IL-4 had no neuroprotective effects (Park et al 2014).

Cyclooxygenases (COX) are enzymes which hydrolyze arachidonic acid (AA) to prostaglandins and thromboxanes which can affect vascular function, either by vasoconstriction or vasodilatation, platelet aggregation and inflammatory state (Huang et al 2016). After Ca^{2+} influx, the increased levels of Ca^{2+} activate phospholipase A_2 (PLA_2) which then hydrolyze AA (Wang et al 2007). COX-1 is involved in physiological functions and is steadily expressed while COX-2 is highly expressed during inflammation and is very inducible by cytokines (Huang et al 2016). Ischemic stroke has been observed to induce COX-2 mRNA expression after 6 hours, reaching a peak at 12-24 hours. Also, the COX-2 protein and PGE_2 were upregulated during ischemic stroke. Increased immunoreactivity of COX-2 was observed for 4 days on ipsilateral side (Nogawa et al 1997). IL-13 has been observed to enhance COX-2 activity and driving microglia to apoptosis, for example, through E prostanoid receptor 2 (EP2) activation, thus resolving the inflammation (Fu et al 2015).

2.3.2. Microglia and astrocytes

Astrocytes take up to 50% of human brain volume and thus are the most abundant glial cells. They usually are maintaining the brain homeostasis in many ways, for example, by glutamate uptake, K^+ buffering, free radical elimination, regulating the functions of blood-brain-barrier (BBB), regulating synaptogenesis and providing structural support (Dhungana 2014, Penky & Nilsson 2005). Microglia cells are another important cell group in brain homeostasis, normally involved in synaptic interactions, either pruning or repairing them, and in cell phagocytosis. Environmental changes, such as ischemic stroke, can transform the cell from ramified (resting) state to active state, in which they can no longer visually be distinguished from macrophages (Kim et al 2016).

After two hours of ischemic stroke, the microglia become active. Microglia and macrophages can be roughly classified into two kinds of phenotypes: proinflammatory M1- and anti-inflammatory M2-phenotype (Kim et al 2016). However, the classification of macrophages/microglia is not that simple since the cell activity and function can be, for example, a combination of these two types, making cell classification a lot more complex in reality. In early stages, the M1-type cells are more active and they secrete both cytotoxins, such as superoxide and nitric oxide, and pro-inflammatory cytokines, such as TNF-α, IL-12, IL-6 and IL-1β. The M2-type cells become more active in later stages, and these cells secrete VEGF, promote phagocytosis and produce anti-inflammatory cytokines, such as IL-4 and IL-13. Microglia cells can switch their phenotype from M1 to M2 (Kim et al 2016), possibly by TREM-2 activation which promotes phagocytosis (Kawabori et al 2015) or inducing CD45 receptor to inhibit CD40 receptor induced M1-type cell activity (Salemi et al 2011). Astrocytes also have a role in ischemic stroke, since they produce some neurogenic factors and a glial scar which prevents additional peripheral damage but isolates the ischemic area and interrupts area recovery (Nowicka et al 2008). Also, astrocytes have a potential to release proinflammatory molecules, such as nitric oxide, by activation of inducible nitric oxide synthase (iNOS) as well as to secrete anti-inflammatory cytokines (Kim et al 2016). Mechanisms of action are also introduced on Figure 3 (p. 11).

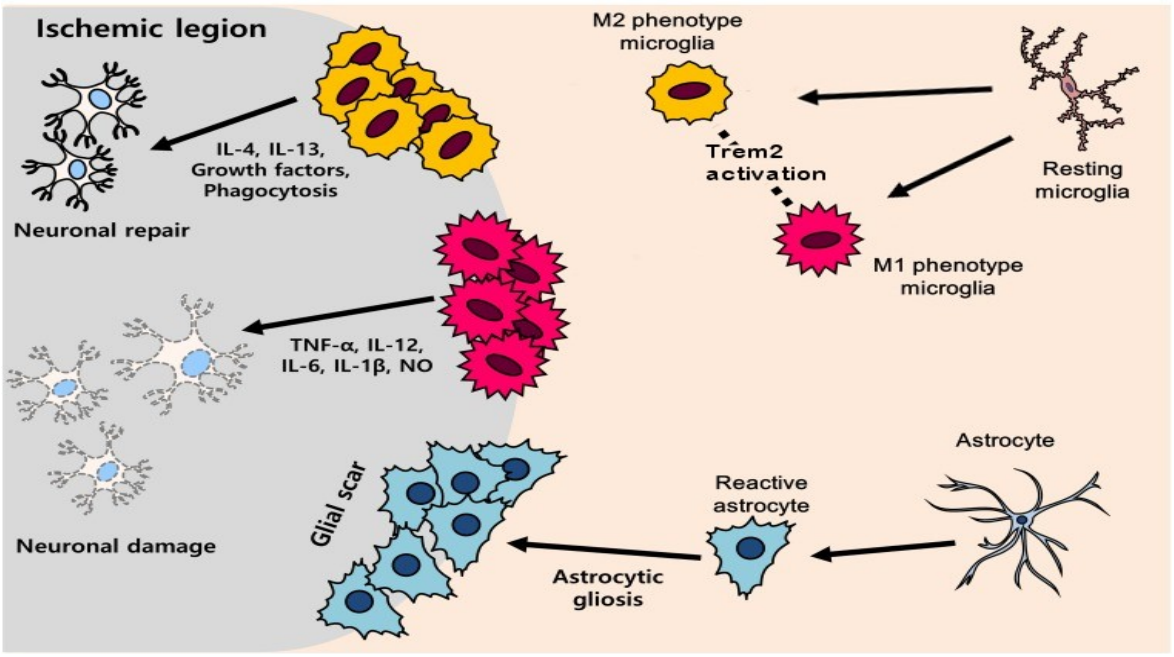

Figure 3: Glial cell activation and differentiation on ischemic stroke. Figure modified from Kim et al (2016).

Triggering receptor expressed on myeloid cells-2 (TREM2) is a microglia receptor which is controlling phagocytosis and it is essential for brain homeostasis (Kawabori et al 2015). Activation of TREM2 receptor inhibits Toll-like receptor (TLR) response (Ito & Hamerman 2012) which normally activates NF-κB and the production of inflammatory cytokines (Hamerman 2016). The amount of TREM2 has been observed to increase during ischemic stroke, peaking at 7 days after ischemia at peri-ischemic area. Mice with TREM2 knocked out have been stated to have a larger infarct lesion than the wildtypes two weeks after ischemia. TREM2 deficiency also seems to affect post-ischemic angiogenesis and overall phagocytic activity, being strongly linked to neurological recovery (Kawabori et al 2015). Since TREM2 activity has anti-inflammatory effect and it supports neurological recovery, it would be interesting to see if IL-13 treatment will increase TREM2 activity after ischemic stroke.

2.3.3. Peripheral lymphatic cells

All cytokines from microglia or astrocytes are thought to have an effect on peripheral lymphatic cells, including neutrophils, monocytes and lymphocytes, within a couple of days. The effects of leucocytes are mostly harmful in ischemic stroke, causing disturbances to the flow of red blood cells, releasing reactive oxygen species and producing vasoconstrictive substances such as prostaglandins or leukotrienes. Neuthophils can arrive at ischemic area

within 15 minutes of the event, reaching highest number after 2-4 days, and they cause mostly harmful effects, such as weakening of BBB and increased infarct area. Lymphocytes usually cause the same effects but a couple days later (Kim et al 2016). These changes in infiltrating leukocytes as well as the time course of these events are again strongly model-dependent.

Chemokines are small molecules which are involved in, for example, cell migration and angiogenesis. They can be classified to four different structural subtypes which are CXC, CC, C and CX_3C, where C describes amino acid cysteine and X is some other amino acid. G-protein coupled chemokine receptors are classified using the same principle (Rossi & Zlotnik 2000). CXC chemokines attract mainly neutrophils whereas CC chemokines affect monocytes and lymphocytes (Kim et al 2016). Monocyte chemoattractant protein-1 (MCP-1, CCL2) is an important chemokine for monocytes, but only a subset of monocytes expresses the receptors for these chemokines. Monocytes with chemokine receptors (CCR2) usually express high levels of Ly6C and they are often referred as inflammatory monocytes (Chu et al 2014). Cells with no CCR2 have also low levels of Ly6C, and they can be referred as resident cells (Geissmann et al 2003). The expression of MCP-1 is increased during cerebral ischemia and is linked to increased damage of the brain (Wang et al 2007). IL-8 (CXCL8) is a proinflammatory cytokine and major chemoattractant to neutrophils (Turner et al 2014).

Peripheral lymphatic cells utilize adhesion molecules expressed by endothelial cells to access the tissue. P- and E-selectins are rapidly expressed on endothelial cell surface after stimulus on which leukocytes expressing L-selectin can bind. Interactions with selectins are reversible but they can lead to expression of other adhesion molecules, integrins and Ig superfamily of adhesion molecules (DeGraba 1998). Adhesion molecules belonging in immunoglobulin superfamily, especially ICAM-1 and VCAM-1, are highly inducible in response to cytokines and expression of ICAM-1 is observed to increase on ischemic stroke, increasing the volume of ischemic infarcts and worsening neurological outcome (Wang et al 2007). β2-integrins, such as LFA-1, Mac-1 and VLA-4, are expressed by different leukocytes (DeGraba 1998) and are activated by cytokines and chemokines (Wang et al 2007). Various studies (Chen et al 1994, Bowes et al 1995, Jiang et al 1995, Yenari et al 1998) have observed

that blocking only Mac-1 or all β2-integrins will reduce neutrophil infiltration and neuronal injury.

By modulation of interleukin functions or concentrations, it might be possible to modify also the functions of peripheral lymphatic cells, thereby making interleukin therapy a proper approach for treating ischemia.

2.4. Previous studies

Mesenchymal stem cells (MSC) with continuous IL-13 secretion have been tested in treating spinal cord injury (SCI). The spinal cord of the mice was cut, the MSC and MSC/IL-13 were transplanted and the effects were analyzed with locomotive tests, immunofluorescence analysis and histological quantifications. The treatment reduced recovery time and lesion size, reduced astrogliosis and increased both number and activity of alternatively activated macrophages while decreasing the number of microglia *in vivo*. (Dooley et al 2016). These results suggest that IL-13 is highly neuroprotective, thus it can be speculated that the same treatment could be beneficial in treating ischemic stroke.

Another recently identified anti-inflammatory cytokine, IL-33, has also been studied in SCI, in which mice received either IL-33 or vehicle treatment intraperitoneally, after the spinal cord injury, four times within 10 days. Treatment effects were analyzed with motor tests, RNA analyses, different histological stainings and MRI imaging. SCI increased the IL-33 mRNA expression in astrocytes and significantly affected the IL-33 receptor subunits. IL-33 treatment also improved functional recovery, showed a strong trend to reduced demyelination, reduced astrogliosis and T-cell infiltration to spinal cord, and promoted a shift towards M2 type microglia with significantly prolonged effect (Pomeshchik et al 2015). When IL-33 was studied in ischemic stroke using permanent surgical method (see Table 1, p.14), IL-33 was found to be protective, possibly partially by inducing IL-4 expression (Korhonen et al 2015). IL-33 can also increase IL-13 production on lymphatic cells, which is usually linked to allergic reactions (Cayrol & Girard 2014). Since the blockage of elevated IL-4 did not entirely attenuate neuroprotective effects of IL-33 (Korhonen et al 2015) and IL-13 is

observed neuroprotective in SCI (Dooley et al 2016), it is reasonable to hypothesize that intraperitoneal IL-13 administration could provide neuroprotection also in ischemic stroke.

Table 1: Most common methods to produce middle cerecral artery occlusion (MCAo) (Sommer 2017).

Model	Method	Details
Intraluminal suture MCAo	Monofilament is transported into desired site to block the artery	Most frequently used model, can be used modelling both permanent and transient ischemia. Requires surgery and also causes damage to striatum, making it possible to analyze motor deficits and recovery. Causes usually large infarcts depending on ischemia duration, fast reperfusion if removed
Surgical MCAo	Burring a small hole into the skull and blocking the circulation temporarily (hooks, clippers, electrocoagulation/cauterization) or permanently (cauterization followed by transection)	Can cause skull traumas, damage brain tissue or cause the inflammation to spread. Fast reperfusion. Damage can be caused proximally to striatum and cortex, possible to analyze motor deficits and recovery.
Endothelin-1 driven MCAo	Applying endothelin-1 directly to brain surface, parenchyma or blood vessel, causing vasoconstriction	Highly modifiable, slow development of ischemia and little edema. Well suited with recovery and lacunar stroke studies
Embolic MCAo	Embolus-like material or clot formation factor introduced in desired area	Great variation in infarct location and size depending on used protocol, size usually correlates with intraluminal MCAo. Lysis is spontaneous process like in humans, most accurate model when comparing to humans. With embolus-like material, occlusion is permanent and depends on material size.

3. Material and Methods

I was only participating in the immunohistochemical and statistical part of the study. Other study materials, methods and results are presented to give a complete comprehension of the study.

3.1. Animals

The experiment was done with 4 months old mice, which were divided to control (n=8) and IL-13 treatment (n=10) groups. The mice were anesthetized during critical procedures. Importantly, the experiments were conducted according to the national regulation of the usage and welfare of laboratory animals, approved by the National Animal Experiment Board of Finland and follow the Council of Europe legislation and regulation for animal protection. The protocol is similar to the previous study (Korhonen et al 2015) where animals were housed in cages under controlled conditions, such as temperature, humidity and 12h light and dark cycles. All the analyzes were performed in blinded study groups. A total of three mice were excluded, one from control and two from IL-13 group, due to missed bifurcation.

3.2. Ischemic surgery

Ischemic surgery was performed by using permanent surgical method, using a thermocoagulator (Table 1, p.14). Animals were anesthetized with 5% isoflurane and then maintained with 2% isoflurane (30% oxygen, 70% nitrogen). Mice were mechanically ventilated and the body temperature was maintained at 37^{o}C with feedback-regulated rectal probe. Surgical incision was done in the right temple and the dura was removed after retracting the temporal muscle and drilling the skull. The MCA was cauterized, skin wound sutured, and after recovering from anesthesia, mice were returned to their cages. Mice were perfused 3 days after the surgery, after anesthetizing with 250mg/kg avertin.

3.3. IL-13 treatment

1µg of IL-13 was added in 100µl of 0.01 M phosphate buffered saline (PBS), with 0.0025% of bovine serum albumin (BSA). IL-13 group received this mixture intravenously through tail vein (1µg IL-13 in 100µl PBS; 0.0025% BSA), and the vehicle group received equal volume of PBS (0.01 M, 0.0025% BSA). The administration was initiated right after the ischemic surgery. No side-effects were observed by IL-13 administration.

3.4. Magnetic resonance imaging

Magnetic resonance imaging (MRI) was performed using 9.4T Oxford NMR magnet at 3-day post ischemia. The images were multislice T2-weighted (repetition time 3000ms, echo time 40ms, matrix size 128*256 and Field of view 19.2*19.2 mm^2, slice thickness 0.8mm and number of slices 12) and were analyzed using Aedes software in MathLab environment. The relative percentage of infarct volume was calculated using the following formula:

Infarct volume= (volume of left hemisphere- (volume of right hemisphere-measured infarct volume))/volume of left hemisphere.

3.5. Immunohistochemistry

The mice were sacrificed 72 hours after ischemia. After removing the brain, they were post-fixed using 4% paraformaldehyde for 18-20 hours, following a cryoprotection with 30% sucrose for two days. Liquid nitrogen was used to freeze the brain, and a total of six 20µm-thick sections with 400µm distance were then cut with cryostat (Leica Microsystems, Wetzlar, Germany).

The markers we used were glial fibrillary acid protein (GFAP) for astrocytes, ionized calcium-binding adapter molecule-1 (Iba-1) for all microglia/macrophages, Arginase-1 (Arg-1) for M2-type macrophages/microglia and Caspase-3 for apoptotic cells. TREM-2 was used to study the mechanisms of possible M2-polarization. Iba-1, Arg-1 and GFAP staining were done as stated earlier (Korhonen et al 2015). At first, the slides were air dried for 1hr and the samples were bordered using ImmEdge Pen (Vector Laboratories, Burlingame, CA, USA) and washed with PB for 2x10 min, PBS for 2x5 min and 0,05% Tween in PBS (PBST) for 2x5 min. The sections were blocked with serum and then exposed to primary antibodies for

overnight incubation (GFAP 1:500 dilution, DAKO, Glostrup, Denmark; Iba-1, 1:250 dilution, Wako Chemicals, Tokyo, Japan; Arg1 (N-20) 1:200 dilution, Santa Cruz Biotechnology, Dallas, TX, USA; Caspase-3 (#9664) 1:200 dilution, Cell Signaling Technology, Danvers, MA, USA; TREM-2 (ab86491) 1:75 dilution, Abcam, Cambridge, UK). Arg-1 staining required processing with sodium citrate dehydrate solution (pH 6) preheated to 92 °C, whereas Caspase-3 required washing in 0.05M sodium citrate for 3x5 min and incubation in 84 °C for 30 min before applying primary antibodies. After washing the slides 3x5min with PBST, the slides Iba-1, Arg-1, GFAP and TREM-2 were incubated for two hours using fluorescent antibodies (Alexa 568) with 1:500 (Arg-1), 1:200 (Iba-1, GFAP) and 1:400 (TREM-2) dilution. Caspase-3 was incubated for 60 minutes with biotinylated antibody with 1:200 dilution. Caspase-3 was then introduced to ABC reagent (1:200 A/B in PBS, Vector Laboratories) for 2 hours and reacted with Ni-DAB to develop color. The samples were dehydrated using increasing concentrations of EtOH (2 minutes each), placed into xylene for 3x5 min and embedded with Depex.

Immunoreactivities were imaged using a digital camera (Color View 12 or F-View; Soft Imaging System, Muenster, Germany) with Analysis Software (Soft Imaging system) attached to an AX70 microscope with 10x magnification (Olympus Corporation, Tokyo, Japan). Images were taken from either the ischemic (Arg-1, Caspase-3) or the peri-ischemic area (GFAP, Iba-1), right next to infarct border, or right center of the infarct border (TREM-2). Corresponding location was imaged from contralateral hemisphere. Relative immunoreactive areas were analyzed from the images using ImagePro Plus Software (Media Cybernetics, Rockville, MD, USA) at specific range for each staining.

3.6. Cytometric bead array

The concentrations of other cytokines (IL-6, IL-10, MCP-1, IFN-γ, TNF, IL-12p70) were measured using cytometric bead array (CBA) kit (BD Biosciences Europe) from mouse plasma samples at 3dpi. The capture beads and detector reagent was mixed either with one sample or with a cytokine standard, and incubated 2 hours on the 96-well microplate. The samples were mixed with wash buffer and centrifuged at 400g for 5 minutes. The supernatant was discarded, beads were broken, wash buffer was added, and the samples were analyzed

with a flow cytometer. The results from cytometry were obtained using FCCAP Array Software (BD™ Cytometric Bead Array (CBA)).

3.7. Statistical analysis

The relative immunoreactive areas acquired from ImagePro Plus Software were exported to Excel software, the lesion volume was computed from MRI images using the formula (see p. 16). The mean value was computed from 6 images for each animal, ipsi- and contralateral side separately, and then arranged by study group (IL-13 and PBS-treated control group) to compare them in two-way t-test. For CBA analysis, the final concentrations of cytokines were transferred from FCAP Array to Excel, divided in treatment groups and the groups were compared using two-way t-test for each cytokine separately. All data in diagrams are expressed as mean +- SEM.

4. Results

4.1. IL-13 treated mice had significantly reduced lesion size in MRI

MRI scan was done to mice at 3-day post-ischemia to see, if there is any difference in lesion size between IL-13-treated group and PBS-treated control group. IL-13 treatment was proven effective, since the lesion size analyzed from MRI images was significantly diminished in IL-13 group, compared to control group, at 3 dpi (Figure 4). Thus IL-13 is at least somehow neuroprotective.

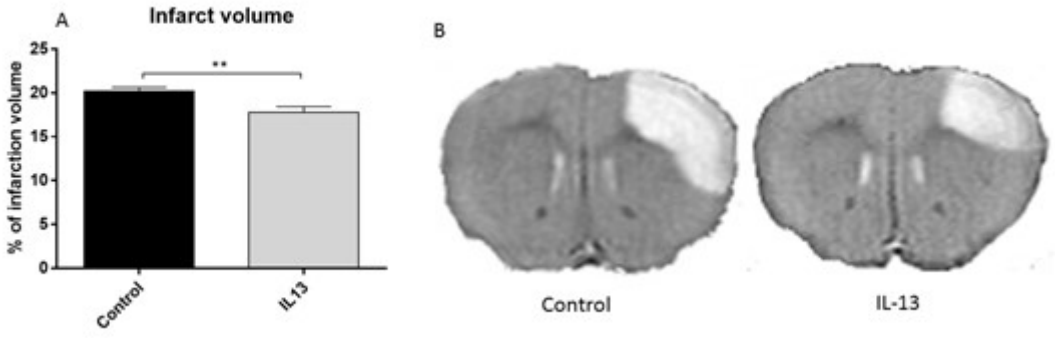

Figure 4: MRI analyses between study groups, 3 days after ischemia. (A) Statistical analysis showed significant reduction in infarct volume in IL-13 treated group (n=8), comparing to PBS-treated control group (n=7). (B) MRI images of the brain where ischemic lesion is represented as white area, from both control and IL-13 groups respectively. Data is expressed as mean +- SEM, **p<0.01

4.2. IL-13 treatment increases M2-type microglia/macrophage in ischemic lesion

Since microglia and macrophages are the major modulators of inflammation in stroke (Kim et al 2016) and IL-13 is an anti-inflammatory cytokine (Park et al 2014), we proposed that IL-13 treatment promotes the shift towards anti-inflammatory M2-type cells. In IL-13 group, there was indeed significantly more M2-type cells on ischemic area than in control group ($p = 0.038$, Figure 5, p. 20). There was no M2-type cell activity on contralateral side (figure not shown).

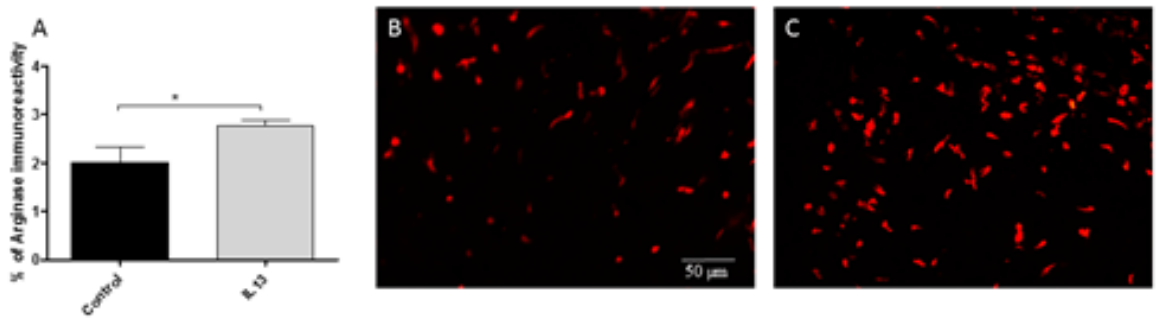

Figure 5 Comparing IL-13 and control group in Arginase-1 activity. (A) IL-13 treated group had significantly more M2-type cell activity in the ipsilateral hemisphere than the control group. The images are from control (B) and IL-13 (C) group respectively. Data is expressed as mean +- SEM. *p<0.05

However, the mechanisms of increased M2-type cell activity remain unclear, since there was no significant difference on neither TREM-2 activation (p = 0.91) nor total number of microglia/macrophages (Iba-1, p = 0,85) between the study groups. The immunoreactivity of TREM2 in negative control was subtracted from samples. and some resulting negative values were marked as 0.

4.3. Unaltered activation of astrocytes between the treatment groups

After ischemic stroke, both study groups showed significantly increased astrocytic activation on ipsilateral side, compared to the contralateral side. There was observed a trend towards decreased astrocytic activation in IL-13 group, comparing to the control group. However, the results were not statistically significant (p = 0.28, Figure 6, p. 21).

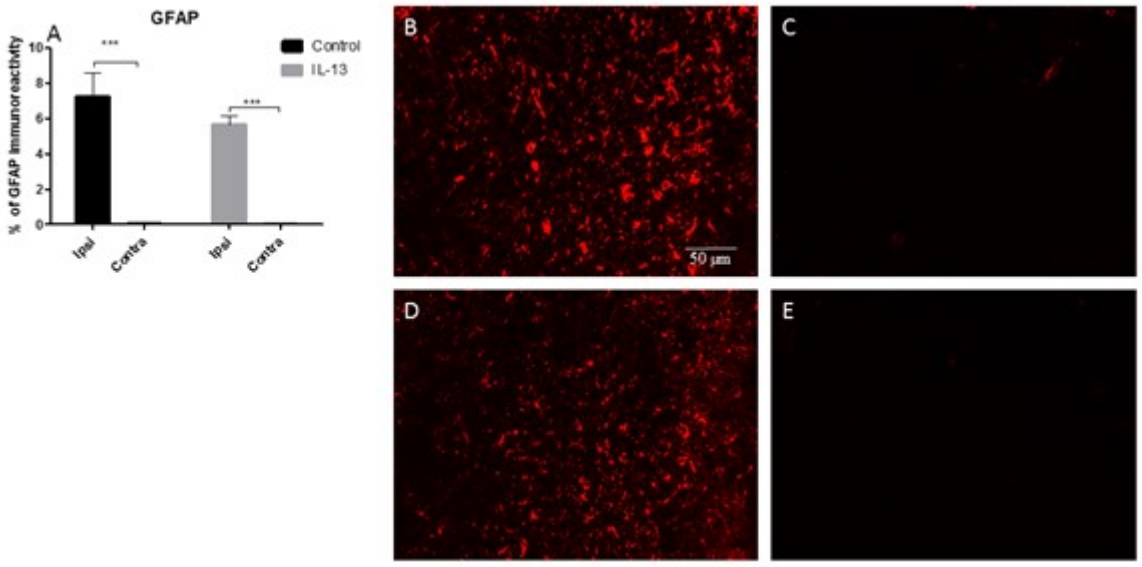

Figure 6: Astrocyte activation in study groups. (A) There was a significant difference in astrocytic activation between hemispheres, and also a trend towards decreased activation in IL-13 group. However, there was no statistical difference in the ipsilateral hemisphere between the control and IL-13 treatment groups. Figures B and D demonstrate the GFAP immunoreactivity, in IL-13 and control group respectively, in peri-ischemic area, and figures C and E show the activation on contralateral side. Data is expressed as mean +- SEM.

4.4. CBA did not show significant changes on cytokine profile

The concentrations of other cytokines (IL-6, IL-10, MCP-1, IFN-γ, TNF, IL-12p70) was not affected by IL-13 treatment. The concentrations of IL-10, IFN- γ and IL-12p70 were not contrasted because of several undetectable concentrations amongst study group. P-values from unpaired t-test were not statistically significant: 0.25 for IL-6, 0.28 for TNF- α, and 0.74 for MCP-1 (Figure 7).

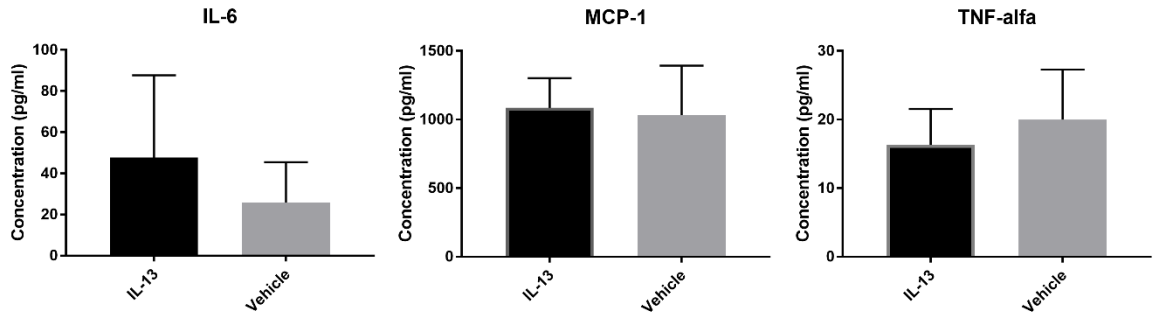

Figure 7: Cytokine concentrations on mouse plasma at 3 dpi. The differences on cytokine concentrations were not statistically significant between the study groups. IL-10, INF-γ and IL-12p70 are not presented due their minimal concentrations. Data is expressed as mean +- SEM.

4.5. IL-13 treatment did not attenuate apoptosis

Caspase-3 was used as a marker to study the effects on apoptosis induced on ischemic stroke. However, caspase-3 immunoreactivity was not significantly different between the study groups ($p = 0.62$).

5. Discussion

On this study, IL-13 treatment was observed to induce M2-type polarization in peri-ischemic area, which was one of the main study hypotheses. The fact whether IL-13 induces this in periphery or in the brain is still a mystery. Cytokines can cross the BBB with specific transporter molecules which makes the transport slower (Banks 2015). It has been observed that at least IL-1, IL-6 and TNF-α have their own saturable transport system. The amount of cytokines passing through the BBB is minimal but sufficient to alter brain function (Banks et al 1995). However, ischemic stroke usually disrupts the BBB by activating matrix metalloproteases (MMPs), which is regulated and contributed in the early phase by MMP-2 and its inhibitory trimolecular complex produced by astrocytes, or cytokine- and COX-2-inducible MMP-3 and MMP-9 produced in later phase, after 24-72 hours of ischemia (Yang & Rosenberg 2011). One does not know whether IL-13 treatment during ischemic stroke induces COX-2 expression, the disruption of the BBB, and thus improve the drug transport to the brain. BBB disruption would also increase the transport of other substances, such as tPA, to the brain tissue, inducing cytokines and raising the risk of brain hemorrhage (Yang and Rosenberg 2011). To conclude whether IL-13 would infiltrate through the BBB, one should measure IL-13 concentrations from the brain. Since the amount of IL-13 used on this study did not increase the TREM-2 immunoreactivity ($p = 0.91$) or alter the concentrations of other cytokines, the mechanisms of M2-type polarization remain somehow unclear. However, it should be noted that we only analyzed the TREM2 protein immunoreactivity, not the actual activity of the receptor.

Crossing the BBB is not the only way for cytokines to alter glial cell function, since cytokines can signal through sensory nerves, such as *n. vagus*, inducing the cytokine production in the brain (Goehler et al 2000). The neuroprotective effects can also be mediated through macrophage or T-cell modulation. Interferon-γ or LPS induce M1-type polarization in macrophages, whereas IL-4 or glucocorticoids induce M2-type polarization, possibly by activating PI3K/Akt pathway, which eventually results in negative regulation of NF-κB and TLRs (Vergadi et al 2017). The M2-type polarization can also be mediated by regulatory T cells (Tregs), possibly by IL-10 secretion which suppresses TNF-α and IL-6 gene expression, or by affecting molecules upstream of PI3K/Akt pathway (Zhou et al 2017). Specific markers

are needed for T-cells and Tregs, CD3/CD9 and CD4/CD25/FoxP3, respectively (Lan et al 2017), to study the effects of IL-13 on these cells. Since lymphocytes are activated and infiltrated to the brain on later time points (Kim et al 2016), measuring the activation of these cells on 3dpi and later time points might be reasonable.

Since ischemic stroke is usually caused by atherosclerotic plague or by a blood clot, and most patients experiencing ischemic stroke are above working-age (Roine 2016), our model with healthy and young male mice is not very accurate. Moreover, ischemic stroke is usually contributed by diabetes, by disrupting endothelium and promoting atherosclerosis with inflammation (Chen et al 2016), hypertension (McManus & Liebeskind 2016) and dyslipidemia (Dhungana 2014), all of which either contribute to stroke pathogenesis or have a negative impact on prognosis. Co-morbidities, especially atherosclerosis and diabetes, might attenuate the response to the IL-13 treatment due the systemic inflammation (Chen et al 2016). One has to remember that these co-morbidities are treated and secondary prevention is done with various drugs which can have various interactions together (Roine 2016, Kervinen 2016). Adding IL-13 treatment to stroke patient's medication could have unexpected effects. Thus, these possible interactions need to be studied at some point, perhaps in later studies.

Gender seems to be somehow affecting the risk of ischemic stroke. It has been observed that incidence of stroke for males is 33% higher than females and males experience their first stroke at a little earlier age than females (Appleros et al 2009). These differences may be caused by higher estrogen concentrations in women, which promotes vasodilatation by, for example, increasing NO production in endothelium, whereas testosterone can promote vasoconstriction (Krause et al 2006). However, women with diabetes mellitus have been observed to have ischemic strokes at younger age, apart from the males where there was no corresponding age difference (Madsen et al 2017).

Other factors that may have affected the results of this study are small study size, consisting only on 15 mice, time points, ischemic model and IL-13 concentrations. We observed that IL-13 treatment reduced lesion size and increased the number of M2-type cells, compared

to the control group. Perhaps with more mice, we could have reached required statistical difference in decreased astrocytic activation which we observed as a trend. However, our results suggested that IL-13 treatment did not affect the caspase-3 induced apoptosis ($p = 0.62$) or total number of microglia/macrophages on peri-ischemic area determined with Iba-1 ($p = 0.85$). These results could suggest that IL-13 treatment does not provide direct neuroprotection and treatment induces a shift towards M2-type glial cells, rather than induce the synthesis of M2-type glial cells, by some other mechanism than increased TREM-2 expression. There are various ischemic models (see table 1, p. 14), thus studying the effects on a different model than surgical one could provide useful information about effectiveness of the treatment. With a model allowing reperfusion, one could observe how IL-13 would contribute to reperfusion damage and motor recovery. The mice also received only one type of treatment during the same time period, and the results were also analyzed only 3 dpi, which can be noted by observing various time point and applying different concentrations of IL-13 on following studies.

One has to remember that this was a pilot study with a goal to see if IL-13 treatment is beneficial in any way on ischemic stroke. Our results support this theory but more research is needed to strengthen our hypothesis.

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