University of Veterinary Medicine Hannover

Department of Pathology

Center for Systems Neuroscience Hannover

Pathogenetic role of cholesterol biosynthesis and STAT3 signalling in chronic demyelinating diseases

Thesis

Submitted in partial fulfilment of the requirements for the degree

DOCTOR OF PHILOSOPHY

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To my family
Parts of the thesis have already been published:

**Publication:**

1. Aims

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS) in humans with unknown and presumably multiple etiologies, possibly initiated by a virus or virus-triggered immunopathology (Lucchinetti et al. 2004; Lassmann et al. 2001; Owens et al. 2011). The pathologic hallmarks of MS include inflammation, demyelination, gliosis and axonal damage.

Theiler’s murine encephalomyelitis virus (TMEV) strains may induce demyelinating Theiler’s murine encephalomyelitis (TME) in susceptible SJL mouse strains that represents a model to study the pathogenesis especially of the chronic progressive course of MS and to apply potential treatments (Olesyak et al. 2004; Ulrich et al. 2008 and 2010). The failure of remyelination in MS is thought to be contributed to axonal injury and degeneration, a major cause for the neurological disability in humans (Irvine and Blakemore 2008). Recent evidence suggests that a block of oligodendroglial differentiation causes remyelination failure in MS (Franklin and Ffrench-Constant 2008; Kuhlmann et al. 2008). Similar results were encountered in TME. Here an increased number of oligodendroglial precursor cells (OPC) and a lack of differentiation to mature oligodendrocytes was present (Ulrich et al. 2008). The cause for this arrest in OPC differentiation is unknown. The objectives of this research project were therefore to clarify the mechanisms leading to the endogenous blockade of OPC differentiation and to identify whether this differentiation arrest can be overcome with therapeutic interventions in this TME model for MS.
2. Introduction

2.1 Paigen diet

2.1.1 General comments about the Paigen diet

Numerous studies have found an association between the consumption of certain lipid-enriched diets and the rate of atherosclerotic lesion development in murine models of atherosclerosis (Getz and Reardon 2006). A variety of different lipid-enriched diets have been used for studies of atherosclerosis (Getz and Reardon 2006). Diets vary in the amount of cholesterol, the type and amount of fatty acids, and the absence or presence of cholate. Each of these components as well as the protein source have been shown to influence the lipoprotein level and/or the development of atherosclerosis, however, dietary cholesterol seems to represent the major atherogenic component (Getz and Reardon 2006).

A lipid-rich diet that is widely applied in murine models of atherosclerosis is called Paigen diet (Paigen et al. 1985, 1987 and 1990; Paigen 1995). This diet originated from the Thomas-Hartroft diet which contained 30% cocoa butter, 5% cholesterol, 2% sodium cholate, 30% casein, 5% alphacel, 4% vitamin mixture, 4% salt mixture, 6.5% sucrose, 6.5% dextrose, 6.5% dextrin, and 0.5% choline chloride (Morrisett et al. 1982). Thomas and Hartroft (1959) used this high-fat diet to induce arterial damage and myocardial infarction in rats. A significant number of myocardial and renal infarcts were induced in rats fed large amounts of either butter or lard (Thomas and Hartroft 1959). Along with this cholesterol-elevating regimen, thrombosis occurred before the appearance of severe structural changes in the arterial walls compared to a diet with high level of less saturated fat, e.g. corn oil (Thomas and Hartroft 1959).

A modified diet based on the Thomas-Hartroft diet was applied by Paigen et al. (1985), however, this diet caused considerable mortalities (Paigen et al. 1985). Deaths, due to respiratory infections only occurred in mice fed the atherogenic diet (Paigen et al. 1985). It was assumed that the atherogenic diet caused a suppression of the immune response (Paigen et al. 1985). To overcome the mortality problem, a diet
containing 1 part Thomas-Hartroft high-fat diet and 3 parts breeding chow, the so-called Paigen diet was created containing 15% fat, 1.25% cholesterol, and 0.5% sodium cholate with a ratio of polyunsaturated to saturated fatty acids of 0.69 (Paigen et al. 1985; Nishina et al. 1990; Paigen 1995; Getz and Reardon 2006). The predominant fat in the Paigen diet are saturated fatty acids derived from either cocoa butter or butter fat. 1% corn oil is often added to avoid a polyunsaturated fatty acid deficiency (Getz and Reardon 2006). Feeding such a diet reproducibly induces lesions within 10-14 weeks in susceptible mouse strains without any increased mortality (Ishida et al. 1991).

Saturated fatty acids are derived from either cocoa butter or butter fat (Getz and Reardon 2006). The predominant lipid composition of cocoa butter is triacylglycerol (TAG) (Table 1). Most cocoa butters have similar triacylglycerol compositions. Palmitic-oleic-palmitic acid (POP), palmitic-oleic-stearic acid (POS) and stearic-oleic-stearic acid (SOS) are the major triacylglycerols in cocoa butter (Table 2; Chaiseri and Dimick 1989; Hernandez and Castellote 1989; Lipp et al. 2001; Guyon et al. 2003). The main fatty acids of cocoa butter are palmitic acid (range 22.5%-23.1%), stearic acid (range 24.51%-37.4%) and oleic acid (range 28.74%-38.4%). Compositions of the fatty acids of the cocoa butter are shown in Table 3 (Lipp et al. 2001; Rao and Lokesh 2003).

Cholesterol is necessary to elevate plasma cholesterol levels in laboratory mice fed Paigen diet (Paigen et al. 1985). Cholate is not used in atherogenic diets, unless its complex effect is being investigated specifically (Getz and Reardon 2006). As a bile salt, cholate can facilitate cholesterol absorption and exert a feedback control on cholesterol transformation to bile acid within the hepatobiliary system (Getz and Reardon 2006). Thus, a diet with cholate will increase cholesterol loading and hence hypercholesterolemia (Getz and Reardon 2006). In addition, cholate induced hepatic gene expression was particularly related to fibrosis (Boisvert et al. 1999). Moreover,
feeding of a diet containing 0.5% cholate for 5 to 7 months resulted in skin xanthomatas in LDLR/- mice (Ishibashi et al. 1994).

Table 1: Range of lipid composition of cocoa butter from different countries*

<table>
<thead>
<tr>
<th>Lipid classes</th>
<th>Range (wt%)</th>
<th>Mean (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerols</td>
<td>96.21-97.30</td>
<td>96.97</td>
</tr>
<tr>
<td>Diacylglycerols</td>
<td>0.80-1.79</td>
<td>1.30</td>
</tr>
<tr>
<td>Monoacylglycerols</td>
<td>0.02-0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>0.88-1.46</td>
<td>1.17</td>
</tr>
<tr>
<td>Sterols</td>
<td>0.10-0.14</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*according to Widlak 1999; wt% = weight percentage

Table 2: Average of triacylglycerol compositions of cocoa butter from different countries*

<table>
<thead>
<tr>
<th>Triacylglycerol (%)</th>
<th>PLiP</th>
<th>POO</th>
<th>PLiS</th>
<th>POP</th>
<th>SOO</th>
<th>SLiS</th>
<th>POS</th>
<th>SOS</th>
<th>SOA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>0.68</td>
<td>2.65</td>
<td>3.33</td>
<td>17.99</td>
<td>4.78</td>
<td>2.54</td>
<td>39.04</td>
<td>25.65</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* according to Chaiseri and Dimick 1989

PLiP = palmitic-linoleic-palmitic acid; PLiS = palmitic-linoleic-stearic acid;
POO = palmitic-oleic-oleic acid; POP = palmitic-oleic-palmitic acid;
POS = palmitic-oleic-stearic acid; SLiS = stearic-linoleic-stearic acid;
SOA = stearic-oleic-arachidic acid; SOO = stearic-oleic-oleic acid;
SOS = stearic-oleic-stearic acid

2.1.2 Pathologic changes induced by Paigen diet
Introduction

Susceptible C57BL/6 mice fed with Paigen diet developed quite reproducible small atherosclerotic lesions in the aortic arch after 14 weeks (Paigen et al. 1985). At week 14, large accumulation of intracellular fat in many foam cells was observed within lesion and they bulged slightly into the lumen of the aorta (Paigen et al. 1985). Massive atherosclerotic lesions were found in the coronary arteries after 6 months and in the aortic arch after 9 months in susceptible mice (Paigen et al. 1985, 1987 and 1990). No lesions were found in control mice from different strains maintained on breeder chow which contains 10-11% fat, and no added cholesterol and cholate (Paigen et al. 1985 and 1990).

Table 3: Fatty acid composition of cocoa butter (individual fatty acid composition % / total fatty acid of cocoa butter)*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capric C10:0</td>
<td>trace-12.5</td>
</tr>
<tr>
<td>Lauric C12:0</td>
<td>trace</td>
</tr>
<tr>
<td>Myristic C14:0</td>
<td>trace-4.32</td>
</tr>
<tr>
<td>Myristoleic C14:1 (ω-5)</td>
<td>1.29</td>
</tr>
<tr>
<td>Palmitic C16:0</td>
<td>22.5-23.1</td>
</tr>
<tr>
<td>Palmitoleic C16:1 (ω-7)</td>
<td>0.95</td>
</tr>
<tr>
<td>Margaric C17:0</td>
<td>trace</td>
</tr>
<tr>
<td>Stearic C18:0</td>
<td>24.51-37.4</td>
</tr>
<tr>
<td>Oleic C18:1 (ω-9)</td>
<td>28.74-38.4</td>
</tr>
<tr>
<td>Linoleic C18:2 (ω-6)</td>
<td>1.5-3.93</td>
</tr>
</tbody>
</table>

*according to El-Saied et al. 1981 and Rao and Lokesh 2003
Typical fatty streaks were also observed in other animal species such as rabbits and rats fed an atherogenic diet (Paigen et al. 1985). In the rabbit, foam cell-filled fatty streak type lesions progressed to fibrous plaques, reminiscent of human lesions, if the atherogenic diet was alternated weekly with normal chow (Constantinides 1960; Paigen et al. 1985).

The total plasma cholesterol level was elevated in mice after 5 weeks feeding of the Paigen diet. However, there was little correlation between plasma cholesterol levels and presence of aorta lesions (Paigen et al. 1985; Vergnes 2003).

2.1.3 Paigen diet induced lesions in mice

Inbred mouse strains show considerable differences in the rate of lesion formation following an atherogenic diet (Paigen et al. 1985). So far, most experiments that used the Paigen diet as an atherogenic diet employed the particularly atherosclerosis susceptible mouse strain C57BL/6 (Paigen et al. 1985; Getz and Reardon 2006). This strain, however, does not develop chronic-demyelinating demyelination after infection with Theiler’s murine encephalomyelitis virus (Lipton and Dal Canto 1979). The Theiler’s murine encephalomyelitis susceptible mouse strain SJL/J mice fed Paigen diet developed, however, almost no atherosclerotic plaques in the aortic arch after 18 weeks compared to the C57BL/6 mice. Interestingly 20% of these SJL/J mice had gallstones compared to only 14% of Paigen diet fed C57BL/6 mice (Paigen et al. 1990).

2.2 Lipids

Lipids are water-insoluble biomolecules that are highly soluble in organic solvents such as chloroform (Berg et al. 2002; Stockham and Scott 2002). Lipids have many functions in the body, particularly as an energy source e.g. triglycerides and fatty acids, as structural components of cell membranes e.g. phospholipids and cholesterol, and as substrates for hormones and second messengers (Stockham and Scott 2002).
Introduction

Based on the chemical composition, lipids are classified in the seven major lipid classes fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides (Fahy et al. 2005; Fahy et al. 2009).

**Fatty acids** are particular components of lipids and possess the most biologically significant properties of lipids in cell membranes (Berg et al. 2002). Fatty acids are divided into either saturated fatty acids which do not contain any double bonds or unsaturated fatty acids which contain 1-6 double bonds with carbon chains varying between 2 and 36 carbon atoms. Thus fatty acid chains differ by length, often categorized as short-, medium-, and long-chain fatty acids, and prostaglandins (fatty-acid derivatives; Stockham and Scott 2002; Fahy et al. 2005; Fahy et al. 2009; Tvrzicka et al. 2011).

**Glycerolipids** are esters of glycerol and fatty acids, divided into TAG, diacylglycerols and monoacylglycerols (Stockham and Scott 2002; Fahy et al. 2005; Fahy et al. 2009).

**Glycerophospholipids** and **sphingolipids** are abundant in all biological membranes (Fahy et al. 2005; Fahy et al. 2009). A glycerophospholipid molecule normally contains four components: fatty acids, a backbone (glycerol) to which the fatty acids are attached, a phosphate and a small hydrophilic compound such as choline, serine, ethanolamine or inositol linked to the phosphate (Berg et al. 2002; Fahy et al. 2005; Fahy et al. 2009). Sphingomyelin (ceramide phosphocholine), which represents a prototypic sphingolipid is composed of two fatty acids bound to a sphingoid base backbone which is coupled to a hydrophilic choline by phosphate (Fahy et al. 2005; Fahy et al. 2009).

**Cerebrosides** (ceramide monosaccharide), such as cerebroside sulphate, ceramide oligosaccharide and ganglioside also belong to the group of sphingolipids (Berg et al. 2002; Fahy et al. 2005; Fahy et al. 2009). The common structural component of glycosphingolipids is ceramide (N-acylsphingosine). They are similar to sphingomyelin, both are derivatives of a ceramide, but they lack the phosphodiester-bound polar head groups (Berg et al. 2002; Fahy et al. 2005; Fahy et al. 2009).
Sterols are cholesterol, cholesterol esters, bile acids, steroid hormones, and vitamin D (Stockham and Scott 2002; Fahy et al. 2005; Fahy et al. 2009).

Other lipids include terpenes (Vitamins A, E and K) and wax aliphatic alcohols (Stockham and Scott 2002; Fahy et al. 2005; Fahy et al. 2009).

Lipoproteins are lipids combined with other classes of compounds including very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL).

Derived lipids are molecules, soluble in lipid solvents that are produced by hydrolysis of natural lipids.

**2.2.1 Physiological and pathophysiology roles of fatty acids**

Fatty acids are substantial components of cell membranes (Tvrzicka et al. 2011). Not all fatty acids can be produced endogenously in humans and animals because of the lack of certain desaturases (Tvrzicka et al. 2011). Dietary sources of fatty acids include animal products (butter, lard) and tropical plant oils (coconut, palm) for saturated fatty acids, as well as vegetable oils (olive, sunflower and soybean oils) and marine products (algae and fish oils) for unsaturated fatty acids (Tvrzicka et al. 2011).

Fatty acids take part in complex metabolic pathways and play an important role in humans and other species (Tvryicka et al. 2011; Kremmyda et al. 2011). Fatty acids in form of TAG from dietary fat are a principal source of energy (Kremmyda et al. 2011). TAG are a substantial component of the adipose tissue, which serves multiple metabolic as well as structural and functional roles such as temperature (e.g. in marine mammals) and mechanical isolator (Kremmyda et al. 2011).

Fatty acids are also structural components of all cell membranes in the form of glycerophospholipids, which can influence the thickness and fluidity of the membrane and the activity of membrane associated proteins including enzymes, ion channels, receptors and transporters (Nelson and Cox 2005; Kremmyda et al. 2011). Fatty acids also form second messengers. Phosphatidylinositol and phosphatidylcholine may play a role as sources of intracellular signals (Kremmyda et al. 2011).
Fatty acids of the cell membrane, especially longer-chain polyunsaturated fatty acids including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid are also precursors of lipid mediators, such as eicosanoids (prostaglandins, thromboxanes and leukotrienes), resolvins and neuroprotectins (Smith et al. 2008; Kremmyda et al. 2011).

Fatty acids influence cell signalling by acting as ligands of receptors or as modulators of gene transcription in signal transcription pathways (Chapkin et al. 2008; Tvryicka et al. 2011). Fatty acids are also involved in receptor interactions in signal transduction pathway. Amide derivatives of fatty acid such as ethanolamine, ammonia and some bioactive amines interact with cannabinoid as well as vaniloid receptors. The brain cannabinoid receptors takes part in signal pathways of memory, movement, emotional and nociceptive processes. The peripheral cannabinoid receptors are involved in the modulation of immunocompetent cells (Pertwee 2008; Kremmyda et al. 2011).

The fatty acid status and the fatty acid composition of blood plasma and adipose tissue can be influenced by the dietary fat. Moreover, variation of fatty acid composition due to increased intake of animal fat and decreased intake of vegetable and marine origin oils may induce a variety of pathological processes (Kunesová et al. 2006; Vecka et al. 2006; Hlavatý et al. 2008; Asp et al. 2011; Gillingham et al. 2011; Teng et al. 2010 & 2011).

Essential fatty acids (EFA) are specific fatty acids which need to be taken from the diet, e.g. linoleic, alpha-linolenic (Tvrzicka et al. 2011). A number of disorders are induced by a deficiency of EFA, summarized in Table 4. The pathophysiological and clinical differences between n-3 and n-6 polyunsaturated fatty acids deficiencies are also shown in Table 5.
Table 4: Essential fatty acid deficiency syndrome*

<table>
<thead>
<tr>
<th>Essential fatty acid deficiency syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth disturbances</td>
</tr>
<tr>
<td>Increased transepidermal losses of water (increased skin permeability)</td>
</tr>
<tr>
<td>Increased predisposition to bacterial infections</td>
</tr>
<tr>
<td>Male and female infertility</td>
</tr>
<tr>
<td>Decreased status of AA/ increased status of mead acid (20:3 n-9)</td>
</tr>
<tr>
<td>Disturbed stability of biomembranes</td>
</tr>
<tr>
<td>Disturbed cholesterol transport</td>
</tr>
<tr>
<td>Increased fragility of capillaries</td>
</tr>
<tr>
<td>Kidney failure (hematuria, hypertension)</td>
</tr>
<tr>
<td>Lower contractility of myocardium</td>
</tr>
<tr>
<td>Abnormal QRS in electrocardiogram</td>
</tr>
<tr>
<td>Lowered production of ATP (myocardium, liver)</td>
</tr>
<tr>
<td>Dysopsia (lowered visual acuity, disturbed adaptation to darkness)</td>
</tr>
<tr>
<td>Neurological disturbances (sensor and motor neuropathies)</td>
</tr>
<tr>
<td>Increased food demand with negative nitrogen balance</td>
</tr>
<tr>
<td>Disturbed synthesis of eicosanoids</td>
</tr>
</tbody>
</table>

*according to Kremmyda et al. 2011

AA = Arachidonic acid; ATP = Adenosine triphosphate; QRS = QRS complex

Excess intake of fat leads to higher risk of cardiovascular disease (Bysted et al. 2005; Astrup et al. 2011; Kremmyda et al. 2011). Observational studies have found that increased saturated fatty acids intake is linked to an increased plasma low-density lipoprotein cholesterol (LDL-cholesterol) level and increased cardiovascular disease. Moreover high monounsaturated fatty acid intake from olive oil results in increased high-density lipoprotein cholesterol (HDL-cholesterol) and decreased LDL-cholesterol (Astrup et al. 2011; Kremmyda et al. 2011). Furthermore, unsaturated fatty acids may also be able to modulate the immune system through several mechanisms including reduction of T lymphocyte proliferation, reduction of cytokine synthesis, such as IL-1 and IL-2, increased phagocytic activity, and modification of
Introduction

Natural Killer (NK) cell activity (Wang et al. 1994; De Pablo and De Cienfuegos 2000; Gupta et al. 2012). The influence of different families of fatty acids on immune system functions in both experimental animals and humans is shown in Table 6.

**Table 5: n-3 and n-6 polyunsaturated fatty acids (PUFAs) deficiencies in human and animals**

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>n-3 PUFAs</th>
<th>n-6 PUFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinic</strong></td>
<td>Normal skin, growth and reproduction;</td>
<td>Growth retardation;</td>
</tr>
<tr>
<td></td>
<td>Abnormal electroretinogram;</td>
<td>Skin lesions;</td>
</tr>
<tr>
<td></td>
<td>Dysopsia;</td>
<td>Disturbed reproduction;</td>
</tr>
<tr>
<td></td>
<td>Polydipsia</td>
<td>Steatosis;</td>
</tr>
<tr>
<td><strong>Biochemical parameters</strong></td>
<td>↓18: 3n-3 and 22:6n-3;</td>
<td>↓18:2n-6 and 20:4n-6;</td>
</tr>
<tr>
<td></td>
<td>↑22:4n-6 and 22:5n-6</td>
<td>↑20:3n-9 (only with parallel ↓ of PUFAs n-3)</td>
</tr>
<tr>
<td></td>
<td>↑20:3n-9 (only with parallel ↓ of PUFAs n-6)</td>
<td></td>
</tr>
</tbody>
</table>

*according to Kremmyda et al. 2011

Fatty acid can cross the blood-brain barrier via penetration or through direct uptake of lipoprotein particles mediated by lipoprotein receptors (Dhopeshwarkar and Mead 1973; Smith and Nagura 2001). High fat diets might increase the uptake of fatty acids into the brain from the plasma (Patil et al. 2005 and 2006; Laine et al. 2007; Gupta et al. 2012). Alterations or elevations of saturated fatty acids can disrupt and undermine brain function (Patil et al. 2005 and 2006; Laine et al. 2007; Gupta et al. 2012). Saturated free fatty acids (palmitic acid, lauric acid and stearic acid) could trigger inflammation in cultured macrophages and release TNFα and IL-6 in cultured astrocytes. Furthermore, they modulate amyloid processing in neurons and astrocytes. However unsaturated fatty acids were unable to induce cytokine release from cultured astrocytes (Patil et al. 2005 and 2006; Laine et al. 2007; Gupta et al. 2012). To
conclude, animal models with increased serum lipids might be associated with increased brain inflammation (Mattson et al. 2003; Perry et al. 2003).

**2.2.2 Cholesterol biosynthesis in the central nerves system (CNS)**

In healthy mice, rats and humans, cholesterol is produced in the CNS by local *de novo* synthesis and it is not derived from the blood plasma due to the closed blood-brain barrier (Adibhatla and Hatcher 2008; Hayashi 2011). While astrocytes synthetize cholesterol and release it together with apolipoprotein E, neurons degrade cholesterol to 24(S)-hydroxycholesterol (Chen et al 2013). Investigations conducted by Saher et al. (2005) and Verheijen et al. (2009) indicated that oligodendrocytes and Schwann cells are primarily responsible for the synthesis of cholesterol required for myelination. However, in an experimentally-induced conditional transcriptional blockade of key enzymes of cholesterol biosynthesis, these cells used cholesterol from the extracellular space for the synthesis of myelin membranes (Saher et al. 2005; Verheijen et al. 2009). Saher et al. (2005) showed that the delayed myelination observed in the oligodendrocyte-specific squalene-synthase knock-out mice cannot be influenced positively by a cholesterol enriched diet. However, other studies suggested that an experimental change in serum cholesterol concentration may have an effect on the cholesterol metabolism of the brain (Eckert et al. 2001). Thus, for example the statin “Lovasatin” can induce a reduction of cholesterol in the brain of C57BL/6-mice (Eckert et al. 2001). It is also known that elevated serum cholesterol levels positively correlate with an increased expression of apolipoprotein E (ApoE) in the brain (Petanceska et al. 2003). ApoE is the major cholesterol-transporting protein in the CNS. It is mainly expressed by astrocytes and ApoE is responsible for the formation of cholesterol-rich lipoproteins (high-density lipoprotein, HDL). This is mediated by ATP-binding transporter molecules (Hayashi 2011). HDL is responsible for the transport of cholesterol to neighboring cells (Hayashi 2011). An up-regulation of the transcription of the *Apoe*- and the ATP-binding cassette, sub-family G (WHITE), member 1 (*Abcg1*) genes was show in the spinal cord of TMEV-infected mice (Ulrich et al. 2010).
Introduction

2.3 Methods for cholesterol analysis

Detection for cholesterol analysis mainly includes histological, chromatographic and colorimetric methods. To visualize cholesterol in tissues and cells, various previous reports used filipin staining as the standard method for histology (Kruth 1984; Kruth and Fry 1984; Lee and Kruth 2003). Filipin is a polyene antifungal antibiotic produced naturally by the bacteria *Streptomyces filipinensis* (Arthur et al. 2011). Filipin has been shown to mediate a cellular leakage in membrane permeability in natural and artificial biological membranes, when an interaction between filipin and sterol, preferably cholesterol, occurred (Norman et al. 1972). Filipin specifically binds 3β-hydroxysterols, e.g. free cholesterol within artificial phospholipid bilayers and biological membranes. Due to this feature, filipin has been used as a histochemical stain for cholesterol in biomembranes (Williamson 1969; van Leeuwen et al. 2008). The filipin stain appears blue when viewed under the fluorescence microscope in the UV range between 360-460nm (Reid et al. 2004). However, detection of filipin staining under confocal microscopy is difficult, because filipin staining exhibits rapid photobleaching (Reid et al. 2004). In addition, filipin staining detects only unesterified cholesterol but does not react with cholesteryl ester (Kruth 1984; Kruth and Fry 1984).

The lipid-soluble dye oil red O, which does not stain unesterified cholesterol, was used to detect hydrophobic lipids, including esterified cholesterol (Kruth 1984; Kruth and Fry 1984; Lee and Kruth 2003). Dye specificities are shown in Table 7.
Table 6: Influence of different families of dietary fatty acids on immune system functions in both experimental animals and humans*

<table>
<thead>
<tr>
<th>Immune function</th>
<th>n-3</th>
<th>n-6</th>
<th>n-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte proliferation</td>
<td>Decreased in humans and animals</td>
<td>No effect in rats</td>
<td>Decreased in rats</td>
</tr>
<tr>
<td>IL-1 production</td>
<td>Decreased in patients suffering from rheumatoid arthritis</td>
<td>No effect in rats from rheumatoid arthritis, decreased in Balb/c mice</td>
<td></td>
</tr>
<tr>
<td>IL-2 production</td>
<td>Decreased in humans and animals</td>
<td>Increased in mice</td>
<td>No effect in rats</td>
</tr>
<tr>
<td>TNF production</td>
<td>Decreased in humans, decreased in macrophages of mice</td>
<td>Decreased in rats</td>
<td>No effect in Balb/c mice</td>
</tr>
<tr>
<td>NK cell activity</td>
<td>Decreased in rats and humans</td>
<td>Decreased in rats</td>
<td>Decreased in Balb/c mice and rats</td>
</tr>
</tbody>
</table>

*according to De Pablo and Cienfuegos 2000

IL = interleukin. NK = natural killer cells. TNF = tumor necrosis factors

Table 7: Dye specificities for detection different lipid classes*

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Oil Red O</th>
<th>Filipin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fatty acids</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Unesterified cholesterol</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Esterified cholesterol</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*according to Rudolf and Curcio 2009

+: reliably detectable, -: not detectable
To detect lipids, thin-layer chromatography (TLC) and gas chromatography (GS) are commonly used chromatographic analysis techniques (Hamilton and Hamilton 1992). In the last twenty years, high-performance liquid chromatography (HPLC) replaced preparative gas chromatography as a method for lipid analysis (Volin 2001). Several advantages make TLC particularly useful for lipid analysis. TLC is an easy to perform technique, inexpensive and easily established (Hamilton and Hamilton 1992). Analyses are performed rapidly and many samples can be screened simultaneously together with standards (Hamilton and Hamilton 1992; Fuchs 2011). In addition, samples can be recovered for further analysis (Hamilton and Hamilton 1992; Fuchs 2011). An overview of important techniques of lipid analysis, and the advantages and drawbacks of the various methods are listed in Table 8 (Fuchs et al. 2011).

The principle of TLC is the separation of different lipid classes. This is achieved on a stationary phase due to polarity differences of the analytes (Fuchs et al. 2011). The most popular stationary phases for lipid separations are silica gel, alumina or kieselgur (Fuchs et al. 2011). Generally, the lipid mixture is applied to the stationary phase, and then the mixture is resolved by differential migration of its components, as a stream of solvent passes through the layer of the adsorbent by capillary action. Each lipid component has a characteristic mobility which can be described as its retention factor ($R_f$) value. The $R_f$ value is defined as the distance travelled by the component divided by the distance travelled by the solvent front.

$$R_f = \frac{\text{distance traveled by sample}}{\text{distance traveled by solvent}}$$

Since lipids are generally colorless, the separated lipid components have to be rendered visible by chemical reagents. For quantitative analysis, the proportions of the individual components are then determined by various techniques available (Hamilton and Hamilton 1992).

TLC is currently used for two different methods of lipid analysis (Lederkremer and Johnson 1965; White et al. 1998). Firstly, the different classes of lipids are separately extracted and then each class of lipid is analyzed via unique TLC methodology. Alternatively, a complex mixture of lipids is separated on TLC plates and then further
characterized. The lipid classes are divided into neutral lipids such as triglycerides, polar lipids such as phospholipids, and cholesterol. Ideally, lipids are chromatographed on a single alumina or silica gel TLC plate using sequential solvent systems running in the same dimension. Relatively nonpolar lipids such as neutral lipids, fatty acids and cholesterol migrate to unique positions in the upper half of the chromatogram, whereas relatively polar lipids like phospholipids and sphingolipids are separated on the lower half of the chromatogram (Lederkremer and Johnson 1965; White et al. 1998).

Serum cholesterol and triglyceride are the most frequently measured lipid compounds in blood or other body fluids (Stockham and Scott 2002). The colorimetric assay technique is assumed to be a fast and economic method to determine cholesterol concentrations in serum and other tissues (Viturro 2010; Li et al. 2012). Furthermore, the colorimetric assay has its advantages in its simplicity in both the instrumentation and performance (Jiang et al. 2010).

2.4 Cholesterol in the Theiler’s murine encephalomyelitis

Cholesterol is an important constituent of most mammalian cell membranes and its concentration in various cellular membranes is tightly controlled by homeostatic processes (Maxfield and Wüsten 2012). Cholesterol plays an important role in determining the biophysical properties of biological membranes, as well as a precursor for the synthesis of steroid hormones, bile acids, and lipoproteins (Liscum and Underwood 1995). Cholesterol is present in tissues and plasma as free cholesterol or as cholesterol ester, which can either be a degradation form or a storage form of cholesterol (Mukherjee et al. 1998). Cholesterol belongs to the family of steroids, is composed of an approximately planar steroid ring system, with a 3β-hydroxysterol function on one end and a hydrophobic alkyl tail on the other (Mukherjee et al. 1998). The cholesterol metabolism of the CNS has many unique features, such as a predominantly local de novo synthesis and intercellular transport with high-density lipoproteins (HDL; Adibhatla and Hatcher 2008; Hayashi 2011). However, many brain-specific aspects of cholesterol biosynthesis and metabolism such as the
excretion of excess cholesterol from the brain are poorly understood (Hayashi 2011). Due to the high lipid and cholesterol levels in the myelin sheaths, it is not surprising that a reduced concentration of phospholipids and total cholesterol, and an increase of cholesterol esters have been seen in the foci of demyelination in multiple sclerosis (MS; Cumings 1955; Wender et al. 1974). Notably, there is a lower but similar reduction of cholesterol in the normal appearing white matter of MS patients (Cumings 1955; Wender et al. 1974). The analysis of transcriptional changes in MS and models of virus-induced murine myelin loss such as Theiler's murine encephalomyelitis (TME) and experimental autoimmune encephalomyelitis (EAE), showed a down-regulation of cholesterol biosynthesis with a particularly close association to demyelination (Lock et al. 2002; Muller et al. 2008; Ulrich et al. 2010). A primary transcriptional change of cholesterol biosynthesis can be postulated as an important factor in the pathogenesis of demyelinating diseases (Lock et al. 2002; Ulrich et al. 2010). In particular, the initially unexplained therapeutic failure of statins in clinical trials in MS patients provided further evidence of an association between cholesterol metabolism and demyelination (Weber et al. 2007; Klopfleisch et al. 2008; Miron et al. 2009). By now, no biochemical basic data regarding cholesterol metabolism exist in the model of TMEV-induced demyelination. In contrast to the toxic cuprizone-induced model of MS and experimental autoimmune encephalomyelitis (EAE), the TME model is characterized by a progressive chronic course with poor remyelination and thus provides ideal conditions for the analysis of the degradation of cholesterol and phospholipids (Ulrich et al. 2008). The effect of a statin therapy with cholesterol biosynthesis-inhibiting, anti-inflammatory and immunomodulatory effects has been described in other models of MS (Klopfleisch et al. 2008; Miron et al. 2009; Paintlia et al. 2005; Youssef et al. 2002). However, so far an opposite cholesterol-increasing effect on the metabolism has not yet been studied in any MS model. Cholesterol increasing diets such as the Paigen diet represent standard models of atherosclerosis (Getz and Reardon 2006) and will be transmitted to an MS model for the first time in the planned investigations of this thesis.
Table 8: Overview of important techniques of lipid analysis. The advantages and drawbacks of the various methods are listed (according to Fuchs et al 2011)

<table>
<thead>
<tr>
<th>Technique</th>
<th>Principle</th>
<th>Advantages</th>
<th>Drawbacks</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>Thin-layer chromatography (TLC)</td>
<td>Separation is achieved on a “stationary phase” (normally silica gel) due to polarity differences of the analyses.</td>
<td>TLC is quite inexpensive and fast. Variations of the mobile phase enable separation of even complex mixtures. Different stainings can be easily performed.</td>
<td>Oxidation (of unsaturated lipids) may occur if the TLC plate is stored for a while since a large (lipid) surface is exposed to atmospheric oxygen. Preparative applications are limited.</td>
<td>Often used as initial method if a complex lipid mixture has to be analyzed.</td>
</tr>
<tr>
<td>Gas chromatography (GC)</td>
<td>Separation of volatile compounds on a carrier gas. Detection often performed by means of mass spectrometry.</td>
<td>Highly established in fatty acid analysis. Automated devices are commercially available.</td>
<td>Only volatile compounds can be analyzed. Thus, derivatization of the analyte is required.</td>
<td>Most widely applied technique for examination and quantifying fatty acyl compositions of lipids. However, increasingly replaced by soft ionization mass spectrometry techniques.</td>
</tr>
<tr>
<td>High-performance liquid chromatography (HPLC)</td>
<td>Separation on a “stationary phase” under high pressure by elution with different solvents.</td>
<td>High quality separations are achievable. Also applicable on a preparative scale.</td>
<td>More time-consuming and expensive than TLC. Detection of saturated lipids (lack of UV absorptions) is difficult. Post-column derivatization is challenging.</td>
<td>Routine method of lipid isolation in many laboratories. However, “fine-tuning” of the composition of the mobile phase to the lipid mixture of interest is challenging.</td>
</tr>
</tbody>
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3. STAT3 represents a molecular switch possibly inducing astroglial instead of oligodendroglial differentiation of oligodendroglial progenitor cells in Theiler's murine encephalomyelitis

Yanyong Sun*, Annika Lehmecker*, Arno Kalkuhl†, Ulrich Deschl†, Wenhui Sun*, Karl Rohn§, Iva D. Tzvetanova¶, Klaus-Armin Nave§, Wolfgang Baumgärtner*, Reiner Ulrich*

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Abstract

Aims: Insufficient oligodendroglial differentiation of oligodendroglial progenitor cells (OPCs) is suggested to be responsible for remyelination failure and astroglial scar formation in Theiler's murine encephalomyelitis (TME). The aim of the present study is to identify molecular key regulators of OPC differentiation in TME, and to dissect their mechanism of action in vitro. Methods: TME virus (TMEV) infected SJL/J-mice were evaluated by rotarod analysis, histopathology, immunohistology, and gene expression microarray analysis. The STAT3 pathway was activated using meteorin and inhibited using STAT3 inhibitor VII in the glial progenitor cell line BO-1 and in primary rat OPCs in vitro. Results: As expected, immunohistology demonstrated progressively decreasing myelin basic protein-positive white matter in TME. In contrast, intraleSIONal NG2-positive OPCs as well as GFAP-positive
astrocytes were increased. Gene Set Enrichment Analysis revealed 26 Gene Ontology terms including “JAK-STAT cascade” to be significantly positively correlated with the density of NG2-positive OPCs. Immunohistology revealed an increased amount of activated, phosphorylated STAT3-expressing astrocytes, OPCs, and microglia/macrophages within the lesions. Meteorin-induced activation of STAT3-signalling in BO-1 cells and primary rat OPCs resulted in an enhanced GFAP- and reduced CNPase-expression. In contrast, an oppositional result was observed in BO-1 cells treated with STAT3 inhibitor VII. Conclusions: The STAT3 pathway is a key regulator of OPC-differentiation, suggested to shift their differentiation from an oligodendroglial towards an astrocytic fate, thereby inducing astrogliosis and insufficient remyelination in TME.


4. Central nervous system de- and remyelination is independent from systemic cholesterol level in Theiler’s murine encephalomyelitis.

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Abstract

High fat intake has been described as a risk for developing multiple sclerosis (MS). Since then, no final conclusion about the effect of high fat diets and hypercholesterolemia on MS was drawn. Cholesterol biosynthesis is a key pathway during myelination and disturbances of the cholesterol biosynthesis are described in demyelinating diseases. To address the possible interaction of cholesterol and dyslipidemia in MS, cholesterol biosynthesis, lipid composition of the major lipid repositories in the body and experimentally-induced, systemic hypercholesterolemia were examined in Theiler’s murine encephalomyelitis (TME), a model for MS, using DNA microarrays, histology, immunohistochemistry, serum clinical chemistry, and high performance thin layer chromatography.

An overall down-regulation of genes associated with cholesterol biosynthesis was present on the transcriptional level. The levels of galactocerebroside and sphingomyelin in the spinal cord were reduced in the advanced TME stage. De- and remyelination were independent from serum cholesterol levels. Serum hypercholesterolemia exhibits no negative effect on virus-induced, inflammatory demyelination of the central nervous system in the atherosclerosis-resistant SJL/J mouse strain.

The findings indicate that the inconclusive reports regarding dyslipidemia and MS could result from an indirect pathomechanistic relation and/or confounding influence of genetic predisposition towards atherosclerosis.
Introduction:

Half a century ago a positive correlation between high fat intake and the risk for developing multiple sclerosis (MS) was described (Swank, 1950). Since then numerous studies have been conducted investigating the relationship between high fat diet, obesity and hypercholesterolemia as possible etiological factors for MS (Alter et al., 1974; Esparza et al., 1995; Ghadirian et al., 1998; Lauer, 1997; Ozay et al., 2014; Swank, 1954; Warren et al., 1982; Westlund and Kurland, 1953; Zhang et al., 2000). However, conflicting findings of various studies allowed no final conclusion about the possible beneficial or detrimental effect of high fat diets on initiation and progression of demyelinating diseases. Increasing evidence suggests that obesity and subsequent dyslipidemia is an important comorbidity in MS (Marrie and Horwitz, 2010). Changes in lifestyle due to physical and mental impairment were suggested as possible contributing factors (Marrie and Horwitz, 2010). In addition, recent studies revealed an association between an adverse lipid profile (high serum levels of total cholesterol, low density lipoproteins (LDL) and triglycerides) and progressing disease severity (Giubilei et al., 2002; Tettey et al., 2014a; Tettey et al., 2014b; Weinstock-Guttman et al., 2013; Weinstock-Guttman et al., 2011). Adverse lipid profiles, especially low HDL and high LDL levels, are known to act as pro-inflammatory mediators either initiating or exacerbating inflammatory diseases such as atherosclerosis, cardiovascular disease, metabolic syndrome and obesity (Esteve et al., 2005).

The hallmark of the progressive form of MS is ongoing myelin destruction and a failure of sufficient remyelination (Ferguson et al., 1997; Podbielska et al., 2012; Trapp and Nave, 2008; Trapp et al., 1999). Only about 20% of MS patients display prominent remyelination (Patrikios et al., 2006). Paradoxically, the presence of oligodendrocyte precursor cells (OPCs), predecessors of myelin-forming oligodendrocytes, was repeatedly described in MS lesions (Chang et al., 2000; Franklin, 2002; Horner et al., 2002; Scolding et al., 1998). The differentiation and maturation process of OPCs seems to be dysregulated by unknown factors leading to a failure of remyelination. This has been shown for MS and in some related animal models (Chang et al., 2000; Franklin, 2002; Kotter et al., 2006; Kuhlmann et al., 2008; Sun et al., 2014; Ulrich et al., 2008).

Theiler’s murine encephalomyelitis (TME) is an experimentally, virus-induced, inflammatory, demyelinating disease of the spinal cord. Observations in TME indicate a robust association of down-regulated cholesterol biosynthesis with demyelination and deterioration of the
clinical score (Ulrich et al., 2010). Since cholesterol biosynthesis is described as a rate-limiting step in myelin synthesis (Saher et al., 2011), an altered cholesterol biosynthesis is suggested as a possible pathomechanistic factor inhibiting remyelination (Ulrich et al., 2010). Imbalances of the lipid and cholesterol metabolism in MS and several animal models of demyelinating diseases have been frequently been described (Confaloni et al., 1988; Cumings, 1955; Gerstl et al., 1961; Wender et al., 1974). Decreased cholesterol levels were observed in MS lesions as well as in the normal appearing white and grey matter of MS patients (Cumings, 1955; Gerstl et al., 1961; Wender et al., 1974). Under physiological conditions cholesterol is synthesized locally de novo in the central nervous system (CNS) and the blood brain barrier shields the brain cholesterol pool from the circulatory cholesterol pool. Nonetheless, brain endothelial cells have the possibility of an LDL uptake through luminal receptors (Bjorkhem and Meaney, 2004; Heverin et al., 2005). Interestingly, under pathological conditions the interaction between the CNS and circulatory cholesterol seems to be enhanced (Balazs et al., 2004; Baron and Hoekstra, 2010; Chrust et al., 2011; de Preux et al., 2007; Karasinska et al., 2009; Leoni and Caccia, 2013; Saher et al., 2005; Saher and Simons, 2010; Zhao et al., 2007). Moreover, a physiologic hypercholesterolemia is observed during the peak of the myelination process (Dietschy and Turley, 2004; Uranga and Keller, 2010). Similarly, administration of dietary lipids during pregnancy and lactation apparently had an accelerating effect on the myelination of the CNS (Salvati et al., 1996). Furthermore, feeding high levels of cholesterol resulted in an increased brain cholesterol level in animal models (Dufour et al., 2006; Sparks et al., 1994).

The aforementioned diverse observations display the complex and currently still elusive interactions between MS and cholesterol metabolism. Due to the controversial discussion about the efficacy of statins, 3-hydroxy-3 methylglutaryl-coenzyme-A reductase (HMG-CoA-reductase)-inhibitors (Weber et al., 2007), as possible cholesterol-lowering drugs for the treatment of MS, further studies investigating the interaction between demyelinating diseases such as MS and cholesterol biosynthesis are required. Especially studies that address the role of cholesterol biosynthesis on disease progression of MS, with particular emphasis on its potential beneficial effect on remyelination are required. To further elucidate the interaction between initiation and progression of myelin loss and remyelination, the aims of the present study were threefold and included (i) a detailed analysis of the cholesterol biosynthesis pathway on the transcriptional level, (ii) a quantitative analysis of the lipid composition of
spinal cord, blood serum, and liver, and (iii) a determination of the effect of experimentally-induced, systemic hypercholesterolemia on de- and remyelination in a virus model of MS.
Results

Analysis of the cholesterol biosynthesis pathway on the transcriptional level

In order to focus on cholesterol biosynthesis, a subset of 21 genes representing the Metacore™ cholesterol biosynthesis pathway was analyzed in detail (Figure 1; Supplemental table 1). The low level and pathway analysis of the complete dataset has been described in detail in a previous publication (Ulrich et al., 2010). Notably, nearly all genes involved in cholesterol biosynthesis showed a mild down-regulation in TMEV-infected animals compared to mock-infected animals (Figure 1; Supplemental table 1). 8, 18, 20, and 18 differentially regulated genes were detected at 14, 42, 98 and 196dpi, respectively (Figure 1; Supplemental table 1). 7 genes were differentially regulated at all time-points from 14-196dpi.

When ranked according to their fold change, these 7 genes were among the genes with the most severe down-regulated transcripts of the analyzed subset in animals with advanced TME and histological prominent demyelination at 98 and 196dpi. The most severe down-regulation was detected for isopentenyl-diphosphate delta isomerase (Idi1). Additional analysis of single, manually selected genes involved in cholesterol metabolism and transport showed, that 7-dehydrocholesterol reductase (Dhcr7), and cytochrome P450, family 46, subfamily a, polypeptide 1 (Cyp46a1) were significantly down-regulated beginning with day 42, the first day of significant demyelination in TME. In contrast, apolipoprotein E (Apoe) and ATP-binding cassette, sub-family A (ABC1), member 1 (Abca1) were significantly up-regulated.

Quantitative analysis of the lipid composition of blood serum, liver and spinal cord

The lipid composition of liver, blood serum, and spinal cord was measured in order to detect the influence of TMEV-infection on the lipid composition of the major storage pools of lipids in the body. No significant influence of infection on the serum levels of total cholesterol, LDL, HDL, triglycerides and FFA was observed (Figure 2). Similarly, TMEV infection did not influence levels of triglycerides, FFA, cholesterol, monoacylglycerol, phosphatidylethanolamine, cardiolipin, phosphatidylinositol, phosphatidylserine, phosphatidylcholine and sphingomyelin compared to mock-infected animals in the liver (Figure 2). In the spinal cord, galactocerebroside and sphingomyelin levels were significantly decreased in TMEV-infected animals at 196dpi (Figure 2). Free fatty acids, cholesterol, monoacylglycerol, cardiolipin, phosphatidylethanolamine phosphatidylserine, phosphatidylcholine and sphingomyelin compared to mock-infected animals in the liver (Figure 2). The most severe down-regulation was detected for isopentenyl-diphosphate delta isomerase (Idi1). Additional analysis of single, manually selected genes involved in cholesterol metabolism and transport showed, that 7-dehydrocholesterol reductase (Dhcr7), and cytochrome P450, family 46, subfamily a, polypeptide 1 (Cyp46a1) were significantly down-regulated beginning with day 42, the first day of significant demyelination in TME. In contrast, apolipoprotein E (Apoe) and ATP-binding cassette, sub-family A (ABC1), member 1 (Abca1) were significantly up-regulated.

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phosphatidylserine, and phosphatidylcholine were not affected by TMEV infection at any time-point (Figure 2).

**Influence of hypercholesterolemia on TMEV-infection**

**Peripheral metabolic and pathomorphological changes**

Paigen diet significantly increased the body weight in TMEV-infected animals beginning from -7dpi till 140dpi compared to the control diet group, and mock-infected animals receiving the Paigen diet had a significant higher body weight compared to TMEV-infected animals at all time-points post infection (Figure 3).

In order to detect the influence of the Paigen diet on the systemic cholesterol repositories level, blood serum and liver were analyzed. Over the entire investigated period (7dpi till 196dpi), mice fed the Paigen diet displayed a significant increase in serum total cholesterol levels compared with control diet mice (Figure 3). TMEV-infected, Paigen diet mice showed a trend towards lower total cholesterol levels compared to mock-infected. TMEV-infected, Paigen diet mice displayed a trend towards lower total cholesterol levels beginning at 42dpi with a significant difference observed at 98dpi (Figure 3). LDL serum levels were also significantly increased in TMEV- and mock-infected mice fed the Paigen diet. A significant increase in serum HDL levels was measured on day 21, 42, 98dpi in TMEV- and mock-infected mice on Paigen diet (Figure 3). Paigen diet, TMEV–infected mice showed a trend towards lower serum triglyceride levels with a statistical significance at 98 and 196dpi compared to Paigen diet, mock-infected mice (Figure 3). Additionally, a trend towards decreased serum triglyceride levels with statistical significance at 21dpi, 42dpi and 196dpi was observed in Paigen diet, TMEV-infected mice compared to control diet, TMEV-infected animals (Figure 3). Paigen diet, TMEV-infected mice showed a trend towards lower free fatty acids serum levels as compared to Paigen diet, mock-infected mice, with a statistical significance at 42 and 98dpi. Serum albumin levels showed a trend to lower levels in Paigen diet, TMEV-infected mice compared to control diet, TMEV-infected mice with a statistical significance at 7 and 98dpi (Figure 4). No differences were detected between feeding groups or between TMEV- and mock-infected mice for ALT, GLDH (Figure 4), GGT, total bilirubin, and direct bilirubin. GGT, total bilirubin, and direct bilirubin were under the detection limit of 3 IU/L, 1.7µmol/L, and 1.5µmol/L respectively, in the majority of animals.
Paigen diet induced a significantly higher liver weight compared to the control diet in TMEV- and mock-infected animals. Control diet, TMEV-infected mice exhibited a decreased liver weight at 98 and 196dpi compared to control diet, mock-infected mice (Figure 4). Histological examination of the liver revealed a moderate to severe, multifocal to diffuse, centrolobularly accentuated, microvesicular hepatic lipidosis in 96.6% respectively 83.0% of all mock-infected respectively TMEV-infected, Paigen diet mice (Figure 4). In addition, the oil red O positive area was significantly higher in TMEV- and mock-infected, Paigen diet mice compared to control diet mice (Figure 4). A mild, multifocal, periportally accentuated, hepatic fibrosis was associated with the fatty degeneration in Paigen diet mice in 31% of the mock-infected and 13.3% of TMEV-infected mice, beginning with 98dpi (Figure 4). The inflammatory response in the liver showed no significant difference between the feeding groups. No necrosis or cholestasis was detectable in any of the groups. HPTLC analysis of the lipid content of the liver revealed a significant increase in the amount of triglycerides and cholesterol in TMEV-, and mock-infected, Paigen diet mice at 98dpi. Phosphatidylinositol was increased only in TMEV-infected, Paigen diet fed mice compared to TMEV-infected control diet fed mice. Sphingomyelin, phosphatidyserine, phosphatidylcholine, monoacylglycerol, phosphatidylethanolamine and cardiolipin showed comparable levels in the Paigen diet and control diet groups (Figure 4).

Histological examination of the heart, aorta and large pulmonary arteries was performed to exclude Paigen diet induced atherosclerotic changes. In HE-stained sections of the heart, 6% of all animals showed a mild, multifocal lymphohistioplasmacytic infiltration with no significant influence due to TMEV infection or the feeding regimen. A mild, focal, lymphohistiocytic, subintimal or intramural infiltration was found in the aorta of 3% of the animals and in large pulmonary arteries in 1% of the animals with no significant influence of infection or feeding regimen.

**CNS metabolic and pathomorphological changes**

The feeding regimen had no influence on the motoric performance of the animals as determined by the Rotarod assay. Mock-infected, Paigen diet mice attained a significant higher number of rpm compared to TMEV-infected, Paigen diet mice beginning with 28dpi. Rotarod performance in TMEV-infected, Paigen diet mice was reduced by about 75.5 % at 196dpi compared to their performance at 0dpi (Figure 5).
Hypercholesterolemia in TME

Scoring of the degree of meningitis and leukomyelitis revealed no significant differences between the feeding groups. TMEV-infection induced a mild to moderate, mononuclear infiltration in the meninges and the perivascular space of the white matter beginning at 7dpi (Figure 5). Mock-infection caused minimal meningitis at all time-points, and a minimal leukomyelitis was observed only at day 7dpi. The infiltrates were composed of CD3-positive T-lymphocytes, IgG producing B-lymphocytes and few CD107b-positive macrophages in the meninges and perivascular spaces (Figure 5). The inflammation in the parenchyma of the white matter was dominated by CD107b-positive macrophages and to a lesser extent by CD3-positive T-lymphocytes and IgG producing B-lymphocytes. Single CD3-, CD107b- and IgG-positive cells were detectable in mock-infected animals (Figure 5). CD3-positive T-lymphocytes represented the first cellular response to TMEV-infection with a significantly higher cell density as early as 7dpi in TMEV-infected animals compared to mock-infected animals (Figure 5). A significantly higher amount of IgG producing B-lymphocytes and CD107b-positive macrophages was detectable beginning with 42dpi in all TMEV-infected mice compared to mock-infected mice. The feeding regimen had no influence on the amount and quality of the inflammatory response (Figure 5).

A severe progressive demyelination was present in all TMEV-infected animals beginning at 42dpi as assessed in LFB-CV-stained spinal cord sections with no difference between the feeding groups (Figure 6). No demyelination was observed in mock-infected animals. Evaluation of HE- and toluidine blue stained specimens confirmed the results obtained in LFB-CV-stained spinal cord sections with a strong correlation of the three independent evaluations (Spearman's correlation, HE, r = 0.95, p ≤ 0.05; toluidine blue, r= 0.89, p≤0.05).

The amount of demyelination, as determined by the MBP-immunopositive white matter area was in accordance with the semi-quantitative histological evaluation using LFB-CV. Beginning at 98dpi, TMEV-infected animals showed a progressively decreasing MBP-immunoreactivity of the white matter compared to mock-infected animals. No significant difference was detectable between the two feeding groups (Figure 7). NG2-positive cell density was significantly increased in TMEV-infected animals compared to mock-infected animals, starting at 42dpi in Paigen and control diet group. At 196dpi TMEV-infected, Paigen diet mice had a significantly decreased number of NG2-immunopositive cells compared to TMEV-infected, control diet mice (Figure 7). Remyelination, semi-quantitatively assessed in semi-thin toluidine blue-stained spinal cord sections, was progressively increasing from 42dpi to 196dpi in TMEV-infected animals (Figure 6). PRX-immunohistochemistry indicated an
involvement of Schwann cell remyelination in this process by an increasing number of PRX-immunopositive cells at 98 and 196dpi (Figure 7). The feeding regimen had no influence on the amount and timing of remyelination or the amount of Schwann cell remyelination. The quantitative analysis of the lipid content of the spinal cord detected a significantly higher level of sphingomyelin only at 98dpi in TMEV-infected, Paigen diet fed mice. No effect of the feeding regimen was detectable on 21, 42, 196dpi (Figure 7). Similarly, the quantity of cholesterol, free fatty acid, monoacylglycerol, galactocerebroside, phosphatidylethanolamine, cardiolipin, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine was not influenced by the feeding regimen (Figure 7).

The amount of astrogliosis assessed by immunohistochemistry showed an increase in the GFAP-immunopositive area with a statistical significance at 42dpi and 196dpi (Figure 7). No difference was observed between the two feeding groups. Immunohistochemistry confirmed the presence of viral antigen in the spinal cords of the TMEV-infected animals in association to inflammatory and demyelinating changes with a prominent expression starting at 42dpi. In mock-infected animals no virus was present in the spinal cords (Figure 7).
Hypercholesterolemia in TME

Discussion

Previous studies reported inconsistent results concerning the role of cholesterol as a pathomechanistic factor (Swank, 1950) or comorbidity of MS (Marrie and Horwitz, 2010). However, cholesterol biosynthesis is also a key pathway during the physiologic myelination process (Saher et al., 2005), and transcriptional down-regulation of cholesterol biosynthesis was the most important biological function associated with demyelination in TME (Ulrich et al., 2010). Here, we addressed the question, whether dietary factors might contribute to increased remyelination and examined potential changes along the cholesterol biosynthetic pathway at the transcriptional level. Furthermore, the lipid composition of the main cholesterol repositories of the body as well as the effect of experimentally-induced systemic hypercholesterolemia on de- and remyelination in the TME model of MS were assessed.

Transcriptional profiling of Cholesterol biosynthesis

On the transcriptional level, we observed an overall down-regulation of genes associated with cholesterol biosynthesis, comparable to observations in myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) in rats (Mueller et al., 2008) and in MS patients (Lock et al., 2002). This down-regulation was suggested to be a transcriptional representation of a reduced capacity for myelin repair (Lock et al., 2002). The majority of the examined genes showed progressive down-regulation, indicating a continuous decline in the ability to synthesize cholesterol. This correlates with the chronic progressive clinical course of TME. Seven genes of the cholesterol biosynthesis pathway were down-regulated at all examined time-points. The rather early decrease in their expression, already at 14dpi, indicates their regulatory importance or a special vulnerability of oligodendrocytes triggered by the viral infection or the inflammatory changes in the environment. \textit{Idil1} was identified as the gene with the strongest down-regulation. It encodes an enzyme that catalyzes the conversion of isopentenyl diphosphate to dimethylallyl diphosphate, a substrate for farnesyl synthesis (Maglott et al., 2011). Reduced activity of the enzyme is known to be the main pathomechanism in the Zellweger syndrome or neonatal adrenoleukodystrophy (Maglott et al., 2011). The neuropathological lesions in these disorders include an inflammatory demyelination, non-inflammatory dysmyelination and non-specific reduction of the myelin volume in the white matter. Mutations in \textit{Dhcr24} diminish the reduction of the delta-24 double bond of sterol intermediates during cholesterol biosynthesis (Waterham et al., 2001).
Mutations in \textit{Sc5d} diminish the transformation of lanosterol into 7-dehydrocholesterol (Kanungo et al., 2013). Both result in a phenotype similar to the human Smith–Lemli–Opitz syndrome (SLOS; Saher et al., 2005). SLOS is elicited by an inherited mutation in \textit{DHCR7}, a gene responsible for the conversion of 7-dehydrocholesterol to cholesterol. The disorder is characterized by dysmyelination (Caruso et al., 2004; Elias et al., 1997; Irons et al., 1997). Dietary cholesterol supplementation is one of the standard therapies in this disease (Caruso et al., 2004; Elias et al., 1997; Irons et al., 1997). Down-regulation of \textit{DHCR7} as observed in the present study, was described not only in MS lesions, but also in the normal appearing white matter of affected patients. This finding suggests a down-regulation of cholesterol biosynthesis prior to demyelination (Lindberg et al., 2004). Similarly, a mutation in \textit{Hmgcs1}, a gene important for the conversion of (S)-3-hydroxy-3-methylglutaryl-CoA and CoA to acetyl-CoA, seems to be responsible for a misdirected migration of OPCs causing a lack of interaction between glial cells and axons resulting in a failure to express myelin genes (Mathews et al., 2014). Changes in CNS cholesterol homeostasis occur in all likelihood due to the down-regulation of \textit{Cyp46a1}, encoding an important cholesterol-removing enzyme, and the increased gene expression of \textit{Apoe} and \textit{Abca1}, both of which are involved in the efflux and transport of cholesterol (Martin et al., 2010).

Based on these observations, it can be concluded that the observed failure of sufficient remyelination might be caused by a change in the genes described above causing a severe dysregulation during myelination. Observations during developmental myelination suggested the presence of specific checkpoints to ensure sufficient production of cholesterol to precede brain myelination (Fünfschilling et al., 2012; Herz and Farese, 1999). Considering the concept of an orchestrated remyelination process, it seems plausible that the suggested dysregulation of OPCs in MS and its animal models may be caused by insufficient cholesterol biosynthesis. However, a down-regulation of the genes associated with cholesterol biosynthesis secondary to demyelination or loss of oligodendrocytes cannot be excluded. To further elucidate the underlying processes, the lipid composition of the main cholesterol repositories in the body was analyzed in the second part of the study.

\textbf{Changes in the lipid composition of blood serum, liver, and spinal cord}

Recent studies indicate that serum dyslipidemia could be a comorbidity of MS (Marrie and Horwitz, 2010). Different MRI studies in MS patients showed an association with an adverse
lipid profile and disability progression (Giubilei et al., 2002; Tettey et al., 2014a; Tettey et al., 2014b; Weinstock-Guttman et al., 2013; Weinstock-Guttman et al., 2011), an effect we could not reproduce in the present study. It can eventually be related to the fact that mice have relatively high HDL and low LDL levels under physiological conditions (Getz and Reardon, 2006). Therefore, the mouse is generally not as susceptible as humans to a disruption of this lipoprotein balance (Getz and Reardon, 2006). In one study, MS patients’ plasma levels of triglycerides were increased, whereas LDL levels decreased in comparison to healthy controls (Palavra et al., 2013). Another study reported increased total cholesterol, HDL, LDL, and triglyceride serum levels in MS patients (Sternberg et al., 2013).

In the spinal cord, galactocerebroside (galactosylceramide; GaIC) and sphingomyelin levels were significantly decreased in TMEV-infected animals compared to mock-infected animals at 196dpi. GaIC is the most typical myelin lipid and is used as a marker for mature oligodendrocytes (Baumann and Pham-Dinh, 2001). In addition, GaIC is proportional to the amount of myelin during development (Norton and Poduslo, 1973). GaIC-deficient mice show impaired insulator function of the myelin sheath (Baumann and Pham-Dinh, 2001). Sphingomyelin is involved in cell adhesion and forms lipid rafts with cholesterol for signal transduction (Podbielska et al., 2012; for a review see Lindner and Naim, 2009). Interestingly, cholesterol levels were not different between TMEV- and mock-infected animals, although a decrease in cholesterol is described in lesions and normal appearing white matter in MS patients (Cumings, 1955; Gerstl et al., 1961; Wender et al., 1974). However, our findings are in line with a study in EAE. In the latter, transcriptional changes in cholesterol transporters, but no alterations in spinal cord cholesterol levels were detected (Mueller et al., 2008). A plausible explanation could be the different degradation capacities for various lipid components in the myelin sheath and the sustained duration of the disease in human patients. Under physiological circumstances, cholesterol has by far the longest half-life of about 300 days. In contrast, cerebrosides have a half-life of about 20 days (Ando et al., 2003). Furthermore, hyperactivities of degradation enzymes as described for the hydrolysis of sphingomyelin may play a central role (Wheeler et al., 2008). However, an impaired response or clearing activity of macrophages might represent a possible cause for the poor clearance of myelin debris leading to dysregulation of OPC differentiation (Kotter et al., 2006; Robinson and Miller, 1999).
Hypercholesterolemia in TME

**Influence of hypercholesterolemia on TMEV-infection**

Since we did not detect serum dyslipidemia in a lipid balanced diet, we were interested in the influence of hypercholesterolemia in TMEV-infected animals. A possible beneficial effect of cholesterol supplementation appeared to be plausible in view of our microarray study and an *in vitro* study, in which treatment with statins that lead to the formation of abnormal myelin membranes (Maier et al., 2009) or retraction of processes and cell death of OPCs and oligodendrocytes, that could be rescued by cholesterol supplementation (Miron et al., 2007). Cholesterol supplementation has never been offered as a potential treatment for MS likely due to controversially discussed negative impact of high-fat diets as a possible etiological factor for MS (Alter et al., 1974; Lauer, 1997; Ozay et al., 2014; Swank, 1954; Warren et al., 1982; Weinstock-Guttman et al., 2005; Westlund and Kurland, 1953). However, as demonstrated in SLOS, dietary cholesterol supplementation can have a beneficial effect with respect to remyelination (Caruso et al., 2004; Elias et al., 1997; Irons et al., 1997).

In the present study, we could neither see an anticipated beneficial effect due to the higher circulatory availability of cholesterol, nor detect a negative impact of a high cholesterol diet on the development of the disease. This was substantiated by clinical assessment, histology and immunohistochemistry. The amount, onset or duration of meningitis, leukomyelitis, demyelination or remyelination remained unchanged. The reason for the significantly decreased density of NG2-positive cells in the Paigen diet fed mice compared with control diet fed animals at 196dpi remains elusive. These observations cannot be explained by an increased differentiation of OPCs to myelinating oligodendrocytes, because there was no consecutive increase in the myelinated area. Similar to previous studies in TME, an increased number of OPCs without maturation to myelinating oligodendrocytes was demonstrated at earlier time points (Ulrich et al., 2008). Interestingly, a similar differentiation arrest was observed in a cuprizone experiment in simvastatin-treated animals (Miron et al., 2009).

In the present study, the amount of sphingomyelin was increased at 98dpi in Paigen diet mice compared to control diet mice. However, the underlying mechanism is undetermined.

Surprisingly, our results are in contrast to a similar feeding experiment using a Western-type lipid-rich diet conducted in MOG-induced EAE in C57BL/6J mice (Timmermans et al., 2014). It was shown that high fat diet increased the immune cell infiltration, inflammatory mediator
Hypercholesterolemia in TME

production and exacerbated neurologic symptoms in this EAE model (Timmermans et al., 2014). The difference may be due to the different mouse strains used or the mode in which myelin loss is induced. The C57BL/6J mice used by Timmermans et al. (2014) are known to be one of the most atherosclerosis sensitive strains (Getz and Reardon, 2006), whereas the SJL/J mouse strain develops a significant hypercholesterolemia, despite a relative resistance to atherosclerosis, when fed with Paigen diet (Nishina et al., 1993; Paigen et al., 1990). In contrast to the Paigen diet, the Western-type diet is known to have a higher atherogenic potential (Getz and Reardon, 2006). Thus, the increased inflammatory reaction in Timmermans et al. (2014) could be a secondary effect of atherosclerotic changes. Clinical chemistry showed significantly elevated total cholesterol, HDL, LDL and free fatty acids in the blood serum of all Paigen diet fed animals. The observed down-regulation of triglycerides as an effect of the Paigen diet is a well-known fact in atherosclerosis research (Getz and Reardon, 2006; Nishina et al., 1993; Paigen et al., 1990). The increased fat content of the diet resulted in a severe hepatic lipidosis, predominantly consisting of triglycerides and cholesterol.

Based on the insights gained in this study it can be concluded that down-regulation of cholesterol biosynthesis is a robust transcriptional marker for demyelinating conditions like TME, EAE (Mueller et al., 2008) and MS (Lock et al., 2002). Furthermore, de- and remyelination in the chronic progressive TME model represent two processes that develop and precede independently from serum cholesterol levels, most likely due to the inability of the circulatory cholesterol to enter the CNS due to a tight blood brain barrier. Moreover, serum hypercholesterolemia and dyslipidemia exhibit no negative effect on virus-induced, inflammatory demyelination in the CNS in this atherosclerosis-resistant mouse strain. The reported findings could indicate that the inconclusive reports regarding dyslipidemia and MS are maybe influenced by rather indirect pathomechanistic factors and/or the confounding influence of the respective genetic predisposition towards atherosclerosis.
Materials and Methods:

Microarray experiment:

Microarray analysis of transcriptional changes was performed with SJL/JHanHsd mice (Harlan Winkelmann, Borchen, Germany) intracerebrally infected with $1.63 \times 10^6$ Plaque forming units (PFU) per mouse of the BeAn strain of Theiler’s murine encephalomyelitis virus (TMEV) in comparison with mock-infected mice at 14, 42, 98, 196 days post infection (dpi), as described previously (Ulrich et al., 2010). Briefly, six biological replicates were used per group and time point, except for 5 TMEV-infected mice at 98dpi. RNA was isolated from frozen spinal cord specimens using the RNeasy mini kit (Qiagen, Hilden, Germany), amplified and labeled with the Message-Amp II-Biotin enhanced kit (Ambion, Austin, TX, USA) and hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA). Quality control and low level analysis of raw fluorescence intensities were performed with RMAExpress (Bolstad et al., 2003). MIAME-compliant data sets are deposited in the ArrayExpress database (E-MEXP-1717; http://www.ebi.ac.uk/arrayexpress).

In order to focus on the transcriptional changes related to the cholesterol biosynthesis, 21 genes represented in the canonical cholesterol biosynthesis pathway of the MetacoreTM database (version 6.5; GeneGo, St. Joseph, MO, USA) and 24 manually selected individual genes involved in cholesterol metabolism and transport were individually analyzed using pairwise Mann-Whitney-U-tests (IBM SPSS Statistics, Version 21, IBM, Chicago, USA). Statistical significance was generally accepted as $p \leq 0.05$.

Feeding Experiment:

Three weeks old female SJL/J mice (Charles River, Sulzfeld Germany) were randomly grouped into two feeding groups and fed conventional mouse diet (low fat control diet; product number: S2205-E010, ssniff Spezialdiäten GmbH, Soest, Germany; Table I), or Paigen diet (high fat Paigen 1,25% cholesterol diet; product number: S2205-E015, ssniff Spezialdiäten GmbH, Soest, Germany; Table I; Paigen et al., 1985), beginning 13 days prior to the infection (-13dpi) over the complete time of the experiment either ad libitum with free access to tap water. The animals were housed in isolated ventilated cages (Tecniplast, Hohenpeißenberg, Germany). At an age of five weeks animals from the two feeding groups were inoculated into the right cerebral hemisphere with $1.63 \times 10^6$ PFU/mouse of the BeAn-
strain of TMEV as previously described (Ulrich et al., 2006; Ulrich et al., 2010; Ulrich et al., 2008). Mock infection was performed with solvent only (Ulrich et al., 2006; Ulrich et al., 2010; Ulrich et al., 2008). At 7, 21, 42, 98 and 196dpi, six animals per group (except 5 mice at 42dpi in mock-infected, Paigen diet fed group) were sacrificed after general anesthesia as described (Ulrich et al., 2006).

Immediately after death, brain, liver, spinal cord, heart and lungs were removed and weighted (Sartorius TE13S-DS, Sartorius AG, Göttingen, Germany). For histology and immunohistochemistry the organs was fixed in 10% formalin for 24 h and embedded in paraffin wax (formalin-fixed and paraffin embedded, FFPE). For lipid analysis specimens were immediately snap-frozen and stored at -80°C. For cryo-sections liver was embedded into Optimal Cutting Temperature compound (OCT; Tissue-Tek® O.C.T.™ compound, Sakura, Alphen aan den Rijn, Netherlands). Additionally, spinal cord segments were fixed with 5% glutaraldehyde/cacodylate buffer for 24 h, post-fixated with 1% osmium tetroxide, dehydrated, and embedded in epoxy resin (Baumgärtner et al., 1987; Ulrich et al., 2008). The animal experiments were authorized by the local authorities (Regierungspräsidium Hannover, Germany, permission number: AZ: 33.14-42502-04-11/0517).

Clinical examination and rotarod analysis

The clinical course was evaluated weekly employing weight measurements (Sartorius TE13S-DS, Sartorius AG, Göttingen, Germany) and Rotarod testing (RotaRod Treadmill, TSE Technical & Scientific Equipment, Bad Homburg, Germany). Prior to infection mice were trained twice at –13 and –7dpi for 10 minutes at a constant speed of 5 or 10 rounds per minute (rpm), respectively. For the measurements, the rod speed was linearly increased from 5 rpm to 55 rpm over a time period of 5 min and the attained rpm at drop was analyzed for significant differences between the groups using three-factorial ANOVA with repeated measures and post-hoc independent t-tests for the factors status of infection, time-point post infection and feeding regimen using IBM SPSS Statistics (IBM SPSS Statistics, Version 21, IBM, Chicago, USA). A mean score per mouse was calculated from three trials per day (Ulrich et al., 2010).

Clinical chemistry of blood serum
Blood was collected immediately after death from the caudal V. cava and serum stored at -80°C. Serum concentration of total cholesterol, low density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, alanine transaminase (ALT), gamma-glutamyl transpeptidase (GGT), glutamate dehydrogenase (GLDH), total bilirubin, direct bilirubin, albumin, urea (Cobas®, Roche Diagnostics GmbH, Mannheim, Germany) and free fatty acids (FFA; Wako chemicals GmbH, Neuss, Germany) were measured with a Hitachi Automatic Bioanalyzer (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. Arithmetic means with 5-95% confidence interval of the mock-infected animals fed with normal diet were used as reference values.

**Histological examination**

Serial sections of FFPE liver specimens were stained with hematoxylin and eosin (HE), Heidenhain's azan trichrome stain and Fouchet’s stain, and were semi-quantitatively scored for lipidosis (0 = no lipidosis; 1 = single foci of lipidosis; 2 = mild lipidosis; 3 = moderate lipidosis; 4 = marked lipidosis; 5 = severe, diffuse lipidosis), type of intracytoplasmic lipid droplets (micro- or macrovesicular), fibrosis (0= no fibrosis; 1= mild; 2= moderate; 3= severe), and cholestasis (0= no cholestasis; 1= mild; 2= moderate; 3= severe) respectively (Thoolen et al., 2010). Cryo-sections of the liver were stained with Oil red O stain and the percentage of Oil red O positive areas was morphometrically assessed using the analysis 3.1 software package (SOFT Imaging system, Münster, Germany; Haist et al., 2012).

FFPE transversal sections of the ascending aorta, heart and large pulmonary arteries were stained with HE and examined for changes by light microscopy.

FFPE transverse sections of cervical, thoracic and lumbar spinal cord segments were stained with HE and Luxol fast blue-cresyl violet (LFB-CV) and evaluated semi-quantitatively for meningitis, leukomyelitis (0 = no changes; 1 = scattered infiltrates; 2 = 2-3 layers of inflammatory cells; 3 = more than 3 layers of inflammatory cells) and demyelination (0 = no changes; 1 = ≤25%; 2 = 25-50%; 3 = 50-100% white matter affected; Hansmann et al., 2012; Ulrich et al., 2006; Ulrich et al., 2010). Remyelination in the spinal cord was assessed semi-quantitatively in semi-thin, epoxy resin embedded, toluidine blue stained, transverse sections of cervical, thoracic and lumbar spinal cord (0 = no changes; 1 = single focus up to 25%; 2 =
25-50%; 3 = 50-100% of white matter affected; Ulrich et al., 2008). A mean score per mouse was calculated from cervical, thoracic and lumbal spinal cord scores.

**Immunohistochemistry**

Immunohistochemistry was performed on FFPE transverse serial spinal cord sections, using the avidin-biotin-peroxidase complex (ABC) method (Vector Laboratories, Burlingame, CA, USA) with 3,3’-diaminobezidine-tetrahydrochloride (DAB) as chromogen (Ulrich et al., 2008). The antibodies applied were anti-CD3 (polyclonal rabbit anti-human, diluted 1:1,000, Dako A0452, Hamburg, Germany) for T lymphocytes, anti-CD107b (monoclonal rat antimouse biotinylated, clone M3/84, diluted 1:800, Serotec MCA2293B, Oxford, UK) for microglia/macrophages, anti-IgG (goat anti-mouse-IgG, diluted 1:200, Vetor Laboratories, BA9200, Burlingame, CA, USA) for plasma cells, anti-glial fibrillary acidic protein (GFAP; polyclonal rabbit anti-cow, diluted 1:1,000, Dako Z0334, Hamburg, Germany) for astrocytes, anti-myelin basic protein (MBP; polyclonal rabbit anti-human, diluted 1:1,600, Merck/Millipore AB980, Darmstadt, Germany) for myelin, anti-periaxin (PRX; polyclonal rabbit anti-human; diluted 1:5,000; Sigma-Aldrich, St. Louis, USA) for peripheral myelin, anti-neural/glial antigen 2 (NG2; polyclonal rabbit anti-rat, diluted 1:400, Merk/Millipore AB5320, Darmstadt, Germany) for OPCs, and anti-TMEV (polyclonal rabbit anti-TMEV capsid protein VP1, diluted 1:2,000) for virus detection. The density of GFAP, NG2, MBP, CD3, IgG, CD107b, PRX and TMEV immunopositive cells per square millimeter was calculated by dividing the number of immunopositive cells obtained from the complete thoracic spinal cord section through the measured area of the spinal cord (Sun et al., 2014; Ulrich et al., 2008). The percentage of MBP-positive white matter areas was calculated by dividing the computationally detected immunopositive areas by the white matter area employing the program Analysis 3.1 (Sun et al., 2014; Ulrich et al., 2010; Ulrich et al., 2008). For clinical chemistry, histology, immunohistochemistry and lipid analysis Kruskal-Wallis-Test followed by pair-wise post-hoc Mann-Whitney-U-Test with Bonferroni-correction were calculated independently for the factors infection, time-point post infection and feeding group (IBM SPSS Statistics). Statistical significance was generally accepted as p ≤ 0.05.

**High performance thin layer chromatography (HPTLC) of liver and spinal cord**
HPTLC was performed to analyze liver and spinal cord cell membrane lipids. Lipids were extracted and prepared for HPTLC with minor modifications as previously described (Brogden et al., 2014). Briefly, liver and spinal cord samples were homogenized in multiple steps and dissolved in methanol and chloroform (2:1). The upper aqueous layer was removed and the remaining fraction was vacuum-dried and stored at -20°C. Liver and spinal cord lipid samples were dissolved in chloroform/methanol (1:1) solution, applied on HPTLC Silica gel 60 glass plates (Merck, Darmstadt, Germany) and run with three subsequent running solutions consisting of acetic acid ethyl ester/1-propanol/chloroform/methanol/0.25% potassium chloride (27:27:11:10), n-hexane/diethyl ether/acetic acid (75:23:2), and 100% n-hexane. For visualization of the lipid bands, plates were stained in phosphoric acid/copper sulphate (10:7.5) solution. Lipid bands were identified by comparison to authentic standards (Sigma Aldrich, St. Louis, USA) substances and analyzed using the CP ATLAS software (Lazarsoftware). The intensity of each lipid detected by CP ATLAS software was divided by the weight of the respective sample. An average intensity of two repeats for each sample was calculated. Dividing the intensity/weight value by a correction factor normalized differences between the plates. The correction factor was calculated by dividing the geometric mean of the standards from the respective plate by the geometric mean of all standards. Statistical significance was calculated by three-factorial ANOVA and post-hoc independent t-tests (IBM SPSS Statistics).
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Author contribution:


The authors have no conflicting financial interests.

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Hypercholesterolemia in TME


Hypercholesterolemia in TME


Hypercholesterolemia in TME


Figures legends:

Figure 1: Canonical cholesterol biosynthesis pathway map in Theiler’s murine encephalomyelitis

Transcriptional changes associated with cholesterol biosynthesis pathway in Theiler’s murine encephalomyelitis virus (TMEV)-infected mice in comparison with mock-infected control mice, illustrated in the canonical cholesterol biosynthesis pathway map from Metacore™ database (GeneGO, St. Joseph, USA). The bars labeled from one to four display the fold changes of significantly differentially regulated genes in TMEV-infected animals (N=6) compared with mock-infected animals (N=6) on the four days post infection (dpi) employing pair-wise Mann-Whitney U-tests (p ≤ 0.05) (1 = 14dpi; 2 = 42dpi; 3 = 98dpi; 4 = 196dpi). The blue indicator scale of the bar marks down-regulation and displays a comparable measurement of the magnitude of the negative fold change.

Green arrows = positive functional interaction; Z = catalysis; grey arrows = technical links; orange icons = enzymes; purple hexagons = generic compound; blue rectangle = normal process; grey rectangle = reaction; Acat1 = acetyl-Coenzyme A acetyltransferase 1; Cyp51 = cytochrome P450, family 51; Dhcr24 = 24-dehydrocholesterol reductase; Dhcr7 = 7-dehydrocholesterol reductase; Ebp = phenylalkylamine Ca2+ antagonist (emopamil) binding protein; Fdft1 = farnesyl diphosphate farnesyl transferase 1; Fdps = farnesyl diphosphate synthetase; Ggps1 = geranylgeranyl diphosphate synthase 1; Hmgcr = 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; Hmgcs1 = 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1; Idi1 = isopentenyl-diphosphate delta isomerase; Idi2 = isopentenyl-diphosphate delta isomerase 2; Lss = lanosterol synthase; Mvd = mevalonate (diphospho) decarboxylase; Mvk = mevalonate kinase; Nsdhl = NAD(P) dependent steroid dehydrogenase-like; Pmvk = phosphomevalonate kinase; Sc4mol = sterol-C4-methyl oxidase-like; Sc5d = sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S. cerevisiae); Sqle = squalene epoxidase; Tm7sf2 = transmembrane 7 superfamily member 2;

Figure 2: Quantitative analysis of the lipid composition of liver, blood serum and spinal cord in Theiler’s murine encephalomyelitis
Lipid composition of blood serum, liver and spinal cord of Theiler’s murine encephalomyelitis virus-infected female SJL/J mice (N=6; except 7dpi in B, C, D, E were only 3 animals are used) compared to mock-infected female SJL/J mice (N=6) was analyzed on five days post infection (7dpi, 21dpi, 42dpi, 98dpi, 196dpi). Displayed are serum lipid values, as measured with Hitachi Automatic Bioanalyzer (Roche Diagnostics GmbH, Mannheim, Germany), of (A) total Cholesterol, (B) low density lipoprotein, (C) high density lipoprotein, (D) triglycerides and (E) free fatty acids in mmol/L. Furthermore (F) liver (only at 98dpi) and (G) spinal cord (21dpi, 42dpi, 98dpi, 196dpi) lipid intensity values per sample weight, as measured with high performance thin layer chromatography, are shown. The data are displayed as bars or box-and-whisker plots showing the median and 95% percentile. Statistically significant differences (p ≤ 0.05) as detected by pair-wise Mann-Whitney U-tests variance or three-factorial ANOVA with post-hoc independent t-tests are marked with asterisks (★).

Figure 3: Body weight and quantitative analysis of the lipid composition of the blood serum in Theiler’s murine encephalomyelitis after experimentally-induced hypercholesterolemia

(A) Mean body weight in gram (g) over time (days post infection; dpi) of Theiler’s murine encephalomyelitis virus (TMEV)-infected female SJL/J mice (N=6; except in Figure 3 D, E, F: TMEV-infected, control diet group: 7dpi N=3) compared to mock-infected female SJL/J mice (N=6; except mock-infected; Paigen diet group: 42dpi N=5) grouped into two feeding groups (control diet; Paigen diet).

(B - F) Serum lipid levels at 7, 21, 42, 98 and 196dpi of the animals described in (A) as measured with Hitachi Automatic Bioanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). Data of (B) total cholesterol, (C) low density lipoprotein, (D) high density lipoprotein, (E) triglycerides and (D) free fatty acids are displayed in mmol/L as box-and-whisker plots showing the median and 95% percentile. Gray areas show the median and 95% percentile of the mock-infected animals fed with control diet, used as reference values. Statistical significant differences (p ≤ 0.05) as detected by pair-wise Mann-Whitney U-tests are marked with asterisks (★) for the comparison of TMEV-infected animals with mock-infected animals of the same feeding group. Statistically significant differences (p ≤ 0.05)
between Paigen diet and control diet fed animals of the same infection group are marked with a dot (●).

**Figure 4: Analysis of liver-weight, histology, quantitative lipid composition of the liver and serum liver values in Theiler's murine encephalomyelitis after experimentally-induced hypercholesterolemia**

(A) Liver weight in gram (g) on 7, 21, 42, 49, 98, and 196dpi of Theiler’s murine encephalomyelitis virus-infected female SJL/J mice (N=6) compared to mock-infected female SJL/J mice (N=6; except mock-infected; Paigen diet group: 42dpi N=5) grouped into two feeding groups (control diet; Paigen diet). (B-D) Serum lipid levels of animals and time-points described in (A) as measured with Hitachi Automatic Bioanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). Data of (B) glutamate dehydrogenase in IU/L, (C) alanine transaminase in IU/L, and (D) albumin in g/dl, are displayed as box-and-whisker plots showing the median and 95% percentile. Gray areas show the median and 95% percentile of the mock-infected animals fed with control diet, used as reference values. (E) Histological examination of the liver stained with (I) hematoxylin and eosin-stain, (II) Heidenhain's azan trichrome stain, (III) Fouchet's stain and (IV) oil red O stain revealed a moderate to severe, multifocal to diffuse, centrolobularly accentuated, microvesicular hepatic lipidosis in all Paigen diet fed mice (I). A mild, multifocal, hepatic fibrosis was associated with the fatty degeneration in Paigen diet fed animals (II). No cholestasis was detectable in any of the groups (III). Oil red O positive areas were significantly higher in TMEV- and mock-infected, Paigen diet mice compared to control diet mice (IV). Exemplary displayed is the liver of a TMEV-infected control diet fed mouse (left) and Paigen diet fed mouse (right) at 98dpi. Pictures were taken with a 10-fold objective for (I), 20-fold objective for (II-III) and 40-fold objective for (IV). The bar indicates 100 μm in (I), 50 μm in (II-III) and 20 μm in (IV). (F) Lipid composition of the liver (only at 98dpi) of the animals described in (A). Displayed are the lipid intensity values per sample weight, as measured with high performance thin layer chromatography as bars showing the median and 95% percentile. Statistically significant differences (p ≤ 0.05) as detected by pair-wise Mann-Whitney U-tests or three-factorial ANOVA with post-hoc independent t-tests are marked with asterisks (★) for the comparison of TMEV-infected animals with mock-infected animals of the same feeding group.
Statistically significant differences (p ≤ 0.05) between Paigen diet and control diet fed animals of the same infection group are marked with a dot (•).

**Figure 5: Rotarod performance and inflammatory changes in the spinal cord in Theiler’s murine encephalomyelitis after experimentally-induced hypercholesterolemia**

(A) Rotarod performance on 0-195dpi of Theiler’s murine encephalomyelitis virus (TMEV)-infected female SJL/J mice (N=6) compared to mock-infected female SJL/J mice (N=6; except mock-infected; Paigen diet group: 42dpi N=5) grouped into two feeding groups (control diet; Paigen diet). (B) Hematoxylin and eosin-stained thoracic spinal cord section of a mock-infected, control diet fed mouse (I), mock-infected, Paigen diet fed mouse (II), TMEV-infected, control diet fed mouse (III), and a TMEV-infected, Paigen diet fed mouse (IV) at 196dpi. (III-IV) TMEV-infection induced a mild to moderate, mononuclear infiltration in the meninges and the perivascular space of the white matter and a progressive demyelination. (I-II) Mock-infected animals show no changes. Pictures were taken with a 20-fold objective. The bar indicates 50 µm. (C-D) Cell density of the inflammatory infiltrates characterized by immunohistochemistry of the animals described in (A) on day 7, 21, 42, 98 and 196dpi for (C) CD3-positive T-lymphocytes; (D) IgG-producing B-lymphocytes and (E) CD107b-positive macrophages. The cell density is displayed as box-and-whisker plots showing the median and 95% percentile. Statistical significant differences (p ≤ 0.05) as detected by pair-wise Mann-Whitney U-tests are marked with asterisks (★) for the comparison of TMEV-infected animals with mock-infected animals of the same feeding group. Statistically significant differences (p ≤ 0.05) between Paigen diet and control diet fed animals of the same infection group are marked with a dot (•).

**Figure 6: Semi-quantitative assessment of de- and remyelination in the spinal cord in Theiler’s murine encephalomyelitis after experimentally-induced hypercholesterolemia**

(A) Luxol fast blue-cresyl violet-stained thoracic spinal cord section of a mock-infected, control diet fed mouse (I), a mock-infected, Paigen diet fed mouse (II), a TMEV-infected, control diet fed mouse (III), and a TMEV-infected, Paigen diet fed mouse (IV) at 196dpi. (III-IV) TMEV-infection induced a severe progressive demyelination. No demyelination was observed in mock-infected animals. Pictures were taken with a 20-fold objective. The bar
Hypercholesterolemia in TME indicates 50 µm. (B) Semi-thin toluidine blue-stained thoracic spinal cord section of the groups described in (A). Remyelinated axons are characterized by thinner myelin sheaths than normal. Myelin debris can be found intra-cytoplasmatically in macrophages. Pictures were taken with a 60-fold immersion oil objective. The bar indicates 10 µm. (C-D) Semi-quantitative scoring (0-3) of (C) demyelination, assessed in Luxol fast blue-cresyl violet-stained spinal cord section and (D) remyelination, assessed in semi-thin toluidine blue-stained spinal cord sections at 7, 21, 42, 98 and 196dpi. The scores are displayed as box-and-whisker plots showing the median and 95% percentile. Statistically significant differences (p ≤ 0.05) as detected by pair-wise Mann-Whitney U-tests are marked with asterisks (★) for the comparison of TMEV-infected animals with mock-infected animals of the same feeding group. Statistically significant differences (p ≤ 0.05) between Paigen diet and control diet fed animals of the same infection group are marked with a dot (●).

**Figure 7: Immunohistochemical assessment of de- and remyelination and lipid composition of the spinal cord in Theiler’s murine encephalomyelitis after experimentally-induced hypercholesterolemia**

(A) Myelin basic protein-immunopositive positive white matter area; (B) density of neuron/glial-antigen 2-immunopositive cells; (C) density of periaxin-immunopositive cells, and (D) glial fibrillary acid protein-immunopositive spinal cord area of Theiler’s murine encephalomyelitis virus (TMEV)-infected female SJL/J mice (N=6) compared with mock-infected female SJL/J mice (N=6; except mock-infected; Paigen diet group: 42dpi N=5) grouped into two feeding groups (control diet; Paigen diet) at 7, 21, 42, 98 and 196dpi. The scores are displayed as box-and-whisker plots showing the median and 95% percentile. (E) Spinal cord lipid intensity values per sample weight, as measured with high performance thin layer chromatography, are shown for the animals described above. Statistically significant differences (p ≤ 0.05) as detected by pair-wise Mann-Whitney U-tests or three-factorial ANOVA with post-hoc independent t-tests are marked with asterisks (★) for the comparison of TMEV-infected animals with mock-infected animals of the same feeding group. Statistically significant differences (p ≤ 0.05) between Paigen diet and control diet fed animals of the same infection group are marked with a dot (●).
Hypercholesterolemia in TME

Tables:

Table I: Diet composition

Diet composition in percent dry weight of the conventional low-fat diet, used as control diet, and the cholesterol-rich, high-fat Paigen diet, used to induce hypercholesterolemia in female SJL/J mice applied as a maintenance diet from 14 days prior to the infection with Theiler’s murine encephalomyelitis virus or mock substance over the complete studied period of 196 days post infection.

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^aNfE = Nitrogen-free extract; ^bME= metabolized energy;
Supplemental Material:

Supplemental Table 1: Fold changes and p-values of genes associated with cholesterol synthesis, metabolism and transport

The table displays the 48 probe sets (21 genes) represented in the canonical cholesterol biosynthesis pathway map in the Metacore™ database (version 6.5; GeneGo, St. Joseph, MO, USA) and manually selected 43 probe sets (24 genes) used for the analysis of the cholesterol biosynthesis, metabolism and transport in TME. Shown are the fold changes (FC) and p-values. Fold changes are calculated as the ratio of the inverse-transformed arithmetic means of the expression values of Theiler’s murine encephalomyelitis virus (TMEV)-infected animals versus mock-infected mice animals on 14, 42, 98, and 196dpi. Down-regulation is shown as negative reciprocal values (Ulrich et al., 2010). P-values were calculated by performing pair-wise Mann-Whitney-U-tests in IBM SPSS Statistics (Version 21, IBM, Chicago, USA). MIAME-compliant row data sets are deposited in the ArrayExpress database (E-MEXP-1717; http://www.ebi.ac.uk/arrayexpress).
Hypercholesterolemia in TME

Figure 1:
Figure 2A-F:
Figure 2G:
Hypercholesterolemia in TME

Figure 3A-B:
Figure 3C-F:
Figure 4A-D:
Figure 4E:
Figure 4F:
Figure 5A-B:
Figure 5C-E:
Figure 6A-B:
Figure 6C-D:
Figure 7A-D:
Figure 7E:
## Supplemental Table 1: Cholesterol Biosynthesis Pathway

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5. Discussion

5.1 Role of JAK-STAT signalling in TME

Gene Set Enrichment Analysis was performed to combine the data of gene expression microarray studies with the morphometrically assessed density of OPCs within the spinal cords from the same animals in order to search for transcriptional changes intimately associated with the OPCs at the pathway level (Subramanian et al. 2005; Ulrich et al. 2010). Accordingly, a cluster of 6 GO terms related to tyrosine phosphorylation and the JAK-STAT cascade represented the most interesting finding from the list of 26 GO terms significantly positively correlated to the density of OPCs. Notably, a recent meta-analysis identified the JAK-STAT cascade to be one out of 15 GO terms significantly positively correlated with demyelination in MS, TME, EAE and a transgenic TNF-overexpressing mouse model of demyelination (Raddatz et al. 2014), highlighting that JAK-STAT signalling is of general importance in demyelinating conditions. The JAK-STAT cascade is involved in a plethora of physiological and pathological conditions including inflammatory and immune responses (Rodriguez et al. 2006; Darnell et al. 1994), and tumorigenesis (Brantley and Benveniste 2008; Hansmann et al. 2012). Binding of many extracellular ligands such as cytokines, growth factors and hormones to their cell surface receptors can lead to a recruitment of JAKs followed by activation of a context dependent set out of the seven different STATs by tyrosine phosphorylation. The activated STATs homo- or heterodimerize, translocate to the nucleus, bind to specific DNA motifs and induce the expression of their target genes (Kisseleva et al. 2002; Rawlings et al. 2004; Harrison 2012).

With the JAK-STAT cascade being an interesting candidate pathway, we focused the analysis on a manually curated list of genes involved in JAK-STAT signalling. The demonstration of up-regulated expression of Stat1, -2, -3, -4, and -6 as well as several upstream activators and downstream targets in TME did not unequivocally indicate which of the multiple STAT cascades is most important in the setting of OPC dysregulation. Furthermore, the transcriptional profiles give no information concerning the phosphorylation dependent activation status of the various JAK-STAT pathways (Harrison 2012). However, the different STAT proteins are activated by various upstream events with considerable specificity and are known to be involved in different processes (Akira 1999). STAT1 and -2 are both participating in interferon signalling and the activation of the inflammatory response (Darnell et al. 1994; Liu et al. 1998). STAT4 and -6 are essential for the differentiation of T cells into
the Th1- or Th2-phenotype, respectively, and thus the activation of the adaptive immune response (Liu et al. 1998; Rodriguez et al. 2006). Remarkably, the STAT3 pathway is known to be a key regulator that promotes astrocytic differentiation in neuroglial progenitor cells during embryogenesis (Bonni et al. 1997; Rajan and McKay 1998; He et al. 2005; Cao et al. 2010), and represents a molecular hub for signalling pathways in glial tumors such as glioblastoma (Brantley and Benveniste 2008; Hansmann et al. 2012). Furthermore, multiple genome-wide association studies implicate the STAT3 gene as a MS susceptibility locus (Jakkula et al. 2010; Lill et al. 2012). Based on this knowledge, STAT3 was selected for further experimental analysis.

The identification of intralesional spindloid cells co-expressing p-STAT3 and NG2 supports the GSEA results, suggesting that the JAK-STAT cascade is an important signalling pathway within intralesional OPCs. It seems reasonable that this active intranuclear p-STAT3 shifts the differentiation of the intralesional OPCs towards an astrocytic fate, since STAT3 is known to induce astrocytic differentiation in neuroglial progenitor cells during embryogenesis (Bonni et al. 1997; Rajan and McKay 1998; He et al. 2005; Cao et al. 2010). In agreement with this, a minor amount of intralesional cells exhibiting NG2 and GFAP colocalization has been described in a previous TME study (Ulrich et al. 2008), MHV-induced demyelination (Redwine and Armstrong 1998), as well as traumatic CNS injury (Alonso 2005). Furthermore, a genetic fate-mapping study employing a freeze-injury of the brain displayed a high amount of OPCs differentiating into astrocytes (Tatsumi et al. 2008). Additionally, a low but distinct percentage of OPCs is known to differentiate into astrocytes in demyelinating lesions induced by ethidium bromide and in experimental autoimmune encephalomyelitis (Zawadzka et al. 2010; Tripathi et al. 2010). In contrast to these demyelinating conditions, there is progressive demyelination and astrogliosis with only delayed and insufficient remyelination in TME (Ulrich et al. 2008), which is suggested to result from a different micromilieu within the lesions reducing the capability of the OPCs to differentiate into oligodendrocytes. Obviously, not all astrocytes within the sclerotic lesions of chronic progressive TME are derived from OPCs, since in analogy to other pathological conditions the majority of astrocytes is generated by parenchymal astrocytes that re-enter the cell cycle (Young et al. 2010; Zawadzka et al. 2010; Richardson et al. 2011).

In summary, TMEV-induced chronic progressive demyelinating leukomyelitis was accompanied by an increased population of intralesional NG2-positive OPCs and astrogliosis. GSEA suggested an association between OPCs and the JAK-STAT signalling cascade.
Immunohistochemistry revealed an increased intralesional number of p-STAT3-positive cells co-expressing either GFAP, NG2, or CD107b. Meteorin-induced activation of STAT3-signalling in BO-1 cells and primary rat OPCs resulted in an enhanced GFAP- and reduced CNPase-expression in some of the tested media. In contrast, an oppositional result was observed in BO-1 cells treated with STAT3 inhibitor VII. These results suggest that STAT3 signalling represents an important factor shifting the differentiation of OPCs from an oligodendrocytic to an astrocytic fate, thereby simultaneously inducing remyelination failure and glial scarring in chronic progressive demyelinating TME.

5.2 Influence of dietary cholesterol supplementation in TME

On the transcriptional level we observed an overall down-regulation of genes associated with cholesterol biosynthesis, comparable to observations in MOG-induced experimental autoimmune encephalomyelitis (EAE) in rats (Mueller et al. 2008) and in MS patients (Lock et al. 2002). This down-regulation was suggested as a transcriptional representation of a reduced capacity for myelin repair (Lock et al. 2002). The majority of the examined genes showed progressive down-regulation, indicating a continuous decline in the ability to synthesize cholesterol. This correlates with the chronic progressive clinical course of TME. Seven genes of the cholesterol biosynthesis pathway were down-regulated at all examined time-points. The rather early decrease of their expression, already at 14dpi, indicates their regulatory importance or a special vulnerability of oligodendrocytes triggered by the virus infection or the inflammatory changes in the environment. Based on these observations, it can be concluded that the observed failure of sufficient remyelination might be caused by a change in the above-described genes causing a severe dysregulation during myelination. Observations during developmental myelination, suggested the presence of specific checkpoints to ensure sufficient production cholesterol to proceed brain myelination (Fünschilling et al. 2012; Herz and Farese 1999). Considering the concept of an orchestrated remyelination process, it seems plausible that the suggested dysregulation of OPCs in MS and its animal models could be caused by insufficient cholesterol biosynthesis. However, a down-regulation of the genes associated with cholesterol biosynthesis secondary to demyelination or loss of oligodendrocytes cannot be excluded. To further elucidate the underlying processes the lipid composition of the main cholesterol repositories in the body was analyzed in the second part of the study.
Recent studies indicate that serum dyslipidemia could be a comorbidity of MS (Marrie and Horwitz 2010). Different MRI studies in MS patients showed an association with an adverse lipid profile and disability progression (Giubilei et al. 2002; Tettey et al. 2014a; Tettey et al. 2014b; Weinstock-Guttman et al. 2011; Weinstock-Guttman et al. 2013), an effect we could not reproduce in the present study. It can eventually be related to the fact that mice have relative high HDL and low LDL levels under physiological conditions (Getz and Reardon 2006). Therefore the mouse is generally not as susceptible as humans to a disruption of this lipoprotein balance (Getz and Reardon 2006). In the spinal cord, galactocerebroside and sphingomyelin levels were significantly decreased in TMEV-infected animals compared to mock-infected animals at 196 dpi. Galactocerebroside (galactosylceramide; GalC) is the most typical myelin lipid and is used as a marker for mature oligodendrocytes (Baumann and Pham-Dinh 2001). In addition GalC is proportional to the amount of myelin during development (Norton and Poduslo 1973). GalC-deficient mice show impaired insulator function of the myelin sheath (Baumann and Pham-Dinh 2001). Sphingomyelin is involved in cell adhesion and forms lipid rafts with cholesterol for signal transduction (Podbielska et al. 2012). Interestingly cholesterol levels are unchanged between TMEV- and mock-infected animals, although a decrease in cholesterol is described in lesions and normal appearing white matter in MS patients (Cumings 1955; Gerstl et al. 1961; Wender et al. 1974). However, our findings are in line with a study in EAE. In the latter transcriptional changes in cholesterol transporters, but no alterations in spinal cord cholesterol levels were detected (Mueller et al. 2008). A plausible explanation could be the different degradation capacities for various lipid components in the myelin sheath and the sustained duration of the disease in the human patients. Under physiological circumstances cholesterol has by far the longest half-life of about 300 days. In contrast cerebrosides have a half-life of about 20 days (Ando et al. 2003). Furthermore hyperactivities of degradation enzymes as described for the hydrolysis of sphingomyelin may play a central role (Wheeler et al. 2008). However, an impaired response or clearing activity of macrophages might represent a possible cause for the poor clearance of myelin debris leading to dysregulation of OPC differentiation (Kotter et al. 2006; Robinson and Miller 1999).

In the present study we could not see an anticipated beneficial effect due to the higher circulatory availability of cholesterol, neither could we detect a negative impact of a high cholesterol diet on the disease course. This was substantiated by clinical assessment, histology, and immunohistochemistry. The amount, onset or duration of meningitis, leukomyelitis,
demyelination or remyelination remained unchanged. The reason for the significantly decreased density of NG2-positive cells in the Paigen diet fed mice compared with control diet fed animals at 196dpi remains elusive. The observation cannot be explained by an increased differentiation of OPCs to myelinating oligodendrocytes, because there was no consecutive increase in myelinated area. Similar to previous studies in TME an increased number of OPC without maturation to myelinating oligodendrocytes was observable at earlier time points (Ulrich et al. 2008). Interestingly, a similar differentiation arrest was observed in a cuprizone experiment in simvastatin-treated animals (Miron et al. 2009).

Surprisingly our results are in contrast to a similar, feeding experiment using a Western-type lipid rich diet conducted in MOG-induced EAE in C57BL/6J mice (Timmermans et al. 2014). It was shown that high fat diet increased the immune cell infiltration, inflammatory mediator production and exacerbated neurologic symptoms in this EAE model (Timmermans et al. 2014). The difference may be due to the different mouse strains used or the mode myelin loss is induced. The C57BL/6J used by Timmermans et al. (2014) are known to be one of the most atherosclerosis sensitive strains (Getz and Reardon 2006), whereas our SJL/J mouse strain develops a significant hypercholesterolemia, despite a relative resistance to atherosclerosis, when fed with Paigen diet (Nishina et al. 1993; Paigen et al. 1990). In contrast to the Paigen diet, the Western-type diet is known to have a higher atherogenic potential (Getz and Reardon 2006). Thus, the increased inflammatory reaction in Timmermans et al. (2014) could be a secondary effect of atherosclerotic changes. Clinical chemistry showed significantly elevated total cholesterol, HDL, LDL and free fatty acids in the blood serum of all Paigen diet fed animals. The observed down-regulation of triglycerides as an effect of Paigen diet is a well-known fact in atherosclerosis research (Getz and Reardon 2006; Nishina et al. 1993; Paigen et al. 1990). The increased fat content of the diet resulted in a severe hepatic lipidosis, predominantly consisting of triglycerides and cholesterol.

Based on the insights gained in this study it can be concluded, that down-regulation of cholesterol biosynthesis is a robust transcriptional marker for demyelinating conditions like TME, EAE (Mueller et al. 2008) and MS (Lock et al. 2002). Furthermore, de- and remyelination in the chronic progressive TME model represent two processes that develop and precede independent from serum cholesterol levels, most likely due to the inability of the circulatory cholesterol to enter the CNS due to a tight blood brain barrier. Moreover, serum hypercholesterolemia and dyslipidemia exhibit no negative effect on virus-induced, inflammatory demyelination in the CNS in this atherosclerosis-resistant mouse strain. The
reported findings could indicate that the inconclusive reports regarding dyslipidemia and MS maybe influenced by rather indirect pathomechanistic factors and/or the confounding influence of the respective genetic predisposition towards atherosclerosis.

5.3 Interaction between STAT3 and cholesterol biosynthesis in TME

The basis for the progressive nature of neurological disabilities in MS is ongoing myelin destruction and a failure of remyelination, resulting in a progressive axonal damage with limited remyelination (Ferguson et al. 1997; Trapp et al. 1999; Trapp and Nave 2008; Podbielska et al. 2012). Observations in MS and its animal models suggest a block of oligodendroglial differentiation as a possible cause for the insufficient remyelination in over 80% of MS patients (Patrikios et al. 2006; Franklin and Ffrench-Constant 2008; Kuhlmann et al. 2008; Ulrich et al. 2008). Despite the presence of multiple oligodendrocyte precursor cells in the demyelinated lesion, these cells fail to differentiate into myelinating oligodendrocytes (Chang et al. 2000; Kuhlmann et al. 2008; Ulrich et al. 2008). Transcriptional analysis and preliminary observations in TME directed in two directions (Ulrich et al. 2008; Ulrich et al. 2010). On the one hand the observation, that some OPCs underwent astrocytic differentiation (Ulrich et al. 2008) is an indication for an abnormal lineage decision of OPCs. On the other hand transcriptional changes in the cholesterol biosynthesis (Ulrich et al. 2010), interpreted together with similar observations in MS and its animals models (Lock et al. 2002; Mueller et al. 2008; Ulrich et al. 2010), the unsatisfying effects of statin treatment (Miron et al. 2007; Klopfleisch et al. 2008; Miron et al. 2009), and the decreased cholesterol level in normal appearing white and grey matter in MS patients (Cumings 1955; Gerstl et al. 1961; Wender et al. 1974) pointed to an involvement of the cholesterol biosynthesis in the insufficient differentiation process in OPCs.

Both reasonable implications were further characterized in two independent studies. The first study revealed a correlation of JAK-STAT signaling with the amount of NG2-positive OPCs on the transcriptional level. In subsequent experiments STAT3 was identified as a key regulator of OPC- differentiation and suggested to induce astrogliosis due to a shift in OPCs towards astrocytic fate. The second study displayed an over-all down-regulation of genes associated with cholesterol biosynthesis on the transcriptional level. In the following feeding experiment, no substantial influence of cholesterol supplementation on the inflammatory response, astrogliosis, lipid composition, demyelination and remyelination was observed.
The involvement of astrocytes in the pathophysiology of MS in general and their interaction with OPCs in particular is still not fully understood. Astrocytes are assumed to be beneficial in initial stages of MS, but in chronic stages a switch towards negative effects was supposed (Kipp et al. 2011). On the one hand a contribution of astrocytes to various degenerative and inflammatory reactions was described, but on the other hand they are suggested to create a permissive environment for remyelination (Kipp et al. 2011). The glial scar formation in MS is thought to serve as a barrier between the damaged and the surrounding tissue but also may secret factors to inhibit OPC migration and interact with cholesterol biosynthesis (Miron et al. 2011).

Interestingly, in an in vitro model of the blood brain-barrier co-culture of brain capillary endothelial cells with astrocytes lead to an increased number of LDL-receptors and a three-fold enhanced capacity to bind LDL (Dehouck et al. 1994). Due to this observation a modulatory function of astrocytes on the lipid up-take was suggested (Dehouck et al. 1994). Further studies indicated, that astrocytes immediately incorporate and process these LDL particles from the circulatory and are therefore suggested as key regulators for the lipid homeostasis in the brain (Ferretti and Bacchetti 2011). Moreover, it was demonstrated, that the interaction of apolipoproteins with ATP-binding cassette transporter ABCA1 (member 1 of human transporter sub-family ABCA) (ABCA1) activated the JAK2/STAT3 signalling pathway. JAK2 in turn causes two independent effects, lipid export from the cell and STAT3-mediated anti-inflammatory activity (Liu and Tang 2012). However, no differences in the lipid levels of the spinal cord were detectable in the present study.

Summarizing the above a possible explanation for the lack of myelinating oligodendrocyte in TME lesions is a STAT3-mediated dysregulation of OPCs, an involvement of the cholesterol metabolism in this process seems likely, but could not be verified. Dietary cholesterol supplementation had no influence on these processes in TME.
6. Summary

Pathogenetic role of cholesterol biosynthesis and STAT3 signaling in chronic demyelinating diseases

Wenhui Sun

Multiple sclerosis is an immune-mediated demyelinating disease of the central nervous system with still unknown etiology. The basis for the progressive nature of neurological disabilities in MS is ongoing myelin destruction and a failure of remyelination. This is resulting in a progressive axonal damage with limited remyelination. Transcriptional analysis and observations from previous studies in murine Theiler’s virus encephalomyelitis (TME), an experimental, virus-induced animal model of MS lead to different underlying mechanisms. On the one hand oligodendrocyte precursor cells (OPC) are present in the lesion but do not differentiate into myelinating oligodendrocytes and on the other hand, substantial, transcriptional changes are present concerning the cholesterol biosynthesis.

Therefore, the objectives of this thesis were first to identify the underlying mechanisms leading to the dysregulation of OPC differentiation and second to analyze the changes in cholesterol metabolism and study the possible influence on de- and remyelination by dietary cholesterol supplementation.

Therefore five weeks old, female SJL/J mice were infected intracerebrally with the low virulent BeAn strain of TME virus. Mock-infected animals served as controls. The motoric performance of the animals was evaluated weekly by Rotarod test over the studied period from 7-196 days post infection (dpi). At different stages of the disease 6 animals per group was necropsied and evaluated by histology, immunohistochemistry and oligonucleotide microarrays. In addition, the effect of activation and inhibition of the STAT3 signalling pathway on glial precursor cells was examined in vitro on BO-1 cells and primary rat OPC cultures for in the first part of the study.

In the second part an additional feeding experiment was performed to clarify the influence of hypercholesterolemia on the disease course in TME. For this purpose one group of animals was fed a conventional mouse diet, the other group received a high-fat and high-cholesterol Paigen-diet throughout the complete period of the experiment. Subsequently, the lipid composition of liver, blood and spinal cord was assessed by high performance liquid chromatography.
Summary

In the first part of the study, a high correlation between the immunohistochemically evaluated OPC-density and the transcriptional change in the JAK-STAT signaling pathway was observed by using microarray technology. Immunohistologically an increased amount of STAT3-expressing astrocytes, OPCs and microglia / macrophages was observed in TME infected animals. In subsequent cell culture experiment in both, BO-1 cells and primary OPC cultures, an increased expression of GFAP and a reduced expression of CNPase was detect, after activating the STAT3 signaling pathway by Meteorin. Inhibiting the STAT3 signaling pathway by STAT3 inhibitor VII initiated the opposite effect in BO-1 cells.

In the second part of the present study, a progressive, transcriptional down-regulation of genes involved in cholesterol biosynthesis was detected. The content of galactocerebrosides and sphingomyelins in the spinal cord was reduced in the advanced stage of TME. The dietary cholesterol supplementation had no impact on the degree and quality of inflammatory changes, de- and remyelination in the TME model. Interestingly, no negative effect of serum hypercholesterolemia was recognizable in atherosclerosis-resistant SJL/J mice.

In summary, the study shows that STAT3 signaling pathway plays a key role in the regulation of OPC differentiation. Due to the changes observed in vitro, it is likely that STAT3 promotes the differentiation of OPCs to astrocytes and is thus leading to a persistent astrogliosis. Moreover, serum hypercholesterolemia exhibits no negative effect on virus-induced, inflammatory demyelination in the CNS in this atherosclerosis-resistant mouse strain. The reported findings could indicate, that the inconclusive reports regarding hypercholesterolemia and MS maybe influenced by rather indirect pathomechanistic factors or the confounding influence of the respective genetic predisposition towards atherosclerosis.
7. Zusammenfassung

Pathogenetische Rolle der Cholesterol-Biosynthese und des STAT3-Signalweges in chronisch-demyelinisierenden Erkrankungen

Wenhui Sun


Die Ziele der vorliegenden These bestanden deshalb darin, die zugrundeliegenden Mechanismen der dysregulierten Vorläuferzell-Differenzierung zu identifizieren und die Veränderungen im Cholesterol-Stoffwechsel sowie die mögliche Beeinflussung der De- und Remyelinisierung durch dietetische Cholesterol-Supplementierung zu analysieren.

Zusammenfassung


Zusammenfassend zeigten die Untersuchungen, dass der STAT3-Signalweg eine Schlüsselrolle in der Regulation der Differenzierung oligodendroglialer Vorläuferzellen einnimmt. Durch die in vitro gezeigten Veränderungen ist es wahrscheinlich, dass STAT3 eine Differenzierung von Oligodendrozyten Vorläuferzellen zum Astrozyten begünstigt und somit die für MS kennzeichnende Astrogliose hervorruft.

Des Weiteren, stellt die Beobachtung, dass der Serum-Cholesterolspiegel keinen Einfluss auf den Krankheitsverlauf der TME hat, eine mögliche Erklärung für die teils widersprüchlichen Aussagen bezüglich eines pathogenetischen Zusammenhangs zwischen der Hypercholesterämie und der MS dar. Die Ergebnisse dieses Fütterungsversuchs deuten
Zusammenfassung

darauf hin, dass keine primäre Beeinflussung des Krankheitsbilds durch fettreiche Diät besteht. Der Eindruck eines pathogenetischen Zusammenhangs entstand wahrscheinlich durch die hohe Sensibilität der Menschen zur Atherosklerose.
8. References


References


months in interferon-beta treated patients following the first demyelinating event. J Neurol Neurosurg Psychiatry 84: 1186-1191.


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