Salt-dependent chemotaxis of macrophages

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Erklärung

Diese Dissertation wurde im Sinne von § 4 der Promotionsordnung vom 03.06.2011 im Zeitraum November 2009 bis Januar 2014 von PD Dr. R. Hemmersbach und Prof. Dr. W. Kolanus betreut und am Deutschen Zentrumfür Luft- und Raumfahrt angefertigt.

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht (gemäß § 6 der Promotionsordnung vom 03.06.2011). Weiterhin erkläre ich, die vorliegende Arbeit an keiner weiteren Hochschule als Dissertation eingereicht zu haben.
Einige Experimente wurden in Kooperation mit anderen Personen durchgeführt, dies ist an den entsprechenden Stellen vermerkt.

Bonn, den

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“To boldly go…”

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1 Introduction

1.1 New perspectives in salt and fluid balance

In the 19th century, the French physiologist Claude Bernard (1878) introduced the physiological concept of homeostasis. He described the importance of maintaining the internal environment ("*milieu intérieur*") in a steady state of nearly constant conditions. In contrast to the fluid contained inside the cells, about one third of the body fluid is present as extracellular fluid in the internal environment.

Salt and fluid homeostasis is achieved by keeping the concentration of osmotically active electrolytes within narrow ranges (Cannon 1929, 1932). In the extracellular space, sodium is the predominating ion and acts together with its accompanying anions to hold water (Walser 1992). Conversely, potassium has the equivalent role in the intracellular space. This particular distribution is maintained by the Na^+/K^+-ATPase pump which moves sodium and potassium ions across the plasma membrane and thus controls cellular volume. Electrolyte imbalances disturb the extracellular fluid volume and affect circulation and blood pressure, for instance resulting in edema (Walser 1992; Katzarski et al. 2003; Guyton and Hall 2010).

The fundamental physiological concept in extracellular volume regulation is the two fluid compartment model: Intravascular and interstitial space equilibrate rapidly to keep body fluid volume and electrolytes within narrow limits. For sodium, the general accepted view is that sodium is mainly present in the extracellular space (140 mM) and that an accumulation of sodium will inevitably result in a simultaneous accumulation of water in order to maintain iso-osmolality and isotonicity in the body (Strauss et al. 1958; Rose 1994). According to this notion, there is no notable difference between sodium concentration in the blood and in the interstitium (Pitts 1974). Also, the kidney is thought to be the main organ to regulate these constant levels of sodium and water in the blood and thus in the extracellular space (Smith 1953).

These fundamental principles were challenged by an experiment performed with an astronaut on board the MIR space station, which showed that sodium can be stored in the skin without accompanying water retention (Gerzer and Heer 2005). Subsequent long-term studies investigating human sodium and water balance confirmed the concept of osmotically inactive sodium storage in the skin (Heer et al. 2000; Titze et al. 2002; Palacios et al. 2004; Rakova et al. 2013). Earlier studies in animals revealed that due to a higher interstitial sodium concentration compared to plasma, a local hypertonic environment exists in the skin

interstitium (Haljamae 1970; Haljamae et al. 1974; Haljamae 1978) and in lymph organs (Szabo and Magyar 1982). When rats were fed a high salt diet, their sodium concentration in the skin increased, while plasma levels remained constant (Titze et al. 2003; Titze et al. 2005; Schafflhuber et al. 2007; Machnik et al. 2009). In addition, no simultaneous increase in water could be found in these studies, which prompted the authors to conclude that contrary to the widely accepted notion, there is no fast compensation between interstitial and plasma sodium levels. Instead, excess sodium eludes excretion through the kidney by accumulating in the skin interstitium. This sodium reservoir can be visualized by ^{23}Na magnetic resonance imaging (Kopp et al. 2012). MRI scans revealed elevated sodium content in the skin of salt-sensitive hypertensive patients, as well as a general increase with age and in men as compared with women. Interestingly, age-dependent water-free sodium storage in muscle tissue was found in men with increasing age, but not in women (Kopp et al. 2012; 2013).

Recently, a long-term study that simulated a space flight to Mars (Mars 500) allowed a unique possibility to investigate sodium metabolism following constant salt intake in controlled conditions for 105 and 250 days. In this study, Rakova et al. (2013) found that sodium is rhythmically stored and released in the interstitium completely independent from salt intake. Under constant salt intake, a weekly rhythm of urinary sodium excretion emerged. This circaseptan variability in sodium excretion was also found earlier in rats under a constant high salt diet (Uezono et al. 1987). Although the underlying mechanism is not completely resolved, an inverse relationship of free cortisol and aldosterone levels indicates hormonal control: Sodium excretion was paralleled by elevated free cortisol (possibly due to sodium-sensitive changes in 11β-HSD2 activity) and was inversely correlated to aldosterone levels. Furthermore, the variability in total body Na^+ was reflected by a several weeks long (infradian) rhythm that was not accompanied by changes in water content or blood pressure. However, the systolic blood pressure in the test subjects significantly decreased 3-4 mmHg over several weeks when the salt intake was reduced from 12 g to 6 g NaCl per day. These findings are inconsistent with the view that sodium levels in the body are kept at narrow limits by rapid urinary excretion.

How is sodium stored without water retention in the skin? Experiments with rats showed that changes in glycosaminoglycan metabolism enable osmotically-inactive sodium storage in the skin interstitium (Titze et al. 2004; Schafflhuber et al. 2007). Glycosaminoglycans are negatively charged polyanions, which comprise of repeating disaccharide units (uronic acids and amino sugars). The negative charge density and therefore cation binding capacity correlates with the degree of sulfatation, as found by Volpi (1999). The fact that glycosaminoglycans, for instance chondroitin sulfate, are able to bind sodium was observed more than 50 years ago by Farber et al. (1957) and Farber (1960). In cartilage,

glycosaminoglycans locally increase sodium concentration to 250 – 350 mmol/l which is beyond the mean sodium concentration in the serum of 142 mmol/l (Mobasheri et al. 1997; Mobasheri 1998; Silbernagl and Despopoulos 2012). Similarly, sodium concentration in the skin of rats rose from 140 mM to 190 mM without a simultaneous increase in skin water content following a high salt diet (Titze et al. 2004). The authors found that gene expression of enzymes involved in glycosaminoglycan chain elongation like chondroitin synthase were increased after 8 weeks of high salt diet compared to low salt diet animals. According to Titze et al. (2004), glycosaminoglycan content and polymerization were significantly elevated. Schafflhuber et al. (2007) further showed that dietary salt intake is directly linked with the polyanionic character of the extracellular matrix due to polymerization and sulfatation of glycosaminoglycans, providing additional binding capacity for sodium.

Taken together, novel evidence suggests that the classical concept of a tightly-regulated sodium metabolism resulting in iso-osmolality in the two compartment model is not adequate. The skin acts as a sodium reservoir and thus creates a local hypertonic environment, which is not readily equilibrated. Instead, macrophage-driven lymphangiogenesis which is mediated by vascular endothelial growth factor C (VEGF-C) secretion is responsible for the removal of osmotically inactive stored salt (Machnik et al. 2009). Consequently, osmolality in the lymphatic system exceeds serum osmolality (Szabo et al. 1982), indicating that the interstitium including the lymph capillary network acts as a third separate and locally regulated compartment.

1.2 A new role for macrophages in salt balance regulation

1.2.1 Macrophages and chemotaxis in the immune system

Together with dendritic cells, blood-circulating monocytes and bone marrow precursor cells, macrophages form the mononuclear phagocytotic system and are present in every peripheral tissue of the body (van Furth 1982; Wiktor-Jedrzejczak and Gordon 1996). Macrophages are derived from common myeloid progenitor cells in the bone marrow, which give rise to blood-circulating monocytes depending on the presence of Colony Stimulating Factor (CSF) (Hume 2006, 2008; Geissmann et al. 2010). Continuous extravasation establishes tissue-specific subpopulations of macrophages, including skin Langerhans cells, lung alveolar macrophages, microglia in the brain, or serosal peritoneal macrophages (Gordon and Taylor 2005).

Macrophages play important roles in both innate and adaptive immune responses (Medzhitov and Janeway 2000; Murray and Wynn 2011). One major function of macrophages is to monitor their environment for invading pathogens like bacteria or viruses and consequent elimination by phagocytosis. In addition, phagocytosing macrophages are essential for the clearance of apoptotic cells and cell debris (Elliott and Ravichandran 2010).
Foreign antigens are identified and internalized by pattern-recognition receptors on the cell surface, including scavenger receptors and Toll-like receptors (Medzhitov et al. 1997; Kawai and Akira 2011). In order to promote an adequate immune response, antigens are processed in the phagosome and presented to other immune cells like antigen-specific T lymphoctyes by major histocompatibility complex (MHC) molecules (Ramachandra et al. 2009). Furthermore, activated macrophages modulate immune responses by expression of cytokines like TNF-α, IL-6 and IFN-γ, and costimulatory molecules, respectively (Sieff et al. 1998; Dale et al. 2008).
Macrophages consist of several subclasses. Additionally to classically activated M1 macrophages functional subsets like anti-inflammatory M2 (Mantovani et al. 2002; Gordon 2003; Mosser 2003; Mantovani et al. 2004), “regulatory” macrophages (Sutterwala et al. 1997, 1998), and tumor-associated macrophages (Mosser and Edwards 2008) have been described. It seems likely that even more polarized macrophage types will be classified in the future.
During acute infections, circulating monocytes are recruited from the bloodstream into inflamed tissues by chemokines like CCL2 (Serbina et al. 2009; Shi et al. 2011). Specifically, monocytes expressing the chemokine receptor CCR2 and high levels of the myeloid marker lymphocyte antigen 6C ($LYC6^{hi}$) follow a CCL2 gradient and extravasate into the infected tissue, where they develop into inflammatory macrophages (Geissmann et al. 2010; Kim et al. 2011). This directional migration response in a gradient of chemotactic molecules is termed chemotaxis, in contrast to chemokinesis, which is involves stimulated, but random, cell movement toward a chemical stimulus (Becker 1977). Monocytes and macrophages recognize to a variety of extracellular chemotactic signals, including bacterial and viral components, chemokines, cytokines and inflammatory mediators (Foxman et al. 1999; Turner et al. 1999; Moser et al. 2004).

1.2.2 Chemotaxis and cell migration are promoted by Rho Family GTPases

The processes underlying cell migration can be described by a five step cycle (Lauffenburger and Horwitz 1996; Rafelski and Theriot 2004; Parsons et al. 2010): First, the cell polarizes

into the direction of movement, where reorganization of the actin cytoskeleton forms a protrusion called lamellipodium. Subsequently, the protrusion is adhered to the surrounding extracelluar matrix proteins by cell-surface intregrins. The next step is the forward movement of the cell body due to actomyosin contraction, which is followed by loosing adhesion at the rear of the cell.

Macrophages form a large number of weak adhesions, which are not classical stress-fiber associated dynamic adhesions (Pixley 2012). Normally, strong focal adhesions link contractile actin and myosin-II-containing filaments to extracellular matrix proteins by integrins (Critchley et al. 1999). Instead, macrophages seem to gain increased dynamic adhesion by a multitude of point contacts and focal complexes, providing a higher traction force and responsiveness during cell migration (Beningo et al. 2001; Gallant et al. 2005). In addition, macrophages form podosomes during migration, which consist of a dense F-actin array enclosed by adhesion proteins (Linder and Aepfelbacher 2003). During interstitial migration, podosomes are sites of extracellular matrix degradation (Evans et al. 2003; Murphy and Courtneidge 2011), as has also been shown in human primary macrophages by Van Goethem et al. (2011).

Cell motility highly depends on rapid reorganization of the actin cytoskeleton. Therefore, chemotactic signaling intracellularly converges on pathways that modulate the actin cytoskeleton. The majority of the chemoattractive signals are recognized by seven transmembrane spanning G-protein coupled receptors (GPCRs) and transduced by heterotrimeric G proteins (Boulay et al. 1997). Experiments with pertussis toxin revealed that these GPCRs are associated with Gαi, but also G12/13 and the Gβγ-subunit are important mediators (Xu et al. 2003). In addition, chemoattractants interact with receptor tyrosin kinases (RTKs) (Weiss et al. 1997).

Eventually, signaling pathways converge on Phosphatidylinositide 3-kinase (PI3-K) and the subsequent activation of small GTPases of the Rho family. PI3-K is responsible for production of phosphatidylinositol 3-phosphate (PIP3), which interacts with a variety of other effector proteins including protein kinases, guanine nucleotide-exchange factors (GEFs) and GTPase-activating factors (GAPs) (Shaw 1996; Bottomley et al. 1998). GEFs and GAPs in turn are essential regulators of the small GTPases of the Rho family, which are also activated by chemokine and cytokine receptors and control polymerization of actin (Machesky and Insall 1999). Furthermore, these small GTPases of the Rho family might be directly activated by PI3-K and following *src*-related kinase Lyn activity (Benard et al. 1999; Jones 2000).

Figure 1-1 shows a graphical summary of known RhoGTPases, their downstream mediators and actin nucleators involved in the actin cytoskeleton response. In macrophages, actin

polymerization is mediated by RhoA, RhoB, Rac1, Rac2 and Cdc42 (Ridley 2008). RhoA, Rac1 and Cdc42 were found in the leading edge of migrating cells, allowing a spatiotemporal integration of chemotactic signals (Machacek et al. 2009; Pertz 2010).

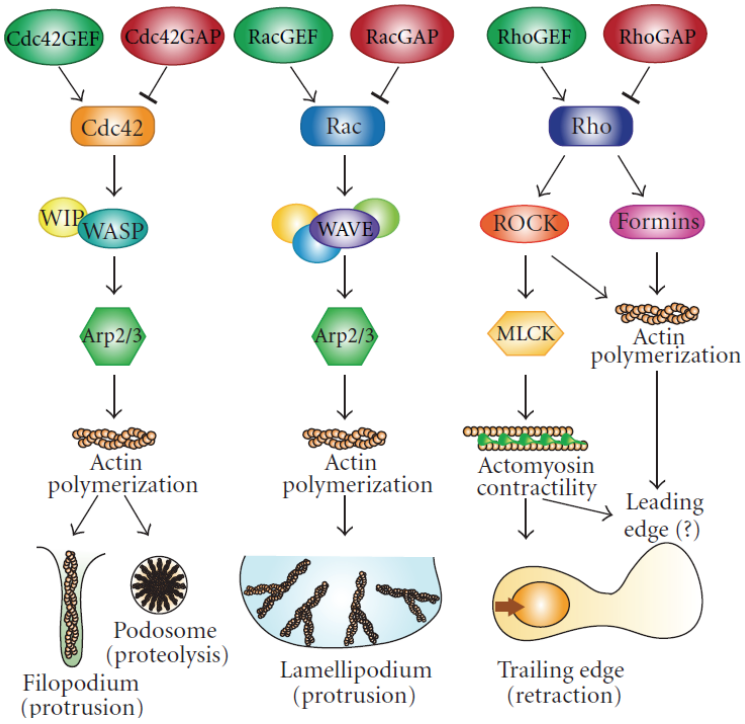


Figure 1-1: Downstream signaling events in cell migration converge on different Rho GTPase-dependent actin cytoskeleton responses

RhoGTPases Cdc42, Rac and Rho are regulated by GEF and GAP activity and can also be directly activated by receptor signaling following interaction with chemoattractive signals. The mediator complexes WASP and WAVE initiate actin polymerization by activating the actin nucleator complex Arp2/3, resulting in differentiated formation of filopodia, podosomes, or lamellipodia. In addition, the effects of Rho on actomyosin contractility are transduced by ROCK and MLCK. (from Pixley 2012).

Activation of Cdc42 leads to podosome and filopodia formation, while Rac promotes lamellipodia spreading (Allen et al. 1997). In addition, Cdc42 is important for macrophage polarization and chemotactic sensing (Allen et al. 1998). It has been shown that the effects of Cdc42 on actin polymerization and chemotaxis are mediated by the WASP complex (Park et al. 2010). Together with its N-terminal binding protein WIP, WASP activates the actin nucleator complex Arp2/3, which is present at the leading edge and in podosomes (Tsuboi 2007; Monypenny et al. 2011). Rac signaling also results in Arp2/3 activation, but uses the downstream effector WAVE2 in macrophages (Kheir et al. 2005; Takenawa and Suetsugu

2007). The Arp2/3 complex binds to sides of existing actin filaments, where it promotes branched polymerization and cross-linking of the actin cytoskeleton (Mullins et al. 1998; Mullins and Pollard 1999; Takenawa and Suetsugu 2007). Linear growth of actin filaments seems to be initiated by Rho-dependent activation of the formin family of actin nucleators (Goode and Eck 2007). In addition, Rho triggers Rho-kinase (ROCK) 1 and 2, which together with Ca^{2+} phosphorylate myosin-light-chain kinase (MLCK) to increase actomyosin contractility and retraction of the trailing edge (Riento and Ridley 2003; Vemula et al. 2010). Furthermore, the same small GTPases are regulators of cell adhesion during migration: Rho and Cdc42 mediate integrin-based focal complex formation, while for instance phosphorylated paxilin in adhesions also recruits Rac downstream effectors to initiate lamellipodium formation (Zaidel-Bar et al. 2007; Vicente-Manzanares et al. 2009).

However, it would be an oversimplification to consider these pathways as completely independent. On the one hand, there is a crosstalk between them: For instance, Cdc42 can trigger Rac-dependent lamellipodium formation, which in turn increases stress fibers mediated by Rho (Ridley and Hall 1992) . On the other hand, these signaling pathways are further influenced by several other pathways before integration converges on the actin cytoskeleton response.

1.2.3 Tissue macrophages control salt balance, interstitial volume and blood pressure

In addition to their prominent functions in the immune system, evidence over the last decade emerged how macrophages are responsible for interstitial volume and blood pressure homeostasis. A correlation between macrophage-derived growth factors and lymphangiogenesis was described previously in association with an inflammatory immune response. Schoppmann et al. (2002) identified tumor-associated macrophages as the major source of lymph vessel growth-promoting VEGF-C and VEGF-D in human cervical cancer samples. A direct contribution of macrophages was shown by Maruyama et al. (2005) in a mouse corneal transplant model: In addition to secretion of VEGF-C, macrophages transdifferentiated into lymphatic endothelial cells which integrated into existing lymph vessels.

When Machnik et al. (2009) investigated the lymph capillary system following a high-salt diet in rodents, they found that the local hypertonic sodium storage in the skin induced lymphangiogenesis. The authors could show that the lymph vessel proliferation depends on tissue-infiltrating macrophages, but is not accompanied by an inflammatory response. Further analysis of the infiltrated macrophages revealed that the signal of osmotic stress

caused by the hypertonic salt-storage environment is translated into an increased tonicity-responsive enhancer binding protein (TonEBP) expression in these cells. TonEBP (also termed NFAT5, nuclear factor of activated T-cells 5) is the only known mammalian transcription factor directly related to osmotic stress and protects cells by inducing expression of osmoprotective genes, like heat-shock proteins and betaine transporter (Takenaka et al. 1994; Miyakawa et al. 1999). In macrophages, TonEBP additionally binds to two sites of the *Vegfc* promoter and thus regulates vascular endothelial growth-factor C (VEGF-C) expression (Machnik et al. 2009). VEGF-C release by macrophages resulted in a local lymphangiogenesis of preexisting lymph capillaries by binding VEGFR3 receptors. In addition, VEGF-C increased interstitial endothelial nitric oxide synthase (eNOS) expression via activation of VEGFR2 receptors. The subsequent release of nitric oxide is thought to compensate blood pressure following a dietary high salt loading (Tolins and Shultz 1994; Leonard et al. 2006; Kang et al. 2007).

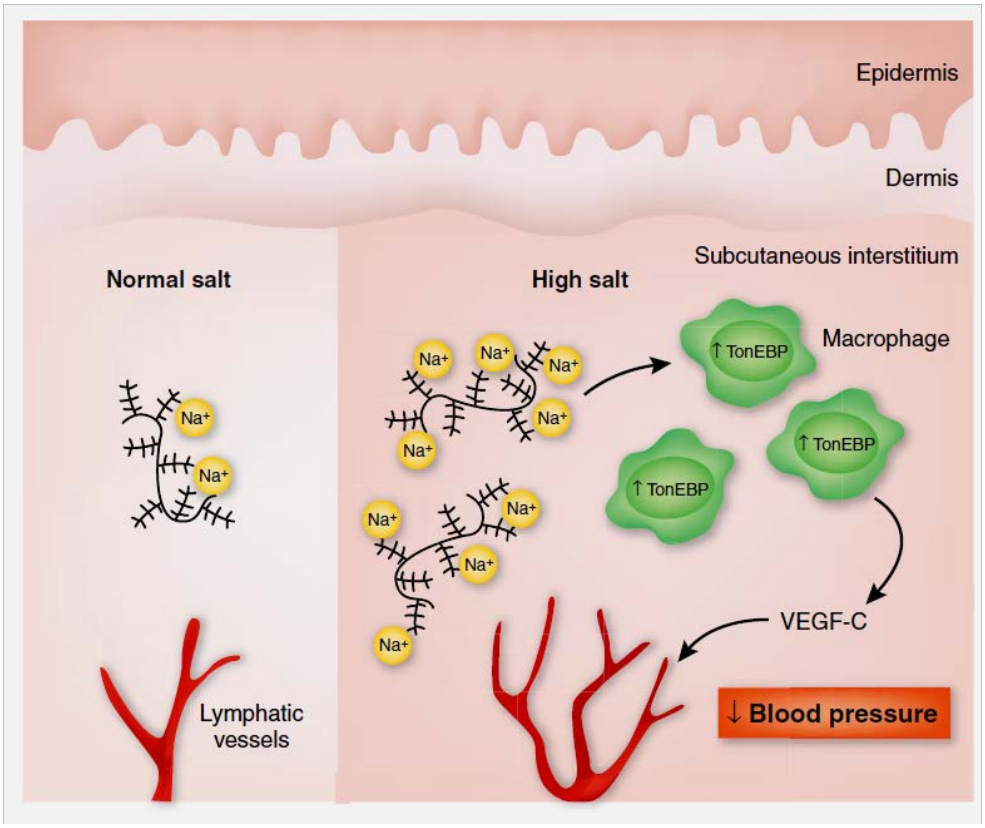


Figure 1-2: Homeostatic role of tissue macrophages

Following high salt intake, excess Na^+ is bound to proteoglycans in the skin interstitium in an osmotically inactive state. Macrophages accumulate in areas of hypertonic sodium storage and express TonEBP, which induces VEGF-C production. Secreted VEGF-C stimulates lymphangiogenesis in order to remove excess sodium and to decrease blood pressure, counteracting the development of salt-sensitive hypertension (Marvar et al. 2009).

Machnik et al. (2009) proposed that TonEBP-induced secretion of VEGF-C from macrophages modifies lymph capillary density and thus augments lymphatic drainage to remove excess sodium from the interstitium (see Figure 1-2). When signaling pathways were abrogated by VEGF receptor inhibition and depletion of macrophages, the interstitial electrolyte concentrations and fluid volume increased. Furthermore, the mean arterial blood pressure rose significantly, suggesting that this system buffers the development of salt-sensitive hypertension. These experiments documented a homeostatic function of macrophages in the skin interstitium (Machnik et al. 2009; Machnik et al. 2010). However, in contrast to earlier reports of a stimulation of macrophages by osmotic stress (Lang et al. 2002), these authors found no sign of an inflammatory response, as was indicated by unchanged TNF-α expression levels in the skin and serum following the high salt diet.

The newly discovered TonEBP/VEGF-C mediated regulation mechanism was termed extrarenal, since it does not directly involve blood pressure or volume regulation by the kidney. Instead, macrophages were recognized to control local tissue environment and blood pressure in the skin interstitium by driving lymphatic vessel hyperplasia and increasing interstitital eNOS expression (see Figure 1-2). Failures in this regulatory axis are associated with the development of salt-sensitive hypertension in both rodents (Machnik et al. 2009; Machnik et al. 2010) and humans, as elevated VEGF-C serum levels in hypertensive patients (Machnik et al. 2009) or following a high-salt diet (Slagman et al. 2012) were reported. These results emphasize clinical importance of a homeostatic macrophage function, which might be beneficial for prospective treatment of hypertension.

1.3 Hypertension poses a threat to global public health

Hypertension is a chronic disease defined by a systolic blood pressure exceeding 140 mmHg and/or diastolic blood pressure above 90 mmHg, compared to normal 120 mmHg and 80 mmHg, respectively. Since elevated blood pressure does not cause any specific symptoms apart from headaches or heart palpitations, it often remains undiagnosed until damages to blood vessels lead to severe conditions like stroke or heart disease. Hypertension is characterized by a rise in total peripheral resistance in the arteries, which is probably due to decreased elasticity and diameter of arterioles (Folkow 1982; Torres et al. 2013) as well as changes in the microcirculation of the capillaries (Struijker Boudier et al. 1992; Jung et al. 2013) and also normally occurs with age. The underlying mechanism might

involve a chronic activation of the sympathetic nervous system (Esler 2010; Parati and Esler 2012) or abnormal activation of the renin-angiotensin system (Navar 2010). In addition, the contribution of a multitude of factors including a proinflammatory role for Angiotensin II (Marchesi et al. 2008) or a dysbalance between nitric oxide (NO) and endothelium-derived contracting factors (Versari et al. 2009) is discussed. However, in more than 95 % of hypertensive patients, the primary cause for the high blood pressure is unknown (Harrison et al. 2005). A genetic predisposition is very likely, but is not suffiecient for manifestation of the disease. Instead, behavioral risk factors including an unhealthy diet containing high salt and fat, alcohol and tobacco abuse, physical inactivity and persistent stress, or metabolic factors like diabetes and obesity play an important role (World Health Organization report "A global brief on hypertension" , WHO 2013).

According to the WHO report “Causes of death” (2008), hypertension accounts for more than 51 % of deaths due to stroke and at least 45 % of deaths due to heart disease. Because it is responsible for the estimated deaths of 9.4 million people every year, the WHO refers to high blood pressure as a “silent killer”. When hypertension is not diagnosed and treated early on, it is a major risk factor for cardiovascular disease (CVD), stroke and kidney failure, which are leading causes for mortality worldwide (WHO report “Global health risks”, 2009). The increasing incidence of hypertension, which is attributed to population growth, ageing and lifestyle risk factors, also poses a huge economic burden. Costs for treatment and its associated complications like cardiac bypass surgery or dialysis have to be accounted for. By the year 2008, about one billion people suffered from hypertension (WHO report “Global status report on noncommunicable diseases”, 2010), creating immense healthcare costs with approximately 76.6 $ billion per year in the US alone (Lloyd-Jones et al. 2009, 2010).

Referring to the WHO report “A global brief on hypertension” (2013), about 40 % of the worldwide population over age 25 are diagnosed with hypertension, but more than 80 % of hypertension-related deaths occur in middle- and low-income countries. High blood pressure either remains undiagnosed or uncontrolled because of extremely high health care costs in these countries. In contrast, early detection and treatment as well as raised awareness have declined the mortality due to hypertension in high-income countries. However, the WHO predicts that mortality due to CVD will increase by at least 5 % until 2030 (WHO report “A global brief on hypertension”, 2013). On the economic level, the losses due to CVD will exceed public health spending by the year 2025. These dramatic expectations make it clear that huge efforts have to be undertaken worldwide to counteract this development.

1.4 The influence of sodium intake on blood pressure

The hypothesis of a connection between salt intake and blood pressure was first proposed in 1904 (Ambard and Beauchard 1904). However, their findings were quickly questioned (Löwenstein 1907) and from then onward the debate concerning a potential effect of high salt intake on hypertension has been going on for decades (Graudal 2005). Early findings already led to a recommendation of salt-reduction for treatment of hypertensive patients in 1925 (Allen 1925). On the other hand, by this time there were no anti-hypertensive drugs available for treatment like diuretics, beta blockers or ACE-inhibitors. However, Allen´s theory remained disputed and was almost forgotten in the following years until 1948. At that time, the idea of salt reduction was reinforced by Kempner (1948) by successfully treating hypertensive patients with a strict low-salt rice diet (0.3 g salt/day). In the 1960s, Lewis Dahl was the first to realize a positive correlation between salt intake of different populations worldwide and the prevalence of hypertension among different cultures (Dahl 1960).

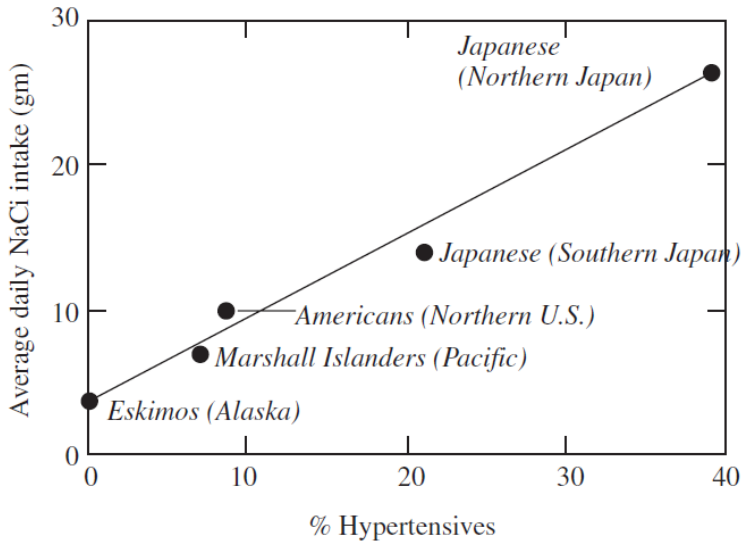


Figure 1-3: Correlation of average daily salt (NaCl) intakes with prevalence of hypertension in different geographic areas and among different human populations (from Dahl, 1960).

Figure 1-3 from Dahl (1960) shows how mean salt intake ranges from 4 g/day (68 mmol sodium) in the Inuit population to an average of up to 27 g (462 mmol) in Japanese, which was mainly attributed to extensive seasoning with soy sauce in this culture. These epidemiological studies in more than 20 different populations were continued by

Gleibermann (1973) and (Froment et al. 1979). Subsequently, internationally cooperated INTERSALT (1985-87; Pietinen et al. (1988)) and INTERMAP (1996-1999; Stamler et al. (2003)) studies estimated sodium intake worldwide by standardized 24-hour sodium urinary excretion. INTERSALT (Pietinen et al. 1988; Rose and Stamler 1989; Elliott et al. 1996) collected data from more than 10,000 individuals in 52 populations and 32 countries, making it the most extensive study until today. It found the lowest sodium consumption in native Indian populations of Brazil, Papua New Guinea and Kenya, with a mean sodium intake below 50 mmol/day (2.9 g salt).

Conversely, the highest sodium intake was again found in Asia with the People's Republic of China and Japan consuming 11.8 – 15.2 g salt/day (200-259 mmol). In general, sodium intake was higher in men (150-199 mmol/day) than women (100-149 mmol/day) and increased with age (Brown et al. 2009). The INTERSALT study calculated a mean urinary sodium excretion of 170 mmol (9.9 g NaCl) per day, which is nearly twice the amount recommended by the current WHO guideline of 90 mmol (5 g NaCl) per day to prevent CVD (WHO report "Prevention of cardiovascular disease: guidelines for assessment and management of cardiovascular risk", 2007).

Animal studies reported that a chronic high salt diet induces hypertension in rats and primates (Dahl, 1960, Denton et al. 1995; Elliott et al. 2007). In their experiments, Dahl et al. (1962) observed that not all rats developed hypertension following a high salt diet and therefore proposed the involvement of genetic factors. Breeding salt-sensitive hypertensive rats (so-called ´Dahl rats´) led to an animal strain that is still used today to find hypertension-responsive genes and genetic markers of salt sensitivity. Concerning humans, after many years of clinical studies there is a broad agreement that a reduction in salt intake leads to a beneficial decrease in systolic blood pressure of about 4 mmHg, while diastolic blood pressure is reduced about 2 mmHg in hypertensive patients (Cutler et al. 1997; Graudal et al. 1998). In addition, even healthy subjects with a normal blood pressure show a decrease of at least 1 mmHg for both systolic and diastolic values. The recently issued WHO review ("Effect of reduced sodium intake on blood pressure, renal function, blood lipids and other potential adverse effects", 2013) summarized 35 studies that investigated dietary effects of salt intake on blood pressure and other health parameters (until August 2011). The report found that overall systolic blood pressure was reduced 4-5 mmHg, whereas diastolic blood pressure decreased 1-2 mmHg in individuals with hypertension. In line with earlier studies, the decrease was lower in normal healthy individuals (1-2 mmHg reduction). Although the effect of reduced salt intake was not always statistically significant, the overall tendency of a blood pressure reduction cannot be ignored (He and MacGregor 2004, 2008). Since hypertension increases the risk of CVD, studies also analyzed the long-term effects of salt-reduction on

the incidence of CVD. Cook et al. (2007, 2008, 2009) found a linear direct effect of reduced sodium intake on CVD, with a significant 25% lower risk after 10 – 15 years.

However, a reduced salt diet does not necessarily lead to a significant decrease in blood pressure in all individuals, and all the factors influencing salt-sensitive hypertension are not completely resolved. For instance, a genetic component for salt-sensitivity as known in animal models is discussed (Beeks et al. 2004; Sanada et al. 2011). Furthermore, various parameters including other food components such as potassium and calcium might have a potentially greater influence on blood pressure development than sodium alone (He and MacGregor 2008; Cook et al. 2009). This raises the question whether the effect of salt reduction is negligible compared to other dietary impacts (Appel et al. 1997; WHO report "Effect of reduced sodium intake on blood pressure, renal function, blood lipids and other potential adverse effects", 2013). For instance, a study by Chang et al. (2006) using potassium-enriched salt as a substitute for sodium chloride described a 41 % reduction in CVD mortality. On the other hand, excess sodium intake has deleterious effects on the heart, kidneys and blood vessels completely independent of its effects on blood pressure (Frohlich 2007).

The high amount of sodium that is consumed especially in processed or restaurant-prepared food in the developed countries dramatically exceeds the 10 – 20 mmol/day our body physiologically needs (Brown et al. 2009; Table 1-1). As Table 1-1 shows, the worldwide daily sodium intake is well above the limits that are recommended by health organizations like the WHO and the American Heart Association. However, the question is to which level the amount of sodium intake needs to be reduced and if it is implementable. Since most of the consumed sodium is hidden in processed foods, a population-wide decrease in sodium intake is only possible in cooperation with the food industry. In addition, consumer willingness and inconvenience to get accustomed to low sodium levels has to be overcome.

In summary, a multitude of studies over several decades suggest a beneficial effect of salt reduction on blood pressure and risk for CVD. Growing evidence that a high salt intake will lead to increasing health and economic problems throughout the world emphasizes the need for an individual awareness of salt intake in the population.

Table 1-1: Mean amount of sodium intake recommended and consumed in selected countries worldwide according to INTERSALT/INTERMAP study

Country	Sodium intake [mmol/d]	Corresponding NaCl intake [g/day]
Physiologically needed	10 – 20	0.6 -1.2
American Heart Association	< 64	3.8
Tanzania	≈ 69	4.1
Cameroon	≈ 71	4.2
Venezuela	≈ 77	4.5
WHO	90	5.3
Spain	≈ 93	5.5
EU mean*	≈ 136 – 204	8 -12
Canada	≈ 135	8
Germany	≈ 139 ≈ 107*	8.2 6.3
Australia	≈ 144	8.5
USA	≈ 163	9.6
Brazil	≈ 171	10
Japan	≈ 189	11.1
China	≈ 272	16

from Brown et al. (2009) and *European Union salt-reduction framework (2012)

1.5 Aims of this study

In addition to their established roles in immune defense, macrophages were recently found to regulate interstitial salt and fluid balance, as well as blood pressure (Machnik et al. 2009). The authors of that study found an accumulation of macrophages in the skin interstitium of rats that were fed a high salt (NaCl) diet. These results raised questions about a possible chemotactic response of macrophages toward a hypertonic salt environment, which would be present in the skin interstitium due to excess stored Na^+ (Titze et al. 2004; Schafflhuber et al. 2007). Therefore, the study at hand investigated and characterized the novel concept of salt-dependent chemotaxis of macrophages by means of *in vitro* transwell migration assays.

Migration of RAW264.7 macrophages, peritoneal macrophages and bone marrow-derived macrophages in a hypertonic NaCl environment was analyzed to confirm specificity of the chemotactic response toward NaCl. In order to unravel the responsible molecular mechanism for salt-dependent chemotaxis, the effects of excess NaCl on cell morphology and migration machinery like the actin cytoskeleton were investigated. In addition, a potential NaCl-dependent chemokine expression was studied as previous studies demonstrated for instance for CCL-2 (Kojima et al. 2010). Since the osmoprotective transcription factor TonEBP is activated during osmotic stress, the present study aimed at elucidating the involvement of TonEBP in salt-dependent chemotaxis.

Such investigations should give further insight into the development of salt-sensitive hypertension and might lead to prospective new drug targets for hypertension therapy.

2 Material and Methods

2.1 Material

2.1.1 Laboratory equipment

Centrifuges	BioFuge primo R , Fresco 21, Heraeus, Hanau
Developing machine	SRX-101A, Konica Minolta, Langenhagen
Digital heatblock	VWR, West Chester, USA
Electroporator	GenePulser Xcell, Biorad, Hercules, USA
Flame photometer	EFOX 5053, Eppendorf, Hamburg
Flow cytometer	FACS Canto II, BD Biosciences, Franklin Lakes, USA
Freezing container	Nalgene Thermo Scientific, USA
Horizontal shaker	WS-5, Edmund Bühler, Hechingen
Incubator	C60 / C200, Labotect, Göttingen Binder, Tuttlingen
Laminar flow hood for cell culture	Telstar Bio-II-A, Labotect, Göttingen
Magnetic stirrer	Roth, Karlsruhe
Microliter centrifuge	Hermle Labortechnik, Wehingen
Microplate reader GloMaxMulti+	Promega, Madison, USA
Microscopes (inverted)	Nikon ECLIPSE TE-2000E, Nikon, Tokyo, Japan Nikon ECLIPSE i80, Nikon, Tokyo, Japan Confocal laser-scanning FV-1000, Olympus, Tokyo, Japan Axiovert 10, Zeiss, Jena
Neubauer hemocytometer	Roth, Karlsruhe
Osmometer	Vogel Löser Messtechnik, Gießen
pH-meter	Schott Instruments, Mainz
Pipettes	Eppendorf, Hamburg Gilson, Middleton, USA
Shaker KL2	Bühler Laborgeräte, Tübingen

Roll mixer	VWR, West Chester, USA
Vortex	Vortex Genie, Roth, Karlsruhe
Water bath	Köttermann Labortechnik, Germany
Wet Blot System (Mini-Trans Blot Cell)	Biorad, Hercules, USA

2.1.2 Consumables

Cell culture dishes 60 mm^2	Nunc, Penfield, USA
Cell culture plates (6-well / 24-well)	Corning Costar Corporation, New York, USA
Cell culture flasks (vented)	BD Falcon, Franklin Lakes, USA
Cell scraper	Sarstedt, Nümbrecht
Cryovials	Nunc, Penfield, USA
Electroporation cuvettes (2 mm / 4 mm)	Biorad, Hercules, USA
FACS tubes	Sarstedt, Nümbrecht
Falcon tubes	Greiner Bio-one, Frickenhausen
Filter paper	Whatman Nr. 4, Schleicher & Schuell Bioscience, Dassel
Lab-Tek chamber slides	Nunc, Penfield, USA
Membrane inserts, 8 µm pore filter	BD Biosciences, Franklin Lakes ,USA
Membrane inserts, 5 µm pore filter	Corning Costar Corporation, New York, USA
Microtiter plates (96-well, clear, flat bottom)	Roth, Karslruhe Greiner Bio-one, Frickenhausen
Nylon cell strainers (40 µm)	BD Biosciences, Franklin Lakes, USA
reaction tubes 0.5 – 2 ml	Eppendorf, Hamburg and Starlab, Hamburg
Pasteur pipettes	Roth, Karlsruhe
Pipette tips (10 µl, 200µl, 1000µl)	Roth, Karlsruhe and Eppendorf, Hamburg
PVDF (polyvinylidene difluoride) membrane	Biorad, Hercules, USA
Serological pipettes	VWR, West Chester, USA
Sterile filters (0.2 µm)	Schleicher & Schuell Bioscience, Dassel
Syringes (1 ml, 5 ml)	B.Braun, Melsungen
X-ray film	Amersham Hyperfilm MP, GE Healthcare, Pittsburgh, USA

2.1.3 Chemicals and biochemical reagents

Acetic acid	Roth, Karlsruhe
Acrylamid/Bisacrylamid-Mix 30%	Roth, Karslruhe
Ammonium persulfate (APS)	Roth, Karlsruhe
Autoradiography film Cleaner D	Fujifilm, Tokyo, Japan
Bovine serum albumin (BSA)	Roth, Karlsruhe
Bromphenol blue	Roth, Karlsruhe
Complete Protease Inhibitor Cocktail Tablets	Roche Applied Science, Mannheim
Coomassie Brillant Blue	Sigma-Aldrich, Taufkirchen
DABCO (1,4-Diazabicyclo[2.2.2]octane)	Roth, Karlsruhe
Dimethyl sulfoxide (DMSO)	Roth, Karlsruhe
Dithiothreitol (DTT)	Roth, Karlsruhe
EDTA (ethylene diamine tetraacetic acid)	Sigma-Aldrich, Taufkirchen
Ethanol	Roth, Karlsruhe
Fibronectin	Harbor Bio-Products Norwood, USA
FluoroShield	ImmunoBioscience Corp., Mukilteo, USA
FuGENE HD transfection reagent	Roche Applied Science, Mannheim
Hydrochloric acid	Roth, Karlsruhe
Isopropanol	Merck, Darmstadt
L-glutamine	PAA, Cölbe
Lipofectamin 2000 Transfection Reagent	Invitrogen, Carlsbad, USA
Lipopolysaccharide (LPS) *E.coli*	Sigma-Aldrich, Taufkirchen
Liquichek urine chemistry control	Biorad, Hercules, USA
Mannitol	Sigma-Aldrich, Taufkirchen
Methanol	Roth, Karlsruhe
Milk powder	Roth, Karlsruhe
N-(1-naphthyl)ethylenediamine (NED)	Roth, Karlsruhe
Ortho-phosphoric acid	Roth, Karlsruhe
Oligofectamine transfection reagent	Invitrogen, Carlsbad, USA
Osmometer standard	Löser Messtechnik, Gießen
Polymyxin B	Sigma-Aldrich, Taufkirchen
Ponceau S	Roth, Karsruhe
Paraformaldehyde (PFA)	Merck, Darmstadt
Pertussis toxin from *Bordetella pertussis*	Sigma-Aldrich, Taufkirchen

Precision Plus Protein Standard	Biorad, Hercules, USA
RotiQuant 5x	Roth, Karlsruhe,
Sodium chloride (NaCl)	Merck, Darmstadt
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe
Sodiumhydroxide	Roth, Karlsruhe
Sulfanilamide	Roth, Karlsruhe
Tetramethylethylendiamin (TEMED)	Sigma-Aldrich, Taufkirchen
Tris-[hydroxymethyl]aminomethan (Tris)	Roth, Karlsruhe
Triton-X 100	Roth, Karlsruhe
Trypan blue staining solution 0,4%	Sigma-Aldrich, Taufkirchen
Tween 20	Roth, Karlsruhe
Urea	Roth, Karlsruhe
Urine Standard	EFK-diagnostic, Magdeburg
Vybrant CFDA-SE	Invitrogen, Carlsbad, USA

2.1.4 Media, sera and buffers

Dulbecco´s Modified Eagle Medium low glucose (DMEM)	PAA, Cölbe
Fetal Calf Serum (FCS)	Biochrom, Berlin ;Sigma Aldrich, Taufkirchen
Iscove's Modified Dulbecco's Medium (IMDM)	PAA, Cölbe
Opti-MEM	Invitrogen, Carlsbad, USA
Penicillin-Streptomycin	Biochrom, Berlin
Phosphate buffered saline (PBS)	PAA, Cölbe
Trypsin/EDTA 10x (0,5% / 0,2%)	Biochrom, Berlin
Very low endotoxin-RPMI 1640 (VLE-RPMI)	Biochrom, Berlin

2.1.5 Commercial kits

Apoptotic/necrotic/healthy cells staining kit	Promokine, Heidelberg
CCL2 ELISA	R&D Duoset, Minneapolis, USA
Cell Titer-Blue Cell Viability Assay	Promega, Madison, USA
ECL Western Blotting Substrate Kit	Pierce, Rockford, USA
Leukotriene B_4 EIA kit	Cayman, Ann Arbour, USA

2.1.6 siRNA oligonucleotides

ON-TARGETplus Mouse Nfat5 siRNA:

L 058868-09	5´- GGGCAAUGAUUCUGGUCGA - 3´	Dharmacon, Chicago, USA
L 058868-10	5´- AGAGUAACUGGGCGAAAUA - 3´	“
L 058868-11	5´- CGGCAGGAGUGGAAGCGUU - 3´	“
L 058868-12	5´- CCACUCAUAUCAAGCAGUA - 3´	“

AllStars Negative siRNA #1027281 Qiagen, Hilden, Germany

2.1.7 Antibodies and fluorescent agents

2.1.7.1 Unconjugated antibodies

Name	Dilution	Host species	Manufacturer
NFAT5-anti-human	1:1000	rabbit	Thermo Fisher Scientific, Waltham, USA
β-Actin-anti-mouse	1:1000	rabbit	Sigma-Aldrich, Taufkirchen
CCL2-anti-mouse	3 µg/ml	rat	RD Systems, Minneapolis, USA
IgG_{2B} isotype control	3 µg/ml	rat	RD Systems, Minneapolis, USA

2.1.7.2 Conjugated antibodies

Name (conjugation)	Dilution	Manufacturer
stabilized goat-anti-rabbit (HRP)	1:800	Pierce, Rockford, USA
CD86 rat-anti-mouse (PE)	1:100 (FACS)	eBioscience, San Diego, USA
CD25 rat-anti-mouse (APC)	1:100 (FACS)	BD Biosciences, Franklin Lakes, USA

2.1.7.3 Fluorescent agents

Name	Dilution	Manufacturer
TRITC-Phalloidin	1:50	Sigma-Aldrich, Taufkirchen
DAPI	1:1000	Sigma-Aldrich, Taufkirchen

2.1.8 Chemokines

Murine CXCL12, CCL2, CCL19, GM-CSF, and C5a were purchased from Peprotech, Rocky Hill, USA

2.1.9 Buffers and solutions

10x PBS (pH 6.8)	1.37 M NaCl 27 mM KCl 101 mM Na_2HPO_4 18 mM KH_2PO_4
Coomassie Staining Solution	0.025 g Coomassie Brillant Blue 25 ml Isopropanol 10 ml Acetic acid ad 100 ml H_2O dest.
Erythrocyte lysis buffer	150 mM NH_4Cl 10 mM $KHCO_3$
Ponceau S Staining Solution	0.1 g Ponceau S 5 ml Acetic acid ad 100 ml H_2O dest.
SDS-sample buffer, 5x	0.28 M Tris-HCl pH 8 30 % (w/v) Glycerol 10 % (w/v) SDS 60 mM DTT 0.0012 % (v/v) Bromphenol blue 3.4 ml A. bidest

Stacking SDS gel, 5%	630 µl 1.0M Tris-HCl, pH 6.8 830 µl 30 % (v/v) Acrylamide 50 µl 10 % (v/v) SDS 50 µl 10 % (v/v) APS 5 µl TEMED
Resolving SDS gel, 10 %	1.9 ml A. bidest 1.3 ml 1.5M Tris-HCl, pH 8.8 1.7 ml 30 % (v/v) Acrylamide 50 µl 10 % (v/v) SDS 50 µl 10 % (v/v) APS 2 µl TEMED
1x Tris buffered saline with Tween (TBST)	0.5 M Tris-HCl, pH 7.5 1.5 M NaCl 0.06 % (v/v) Tween
Western Blot Running Buffer, 10x (Laemmli)	25 mM Tris 200 mM Glycin 0.1 % (w/v) SDS
Western Blot transfer buffer, 20x	200 mM Glycin 25 mM Tris 20 % (v/v) Methanol 0.002 % (w/v) SDS

2.1.10 Software

Cell Profiler	Broad Institute, Cambridge, USA
Corel Draw X4	Corel, Ottawa, Canada
Graph Pad Prism	Graph Pad Software Inc., La Jolla, USA
Image J with ImageJ1.38 plugin	National Institutes of Health, USA
FACS Diva	BD Biosciences, Franklin Lakes, USA

FlowJo	Tree Star, Ashland, USA
NIS Elements	Nikon, Tokyo, Japan
SPSS Statistics 20	IBM, Armonk, USA

2.2 Methods

2.2.1 Animals

All animal experiments were conducted in a licensed animal facility in accordance with the German law on the protection of experimental animals and were approved by local authorities of the state of North-Rhine-Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz NRW). Mice were sacrified by cervical dislocation.

2.2.1.1 Ton(flox/flox)LysM(wt/cre) mice

Bone material for isolation of bone marrow-derived macrophages (BMDMs) from Ton(flox/flox)LysM(wt/cre) mice with a C57BL/6 background in which the *TonEBP* gene had been disrupted specifically in the myeloid cell population were a kind gift from Prof. Dr. Jens Titze, Erlangen University, Germany and Vanderbilt University, USA. These mice were generated by inGenious Targeting Laboratory, Inc. (Ronkonkoma, USA) as described in Wiig et al. (2013). Briefly, the LysM-Cre (Clausen et al. 1999) deleter mouse strain was crossed with *Nfat5*$^{fl/fl}$ mice that harbor two *lox*P sites which target exon 4 of the *Nfat5* gene. Knockout construct control mice (Ton(flox/flox)LysM(wt/wt)) and wildtype C57BL/6 mice were used as controls.

2.2.2 Cell culture

2.2.2.1 Cultivation of RAW264.7 cells

The murine macrophage-like cell line RAW264.7 (ATTC #TIB-71) was a kind gift from Dr. Agnes Schröder, University of Erlangen, Germany. Cells were cultivated in 75 cm² cell culture flasks in Dulbecco's modified Eagle´s Medium (low glucose) supplied with 10 % FCS

(Biochrom) and 1% Penicillin/Streptomycin in a 5 % CO_2 atmosphere with 95 % humidity at 37 °C. Subcultivation occurred twice a week when reaching 90 % cell confluence with the following procedure: First, the old media was aspirated and cells washed with pre-warmed phosphate buffered saline (PBS). Cell detachment was accomplished by incubation with 5 ml of pre-warmed Trypsin/EDTA at 37 °C for 5 to 10 minutes. Trypsin activity was stopped with 10 ml of DMEM containing 10 % FCS and the cell suspension was transferred into a falcon tube, centrifugated at 180 *g* for 5 minutes and the cell pellet solved in 10 ml DMEM. According to cell number determination from a sample of 20 µl in a hemocytometer (Neubauer), the cell suspension was diluted 1:20 or 1:10 (equaling 2-4x10^6 cells) in 25 ml medium in a new cell culture flask. Cells were used for up to 25 passages.

2.2.2.2 Freezing of RAW264.7 cells

Cells were harvested from an up to 90 % confluent-grown cell culture flask as described above and the cell pellet was resolved in 1- 1,5 ml DMEM. 500 µl each (equaling 1 -1.5 x 10^7 cells) were transferred into a cryovial containing 500 µl FCS (Biochrom) with 10 % DMSO to avoid cell damage during the freezing process. Quickly, cryovials were transferred to a Mr. Frosty Freezing Container and stored in a -80 °C freezer, therefore ensuring the temperature decreased by 1 °C per minute. On the following day, cryovials were transferred into the liquid nitrogen container for long-term storage.

2.2.2.3 Recultivation of frozen RAW264.7 cells

Aliquots of RAW264.7 stored in liquid nitrogen were slowly thawed by room temperature until the liquid could be transferred into a falcon tube containing 15 ml of 4 °C cold DMEM. After 15 minutes adaptation, the cell suspension was centrifugated at 180 *g* for 5 minutes and the media replaced with fresh culture medium. Cells were seeded in a 75 cm² cell culture flask and the medium was replaced to remove residues of toxic DMSO not later than 24 hours.

2.2.2.4 RAW264.7 cells with a stable TonEBP overexpression

RAW264.7 macrophages with a stable TonEBP (Tonicity-responsive enhancer binding protein) overexpression were a kind gift from Professor Dr. Jens Titze, University of Erlangen, Germany and Vanderbilt University, USA. Generation of stable TonEBP overexpression was established by Wolfgang Neuhofer as described previously (Machnik et al. 2009; Phd thesis Schröder 2010), RAW264.7 macrophages were transfected with a

construct of murine TonEBP cDNA in the mammalian expression vector pCMV-Tag2 (Stratagene, La Jolla, USA; Figure 2-1) and selected by addition of 800 µg/ml Kanamycin to the DMEM culture medium.

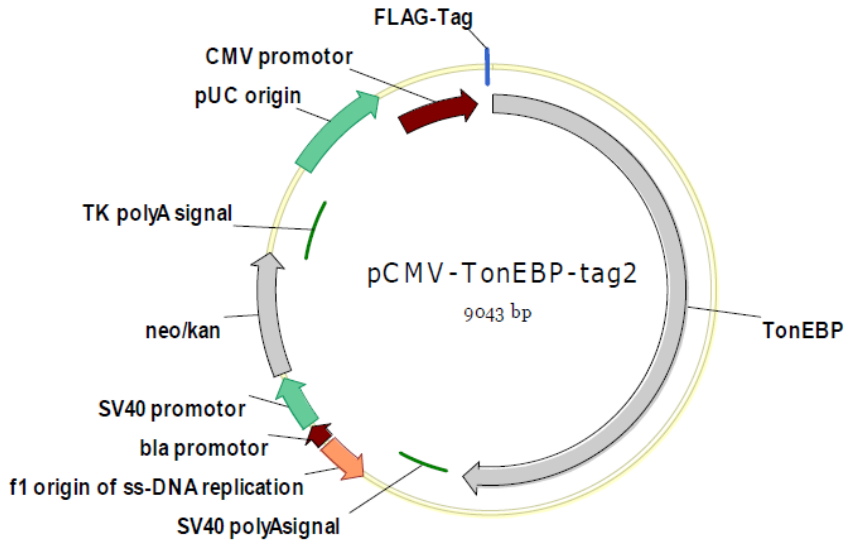


Figure 2-1: Plasmid chart of pCMV-TonEBP-tag2

TonEBP was cloned behind the cytomegalovirus (CMV) promoter to gain constitutive protein expression. In order to allow selection of clones, the plasmid contains a resistance against kanamycin (from PhD thesis Agnes Schröder).

2.2.2.5 Generation of bone marrow-derived dendritic cells (BMDCs) and macrophages (BMDMs)

Murine bone marrow-derived dendritic cells (BMDCs) were differentiated from hematopoietic stem cells as described previously by Quast et al. (2011). Seven week old C57BL/6 wildtype and LysMCreTonEBP (flox/flox) mice were sacrificed by cervical dislocation and the femurs and tibiae retrieved. The bones were opened at both ends and washed with PBS several times under sterile conditions to remove the cells. Filtration with 40 µm pore nylon cell strainers separated cells from the remaining tissues. Following centrifugation (400 *g*, 10 min), hematopoietic stem cells were plated into 10 cm non-treated petri dishes at a concentration of $5x10^6$ cells in 10 ml VLE-RPMI 1640 supplemented with 10 % heat-inactivated FCS (Sigma-Aldrich), 100 u/ml Penicillin, and 0.1 mg/ml Streptomycin. Differentiation into BMDCs was achieved by addition of 10 ng/ml recombinant murine GM-CSF. The cell culture medium was half-renewed every three days until cells could be used for functional assays after 7 – 10

days. In order to stimulate maturation of BMDCs, 200 ng/ml LPS was added to the cells for 48 h.

For differentiation into bone marrow-derived macrophages (BMDMs), hematopoietic stem cells were plated into 10 cm non-treated petri dishes at a concentration of $5x10^6$ cells in IMDM, supplemented with 10% FCS (Sigma-Aldrich), 2 mM L-glutamine, 100 u/ml Penicillin, 0.1 mg/ml Streptomycin and 10 ng/mL recombinant murine M-CSF.

2.2.2.6 Isolation of murine peritoneal macrophages

Peritoneal macrophages were isolated from C57BL/6 mice by peritoneal lavage with 2 mM EDTA/PBS, kept on ice and centrifuged (400 *g*, 8 min). In order to remove the remaining erythrocytes, the cell pellet was resuspended in erythrocyte lysis buffer and incubated for 5 min at 37 °C. The reaction was stopped by addition of 35 ml ice-cold PBS and the remnants washed twice with PBS following centrifugation at 400 *g* for 5 min. Next, cells were plated into 10 cm non-treated petri dishes in IMDM supplemented with 1 % Penicillin/Streptomycin and 10 % FCS. Once macrophages had adhered to the surface, the cell culture medium was replaced after several hours in order to remove remaining non-adherent B-cells and other cells.

2.2.3 Cell-based assays

2.2.3.1 *In vitro* transwell migration assay

Chemotaxis of the RAW264.7 cells, BMDMs and BMDCs, and peritoneal macrophages was analyzed *in vitro* with a transwell assay (modified Boyden chamber) using cell culture membrane inserts with 8 µm pore size (Figure 2-2). $2 x10^5$ cells were placed in duplicates or triplicates in serum-reduced (0.5 % FCS) cell culture media in the upper well while the culture medium of the lower compartment was supplemented with 200 ng/ml chemokines (25 nM CXCL12 or 15 nM CCL2, respectively) or NaCl with concentrations between 10-100 mM (reaching a final concentration of 155 to 255 mM NaCl in the media). Following 20 hours incubation at 37 °C / 5 % CO_2, cells on top of the membrane that had not migrated were removed with cotton swabs. Transmigrated cells on the bottom of the membrane were stained with 5 µM Vybrant CFDA-SE in PBS according to the manufacturer´s protocol, washed in PBS and fixated with 4 % PFA/PBS for 15 min. For quantification of cell migration, 5-10 random fields were observed and photographed in each sample with an inverted Nikon

Eclipse TE 2000-E fluorescence microscope. Later, the number of transmigrated cells in the images was counted using Cell Profiler software as described by Carpenter et al. (2006).
For migration analysis of BMDCs, smaller cell culture inserts with 5 µm pore size (Corning) were used and cells were stimulated with 25 nM CCL19 as a migration control. Transmigrated BMDCs in the lower compartment were counted after incubation at 37 °C / 5 % CO_2 for 4 hours.

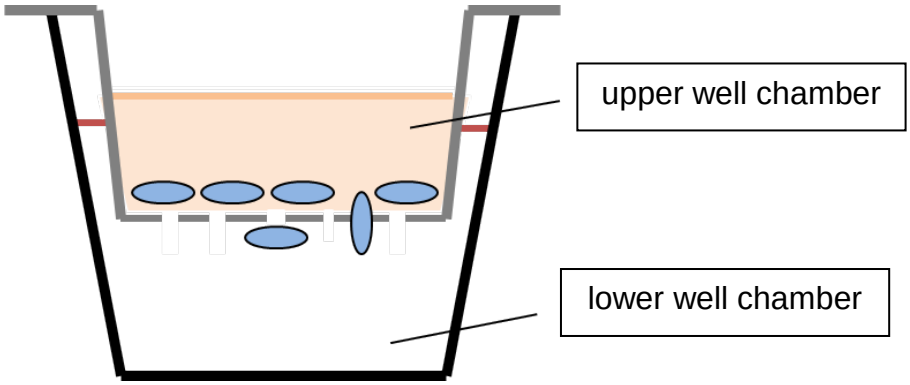


Figure 2-2: Transwell migration assay using a cell culture insert

The cell culture insert (gray) was placed in a 24-well plate (black). Cells (blue) were seeded in the upper chamber and migrated through pores (8 µm for macrophages or 5 µm for DCs) in the membrane toward a chemotactic stimulus in the lower chamber. After 20 hours (4 h for DCs), remaining cells on the top of the membrane were removed and transmigrated cells were stained and quantified.

2.2.3.2 Immunofluorescence TRITC-Phalloidin staining

For microscopical analysis of the actin cytoskeleton, phalloidin-based immunofluorescent staining was used. Phalloidin is a phallotoxin isolated from *Amanita phalloides* that specifically binds to and stabilizes F-actin. Coupled to a fluorochrome (here: TRITC), this staining allows localization of actin cytoskeleton filaments. In order to stain cell nuclei, DNA-binding fluorescent DAPI (4',6-diamidino-2-phenylindole) was used.
$5x10^5$ RAW264.7 cells were placed on fibronectin-coated glass coverslips in serum-reduced (0.5 % FCS) media. Following adhesion, cells were stimulated with 40 mM NaCl, 10 nM C5a, 15 nM CCL2 or 25 nM CXCL12, respectively, for 5 min. Subsequently, the coverslips were washed twice with PBS and the cells fixated in 4 % PFA in PBS for 20 min. For permeabilization, cells were incubated in 0.2 % Triton-X 100 in PBS for 5 minutes. The

staining with TRITC-Phalliodin and DAPI occurred for 45 min in the dark. The remaining staining solution was removed by washing three times with PBS and finally once with A. bidest. To avoid fading of the fluorescent dyes, the coverslips were mounted in FluorShield containing DABCO (1,4-Diazabicyclo[2.2.2]octane), and stored at 4 °C in the dark. Microscopic analysis of cell morphology and the actin cytoskeleton was performed with an inverted confocal laser scanning microscope (Fluoview 1000, Olympus).

2.2.3.3 Dynamics of lamellipodia formation measured by kymograph and line-scan analysis

Lamellipodia are cell membrane protrusions that define the moving edge of the cell. They are formed by rapid reorganization and polymersiation of the actin cytoskeleton. In order to study lamellipodia dynamics in RAW264.7 cells, cells were adhered on a glass surface of a Lab-Tek Chamber Slide in DMEM with reduced FCS (0.5 %) for 20 minutes in a 5 % CO_2 atmosphere with 95 % humidity at 37°C. Following stimulation with 40 mM NaCl, 10 nM C5a, 15 nM CCL2 or 25 nM CXCL12, respectively, RAW264.7 cells were observed over 5 min by taking a time series of phase contrast images every 2 s with an inverted Nikon Eclipse TE 2000-E microscope (supplied with a climate chamber providing 37 °C, 5 % CO_2 and humidity). For analysis, the area of interest was selected in each image by assigning lines that cross the motile lamellipodium of the polarized cell (white line in Figure 3-7 B), resulting in 1-pixel-wide areas. These were cut out and aligned in oder to receive time-space-plots (Figure 3-7 B), so-called kymographs. Since lamellipodia protrusions are now depicted as linear ascending lines, the velocity of lamellipodia movement in µm/min can be determined by the slope of these lines (v = dx /dt, yellow lines in Figure 3-7 B). The kymograph and line-scan analysis was performed using the walking average plug-in for ImageJ1.38 (National Institutes of Health; http://imagej.nih.gov/ij/).

2.2.3.4 Cell Viability Assays

2.2.3.4.1 Cell Titer-Blue Viability Assay

In order to quantify cell viability, the metabolic activity of the cells was measured with Cell Titer-Blue Cell Viability Assay from Promega according to manufacturer´s protocol. The Cell Titer-Blue Reagent contains the dye resazurin which is reduced to resorufin by living cells. Therefore, the amount of resorufin is directly proportional to the number of living cells and can be determined in a fluorometer (560 nm excitation/ 590 nm emission). For this assay,

1x 10^4 RAW264.7 cells or BMDMs per well were transferred to a 96 well plate in serum-reduced media (0.5 % FCS) and following adhesion, Na^+ concentration of the media was increased by adding 10 to 100 mM NaCl. After 20 hours, the amount of reduced resorufin referring to metabolic cell viability was measured with a GloMax-Multi microplate reader.

2.2.3.4.2 Apoptotic/Necrotic/Healthy Cells Detection Kit

Influence of increased salt concentration on cell viability was additionally quantified with an Apoptotic/Necrotic/Healthy Cells Detection Kit from Promokine. FITC-coupled Annexin V allows specific staining of phosphatidylserine-covered apoptotic cells, while an ethidium-based fluorescent dye permeates necrotic cells only and binds to nucleic acid. All nuclei of living cells are stained with cell the membrane-permeable dye Hoechst. Therefore, apoptotic, nectrotic and living cells can be distinguished specifically within the same cell population. For this assay, 1x10^5 RAW264.7 cells were placed on glass coverslips in serum-reduced DMEM (0.5 % FCS) and stimulated with excess 40 mM NaCl. 5 % DMSO stimulus was used as an apoptosis-inducing control. Fixation and staining of cells was performed as described in the protocol for adherent cells provided by the manufacturer. Microscopic analysis was performed with a Nikon ECLIPSE i80.

2.2.3.5 Flow cytometry

Flow cytometry allows analysis and quantification of differently labeled cell populations in a single cell solution. The activation status of BMDMs was studied following staining of CD86 with a PE-conjugated rat-anti-mouse antibody (eBioscience) and staining of CD25 with an APC-conjugated rat-anti-mouse antibody (BD Pharmingen). Briefly, 0.5x10^6 BMDMs were placed in FACS tubes and washed with PBS (centrifugation at 300 *g* for 5 minutes at 4 °C). Staining was performed in 50 µl PBS for 30 minutes in the dark. After washing with 150 µl PBS for 3 times, cells were suspended in 200 µl PBS and analyzed using a FACS CantoII device.

2.2.4 Biochemical assays

2.2.4.1 Flame photometry

The concentration of alkali and alkaline earth metal elements in a solution can be measured by flame photometry. The diluted measuring solution is dispersed into a flame as a fine aerosol, following wavelength analysis which corresponds to a specific element.
For determination of Na^+ concentration in cell culture media, samples of both the upper and the lower wells of the transwell system were collected at different time points after NaCl supplementation (1, 10, 30 minutes, and 1, 2, 4, 8, 12, 16 and 20 hours). The Na^+ content in the supernatants was measured by flame photometry in an EFOX 5053 device in comparison to an urine standard. Liquichek Urine Chemistry Control containing known Na^+ concentrations was used to ensure the accuracy of the photometer.

2.2.4.2 Osmometer measurements

Osmolality is defined by the number of osmoles of solute per kilogram of solvent (usually water) in an aqueous solution (osm/ kg H_2O). It can be measured by determination of freezing-point depression, since osmotically active particles like salt decrease the freezing point of an aqueous solution. To determine osmolality of media solutions applied in the transwell migration assays, samples from different osmotic stimuli (+40 mM NaCl, +80 mM Urea, +80 mM Mannitol) were analyzed with a freezing point micro-osmometer OM815, after the osmometer had been calibrated with a 300 mosm/kgH_2O standard solution. A sample of 0.9 % NaCl-solution (186 mosm/ kg H_2O) was used to ascertain the accuracy of the measurements.

2.2.4.3 Nitrite determination by Griess reaction

Indirect verification of nitric oxide (NO) production by cells was performed with a Griess diazotization reaction to detect organic nitrite, which is formed under physiological conditions by spontaneous oxidation of NO. When sulfanilic acid is added to a nitrite-containing sample, a diazonium salt is formed. In the second step, binding to N-(1-naphthyl)ethylenediamine (NED) results into a pink azo dye that displays an absorbance at 548 nm and can therefore

be quantified in a spectrophotometer. In this work, the assay was used to test the ability of Polymyxin B to inhibit LPS-dependent NO-production.

2x 10^5 cells were placed in a 96 well plate in 200 µl serum-reduced media (0.5 % FCS). After adhesion, the cells were stimulated for 24 hours with 10 ng/ml, 200 ng/ml or 1 µg/ml of Lipopolysaccharide, in combination with each 1, 10 or 50 µg/ml of Polymyxin B, respectively. Supernatants were collected and stored at -20 °C.

For detection of nitrite according to Griess reaction, 50 µl of supernatants were transferred to a 96 well plate in triplicates and 50 µl of Griess reagent 1 (1 % sulfanilamide and 5 % ortho-phosphoric acid in H_2O) were added. Following 5-10 minutes incubation in the dark at room temperature, 50 µl of Griess reagent 2 (0.1 % NED) were added. Following incubation in the dark, absorbance was measured at 550 nm in the GloMax-Multi microplate reader. As a standard for nitrite, concentrations from 1.56 - 100 µM of sodium nitrite were used.

2.2.5 RNA interference (RNAi) against TonEBP

In order to diminish the amount of TonEBP protein in RAW264.7 cells, RNA interference was used. With this method, introduction of small interfering RNAs (siRNAs) into the cells leads to a decreased protein expression by degrading the specific mRNA coding for the target protein. Different methods of transfecting RAW264.7 cells with siRNA directed against TonEBP were applied:

2.2.5.1 Electroporation

During electroporation the cell membrane is subjected to a high electrical field stimulus and is therefore permeable for entry of siRNA complexes. 10 µg of ON-TARGETplus siRNA duplexes directed against the target sequence of murine TonEBP or 10 µg of AllStars Negative siRNA were transferred to a 4-mm (2-mm for HL60 protocol) cuvette and 2 x 10^6 RAW 264.7 cells in 100µl Opti-MEM were added. After incubation for 3 min, electroporation was performed in a GenePulser Xcell using one of the following pulse conditions:

a) exponential, 400 V, 150 µF,
b) square wave, 1000 V, 2 x 1 ms pulse
c) HL60 preinstalled mammalian protocol for adherent cells (square wave, 140 V, 25 ms pulse, in 2-mm cuvette)

Three days following electroporation, migration behavior of untreated cells, cells that had been electroporated without siRNA (mock), electroporated with ns-siRNA and of cells electroporated with siRNA duplexes directed against TonEBP was analyzed in a transwell assay. Efficiency of the siRNA-mediated silencing was evaluated by Western Blot analysis following stimulation with additional 40 mM NaCl for 24 hours.

2.2.5.2 Transfection with Lipofection agents

Positively charged lipofection agents bind negatively charged nucleic acids and form liposomes, which are taken up by the cellular membranes and are therefore used to introduce siRNA into cells. FuGENE HD (Roche Applied Science), Lipofectamine 2000 and Oligofectamine transfection reagents (both Invitrogen) were tested for transfection with aforementioned siRNA duplexes according to protocols listed below, following 48 h and 72 h incubation. Evaluation of knockdown efficiency was performed by Western Blot analysis after stimulation with additional 40 mM NaCl for 20 hours.

Transfection with FuGENE HD was performed in 6-well plates comparable to 35 mm petri dishes as described by Kawaai et al. (2008) using 30 pmol siRNA in 100 µl Opti-MEM for $1x10^5$ cells and 6 µl FuGENE reagent. The culture medium was replaced following 6 hours incubation. Lipofectamine 2000 and Oligofectamine were used according to the Invitrogen Stealth siRNA Transfection protocol in 6 well or 12 well plates, respectively. For transfection with Lifpofectamine 2000, $3x10^5$ cells were incubated with complexes of 5 µl LF in 250 µl Opti-MEM and 100 pmol siRNA in 250 µl Opti-MEM for 4 hours in DMEM without antibiotics. For transfection with Oligofectamine, $1x10^5$ cells were treated with complexes of 100 pmol siRNA in 85 µl Opti-MEM and 3 µl OF in 10 µl Opti-MEM in serum-free DMEM without antibiotics. Following incubation for 4 hours at 37 °C, cells were washed and the medium was replaced with normal DMEM.

2.2.6 Protein biochemistry

2.2.6.1 Western blot analysis of protein expression

For analysis of TonEBP protein expression, 2×10^6 RAW264.7 cells were placed in cell culture dishes in serum reduced DMEM (0.5 % FCS) and exposed to additional 40 mM NaCl for 20 hours. Afterwards, cells were washed with PBS and lysed in 8 M urea supplied with

10 % Complete Protease Inhibitor Cocktail, followed by incubation for 30 min on ice and centrifugation for 10 min at 13.000 rpm. The supernatant was used for immunoblot analysis and stored at -80 °C. The protein concentration was measured by Bradford assay using Roti-Quant (5x) and a bovine serum albumin (BSA) standard according to manufacturer´s protocol. For immunoblotting, 20 - 40 µg of total protein were separated on 8 % SDS-polyacrylamide gels under reducing conditions including a prestained protein standard. Wet tank electroblotting onto a polyvinylidene diflouride (PVDF) membrane was performed for 2 hours at 80 V in a 4 °C cold room using previously cooled transfer buffer. The blots were cut below the 75 kDa band according to the prestained protein standard and blocked with 5 % nonfat milk in PBS and 0.1 % Tween 20, pH 7.5 (PBST) for 1 h at room temperature. The upper part of the blot was incubated overnight at 4 °C with a rabbit anti-TonEBP antibody diluted 1:1.000 in 5 % nonfat milk in PBST, while the lower part of the blot was incubated with rabbit anti-actin antibody under the same conditions. After three washes with PBST, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Pierce, Rockford, USA) diluted 1:1000 in blocking solution for 90 minutes at room temperature. After washing with PBST for three times, chemiluminescent signals were detected using ECL Western Blotting Substrate on autoradiographic film.

2.2.6.2 Detection of CCL2 production in RAW264.7 cells by ELISA

Analysis of CCL2 production in RAW264.7 cells was performed by enzyme-linked immunosorbent assay (ELISA). Following adhesion of $2x10^5$ cells in 96-well plates in serum reduced (0.5 % FCS) DMEM, the medium was replaced and cells were stimulated with additional 40 mM NaCl. Supernatants of cells cultivated with and without excess NaCl were collected after 1, 2, 4, 8, 16, and 20 hours and quickly stored at -20 °C. Later, the amount of CCL2 was quantified using mouse CCL2 ELISA according to manufacturer´s instructions (R&D Duoset).

2.2.6.3 Detection of Leukotriene B4 production by ELISA

$2x10^5$ RAW264.7 cells were placed in a 96-well plate in serum-reduced DMEM (0.5 % FCS) and stimulated with additional 40 mM NaCl. Supernatants of untreated and NaCl stimulated cells were collected after 8, 16 and 20 hours, while supernatants of cells stimulated with 1 µg/ml LPS for 20 hours were used as positive control. Also, supernatants of cells stimulated with the chemokines CXCL12 and CCL2 (200 ng/ml) for 20 hours were collected. All samples were quickly stored at -20 °C until analysis. Concentration of LTB4 in

supernatants was determined by ELISA with Leukotriene B_4 EIA kit according to the protocol provided by the manufacturer (Cayman).

2.2.7 Statistical analysis

All experiments were repeated at least three times using duplicates or triplicates. For each transwell assay sample, at least 5 visual fields were analyzed microscopically.

All displayed data are expressed as mean ± standard deviation (SD). Testing for normal distribution of sample data was performed with the Kolmogorov-Smirnov test before using two-tailed Student's t-test to compare means. The homogeneity of variances for the t-test was tested with the Levene's test. Values of $p < 0.05$ were considered significant. All tests were performed using IBM SPSS Statistics 20.0 software. Assigned p values were $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$.

3 Results

The results shown in this work describe the process of the discovery and subsequent characterization of salt-dependent chemotaxis of macrophages. At first, migration toward excess NaCl was investigated using transwell migration assays in RAW264.7 cells. This was compared to migration behavior of BMDMs, peritoneal macrophages and BMDCs. On the mechanistic level, the early effects of a NaCl stimulus on cell morphology, actin cytoskeleton reorganization and lamellipodia formation were studied. This was followed by analysis of late processes that require protein synthesis, including the induction of chemoattractant factors by a hypertonic NaCl stimulus and the participation of the osmoprotective transcription factor TonEBP.

3.1 Viability of RAW264.7 cells and BMDMs in hypertonicity

Before using functional assays employing excess NaCl as a stimulus, possible cytotoxic effects of a high NaCl concentration needed to be excluded. According to the previously established data, RAW264.7 cells were stimulated for 24 hours with excess 40 mM NaCl in order to investigate the expression of the osmoprotective transcription factor TonEBP (Machnik et al. 2009, PhD thesis Agnes Schröder). Flame photometric measurements determined that the sodium concentration was raised from 155 mM to 195 mM, which reflected the concentration differences observed in the skin of rats following a high salt diet (Machnik et al. 2009). For migration experiments in this work it needed to be shown that osmotic stress induced by high NaCl concentration does not alter cell viability of macrophages. Therefore, in addition to identifying dead cells by trypsin blue staining, more specific cell viability assays were performed which determined metabolic activity of cells and investigated a possible induction of apoptosis.

The metabolic activity of RAW264.7 cells referring to cell viability was studied with a CellTiter-Blue cell viability assay following application of stimuli from 10 mM to 200 mM NaCl for 20 hours (resulting in final sodium concentrations of 165 mM to 355 mM). Figure 3-1 A shows that there was no significant difference in cell viability compared to untreated RAW264.7 cells following stimuli of 10 mM to 40 mM NaCl. In fact, cell viability of cells treated with 40 mM NaCl was approximately 90 % compared to control cells. However, from excess 60 mM NaCl onwards, cell viability decreased significantly. At a stimulus of 200 mM

NaCl, no more than 15 % of the cells were viable, which was similar to cells treated with 5 % DMSO. In addition, the viability tests were performed with BMDMs treated with a NaCl stimulus of 40 mM to 200 mM. Figure 3-1 B shows that there was no significant difference in cell viability between untreated cells and BMDMs stimulated with 40 mM NaCl. Cell viability compared to unstimulated cells decreased from 80 mM NaCl onwards until less than 20 % of BMDMs were viable following a stimulus of 200 mM NaCl (Figure 3-1 B).

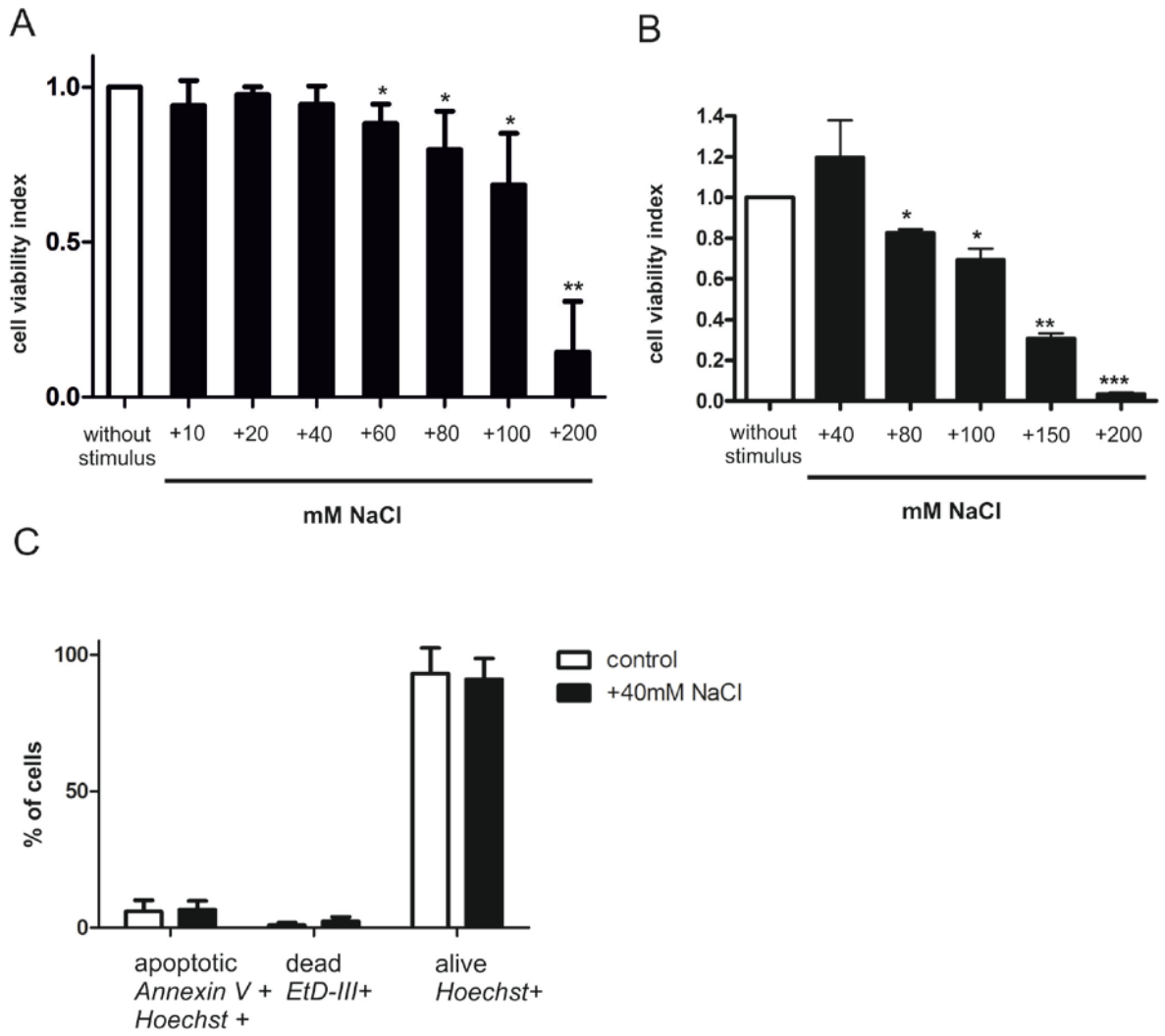


Figure 3-1: Cell Viability of RAW264.7 cells and BMDMs is not disturbed at excess 40 mM NaCl

Metabolic activity of RAW264.7 cells (A) and BMDMs (B) measured with CellTiterblue assay following stimulation with excess 10 mM to 200 mM NaCl for 20 hours. Cell viability index indicates NaCl-stimulated cells compared to untreated control cells. RAW264.7 cells following stimulation with 40 mM NaCl for 20 hours were quantified with Apoptotic/ necrotic/ healthy cell staining kit (C). Data are shown as mean ± SD with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to unstimulated control cells. $n > 3$ in triplicates. Partly modified from Müller et al. (2013).

As shown by others, osmotic stress due to a high NaCl concentration results in cell shrinkage and can lead to protein degradation and apoptosis in PBMCs (Gastaldello et al. 2008). In order to investigate whether excess NaCl might induce apoptosis in RAW264.7 cells, an Apoptotic/necrotic/healthy cell staining kit was used following stimulation with excess 40 mM NaCl for 20 hours. Cell staining with specific fluorescent dyes allowed quantification of living, apoptotic or necrotic cells, respectively. Figure 3-1 C shows that quantification of stained cells revealed no significant increase in apoptotic or dead cells compared to unstimulated cells. Altogether, these results indicate that a NaCl stimulus of 40 mM does not alter cell viability in RAW264.7 cells and BMDMs. Furthermore, this NaCl concentration does not induce apoptosis. Since this stimulus was already established by Machnik et al. (2009) and is based on *in vivo* data, for all following migration studies a stimulus of 40 mM NaCl was applied. If not stated differently, “excess NaCl” describes an additional 40 mM NaCl stimulus resulting in a final NaCl concentration of 195 mM.

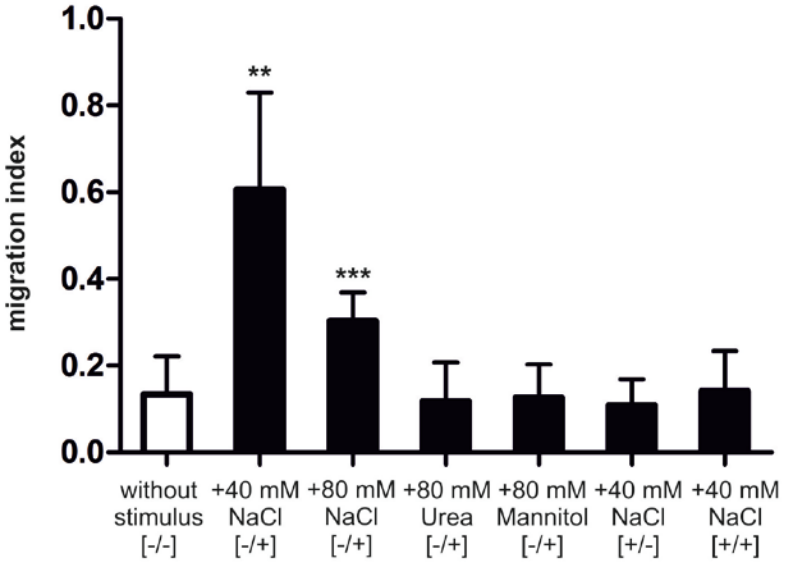
3.2 Characterization of macrophage migration toward excess NaCl

Previous results by Machnik et al. (2009) have shown that macrophages accumulate in the skin of rats that were fed a high salt diet. These macrophages proved to be essential for regulation of interstitial electrolyte composition and blood pressure. The question remained how macrophages sense increasing concentrations of stored sodium in the interstitium and subsequently migrate toward these areas of high sodium concentrations. The hypothesis underlying this work is that a chemoattraction toward a high sodium concentration similar to chemotaxis during immune responses might be the responsible mechanism.
To test this, migration of RAW264.7 cells toward a stimulus of excess 40 mM NaCl and control chemokines was investigated in different cell migration assay systems, including real-time imaging of single cells during the migration process.

3.2.1 Macrophages recognize excess NaCl as chemotactic stimulus

A transwell migration assay (modified Boyden chamber) was used to investigate whether RAW264.7 cells migrate toward an increased NaCl concentration. A stimulus of excess 40 mM NaCl had been established previously by Machnik et al. (2009), who found that this concentration simulates the difference between the combined serum Na^+ and K^+ concentrations and the ratio of the Na^+ and K^+ content relative to water in skin of rats that were fed a high salt diet.

A



B

	mosm/kgH_2O
DMEM Glucose	320
+40 mM NaCl	394
+80 mM NaCl	401
+80 mM Mannitol	399

Figure 3-2: Salt-dependent chemotaxis is specific to increased osmolality by NaCl

Transwell migration assay of the murine macrophage cell line RAW264.7 toward the excess NaCl, urea or mannitol, respectively (A). The migration index compares the number of transmigrated cells to CXCL12-stimulated cells after 20 hours. [-/-] indicates that no stimulus was added to the transwells. The stimulus was added to [-/+] = the lower well of the transwell; [+/-] = to the upper well of the transwell; [+/+] = to both wells of the transwell (A). Osmolality of stimuli determined by osmometer analysis (B). **p < 0.01, ***p < 0.001 as compared to control of cells without stimulus. n < 3 in duplicates (modified from Müller et al. (2013))

After raising the NaCl concentration with additional 40 mM in the lower well of the transwell assay, a significantly increased migration response was found compared to unstimulated control cells after 20 hours (Figure 3-2 A). Quantification and comparison of transmigrated cells to cells stimulated with the chemokine CXCL12 (“migration index”) revealed a migration capacity of NaCl-stimulated cells of about 60 %. A stimulus of excess 80 mM NaCl led to a still significant, but lower migration response compared to excess 40 mM NaCl. Application of control osmolytes showed that hyperosmolarity by NaCl was specifically required to elicit a

migration response, since RAW264.7 cells did not migrate toward increased hyperosmolality by urea or toward increased hypertonicity by mannitol, respectively. This finding was confirmed by cryoscopic (freezing point) measurements of osmolality, which revealed an equal value of approximately 400 mosm/ kgH_2O for all applied osmotically active stimuli (Figure 3-2 B). These results led to the question whether an increasing NaCl gradient is required to induce RAW264.7 cell migration.

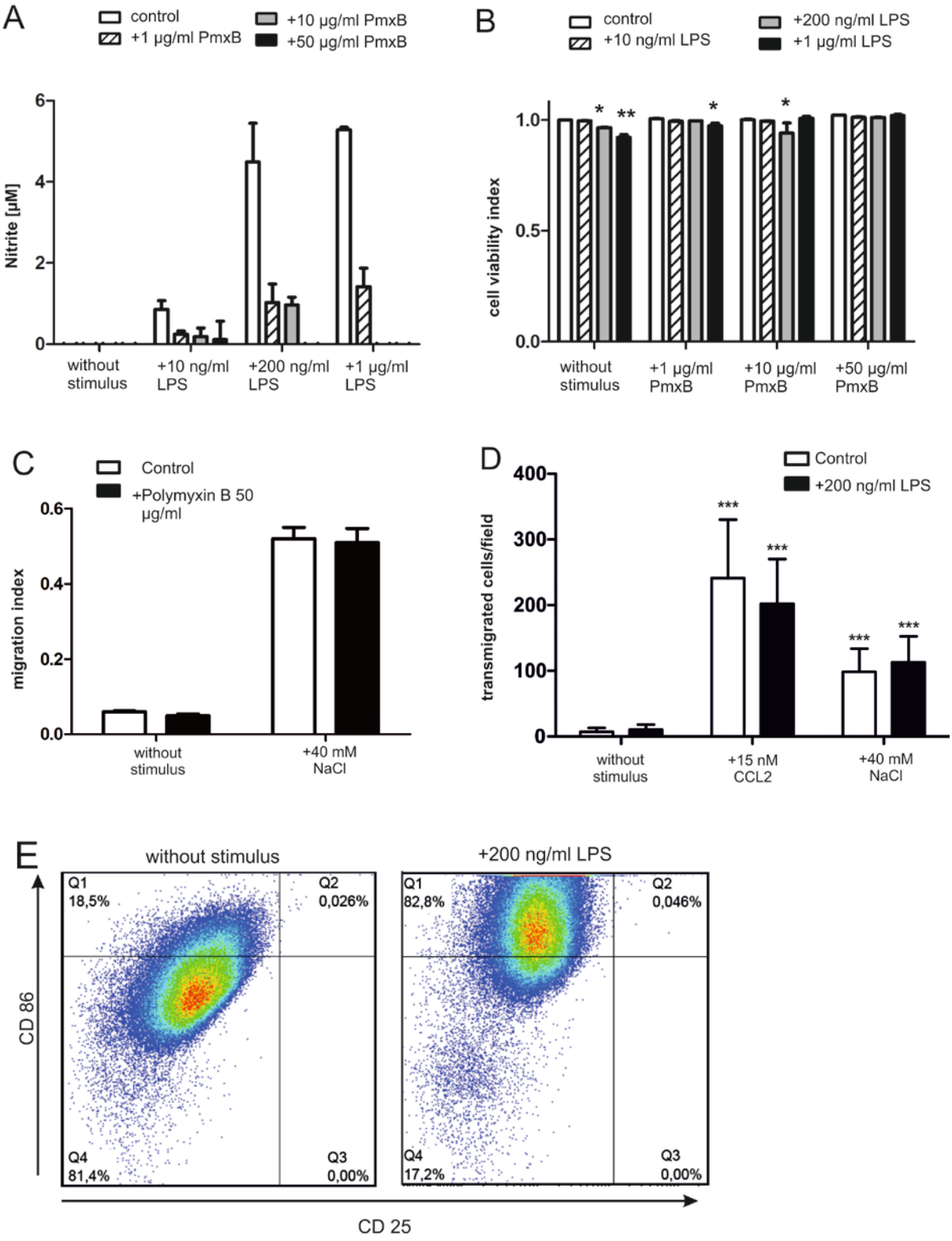
Therefore, migration behavior in a reverse gradient by application of 40 mM NaCl to the upper well of the transwell assay only ((+/-) in Figure 3-2 A) was studied. In addition, the NaCl stimlulus was applied to both wells of the transwell in order to analyze migration in an uniformly increased NaCl environment ((+/+) in Figure 3-2 A). The results show that an increasing gradient is the only setting in which the cells migrated significantly, while both the decreasing as well as the abolished gradient did not lead to a directional migration response. Altogether, these results indicate that an increasing stimulus of NaCl is a prerequisite for salt-dependent chemotaxis.

3.2.2 Migration of RAW264.7 cells toward excess NaCl is not due to contamination by endotoxin

It has been described previously that macrophages recognize endotoxin as a chemotactic stimulus (Tajima et al. 2008). In order to investigate if RAW264.7 migration toward NaCl was influenced by contamination with lipopolysaccharides (LPS), all reagents were treated with Polymyxin B (PmxB), which binds LPS and therefore neutralizes its cell-activating ability.
Different concentrations of PmxB were tested for their ability to abrogate LPS-dependent NO production in RAW264.7 cells, as was confirmed by Griess test (Griess diazotization reaction). Figure 3-3 A shows that although 1 µg/ml of PmxB had an effect on LPS concentrations ranging from 10 ng/ml to 1 µg/ml, only 50 µg/ml PmxB were able to completely diminish a stimulus of 1 µg/ml LPS. This effect was confirmed in a CellTiter-Blue cell viability assay, when addition of 50 µg/ml PmxB rescued LPS-dependent reduction of metabolic viability in RAW264.7 cells (Figure 3-3 B).
When cell migration toward excess NaCl or 25 nM CXCL12 was investigated with and without the presence of 50 µg/ml PmxB, no significant differences were found (Figure 3-3 C). In addition, migration behavior of LPS-activated BMDMs toward 40 mM NaCl or 15 nM CCL2 was not altered compared to untreated cells (Figure 3-3 D). When exposed to control media without a chemoattractive stimulus, LPS-activated BMDMs showed no increased

chemokinesis. Activation of BMDMs was confirmed by upregulation of CD86 by flow cytometry analysis (Figure 3-3 E, F).

Altogether, these results affirm that contamination of cell reagents by endotoxin is not responsible for chemotaxis toward an excess salt stimulus.



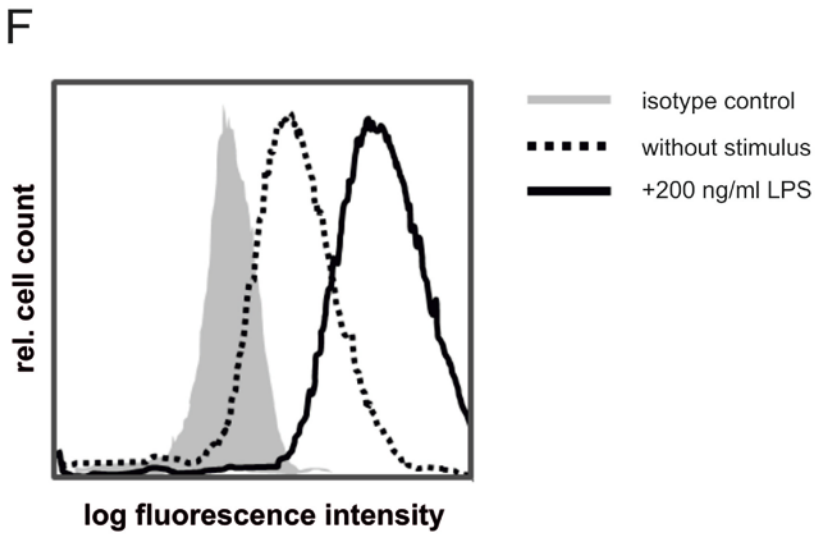


Figure 3-3: LPS-independent migration of macrophages

Nitrite production of RAW264.7 cells measured by Griess reaction (A) and cell viability determined with CellTiter-Blue cell viability assay (B) following stimulation with different concentrations of LPS and PmxB. Transwell migration assay of RAW264.7 cells in the presence of PmxB (C) and of BMDMs stimulated with 200 ng/ml LPS (D) toward excess 40 mM NaCl, respectively. CD86 upregulation in LPS-stimulated BMDMs (E, F). Cell viability index indicates cell viability compared to untreated control cells (B). Migration index is determined by transmigrated cells compared to migration toward CXCL12 (C) or CCL2 (D). "+" indicates that the stimulus was added to the lower well of the transwell (C, D). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to unstimulated control cells. n = 3 (partly modified from Müller et al. (2013))

3.2.3 Migration of RAW264.7 cells toward a hypertonic NaCl stimulus is dose-dependent

Since the migration response toward excess 80 mM NaCl was lower than toward 40 mM NaCl, the question arose whether salt-dependent chemotaxis followed a dose-dependency and where the maximum of the cell migration was located. For this reason, migration response in a transwell assay was investigated following a stimulus of excess 10 mM to 100 mM NaCl. Quantification of transmigrated RAW264.7 cells in comparison to CXCL12-stimulated cells showed that RAW264.7 cells also significantly migrated toward a stimulus of excess 10 mM NaCl (Figure 3-4).

However, the maximum of the migration response was observed at a stimulus of additional 40 mM NaCl. At higher concentrations, cell migration decreased, and from 100 mM NaCl

onward did not differ from the migration response of untreated cells. In order to test if decreasing cell viability was responsible for a lower migration response, the same NaCl concentrations were used in a CellTiter-Blue cell viability assay (see Figure 3-1 A).

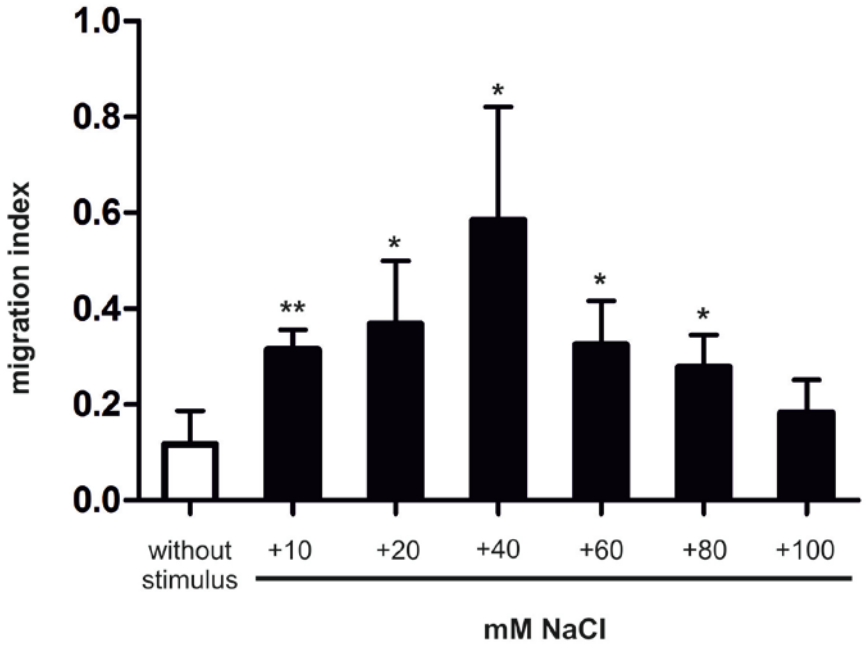


Figure 3-4: Dose-dependent migration of RAW264.7 cells toward excess NaCl

Transwell migration assay of RAW264.7 cells toward excess 10 to 100 mM NaCl in relation to migration of CXCL12-stimulated cells (migration index). "+" indicates that the stimulus was added to the lower well of the transwell. *p < 0.05, **p < 0.01 n = 3 in duplicates (from Müller et al. (2013)).

Figure 3-1 shows that RAW264.7 cells stimulated with additional 40 mM NaCl for 20 hours were about 90 % viable compared to untreated control cells. Nevertheless, from 60 mM onward cell viability was significantly reduced. These results indicate that it is not possible to clearly differentiate between decreased viability and cell migration from 60 mM onward. Altogether, since the stimulus of excess 40 mM NaCl elicited the maximum migration response (Figure 3-3), had no effect on cell viability (Figure 3-1) and corresponded to previously measured *in vivo* data (Machnik et al. 2009), it was used in all following experiments.

3.2.4 Migration of primary macrophages and dendritic cells toward excess NaCl

In order to investigate if other macrophages apart from the RAW264.7 cell line also recognize a high NaCl concentration as a chemoattractant stimulus, bone marrow derived macrophages and peritoneal macrophages were analyzed. After applying a stimulus of excess NaCl in a transwell migration assay, a highly significant number of both transmigrated BMDM cells and peritoneal macrophages compared to untreated cells could be found. Migration capacity of BMDMs was approximately 75 % as compared to CCL2-stimulated cells (Figure 3-5 A), while migration capacity of peritoneal macrophages was approximately 50 % (Figure 3-5 B). With both BMDMs and peritoneal macrophages showing a significant and robust migration response toward a high NaCl stimulus which is comparable to RAW264.7 data, these results indicate that salt-dependent chemotaxis is not restricted to the RAW264.7 cell line.

Next, the hypothesis needed to be tested whether only macrophages recognize a high NaCl concentration as a chemoattractant stimulus or if other migratory cells are also capable of salt-dependent chemotaxis. Therefore, migration behavior of both motile immature dendritic cells (imDCs) and mature dendritic cells (mDCs) derived from bone marrow stem cells was investigated. Upon stimulation with LPS, moderately migrating imBMDCs matured into highly motile mBMDCs. Transwell migration assays revealed that both imBMDCs and mBMDCs migrated toward the chemokine control (CXCL12 or CCL19), but neither showed a migration response toward the excess NaCl stimulus (Figure 3-5 C and D). Therefore, it appears that migration toward a hypertonic NaCl concentration remains a specific feature of macrophages and is not a common characteristic of migratory cells of the myeloid lineage.

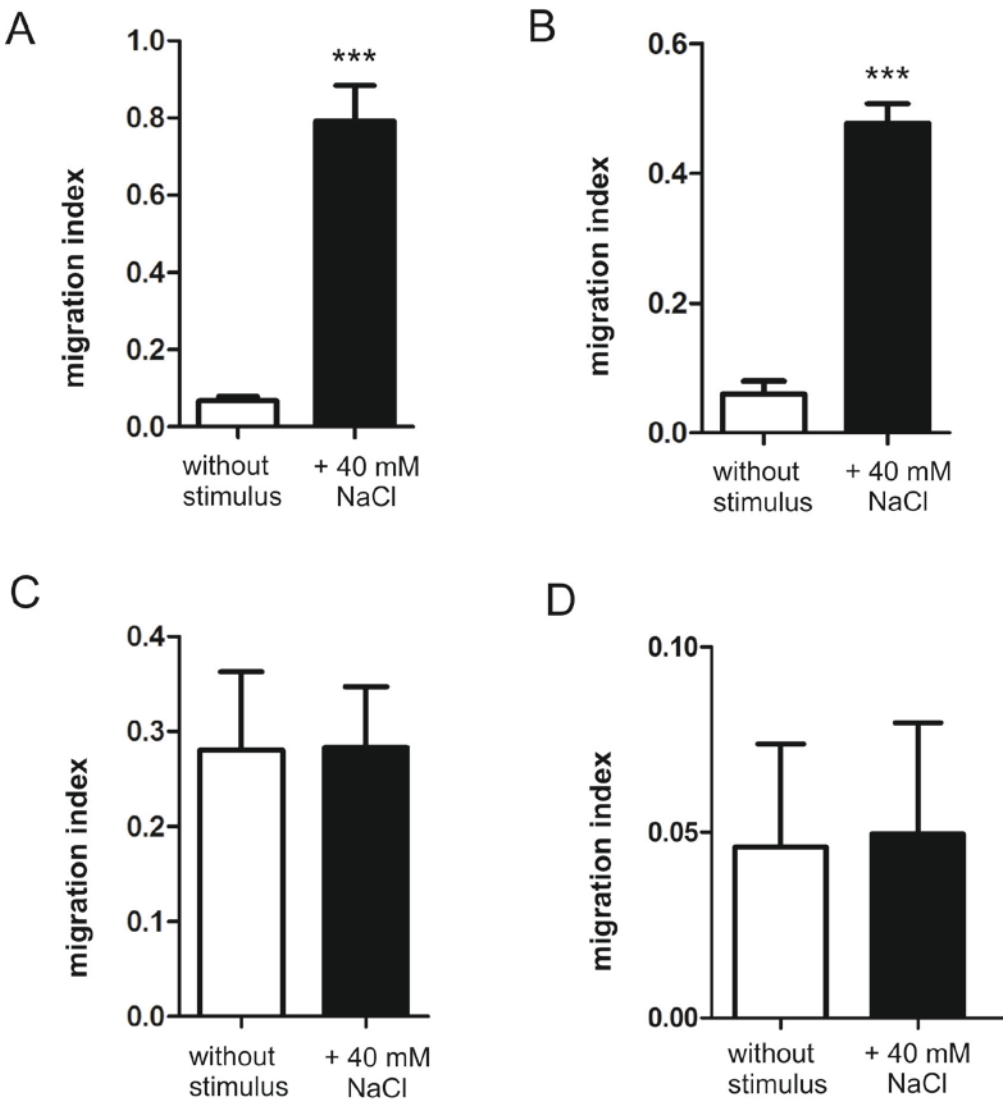


Figure 3-5: Migration of primary macrophages and dendritic cells toward excess NaCl

Transwell migration assay of BMDMs (A), peritoneal macrophages (B), mBMDCs matured with 200 ng/ml LPS (C) and unstimulated (immature) imBMDCs (D). The migration index was determined by the number of transmigrated cells in relation to cells transmigrated toward CCL2 (A) and (B), CCL19 (C) or CXCL12 (D). "+" indicates that the stimulus was added to the lower well of the transwell. Error bars indicate ± SD, n = 3, ***$p < 0.001$ as compared to cells without stimulus. (BMDM data by Thomas Quast, University of Bonn. Partly modified from Müller et al. (2013)).

3.2.5 Delayed migration kinetics of macrophages in the Na^+ gradient

Since RAW264.7 cells, BMDMs and peritoneal macrophages are slow migrating cells, it was unclear whether the NaCl gradient was stable for the duration of the transwell experiment. Therefore, media samples from both the upper and lower wells of the transwell assay were collected over 20 hours and the Na^+ concentration was determined by flame photometry. Figure 3-6 shows that although the Na^+ gradient started at Δ 40 mM Na^+, it dissolved quickly. 8 hours into the experiment, the difference between the upper and lower well was no more than Δ 10 mM Na^+. Interestingly, when cell migration kinetics of RAW264.7 cells was analyzed, cell migration did not significantly increase until 8 hours. This data indicates that per se slow migrating RAW264.7 macrophages display a delayed migration response to a chemotactic NaCl stimulus. However, at the time point when cell migration is initiated, the Na^+ concentration gradient has already disappeared.

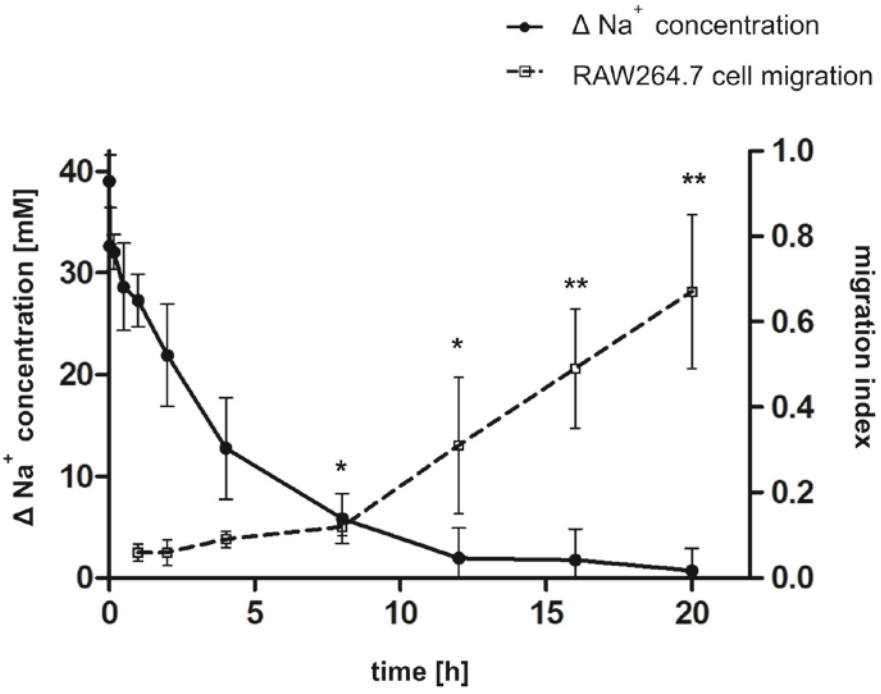


Figure 3-6: Migration kinetics of RAW624.7cells in the Na^+ gradient

Measurement of migration capacity toward excess 40 mM NaCl compared to CXCL12 (migration index) in a transwell migration assay over 20 hours. Δ Na^+ indicates the difference in sodium concentration between the upper and lower wells of the transwell chamber as determined by flame photometry. Migration index compares transmigrated cells to CXCL12-stimulated cells. (n = 3 in duplicates) p* < 0.05, p** < 0.01 as compared to migration at 1 min. Partly modified from Müller et al. (2013)

3.3 Investigation of lamellipodial dynamics in RAW264.7 cells: Excess NaCl has no direct effect on actin cytoskeleton reorganization

One of the first fundamental events in cell migration consists of reorganization of the actin cytoskeleton and formation of membrane protrusions (lamellipodia). Because of the delayed migration of RAW264.7 cells, the question remained if during salt-dependent chemotaxis, a fast reorganization of the actin cytoskeleton and cell polarization would also take place during the first minutes following application of the stimulus. This hypothesis was tested in RAW264.7 cells by phalloidin-based staining of F-actin following a stimulus of excess NaCl. Analysis by confocal laser-scanning microscopy revealed a high variability in cell morphology and actin cytoskeleton structure, which did not allow quantification of differences between salt-stimulated and untreated control cells (Figure 3-7 A). Moreover, no considerable changes in lamellipodia formation in RAW264.7 cells treated with the chemokines CXCL12, CCL2 or the complement factor C5a, respectively, could be observed.

In order to precisely investigate potential differences in actin cytoskeleton mobilization, a more sensitive method was applied that analyzed morphological changes and dynamics of lamellipodia formation in RAW264.7 cells by time-lapse phase contrast video microscopy (Methods 2.2.3.3). Velocity of membrane protrusion formation was determined by computer-assisted kymograph and line-scan analysis, using ImageJ software (Figure 3-7 B, C). Figure 3-7 shows that CCL2, CXCL12 and C5a significantly increased the mean lamellipodia formation velocity during 5 minutes following the applied stimuli. Particularly, CCL2 and CXCL12 increased lamellipodia formation to approximately 1-2 µm/min, while stimulation with C5a resulted in a response of about 1 µm/min. However, a stimulus of excess NaCl was unable to induce a significant change in lamellipodial dynamics compared to the untreated control. These results indicate that excess NaCl is not directly involved in the early events of actin cytoskeleton reorganization during salt-dependent chemotaxis.

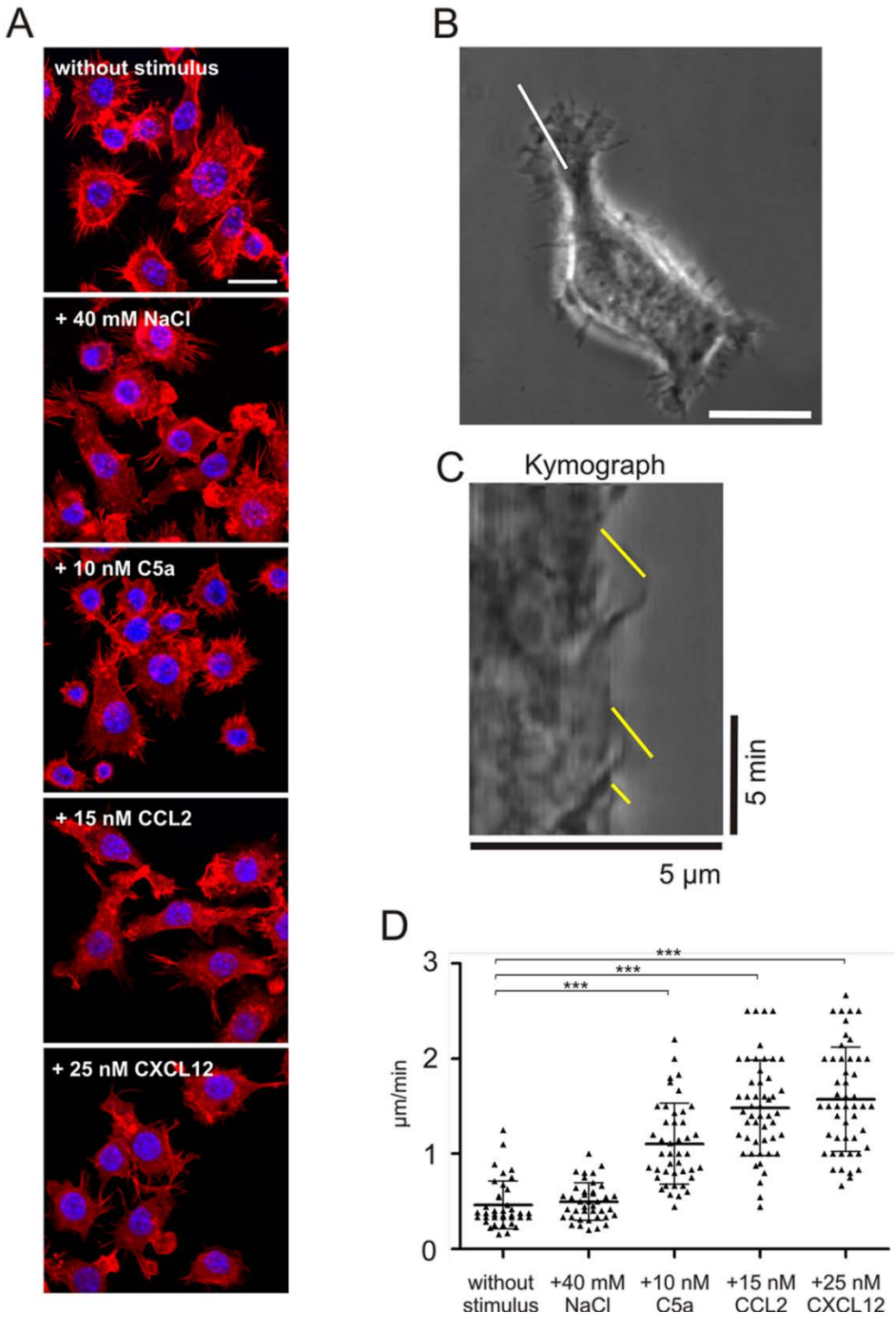


Figure 3-7: Excess NaCl does not influence dynamics of lamellipodia formation

Microscopic analysis of TRITC-Phalloidin stained F-actin (red) in unstimulated RAW264.7 cells and cells treated with excess 40 mM NaCl, 10 nM C5a, 15 nM CCL2, or 25 nM CXCL12 (A). Cell nuclei were visualized by DAPI staining (blue). Images were captured with an inverted confocal laser scanning microscope focused to the basal plasma membrane of the cells. Microscopic analysis of lamellipodial dynamics in RAW264.7 cells on a glass surface stimulated with excess 40 mM NaCl, 10 nM C5a, 15 nM CCL2, or 25 nM CXCL12 (B-D). A time-series of lamellipodial dynamics was created using phase contrast over a period of 5 min at 2 sec per frame. The area of interest in the polarized cell was marked on each

image by lines that cross the motile lamellipodium (white line in B), resulting the yellow line in the kymograph analysis (C). The slope of the yellow line was analyzed by line-scan analysis using Image J to determine the velocity of lamellipodia protrusion formation. Quantification of dynamics of lamellipodia formation in motile RAW264.7 cells (D). Three kymographs per cell were analyzed; each dot represents the value of one single kymograph (C). Data are representative for one experiment out of three. Error bars indicate ± SD. ***p < 0.001. Bars in microscopic images represent 10 µm (A, B).
Data by Thomas Quast, University of Bonn. (from Müller et al. (2013))

3.4 Salt-dependent chemotaxis of macrophages depends on protein biosynthesis and Gαi-coupled receptors

Since RAW264.7 macrophages migration occurred with a delay of about 8 hours into the transwell migration assay, the question arose whether this observation also reflects the time required for *de novo* protein synthesis preceding the migratory activity of the cells. In order to test if salt-dependent chemotaxis relies on *de novo* protein synthesis, the small molecule inhibitor cycloheximide was applied. In addition, the participation of Gαi-coupled GPCRs, to which also includes the family of chemokine receptors, was investigated by the presence of pertussis toxin during a transwell assay.

3.4.1 Cycloheximide inhibits salt-dependent chemotaxis in RAW264.7 cells

Cycloheximide is derived from the bacterium *Streptomyces griseus* and is widely used for *in vitro* experiments to arrest eukaryotic protein biosynthesis. In particular, binding of cycloheximide to the large ribosomal subunit inhibits the elongation phase of translation by blockade of tRNA translocation (Schneider-Poetsch et al. 2010).
RAW264.7 macrophages were preincubated in 10 µg/ml cycloheximide for one hour before the start of the transwell assay. Analysis of the migration behavior in the presence of cycloheximide showed that cycloheximide impaired migration of RAW264.7 cells toward excess NaCl, as well as migration toward the chemokine CXCL12 (Figure 3-8). Untreated cells had a normal migration capacity toward excess NaCl of approximately 50 % compared to migration toward the CXCL12 stimulus. These data indicate that *de novo* protein synthesis is a requirement for cell migration toward both chemokines like CXCL12 and toward a high NaCl concentration.

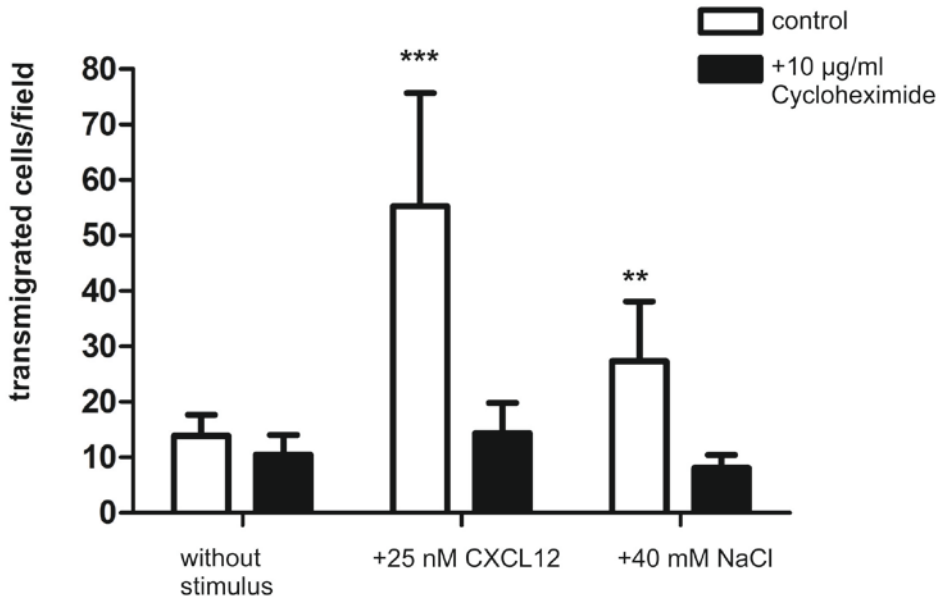


Figure 3-8: Migration behavior of RAW264.7 cells in the presence of cycloheximide

Transwell migration assay of RAW264.7 cells in the presence and absence of 10 µg/ml cycloheximide. "+" indicates that the stimulus was applied to the lower well of the transwell assay. (n = 3 in duplicates). Error bars indicate ± SD, p** < 0.05, p*** < 0.01 as compared to unstimulated control.

3.4.2 Salt-dependent chemotaxis of macrophages requires Gαi - coupled receptors

The extracellular concentration gradient of chemokines is conveyed into the cell by binding to chemokine receptors, which belong to the G-protein-coupled receptor (GPCR) family. According to literature (Katada and Ui 1982; Burns 1988), the exotoxin produced by the bacterium *Bordetella pertussis* selectively inhibits the heterotrimeric G-proteins of the chemokine receptor by ADP-ribosylation of the Gαi subunit.

In order to elucidate the role of Gαi-coupled GPCRs and in particular chemokine receptors in salt-dependent chemotaxis RAW264.7 macrophages were incubated with pertussis toxin during a transwell migration experiment. Migration behavior of RAW264.7 cells toward

CXCL12 and NaCl in the presence of 100 ng/ml or 200 ng/ml pertussis toxin was investigated in a transwell migration assay. Figure 3-9 shows that both migration toward CXCL12 and excess NaCl was inhibited to chemokinesis level of unstimulated cells with increasing concentrations of pertussis toxin. This result suggested that a pertussis toxin-sensitive GPCR with Gαi subunit is involved in salt-dependent chemotaxis.

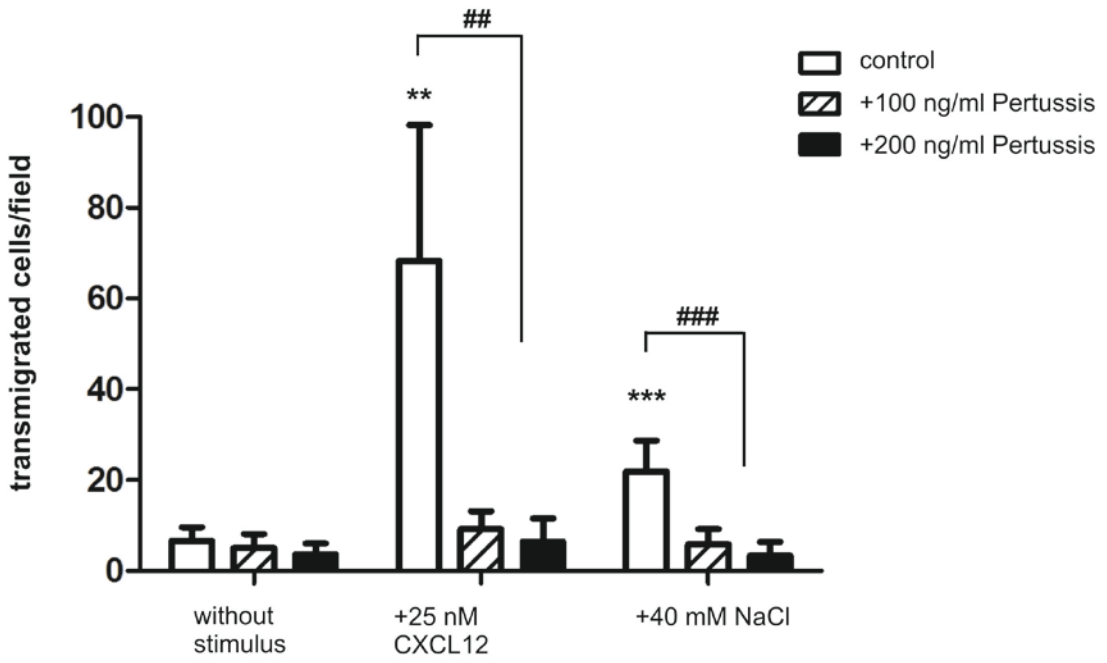


Figure 3-9: Migration of RAW264.7 cells toward NaCl is inhibited by pertussis toxin

Transwell migration assay of RAW264.7 cells in the presence of 100 ng/ml or 200 ng/ml pertussis toxin, respectively. “+” indicates that the stimulus was applied to the lower well of the transwell assay. (n = 3 in duplicates). Error bars indicate ± SD, p** < 0.05, p*** < 0.01 as compared to unstimulated control. # compares control with pertussis toxin treated cells under the same conditions.

3.5 Investigation of protein expression-dependent mechanisms in salt-dependent chemotaxis of macrophages

3.5.1 NaCl-dependent induction of CCL2 in RAW264.7 cells

The data so far indicated that a hypertonic NaCl stimulus had no direct effect on the early migration event of actin cytoskeleton reorganization. However, the delayed migration response of macrophages (see Figure 3-6) depended on protein expression machinery (see Figure 3-8), the participation of $G_{\alpha i}$-receptors and thus possibly also chemokine receptors (Figure 3-9). Since previous observations showed a NaCl-dependent expression of chemokines, especially CCL2 (Kostyk et al. 2006; Kojima et al. 2010), a possible involvement of salt-dependent CCL2 induction was investigated in RAW264.7 cells. First, CCL2 needed to be confirmed as a chemoattractant stimulus for RAW264.7 cells. Subsequently, influence of CCL2 in salt-dependent chemotaxis was studied by measuring a CCL2 concentration in supernatants of RAW264.7 cells following stimulation with NaCl. In a final approach, RAW274.7 cells were incubated with different concentrations of an anti-CCL2 antibody and an IgG isotype control during the transwell migration assay.

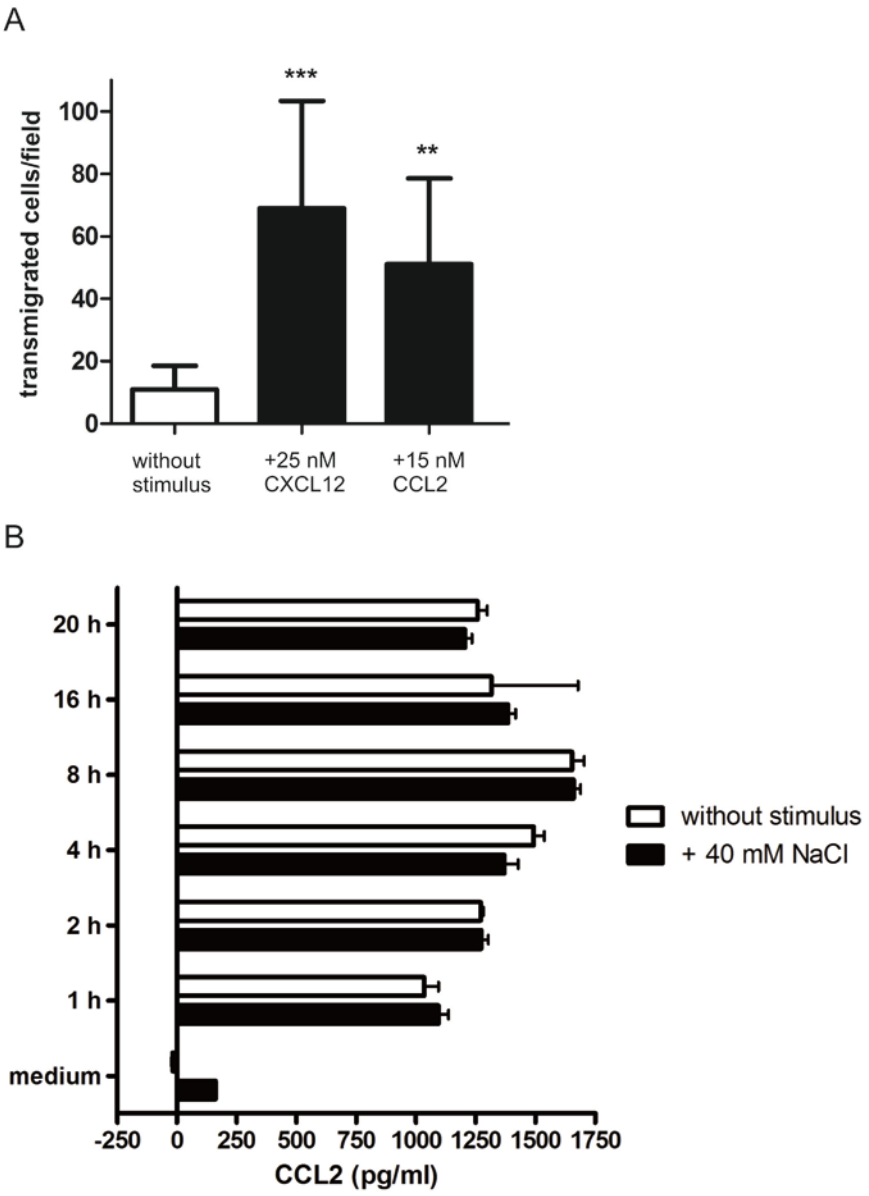


Figure 3-10: CCL2 production in RAW264.7 cells is not increased by excess NaCl

Transwell assay comparing migration of RAW264.7 cells toward 15 nM CCL2 to 25 nM CXCL12 (A). RAW264.7 cells were stimulated with additional 40 mM NaCl over 20 hours and CCL2 secretion in supernatants was determined by ELISA (B). "+" indicates that the stimulus was applied to the lower well of the transwell assay. Error bars indicate ± SD. p** < 0.05, p*** < 0.01 as compared to unstimulated control. ELISA analysis performed by Luisa Klotz and Stephanie Hucke, University of Münster. Partly modified from Müller et al. (2013).

3.5.1.1 RAW264.7 cells produce CCL2 independently from a NaCl stimulus

Before investigating a possible role of CCL2 in salt-dependent chemotaxis, the migration sensitivity of RAW264.7 cells toward a CCL2 stimulus needed to be confirmed. Figure 3-10 shows that RAW264.7 cells significantly migrated toward CCL2, although the migration capacity was 25% lower than compared to CXCL12. Next, the hypothesis was tested whether a NaCl-stimulus leads to an increased CCL2 production, as described by Kojima et al. (2010) in NRK52E cells. For this reason, RAW264.7 cells were stimulated with excess NaCl and the CCL2 concentration in the supernatant was determined by ELISA measurement over 20 hours. However, no difference between NaCl-stimulated and unstimluated RAW624.7 cells could be found at all investigated time points, even though the amount of CCL2 increased in both settings during the 20 hours (Figure 3-10 B). These results indicate that while RAW264.7 cells produce CCL2, it is not induced by a high NaCl concentration and therefore cannot be accounted for as responsible mechanism in salt-dependent chemotaxis.

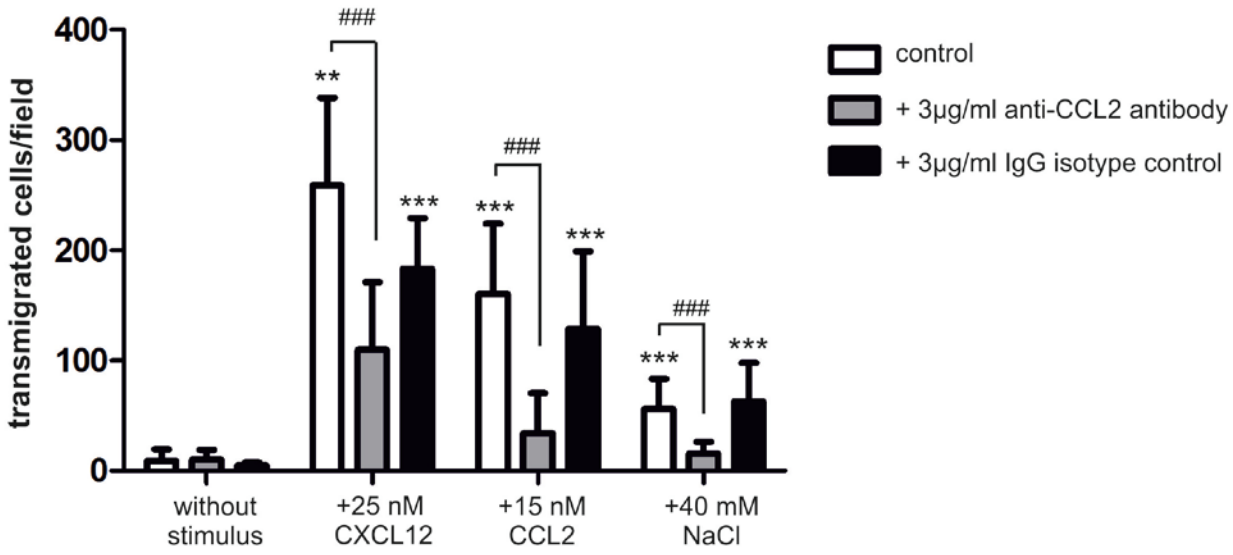


Figure 3-11: Inhibition of free CCL2 leads to decreased migration of RAW264.7 cells

Transwell migration assay of RAW264.7 cells in the presence of an anti-CCL2 antibody including IgG isotype control. "+" indicates that the stimulus was applied to the lower well of the transwell assay. Error bars indicate ± SD. p** < 0.05, p*** < 0.01 as compared to unstimulated control, # compares control cell migration with anti-CCL2 antibody treated cells under the same conditions.

3.5.1.2 Inhibition of free CCL2 leads to decreased migration of RAW264.7 cells

Although no direct influence of a NaCl stimulus on CCL2 production could be found in RAW264.7 cells, ELISA measurements revealed that CCL2 is present in increasing amounts during salt-dependent chemotaxis. Therefore, an antibody directed against murine CCL2 was used to investigate if migration toward a high NaCl concentration might depend on the presence of CCL2. Therefore, all cell reagents were incubated with 3 µg/ml anti-CCL2 antibody or IgG isotype control antibody, respectively, in order to deprive RAW264.7 cells of free CCL2 in the media. Figure 3-11 shows that in the presence of an anti-CCL2 antibody, migration toward 15 nM CCL2 was reduced to approximately 20 % compared to untreated control cells. Furthermore, migration toward CXCL12 decreased to approximately 40 %, while migration capacity toward excess NaCl was abrogated to 30 %. RAW264.7 cells that had been treated with the IgG isotype control showed no significant differences in migration behavior toward all applied stimuli. These data indicate that although CCL2-production is not induced by a high NaCl concentration in RAW264.6 cells, migration toward both excess NaCl and chemokines depends on the presence of free CCL2.

3.5.2 The role of TonEBP in salt-dependent chemotaxis of macrophages

Subjecting cells to osmotic stress such as a high NaCl concentration leads to expression of the osmosensitive transcription factor TonEBP. It has been shown previously that in addition to protecting cells from osmotic stress, TonEBP is involved in a variety of other essential cell functions, including cell migration (Jauliac et al. 2002; O'Connor et al. 2007) and transcription of chemokines (Kostyk et al. 2006; Kojima et al. 2010). In order to investigate a possible role of TonEBP in salt-dependent chemotaxis, cell migration of RAW264.7 cells with a stable TonEBP overexpression was analyzed. In addition, RNAi against TonEBP was established in RAW264.7 cells to study the influence of a reduced TonEBP protein level during salt-dependent chemotaxis. For further support of RNAi data, migration behavior of BMDMs lacking a functional TonEBP gene was investigated.

3.5.2.1 Overexpression of TonEBP in RAW264.7 cells does not affect cell migration toward excess NaCl

The first setting to assess whether TonEBP is involved in salt-dependent chemotaxis was investigation of migration behavior in RAW264.7 cells with a stable TonEBP overexpression,

which were a kind gift from Professor Jens Titze (University of Erlangen/ Vanderbilt University, USA). TonEBP protein expression was strongly increased following a high NaCl concentration (Figure 3-12 A). TonEBP overexpressing RAW264.7 cells displayed a high amount of TonEBP protein without an additional salt stimulus. However, upon stimulation with excess NaCl protein expression did not increase much further in these cells. Concerning migration behavior, if TonEBP played a positive role in salt-dependent chemotaxis, the migration response was expected to be much higher and earlier in TonEBP overexpressing than in wildtype RAW264.7 cells. Nevertheless, when comparing migration capacity of RAW264.7 wildtype cells to TonEBP overexpressing cells over 20 hours, no significant difference in migration kinetics was observed (Figure 3-12 B). These results indicate that TonEBP is not responsible for salt-dependent chemotaxis.

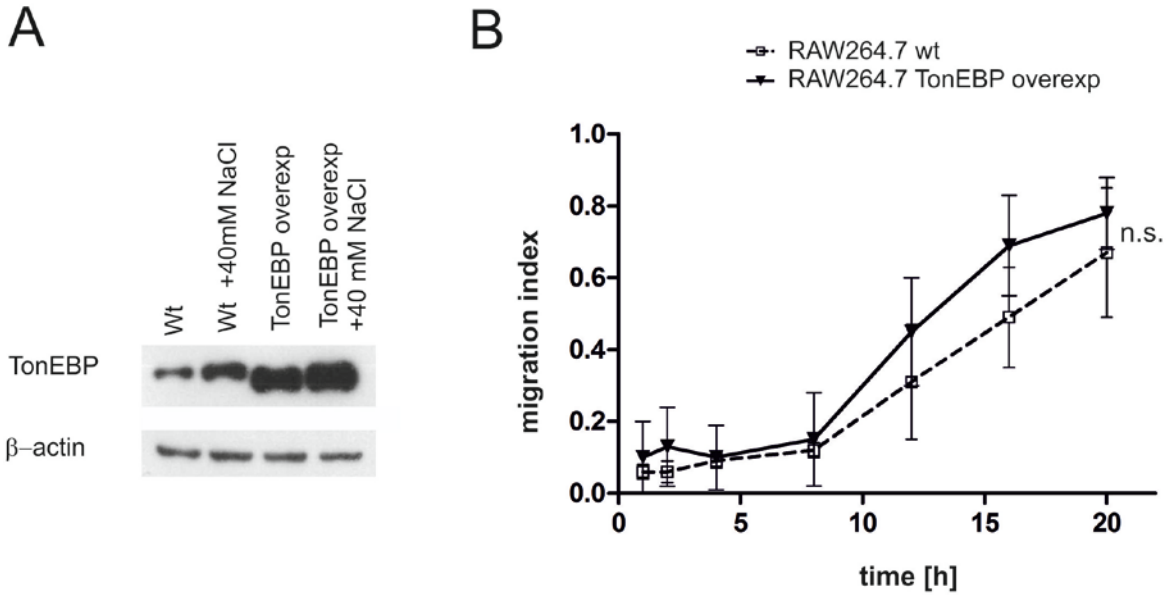


Figure 3-12: TonEBP overexpression has no influence on salt-dependent chemotaxis

Western Blot analysis of TonEBP protein expression in RAW264.7 wildtype and TonEBP-overexpressing cells (TonEBP overexp), with or without an excess 40 mM NaCl stimulus. β-actin protein expression as loading control (A). Kinetics of RAW264.7 wildtype and TonEBP overexpressing cells toward 40 mM excess NaCl over 20 hours, studied in transwell migration assays (B). Migration index indicates the number of transmigrated cells in relation to CXCL12 stimulated cells. mean ± SD from 3 experiments performed in duplicate. n.s. = non-significant. From Müller et al. (2013).

3.5.2.2 RNAi against TonEBP in RAW264.7 cells

RNAi against TonEBP was applied to investigate any effects of decreased TonEBP protein amount on salt-dependent chemotaxis. First, the best method for transfection of RAW264.7 cells with the adequate siRNA duplexes needed to be established. Therefore, transfer of siRNA into cells via different electroporation and lipofection protocols (see 2.2.5) was tested. The efficiency of the reduction in protein levels ("knockdown") was determined in Western Blot analysis following a 24 h stimulus of excess NaCl.

3.5.2.2a siRNA transfection using electroporation impairs migration in RAW264.7 cells

In order to bring siRNA duplexes for RNAi against TonEBP into RAW264.7 cells, various electroporation protocols were tested. At first, the most efficient of four different siRNAs (#9, #10, #11, #12, and a Mix of #9-12) was identified by varying concentrations of applied siRNA and incubation periods (24 to 72 h).

Initially, an exponential pulse protocol (400 V) was applied and after incubation for 72 h, TonEBP protein expression showed that 6 µg of siRNA#12 proved to be most effective, resulting in the strongest decrease in protein level (Figure 3-13 A, B). This effect was also visible after 48 h of incubation, but less prominent than after 72 h (data not shown). However, when cell migration was analyzed in transwell assays, changes in migration ability of electroporated RAW264.7 cells were found. Although cell viability and morphology was comparable to untreated cells (data not shown), migration toward excess NaCl was significantly decreased (Figure 3-13 C). In some cases, migration toward CXCL12 was also impaired. Figure 3-14 shows that RAW264.7 cells that had been electroporated without siRNA (mock) or non-silencing control siRNA (ns) were unable to migrate toward the excess NaCl stimulus, therefore indicating that electroporation conditions were responsible for disturbed cell migration. Since the migration data demonstrate that the applied electroporation conditions interfered with cell migration, a square wave protocol using two pulses of 1 ms and a pre-installed protocol for mammalian adherent cells (HL60) were tested.

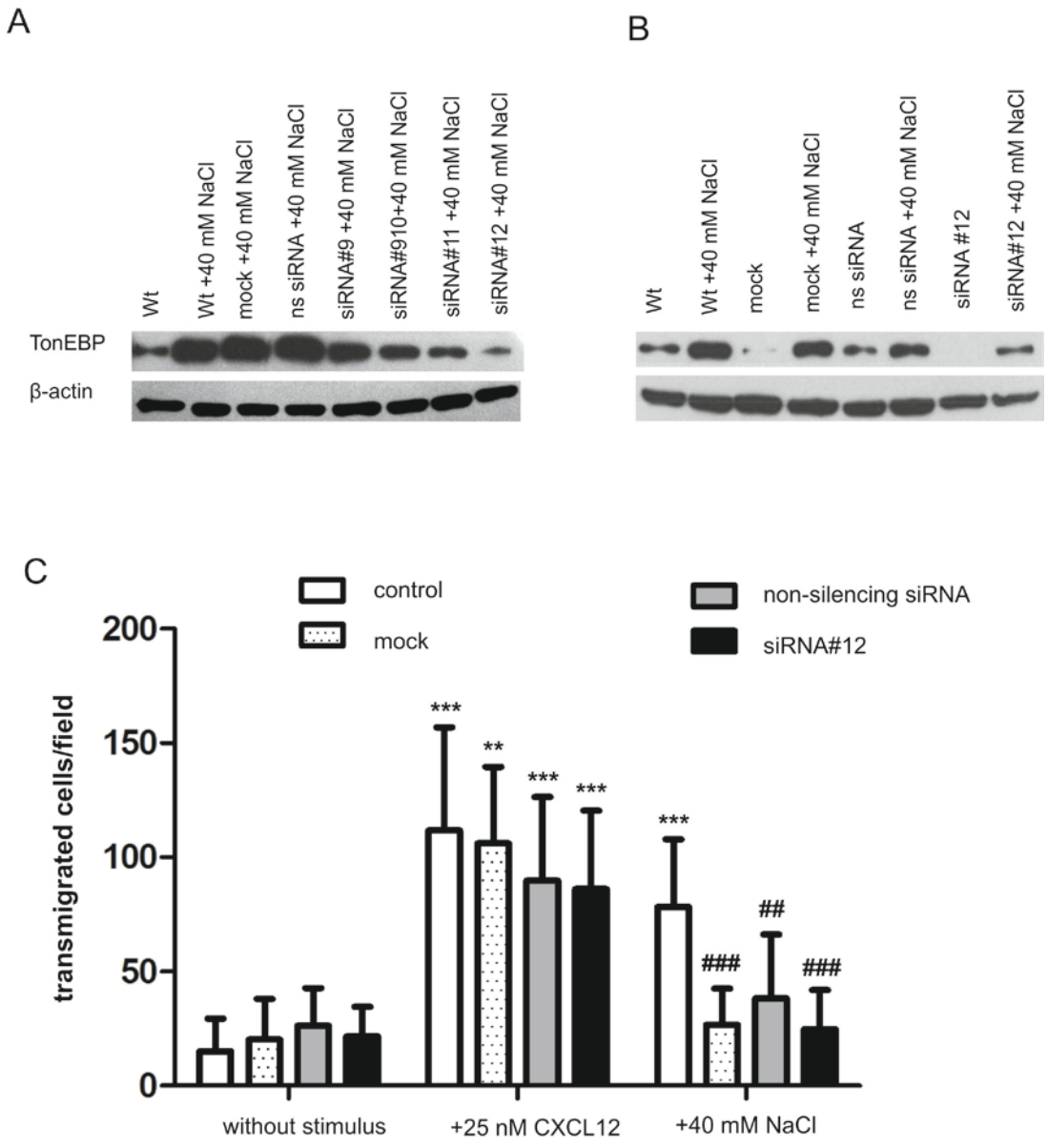


Figure 3-13: RNAi of TonEBP in RAW264.7 cells using exponential electroporation protocols

Western Blot analysis of TonEBP protein expression following transfection with TonEBP siRNA#9-12 (A) and with non-silencing (ns) siRNA or siRNA#12, respectively (B). Migration of electroporated RAW264.7 cells toward CXCL12 and NaCl in a transwell assay (C). For all experiments, electroporation with an exponential protocol of 400 V was applied. β-actin protein expression served as loading control (A, B). Cells were incubated for 72 h following transfection and stimulated with 40 mM excess NaCl for 24 h to assess TonEBP protein levels (A, B). mock = electroporation without siRNA transfection. migration data (C) exemplary for 5 individual experiments in duplicate. "+" indicates that the stimulus was added to the lower well of the transwell. p** < 0.01, p*** < 0.001 as compared to respective unstimulated cells. # relates cell migration of electroporated cells to migration of control cells toward the NaCl stimulus.

Under square wave conditions using two 1 ms long pulses, siRNA#12 and, to a lesser degree, siRNA#9 showed the highest knockdown ability in Western Blot analysis, while the HL60-protocol revealed the best reduction in TonEBP protein level with siRNA#12 (Figure 3-14 A, B). The fact that the siRNA Mix containing all four single siRNAs showed comparable results to TonEBP knockdown in RAW264.7 cells treated with siRNA#12 only, leads to the conclusion that siRNA#12 was the most effective component under these conditions in the mix. Again, effects of these square wave electroporation protocols on cell migration were investigated in transwell assays using RAW264.7 cells that had been electroporated without the presence of siRNA (mock) or in the presence of non-silencing siRNA (ns). Figure 3-14 shows that following both twice 1 ms pulse and HL60 protocol treatment, RAW264.7 cells without transfection of siRNA (mock) migrated toward CXCL12 comparable to untreated cells, but did not migrate toward NaCl. Subsequent to application of non-silencing control siRNA, RAW264.7 cells additionally failed to migrate toward CXCL12 with both protocols. These results emphasize that siRNA transfection by means of electroporation could not be used without altering cell migration of RAW264.7 cells.

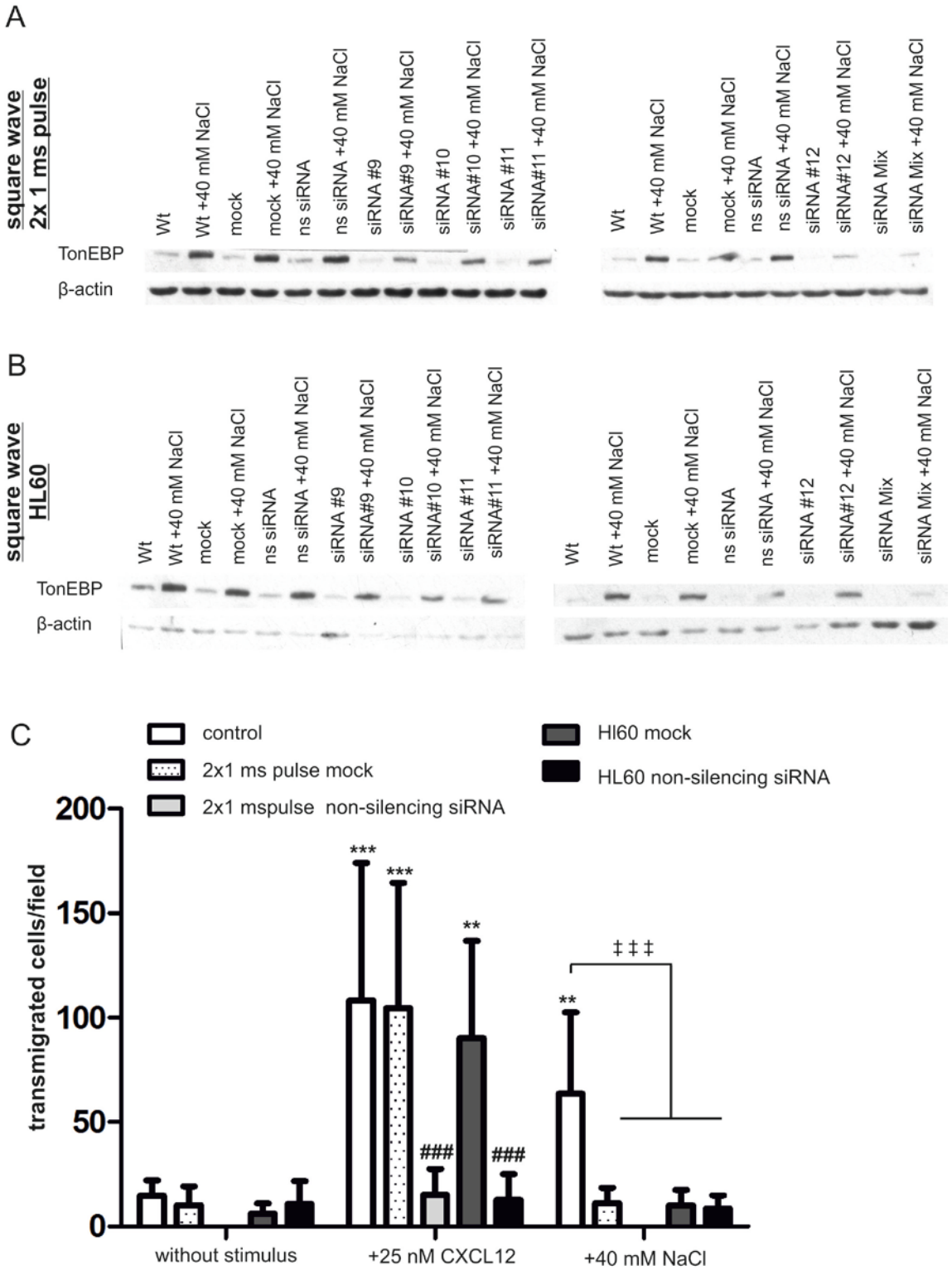


Figure 3-14: RNAi against TonEBP in RAW264.7 cells using square wave electroporation protocols

Western Blot analysis of TonEBP protein expression following electroporation according to square wave protocol with two 1ms pulses (A) and pre-installed HL60 protocol (B). Migration of electroporated RAW264.7 cells toward CXCL12 and NaCl in a transwell assay (C). Cells were incubated for 72 h following transfection and stimulated with 40 mM excess NaCl for

24 h to assess TonEBP protein levels (A,B). β-actin protein expression served as loading control (A, B). mock = electroporation without siRNA transfection. ns = non-specific siRNA. "+" indicates that the stimulus was added to the lower well of the transwell. p** < 0.01, p*** < 0.001 as compared to respective unstimulated cells. # relates cell migration of electroporated cells to migration of control cells toward the CXCL12 stimulus. ‡ compares transmigrated electroporated cells to migration of control cells toward the excess NaCl stimulus.

3.5.2.2b Optimization of siRNA transfection using lipofection in RAW264.7 cells

In order to establish efficient siRNA transfection without disturbed cell migration, different lipofection agents were tested with varying reagent and siRNA concentrations for different incubation periods. First, RAW264.7 cells were treated with FuGENE transfection reagent as described in the protocol for siRNA transfection by Kawaai et al. (2008). Western Blot analysis of TonEBP protein levels following 48 h and 72 h of incubation showed no significant changes compared to controls with any of the applied siRNAs (Figure 3-15 A). Using Oligofectamine reagent according to the protocol provided by the manufacturer, siRNA#12 led to a reduced TonEBP protein level after 72 h, but knockdown efficiency was still improvable (Figure 3-15 B).

Finally, lipofectamine 2000 reagent revealed a significant knockdown for TonEBP protein with both siRNA#9 and siRNA#12 after 48 h and 72 h of incubation, with the highest knockdown achieved using siRNA#9 after 72 h incubation (Figure 3-15 C). Investigation of cell migration following lipofectamine 2000 treatment showed no differences between control, lipofectamine-only treated (mock) and RAW264.7 cells treated with non-silencing siRNA. Therefore, transfection by lipofectamine 2000 reagent proved to be the most adequate method for transfection of TonEBP siRNA in RAW264.7 cells without any influence on cell migration behavior.

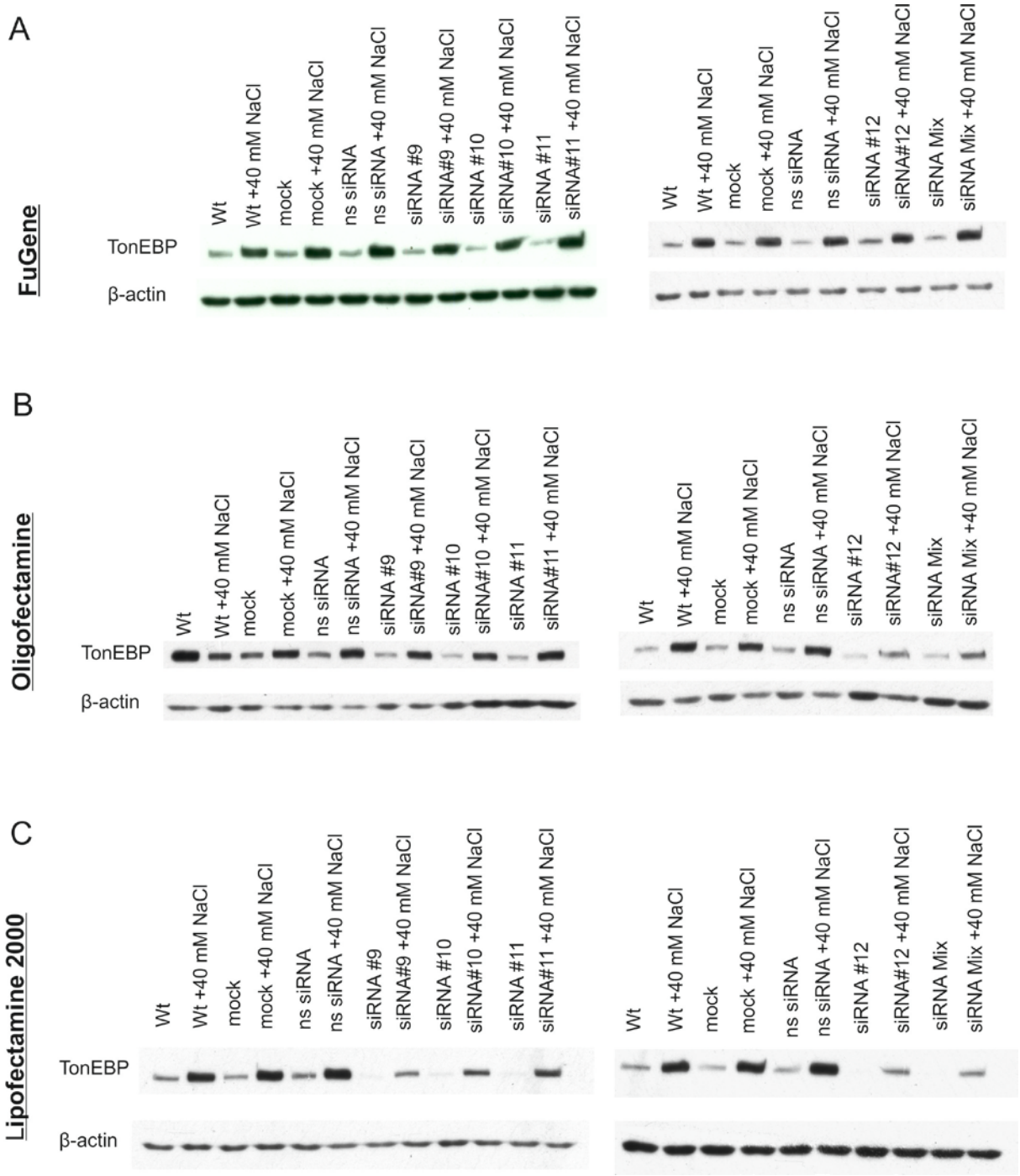


Figure 3-15: RNAi against TonEBP using lipofection in RAW264.7 cells

Western blot analysis of TonEBP protein expression following transfection of TonEBP siRNA using FuGene (A), Oligofectamine (B) and Lipofectamine 2000 (C) in RAW264.7 cells after 72 h of incubation. Cells were stimulated with 40 mM excess NaCl for 24 h to assess TonEBP protein levels. β-actin protein expression served as loading control. mock = transfection without siRNA. ns = non-specific siRNA.

3.5.2.3 Migration following RNAi against TonEBP in RAW264.7 cells and in BMDMs derived from Ton(flox/flox)LysM(wt/cre) mice

After establishing RNAi against TonEBP in RAW264.7 cells, the influence of a decreased TonEBP protein amount on salt-dependent chemotaxis was investigated in a transwell assay. Western blot analysis revealed a significant decrease in TonEBP protein following transfection of TonEBP siRNA #9 with lipofectamine 2000 (Figure 3-16 A). However, cell migration toward CXCL12 and excess NaCl of cells treated with TonEBP siRNA was similar to controls (Figure 3-16 B). No significant difference could be found in migration behavior of untreated RAW264.7 cells, cells that were transfected with non-silencing control siRNA (ns), and cells that were subjected to lipofectamine reagent only (mock).

These results could be confirmed by investigation of cell migration of BMDMs derived from mice in which the TonEBP gene was specifically not functional in myeloid cells (Ton(flox/flox)LysM(wt/cre). The material was generously shared by Jens Titze's group.

The absence of TonEBP protein in BMDMs isolated from these mice was confirmed by Western blot analysis (Figure 3-16 C). Figure 3-16 D shows that BMDMs in which the TonEBP gene was disrupted showed no differences in cell migration behavior compared to BMDMs derived from knock-out construct control mice (Ton(flox/flox)LysM(wt/wt)) and wildtype mice. Therefore, both RNAi and functional knockout experiments illustrate that TonEBP has no function in salt-dependent chemotaxis of macrophages.

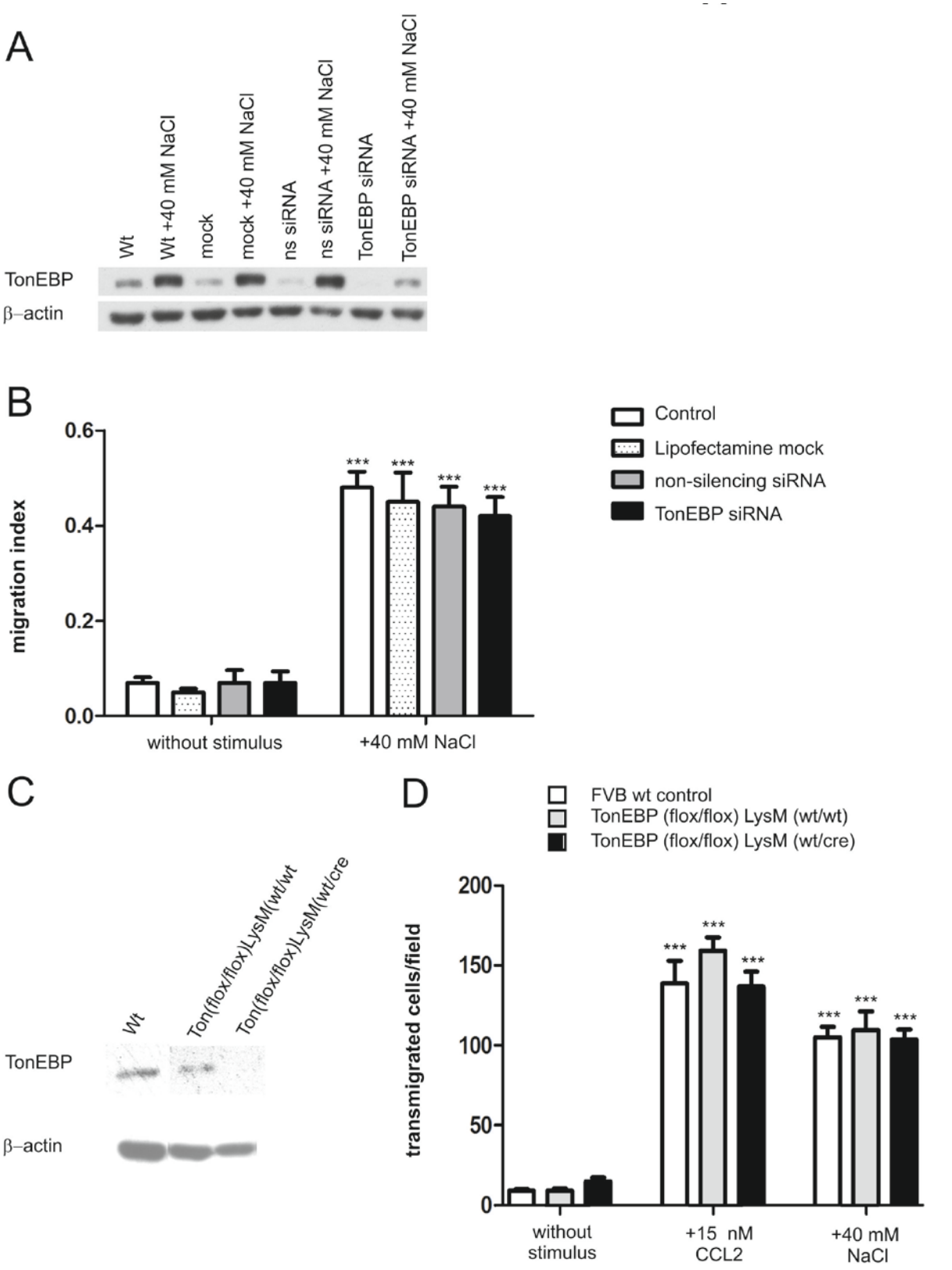


Figure 3-16: TonEBP is not involved in salt-dependent chemotaxis of macrophages

Western blot analysis of TonEBP protein expression (A) and transwell migration assay (B) of RAW264.7 cells transfected with lipofectamine 2000. Lipofection was incubated for 72 h days including a 40 mM NaCl stimulus for 24 h to assess TonEBP protein expression. Western

blot analysis of TonEBP expression in BMDMs derived from FVB wildtype control mice (wt), TonEBP(flox/flox)LysM(wt/wt) control mice and TonEBP(flox/flox)LysM(wt/cre) myeloid cell population specific knockout mice following stimulation with excess 40 mM NaCl (C). Transwell migration assay of the aforementioned BMDMs toward 15 nM CCL2 or 40 mM NaCl,(D). migration index shows transmigrated RAW264.7 cells toward excess NaCl compared to migration toward a 25 nM CXCL12 stimulus (B). mock = transfection without siRNA, ns = non-specific siRNA (B). β-actin protein expression was used as a loading control (A, C). ***$p < 0.001$ as compared to cells without stimulus. BMDM migration data by Thomas Quast. Partly modified from Müller et al. (2013).

3.5.3 A possible role for Leukotriene B4 in salt-dependent chemotaxis of macrophages

Recently published data showed that following autocrine secretion, Leukotriene B4 (LTB4) exerts a strong chemotactic response in neutrophils (Chtanova et al. 2008; Lämmermann et al. 2013). Since RAW264.7 cells were described to produce small amounts of LTB4 upon stimulation with LPS (Hong et al. 2004; Choi et al. 2011), the hypothesis was tested whether salt-dependent chemotaxis of macrophages could rely on a similar mechanism. For that reason, LTB4 production in RAW264.7 macrophages was determined by ELISA following stimulation with 40 mM NaCl over 20 hours.

Figure 3-17 shows that although LPS-stimulated RAW264.7 cells produced the highest amount of LTB4, no differences between NaCl-stimulated and control cells could be detected, since all measured data showed no significant differences compared to the media controls. In addition, stimulation with CCL2 or CXCL12, respectively, did not induce LTB4 production. According to the manufacturer, the measured LTB4 concentration was below the given detection limit of 13 pg/ml and therefore out of the linear area of the standard curve, although internal assay controls were within the optimal parameters. Nevertheless, it can be concluded that NaCl has no influence on LTB4 expression in RAW264.7 cells and seems therefore unlikely to be involved in salt-dependent chemotaxis.

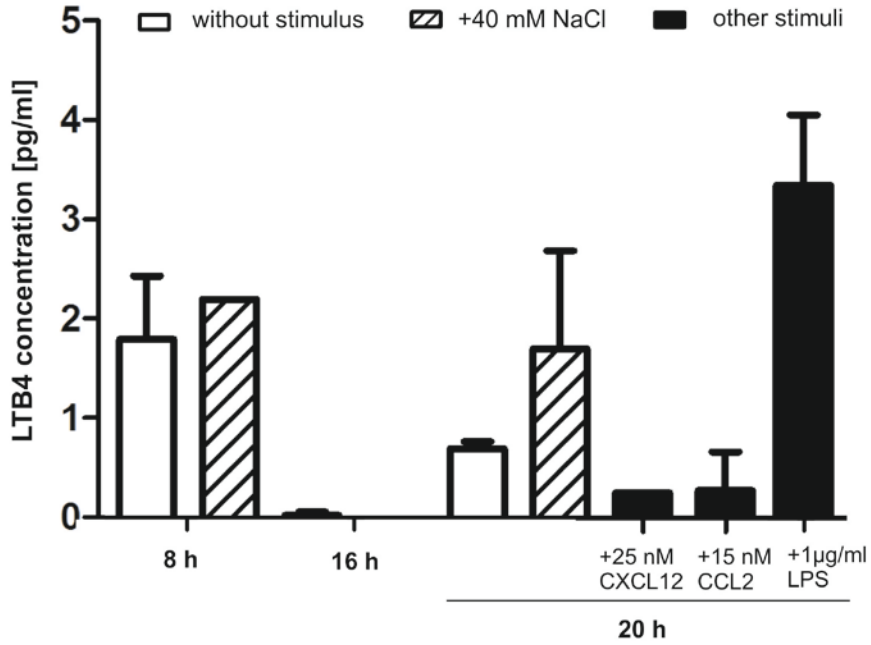


Figure 3-17: LTB4 production in RAW264.7 cells is independent from a NaCl-stimulus

ELISA measurement of LTB4 concentration in supernatants of RAW264.7 cells following stimulation with excess 40 mM NaCl, 25 nM CXCL12, 15 nM CCL2 or 1 µg/ml LPS, respectively, for 20 hours. All data points from duplicates.

4 Discussion

4.1 Macrophages recognize NaCl as a chemoattractive signal

This work postulates the novel concept of salt-dependent chemotaxis of macrophages, in which the migration response is specifically induced by a hypertonic NaCl stimulus. *In vitro* cell migration experiments revealed that RAW264.7 cells perceive a hypertonic NaCl stimulus as a chemoattractive signal. Stimuli of the osmolytes urea or mannitol, that raised osmolality to the same level of about 400 mosm/ kgH_2O as the NaCl stimulus, were unable to elicit cell migration. Therefore, hypertonicity and hyperosmolarity in general are not responsible for salt-dependent chemotaxis. The specificity of the salt-dependent migration response was further emphasized by migration data from LPS-activated BMDMs and migration of RAW264.7 cells in the presence of PmxB. These experiments excluded an underlying unspecific activation by endotoxin of the migrating macrophages, as described by Tajima et al. (2008).

In addition, cell migration occurred only into the direction of an increasing NaCl-gradient, since a decreasing gradient and the absence of a concentration gradient of excess NaCl did not induce a migration response in RAW264.7 cells. This directional chemotactic, instead of random chemokinetic activity was also found in the same cell type in the migration response toward CCL2 (Kanellis et al. 2004; Tajima et al. 2008; Nishimura et al. 2009)).

Salt-dependent chemotaxis was further characterized by a dose-dependent migration response toward various excess NaCl concentrations. The maximum migration response was observed toward excess 40 mM NaCl, which is in line with *in vivo* data described by Machnik et al. (2009), who found this concentration difference in skin electrolytes in rats receiving a high salt diet.

However, for increasing NaCl concentrations starting from excess 60 mM NaCl, it was impossible to distinguish between effects of reduced cell viability and a possible dose-dependent decrease in migration sensitivity. A decrease in cell viability is likely, as increased hypertonicity leads to cell shrinkage and an osmotic stress response (Gastaldello et al. 2008). A possible induction of cell death by apoptosis was demonstrated by Malek et al. (1998) in endothelial cells following treatment with 300 mosm/ kgH_2O NaCl, mannitol and urea. This probably occurs due to multimerization of cell surface receptors and subsequent activation of c-Jun amino-terminal protein kinase (JNK) by osmotic stress, as shown in HeLa cells (Rosette and Karin 1996). Nevertheless, an environment of 400 mosm/ kgH_2O following

the excess 40 mM NaCl stimulus does not compromise RAW264.7 cells or the used primary macrophages, as confirmed by different cell viability experiments.

In vivo, Na^+ is bound by negatively charged polymerized glycosaminoglycans (GAG) in the interstitium of the skin (Titze et al. 2004; Schafflhuber et al. 2007). This osmotically inactive binding acts as a reservoir for Na^+ and creates a local hypertonic microenvironment (Wiig et al. 2013). In the present work, investigation of salt-dependent chemotaxis has been restricted to the migration of macrophages toward free Na^+ in the culture medium by transwell migration assays. In order to use a chemotaxis assay that resembles the situation in the interstitium *in vivo*, macrophage migration toward Na^+ that is bound to glycosaminoglycans should be studied in a follow-up study.

4.2 Cell-specificity of salt-dependent chemotaxis in macrophages

The question arose whether other motile cells, or cells closely related to macrophages such as dendritic cells could also perform salt-dependent chemotaxis or if it defines a specific characteristic of macrophages. For that reason, cell migration of bone marrow-derived macrophages and dendritic cells toward an excess NaCl concentration was evaluated. Both peritoneal primary macrophages and BMDMs showed that migration toward a hypertonic NaCl stimulus was not exclusive to RAW264.7 macrophages. However, not all highly motile cells of the myeloid lineage recognize excess NaCl as a chemoattractive signal, since highly motile LPS-matured BMDCs did not migrate toward a NaCl stimulus. Therefore, it seems that salt-dependent chemotaxis is limited to macrophages and is not a common feature of migratory cells.

On the other hand, it cannot be excluded that other types of motile immune cells or even non-immune cells that were not tested might perform salt-dependent chemotaxis. Immune cell candidates are for example neutrophil granulocytes, or cells of the lymphoid lineage such as T cells. However, for neutrophils it is known that a hyperosmolar environment interferes with several cell functions including cell migration (Rosengren et al. 1994), exocytosis of granules (Kazilek et al. 1988; Rizoli et al. 2000) and phagocytosis (Hampton et al. 1994). Rosengren et al. (1994) reported that cell migration of human neutrophils toward bacterial peptides (fMLP) was completely abrogated in hyperosmolar conditions from 350 to 410 mosm/ kgH_2O. In contrast, treatment with additional 40 mM NaCl significantly enhanced proliferation rate and interleukin-2 expression in mitogen-stimulated human PMBCs and Jurkat T cells (Junger et al. 1994, 1997). Moreover, recent results demonstrated that

following stimulation with 40 mM NaCl, naïve $CD4^+$ T cells show an increased differentiation into an inflammatory T_H17 phenotype that was mediated by p38/MAPK, TonEBP and SGK1 (Kleinewietfeld et al. 2013). Physiologically, these results imply a correlation of high salt-intake and autoimmunity.

Even though a distinct function in hypertonic environments like regulating salt balance for macrophages or presumably T_H17-differentiation for naïve T cells has not yet been described for other cell types, beneficial effects of hypertonicity on other immune cells, especially T-cells have been found. In this context, it needs further elucidation whether other cells in addition to macrophages might also be attracted to a high salt concentration.

4.3 Secondary factors might contribute to salt- dependent chemotaxis

4.3.1 The osmoprotective transcription factor TonEBP

The investigation of the early process of lamellipodia formation in RAW264.7 cell migration revealed that an excess NaCl stimulus does not immediately influence cell polarization and formation of membrane protrusions. Therefore, the chemotactic signaling and actin cytoskeleton reorganization following the hypertonic NaCl stimulus is likely conveyed by secondary factors and is not directly initialized by excess NaCl.

A promising candidate is the osmoprotective transcription factor TonEBP, being the only known mammalian osmosensitive protein which is induced by a hypertonic salt stimulus (Küper et al. 2007). The expression of TonEBP takes about 8 hours, which was speculated to correlate with the observed delayed migration of the macrophages. Of note, TonEBP has also been associated with a variety of other cell functions, including cell migration. Jauliac et al. (2002) described the role of TonEBP in integrin-dependent migration and invasion of breast carcinoma cells, while O'Connor et al. (2007) observed a positive effect on murine myoblast migration by TonEBP-dependent regulation of cysteine-rich CCN (connective tissue growth factor) matrix protein Cyr61. In addition, TonEBP-deficient BMDMs from TonEBP +/− ApoE−/− mice showed a decreased migration toward M-CSF (Halterman et al. 2012). However, in the present work migration toward CXCL12 or excess NaCl was not altered in RAW264.7 cells with a stable TonEBP-overexpression or following RNAi against TonEBP.

Subsequently, the results gained from RNAi against TonEBP were confirmed by cell migration studies of BMDMs from TonEBP(flox/flox)LysM(cre/wt) mice that lacked a functional TonEBP gene specifically in the myeloid cell population. Migration of these cells

was not significantly different from BMDMs derived from knockout-construct control (TonEBP(flox/flox)LysM(wt/wt)) or wildtype mice.

Data from Wiig et al. (2013) support a nonessential role of TonEBP in cell migration, since the infiltration of macrophages in the skin of TonEBP(flox/flox)LysM(cre/wt) mice following a high salt diet was not changed. In contrast, several non-migratory macrophage functions like control of electrolyte composition and VEGF-C expression were affected in these mice (Wiig et al. 2013). Altogether, although TonEBP is important for the osmotic stress response and the effective clearance of excess sodium *in vivo*, it is not involved in the induction of cell migration during salt-dependent chemotaxis.

4.3.2 Autocrine/paracrine loops of chemokine/ chemoattractant soluble factors

A hypertonic NaCl gradient was essential for migration toward NaCl, but cells did not start to migrate until the gradient was reduced to less than 10 mM excess NaCl by diffusion after 8 hours. This observation may be interpreted as an initial triggering of the migratory response of cells by the NaCl gradient, the former of which persists even if the NaCl concentration is equilibrated as was described earlier for CSF-1 dependent migration of macrophages *in vitro* (Webb et al. 1996).

On the other hand, the delayed migration of macrophages probably reflects the requirement to perform protein biosynthesis in order to produce pro-migratory or even autocrine/paracrine acting soluble factors. The inhibited migration of cycloheximide-treated RAW264.7 cells confirms that protein biosynthesis is needed for salt-dependent chemotaxis. Although the molecular mechanisms for salt-dependent chemotaxis remain to be determined, the results suggest that secondary events in the cell account for a delayed migration response which requires protein synthesis.

It has been shown by several studies that NRK52E cells and human PMBCs produce chemokines (CCL2) and cytokines (IL-8) following a hypertonic NaCl stimulus (Shapiro and Dinarello 1995; Kojima et al. 2010). This production was without simultaneous expression of inflammation marker IL-1α (Shapiro and Dinarello 1995) and significantly increased after 8 to 9 hours, which is similar to the kinetics of salt-dependent chemotaxis. While RAW264.7 macrophages migrated toward a CCL2 stimulus and produced CCL2, this chemokine was not induced by excess NaCl. A direct autocrine or paracrine function for CCL2 in salt-dependent chemotaxis can therefore be excluded. However, migration experiments in the presence of an anti-CCL2 antibody demonstrated that both migration toward CXCL12 as well

as toward the salt stimulus depended on free CCL2, which is produced NaCl-independently during the migration process. These results indicate an indirect effect of free CCL2 during both salt-dependent and chemokine-related chemotaxis.

Alternatively, it can be speculated that other chemokines with autocrine or paracrine function could be secreted by a population of initially migrating cells followed by a delayed, but amplified migration of remaining cells. This migration dynamic of “pioneering” cluster forming cells which later attract a massive influx of cells into that cluster has been described for neutrophils swarms during infection or in wounded tissues (Chtanova et al. 2008; Ng et al. 2011; Lämmermann et al. 2013). In detail, during the first phase (< 15 min) single neutrophils that are close to the lesion respond to chemotactic short-range signals and migrate (Lämmermann et al. 2013). The authors observed that LTB4 released by dying neutrophils is the responsible factor for this amplification of the initial chemoattractive signal in the late phase of the swarming migration behavior. LTB4 has been shown to facilitate neutrophil migration by autocrine and paracrine secretion toward fMLP (Afonso et al. 2012). Therefore, LTB4 was considered as a chemoattractive candidate in salt-dependent chemotaxis.

Regarding macrophages, RAW264.7 cells produced LTB4 following stimulation with LPS in agreement with literature (Choi et al. 2011). However, the LTB4 amounts were very low (3.5 pg compared to at least 15 pg in neutrophils (Oyoshi et al. 2012)) and thereby out of the linear range of the applied ELISA assay. In this work, no influence of excess NaCl on LTB4 production in RAW264.7 cells could be found. However, this result should be proved with an assay specifically suited for small amounts of LTB4.
Since only CCL2 was investigated for NaCl-dependent induction, it is possible that a hypertonic NaCl stimulus leads to secretion of other chemokines or chemoattractive factors by macrophages. For example, CXCL12 has been shown to act in a NFκB-dependent autocrine loop to ensure migration toward HMGB1 in BMDMs and mouse embryo fibroblasts (Kew et al. 2012).

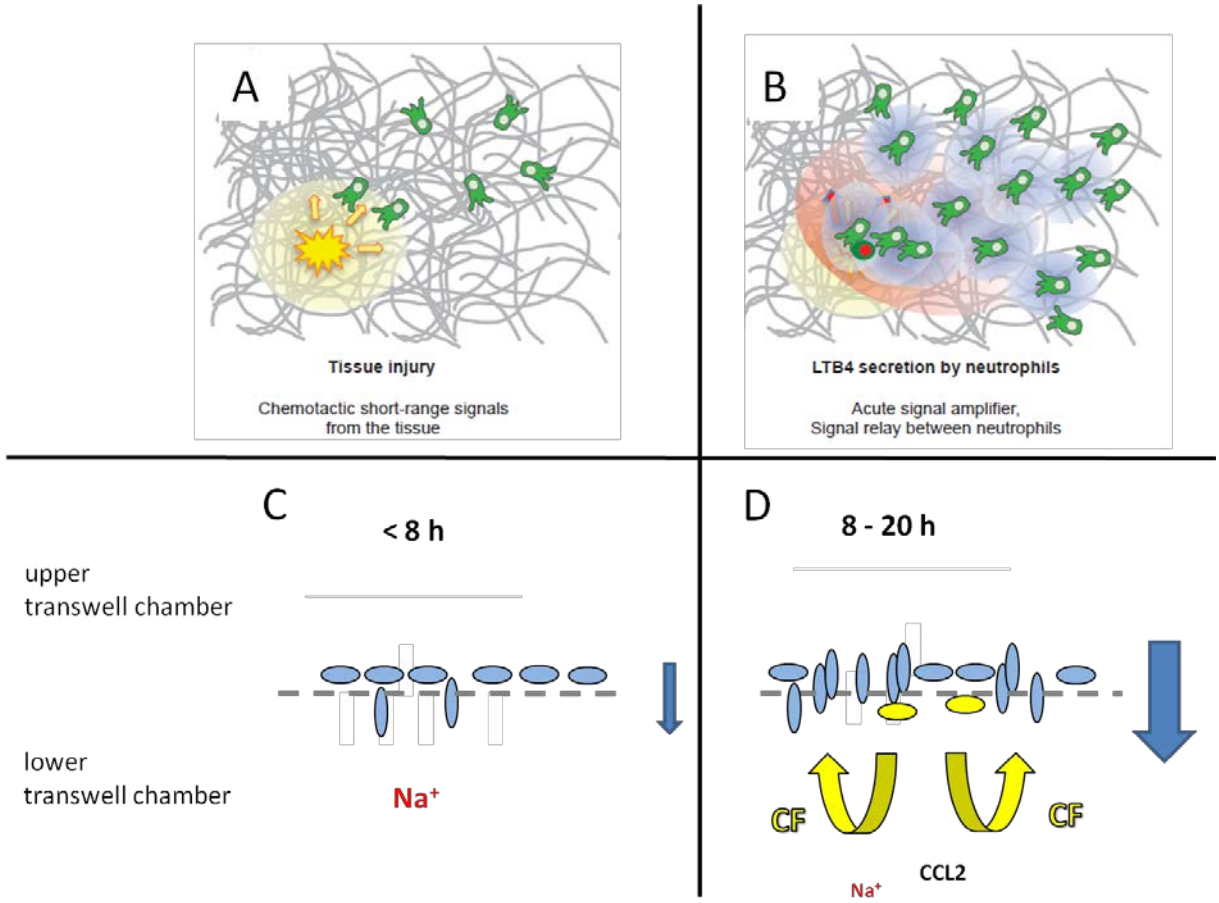


Figure 4-1: Comparison of LTB4-driven neutrophil swarming to events in salt-dependent chemotaxis

Pioneering neutrophil migration through the interstitium toward a tissue lesion (A), and following amplification of migration by autocrine and paracrine secretion of LTB4 (B). ”Pioneering” macrophages migrating toward the hypertonic Na^+ stimulus in a transwell assay (C). Possible secretion (yellow arrows) of a soluble autocrine/paracrine chemotactic factor (CF) leads to enhanced migration of macrophages even after the initial Na^+ gradient has dissolved. The presence of CCL2 seems to be required (D).
Blue arrows indicate migration intensity of macrophages toward the lower transwell chamber.
A, B modified from Lämmermann et al. (2013).

Figure 4-1 provides a mechanism for salt-dependent chemotaxis that is similar to swarming migration behavior of neutrophils. Comparable to the individual migration of neutrophils toward a lesion (A), single macrophages migrate toward the hypertonic Na^+ stimulus during the first 8 hours of the transwell migration assay (C). While autocrine and paracrine secretion of LTB4 massively enhances neutrophil migration (B), salt-dependent chemotaxis is likely to depend on the autocrine/paracrine secretion of a yet unknown chemotactic acting factor (CF) (D). This model also takes account of the fact that macrophage migration toward the

hypertonic NaCl-stimulus is increased by a time when the original Na^+ gradient has already dissolved in the transwell assay.

If the migration of individual pioneering macrophages only depends on the hypertonic Na^+ environment, the following increased migration into the lower transwell chamber occurs primarily toward the autocrine/paracrine acting chemotactic factor. This hypothesis could be tested by transferring the upper transwell chambers including the pioneering migrated macrophages into fresh unstimulated culture medium after 8 hours. Additionally, fresh, differently fluorescence-labelled cells could be placed in the upper transwell chamber after 16 to 20 hours to study whether the salt-dependent chemotaxis is faster when the pioneering macrophages already produce the presumed chemotactic factor.

Because salt-dependent chemotaxis of RAW264.7 cells was abrogated in the same way as migration toward CXCL12 in the presence of pertussis toxin, the participation of a Gαi-coupled GPCR in salt-dependent chemotaxis can be assumed. The majority of chemokine receptors are Gαi protein-coupled receptors; therefore it is likely that a chemokine receptor is involved. However, the Gαi-coupled GPCR family is vast and the pertussis toxin results could also point to a different non-chemokine receptor using Gαi, for instance a prostaglandine receptor.

All in all, to further elucidate the underlying mechanisms of salt-dependent chemotaxis, the following steps could be undertaken: A broad investigation of known soluble chemotactic factors that attract macrophages could be performed with supernatants of NaCl-stimulated macrophages or supernatants from the transwell migration assay. Alternatively, the pioneering migrating cells could be isolated and screened for expression of chemotactic factors, for instance using microarray analysis.

4.3.3 A potential role for ion channels and transporters

Since the initial recognition and signal transduction of high extracellular sodium is unclear, the possible involvement of a potential member of the sodium ion channel family could be proposed. Ion channels and transporters play a prominent role in cell migration by modulating cell volume, the actin cytoskeleton and cell polarization (Schwab 2001). For example, the epithelial Na^+ channel (ENaC) has been described as essential for wound healing associated migration in vascular smooth muscle cells (Grifoni et al. 2006) and in glioma cells (Vila-Carriles et al. 2006). What makes this ion channel even more interesting in respect to cell migration is the fact that the ENaC α-subunit is associated with the actin cytoskeleton (Mazzochi et al. 2006). In detail, ENaC is linked to actin-binding protein

α-spectrin, and this interaction seems to be important for localization of the ion channel in the apical membrane (Rotin et al. 1994). Several authors have reported that short actin filaments facilitate ENaC activation (Cantiello et al. 1991; Prat et al. 1993; Berdiev et al. 1996) and that direct interaction of actin with the COOH terminus of α-ENaC is required for insertion of ENaC into the plasma membrane (Mazzochi et al. 2006).

So far, ENaC expression has not been observed in macrophages, although for example alveolar macrophages are able to decrease ENaC expression and activity in lung endothelial cells (Dickie et al. 2000). However, expression of ENaC β- and γ-subunits beyond epithelial cells has been found to be involved in mechanotransduction of neurons innervating the aortic arch and of vascular smooth muscle cells (Drummond et al. 2001, 2008). Therefore, a possible involvement of ENaC or other sodium ion channels in salt-dependent chemotaxis needs to be emphasized. This hypothesis could be tested by application of the small molecule inhibitor amiloride to specifically block ENaC function during cell migration.

In respect to ion transporter proteins, the Na^+/H^+ exchanger (NHE) is essential for cell migration of neutrophils (Rosengren et al. 1994; Ritter et al. 1998), human melanoma and renal epithelial MCDK-F cells (Klein et al. 2000). NHE localizes at the lamellipodium of migrating cells and controls local cell volume (Klein et al. 2000). Consequently, NHE or other ion transporters could be investigated for a potential role in salt-dependent chemotaxis.

4.4. Salt-dependent chemotaxis of macrophages: Implications for human health

Elevated blood pressure poses a major risk for cardiovascular disease, stroke and kidney failure, which are leading causes for mortality worldwide (WHO report “Global health risks, WHO 2009). To summarize, there is growing evidence that increased salt intake, especially by rising consumption of sodium-rich fast food, leads to major health and economic problems throughout the world (WHO 2009).

How is salt-dependent chemotaxis involved in this problem?

When looking closer at blood pressure regulation in the body, evidence has emerged over the last years that blood pressure is not only regulated systemically by the kidney, the brain or the blood vessels by keeping sodium and volume levels at a narrow range. Instead, a high salt loading leads to a sodium storage in the skin interstitium and creates a local hypertonic microenvironment (Schafflhuber et al. 2007; Machnik et al. 2009; Wiig et al. 2013). Apart from their function in the immune system, macrophages control the removal of excess sodium by a TonEBP/VEGF-C-dependent lymphangiogenesis, as reported by Machnik et al. (2009). These authors also found that if this regulatory axis is inhibited by deletion of

macrophages or blockade of lymphangiogenesis-driving VEGF-C, respectively, blood pressure is increased in animals (Machnik et al. 2010). In addition, an elevated VEGF-C level has been found in the blood of patients with a salt-sensitive hypertension (Machnik et al. 2009). A high salt diet also induced VEGF-C in healthy subjects, although blood pressure was not affected (Slagman et al. 2012). Human sodium storage in the skin tissue was visualized for the first time by use of ^{23}Na magnetic resonance imaging by Kopp et al. (2012, 2013). These studies showed that sodium accumulates during lifetime in the skin and interestingly, this accumulation occurs earlier in hypertensive patients. According to Wiig et al. (2013), macrophage homeostatic function was disrupted by genetic depletion of TonEBP in the myeloid cell population (TonEBP(flox/flox)LysM(cre/wt)) and by blockade of VEGFR3 following high salt diet of the mice. This abrogation of the TonEBP/VEGF-C regulatory axis resulted in addition to an increase in sodium storage in an accumulation of chloride in the skin and the development of salt-sensitive hypertension in the mice. These new findings propose a so far undescribed role for chloride in salt-sensitive hypertension, as chloride and blood pressure increase was directly correlated (Wiig et al. 2013). All in all, these recent studies underline the importance of macrophage homeostatic function in controlling local electrolyte composition in the skin and its connection with systemic blood pressure.

Two important conclusions about the involvement of salt-dependent chemotaxis can be drawn: First, any malfunction in salt-dependent chemotaxis of macrophages toward areas of high sodium storage might lead to development of hypertension. As the sensing, migration toward and resulting accumulation of macrophages in areas of high sodium storage precedes the molecular regulating mechanism to remove sodium from the interstitium, abrogation of chemotaxis might lead to an accumulation of sodium in the skin and increase blood pressure.

Second, identification of the underlying molecular mechanism of salt-dependent chemotaxis might provide new targets for hypertension therapy. Existing antihypertensive treatment consists of beta-blockers, aldosterone antagonists (ACE inhibitors), diuretics and calcium channel blockers. Most patients have to take high blood pressure medication for many years and suffer from side effects like dry cough, asthma or diabetes (Zillich et al. 2008). In addition, the majority requires a combination therapy with several agents to effectively lower their blood pressure (Chobanian et al. 2003; Mancia et al. 2007). These facts show that a small molecule that would specifically act on salt-dependent chemotaxis, or on the sensor for the high sodium environment and that would stay immunological inert could be a promising lead for new medications against hypertension.

4.5 Outlook

The present work characterizes salt-dependent chemotaxis of macrophages and thus provides an explanation for the accumulation of macrophages in areas of high salt storage in the skin interstitium (Machnik et al. 2009). It could be shown here that macrophages specifically recognize a hypertonic NaCl stimulus as chemoattractive signal and the data further suggest that salt-dependent chemotaxis is restricted to macrophages.

However, direct measurements of the lymphoid tissue demonstrated that immune cells in this microenvironment are exposed to osmotic stress (Haljamae et al. 1974; Szabo and Magyar 1982). Several studies described functional effects of osmotic stress for instance on T-lymphocytes (Bortner et al. 2012; Kleinewietfeld et al. 2013), but it can additionally be speculated that immune cells use hypertonic environments for migration into lymphoid tissues. Therefore, it would be interesting to investigate *in vitro* whether T-cells and also other non-immune cells are capable of salt-dependent chemotaxis.

Since the molecular signaling pathway responsible for salt-dependent chemotaxis is unknown, it might be useful to systematically screen for NaCl-induced macrophage attracting factors such as chemokines and cytokines. In addition, Na^+ - selective ion channels like ENaC might be involved. Hence, further studies could include high-performance liquid chromatography of transwell supernatants or microarray analysis of migrating cells in order to identify candidates.

The next step should also focus on salt-dependent chemotaxis in *in vivo* model systems. Macrophage migration toward excess sodium that is bound to glycosaminoglycans needs to be studied. In addition, the clinical relevance of salt-dependent chemotaxis could ultimately be verified by the use of mouse models.

5 Summary

Macrophages exert a prominent function in immune system host defense, but in the recent years increasing evidence emerged that these cells are in addition potent regulators of salt balance. At the outset, this work was based on previous findings that had demonstrated an accumulation of macrophages in the skin tissue of rats, which had been fed on a high salt diet. The question arose whether this was possibly due to a chemotactic response of the macrophages to the hypertonic environment of skin that had sequestered high amounts of Na^+ to the interstitial glycosaminoglycans.

Chemotaxis is an essential process of immune defense to attract immune cells to sites of pathogenic infection. Monocytes/macrophages have been described to recognize many substances among which are chemokines, bacterial components, complement factors and leukotrienes as chemoattractive signals. In contrast, cell migration toward a hypertonic NaCl stimulus, which might represent a potential harmful environment for the cell by causing hypertonic stress, is a completely novel concept.

In vitro transwell migration assays revealed that RAW264.7 macrophages, peritoneal macrophages and bone marrow-derived macrophages, but not bone marrow-derived dendritic cells show salt-dependent chemotaxis toward a hypertonic NaCl stimulus. This dose-dependent migration response was specific to hypertonicity by excess NaCl, as it could not be induced by other osmo-active agents like urea or mannitol. Subsequently, the underlying molecular mechanism of salt-dependent chemotaxis was investigated with respect to early and late events in cell migration. Many potential candidates were addressed, demonstrating that a hypertonic NaCl stimulus did not directly affect actin cytoskeleton reorganization and was unable to induce expression of chemoattractive CCL2 or LTB4 in RAW264.7 cells. Furthermore, migration toward excess NaCl was abrogated by the presence of cycloheximide and pertussis toxin, indicating a dependence on protein synthesis and Gαi-coupled GPCRs, respectively. While the osmoprotective transcription factor TonEBP is a central regulator in macrophages for the removal of excess Na^+ in the interstitium, it was not required for salt-dependent chemotaxis.

Although the complete underlying signaling pathway could not be elucidated, the participation of a NaCl-induced chemotactic factor that acts in an autocrine/ paracrine way, similar to swarming migration behavior of neutrophils, might underlie theses observations. In addition, the role of sodium ion channels in salt-dependent chemotaxis should be considered. Taken together, it is proposed that salt-dependent chemotaxis plays a crucial role in clearance of excess salt *in vivo* and that any defects in macrophage migration toward areas of high salt storage might result in development of hypertension, as recent studies in rodents and also humans imply.

6 List of Figures

7 References

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8 Abbreviations

11β-HSD2	11β-Hydroxysteroid dehydrogenase
µ	micro
A. bidest	aqua bidest
APC	allophycocyanin
APS	ammonium persulfate
BMDC	bone marrow-derived dendritic cells
BMDM	bone marrow-derived macrophages
BSA	bovine serum albumine
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
CFDA	carboxyfluorescein diacetate
CVD	cardiovascular disease
CXCL	C-X-C motif chemokine ligand
DABCO	1,4-Diazabicyclo[2.2.2]octane
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco´s modified Eagle medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ECL	electrochemiluminescence
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENaC	epithelial sodium channel
EtD-III	ethidium-homodimer III
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
g	gravity (9.81 m/s^2)
GAG	glycosaminoglycans
GAP	GTPase-activating factor
GEF	guanine nucleotide exchange factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
GTP	guanosine-5'-triphosphate

h	hour
HRP	horseradish peroxidase
IgG	Immunglobuline
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
kDa	kilodalton
l	liter
LTB4	leukotriene B_4
LPS	lipopolysaccharide
m	meter; milli
M	molarity (mol/l)
m/v	mass per volume
min	minutes
mol	mole
MCLK	myosin-light-chain kinase
n	nano; number of experiments
NED	N-(1-naphthyl)ethylenediamine
NFAT5	nuclear factor of activated T cells 5
NO	nitric oxide
p	pico; p-value
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE	phycoerythrin
PFA	paraformaldehyde
PI3-K	phosphatidylinositide 3-kinase
PIP-3	phosphatidylinositol (3,4,5)-triphosphate
PmxB	polymyxin B
Rac1	ras-related C3 botulinum toxin substrate 1
Rho	ras homologue gene family
RNA	ribonucleic acid
RNAi	RNA interference
ROCK	Rho kinase
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
RTK	receptor tyrosin kinase
SDS	sodium dodecyl sulfate

siRNA	small interfering RNA
TEMED	tetramethylethylendiamin
TBS-T	tris-buffered saline Tween-20
TNF-α	tumor necrosis factor alpha
TonEBP	tonicity-responsive enhancer binding protein
TRITC	tetramethylrhodamine
v / v	volume per volume
VEGF	vascular endothelial growth factor
VLE	very low endotoxin
WASP	Wiskott Aldrich syndrome protein
WAVE	WASP-family verprolin homologous
WHO	World Health Organization
WIP	WASP-interacting protein
wt	wildtype

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