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The significance of ecDNA in osteoclastogenesis from peripheral blood precursors – an *in vitro* study

Význam extracelulárnej DNA v procese vzniku osteoklastov z prekurzorov v periférnej krvi – štúdia *in vitro*

Diploma thesis

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Prague, 2020

Declaration:

I declare that this thesis presented for the master's degree has been composed entirely by myself, using referenced sources. This thesis, in whole or in part, has not been submitted for any other previous degree or professional qualification.

Prague, 10.08.2020

Čestné vyhlásenie:

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Abstract

Introduction: Extracellular DNA (ecDNA) is a common component of blood plasma. Increased levels of ecDNA in plasma can be found in some autoimmune diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis or celiac disease which are associated with inflammatory processes. These diseases are also associated with an increased risk of osteoporosis. Bone is a dynamic structure undergoing constant modelling caused by osteoblasts, osteocytes and osteoclasts. Shifting their equilibrium can lead to pathological conditions such as osteoporosis. In this thesis we focused on elucidating whether ecDNA, an inflammatory agent with proven immunoregulatory effects can alter differentiation potential of monocytes and alternatively lead to osteoclastogenesis via TLR9.

Material and methods: We obtained monocytes from peripheral blood of healthy donors and cultivated them with four types of ODNs control (CO), stimulatory (ST), inhibitory (INH, telomeric (TLM) with phosphodiester (-pO) or phosphorothioate (-pS) backbone for two weeks to establish their effect on differentiation potential of monocytes into osteoclasts. Osteoclastogenesis was evaluated by number of yielded osteoclasts observed on a light microscope. To establish the effect of ODNs on osteoclast activity samples were analysed by qPCR for expression of *ACP5, CTSK, TLR9* and *NFATc1*.

Results: No significant differences were found in experiments that tested -pO ODNs when compared to their respective blanks. Out of three control ODNs tested, only CO-ODN-2395 (10 nM) was proven neutral and appropriate as a control ODN for further experiments. ST-ODN-2006 (0.1 nM) proved to stimulate osteoclastogenesis. Both INH-ODN-4347 and TLM-ODN (TTAGGG)4 did not inhibit osteoclastogenesis when tested alone. Combination of INH-ODN (100 nM) + ST-ODN (0.1 nM) exhibited significant difference when compared to ST-ODN (0.1 nM). Combined effect of TLM-ODN (10 nM) + ST-ODN (0.1 nM) did not significantly differ from the effect of ST-ODN (0.1 nM). Changes in mRNA expression did not show significant differences in any gene when the cells were cultivated with various ODNs, their combinations or when the cells were harvested at different times of cultivation.

Conclusion: Our results suggest that -pS ODNs are appropriate for long-term cultivations. The CO-ODN-2395 (10 nM) seems to be a suitable control in cultivations of monocytes. ST-ODN-2006 (0.1 nM) significantly stimulates osteoclastogenesis. Lone INH-ODN and TLM-ODN do not inhibit osteoclastogenesis when added into the culture. When ST-ODN-2006 (0.1 nM) is combined with INH-ODN-4347 (100 nM) the osteoclastogenesis is significantly inhibited. TLM-ODN (10 nM) + ST-ODN (0.1 nM) do not significantly inhibit osteoclastogenesis, but more robust data is needed to verify this effect. Though ST-ODN produces more osteoclasts, their activity was not increased; nevertheless, more experiments are needed to verify this effect. *TLR9* might possibly not be involved in alternatively activating differentiation of monocytes into osteoclasts, but further tests are required to prove its role in osteoclastogenesis.

Key words: oligodeoxynucleotide, ODN, extracellular DNA, ecDNA, inflammation, osteoclastogenesis, monocyte, osteoclast, TLR9, NF-κB

Abstrakt

Úvod: Extracelulárna DNA (ecDNA) je bežnou súčasťou krvnej plazmy. Zvýšená hladina ecDNA sa v krvi vyskytuje u pacientov s niektorými autoimunitnými ochoreniami spojenými so zápalom, ako sú napríklad systematický lupus erythematosus, reumatoidná artritída alebo celiakia. Tieto choroby sú tiež spojené s vyšším rizikom vzniku osteoporózy. Kosť je dynamická štruktúra, ktorá prechádza neustálymi remodeláciami, za ktoré sú zodpovedné osteoblasty, osteocyty a osteoklasty. Vychýlenie ich rovnováhy môže viesť k patológiám, ako je napríklad osteoporóza. V tejto diplomovej práci sme sa zamerali na skúmanie ecDNA (zápalový faktor s preukázanými imunoregulačnými účinkami) a jej schopnosť meniť diferenciačný potenciál monocytov na osteoklasty a alternatívne tak spúšťať osteoklastogenézu.

Materiál and metódy: Monocyty sme izolovali z periférnej krvi zdravých darcov a kultivovali v prítomnosti štyroch typov ODN, kontrolné (CO), stimulačné (ST), inhibičné (INH), telomerické (TLM), s fosfodiesterovými (-pO) alebo fosforotioátovými (-pS) kostrami počas dvoch týždňov, aby sme overili ich efekt na ľudské monocyty. Osteoklastogenézu sme vyhodnotili počtom osteoklastov spočítaných na svetelnom mikroskope, a ich aktivitu pomocou qPCR analýzy, kde sme analyzovali gény *ACP5, CTSK, TLR9* a *NFATc1*.

Výsledky: Pri testovaní -pO ODN a ich porovnaní so vzorkou bez pridaných ODN sme nenašli žiadne významné rozdiely. Z troch kontrolných ODN sa ako neutrálne potvrdilo len CO-ODN-2395, ktoré je ako kontrola vhodné pre ďalšiu aplikáciu. ST-ODN-2006 (0.1 nM) významne stimulovalo osteoklastogenézu. INH-ODN-4347 ani TLM-ODN (TTAGGG)4 neinhibovali osteoklastogenézu, keď boli k bunkám pridané samostatne. INH-ODN (100 nM) + ST-ODN (0.1 nM) signifikantne inhibovali vznik osteoklastov v porovnaní s ST-ODN (0,1 nM). TLM-ODN (10 nM) + ST-ODN (0.1 nM) vznik osteoklastov významne neihnibovali. Nepreukázali sme významné zmeny v expresii pri žiadnom z testovaných génov, ich kombinácií ani pri analýze, kde boli vzorky odobraté v rôznych časoch počas kultivácie.

Záver: Naše výsledky naznačujú, že -pS ODN sú vhodné na použitie v dlhodobých kultiváciách. Taktiež naznačujú, že CO-ODN-2395 (10 nM) je vhodná kontrola pre kultivácie s monocytmi. ST-ODN-2006 (0,1 nM) významne stimuluje osteoklastogenézu. Samotné INH-ODN a TLM-ODN neinhibujú osteoclastogenézu. Signifikatná inhibícia osteoklastogenézy nastane až pri kombinácii ST-ODN (0,1 nM) a INH-ODN (100 nM). Pri spoločnej kultivácii TLM-ODN (10 nM) a ST-ODN (0,1 nM) nedochádza k signifikatnej inhibícii osteoklastogenézy. Na overenie tohto efektu je však potrebné vykonať ďalšie experimenty. Napriek tomu, že ST-ODN vedie k zvýšenej produkcii osteoklastov, ich aktivita sa nezvyšuje. Podľa našich doterajších výsledkov sa javí, že TLR9 nie je zapojené do aktivácie osteoklastogenézy pomocu ODN. Ďalšie experimenty sú ale potrebné na ozrejmenie výsledkov pochádzajúcich z qPCR.

Kľúčové slová: oligodeoxynucleotidy, ODN, extracelulárna DNA, ecDNA, zápal, osteoklastogenéza, monocyt, osteoklast, TLR9, NF-κB

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1. Introduction

1.1 Extracellular DNA

Deoxyribonucleic acid is a double-stranded molecule, which under normal circumstances is contained in the nucleus of a cell and carries genetic information. Mandel and Métais detected nucleic acid in plasma for the first time in 1948 (Mandel & Métais, 1948). Extracellular DNA (ecDNA) is common component of blood in healthy individuals with levels of less than 50 ng/ml (Klemp et al., 1981). Elevated levels of ecDNA are present in patients suffering from autoimmune diseases e.g. systemic lupus erythematosus (SLE) (Rumore & Steinman, 1990), rheumatoid arthritis (Rykova et al., 2017) or celiac disease (Brynychova et al., 2019), which are associated with inflammatory processes. Increased levels of ecDNA were also found in the plasma or serum of cancer patients (180 ng/ml) (Leon et al., 1977). A major part of this ecDNA was found to originate from tumor cells, therefore making it an important marker for early diagnosis or prognostic purposes (Stroun et al., 1989; Anker et al., 1999).

1.1.1 The structure and cellular origin of ecDNA

DNA can be released to circulation from various cell sources, predominantly after undergoing some type of cell death such as apoptosis, necrosis (Giacona et al., 1998), or NETosis (Brinkmann et al., 2004). Apart from undergoing cell death another source of ecDNA in human blood is its spontaneous release by lymphocytes in extracellular vesicles – exosomes. *In vitro* experiments following the release of ecDNA into the supernatant showed that the amount of DNA was at the same level after 2 hours as well as 8 hours or 16 hours, which confirms that it is also released actively. This DNA is double-stranded and shows typical characteristics such as UV absorption curve and sensitivity to DNase (Anker et al., 1975).

The DNA molecule situated in the nucleus is wrapped up in a protein-DNA complex called the nucleosome. The nucleosome is made up of protein octamer consisting of two copies

of histones H2A, H2B, H3, H4 binding 147 base pairs (bp) of DNA (Fig. 1) and also H1 organizing 10–90 bp of linker DNA (Richmond & Davey, 2003). The DNA released to the circulation thus has the specific nucleosome-like composition.

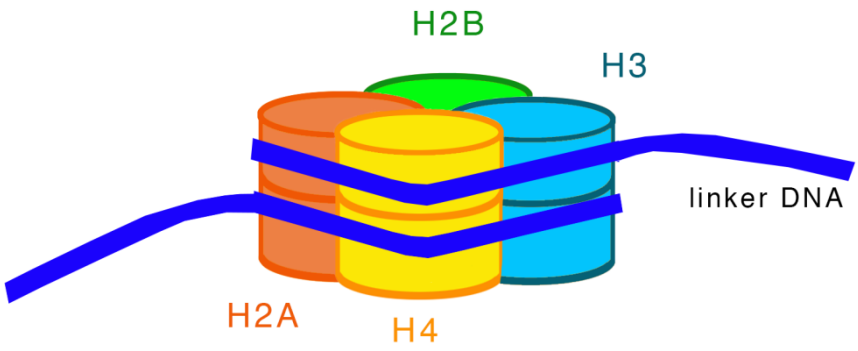

Figure 1: nucleosome structure. *Author*

Apoptosis is a highly coordinated process that involves the activation of cysteine proteases called caspases. It is also a highly energy-dependent task that starts a complex cascade of processes leading from the primary stimuli to the final eradication of the cell (Elmore, 2007). Apoptosis can occur under normal circumstances during development and ageing. It can also be triggered to eliminate disease damaged cells or cells damaged by external factors. Disease or external stimuli can damage DNA structure and endanger genomic integrity and proper cell function by proliferating without DNA repair. Apoptosis is also an integral part of the immune system, ensuring that immune cells possess functional receptors. Those with non-functional, non-rearranged or aberrantly rearranged receptors are eradicated. It is also important for the removal of autoreactive T cells and downregulation of excess immune response (Krammer et al., 1994).

Apoptosis can be described by multiple morphological characteristics such as cell shrinkage, chromatin condensation, apoptotic body formation with intact plasmatic membrane and DNA degradation. DNA is broken down by Ca^{2+}- and Mg^{2+}-dependent endonucleases during apoptosis, resulting in 180 to 200 bp long fragments (Bortner et al., 1995).

Necrosis is a process characterized by cell swelling and chromatin condensation leading to cellular and nuclear lysis and inflammation (Wyllie et al., 1980). During necrosis, DNA is left intact in contrast with fragmented DNA released during apoptosis (Jahr et al., 2001). It can occur under pathological conditions, but also under normal physiological conditions or in development. Necrosis was believed to be a passive process but it is in fact an active choice of the cell that is usually overridden because of its potential dangerous impact on its immediate surroundings. Necrotic cells are able to induce immune response by releasing signals that can stimulate dendritic cells (Gallucci et al., 1999; Leist & Jäättelä, 2001). One of the proinflammatory signals, made available by the loss of membrane intergrity, is the release of DNA that acts as damage-associated molecular pattern (DAMP) and can mediate immunomodulatory effects (Kustanovich et al., 2019).

Apoptosis and necrosis can appear independently, sequentially or simultaneously (Zeiss, 2003). Whether a cell dies by apoptosis or necrosis depends on the type of the stimulus and its level. Stimuli such as heat, radiation or drug use can lead to apoptosis but in higher doses, they lead to necrosis (Elmore, 2007). Whether the cell underwent apoptosis or necrosis, its remains are phagocytosed by macrophages (Majno & Joris, 1995).

Another cell death pathway leading to the release of DNA into circulation is NETosis. It requires the presence of innate immunity cells called polymorphonuclear leukocytes, namely neutrophils. These are in two distinct ways able to release histone bound DNA. The first method is a suicidal NETosis, in which chromatin decondenses, nucleus swells and its nucleoplasm is spilled into the cytoplasm. The final step is perforation of the plasmatic membrane (Yipp & Kubes, 2013). Web-like structures of DNA, called NETs, are released to the extracellular space (Kaplan & Radic, 2012). This process was observed after *in vitro* stimulation of the cell with either interleukin-8 (IL-8) or phorbol 12-myristate 13-acetate (PMA) (Brinkmann et al., 2004). NETosis is described as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent process (Fuchs et al., 2007) that serves as a form of host defence since it can bind gram-negative as well as gram-positive bacteria before the cell death (Steinberg & Grinstein, 2007). The second type of NETosis does not require cell suicide, allowing the cell to preserve its functions and therefore it is

called vital NETosis (Clark et al., 2007). One of the explanations for a different type of NETosis is that neutrophils consist of subsets and these subsets react distinctively, one of the subsets reacts primarily by suicide NETosis and the other subset reacts in a form of vital NETosis. Nevertheless, this explanation does not have enough evidence, to date, to be fully supported. It is still possible that neutrophils survive the suicidal NETosis and continue to perform their function (Yipp & Kubes, 2013).

1.1.2 Intercellular communication

Human lymphocytes are also able to release DNA actively without undergoing any type of cell death – in extracellular vesicles called exosomes. Exosomes are membrane vesicles that are commonly 40–100 nm in diameter (Simons & Raposo, 2009). They are formed by a process of inward budding into large multivesicular bodies. These multivesicular bodies are fused with the plasma membrane after budding and then they are released into the extracellular space (Record et al., 2011). Secreted vesicles, like exosomes, carry important genetic and proteomic information making them vital for intercellular communication, carrying various proteins, lipids, DNAs and RNAs that have regulatory effects on their recipient cells (Valadi et al., 2007; Hong et al., 2009; Kim & Kim, 2009). They can activate the target cells by presenting ligands on their surfaces (Théry et al., 2002) or by transporting surface receptors to different cells by budding, followed by fusion of plasma membrane (Clark et al., 2007).

Interleukins (IL) are a group of cytokines expressed by a wide variety of immune and non-immune cells that exercise a great diversity of functions such as affecting proliferation, activation, differentiation, maturation, migration and adhesion. According to their function interleukins can be categorized into 8 subgroups: IL-1, common γ chain receptor cytokine, cytokines of type 2 immune responses, interleukins with chemokine activity, the IL-10, IL-12 and IL-17 families and others (Akdis et al., 2016). Interleukin 26 (IL-26) is a member of the IL-10 family, the family of interleukins that is present during chronic inflammatory diseases serving as an inflammatory mediator (Knappe et al., 2000; Donnelly et al., 2010). One of IL-26's most notable features is that it is capable of binding

to DNA released from damaged cells and acts as its carrier, which links DNA to inflammation. As a carrier, it is internalized by plasmacytoid dendritic cells and then proceeds to activate them via intercellular DNA receptor Toll-like receptor 9 (TLR9) (Meller et al., 2015). Human monocytes are activated by IL-26/DNA complexes using inflammasomes and cytosolic stimulators of interferon genes (STING) pathways (Poli et al., 2017).

1.1.3 The role of ecDNA in inflecting the immune response

Toll-like receptors (TLRs) are a set of phylogenetically conserved mediators of innate immunity. These receptors are called Toll-like receptors because of their similarity with Toll molecules in fruit flies. The fact that these receptors kept their structure between insects and humans, further proves their critical role in the innate immune response. Several TLR groups can be distinguished, each of them recognizing a different molecular pattern present in pathogens, that is either rare or lacking in humans. TLR9 recognizes unmethylated CpG pattern, which is much rarer in humans than in microbial DNA (Krieg et al., 1995). TLRs 3, 7, 8 and 9 recognize nucleic acid ligands (Takeda et al., 2003). Unlike other TLRs, the four ones mentioned above are expressed inside of the cell in the endoplasmic reticulum and use endosomes for signalling (Latz et al., 2004). Myeloid differentiation primary response 88 protein (MyD88) is a universal adapter protein that mediates the TLR-dependent activation of transcription factor NF-κB. MyD88 recruits the ubiquitin ligase TRAF6 and several kinase complexes such as interleukin-1 receptor associated kinases (IRAKs), transforming growth factor beta-activated kinase 1 (TAK1), inhibitory-kappa B kinase αβγ (IKKαβγ) and mitogen-activated protein kinases (MAPKs). This recruitment is subsequently followed by phosphorylation and ubiquitination promoting translocation of transcription factors, NF-κB and activator protein-1 (AP-1), which can induce production of messenger RNA (mRNA) for tumour necrosis factor (TNF), prointerleukin-1β (proIL-1β) and other inflammatory molecules such as Interferon α/β (IFN α/β) or IL-6 (Ashman & Lenert, 2007; Takagi, 2011). Depending on its primary sequence, ecDNA can pose as a stimulatory or inhibitory agent. In B cells the structural requirements for the stimulatory effect of DNA are as follows: unmethylated CpG

(Klinman et al., 1996), the occurrence of TCC at the 5′ end and the absence of Cs one or two bases 5′ to CG. Upon studying the properties of stimulatory effects of DNA, research showed that changing just two to three bases results in inactivating the stimulatory effect, posing them in contrast as inhibitory. The most effective inhibitory sequence proved to be 'xCC N D D NN GGG NNN' where N is any base and its presence is mandatory, x is optional base but its presence is not required, and D is any base except C (Ashman & Lenert, 2007). When B cells are stimulated by sequences that possess the stimulatory structures, proliferation and expression of CD86 protein are upregulated, establishing this upregulation as a sensitive indicator of the stimulatory effect of the sequence. Upregulation of lytic activity and CD69 expression demonstrates the stimulatory effect of the sequence in natural killer (NK) cells (Hartmann et al., 2000). Inhibitory sequences block the response to stimulatory sequences in B cells (Stunz et al., 2002).

1.2 Bone formation and remodelling

Skeleton is a supporting framework of the body. It also provides many vital functions such as it allows movement, posing as a lever for muscles, it protects vital organs, serves as a reservoir of calcium, growth factors and cytokines, provides an environment for haematopoiesis and also plays a role in maintaining acid-base balance (Clarke, 2008).

Bone is made up of extracellular and cellular components. The extracellular component can be divided into two main fractions – inorganic and organic. The main inorganic compounds are hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), which makes up 60%-70%, and water. Further, the inorganic component also includes inorganic salts. The organic fraction of the bone is made up of type I collagen (90% of the organic matter), non-collagenous proteins and lipids. Type I collagen is an abundant protein in the bone and it provides elasticity to the tissues, stabilizes the extracellular matrix, supports mineral deposition and binds other molecules. Non-collagenous proteins also possess important functions – organizing the bone matrix, cell signalling, metabolism and mineralization. Lipids' function is inevitable because they provide the flow of the ions and signalling molecules in and out of the cell (Boskey, 2013). The cellular component of the bone is made up of three main types of

cells - osteoblasts, osteocytes and osteoclasts that are vital for modeling and remodelling of the bone.

In the process of bone modelling and remodelling an extracellular organic matrix is synthesized first, then the matrix mineralizes leading to bone formation. Osteoblasts are responsible for both. When osteoblasts become embedded in the bone matrix they are referred to as osteocytes. The last step is bone remodelling and reformations by bone resorption provided by osteoclasts (Clarke, 2008; Kini & Nandeesh, 2012).

Despite the common misconception, bone is a highly dynamic structure, undergoing constant remodelling by cause of osteoblasts and osteoclasts. This allows the bone to repair, to adapt to the force placed on it (Datta et al., 2008) or change shape during growth (Turner, 1998). The process can be induced by mechanical forces, hormones (parathyroid hormone), growth factors, vitamin D and cytokines (Datta et al., 2008). The process of remodelling the bone can be divided into six stages: quiescent phase, activation phase, resorption phase, reversal phase, formation phase and mineralization phase (Fig. 2).

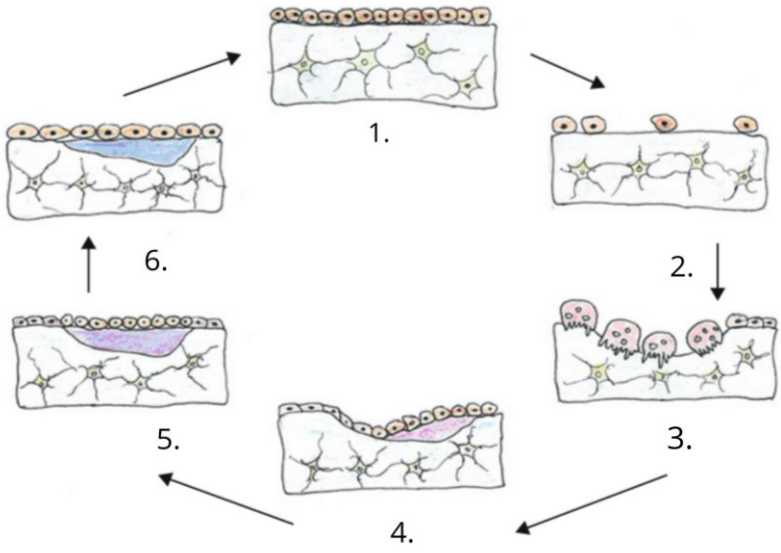

Figure 2: Stages of bone remodelling. *Adapted from Kini & Nendeesh (2012)*
1. quiescent phase – the bone is at rest 2. activation phase – the activation of bone resorption by osteoclasts 3. resorption phase – osteoclasts are fully activated 4. reversal phase – osteoclasts are replaced by osteoblasts 5. mineralization phase – mineralization of the osteoid matrix 6. formation phase – formation of bone structure progressing again to quiescent phase

1.2.1 Osteoclasts

Osteoclasts are typically multinucleated cells that are formed by the fusion of mononuclear precursors. Osteoclasts are capable of resorbing the bone matrix by secreting acid and lytic enzymes during physiological and pathological bone turnover (Aubin & Bonnelye, 2000; Boyle et al., 2003). Their predominant morphological characteristic is a well-developed Golgi apparatus around the nucleus and also abundant lysosomal vesicles. Other distinctive features of the osteoclast are the finger-like protrusions called the ruffled border, which is formed by the cell membrane and cytoplasmic undulations (a lateral expansion of the plasma membrane). The sealing zone is made up of podosomes (specialized adhesion structures) that are in contact with the bone surface (Fig. 3) (Aubin, 1992; Roodman, 1999). To identify mature osteoclast, molecular markers such as tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CTR), vitronectin receptors, carbonic anhydrase II, cathepsin K (CTSK) and vacuolar-type H^+-ATPase are used (Aubin & Bonnelye, 2000). The TRAP enzyme is produced by acid phosphatase 5 (*ACP5*) gene (Behrens & Graham, 2011). The *CTSK* gene encodes the cathepsin K enzyme responsible for degrading the bone matrix. *CALCR* gene product is a calcitonin receptor which is in charge of maintaining calcium homeostasis and in regulating osteoclast-mediated bone resorption (Boyle et al., 2003).

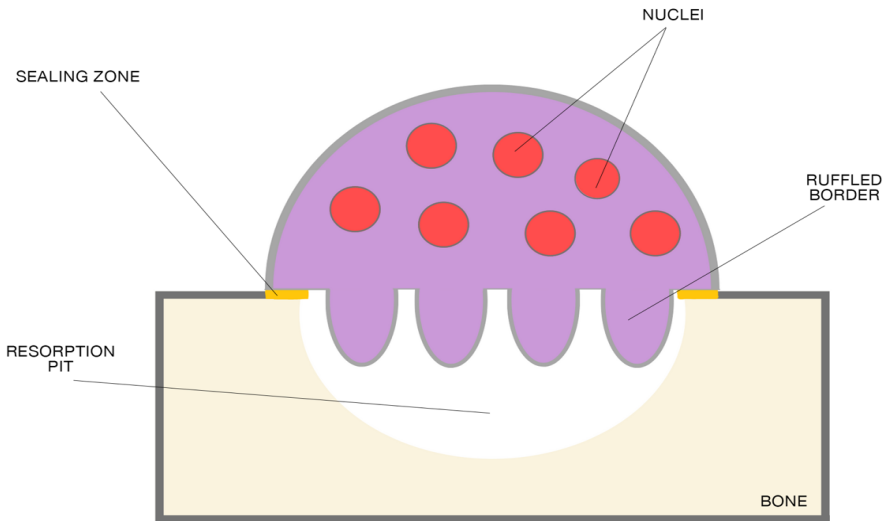

Figure 3: Osteoclast morphology. *Author*

Monocyte-macrophage lineage cells are derived from hematopoietic stem cells (HSCs) and serve as precursors of osteoclasts (Fig. 4). Monocytes are blood circulating leukocytes that represent immune effector cells. By cause of their chemokine and adhesion receptors, they are capable of mediation of migration between blood and tissues during infection. One of the characteristics of monocytes is that they generate inflammatory cytokines and they pursue cells as well as toxic molecules. During inflammation, monocytes can differentiate into dendritic cells or macrophages (Nakagawa et al., 1998; Serbina et al., 2008).

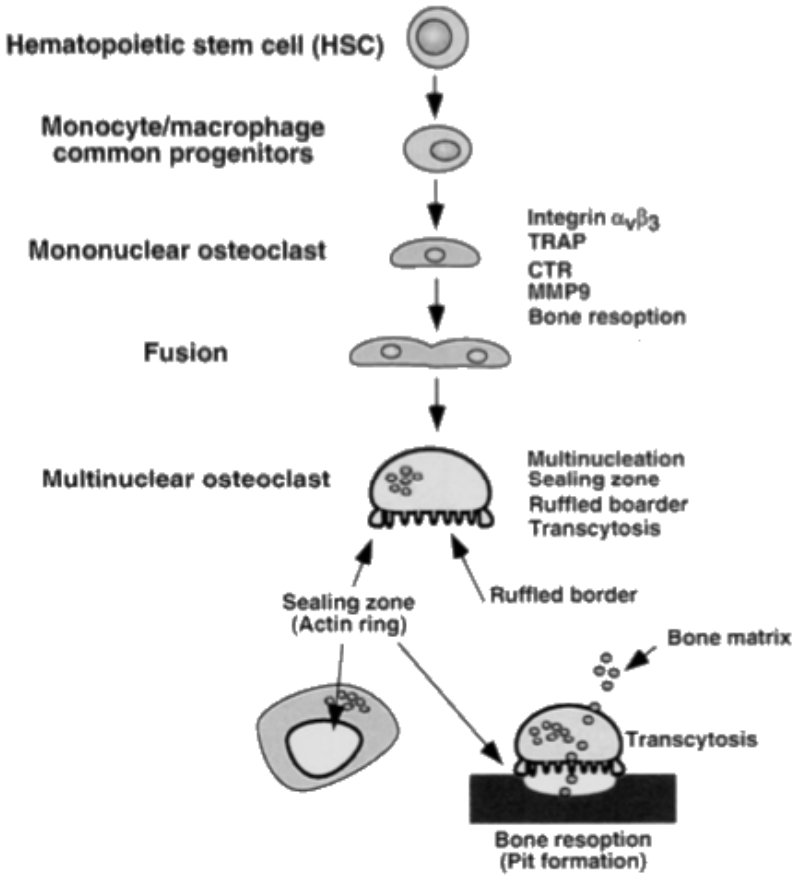

Figure 4: Development of multinucleated osteoclast from HSCs. *Adapted from Miyamoto (2001)*
Precursors of osteoclasts, the monocyte-macrophage lineage cells are derived from HSCs. They first develop into mononuclear osteoclasts and by fusion form multinucleated osteoclasts with distinctive physiology and they produce osteoclastogenic factors, such as TRAP and CTR (Miyamoto, 2001).

Bone resorption is made up of a series of consecutive regulatory steps: osteoclast development, migration of osteoclasts to the resorption site, attachment of osteoclasts to calcified tissues and the development of a ruffled border and a clear zone, followed by the

secretion of the acids and lysosomal enzymes into space beneath the ruffled border (Nakamura et al., 2003).

1.2.1.1 Osteoclastogenesis

Osteoclastogenesis is crucially dependent on two cytokines – receptor activator of nuclear factor κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) (Datta et al., 2008). These cytokines are required to induce expression of genes that are specific for the osteoclast lineage producing tartrate-resistant acid phosphate and cathepsin K leading to mature osteoclasts (Boyle et al., 2003).

M-CSF is a growth factor of cells of the mononuclear phagocyte system that possess the capacity of development into osteoclasts (Stanley & Heard, 1977). It is a tyrosine kinase encoded by the c-*fms* gene. By binding of M-CSF to its receptor CSF1R expressed on the monocyte-macrophage lineage cells, the tyrosine becomes phosphorylated, thereby M-CSF can exercise its role in regulating the development, proliferation and differentiation of osteoclasts (Yeung et al., 1987; Sengupta et al., 1988; Guo et al., 2019).

RANKL is a type 2 transmembrane protein of the tumour necrosis factor α (TNFα) superfamily. It was identified during an *in vitro* study of osteoclastogenesis, where osteoclast precursors were co-cultured with stromal cells acquired from bone marrow or spleen cells in the presence of osteotropic factors such as 1.25-dihydroxy-vitamin D_3, parathyroid hormone (PTH), dexamethasone, prostaglandin E_2, IL-6, IL-11 and IL-1. In this study cell-to-cell contact proved to be of vital importance because of stromal cells that express RANKL when stimulated by osteotropic factors (Takahashi et al., 1988; Udagawa et al., 1989; Anderson et al., 1997). RANKL is responsible for osteoclast formation and differentiation and also increases mature osteoclast activity (Lacey et al., 1998; Burgess et al., 1999). By binding to its receptor RANK that is expressed via M-CSF stimulation on the surface of osteoclast precursor cells, RANKL initiates a signal transduction cascade (discussed in more detail in section 1.2.1.2) and therefore exercises its biological effect in the process of osteclastogenesis (Nakagawa et al., 1998; Park et al., 2017).

Osteoclastogenesis occurs after stimulation of monocyte-macrophage lineage cells to form mononuclear cells called preosteoclasts (Fig.5). The family transcription factor PU.1 and M-CSF are crucial in this step (Yoshida et al., 1990; Tondravi et al., 1997). Both M-CSF and RANKL are then required in fusion of mononuclear preosteoclasts into polykaryon (immature osteoclast) near the resorption sites (Franzoso et al., 1997). Further survival and bone-resorbing function of the osteoclast is regulated by RANKL. Mature osteoclast exerts its function by attachment to the bone matrix by β3 integrin (McHugh et al., 2000). A compartment is enclosed by the ruffled border of the osteoclast and the bone surface. The mineral components of the bone are dissolved by the H^+ ions pumped into the alcove by the osteoclast, followed by protease degradation of the organic matrix leaving behind a characteristic Howship's lacuna (Väänänen et al., 2000).

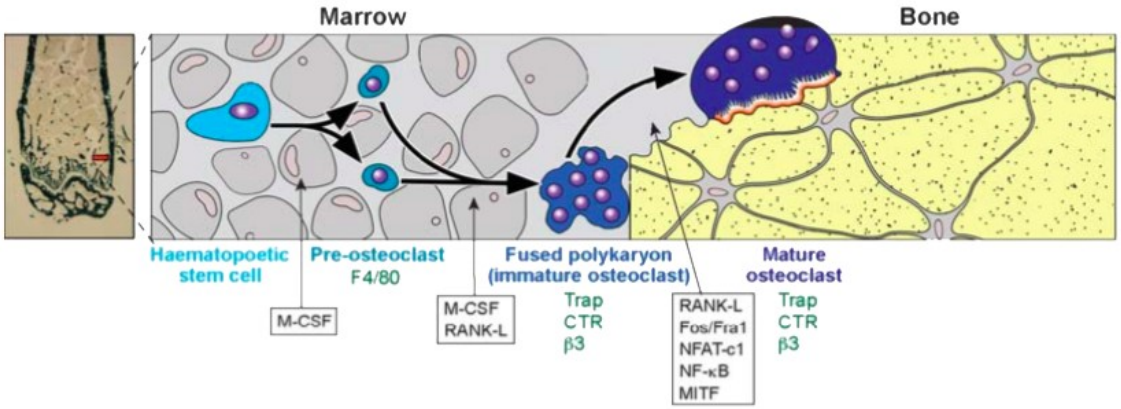

Figure 5: Development of preosteoclast to mature osteoclast. *Adapted from Robling et al. (2006)*
Differentiation of the immature osteoclast arises only under specific circumstances such as the continual presence of RANKL as well as the expression of AP-1 member c-Fos by monocyte-macrophage lineage cells (Matsuo et al., 2004), microphthalmia-associated transcription factor (MITF) (Partington et al., 2004) and nuclear factor of activated T cells calcineurin-dependent 1 (NFATc1) (Matsuo et al., 2004).

1.2.1.2 RANK/RANKL signalling pathway

To form an osteoclast, RANKL uses NF-κB which is transported to the nucleus by tumour necrosis factor receptor-associated factor (TRAF) intermediates (Wong et al., 1998). Various TRAF proteins bind within the specific cytoplasmic domain of RANK (Hsu et al., 1999). TRAF2, TRAF5 and TRAF6 were all shown to bind to RANK, but only TRAF6 has been shown to have fatal effects on osteoclast activity because only by the deficiency of TRAF6 osteoclasts lose their function resulting in osteopetrosis

(Lomaga et al., 1999). TRAF6 assembles signalling proteins that lead to osteoclast-specific gene expression, differentiation and activation. The two most closely studied pathways are the activation of transcription factors NF-κB and AP-1 (Boyle et al., 2003) (Fig. 6). NF-κB activation is induced by signalling cascades mediated by IκB kinase (IKK) (Karin et al., 2002); the AP-1 pathway is induced by jun N-terminal kinase 1 (JNK1) (David et al., 2002). As discussed in section 1.1.3 in more detail, NF-κB and AP-1 pathways can also be activated in a TLR-dependent manner, after ecDNA motif is recognized by TLR. MyD88 protein then recruits TRAF6 and activates kinase complexes such as IKK that are important for osteoclast differentiation and activation. After activation of AP-1 and NF-κB, activation of nuclear factor NFATc1, important for osteoclast formation, may be induced (Fig. 6).

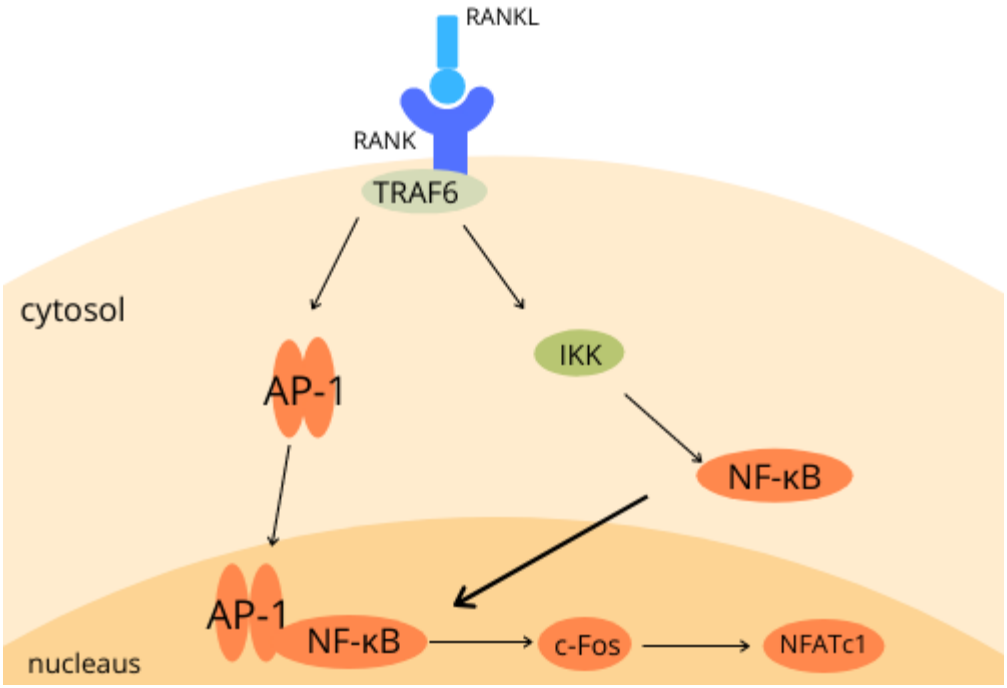

Figure 6: RANK/RANKLsignalling pathway. *Author*
RANKL binds to RANK and activates TRAF6. IKK is activated by TRAF6 via multiple proteins. The NF-κB pathway is activated after the IKK complex is phosphorylated (Ninomiya-Tsuji et al., 1999). Via the induction of AP-1 transcription factor, c-Fos, family members are also activated (Grigoriadis et al., 1994). This signalling process induces the activation of NFATc1 by cause of activated AP-1 resulting in transcription of osteoclastogenic genes that regulate multi-nucleation and bone resorption (Takayanagi et al., 2002; Park et al., 2017).

NF-κB can be activated by the RANK/RANKL signalling pathway. However, since the NF-κB transcription factor can also be alternatively activated after ecDNA is recognized by TLR (Takagi, 2011), the question arises whether the stimulation of monocyte-

macrophage cells by ecDNA with proven stimulatory effect (ST-ecDNA) will result in osteoclast formation (Fig. 7). Similarly, with ecDNA with proven inhibitory effect (INH-ecDNA) (Stunz et al., 2002) the question is raised whether such ecDNA will exercise its effect in blocking osteoclastogenesis (Fig. 8).

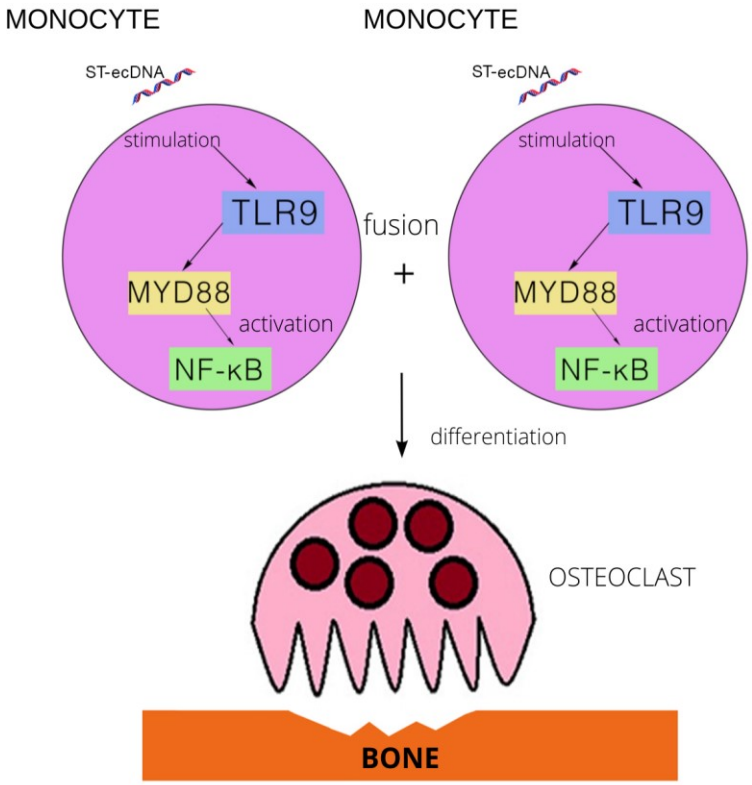

Figure 7: ST-DNA's supposed effect on monocytes, leading to fusion and differentiation into osteoclast. *Author*

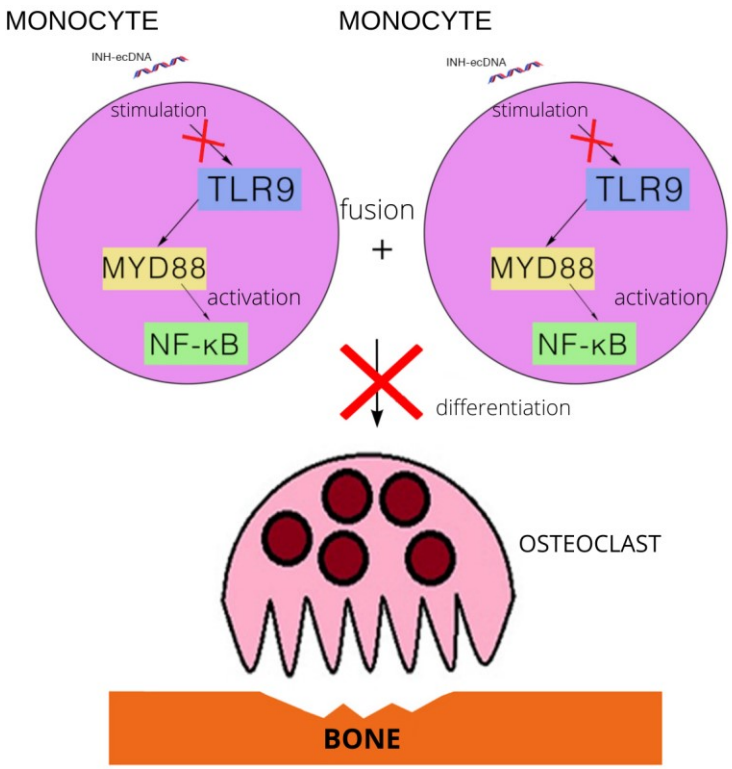

Figure 8: INH-DNA's supposed effect on monocytes, blocking osteoclastogenesis.
Author

1.2.2 Osteoblasts

Osteoblasts are the cells found on the bone surface that are responsible for the bone formation. In contrast to osteoclasts derived from hematopoietic stem cells, the osteoblasts develop from mesenchymal stem cells. Osteoblasts are responsible for secreting non-mineralized bone matrix (osteoid) before their incorporation into the bone matrix as osteocytes (Frost, 1960). Apart from their distinctive morphology and function, osteoblasts can be characterized by their PTH, 1.25-dihydroxyvitamin D3, oestrogen and glucocorticoid receptors, which are vastly involved in their differentiation (Sampath et al., 1992).

Bone formation and resorption are very closely related processes and their equilibrium defines both bone mass as well as its quality. Both osteoblasts' and osteoclasts' proliferation and differentiation are regulated via a number of hormones and transcription factors. Osteoblasts were found to be involved in osteoclastogenesis through a mechanism of cell-to-cell contact (Fig. 9). During osteoblast and osteoclast interaction gap junctions are created and small water-soluble molecules are able to pass between these two types of

cells (Everts et al., 2002). RANKL is expressed on the osteoblast surface and M-CSF is also a product of osteoblasts as well as osteoprotegerin (OPG) (Tan et al., 1997; Udagawa et al., 1999). OPG is a member of the TNF receptor family and it plays a vital role in regulating osteoclast proliferation and differentiation but in contrast with RANKL and M-CSF it influences osteoclastogenesis negatively (Tan et al., 1997) (Fig. 9).

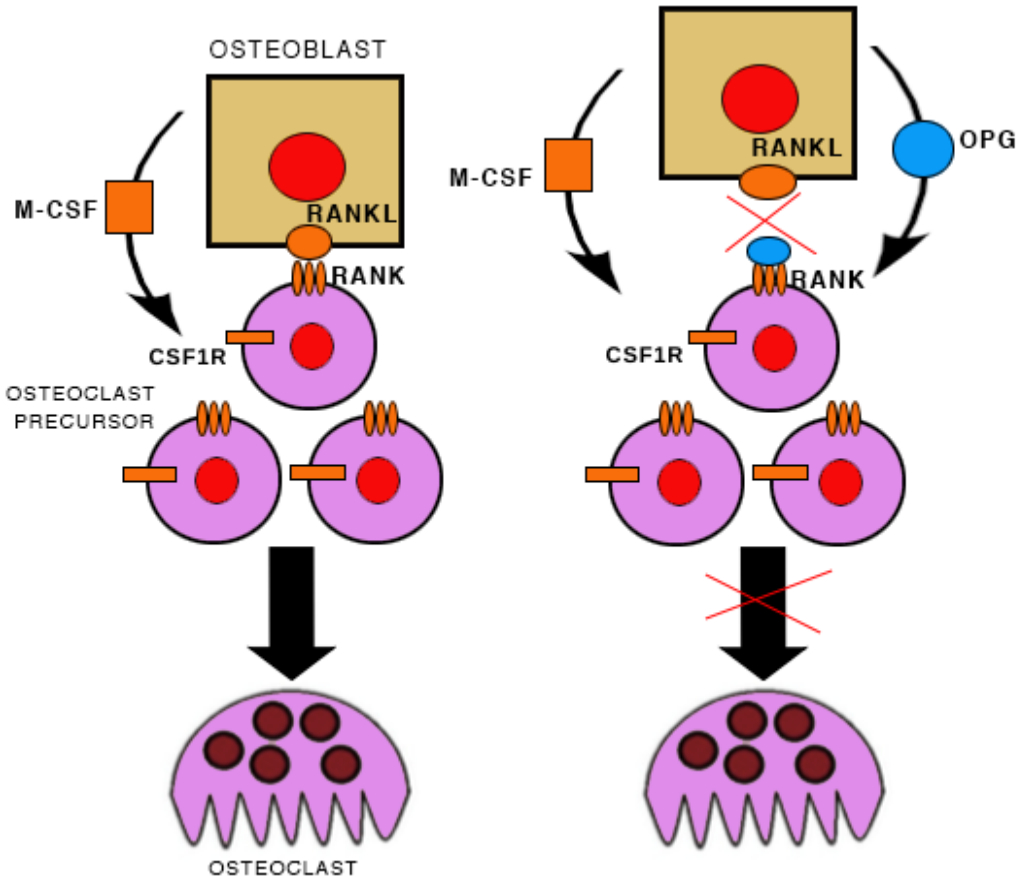

Figure 9: RANK/RANKL/OPG signalling pathway. *Adapted from Škubica (2018)*
OPG is able to bind to RANK on the surface of osteoclast precursors and therefore obstructs the binding of RANKL and subsequently blocks osteoclastogenesis.

1.2.3 Osteocytes

After their incorporation into the mineralized bone matrix, osteoblasts stop producing osteoid and become osteocytes. Osteocytes are the most abundant cell types in adult bone (Wang et al., 2005). They are typically single-nucleated cells (Palumbo, 1986).

RANKL expression makes osteocytes a major component of osteoclast formation. Two independent laboratories tested various cell types and their importance of producing RANKL in mice, using the *Tnfsf11* gene that encodes RANKL and can be knocked-out.

Mice lacking RANKL in various stages of osteocyte differentiation from the osteoblast progenitors to mature osteocytes resulted in severe osteopetrosis, due to complete lack of osteoclasts. This experiment further proves that mesenchymal-derived cells such as osteocytes are crucial in osteoclastogenesis because they provide important cytokines in the system (Nakashima et al., 2011; Xiong et al., 2012).

Osteoclasts and osteoblasts together with osteocytes are all important components in bone modelling and remodelling. Shifting their equilibrium to either side can result in pathological conditions. In this thesis we focused on elucidating whether ecDNA is able to influence monocyte differentiation potential into osteoclasts and therefore influence this shift.

2. Thesis objectives

This thesis deals with a question of the role of ecDNA in affecting human monocyte differentiation into mature osteoclasts by activating *in vitro* the NF-κB signalling pathway, via TLR9. Since ecDNA is found in increased concentrations in patients with chronic inflammatory diseases, potential findings of this study may subsequently lead not only to better understanding of the cellular processes that take place in osteoclast formation, but also to better prevention and prognosis of osteoporosis caused by chronic inflammation.

Our main thesis objective is:

- to establish *in vitro* whether and how a chosen ecDNA sequence is able to influence the differentiation potential of monocytes into osteoclasts

Partial thesis objective:

- application of the experimental procedure to long-term cultivations

Based on literature research we formulated these hypotheses:

- sequence with proven stimulatory effects in innate immune response will exhibit stimulatory effects on monocyte differentiation into osteoclasts
- sequence with proven inhibitory effects in innate immune response will exhibit inhibitory effect on monocyte differentiation into osteoclasts

3. Material and methods

3.1 Blood donors

Mononuclear cells for osteoclast differentiation and cultivation were obtained from healthy blood donors (Table 1), women ages 20–42 and men ages 21–51. Donors were only considered healthy if they met the following criteria:

- they do not suffer any autoimmune disease
- they have not suffered any infectious disease in the 14 days before the blood sampling at minimum
- they are not obese (according to body mass index)
- they are not diagnosed with or suspected for osteoporosis

All donors were acquainted with the study before their blood was taken. They were asked to sign an informed consent according to GDPR standards prior to the study. They were also asked to fill out a questionnaire (Annex A) to map any possible source of subclinical inflammation in the prospective participant in order to eliminate the potential bias in data.

Table 1: Number of donors included in the study

Donors	Number of donors	Age mean (SD)	BMI average (SD)
Women	18	26.7 (5.2)	22.7 (2.4)
Men	17	29.4 (7.6)	22.7 (2.3)

SD ~ standard deviation

3.2 Oligodeoxynucleotides (ODNs)

All standard desalted oligonucleotides used in this thesis were synthesized by Integrated DNA Technologies. We used four main categories for the ODNs: stimulatory (ST), inhibitory (INH), telomeric (TLM) and control (CO) ODNs. At first we used ODNs with phosphodiester (-pO) backbones, which can also be found *in vivo*. Sequences are shown in Table 2.

Table 2: -pO ODN sequences

ODN	Sequence
ST-ODN-2080 (CpG ODN) (Sackesen et al., 2013)	5′TCGTCGTTCCCCCCCCCCC
IHN-ODN-4329 (Ashman and Lenert, 2007)	5′CCTGGAGGGGAAGT
CO-ODN-2310 (Ashman et al., 2011)	5′TCCTGCAGGTTAAGT
TLM-ODN (TTAGGG)3 (Gursel et al., 2003)	5′TTAGGGTTAGGGTTAGGG

ODN ~ oligodeoxynucleotide

Conventional -pO backbone as found in native-state DNA, is very prone to degradation by external and internal nucleases and therefore, poses a problem when used in *in vitro* studies. To extend the effective molecular lifetime of an ODN other phosphate backbones were developed with phosphorothioate (-pS) (Fig. 10) proving as the most effective. The activity of external and internal nucleases has been found to be reduced majorly by using an ODN with -pS internucleotide linkages (Putney et al., 1981). Consequently after testing the -pO ODNs and proving that they are not compatible with our experimental design, we decided to test -pS ODNs. Sequences are shown in Table 3.

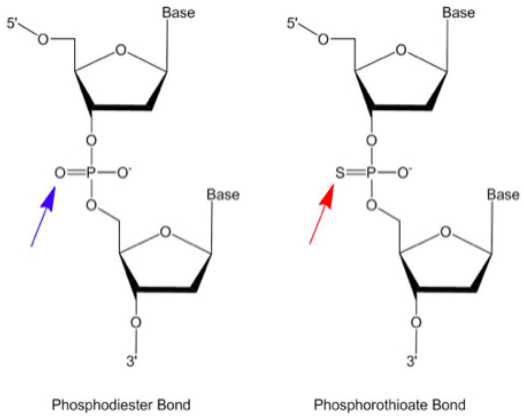

Figure 10: Phosphodiester and phosphorothioate backbones. *Adapted from URL 1*

Table 3: -pS ODN sequences

ODN	Sequence
ST-ODN-2006_pS (CpG ODN) (Sackesen et al., 2013)	5′T*C*G*TCGTTT*TGTCGT*TTTGTC*G*T*T
IHN-ODN-4347_pS (Ashman et al., 2011)	5′C*C*T*ATCCTG*GAGGGG*A*A*G
TLM (TTAGGG)4-ODN_pS (Sackesen et al., 2013)	5′T*T*A*GGGTTA*GGGTTA*GGGTTA*G*G*G
CO-ODN-2310_pS (Ashman et al., 2011)	5′T*C*CTG**C**A*G*GTTAA*G*T
CO-ODN-2114R (Ashman et al., 2011)	5′T*G*AAGG*GGAG*GTC*C*T
CO-ODN-2395-RM (Goldfarb et al. 2017)	5′T*G*CTGCTT*TTGGGG*GGCCCC*C*C

* -pS bond; ODN ~ oligodeoxynucleotide

CO-ODNs were used as controls to eliminate the effect of the ODN as a carrier of the biological effect on the monocytes, proving whether the distinct sequence is responsible for the effect.

3.3 Monocyte isolation from full blood and osteoclast differentiation

Blood (32 ml) was taken using 4 × 9 ml VACUETTE® CTAD Tubes (further referred to as sampling tube) with 3.2% anticlotting agent sodium citrate. Monocytes were separated from full blood following this protocol:

1. Initially, 3 ml Ficoll-PaqueTM PLUS (GE Healthcare) were prepared in 15 ml tubes and left to heat to room temperature (RT).
2. 50 ml Falcon tube was filled with blood from the sampling tube and the sampling tube was then washed two times with HBSS (EuroClone) which was then added to the 50 ml Falcon tube. The final ratio of blood to HBSS was 1:1.
3. The blood/HBSS suspension was then layered on top of 3 ml Ficoll.

4. Ficoll filled tubes with the diluted blood layered on top was centrifuged at 360 rcf for 30 minutes at RT. Components of the sample were separated according to their density.
5. Mononuclear cells were transferred to a new 50 ml Falcon tube filled with 5 ml HBSS, and then completed with HBSS to final volume of 25 ml.
6. Peripheral blood mononuclear cells (PBMCs) were washed of Ficoll by centrifugation at 1 500 rpm, for 10 minutes at RT.
7. After washing, supernatant was poured away and the pellet was resuspended by vortexing. Cell suspension of 10 µl was diluted 10 times with PBS and transferred to Bürker chamber for counting. The 50 ml Falcon tube with the rest of the cell suspension was filled to 25 ml with HBSS and PBMC were washed for the second time by centrifugation at 1 250 rpm for 10 minutes at RT.
8. Supernatant was poured away, cells were resuspended and 25 ml HBSS was added and the suspension was washed for the third time by centrifugation at 800 rpm for 10 minutes at RT with the intention of removing platelets.
9. Supernatant was poured away and the cells were resuspended.
10. Needed amount of complete DMEM medium (EuroClone: protocol can be found in section 3.3.1) was added to the cell suspension to seed 8×10^5 PBMC in 100 µl of DMEM into one well of the Microwell™ 96-well Microplates (Nunc™).
11. Cells were left to attach to the bottom of the plate for 2-3 hours in an incubator at 37 °C and with 5% of CO_2. We obtained adherent monocytes by removing the medium.
12. Every well with adherent monocytes was filled with 100 µl of new complete DMEM medium with M-CSF (Peprotech) (25 ng/ml) and cultivated for 24 hours (Fig. 11).
13. Medium was changed after 24 hours with the addition of M-CSF (25 ng/ml) and RANKL (0.5 ng/ml) and also the studied ODN (-pO: 0.033 µM; 1 µM; 10 µM. -pS: 0.1 nM; 10 nM; 100 nM). One triplet in every experiment was left without ODN (blank) as a control sample.
14. Medium was changed every 3 days and it contained M-CSF, RANKL and the studied ODN.

15. The cultivation took 14 days (Fig. 11) and resulted in matured multinuclear osteoclasts (Fig. 12), which were then visualized using TRAP staining (section 3.4) and microscopically evaluated. Two out of 43 experiments were analysed using quantitative polymerase chain reaction (qPCR).

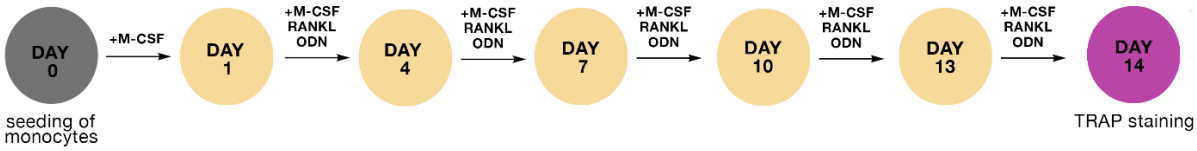

Figure 11: The course of the experiment, *Author*
This diagram shows the course of the experiment from day 0, when the monocytes are obtained, seeded and left to attach to the well. After the two hours that the cells are left to attach, M-CSF is added and cells are cultivated for 24 hours. On day 1 medium is changed and M-CSF, RANKL and ODN are added and the cells are then left to cultivate for three days. Change of the medium with M-CSF, RANKL and ODN is done every three days until day 14 when the cells are fixated and stained.

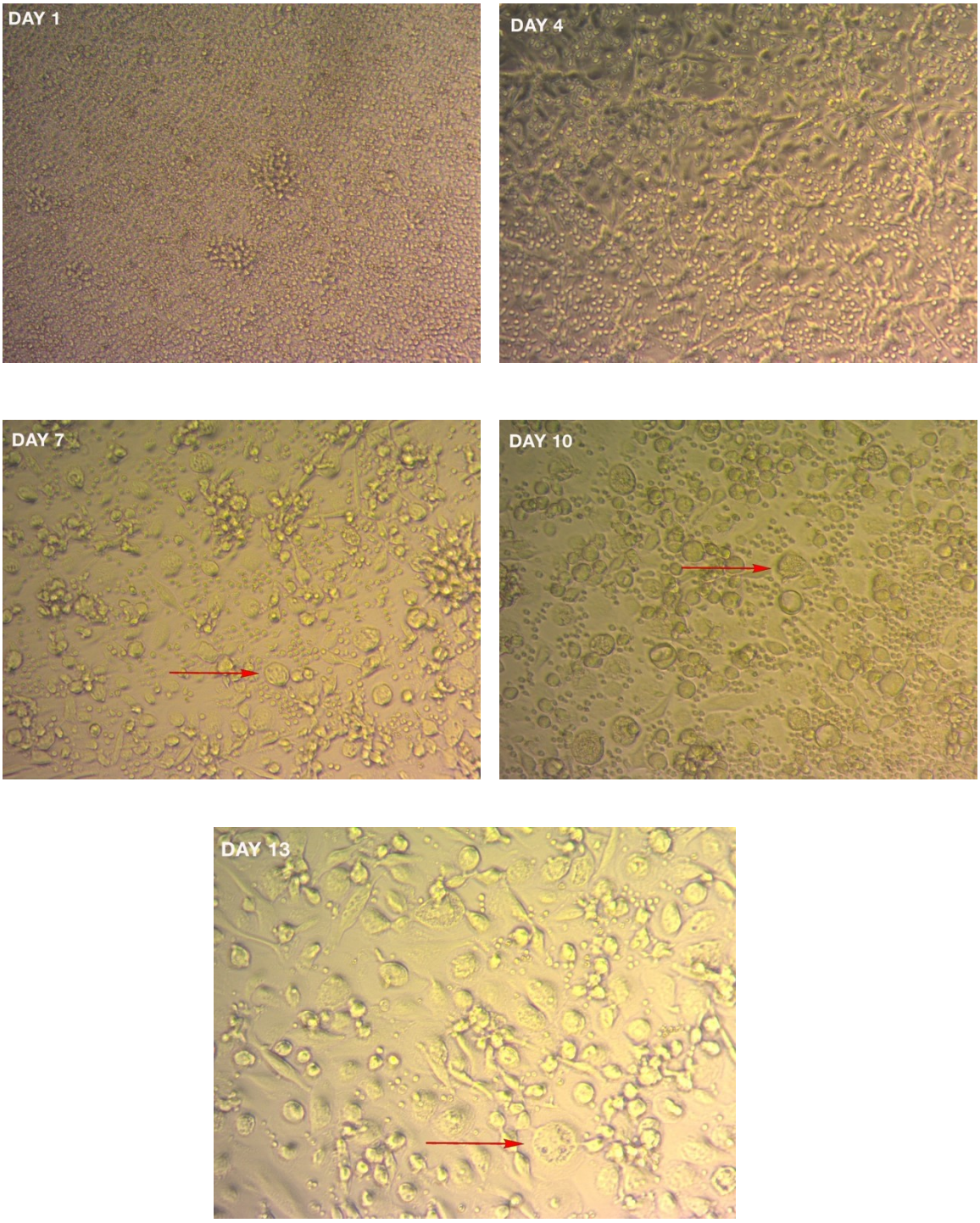

Figure 12: Visualization of progression of osteoclast differentiation during 14-day cultivation. *Author*
Monocyte fusion started at day 1 and continued through day 4 until initial cells with osteoclast morphology can be seen at day 7 (red arrow indicates an osteoclast). More cells with osteoclast morphology can be seen at day 10 and day 13. After day 13 cells were incubated for 24 hours and stained. Carl Zeiss Primovert, magnified 200×.

3.3.1 Preparation of full DMEM medium

1. Foetal bovine serum (FBS) (5 ml aliquot) (ultra-low endotoxin; Biosera was kept at −20 °C and left to unfreeze at 4 °C.
2. Penicillin-Streptomycin aliquot (PS; Sigma-Aldrich) and L-glutamine aliquot (LG; Sigma-Aldrich) were left to thaw at RT.
3. DMEM (44 ml), FBS (5 ml), LG (1 ml of 200 mM) and PS (0.5 ml) were mixed in 50 ml Falcon tube (the final volume of FBS in the medium is 10%).
4. Medium was filtered through 0.22 μm filter (TPP) and kept at 4 °C. This medium was used for three weeks at maximum.

3.4 TRAP staining

To visualize osteoclasts after 14 days of cultivation we followed this protocol:

1. Medium was pipetted away from the wells and the cells were washed using PBS at RT.
2. Paraformaldehyde (PFA) (4%) in PBS was added for 10 minutes to fixate cells in their current state.
3. PFA solution was pipetted away and the fixated cells were washed by PBS.
4. The staining solution was prepared using the Leukocyte Acid Phosphate (TRAP) kit (Sigma-Aldrich). The solution was prepared in two separate tubes (in case of a greater number of sample wells the amount of solution volume was multiplied):
    1. tube: 40 μl Fast Garnet GBC Base Solution
       40 μl Sodium Nitrite Solution
    2. tube: 40 μl Naphthol AS-BI Phosphoric Acid Solution
       160 μl Acetate Solution
       160 μl Tartrate Solution
       3,560 μl Millipore water (Millipore Milli-Q Gradient Water Purification System; Millipore)

5. Solutions from both tubes were mixed together and immediately applied to well with fixated cells. Cells were incubated at 37 °C and 5% CO_2 for 1 hour.
6. The staining solution was pipetted away after incubation and the cells were washed with Millipore H_2O.
7. During the cultivation and for tentative cell inspection the cells were observed using Zeiss Primovert (Carl Zeiss) light microscope. IX71 (Olympus) inverted fluorescent microscope was used for definite cell counting, because of its precise x and y axis table shift. Every TRAP positive cell that possessed more than 3 nuclei was considered an osteoclast.
8. To objectively evaluate the number of osteoclasts a relative number of osteoclasts was derived from each well. First we made a mark on the y axis of the well 3 mm away from the bottom most edge of the well. Then we moved the plate according to its x axis and counted every osteoclast in the field of vision along the diameter of the well, magnified 20 × 1.6. This way the same surface area was evaluated in each well yielding relative osteoclast number (Fig. 13).

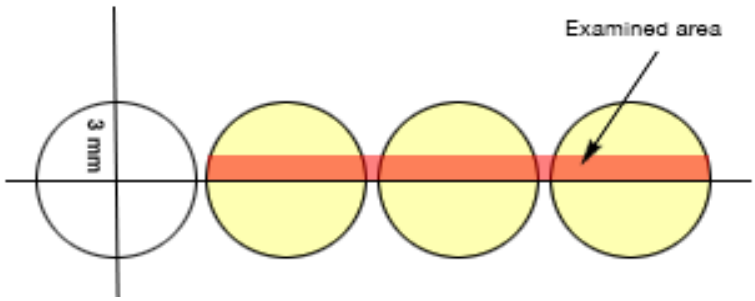

Figure 13: Examined surface area. *Adapted from Škubica (2018)*

3.5 Gene expression evaluation

3.5.1 RNA isolation

GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) was used to isolate RNA.

1. After 14 days of cultivation medium was pipetted away and cells were washed with PBS tempered at RT.

2. Lysis solution (200 µl) was added to each qPCR designated well and left to process for 1 minute. Cell lysate was pipetted into 1.7 ml microtube and 4 µl of 2-mercaptoethanol was added, then it was vortexed. Lysate either underwent immediate further processing or was kept at −80 °C and later underwent the following steps.
3. Lysate was pipetted into a filtration column (component of the kit) and centrifuged at 14 000 rpm for 2 minutes.
4. The filtration column was discarded and 500 µl of 70% ethanol (4 °C) was added to the filtrate and then it was vortexed.
5. We added 500 µl of the solution into the RNA binding column placed in a clean collection tube, centrifuged at 14 000 rpm for 15 seconds and discarded the filtrate. This step was executed twice.
6. 500 µl of wash solution 1 was added to the column and centrifuged at 14 000 rpm for 15 seconds.
7. Filtrate was discarded and 500 µl of wash solution 2 was added. Then it was centrifuged at 14 000 rpm for 15 seconds.
8. Filtrate was discarded and 500 µl of wash solution 2 was added again. Then it was centrifuged at 14 000 for 2 minutes.
9. After we discarded the filtrate for the last time the column was centrifuged at 14 000 rpm for 1 minute to fully discard the solution.
10. The column was transferred to a clean collection tube and 40 µl of Millipore water was added and left to incubate for 1 minute at RT.
11. Then the tube was centrifuged at 14 000 rpm for 1 minute. The RNA was inherent in the filtrate. Its final concentration and purity was evaluated using NanoPhotometer Pearl (Implen) and then it was kept at −80 °C.

3.5.2 Reverse transcription

High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. We followed this protocol:

1. Each component of the kit, but the reverse transcriptase was left to unfreeze on ice.

2. The amount of reaction solution was mixed according to the number of reactions. The amount listed below is needed for one reaction:

   10× Reverse transcription buffer solution..... 2 µl

   25× dNTP mix..... 0.8 µl

   10× Reverse transcription random primers..... 2 µl

   Reverse transcriptase..... 1 µl

   Millipore H2O..... 4.2 µl

3. After the solution was mixed it was vortexed and kept on ice.
4. 10 µl of the reaction solution were pipetted into 200 µl microtubes.
5. We added water and RNA so that the final amount of the reaction solution was 20 µl and the amount of RNA was 20 ng.
6. Microtubes were centrifuged and then transferred into C1000^{TM} Thermal cycler (BioRad) and the setting were set accordingly:

   step 1 (hybridization)..... 10 minutes at 25 °C

   step 2 (elongation)..... 120 minutes at 37 °C

   step 3 (enzyme denaturation)..... 5 seconds at 85 °C

7. cDNA samples were kept at –20 °C until analysis.

3.5.3 qPCR

qPCRBIO Probe Mix No ROX (PCR Biosystems) and probe/primer mix TaqMan® Gene Expression Assays (Applied Biosystems) were used during qPCR. The analysis was done using LightCycler® 480 Instrument II (Roche).

1. To dilute cDNA, 60 µl of Millipore H_2O was added to the cDNA yielded from reverse transcription, making the volume of the solution 80 µl.
2. qPCRBIO Probe Mix No ROX and probe/primer mix were left to unfreeze at RT, under aluminum foil to obstruct them from light, which can compromise the fluorescent dye in the mix.

3. The amount of reaction solution was mixed according to the number of reactions. The amount listed below is needed for one reaction:

   qPCRBIO Probe Mix No ROX..... 7 µl

   probe with primers..... 0.7 µl

   Millipore H_2O..... 2.3 µl

4. 10 µl of this solution was pipetted into wells of 384-well plate LightCycler® 480 Multiwell Plate 384 transparent (Roche) and 4 µl of diluted cDNA were added.

5. The plate was sealed with a sealing foil and centrifuged at 2 500 rpm for 2.5 minutes and then transferred into the plate holder of the LightCycler 480. Following protocol was used:

   Step 1 (hot start)..... 10 minutes at 95 °C

   Step 2 (denaturation)..... 15 seconds at 95 °C

   Step 3 (annealing, extension)... 1 minute at 60 °C

   Step 4 (cooling)..... 30 seconds at 40 °C

   Steps 2. and 3. were repeated 50×

After the reaction we used the Abs Quant/2nd Derivative Maximum method to calculate Ct value (cycle threshold value). Every sample was evaluated using technical triplets. Mean Ct was calculated from Ct values of three wells. If the standard deviation (SD) of Ct values was greater than 0.3 the most outlined well Ct value was discarded and mean Ct was calculated from the remaining two Ct values.

We evaluated the expression of *ACP5, CTSK, TLR9* and *NFATc1* genes and used *B2M* and *HPRT1* as internal controls, which are one of the most stable internal controls used in osteoclast experiments (Stephens et al., 2011).

3.6 Statistical analysis

After evaluation of the number of osteoclasts in each well variation coefficient was established to identify outliers. Identifying outliers was based on removal of the value from

a triplet which exceeded variation coefficient of 0.55. This value was established based on histogram of all variation coefficients where most triplets' variation coefficients were in the 0–0.55 range. If the variation coefficient did not meet the criteria even after removal of the outlying value, the triplet was not used in further analyses. GraphPad Prism 8 (GraphPad Inc.) was used to analyse the data. Normality of the data was established using D'Agostino-Pearson test, Shapiro-Wilk test and Kolmogorov-Smirnov test. To determine the effect of the ODNs, they were compared to blank (sample without added ODNs). Data were analysed using ordinary one-way ANOVA to compare multiple groups. To compare two unpaired groups we used t-test with Welch's correction for parametric data and Mann-Whitney test for non-parametric data. P value was set to 0.05 in all analyses. Data will be presented as mean with SD.

Material for qPCR was obtained from mix of cell lysates of two separate wells to average the gene expression. qPCR was done in technical triplets and SD for the triplet was set to 0.3. If the analysed triplet did not meet the criteria, the outlier was removed. If the SD value did not meet the criteria after removal of the outlying value, the triplet was not used in further analyses. If a sample resulted as negative, it was deemed to have crossed the threshold in the last cycle (cycle 50). Mean Ct values were compared to geometric mean of endogenous controls *B2M* and *HPRT1* or to mean Ct *B2M.* Fold change is presented as $2^{-\Delta Ct}$. Paired data were analysed by Wilcoxon test and P value was set to 0.05. Levels of gene expression are presented in arbitrary values

4. Results

4.1 The effect of -pO ODNs on osteoclast formation

Four types of ODNs (CO, ST, INH, TLM) with -pO backbones were tested to determine their effects on differentiation of osteoclasts from healthy donor monocytes. All ODNs were tested in three concentrations 0.033 μM, 1 μM, 10 μM to establish which one would prove most effective and whether the effect is dose dependent (Table 4). All samples were compared using ordinary one-way ANOVA. No significant differences were found when the samples were compared to their respective blanks. Data for TLM-ODN and INH-ODN are not shown because these experiments did not yield any osteoclasts.

Table 4: Average number and SD of osteoclasts yielded from experiments with -pO-ODN

Concentration	**0.033 μM**		**1 μM**		**1 μM**		**blank**	
ODN	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
CO-ODN-2310	29.18	25.55	33.90	22.09	28.28	11.15	20.54	14.17
ST-ODN-2080	37.4	11.29	38.33	21.08	42.5	17.18	24.85	15.91

CO-ODN-2310: 0.033 μM (n=6); 1 μM (n=6); 10 μM (n=2); blank (n=5)
ST-ODN-2080: 0.033 μM (n=4); 1 μM (n=5); 10 μM (n=2); blank (n=5) n ~ number of experiments
ODN ~ oligodeoxynucleotide; SD ~ standard deviation

4.2 The effect of -pS ODNs on osteoclast formation

ST-ODN-2006, INH-ODN-4347, TLM-ODN (TTAGGG)4 and CO-ODNs -2310; -2114R; -2395 were tested in three concentrations 0.1 nM, 10 nM and 100 nM, with the exception of CO-ODN 2114R which was only tested in concentration of 10 nM, to determine their effect on osteoclast differentiation from healthy donor monocytes.

4.2.1 The effect of -pS CO-ODN

Three types of CO-ODN (2310, 2114R, 2395) were tested to determine their effect on monocytes and establish whether they can serve as a control for further experiments (Table 5). Both CO-ODN-2310 and CO-ODN-2395 were tested in three concentrations

and compared to blank using one-way ANOVA. CO-ODN-2114R was tested in one concentration and compared to blank using unpaired t test with Welch's correction. No significant differences were found in these analyses. Since CO-ODN is used to remove the effect of the carrier of the effect, to establish that the effect is caused by the sequence its difference from the blank was not desirable.

Table 5: Average number and SD of osteoclasts yielded from experiments with three types of CO-ODN with phosphorothioate backbone

Concentration	**0.1 nM**		**10 nM**		**100 nM**		**blank**	
ODN	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
CO-ODN-2310	35.18	20.30	31.17	15.75	36.33	21.27	21.57	12.35
CO-ODN-2114R	ND	ND	35.44	10.74	ND	ND	42.44	11.40
CO-ODN-2395	51.11	13.72	52.11	14.52	45.67	20.52	51.11	9.64

CO-ODN-2310: 0.1 nM (n=8); 10 nM (n=4); 100 nM (n=8); blank (n=8)
CO-ODN-2114R: 10 nM (n=3); blank (n=3)
CO-ODN-2395: 0.1 nM (n=3); 10 nM (n=3); 100 nM (n=3); blank (n=3)
ODN ~ oligodeoxynucleotide; ND ~ not done; SD ~ standard deviation

4.2.2 The effect of -pS ST-ODN-2006

Three concentrations of ST-ODN-2006 and blank were compared using one-way ANOVA to determine ST-ODN-2006's effect on monocytes. Significant difference (p=0.0047, Fig. 14) was found between ST-ODN (0.1 nM) and blank.

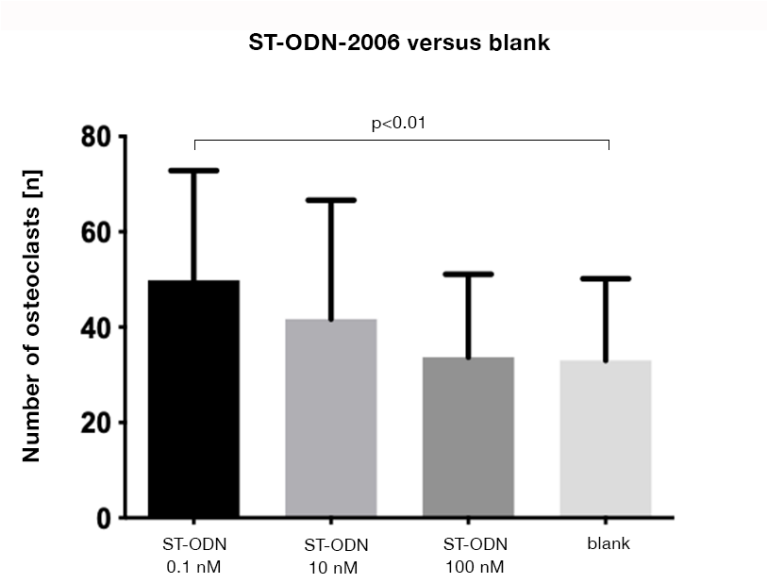

Figure 14: Comparison of number of osteoclasts obtained from cultivation of monocytes with three concentrations of -pS ST-ODN and blank.
The whiskers show the mean with SD for each data set. Column values: **ST-ODN 0.1 nM** (n=10): mean 49.96, SD 22.65; **ST-ODN 10 nM** (n=4): mean 41.67, SD 24.94; **ST-ODN 100 nM** (n=7)**:** mean 33.71, SD 17.38; **blank** (n=10)**:** mean 33.02, SD 16.97. ODN ~ oligodeoxynucleotide; SD ~ standard deviation

ST-ODN-2006 (0.1 nM) was also compared to CO-ODN-2395 (10 nM) (Fig. 15) using unpaired t test with Welch's correction and a significant difference (p=0.0227) was found.

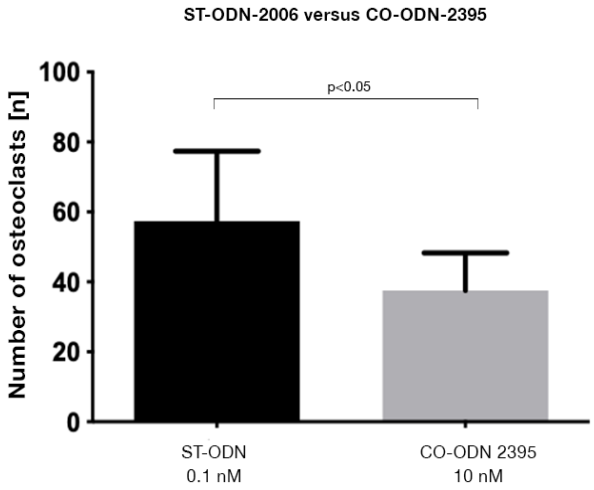

Figure 15: Comparison of number of osteoclasts obtained from cultivation of monocytes with ST-ODN and CO-ODN.
The whiskers show the mean with SD for each data set. Column values: **ST-ODN 0.1 nM** (n=3): mean 57.33, SD 20.07; **CO-ODN 10 nM** (n=3): mean 52.11, SD 14.52. ODN ~ oligodeoxynucleotide; SD ~ standard deviation

We also tested whether and how different time of the first administration of the ST-ODN to the cell culture affects osteoclastogenesis (Table 6). The tested times were day 0, day 1 and day 4. Data were analysed by ordinary one-way ANOVA. No significant difference was found when compared to blank.

Table 6: Average number and SD of osteoclasts yielded from experiments when ODN was added into the cell culture at different times for the first time

Day of administration	**day 0**		**day 1**		**day 4**		**blank**	
ODN	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
ST-ODN-2006	97.44	18.04	78.22	16.84	88.56	23.96	77.22	27.46

ST-ODN-2006: 0.1 nM; day 0 (n=3); day 1 (n=3); day 4 (n=3); blank (n=3)
ODN ~ oligodeoxynucleotide; SD ~ standard deviation

4.2.3 The effect of -pS INH-ODN-4347

The effects of three concentrations of INH-ODN-4347 on the osteoclastogenesis were compared to blank culture using one-way ANOVA; no significant difference was found (Table 7).

Table 7: Average number and SD of osteoclasts yielded from experiments with three concentrations of INH-ODN

Concentration	**0.1 nM**		**10 nM**		**100 nM**		**blank**	
ODN	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
INH-ODN-4347	41.25	20.61	44.64	19.47	39.82	27.39	38.25	19.45

INH-ODN-4347: 0.1 nM (n=3); 10 nM (n=3); 100 nM (n=3); blank (n=3)
ODN ~ oligodeoxynucleotide; SD ~ standard deviation

Further, we co-cultured ST-ODN with INH-ODN to assess their combined effect on osteoclastogenesis, under stimulatory conditions mediated by ST-ODN. We compared their effects using ordinary one-way ANOVA (Fig. 16). Significant difference ($p=0.0037$) was found between ST-ODN (0.1 nM) + INH-ODN (100 nM) and ST-ODN (0.1 nM) and also between ST-ODN (0.1 nM) + INH-ODN (100 nM) and ST-ODN (0.1 nM) + INH-ODN (0.1 nM) ($p=0.0251$).

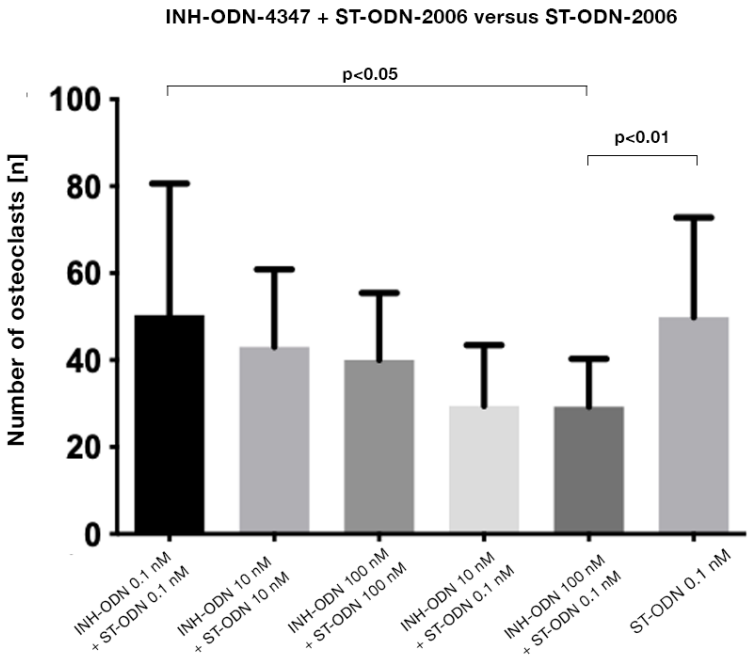

Figure 16: Comparison of number of osteoclasts obtained from cultivation of monocytes with five combinations of concentrations of -pS INH-ODN and ST-ODN.
The whiskers show the mean with SD for each data set. Column values: **INH-ODN 0.1 nM + ST-ODN 0.1 nM** (n=6)**:** mean 50.33, SD 30.30 **INH-ODN 10 nM + ST-ODN 10 nM** (n=5)**:** mean 43.00, SD 17.89 **INH-ODN 100 nM + ST-ODN 100 nM** (n=5)**:** mean 40.00, SD 15.48 **INH-ODN 10 nM + ST-ODN 0.1 nM** (n=3)**:** mean 29.44, SD 14.05 **INH-ODN 100 nM + ST-ODN 0.1 nM** (n=6)**:** mean 27.89, SD 12.24 **ST-ODN 0.1 nM** (n=16)**:** mean 49.96, SD 22.65. ODN ~ oligodeoxynucleotide; SD ~ standard deviation

Results of analysing effect of lone IHN-ODN versus the co-culture of INH-ODN + ST-ODN tested by Mann-Whitney test show no significant difference (Fig. 17).

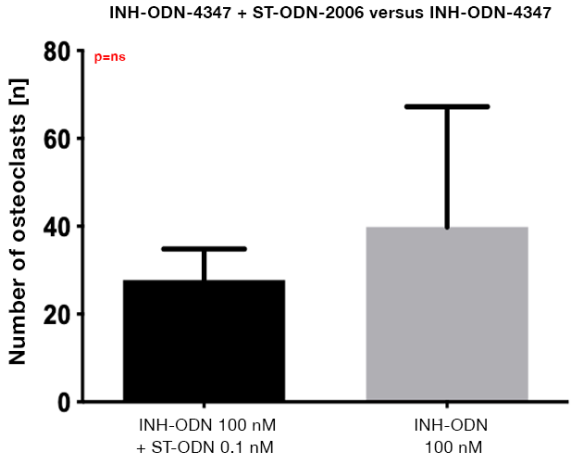

Figure 17: Comparison of number of osteoclasts obtained from cultivation of monocytes with INH-ODN 4347 + ST-ODN and INH-ODN.
The whiskers show the mean with SD for each data set. Column values: **INH-ODN 100 nM + ST-ODN 0.1 nM** (n=3): mean 25.11, SD 10.33 **INH-ODN 100 nM** (n=4)**:** mean 37.92, SD 26.93. ODN ~ oligodeoxynucleotide; SD ~ standard deviation

We also observed changes in osteoclast size when cultivated with INH-ODN, alone as well as in combination with ST-ODN (Fig. 18).

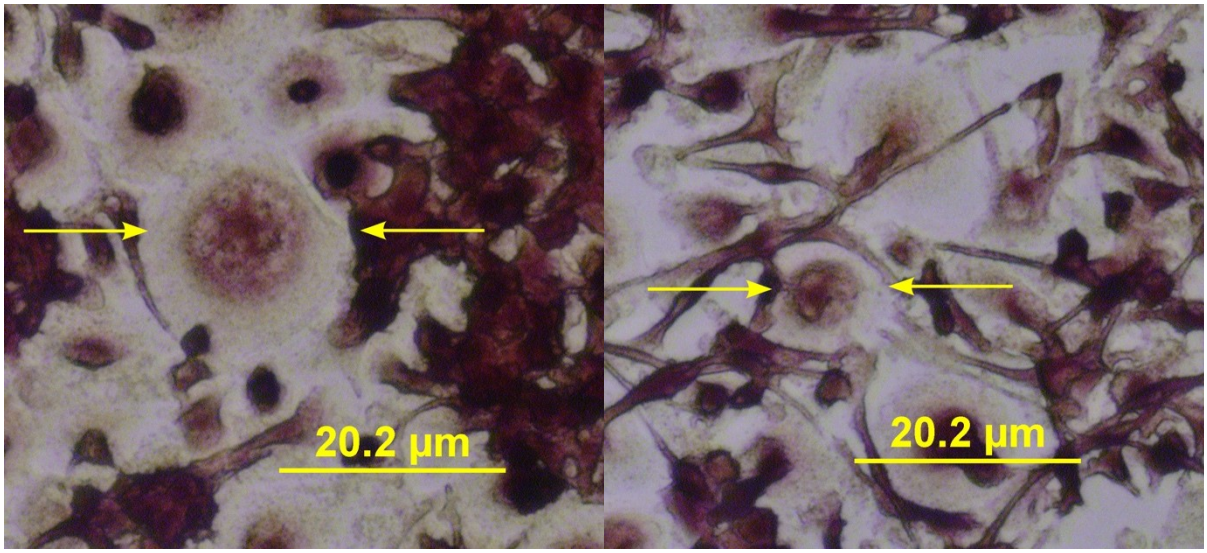

Figure 18: Osteoclasts cultivated with ST-ODN 0.1 nM (left) and ST-ODN 0.1 + INH-ODN 100 nM (right) at 20 × 1.6 magnification.

4.2.4 The effect of -pS TLM-ODN (TTAGGG)4

Effect on osteoclastogenesis of three concentrations of TLM-ODN (TTAGGG)4 and blank were compared using one-way ANOVA (Table 8). No significant difference was found.

Table 8: Average number and SD of osteoclasts yielded from experiments with three concentrations of TLM-ODN

Concentration	**0.1 nM**		**10 nM**		**100 nM**		**blank**	
ODN.	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
TLM-ODN (TTAGGG)4	38.67	7.51	40.67	4.04	55.00	4.58	50.33	8.39

TLM-ODN (TTAGGG)4: 0.1 nM (n=1); 10 nM (n=1); 100 nM (n=1); blank (n=1)
ODN ~ oligodeoxynucleotide; SD ~ standard deviation

ST-ODN + TLM-ODN were co-cultured to assess the effect they have on osteoclast differentiation. Effect of this co-culture was compared to the effect of lone ST-ODN by ordinary one-way ANOVA and no significant difference was found in the analysis (Fig. 19).

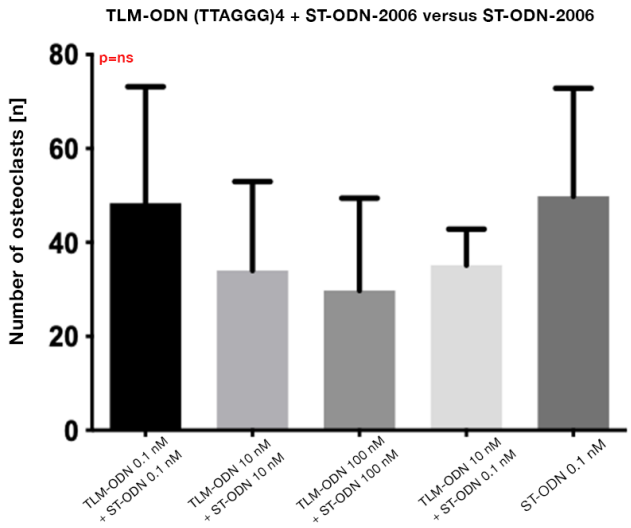

Figure 19: Comparison of the number of osteoclasts obtained from cultivation of monocytes with five combinations of concentrations of -pS TLM-ODN and ST-ODN.
The whiskers show the mean with SD for each data set. Column values: **TLM-ODN 0.1 nM + ST-ODN 0.1 nM** (n=3)**:** mean 51.78, SD 25.33 **TLM-ODN 10 nM + ST-ODN 10 nM** (n=3)**:** mean 34.00, SD 18.93 **TLM-ODN 100 nM + ST-ODN 100 nM** (n=3)**:** mean 29.78, SD 19.65 **TLM-ODN 10 nM + ST-ODN 0.1 nM** (n=6)**:** mean 32.33, SD 11.30 **ST-ODN 0.1 nM** (n=16)**:** mean 49.96, SD 22.65.
ODN ~ oligodeoxynucleotide; SD ~ standard deviation

Results of analysing the effect of lone TLM-ODN versus the combination of ST-ODN + TLM-ODN tested by Mann-Whitney test also show no significant difference (Fig. 20).

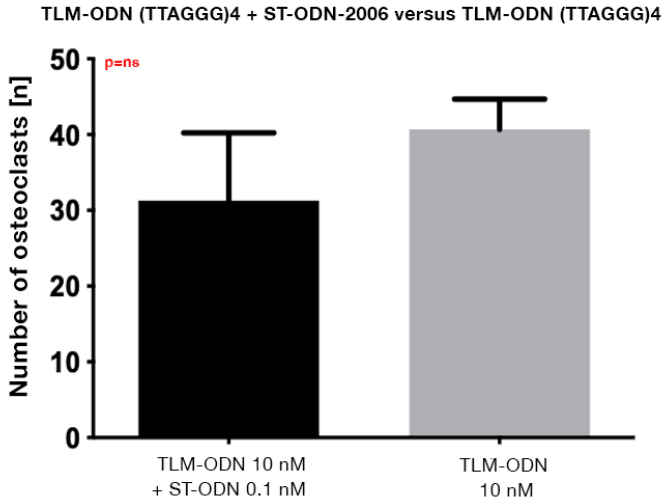

Figure 20: Comparison of the number of osteoclasts obtained from cultivation of monocytes with TLM-ODN + ST-ODN and TLM-ODN.
The whiskers show the mean with SD for each data set. Column values: **TLM-ODN 10 nM + ST-ODN 0.1 nM** (n=3)**:** mean 27.78, SD 14.05 **TLM-ODN 10 nM** (n=1): mean 40.67, SD 4.04.
ODN ~ oligodeoxynucleotide; SD ~ standard deviation

4.3 The effect of -pS ODNs on gene expression of osteoclastic markers

In addition to testing monocyte differentiation potential and the final number of osteoclasts that they produced when cultivated in the presence of -pS ODNs, we also tested gene expression of osteoclastic markers. We assumed that the measure of expression of these markers might describe the cell activity. We compared the Ct values of reference genes (*B2M, HPRT1*) to Ct values of osteoclastic markers *ACP5, CTSK* and *NFATc1* resulting in $2^{-\Delta Ct}$ values which show the fold-change in expression versus the reference genes. We then also tested gene expression of *TLR9* to further demonstrate that ODNs activate osteoclastogenesis via this receptor. We tested all markers in the presence of ST-ODN-2006 (0.1 nM), CO-ODN-2395 (10 nM), ST-ODN-2006 (0.1 nM) + INH-ODN-4347 (100 nM), and ST-ODN-2006 (0.1 nM) + TLM-ODN (TTAGGG)4 (10 nM) to establish what their effect on them is. Since CO-ODN is used to remove the effect of the carrier we first calculated its difference from blank to assess its effect. After this calculation we subtracted this value from the remaining ODNs to determine their true effects on expression. We compared the $2^{-\Delta Ct}$ of ST-ODN to blank. ST-ODN + INH-ODN and ST-ODN + TLM-ODN were compared to ST-ODN. Gene expression was tested at three different times (day 0, day 1, day 4) in order to monitor the process of monocyte-to-osteoclast differentiation and capture the kinetics of the osteoclastogenic process. We compared the levels of expression of every gene in blanks and also in stimulated samples versus their respective blank. The last set of experiments analysed at different times uses only *B2M* as reference. In order to present data with a better resolution, values in the *TLR9* assay were multiplied by 10^2 and values in the *NFATc1* assay by 10^3.

4.3.1 Expression of *ACP5*

We tested the expression of *ACP5* gene in samples where the cells were cultivated with ST-ODN-2006 (0.1 nM), CO-ODN-2395 (10 nM), ST-ODN-2006 (0.1 nM) + INH-ODN-4347 (100 nM), ST-ODN-2006 (0.1 nM) + TLM-ODN (TTAGGG)4 (10 nM) and blank. The geometric mean of *B2M* and *HPRT1* genes served as a reference. As seen in the Fig. 21, 1.69× increase of the *ACP5* expression was detected in osteoclasts cultured with ST-ODN (0.1 nM) compared to blank (p=ns, Wilcoxon test); this expression was non-significantly lowered 0.79× by ST-ODN (0.1 nM) + INH-ODN (100 nM) treatment

and same non-significant decrease was observed under the ST-ODN (0.1 nM) + TLM-ODN (10 nM) influence.

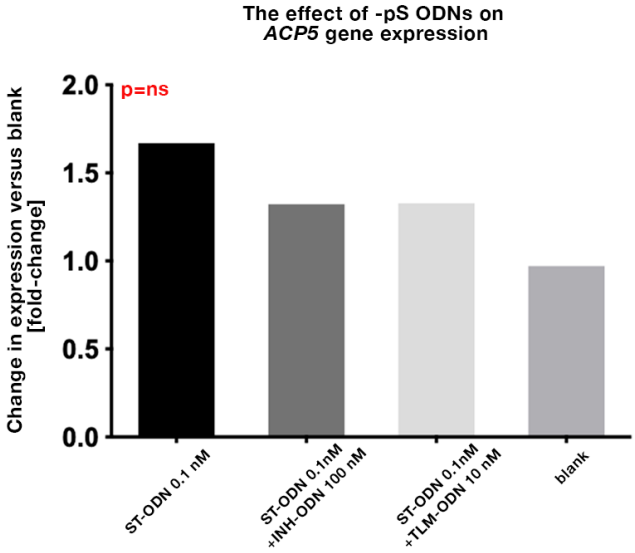

Figure 21: The effect of -pS ODNs on *ACP5* gene expression.
When sample was treated by ST-ODN the expression of *ACP5* increased 1.69× when compared to blank. This expression was lowered 0.79× under ST-ODN + INH-ODN influence when compared to ST-ODN. It was also reduced 0.79× when influenced by ST-ODN + TLM-ODN in comparison to lone ST-ODN. (p=ns) (n=1)

We also tested the expression of *ACP5* in culture treated with ST-ODN in different times of the cultivation. We tested its expression on day 1 without ODNs, then on day 2 with ST-ODN (0.1 nM) already present and at the end of cultivation with their blank counterparts. No significant difference was found among the blanks tested at different times or between treated samples and their blank counterparts when tested with Wilcoxon test (Fig. 22).

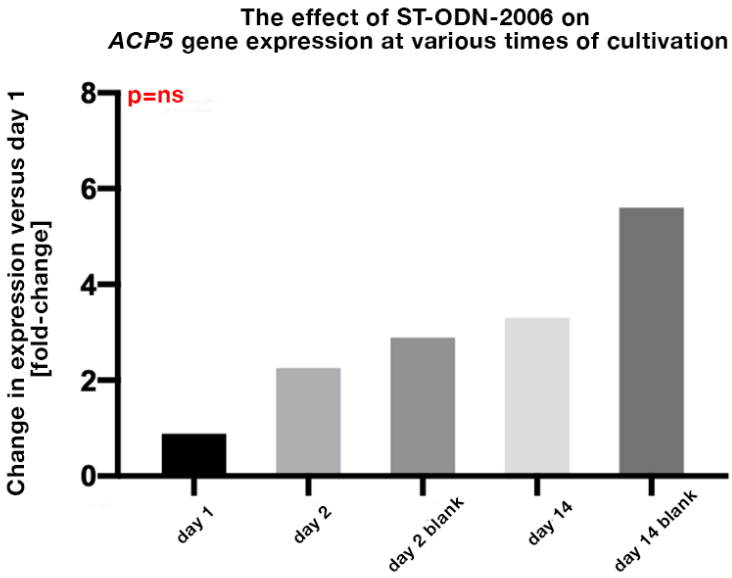

Figure 22: The effect of ST-ODN-2006 on *ACP5* gene expression at various times of cultivation.
On day 2 the expression of *ACP5* by pre-osteoclasts increased 3.01× even without ODN treatment and 5.71× on day 14 (p=ns). In contrast to that, the *ACP5* expression influenced by the ST-ODN increased only 2.37× on day 2 and 3.42× on day 14 compared to day 1. (p=ns) (n=1)

4.3.2 Expression of *CTSK*

Same as *ACP5* we tested gene *CTSK*. Its expression was tested after cultivation with ST-ODN-2006 (0.1 nM), CO-ODN-2395 (10 nM), ST-ODN-2006 (0.1 nM) + INH-ODN-4347 (100 nM), ST-ODN-2006 (0.1 nM) + TLM-ODN (TTAGGG)4 (10 nM) and blank. The geometric mean of *B2M* and *HPRT1* genes served as reference. No significant difference was found when ST-ODN was compared to blank and ST-ODN + INH and ST-ODN + TLM-ODN were compared to ST-ODN, when tested with Wilcoxon test (Fig. 23).

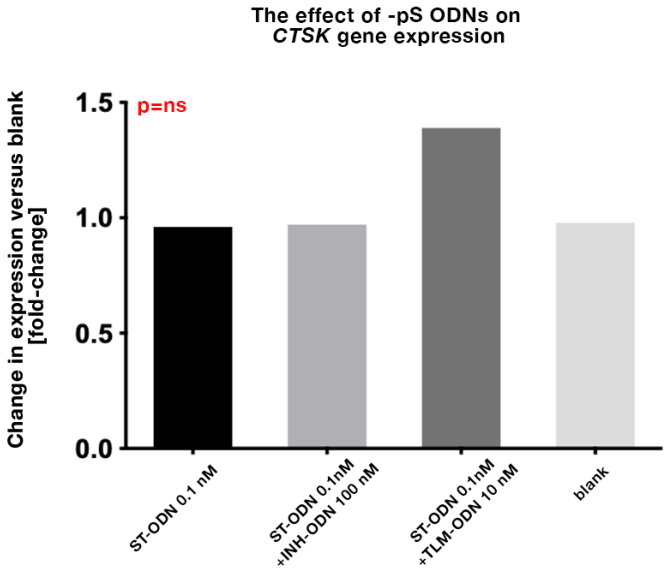

Figure 23: The effect of -pS ODNs on *CTSK* gene expression.
The expression of *CTSK* gene is not influenced by ST-ODN when compared to blank nor by ST-ODN + INH-ODN treatment when compared to ST-ODN. Interestingly, the ST-ODN + TLM-ODN treatment seems to non-significantly increase the expression of *CTSK* 1.41× in comparison to lone ST-ODN. (p=ns) (n=1)

CTSK gene expression was also tested at different times of the cultivation in cultures treated with ST-ODN. Same as with testing *CTSK* we tested on day 1 without ODNs, then on day 2 with ST-ODN (0.1 nM) already present and at the end of cultivation and their respective blank counterparts. No significant difference was found among the blanks tested at different times or between treated samples and their blank counterparts when tested with Wilcoxon test (Fig. 24).

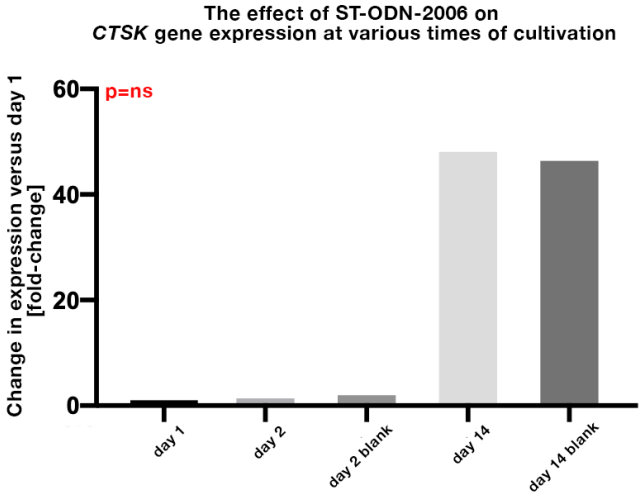

Figure 24: The effect of ST-ODN-2006 on *CTSK* gene expression at various times of cultivation.
The expression of *CTSK* by osteoclasts increased by 2.87× on day 2 without ODN treatment and 47.28× on day 14 (p=ns). *CTSK* expression influenced by the ST-ODN increased 2.29× on day 2 and 48.95× on day 14 (p=ns) compared to day 1. (p=ns) (n=1)

4.3.3 Expression of *TLR9*

We cultivated monocytes with ST-ODN-2006 (0.1 nM), CO-ODN-2395 (10 nM), ST-ODN-2006 (0.1 nM) + INH-ODN-4347 (100 nM), ST-ODN-2006 (0.1 nM) + TLM-ODN (TTAGGG)4 (10 nM) and blank. *B2M* and *HPRT1* were used as reference genes in these experiments. No significant difference was found when ST-ODN was compared to blank and ST-ODN + INH and ST-ODN + TLM-ODN were compared to ST-ODN, when tested with Wilcoxon test (Fig. 25).

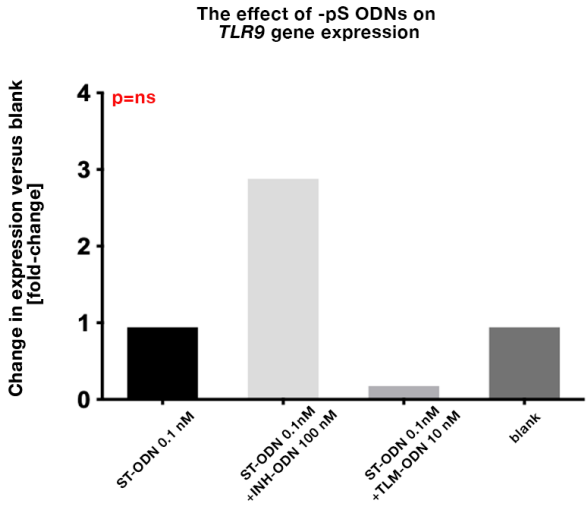

Figure 25: The effect of -pS ODNs on *TLR9* gene expression.
ST-ODN did not influence the expression of TLR9 when compared to blank. The expression of *TLR9* was increased 2.93× under ST-ODN + INH-ODN influence when compared to ST-ODN. It was also decreased 0.23× under the influence of ST-ODN + TLM-ODN when compared to ST-ODN. (p=ns) (n=1)

To determine *TLR9* gene expression we also tested it during different times throughout the cultivation in the presence of ST-ODN. We tested *TLR9* expression on day 1 without ODNs, then on day 2 with ST-ODN (0.1 nM) already present and at the end of cultivation and their respective blank counterparts. Data for day 2 treated with ST-ODN are not shown because they were not detected by the qPCR. No significant difference was found among the blanks tested at different times or between treated samples and their blank counterparts when tested with Wilcoxon test (Fig. 26).

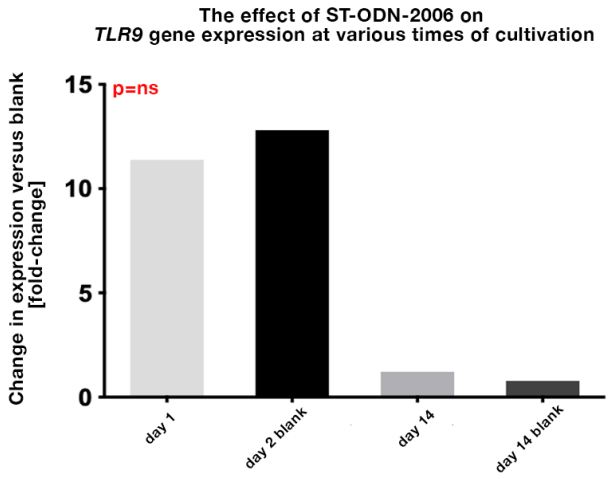

Figure 26: The effect of ST-ODN-2006 on *TLR9* gene expression at various times of cultivation.
The expression of *TLR9* by osteoclasts increased by 1.12× on day 2 without ODN treatment and decreased 0.08× on day 14 without ODN treatment when compared to day 1 (p=ns). *TLR9* expression influenced by the ST-ODN on day 2 was not detected by qPCR and its expression was decreased 0.12× on day 14 when compared to day 1. (p=ns) (n=1)

4.3.4 Expression of *NFATc1*

Same as the previous genes we tested *NFATc1* gene expression first with ST-ODN-2006 (0.1 nM), CO-ODN-2395 (10 nM), ST-ODN-2006 (0.1 nM) + INH-ODN-4347 (100 nM), ST-ODN-2006 (0.1 nM) + TLM-ODN (TTAGGG)4 (10 nM) and blank and used both *B2M* and *HPRT1* as reference genes. No significant difference was found when ST-ODN was compared to blank and ST-ODN + INH and ST-ODN + TLM-ODN were compared to ST-ODN, when tested with Wilcoxon test (Fig. 27).

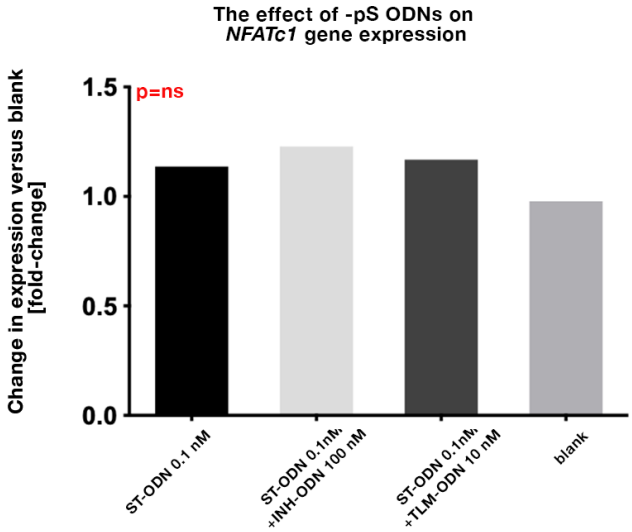

Figure 27: The effect of -pS ODNs on *NFATc1* gene expression.
When sample was treated by ST-ODN the expression of *NFATc1* increased 1.15× when compared to blank. This expression was increased 1.25× under ST-ODN + INH-ODN influence when compared to ST-ODN. It was also increased 1.19× when influenced by ST-ODN + TLM-ODN in comparison to lone ST-ODN. (p=ns) (n=1)

We also tested the mRNA expression of *NFATc1* during different times of cultivation when it was influenced by ST-ODN. Same as for all the other described genes we tested on day 1 without ODNs, then on day 2 with ST-ODN (0.1 nM) already present and at the end of cultivation and their respective blank counterparts. No significant difference was found among the blanks tested at different times or between treated samples and their blank counterparts when tested with Wilcoxon test (Fig. 28).

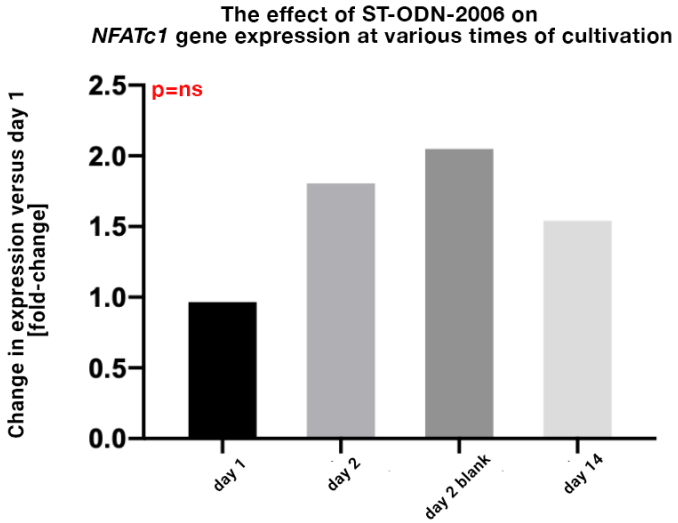

Figure 28: The effect of ST-ODN-2006 on *NFATc1* gene expression at various times of cultivation.
The expression of *NFATc1* by pre-osteoclasts increased 2.08× on day 2 without ODN treatment when compared to day 1, but was not detected by qPCR on day 14 without ODN treatment. *NFATc1* expression influenced by the ST-ODN on day 2 increased 1.84× and 1.58× on day 14 when compared to day 1. (p=ns) (n=1)

5. Discussion

In this thesis we wanted to determine whether ecDNA of a certain sequence can affect the differentiation potential of monocytes into osteoclasts. Since ecDNA is known to bind to TLR9 and activate the NF-κB pathway, we propose the hypothesis that it can also activate the differentiation of osteoclasts in an alternative manner, overriding the common RANK/RANKL pathway. We tested this hypothesis using an *in vitro* model, applying ODNs serving as ecDNA to healthy monocytes.

ecDNA exercises its function only if it meets structural criteria, since TLR9 binds and is subsequently activated by CpG active areas in ecDNA. Our ODNs were chosen according to these criteria. According to Ashman and Lenert (2007) the ecDNA with stimulatory effect should be unmethylated, sequence TCC should be present at the 5′ end and Cs should not occur one or two bases before the CG at 5′ end. Our -pO stimulatory ODN's (ST-ODN-2080's) primary sequence is 5′TCGTCGTTCCCCCCCCCCCC and it is unmethylated. ST-ODN-2080 was also used in experiments by Sackesen et al. (2013) where it showed to enhance proliferation of PBMC.

Also, according to Ashman and Lenert (2007), for a sequence to act as an inhibitor it should follow these requirements for primary sequence: 'xCC N D D NN GGG NNN' where N is any base and its presence is mandatory, x is optional base but its presence is not required, and D is any base except C. The sequence for our -pO inhibitory sequence (INH-ODN-4329) is 5′CCTGGAGGGGAAGT which meets the proposed criteria.

To eliminate the effect of ODN as a carrier of the effect and to prove that the primary sequence of the ODN is responsible, we also used control ODN (CO-ODN-2310). CO-ODN-2310 with the primary sequence 5′TCCTGCAGGTTAAGT was evaluated by Ashman et al. (2011) as neutral in human HEK cells.

Telometric motif TTAGGG is responsible for down-regulation of CpG DNA activated response. According to Gursel et al. (2003) we used the -pO telomeric ODN TLM-ODN (TTAGGG)3 with three repeats of the TTAGGG motif.

Both -pO CO-ODN and ST-ODN did not show any significant difference when compared to their respective blanks (Table 4). -pO INH-ODN and TLM-ODN were not mentioned because we yielded no osteoclasts from the blank samples and therefore could not determine whether there was any effect. The fact that the comparison of -pO CO-ODN and ST-ODN with blank did not result in any significant difference might be caused by the structure of the ODNs and their -pO backbone. Though -pO backbone is present in organisms *in vivo,* its effective lifetime is minimized because it is prone to degradation by extra and intracellular nucleases (Eckstein, 2014). Therefore, -pS backbone was developed to overcome this challenge and extend the effective lifetime of the ODN for *in vitro* experiments (Putney et al., 1981). We decided to carry on with -pS backbone ODNs keeping the sequences of CO-ODN and ST-ODN the same. We switched the INH-ODN sequence according to Ashman et al. (2011) from INH-ODN-4329 to INH-ODN-4347 with a sequence 5′C*C*T*ATCCTG*GAGGGG*A*A*G where * stands for the -pS bond. This type of -pS INH-ODN proved to have strong inhibitory effects on human B cells (Ashman et al., 2011). We also decided to change the TLM-ODN from three repeats to four according to Sackesen et al. (2013).

In experiments with -pS CO-ODN we first tested CO-ODN-2310 which did not show any significant difference when compared to blank, but it yields 63% more osteoclasts at 0.1 nM, 44% more osteoclasts at 10 nM and 68% more osteoclasts at 100 nM proving that it is not neutral and therefore cannot be used as control for further experiments. This is in contrast with Ashman et al. (2011) where it was proven as neutral, this though was proven in human HEK cells and does not mean it remains neutral in monocytes. We then tested CO-ODN-2114R which was also verified as neutral in experiments with human HEK cells by Ashman et al. (2011). In our experiments no significant difference was found when compared to blank, but it yielded 16% less osteoclasts at 0.1 nM than blank, again in contrast with Ashman et al. (2011). This ODN seems not to be neutral in monocytes. We then used CO-ODN-2395 with reversed CpG areas to ST-ODN-2006 to dismantle its stimulatory activity. This ODN yielded 0% more osteoclasts at 0.1 nM, 2% more osteoclasts at 10 nM and 11% less osteoclasts at 100 nM. We decided to use CO-ODN in 10 nM concentration for further experiments to rule out the possibility of the no effect being dose-dependent.

When testing -pS ST-ODN-2006, a significant difference was found at 0.1 nM when compared to both blank (p=0.0047) (Fig. 14) and CO-ODN-2395 (p=0.0227) (Fig. 15). At the concentration of 0.1 nM the experiments yielded 51% more osteoclasts than blank. Experiments with the concentration of 10 nM yielded 26% more osteoclasts than blank culture and experiments with the concentration of 100 nM of ODN yielded 2% more osteoclasts than blank. The stimulatory effect of CpG rich ST-ODN-2006 is in agreement with experiments published by Sackesen et al. (2013) where it showed to enhance the proliferation of PMBC. These experiments also show that higher concentration of ODN might be responsible for blocking its stimulatory effect. Zuo et al. (2002) suggest that when CpG ODN is added into the sample at day 0 it inhibits osteoclastogenesis by blocking M-CSF. By binding of ODN to M-CSF, RANK expression is obstructed and osteoclastogenesis cannot persist. They demonstrate that CpG ODNs exercise their stimulatory effect fully when administered to cell culture for the first time after 72 hours of stimulation carried out with only M-CSF and RANKL. In our experiments ST-ODN was added on day 1 (24 hours after the initial stimulation with M-CSF) but in different concentrations. If ST-ODN at the highest concentration was not able to increase osteoclast production by more than 2% of osteoclasts when compared to blank, it could be caused by M-CSF blockage. We decided to test whether the timing of the ODN administration to culture affected the outcome number of osteoclasts, because Zuo et al. (2002) proved the blockage of M-CSF only on murine bone marrow mononuclear cells. We added ST-ODN into the sample on day 0 after monocytes' initial attachment to the well; these experiments yielded 26% more osteoclasts than blank (Table 6). When ODN was added into the sample on day 1 it yielded only 1% more osteoclasts than blank and when it was added at day 4 it yielded 15% more osteoclasts than blank. This is in contrast with Zuo et al. (2002) where CpG ODN possessed inhibitory activity when added on day 0 because of the M-CSF blockage in case of murine bone marrow mononuclear cells. In our case CpG ST-ODN administered along with M-CSF and RANKL on day 0 increased osteoclast production which would point to a theory that it does not (fully) block M-CSF and does not (completely) obstruct RANK expression. When ODN was added into the sample after pre-stimulation with both M-CSF and RANKL on day 4, osteoclastogenesis already started and ST-ODN only supported this pathway yielding non-significantly more osteoclasts than the blank sample.

-pS INH-ODN was proved to block stimulating activity of ST-ODN when added into the murine B cell sample together (Stunz et al., 2002). We decided to test the effect of INH-ODN on osteoclast differentiation when it is added into the sample alone in three concentrations. No significant difference was found when compared to blank. INH-ODN at the concentration of 0.1 nM yielded 8% more osteoclasts than blank and at concentration of 10 nM it led to 17% more osteoclasts than observed in blank culture (Table 7). INH-ODN at the concentration of 100 nM increased the number of osteoclasts only by 4% when compared to blank. This suggests that when used alone, INH-ODN is not effective in inhibiting the differentiation of monocytes into osteoclasts, probably since it blocks the receptor for DNA but alone has no effect on stimulation of monocytes. Other possibility for its effect might lie in the time of administration into the cell culture. This possibility shall be tested in the future. When co-cultured with ST-ODN we tested 5 combinations of concentrations to establish whether the inhibitory effect only occurs as a result of competition between the ODNs, because the stimulatory effect is pronounced and therefore INH-ODN has a counterpart to inhibit. Difference between ST-ODN (0.1 nM) + INH-ODN (100 nM) and ST-ODN (0.1 nM) proved significant ($p=0.0037$) (Fig. 16). The experiments where ST-ODN and INH-ODN were both tested at the concentration of 0.1 nM they yielded 1% more osteoclasts than when only ST-ODN (0.1 nM) was added into the culture, proving that concentration of 0.1 nM of INH-ODN is not an effective contender for ST-ODN at the same concentration level. Elevating the concentration levels of INH-ODN as well as ST-ODN resulted in decrease in osteoclast numbers. When they were both tested at the concentration of 10 nM there were 14% less osteoclasts than in the sample with lone ST-ODN (0.1 nM) and when they were added into the sample at the concertation of 100 nM these experiments yielded 20% less osteoclasts than experiments with lone ST-ODN (0.1 nM). When we combined INH-ODN (10 nM) with ST-ODN (0.1 nM), these experiments yielded 41% less osteoclasts than culture with ST-ODN (0.1 nM). INH-ODN (100 nM) combined with ST-ODN (0.1 nM) yielded significantly (44%) less osteoclasts than the sample with lone ST-ODN (0.1 nM) and 34% less osteoclasts than lone INH-ODN (Fig. 16). These results show that when INH-ODN is combined with an effective stimulatory ODN in a high enough concentration it is able to block osteoclast production in a dose-dependent manner. INH-ODN did not only block osteoclasts production, but influenced osteoclast morphology as well. Osteoclasts under the influence of INH-ODN looked smaller than osteoclasts derived from experiments

where they were stimulated by ST-ODN. Osteoclasts are recognized as large multinucleated cells; nevertheless, when cultivated with IHN-ODN, they remain multinucleated but shrink in size as visible in the Fig. 18.

Similarly, we expected the -pS TLM-ODN to supress osteoclast production, because it was proven to lower B cell activation by Sackesen et al. (2013). In our case no significant difference was detected, but trend of lowering the osteoclast production by 23% by TLM-ODN at the concentration of 0.1 nM and by 19% at the ODN concentration of 10 nM when compared to blank could be observed (Table 8). At the concentration of 100 nM osteoclast production was increased by 9%. Possible explanations to this trend are that either lone TLM-ODN seems not to exert its effect, or, that the time of administration we used in this experimental design was not suitable for this ODN to exert its function. Further experiments testing the time of administration of the ODN shall also be tested in the future. To clarify the first possibility, we combined TLM-ODN with ST-ODN at various concentrations (Fig. 19) and no significant difference in the number of yielded osteoclasts was found between ST-ODN + TLM-ODN and lone ST-ODN. By adding ST-ODN and TLM-ODN both at the concentration of 0.1 nM the number of osteoclasts increased by 4%, proving the concentration of TLM-ODN too low to block ST-ODN effect on osteoclasts fully. By using both ODNs in a concentration of 10 nM the number of osteoclasts yielded from the experiment was lowered by 32% and using both ODNs in concentration of 100 nM lowered osteoclast production by 40% in comparison with lone ST-ODN (0.1 nM) stimulated cultures. We then also tested the most effective ST-ODN (0.1 nM) and the dose of TLM-ODN (10 nM) that seemed to be inhibiting the process of osteoclastogenesis (Table 8). This experiment yielded 35% less osteoclasts than lone ST-ODN (0.1 nM) influenced cultures. This suggests that TLM-ODN lowers the osteoclast production in competition with ST-ODN. Decrease in osteoclast production, however, was not significant. Presumably, low numbers of performed experiments (n=3 for every concentration/combination of concentrations) may play a role.

In addition to osteoclast numbers we also wanted to determine their activity by assessing the expression of osteoclastogenic markers *ACP5, CTSK* and *NFATc1*. Since our hypotheses are based on ODNs activating osteoclastogenesis through TLR9 we also decided to analyse its expression. *ACP5* gene is responsible for production of tartrate

resistant acid phosphatase 5 used by mature osteoclasts to resorb bone tissue. The *ACP5* gene expression and TRAP production are thus used for mature osteoclasts recognition. *ACP5* gene mRNA expression was increased 1.69× in cells cultivated with ST-ODN (0.1 nM) when compared to blank. Since ST-ODN increases osteoclast production, higher levels of *ACP5* expression are to be expected when compared to inhibitory ODNs or blank. Both cultivation with ST-ODN + INH-ODN and cultivation with ST-ODN + TLM-ODN decreased the *ACP5* mRNA expression in differentiated osteoclasts 0.79× in comparison to the levels affected by lone ST-ODN (0.1 nM). Since these experiments yielded less osteoclasts than blank or ST-ODN sample (data not shown), lower levels of expression are expected. The observed increase of osteoclast *ACP5* expression under the influence ST-ODN and its decrease by INH-ODN and TLM-ODN do not reach statistical significance. The fact that ST-ODN activated osteoclasts expression of *ACP5* to only 1.69× higher levels when compared to blank points to an idea that ST-ODN (0.1 nM) does not change osteoclast activity significantly even though it increases the yielded osteoclast number. When testing the mRNA expression of *ACP5* at various times of cultivation, an increasing trend in expression on each day was observed (Fig. 22). The surprising observation is that mRNA gene expression levels were increased in blank samples when compared to ST-ODN (0.1 nM) stimulated samples. According to Tseng et al. (2014), *ACP5* is a marker of osteoclast maturity and the trend of higher mRNA expression levels in later days can be explained by osteoclasts being more mature by the end of cultivation. The observation of increased mRNA expression levels in blank could be explained by the aforementioned idea that ST-ODN does not increase mRNA expression levels of *ACP5* even though it leads to more osteoclasts or that ST-ODN stunts osteoclast maturity.

Cathepsin K, important for bone matrix degradation by mature osteoclasts, is produced by *CTSK* gene. *CTSK* expression in osteoclasts cultivated with ST-ODN (0.1 nM) does not change when compared to blank culture and similarly, INH-ODN (100 nM) in combination with ST-ODN (0.1 nM) does not affect the *CTSK* mRNA levels in osteoclasts (Fig. 23). Interestingly, cultivation of monocytes with ST-ODN (0.1 nM) + TLM-ODN (10 nM) leads to non-significant 1.41× increase in *CTSK* expression when compared to lone ST-ODN (0.1 nM). The possibility cannot be ruled out that although the TLM-ODN suppresses osteoclastogenesis in monocytes affected by ST-ODN, the differentiated osteoclasts present with non-significantly more activity. When tested at different times

during cultivation, lowest levels of expression were found on day 1, day 2 and day 2 blank. Since cultivation of human monocytes and their differentiation into mature osteoclasts takes 14 days, there might not be osteoclasts that express enough *CTSK* mRNA yet.

Since our hypotheses suggest that ODNs activate the NF-κB pathway via TLR9 we decided to test its expression in the presence of ST-ODN-2006 (0.1 nM), CO-ODN-2395 (10 nM), ST-ODN-2006 (0.1 nM) + INH-ODN-4347 (100 nM), ST-ODN-2006 (0.1 nM) + TLM-ODN (TTAGGG)4 (10 nM) and blank. We also tested for *TLR9* mRNA expression at different times during the cultivation (day 1, day 2, day 14 and their respective blank counterparts). When osteoclasts were cultured with ST-ODN the mRNA gene expression did not change when compared to blank. A non-significant increase 2.87× was observed when cells were influenced by ST-ODN (0.1 nM) + INH-ODN (100 nM) in comparison to lone ST-ODN (0.1 nM). A non-significant decrease in mRNA expression was observed when cells were under the influence of ST-ODN (0.1 nM) + TLM-ODN (10 nM). If this effect is observed in more robust data sets it would suggest that INH-ODN in combination with ST-ODN decreases the number of osteoclasts via TLR9. *TLR9* mRNA expression was increased in the first two days of cultivation and decreased (non-significantly) by the end of cultivation (day 14) when compared to day 1. If confirmed on a larger amount of samples it may suggest that the *TLR9* expression may be important only at the early phases of the differentiation process. However, *TLR9* expression was detected at least 10 cycles later than the reference genes or went undetected in both instances (influenced by various ODNs or analysed at different times of cultivation), meaning that the mRNA expression levels were very low. If mRNA expression levels would remain low, even when larger number of samples was analysed, it would rise a question whether ODNs could change the osteoclastogenic potential of monocytes via a different receptor than TLR9. One of the possibilities of how ODNs could change the osteoclastogenic potential of monocytes is the endoplasmatic reticulum-associated multiple-transmembrane protein, STING (stimulator of interferon genes). STING is a signalling molecule of innate immune system that induces gene expression as a response to stimulatory ligands such as dsDNA and subsequently triggers the NF-κB pathway (Abe and Barber, 2014).

NFATc1 is a specific osteoclastogenic factor, responsible for initiation of transcription of other osteoclastogenic genes. We tested it in the presence of ST-ODN-2006 (0.1 nM), CO-

ODN-2395 (10 nM), ST-ODN-2006 (0.1 nM) + INH-ODN-4347 (100 nM), ST-ODN-2006 (0.1 nM) + TLM-ODN (TTAGGG)4 (10 nM) and blank and also at different times during the cultivation (day 1, day 2 day 14 and their respective blank counterparts). Its expression levels stayed very consistent when tested with various types of ODNs (Fig. 27), which could probably mean that its activity is not affected by administration of ODN. Similarly to *TLR9* expression, *NFATc1* crossed the threshold 10 or more cycles later than the reference genes or was not detected by the qPCR at all, meaning that its expression level was very low. This might mean that on day 14 of osteoclast differentiation, osteoclastogenesis is over, there are not enough precursor cells or pre-osteoclasts present in the culture anymore that could have the capacity to further differentiate into mature osteoclasts and thus to be able to produce higher levels of *NFATc1*. This theory is also supported by the time-experiment that reveals the highest *NFATc1* mRNA levels during day 2 (both in blank culture (2.08×) and ODN-treated culture (1.84×) in comparison to day 1) and drops 1.58× by day 14 (Fig. 28). Nevertheless, since only two experiments were analysed by qPCR for *NFATc1* expression (one when monocytes were stimulated by various ODNs and their combinations and the other one when ST-ODN (0.1 nM) was administered at different times of the cultivation), these results need to be treated with caution and the number of data analysed enlarged.

6. Conclusion

The main focus of this thesis was to elucidate whether ecDNA can change the differentiation potential of monocytes and alternatively induce osteoclastogenesis. We tested the effects of four types of ODNs (CO, ST, INH and TLM) both with their native phosphodiester backbones and their more stable phosphorothioate backbones. These ODNs were chosen because they proved to have either stimulatory or inhibitory effects on the proliferation or function in other types of cells and we wanted to study their effect on human monocytes, peripheral blood precursors of osteoclasts. All studied ODNs were tested in three concentrations (0.033 μM, 1 μM, 10 μM for -pO ODNs and 0.1 nM, 10 nM, 100 nM for -pS ODNs) or combinations. We confronted our findings with current knowledge of ODNs and their effects. Based on presented results we came to these conclusions:

- -pO ODNs did not exercise their effect in monocyte cultures and is therefore not suitable for our experimental design
- CO-ODN-2310 and CO-ODN-2114R are not neutral in monocytes
- CO-ODN-2395 (10 nM) is an appropriate control ODN to use in further experiments
- ST-ODN-2006 (0.1 nM) stimulates monocytes to differentiate into osteoclasts
- lone INH-ODN in either concentration does not inhibit osteoclastogenesis
- INH-ODN (100 nM) + ST-ODN (0.1 nM) prove to inhibit osteoclastogenesis
- lone TLM-ODN in all concentrations does not inhibit osteoclastogenesis
- TLM-ODN (10 nM) + ST-ODN (0.1 nM) treatment of monocytes result in a trend of decreased number of osteoclasts but the observed trend is non-significant
- because of a limited number of experiments, data for qPCR are inconclusive

Further experiments needed to expand our knowledge about the effect ODNs have on osteoclastogenesis are:

- to execute more experiments with TLM-ODN (10 nM) + ST-ODN (0.1 nM) to establish whether this combination inhibits osteoclastogenesis

- to execute experiments where ODNs are administered into the cell sample at various times during cultivation to establish how their initial administration influences their effect in osteoclastogenesis
- to execute more analyses concerning the expression of *ACP5, CTSK, TLR9* and *NFATc1* in cells cultivated with various types of ODNs
- if TLR9 proves not to be involved in inducing the NF-κB pathway, experiments testing for different possible routes such as STING are recommended

List of abbreviations

A	Adenine
ACP5	Gene coding acid phosphatase 5
ANOVA	Analysis of variance
AP-1	Activator protein-1
ATPase	Enzyme that catalyses the hydrolysis of a phosphate bond in adenosine triphosphate
B2M	Gene coding beta-2-microglobulin
BMI	Body mass index
bp	Base pairs
C	Cytosine
CALCR	Gene coding calcitonin receptor
CD (e.g. CD69)	Cluster of differentiation marker
cDNA	Complementary DNA
CO	Control
CpG	*Cytosine-phosphate-guanine*
CSF1R	Colony stimulating factor 1 receptor
Ct	Cycle threshold
CTR	Calcitonin receptor
CTSK	Cathepsin K enzyme
CTSK	Gene coding cathepsin K enzyme
DAMP	Damage-associated molecular pattern
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic *acid*
dNTP	Deoxynucleotide triphosphate

dsDNA	Double-stranded DNA
ecDNA	Extracellular deoxyribonucleic *acid*
FBS	Foetal bovine serum
G	Guanine
GDPR	General data protection regulation
HBSS	Hank's balanced salt solution
HEK	Human embryonic kidney
HPRT1	Gene coding hypoxanthine phosphoribosyltransferase 1
HSC	Hematopoietic stem cell
IFN α/β	Interferon alpha/beta
IKK (e.g. IKKαβγ)	Inhibitory-kappa B *kinase*
IL (e.g. IL-8)	Interleukin
INH	Inhibitory
INH-ecDNA	ecDNA with proven inhibitory effect
IRAK	Interleukin-1 receptor associated kinase
JNK (e.g. JNK1)	Jun N-terminal kinase
LG	L-glutamine
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage-colony stimulating factor
MITF	Microphthalmia-associated transcription factor
mRNA	Messenger ribonucleic *acid*
MyD88	Myeloid differentiation primary response 88 protein
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	Not done
NET	Neutrophil extracellular trap
NFATc1	Nuclear factor of activated T cells calcineurin-dependent 1

NFATc1	Gene coding nuclear factor of activated T cells calcineurin-dependent 1
NF-κB	Nuclear factor-kappa B
NK	Natural killer
ns	Not significant
ODN	Oligodeoxynucleotide
OPG	Osteoprotegerin
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
-pO	Phosphodiester
proIL-1β	Prointerleukin-1 beta
PS	Penicillin-Streptomycin
-pS	Phosphorothioate
PTH	Parathyroid hormone
qPCR	Quantitative polymerase chain reaction
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
rcf	Relative centrifugal force
RNA	Ribonucleic *acid*
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SLE	Systemic lupus erythematosus

ST	Stimulatory
ST-ecDNA	ecDNA with proven stimulatory effect
STING	Stimulator of interferon genes
T	Thymine
TAK (e.g. TAK1)	Transforming growth factor beta-activated *kinase*
TLM	Telomeric
TLR (e.g. TLR9)	Toll-like receptor
TLR9	Gene coding Toll-like receptor 9
TNF (e.g. TNFα)	Tumour necrosis factor
Tnfsf11	Gene coding RANKL
TRAF (e.g. TRAF6)	Tumour necrosis factor receptor-associated factor
TRAP	Tartrate-resistant acid phosphatase
UV	Ultraviolet

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Annex A: Questionnaire for healthy blood donors

Dotazníkové šetření

Název studie: **Úloha monocytů v patogenezi autoimunitních chorob**

Cílová skupina: Zdraví dárci

Kód (vyplní odborné pracoviště): ……..……...

Rok narození: ……..……..

Pohlaví: ……..……

Výška:…….

Váha:……..

Cítíte se zdráv(a)?

ANO

NE

Užíváte pravidelně nějaké léky? (včetně acylpyrinu a hormonální antikoncepce)

ANO Jaké?.....

NE

Užil(a) jste v posledních 4 týdnech nějaké léky (pravidelně užívané léky již neuvádějte)?

ANO Jaké?.....

NE

Léčíte se, nebo jste sledován(a) pro nějaké onemocnění (včetně infekčního)?

ANO Jaké?.....

NE

Trpí někdo ve Vaší blízké rodině osteoporózou nebo jiným onemocněním kostí?

ANO

NE

Prodělal(a) jste v posledních 4 týdnech nějaké onemocnění (nachlazení, průjmové onemocnění apod.)?

ANO Jaké?.....

NE

Podstoupil(a) jste v posledních 7 dnech trhání zubů nebo malý chirurgický výkon?

ANO Jaký?.....

NE

Byl(a) jste v posledních 6 měsících očkován(a)?

ANO Proti čemu?.....

NE

Pro ženy: Byla jste v posledním roce těhotná, nebo jste těhotná?

ANO

NE

Máte či měl(a) jste poruchy imunity, alergie, kožní onemocnění?

ANO Jaké?.....

NE

Máte zaměstnání nebo koníčka se zvýšenou tělesnou zátěží (např. práce ve výškách, horolezectví, potápění)?

ANO Jaké/ý?.....

NE

Dodržujete nějaký speciální způsob stravování (např. vegetariánství, veganství, frutariánství,…)?

ANO Jaký a jak dlouho?.....

NE

3 dny před odběrem jste kouřil(a) (cigarety, marihuanu, vodní dýmku, tabák)?

ANO

NE

3 dny před odběrem jste měl(a) větší fyzickou zátěž? (náročnou práci, či sportovní vytížení)

ANO

NE

3 dny před odběrem jste spal(a) 6 a více hodin každou noc?

ANO

NE

3 dny před odběrem jste konzumoval(a) alkohol (či jiné návykové látky)?

ANO Jaký a kolik?.....

NE

V posledních 14 dnech jste se staral(a) o nemocnou či nesamostatnou osobu (malé dítě, starý člověk)?

ANO

NE

Den před odběrem jste jedl(a) (zaměřte se především na tučná a sladká jídla):

Snídaně:…….

Oběd:…….

Večeře:……..

Svačiny:…….

Čerstvé ovoce a zeleninu:

Nejím

Konzumuji výjimečně

Konzumuji sezónně (spíše v létě, při nemoci,…)

Konzumuji minimálně 1 × měsíčně

Konzumuji minimálně 1 × týdně

Konzumuji minimálně 1 × denně

Ryby:

Nejím

Konzumuji výjimečně

Konzumuji minimálně 1 × měsíčně

Konzumuji minimálně 1 × týdně