Identification of therapeutic targets and antisense oligonucleotide mediated exon skipping based therapies in arthritis

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Chapter 1

Chapter 1- General introduction

1.1 Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is an autoimmune disease characterized by severe chronic painful inflammation of the joints, which results in cartilage-bone destruction and finally loss of function of the affected joints. The exact cause is unknown; it is most likely a matter of chance strongly influenced by a combination of genetic and environmental factors. Immune regulatory factors play important role. The strongest association has found with human leukocyte antigen (HLA) (Gregersen, 1987). Most likely the immune system develops more easily a self-response in the presence of certain HLA variants (Weyand, 1990).

RA is also characterized by the presence of various autoantibodies in the serum and synovial fluid of the patients (Steiner and Smolen, 2002). Rheumatoid factor (RF) is one of these autoantibodies that is specific for Fc portion of immunoglobulin G (IgG). Another autoantibody identified in the sera of RA patients is ACPA (anti-citrullinated protein antibodies), which is specific for epitopes containing the unusual amino acid citrulline. Citrulline is generated by post -translational deimination of arginyl residues by the enzyme peptidyl arginine deiminase (PAD). It is interesting that in some patients ACPA can be detected in the blood even years before the onset of the disease. This implies that, having autoantibodies, such as ACPA, is not sufficient to develop RA; additional changes are required. The factors involved can be environmental like smoking, infection, or mechanical insult but also hormonal mechanisms because women have higher risk to develop RA.

The pathological processes in RA start in the synovium. The synovium encapsulates the joints and provides structural support to the cartilage. Macrophages-like synovial cells and fibroblast-like synovial cells are important cell types in the joint lining of the synovium. During RA, synovium transforms into a hyperplastic tissue with the attack of immune cells resulting in inflammation and synovial thickening; the whole process is called synovitis. T helper cells, autoantibody producing B cells and a variety of innate

effector cells, including macrophages, mast cells, neutrophils and natural killer cells are found in the synovium (McInnes and Schett, 2011).

Synovitis is most likely initiated by autoantibodies that entered the synovium. The autoantibodies while bound to their auto-antigen, forming immune complexes (ICs), are bound by their Fc regions to Fc gamma receptors (FcγR) on the innate effector cells. Cross-linking of the FcR on these cells activates effector mechanism, which at the end results in release of pro-inflammatory cytokines (e.g. TNF-α, IL-1β). These pro-inflammatory cytokines play a crucial role in the regulation of the disease process. However, ICs not only interact with FcγRs but also interact with components of the complement system. This interaction causes activation of both classical and alternative pathways of complement system all of which results in the formation of complement component 5 (C5).

Taking together the complex interactions in the synovium lead to cellular activation by pro-inflammatory agents resulting inflammation, which leads to production of matrix metallo-proteinases (MMPs). MMPs induce death of cartilage producing chondrocytes resulting in cartilage destruction and activate osteoclasts, which, by breaking down the bone, cause deformities in the joint resulting in irreversible loss of function.

Currently, blocking pro-inflammatory cytokines is the key therapeutic strategy against RA. In addition to this, blockade of specific pathways of the complement system also showed protection from arthritis in mice. The mouse models of rheumatoid arthritis are widely used to study pathogenesis of the disease and evaluate potential therapeutic agents. One of the most widely used models is collagen-induced arthritis (CIA). Mice develop polyarthritis when immunized with bovine or chicken collagen type II that results in the production of pathogenic anti-collagen autoantibodies leading to full-blown synovial inflammation and therefore mimicking several aspects of the human disease. Alternatives are the injection of anti-collagen antibodies or serum from arthritic K/BxN mice. In these models the initial phase of the disease (development of autoantibodies) is by-passed and therefore the disease mechanisms are restricted to the downstream antibody effector pathways. Studies with a variety of arthritis models established in transgenic (Tg) or knock-out (KO) mouse strains, lacking important components of the immune system, helps to define the role of these components in

arthritis. Moreover, genetic studies in mice with arthritis enabled identification of the genetic factors that play a major or minor role in this complex disease.

Even though significant progress is made in understanding of the disease mechanism resulting in new therapies such as anti-TNFα therapy, so far none of the treatments resulted in total remission of the disease or was only successful in some patients. Therefore, there is still an urgent need for better medication. So better understanding of the disease mechanisms and identification of new target molecules would result in new and more effective therapies in RA.

1.2 Target molecules in RA

The pathology in RA is mediated by a number of different molecules like cytokines, chemokines, cell adhesion molecules and matrix metalloproteinases. Activated monocytes, macrophages and synovial fibroblasts in the synovium over-produce pro-inflammatory effector cytokines like TNF-α, IL-1β and IL-6 all of which seem to play a pivotal role in the chronic inflammation in RA (Steiner and Smolen, 2002). These pro-inflammatory cytokines induce a variety of signal-transduction cascades and triggers activation of transcription factors. This activation subsequently causes induction of a variety of genes including other cytokines and inflammatory mediators like prostaglandins and MMPs. Production of MMPs destroys the cellular matrix, which is followed by induction of apoptotic pathways in chondrocytes and activation of osteoclasts, which erode the bone. Therefore, pro-inflammatory cytokines and their receptors were selected as therapeutic targets to inhibit the signal transduction cascade, which leads to activation of effector cells of the immune system. Monoclonal anti-TNF antibody and recombinant human TNF receptor have been shown to be effective in reducing the excess of TNF-α found in arthritic joints (Elliott 1993, Moreland 1997). For some patients, TNF blockers stop the progression of rheumatoid arthritis but some others may need combination therapies. Recombinant IL-1 receptor antagonist, which prevents binding of IL-1 to its receptor, is another widely used therapeutic strategy to inhibit the IL-1 pathway. Soluble IL-1 receptors are other candidate IL-1 blocking

agents (Abramson, 2002). In addition other pro-inflammatory cytokines like IL-2, IL-6, IL-15, IL-12, IL-17, IL-18 etc. and their receptors are also potent targets.

Chemokines (like MCP-1, MCP-4, CCL18, etc.), and adhesion molecules (like ICAM-1, VCAM-1, etc.) play a crucial role in the recruitment of immune cells to inflammatory sites. So interfering with these processes, by directly targeting these molecules or by targeting their receptors, can be a valuable tool to reduce inflammation in RA.

Increased production of autoantibodies and their interaction with the autoantigens in the synovium leads to formation ICs that bind with their Fc-part to the FcγRs of the innate effector cells (e.g.: macrophages, mast cells, neutrophils, DCs and NK cells). Cross-linking of the FcγRs on these cells, results in activation of downstream effector mechanism such as release of soluble inflammatory mediators such as TNF-α and IL-1β. Activating FcγRIII has been shown to be the major activating FcγR in different mouse models of arthritis (reviewed in Boross and Verbeek, 2006). So interference with FcγRIII might significantly modulate disease progression in experimental arthritis models in a more upstream level.

ICs not only interact with FcγRs but also activate other components of the immune system such as complement. Activation of the complement system leads to production of complement component 5 that is cleaved to form C5a and C5b. C5a is a strong chemoattractant that recruits inflammatory cells to the synovium and and binding of it to C5aR initiates the inflammatory cascade. It is therefore, considered to be an ideal therapeutic target to decrease inflammation. Another possibility for inhibiting complement-mediated immune responses is interference with complement receptors like C5aR. It has been shown that C5 deficient mice are resistant to collagen induced arthritis (CIA) and KxB/N serum induced arthritis (Wang, 2000 and Ji, 2002).

1.2.1 IL-1 Receptor accessory protein (IL-1RAcP) and soluble IL-1RAcP

Interleukin 1 (IL-1) is a member of the IL-1 family and plays an essential role in arthritis by promoting production of a broad range of pro-inflammatory mediators (Dinarello, 2005). IL-1 binds to the IL-1 receptor (IL-1R) complex that consists of IL-1R type I or type II and IL-1 receptor accessory protein (IL-1RAcP). After binding of IL-1 to the

IL-1R, IL-1RAcP is recruited for stabilization of the ligand-receptor complex. In addition, IL-1RAcP is a crucial co-receptor in this complex that enables recruitment and binding of intracellular proteins such as MyD88 and a series of IL-1R–associated kinases, which finally lead to NF-κB activation (Figure 1). IL-1RII also associates with IL-1RAcP upon binding of IL-1. However, this receptor lacks the intracellular domain present in IL-1RI and cannot generate intracellular signal thus is considered as decoy receptor (Lang, 1998). Both transmembrane and soluble forms of IL-1RI and IL-1RII have been found (Symons, 1995). Another inhibiting molecule of IL-1 signaling is IL-1 receptor antagonist (IL-1Ra). IL-1Ra competes with IL-1 for binding to IL-1RI but it binds weakly to IL-1RII. Likewise, the soluble form of IL-1RI binds stronger to IL-1Ra than to IL-1, lessening the inhibiting effect of IL-1Ra (Burger, 1995).

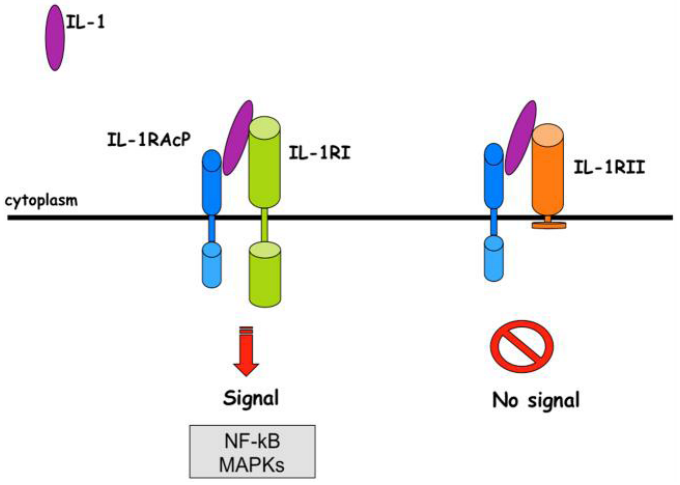

Figure 1. IL-1 receptor family and IL-1 signaling. Upon IL-1 binding to its receptors, IL-1RAcP is recruited to stabilize the complex. However, only binding to IL-1RI generates intracellular signaling, as IL-1RII is a decoy receptor.

IL-1RAcP has also a soluble form (sIL-1RAcP) that lacks the intracellular and transmembrane domains of IL-1RAcP and therefore is unable to facilitate signal transduction. It interacts with the cell surface IL-1RI, IL-1RII, and possibly with

soluble IL-1RII in the extracellular space, forming a high-affinity IL-1 scavenger (Smith, 2003) (Figure 2). sIL-1RAcP has shown to selectively reduces IL-1 activity on cells such as B lymphocytes and chondrocytes, which express more surface type II decoy receptors (Lang, 1998). It has also been shown that using an adenoviral vector encoding sIL-1RAcP can ameliorate collagen-induced arthritis in mice (Smeets, 2005) Thus, increasing sIL-1RAcP levels might be a useful approach to decrease IL-1 related inflammation in RA.

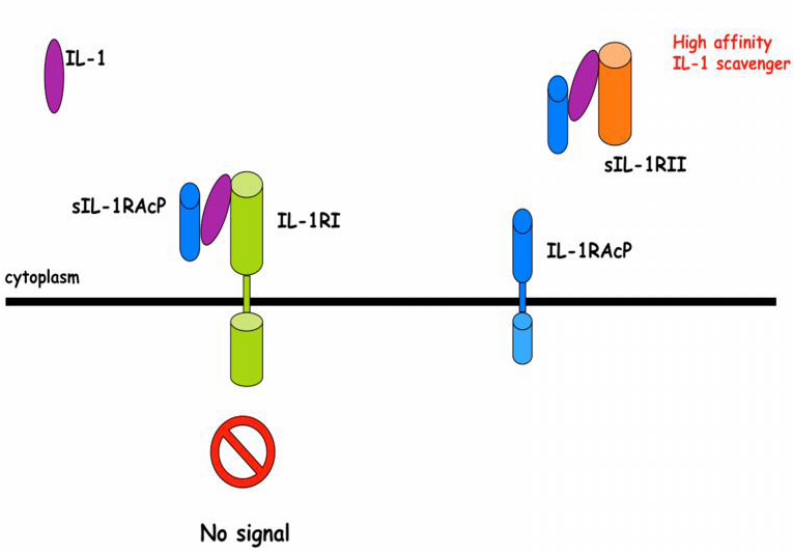

Figure 2. IL-1 inhibition by soluble IL-1RAcP (sIL-1RAcP) Interaction of sIL-1RAcP with IL-RI prevents IL-1 induced signaling as sIL-1RAcP is lacking the intracellular region. When IL-1 binds to sIL-1RII, by the help of sIL-1RAcP, a high affinity IL-1 scavenger is formed to block IL-1 activity.

1.2.2 Complement component 5 (C5)

The complement system is part of the innate immune system, acting to protect the host from pathogens, without need for previous exposure and to remove abnormal cells (e.g. apoptotic cells).

The complement cascade has three pathways; the classical, alternative, and the lectin pathways. The classical and alternative pathways are activated mainly by ICs and the lectin pathway is activated due to recognition of certain saccharides by mannan-binding lectin (MBL). All three pathways of complement activation lead to formation of enzymatic complexes such as C5-convertase. The fifth component of the complement system, C5, is a glycoprotein consisting of 1679 amino acids in two initially connected, but subsequently cleaved, disulfide-linked polypeptide chains, C5α and C5β (Haviland, 1991) When C5 is activated by C5-convertases; C5a and C5b fragments are released (Figure 3). C5a acts as a strong chemoattractant for neutrophils, monocytes, macrophages, and eosinophils, induces production of cytokines and other pro-inflammatory mediators, and enhances vascular permeability (Riedemann, 2003). C5b, on the other hand, initiates assembly of membrane attack complex (MAC). C5b serves as an anchor for the assembly of C6, C7, C8, and C9 (known as C5b-9) and is inserted into the cell membrane of pathogens, leading to cell lysis. However, excessive complement activation leads to elevated C5a and the elevated levels of C5a is known to be associated with many clinical conditions, like rheumatoid arthritis, sepsis, Alzheimer's disease and ischemic heart disease.

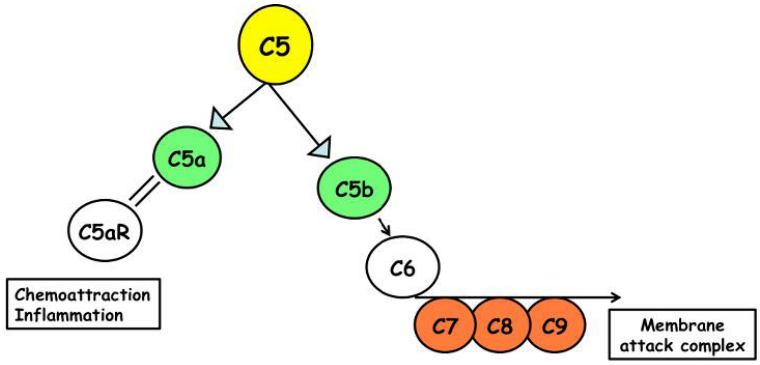

Figure 3. Complement component 5 (C5) is converted to C5a and C5b by C5 convertase. C5a acts as a strong chemoattractant and C5b play role in the formation of membrane attack complex.

It has been shown that in mice, an anti-C5 monoclonal antibody prevents collagen-induced arthritis and ameliorates established disease (Wang, 1995). However, this antibody blocks both C5a and C5b, the decrease of C5b levels that is necessary for formation of membrane attack complex (MAC) that protects against pathogens, is a drawback of this antibody. Therefore there is always need for a more specific therapy only targeting C5a and keeping C5b intact.

1.2.3. CD55 and CD97

Excessive complement activation is connected to human RA because elevated levels of complement components have been observed in the synovial fluid of RA patients (Morgan, 1998). It has also been shown that mice are resistant to arthritis when the complement components or their receptors are targeted (Hielta, 2002). CD55 (also known as decay-accelerating factor- DAF-) is a negative regulator of complement system and the ligand for receptor CD97. The absence of CD55 exacerbates clinical symptoms in several autoimmune disease models (Kaminski, 2004; Miwa, 2002) Additionally; it has been shown that blocking CD97–CD55 interaction by using a CD97 antibody causes amelioration of actively induced arthritis (Kop, 2006). Therefore it is important to know the exact role of CD55 and CD97 or CD55/CD97 interaction in RA, which might have therapeutic potential.

1.2.4 FcγRs

Fc receptors (FcR) are cell surface receptors which bind to the constant regions (Fc) of immonoglobulins (Ig). FcγRs recognize the Fc region of IgG and are expressed on most hematopoietic cells (Nimmerjahn and Ravetch, 2008) (Table 1). FcγR are functionally divided in two classes; the activating receptors (FcγRI, FcγRIII and FcγRIV) and the inhibitory receptor FcγRIIb. In mice, the activating FcγRs are associated with the comman γ-chain which contains an immunoreceptor tyrosine-based activating motif (ITAM) (Isakov, 1997). The activating FcγR are counterbalanced by one single chain inhibitory receptor FcγRIIb which contains an immunoreceptor-based inhibiting motif (ITIM) (Figure 4) (Bolland and Ravetch, 1999). Cross-linking an activating FcγR with the inhibiting FcγRIIb down regulates the activation signal. The activating and inhibitory FcγRs are co-expressed on the same cell and the ratio of the receptors

determines the IC concentration threshold required to trigger cell activation (Clynes, 1999). Crosslinking of FcγR by IgG-Immune complexes initiates intracellular signaling that triggers a wide range of effector functions such as histamine release in mast cells, phagocytosis and cytokine production in macrophages, reactive oxygen species production in neutrophils, release of cytotoxic mediators in NK cells (Takai, 2002) and antigen uptake, promotion of cell maturation and antigen presentation in DCs (van Montfoort, 2012).

Table 1. Expression pattern of murine FcγRs

Name	Expression pattern
FcγRI	Macrophages, monocytes, dendritic cells
FcγRIIb	B cells, mast cells, macrophages, monocytes, neutrophils, dendritic cells
FcγRIII	Monocytes, macrophages, dendritic cells, neutrophils, mast cells, NK cells
FcγRIV	Monocytes, macrophages, dendritic cells, neutrophils

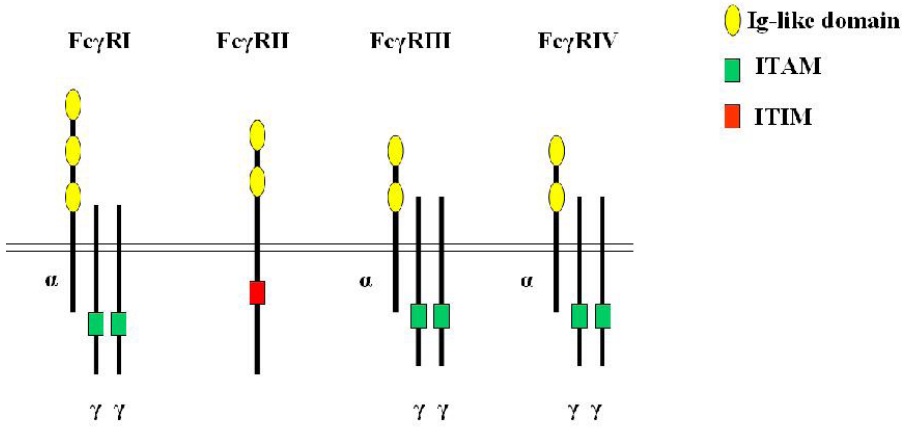

Figure 4. Murine FcγRs. Activating FcγRs, FcγRI, FcγRIII and FcγRIV, are composed of a ligand binding α-chain and a signal transducing γ-chain dimmer which

contains the immunoreceptor tyrosine-based activating motif (ITAM) whereas the inhibitory receptor FcγRIIb contains an immunoreceptor-based inhibiting motif (ITIM)

FcγRI is the only high affinity FcγR and its levels can be up-regulated by some cytokines like interferon gamma. In mice FcγRI is always expressed together with FcγRIII, which is the only activating FcR on NK-, and mast cells.

It has been shown that FcγRs are important players in the pathogenesis of RA (Chen, 2006; Nieto, 2000) and arthritis in mice (Boross, 2008; Kleinau, 2000; Nabbe, 2003; Wipke, 2004). FcR γ-chain KO mice which are deficient for the cell surface expression of all activating FcγRs appeared to be almost completely protected from CIA (Kleinau, 2000) but in AIA the role of FcR γ chain was less striking (van Lent, 2000). Surprisingly FcγRIII KO mice showed greatly diminished disease activity in CIA (Diaz de Stahl, 2002). However, in an FcγRIIb KO background FcγRIII deficiency resulted only in a delay in CIA development (Boross, 2008) illustrating the complexity of the role of the different FcγR in arthritis.

1.2.4.1 FcγRIIB and its role in arthritis

FcγRIIb has the broadest expression pattern compared to all other Fc receptors to provide regulation of immune response in different levels. When FcγRIIb is expressed on myeloid effector cells, and co-engaged with the activating receptors by the ICs, it provides an important control for the activation of these cells. When express on B cells, cross-linking FcγRIIb with B-cell receptor (BCR) forms a negative feedback for the control of B cell activation, proliferation and antibody production. Therefore inactivation of FcγRIIb causes uncontrolled B cell activation, which results in uncontrolled antibody production and increased myeloid cell activation in the effector phase (Bolland and Ravetch, 2000; Takai, 1996). Therefore it is not surprising that in mice FcγRIIb deficiency increases the susceptibility to arthritis. FcγRIIb KO mice on C57BL/6, DBA/1 or 129/C57BL/6 mixed backgrounds, develop more severe CIA compared to wild type mice (Boross, 2011; Kleinau, 2000; Yuasa, 1999). However, these mice lack FcγRIIb on both B cells and myeloid cells. For a better understanding of the cellular basis of the susceptibility to arthritis, an analysis of the cell-type specific

role of FcγRIIb is required. This can be achieved by using cell type specific FcγRIIb KO mice.

1.3 Antisense oligonucleotide (AON) mediated exon skipping

Antisense oligonucleotides (AONs) are short nucleic acid sequences around 20 bp that are designed to recognize and bind to specific regions within messenger RNAs (mRNA). AONs can be used to modulate RNA splicing. When the AON binds to the target exon, it blocks access of the spliceosome and other splicing factors by specific binding to sequences playing role in exon inclusion in the pre-mRNA. This results in exclusion of that target exon from the mature mRNA (exon skipping) with its flanking introns (Figure 5). AONs have been chemically modified to protect them from nuclease activity, to increase bioavailability and to decrease their renal clearance (Kurreck, 2003). The most commonly used modifications are 2'-*O* methyl (2OMe), 2'-*O* methoxyethyl (2OMOE) both with phosphorothioate (PS) modification, peptide nucleic acids (PNA), locked nucleic acids (LNA) and morpholinos. Each modification has advantages and disadvantages. For example 2'-*O* modifications have high affinity for RNA and less toxic for the cells but their half-life is short, where LNA oligonucleotides have great affinity for the target RNA and have very good nuclear uptake but they have less sequence specificity (Dominski, 1993; Sazani, 200; Sazani, 2002; Fluiter 2003)

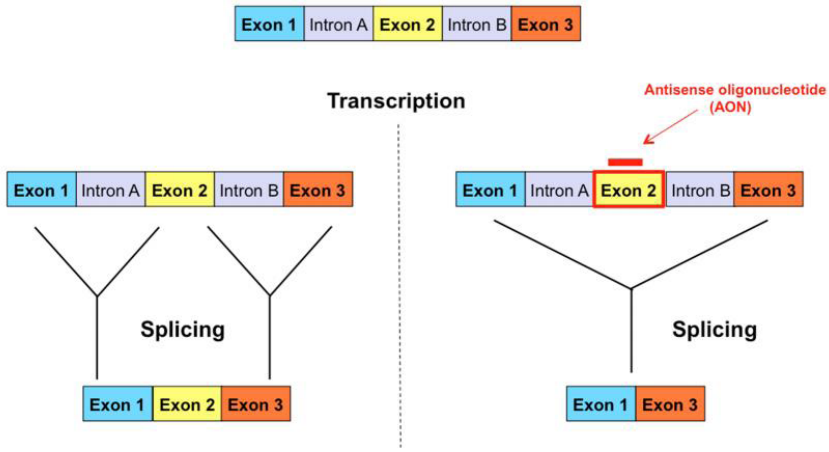

Figure 5. Antisense oligonucleotide (AON) mediated exon skipping. Upon binding of the AON to the target exon, it can not be recognized by the splicing machinery and removed with the flanking introns.

AON-mediated exon skipping can be used to change the ratio of alternative splice products, to restore correct splicing of an aberrantly spliced transcript or to produce a novel splice variant that normally is not expressed. One successful example of the application of this tool is in Duchenne Muscular dystrophy (DMD) to restore the open reading frame by skipping the exon with the nonsense mutation. This allows the generation of internally deleted, yet functional dystrophin proteins converting severe DMD into a milder Becker muscular dystrophy phenotype (Kole, 2001- Aartsma-Rus, 2004a). Another example is changing levels of alternatively splice products of Bcl-x to manipulate the balance from anti- to pro-apoptotic Bcl-x (Mercatante, 2002).

Exon skipping is also a promising strategy to modulate the function of proteins involved in the inflammatory cascades that drive the pathogenic process in inflammatory or auto-immune diseases such as RA. An optimal therapeutic intervention in an immunological disease inhibits exclusively the pathway(s) that cause(s) pathogenesis while maintaining the capacity of the immune system to protect against infections. Treatment of immunological diseases requires a very specific interference inhibiting specifically the immune response that causes the tissue damage but maintaining the capacity of the immune system to protect against pathogens.

1.3.1 Application of AON-mediated exon skipping to convert a membrane bound receptor into a soluble receptor

AON-mediated exon skipping can be applied to induce a switch from a transmembrane form of a receptor to a soluble form. This can be achieved with AONs targeting the exon encoding the trans-membrane region of the receptor. In this way, the number of membrane bound receptors can be reduced and the secreted soluble receptor may act as a decoy receptor to diminish excessively produced ligand. It has already been shown that in this way membrane bound receptors of the cytokines TNF-α and IL-5 can be successfully converted into their soluble forms (Graziewicz, 2008- Karras, 2000). Moreover, it has also been shown that the newly produced soluble proteins have therapeutic effect. For example Δ7TNFR2 has an anti–TNF-α activity and prolonged administration of AONs showed suppression in the severity of arthritis in mice. Beside TNF-α and IL-5, IL-1 is another important pro-inflammatory cytokine. Therefore, molecules in the IL-1 receptor complex, like IL-1R, IL-1RAcP, can be good candidates for the application of AON-base therapy to control IL-1 triggered inflammation in RA and in other chronic inflammatory diseases.

1.3.2 Application of AON-mediated exon skipping on multifunctional proteins

By using AONs, the levels of isoforms of a protein can be changed to favor the production of a desired isoform. For example Bcl-x gene, as mentioned in section 1.3, has two isoforms Bcl-xS (pro-apoptotic) and Bcl-xL (antiapoptotic) and it is possible to shift the splicing patterns toward the production xS isoform with AONs and increase in xS isoform resulted in apoptosis in some cancer cell lines (Mercatante, 2001 and 2002).

mRNAs which encode for a protein with different functional domains can be good targets for AON mediated exon skipping . The advantage of AON mediated exon skipping is, the favorable isoform or functional domain of the protein can be kept intact while the other one is removed. For example, Apolipoprotein B (APOB) has two isoforms; APOB100 and APOB48. APOB100 plays a central role in atherosclerosis

and its knockdown might be a potential treatment against the disease, where the other isoform, APOB48 is important in intestinal fat transport (Chester, 2000). So removal of exon 27 with AONs doesn't disrupt the open reading frame and resulted in a truncated, nonfunctional APOB100 protein, while APOB48 levels were maintained which leads to lower LDL and cholesterol levels (Khoo, 2007). This approach has advantages over RNase-H mediated gene know-down as it allows inhibition of only a specific domain of the gene. The similar approach can be applied to complement component 5 (C5). As mentioned in 1.2.2, C5 is converted into C5a and C5b, where C5a is a chemoattractant in the inflammatory processes and C5b is important to fight against pathogens.

1.3.2.1 Application of AON-mediated exon skipping on complement component 5

Because of the strong chemoattractant property of C5a, specific control of its production without affecting the protective immune responses could gain therapeutic benefit. C5a protein is mostly composed of an anaphylatoxin domain and main part of this domain is encoded by exon 17. AONs targeting exon 17 will initiate the removal of this exon from C5 pre-mRNA which leads to the formation of a truncated, non-functional C5a protein that is probably degraded. Thus by this approach C5a levels can be diminished while C5b is maintained and the formation of MAC is not effected.

1.4 The scope of the thesis

The aim of the present studies is to identify potential therapeutic targets and to investigate different therapeutic strategies against rheumatoid arthritis.

The first part of the thesis aims to present how antisense oligonucleotide mediated exon skipping can be used as a potential novel therapy to fight against inflammation in a variety of diseases like RA. Two different target molecules, a component of the receptor of the effector cytokine IL-1, IL-1RAcP and the complement protein C5 were selected as targets for exon skipping and in vitro their mRNA expression patterns were successfully modulated with AONs resulting in a splice variant that encodes a truncated protein that might have therapeutic potential **(Chapter 2).**

In **Chapter 3,** manipulating the splicing of the mRNA encoding IL-1RAcP with AONs resulting in skipping of the transmembrane encoding exon leading to the formation of a novel soluble form of this molecule very effectively in vivo in mouse liver. In vitro this exon skipping strategy resulted in decrease IL-1 response of IL-1R expressing cells.

FcγRs are located more upstream in the inflammatory process and play important role in RA. In mice the deficiency of the inhibiting FcγRIIb resulted in an increased susceptibility to arthritis. **Chapter 4** investigates the cell type specific contribution of FcγRII to this increased susceptibility by using conditional KO mice on pure C57BL/6 background lacking FcγRII either in the myeloid cell compartment or on B cells.

CD55 and its receptor CD97 are other important complement components that play role in RA. In **Chapter 5** the exact contribution of CD55 in different mouse models of RA was investigated.

Chapter 6 explains in detail how to use AON mediated exon skipping in order to convert membrane bound receptors into secreted, soluble receptors.

Finally, in **Chapter 7** the results obtained with these studies and their implications are discussed.

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