Chapter 7

Chapter 7- Conclusions / Discussion

Rheumatoid arthritis is a heterogeneous, multifactorial, systemic autoimmune disorder. Better understanding of the underlying molecular and cellular disease mechanisms will enable us to develop novel therapeutic strategies targeting the immune system more specifically. In this thesis, we presented a variety of aspects of arthritis research:

a. The development of a novel therapy based on AON-mediated exon skipping to control IL-1 mediated inflammation in the disease
b. The analysis of the cell-type specific contribution of the inhibiting FcγRIIb to the susceptibility to arthritis of mice
c. The identification of the role of CD55/CD97 interaction in arthritis which might be a candidate therapeutic target

These three mechanisms could potentially be targeted to reduce severity of RA. AON-based reduction of pro-inflammatory proteins may be developed as a therapy, while the study of the Fc γ RIIb and CD55/CD97 pathways may yield insights into subsequent therapeutic interventions.

Antisense-oligonucleotide mediated exon skipping based therapies in arthritis

Antisense oligonucleotide (AON) exon skipping is a novel approach that can be used to restore correct splicing of an aberrantly spliced transcript, to produce a novel splice variant with a therapeutic potential or to induce a shift in the ratio between alternative splice products. We used this approach to convert a membrane-bound receptor into its soluble form. This can be achieved by skipping the exon that encodes the transmembrane region as previously shown for the TNFα receptor (Graziewicz, 2008). Like TNFα, IL-1 is also an important inflammatory cytokine in the pathogenesis of arthritis so IL-1R could be targeted in a similar way as was shown for the TNFR However, targeting IL-1R to convert it into its soluble isoform is not the ideal way to diminish IL-1 activity, because the soluble form of the IL-1 receptor binds IL-1R antagonist (IL-1Ra) reducing the activity, of this natural inhibitor of IL-1 signaling. Therefore we selected IL-1RAcP as a target since the natural soluble form of this

molecule (sIL-1RAcP) is another inhibitor of IL-1 signaling. When AONs target exon 9 of both human and mouse IL-1RAcP, they induce skipping of this exon resulting in the formation of a new mRNA, Δ9IL-1RAcP without econ 9. This newly formed transcript will encode a novel soluble form of IL-1RAcP lacking the transmembrane region. Although Δ9IL-1RAcP, in contrast to natural sIL-1RAcP protein, lacks only the transmembrane region but still contains the intracellular regions, in our hands this novel soluble Δ9IL-1RAcP protein also acted as an inhibiting IL-1 scavenger. As a proof of concept we have shown a decrease of the expression of the IL-1 responsive cytokine IL-6 and chemokine ICAM-1 in cells treated with an IL-1RAcP specific AON , indicating that Δ9IL-1RAcP protein is functional *in vitro*.

The key feature of AONs is their ability to prevent access of the spliceosome to the sequences that play a role in exon inclusion in pre-mRNA splicing. To achieve this an AON should be specifically designed based on the criteria reviewed in Aartsma-Rus et al., 2009. However, even though an AON is perfectly designed in silico, it doesn't guarantee its skipping efficiency. So the real potency of an AON can only be determined, empirically, by testing it *in vitro*. For skipping of exon 9 of mouse IL-1RAcP we have designed around 13 AONs and *in vitro* tests showed that they all had different skipping efficiencies. The best one, PS 300, has full 2′-O-MePS chemical modification with skipping efficiency around 50%. However, to be able to detect a biological effect we needed to increase skipping efficiency even further. This can be achieved by increasing the binding efficiency of the AON to its target. Therefore, we designed a new chimeric 2′-O-Me/LNA-PS AON (PS300L) which has 5 LNA (locked nucleic acid) bases that were mainly placed on G and Cs, to increase Tm and especially on a C of an internal CpG, to prevent its recognition as non-self, which could lead to immune-stimulation. With this improvement, skipping efficiency was remarkably increased up to 90% *in vitro*.

In vivo delivery of AONs is very challenging because ideally they should exclusively reach the target organ or cell type, penetrate the cellular and nuclear membranes and reach the pre-mRNA to achieve exon skipping. 2′-O-Me modification protects the oligo's from nuclease activity and PS modification increases their binding efficiency to proteins at the cell surface to facilitate their transfer from the blood to the tissues. However, AONs still need additional conjugations to achieve targeted cell delivery.

Previous studies have showed that unconjugated AONs mainly accumulate in liver, taken up by Kupffer cells and hepatocytes. This was an advantage in our study because we aimed to target liver, as hepatocytes are the main source of natural sIL-1RAcP. However, our *in vivo* experiments in the mouse with unconjugated AONs showed that we had to direct the cellular uptake more in favor of hepatocytes to achieve better skipping of IL-1RAcP exon 9. So we complexed AONs with invivofectamine; lipid-based nanoparticles that allow efficient delivery of synthetic oligonucleotides to hepatocytes. When the most efficient AON, PS300L, was complexed with invivofectamine, skipping efficiency was increased up to 90% even in a very low dose. An alternative to target hepatocytes is conjugation of AONs with galactosamine because galactosamine is a high-affinity ligand for the hepatocyte-specific asialoglycoprotein receptor (ASGPR). It has been shown that galactosamine-conjugated AONs can be transferred very efficiently to hepatocytes (Prakash, 2014). We concluded that AON mediated skipping using an AON that has shown high efficacy *in vitro* can be as effective *in vivo* in a particular cell type depending on the efficacy of the *in vivo* cell type specific targeting.

There are more cell types that express IL-1RAcP making also some other organs and tissues more responsive to IL-1. Myeloid effector cells are also responsive to IL-1 and they play an important role in IL-1 induced inflammation. Thus, targeting directly myeloid effector cells, for example macrophages, with the IL-1Rap specific AONs may provide double effect by reducing the number of membrane bound receptor and by increasing production of Δ9IL-1RAcP protein to neutralize IL-1 activity. However targeting immune cells is challenging, in particular macrophages. Research is ongoing to develop strategies for effective, cell type specific AON delivery such as the use of arginine-rich peptides to target T-cells and dendritic cells and mannosylated chitosan nanoparticles to target macrophages (Shilakari, 2014).

There are some other potential targets in the inflammatory pathways for AON mediated exon skipping approach. For example, activation of the classical and alternative pathways of the complement system can lead to excessive production of C5. C5 is cleaved into C5a and C5b by the protease activity of C5 convertase. C5a acts as a strong chemo-attractant whereas C5b is involved in the formation of the membrane attack complex. The increased production of C5a is associated with a number of inflammatory

diseases including rheumatoid arthritis. C5a binds to its receptor C5aR on the surface of target cells such as macrophages, neutrophils and endothelial cells. So generating a soluble C5aR via the exon-skipping approach would be a way to diminish excessive C5a levels. However, this receptor has seven transmembrane domains, which are all encoded by the last exon, therefore not allowing the application of AON mediated exon skipping. Therefore, as an alternative strategy, we targeted C5a exon 17 that encodes for the anaphylatoxin domain. We hypothesized that skipping of exon 17 specifically reduces C5a action without affecting C5b activity. We designed 2′-O-MePS AONs binding to exon 17 encoding the anaphylatoxin domain of C5a. *In vitro* results showed skipping of exon 17 and formation of a novel C5Δ17 mRNA with an efficiency of around 50%. However we knew from previous experiments that this might not be sufficient to detect a biological effect *in vivo*. So we tried to improve the skipping efficiency of the selected AONs by adding LNA bases as we successfully did with IL-1RAcP AONs. Even though using chimeric AONs, we could only improve skipping efficiency very little *in vitro*. When we targeted mouse liver hepatocytes *in vivo* (the main source of C5) we could hardly detect the skipped products. These results suggest that skipping efficacy strongly varies between targets. However, it cannot be excluded that C5 exon 17 skipping might be improved by other techniques or backbones, or by using oligo's with yet more LNA bases to provide better binding to the target RNA.

In conclusion, we have shown that AONs specific for exon 9 of IL-1RAcP and exon 17 of C5 can modify *in vitro* pre-mRNA splicing. . Additionally, *in vivo*, in mouse liver we could successfully skip exon 9 of IL-1RAcP. We have also shown *in vitro* that skipping of IL-1RAcP exon 9 inhibits IL-1β–mediated signaling. Taken together these results demonstrate the ability of AON mediated exon skipping to induce a novel mRNA splice variant with therapeutic potential. We anticipate that with the improvement of cell/tissue specific targeting of AONs, the application of therapies based on the modification of RNA splicing will increase in the near future.

FcγRIIB in arthritis

Studies so far showed that activating FcγR play an important role in the downstream effector phase of arthritis in mice. FcγRIII plays the most prominent role, but contribution of FcγRI and FcγRIV has also been reported (Boross, 2008; Mancardi, 2011). The dominant role of FcγRIII is probably because of its broad expression pattern and broad ligand specificity compared to the other activating FcγR. The function of FcγRI and FcγRIV seem to be more strictly regulated, implying that their function is more in fine-tuning the downstream antibody effector pathways.

The activating FcgR are counterbalanced by one inhibiting receptor, FcγRIIb, with a broad expression pattern. FcγRIIB plays an inhibitory role in many aspects of the immune response at different stages and acts as a major regulator in arthritis in mice. When FcγRIIB and B-cell receptor (BCR) on B cells are co-cross-linked with the antigen and its specific IgG, it down-regulates antibody production from B cells. Another control mechanism is more downstream, on the level of FcγRIIB-expressing myeloid effector cells. Cross-linking of FcγRIIB with one of the activating FcγRs on these cells by Immune Complexes results in the inhibition of antibody-effector pathways.

It has been shown that FcγRIIb KO mice on pure C57BL/6 background are susceptible to CIA (Boross, 2011) like the previously generated FcγRIIb KO mice on mixed 129/C57BL/6 background (Bolland, 2000). Yet there are some differences in the severity and onset as FcγRIIb KO mice on mixed 129/C57BL/6 background have 129-derived flanking sequences (*Sle16* locus), which induce autoimmunity and increase severity of arthritis. As mice generated from pure C57BL/6 do not have these flanking sequences, they develop milder disease with a delayed onset (Boross, 2011).

In this thesis, we focused on the cell type specific role of FcγRIIb in arthritis. The broad expression pattern of FcγRIIb has made it difficult to define its role in different cell types. Several studies suggest that deficiency of FcγRIIb on B cells is mainly responsible for the increased susceptibility to arthritis of the full FcγRIIb KO mouse (Bolland, 2000; Svensson, 1998) as it leads to uncontrolled B cell activation, which results in uncontrolled antibody production. However, in chapter 4 we show that lack

of FcγRIIb on B cells (CD19Cre mice) did not result in increased disease susceptibility. In contrast inactivation of FcγRIIb on myeloid effector cells (C/EBPαCre mice) significantly increased disease incidence. The explanation might be that in the absence of the inhibiting FcγRIIb activating FcγR expressing effector cells require much less antibodies to become activated. This hypersensensitivity of myeloid effector cells caused by the lower threshold for the activation of downstream antibodies effector pathways together with impaired IC clearance makes C/EBPαCre mice more susceptible to CIA compared to CD19Cre mice. However, we cannot exclude a contribution of the deficiency of FcγRIIb on B cells to the high susceptibility to CIA of the full FcγRII KO mice. Although the incidence of the disease in C/EBPαCre mice significantly increased, we didn't observe a significant increase in disease severity in this genotype compared to disease severity in WT control mice whereas both incidence and severity were significantly increased in full FcγRIIb KO mice. Additionally, when we compared the expression levels of seven different cytokines and chemokines from the synovium of mice with severe CIA, we observed that only two out of seven were significantly increased in C/EBPαCre compared to control group. In contrast in FcγRIIb-/- mice five of these cytokines/chemokines were significantly increased. This suggest, that FcγRIIb deficiency on B cells, although on its own not sufficient to cause a significant increase in disease susceptibility, contributes to the strong disease susceptibility of full FcγRIIb KO mice. However, analysis by FACS of immune cells from C/EBPαCre mice stained with a FcγRIIb specific antibody revealed that the deletion of the FcγRIIb gene is not complete in the myeloid compartment of these mice. (Sharp, 2013). The expression level of the seven cytokines reflects the level of inflammation. The inflammation is mainly determined by the FcgR mediated triggering of the cellular response of myeloid effector cells. It might be that the milder inflammation in C/EBPαCre mice compared to the inflammation in full FcγRIIb KO mice is caused by a higher threshold for IgG Immune complex induced activation of a subset of myeloid effector cells expressing FcγRIIb.

In a spontanous auto-immune disease model, Kanari *et al*., also showed the dominant role of FcγRIIb on effector cells in glomerulonephritis. In this study, mice with the autoimmune-related SLAM locus have higher anti-nuclear autoantibodies (ANA) compared to FcγRIIb KO but no disease was observed, whereas FcγRIIb deletion leads to production of very low ANA titers but still contributes to the pathology of mild

glomerulonephritis. These results also support that FcγRIIb deficiency lowers the threshold for the initiation of the antibody effector pathways in a variety of antibody driven auto-immune responses However, in this study, the contribution of environmental factors and especially gender also seem to play a crucial role, which needs to be further elucidated.

In one recent study using the LysMCre X FcγRIIbfl/fl mice as a model for myeloid specific FcγRIIb deficiency, they could not show a significant increase in susceptibility to CIA (Li, 2014). This could be due to the use of LysMCre mice because this model has very poor deletion of FcγRIIb in myeloid cells. As we show,, the efficacy of C/EBPαCre is much higher compared to LysMCre in deleting FcγRIIbfl/fl alleles in the myeloid compartment, although still less than 100%. In contrast to our own results, the same study of Li et al. showed that the deficiency of FcγRIIb on B cells caused a significantly increased susceptibility to CIA. However in our study we used CD19Cre mice whereas Li et al. used Mb1Cre and Cg1Cre. The expression of CD19Cre and mb1Cre are similar, very early in B cell development in the bone marrow, but it might be that mb1Cre is a bit more efficient. Cg1Cre will delete FcgRIIbfl/fl only after B cells have been activated. Most likely in many but not all activated B cells Cg1Cre will delete FcgRIIbfl/fl. Surprisingly, deletion at an early (Mb1Cre) and a later (Cg1Cre) timepoint in B cell development, resulted in different phenotypes depending on the CIA model. In contrast to Mb1CreX FcgRIIbfl/fl mice Cg1CreX FcgRIIbfl/fl mice are sensitive to cCIA, but not bCIA. We found that CD19CreXFcgRIIbfl/fl developed significantly increased anti-collagen autoantiboy titres in bCIA but not in cCIA. In addition, Li et al found a mild but significant increase in susceptibility to CIA in CD11cCreXFcgRIIbfl/fl mice compared to FcgRIIbfl/fl control mice whereas we did not observe a significant increase in these dendritic cell (DC) specific FcgRIIb KO mice. Besides the mentioned variation in deletion of the FcgRIIb fl/fl alleles in B cells there are many other explanations for the many differences between our results and the results of Li et al. The origin of the ES cells used for the generation of the FcgRIIbfl/fl strain was different as well as the genetic background of the different Cre strains. Also the CIA models used vary between experiments e.g. antigen and adjuvant Furthermore differences in immune status of the mice might play a substantial role in breaking immune tolerance.

Our results with C/EBPαCre X FcgRIIbfl/fl mice in the CIA model indicate that FcgRIIb on myeloid cells plays an important regulatory role in this model. However, it is still unclear on what myeloid subset(s). As already mentioned we observed that DC-specific FcγRIIb KO mice (CD11cCre) didn't have significantly increased disease parameters in CIA compared to control mice. However, although FcγRIIb deficiency on DCs on its own was not sufficient to cause a significant increase in disease susceptibility, it cannot be excluded that DC-specific FcγRIIb deficiency contributes to the strong disease susceptibility of full FcγRIIb KO mice. The contribution of deficiency of FcγRIIb on other myeloid subsets to disease susceptibility still needs to be elucidated. Recently Mcpt5Cre (Scholten, 2008) and hMRP8Cre (Passegue, 2004) mouse strains became available to analyze the role of FcgRIIb on mast cells and neutrophils respectively. However, a transgenic mouse strain, which expresses Cre exclusively in all macrophages, is still not available. We have detected an increase in anti-mouse collagen type II autoantibody titers in both C/EBPαCreX FcgRIIbfl/fl and CD19CreXFcgRIIfl/fl mice. The increase in CD19CreXFcgRIIb fl/fl can be explained by the role of FcgRIIb in a negative feed back mechanism in B cells as one of the controlling mechanisms in antibody production. However it is unclear why also in C/EBPαCreXFcgRIIbfl/fl mice antibody titers are increased. In C/EBPαCre XFcgRIIbfl/fl mice the expression of FcgRIIb on DCs is strongly decreased however in CD11cCreXFcgRIIbfl/fl mice, which lack FcγRIIb expression on the majority of CD11c positive DCs , antibody titers are not increased .

Only mice with substantial anti-CII antibody titers develop arthritis but not all mice with high autoantibody titers developed disease. This suggests that the presence of autoantibodies is essential but not sufficient to develop full-blown disease and there are some additional changes required to initiate the development of severe arthritis. These additional changes can be related to increased inflammatory cytokines, chemokines and activated alternative pathways of the complement system that might be triggered by infections or some other environmental conditions.

The role of CD55/CD97 interaction in arthritis

The main function of CD55 is to regulate complement activation. CD55-/- mice have been shown to enhance complement activation and increased clinical symptoms of auto-immune diseases (Miwa, 2002). However, when CIA is induced in CD55-/- mice, surprisingly, a significant reduction in arthritis activity has been observed. Similarly, CD55-/- mice didn't show increased disease in K/BxN serum–transfer model. These findings suggest that, there might be other complement components to compensate CD55 deficiency or CD55 might have a different role. Therefore, targeting CD55 cannot be effective to ameliorate arthritis. On the other hand, it has been recently shown that (Karpus, 2015) CD55-/-X FcγRIIb-/- mice showed an increase in the severity of arthritis compared to FcγRIIb-/- in the K/BxN serum–transfer model. This means that CD55 deficiency further enhances disease sensitivity in FcγRIIb-/- mice, so it might have a protective role in serum induced arthritis model in mice with susceptible background. Extensive analysis of bone marrow-chimeric mice that express CD55 on either immune or non-immune cells showed that FcγRIIb-/-X $CD55^{host+/+BM-/-}$ and FcγRIIb-/-X $CD55^{host-/-BM+/+}$ mice were equally susceptible to K/BxN serum transfer arthritis and developed slightly more disease compared to FcγRIIb-/-X $CD55^{host+/+BM+/+}$ mice. These results demonstrate that CD55 expression both in immune cells and stromal cells contributes to the protection from K/BxN serum induced arthritis.

The other function of CD55 is acting as a ligand for CD97. It has been shown that treatment of CIA with CD97 antibody, which blocks CD55-CD97 interaction, ameliorates arthritis. (Kop, 2006; De Groot, 2009). In addition to that, CD97-/- mice have lower incidence and severity in CIA compared to WT mice. These results support the idea that interference with binding of CD55 to CD97 has an inhibiting effect on arthritis development. Therefore, although in arthritis the regulatory role of CD55 in complement activation cannot be excluded, the alternative role of CD55 as a ligand for CD97 might be more important in this disease. It is not yet exactly known how CD55–CD97 interaction contributes to the development of arthritis and further studies would reveal the beneficial effect of blocking the CD55–CD97 interaction in RA.

AON mediated exon skipping is a promising approach as a therapy to decrease inflammation in diseases caused by increased production of IL-1. However, the role of

FcγRIIB and CD55 in RA is very complicated and need further studies before these molecules can be targeted or used in therapeutic interventions.

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