Small GTPases : emerging targets in rheumatoid arthritis

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GENERAL DISCUSSION AND SUMMARY
Rheumatoid arthritis (RA) is an auto-immune disease for which no cure is yet available. Recent insights into the pro-inflammatory cytokines and cells present in the inflamed joint, and their contributions to pathology in RA, have led to the development of biological therapies aimed at their neutralization. Anti-TNF-α blockade for example, is one of the most widely used and successful biological therapies. There is a large group of patients however, that don’t respond sufficiently to treatment. Also, therapies with biologicals can have serious side effects as the immune system of treated patients become less efficient in clearing pathogens and opportunistic infections. Therefore, there is still the need for the development of new and more specific therapies.

Cellular activation, proliferation, survival and migration are tightly regulated at the intracellular level by Ras-superfamily small GTPases. Small GTPases are important modulators of cellular responses and crucial mediators of effective immune responses, and initial evidence has suggested these enzymes may contribute to pathology in RA. Ras family GTPase effector pathways such as MAP kinases, PI3K and NF-κB are highly activated in RA synovial tissue (ST). Moreover, strategies that block Ras function are able to suppress fibroblast-like synoviocyte (FLS) proliferation, invasiveness and pro-inflammatory cytokine production in vitro, and exert protective effects in animal models of arthritis in vivo. Similarly, blockage of Rho family small GTPases reduces FLS proliferation, invasiveness, JNK activation and IL-6 production in vitro. Finally, inactivation of the Ras-related small GTPase Rap1 in synovial T cells is associated with increased intracellular oxidative stress. Modulation of small GTPase signaling may therefore be an attractive tool to inhibit the inflammatory process in RA. In this thesis we analyzed the in vitro and in vivo effects of manipulating the activity of representative small GTPases in respect to inflammatory activation of cells relevant to RA, and animal models of this disease.

Much evidence indirectly suggests an active involvement of T cells in RA. Most importantly, the expression of the shared epitope (SE), thought to present self antigen(s) to autoreactive T cells, is one of the strongest indications of TCR involvement in RA. Synovial T cells have a highly differentiated CD45RO+ phenotype, promote the activation of other synovial cells through cell-cell contacts, and in many animal models T cell dependency has been shown. The clinical success of CTLA-4Ig therapy has suggested that TCR engagement may take place in RA, as disruption of the T cell costimulatory CD28 protein with its ligands CD80/86 is of clinical benefit. However, despite this evidence, whether there is TCR triggering in RA is still a mat-
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ter of debate. Several studies have described impaired T cell responses in RA due to deficiencies downstream of the T cell receptor (TCR). Increases in intracellular oxidative stress due to Rap1 inactivation\textsuperscript{10}, impaired phosphorylation of the adaptor protein linker for activation of T cells (LAT)\textsuperscript{15} and chronic downregulation of TCR-zeta expression\textsuperscript{16} are some of the defects described. Based on the inability to detect significant T cell cytokine production and proliferation in the synovium, together with the reported defects in TCR-proximal signaling responses, several studies have proposed that TCR signaling in RA is impaired\textsuperscript{17}. In chapter 2 we analyze synovial T cell responses at a single cell level, and demonstrate that TCR signaling is fully functional and completely capable of initiating T cell proliferation and cytokine production. We provide evidence that the reported hyporesponsiveness in RA synovial T cell bulk cultures is secondary to ex vivo spontaneous apoptosis. Additionally, we find that the increase in susceptibility to apoptosis ex vivo is associated with altered ratios of pro-apoptotic Noxa and anti-apoptotic Mcl-1 expression. These data indicate that although there is no evidence of T cell apoptosis in vivo, once isolated from the synovium T cells are extremely susceptible to apoptosis. It will be interesting to address which soluble factors or specific cellular contacts are responsible for the in vivo inhibition of synovial apoptosis, as abrogation of these signals might exert a therapeutic benefit by selectively promoting T cell apoptosis at the site of inflammation.

The data in chapter 2 clearly shows that synovial T cells are capable of supporting productive TCR signaling. Our results suggest that the inability to detect T cell cytokines and proliferation in the synovium of established RA might be due to the absence of TCR engagement, rather than a consequence of TCR signaling defects. In early stages of disease, where T cell cytokines are observed, TCR engagement may play a prominent role, as autoreactive T cells could initiate inflammation. However in the effector phase of disease, T cells may contribute to inflammation by TCR-independent mechanisms. In this context it will be interesting to investigate the specific mechanism of action by which CTLA-4Ig therapy exerts its effects. In light of our results we propose that alternative mechanisms, independent of effects on classical TCR costimulation, might be responsible for the clinical improvements observed in treated patients. One of these mechanisms could be upregulation of immunomodulatory IDO on CD80/86 expressing cells. Besides antigen presenting cells (APC), T cells in the synovium have also been shown to express functional CD80/86 costimulatory molecules\textsuperscript{18,19}. In vitro studies have shown that triggering of CD80/86 on T cells by CTLA-4 ligation is capable of upregulating IDO expression, reducing their capacity to respond to antigen triggering\textsuperscript{20}. Whether T cells in the synovium,
APCs, or both are targeted in this manner by CTLA-4 Ig therapy remains to be determined.

The small GTPase Rap1 is transiently activated upon TCR triggering and is an important regulator of immune responses. In RA synovial fluid (SF) T cells a block in Rap1 activation is believed to underlie the altered T cell behaviour in this disease\(^{10}\). While RA synovial T cells were first described as hyporesponsive, we demonstrated in chapter 2 that T cells from the SF of RA patients are hyper-responsive to TCR triggering. In chapter 3 we investigate whether inactivation of T cell Rap1 might play an inflammatory role in RA. To accomplish this we used transgenic (Tg) mice that express constitutively active Rap1a, RapV12, within the T cell lineage. We performed a collagen-induced arthritis (CIA) experiment and evaluated the effect of Rap1 activation during experimental arthritis. We observed that arthritis incidence was decreased more than 70\% in RapV12 mice and that both paw swelling as well clinical signs of disease were significantly decreased. Histological evaluation of the paws revealed that cellular infiltration and cartilage destruction were significantly reduced in Tg mice, as was joint damage. Different animal models in which the activity of Rap1 has been genetically modulated have also found defects in T cell responses. In mice expressing the active mutant Rap1E63 defects in T cell responses have been attributed to increased numbers of regulatory T cells (Tregs)\(^{21}\). In RapV12 mice we found no differences in the numbers of Tregs either under homeostatic conditions or after CIA induction, and IL-10 production was equivalent in RapV12 and WT mice. We found however, that TNF-\(\alpha\) production was severely impaired in RapV12 mice, particularly in cytotoxic CD8\(^{+}\) T cells. Defective T cell responses have also been noted in mice lacking Spa1 expression (Spa1\(^{-/-}\))\(^{22}\). In the absence of this GAP, Rap1 activity is elevated in T cells of these mice. These mice show an age-dependent accumulation of unresponsive CD44\(^{\text{high}}\) T cells that drive defective T helper cell responses. Young Spa1\(^{-/-}\) mice with normal primary T cell responses display impaired T cell responses to recall antigens, with reduced antibody production. We investigated whether this was also the case for RapV12 mice. CD44 expression levels in RapV12 mice were comparable to WT mice and no differences were observed between naive, effector memory or central memory populations. We found however, that the circulating levels of specific anti-collagen antibody production were significantly decreased in RapV12 mice. We further investigated the mechanism underlying the defective antibody production and found that RapV12 T cells were impaired in their capacity to upregulate CD40L and ICOS co-stimulatory molecules, which could explain why
RapV12 T cells are less effective in providing help for B cell activation and antibody production. Analysis of B cell responses in experimental models where T cells are dispensable for B cell activation (TNP Ficoll) and in T cell-dependent assays (TNP KLH) may be useful to confirm the defective T helper phenotype of RapV12 mice. Additionally, as CIA is a model of a chronic immune stimulation, it will be of interest to determine if the defective T cell responses in RapV12 Tg mice (lower TNF-α and antibody production) occur only in a chronic setting, or also in primary and secondary acute immune responses. Finally, it is as yet uncertain if the ability of RapV12 to protect against autoimmune disease is specific for CIA, or will be a general effect in other immune-mediated inflammatory models such as EAE.

TCR engagement in the presence of CD28 costimulation is a critical step in the initiation of effector T cell responses, while CTLA-4 ligation is essential at the end of an immune response to dampen cellular activation. Costimulation is therefore a crucial step in determining the fate of T cell responses, however the specific processes downstream of costimulatory molecule ligation are not yet clear. Accumulating studies have shown the involvement of the small GTPase Rap1 in this process. Rap1 is transiently activated upon TCR triggering and further enhanced by CTLA-4 engagement, whereas CD28 ligation blocks Rap1 activation. These data suggests Rap1 as a central regulator of T cell responses to co-stimulatory cues. Rap1 is activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase-activating proteins (GAPs). CD28-dependent inactivation of Rap1 is believed to be the result of Rap1GAP activation, while CTLA-4-dependent Rap1 activation the result of Rap1GAP inactivation. In vivo studies have confirmed the idea of Rap1 as a critical mediator of T cell responses. Different phenotypes of transgenic (Tg) Rap1 GAP animals suggest that each Rap1 GAP may contribute differentially to T cell activation. In chapter 4 we show that the five mammalian Rap1 GAPs are differentially expressed in resting and TCR/CD28 activated T cells. We found Sp1 to be upregulated both at the mRNA and protein levels after TCR/CD28 stimulation in CD4 but not CD8 T cells. We found this upregulation to be PI3-kinase and NF-κB dependent, as inhibitors for each signaling pathway were able to block Sp1 upregulation. We show that rather than dependent on proliferation, Sp1 upregulation is dependent on TCR/CD28 specific signals, as stimulation with IL-15, IL-17 or IL-2/PHA were able to induce proliferation but not Sp1 expression. Additionally, we found Rap1GAP1A and Rap1GAP1B mRNA to be equally upregulated upon TCR/CD28. Surprisingly we found the opposite at the protein level, suppression of Rap-
1GAP1 expression accompanied by protein degradation. Rap1GAP2 was detected at the mRNA level in rested cells, and downregulated upon TCR/CD28 stimulation. At the protein level Rap1GAP2 expression followed the same pattern as Rap1GAP1, expression in rested cells followed by suppression and protein degradation upon TCR/CD28 ligation. Distinct studies have described that post-translational regulation of Rap1GAP protein stability is necessary to avoid proteasomal degradation. The mechanism through which Rap1GAP proteins are degraded during T cell stimulation, and the consequences of inhibiting this process, remains to be determined.

It will be interesting to address which Rap1 GAP regulates Rap1 inactivation in CD8 T cells upon TCR/CD28, as Spa-1 upregulation occurs in CD4 T cells only. RAP1GAP1 would be a good candidate, as it becomes upregulated at the mRNA level. Although we observed RAP1GAP1 protein degradation after 24h of TCR/CD28 stimulation, shorter time-course stimulations may elucidate on the dynamics of RAP1GAP1 activation and degradation.

The findings presented in this chapter suggest that each Rap1 GAP may differentially contribute to T cell activation. Each Rap1 GAP may couple distinct extracellular stimuli to Rap1 regulation, associate to different costimulatory molecules and/or inactivate Rap1 in distinct cellular subcompartments. In this chapter we study how TCR/CD28 stimulation modulates Rap1 GAP expression. It will be interesting to evaluate how other co-stimulatory molecules, either positive (ICOS) or negative (CTLA-4 and PD-1) regulators of T cell activation influence Rap1 activation. Investigating the activation patterns of each Rap1GAP may allow to the development of compounds that will be able to specifically regulate Rap1 activation in distinct T cell compartments, with potential application to diseases like RA where a constitutive block in Rap1 expression is observed.

Fibroblast-like synoviocytes (FLS) from RA patients display proliferative and invasive properties reminiscent of malignant tumor cells. Ras small GTPases play important roles in tumor cell proliferation and invasion. Although no transforming mutations have been detected in RA FLS, pathways mediated by Ras GTPases are known to be de-regulated. Recent evidence suggests that changes in GEF expression or activity might be relevant to disease. This is the case in human melanoma cells, where expression levels of Ras guanine nucleotide-releasing factor (RasGRF) regulates constitutive MMP-9 production. In chapter 5 we analyze the expression patterns of RasGRF1, a specific H-Ras activator, in RA and non-RA ST and FLS. RasGRF1 expression was significantly increased in RA ST and found to
positively correlate and co-localize with MMP-1 and MMP-3 producing cells. Additionally, we found that in vitro modulation of RasGRF1 expression in RA FLS was able to regulate spontaneous MMP-3 production. We could not find an association between RasGRF1 expression and MMP-1 production in RA FLS, which could imply that maybe other cell types, such as macrophages might be more important sources of MMP-1 production. It will therefore be interesting to analyze the effects of knock down and overexpression of RasGRF1 in macrophages in terms of MMP-1 production. In lysates from FLS and ST we observed the expression of post-translationally modified RasGRF1. These truncated fragments are known to be derived from protease-dependent cleavage and these fragments have been shown to have enhanced Ras-activating capacity. The results presented in this chapter suggest that enhanced RasGRF1 expression and activating post-translational modifications in RA FLS sensitize signaling pathways that promote MMP-3 production and consecutively joint damage. It is not yet known which stimuli present in the joint promotes the post-translational modification of RasGRF1, but approaches targeting these activating stimuli may prove of clinical interest in reducing RasGRF1 activation and MMP-3 production.

Cellular migration, proliferation, activation and immunological synapse formation are processes tightly regulated by Rho family Rac small GTPases. In RA these cellular processes are thought to be de-regulated as high numbers of activated immune cells are observed in the synovium, reflecting increased migration into the joints, local proliferation or both. In chapter 6 we investigated whether treatment of arthritic mice with inhibitory Rac1 C-terminal peptide, which specifically blocks Rac1 signaling, would ameliorate disease severity. Different studies have reported the involvement of members of the Rho GTPase family in the pathogenesis of RA. Inhibition of Rac1 signaling in vitro is able to reduce FLS proliferation and invasiveness while modulation of Rho activation regulates FLS proliferation and IL-6 secretion. In vivo, in a model of reactive arthritis, Rac signaling in neutrophils has been shown to regulate joint inflammation. In this chapter we treated arthritic mice during early or chronic disease, with Rac1 C-terminal peptide and found that inhibition of Rac1 signaling in vivo significantly reduces anti-collagen antibody production. The reduction in levels of immunoglobulin (Ig) G1 and IgG2a (early treatment) or IgG2a and IgG2b (treatment during chronic arthritis) correlated with an amelioration of paw swelling in treated animals. Decreased antibody production could be the result of defective T cell priming, as B cell responses are critically dependent on
proper T cell activation. In order to provide help for B cell activation, T cells need to have a productive engagement with APC. Studies in vivo have shown that Rac deficient dendritic cells (DC) have defective migration to secondary lymphoid organs and fail to establish stable contacts with naive T cells\(^43\). In this case, suboptimal T cell priming leads to defective humoral responses to T cell-dependent antigens\(^44\). Rac1 peptide may similarly inhibit anti-collagen antibody production by disturbing T cell-APC interactions. Further studies addressing antibody production in T cell-dependent and -independent assays may elucidate whether treatment with Rac1 C-terminal peptide targets T cell priming or the antibody producing B cells directly. In the first case, it will be interesting to address whether also T cell effector responses, besides T helper, are impaired.

The observed effect on the reduction of paw swelling could be due to effects in edema formation, as inhibition of Rac1-mediated signaling leads to a decrease in leukocyte extravasation\(^45\). However, although in vitro Rac1 C-terminal peptide was able to inhibit actin polymerization, we found no evidence for a decrease in cellular infiltration or joint destruction in vivo. The limited clinical effect of Rac1 peptide treatment may reflect the redundant role of Rac2 in promoting osteoclastogenesis\(^46\)\(^47\) and cellular migration\(^48\), as the inhibitory peptide we used is highly specific for Rac1 and does not interfere with Rac2 signaling\(^49\). Although the data presented in this chapter shows that treatment of arthritic mice with Rac1 peptide can safely be used to reduce paw swelling and the circulating levels of anti-collagen antibodies, future studies will need to directly evaluate the relative contributions of both Rac1 and Rac2 in CIA.

In conclusion, the work presented in this thesis shows that modulation of small GTPase signaling might have good therapeutic potential in the treatment of RA. Strategies aimed at the inhibition or maintenance of small GTPase signaling could be applied at different levels of intracellular signaling cascades and tested for their efficacy in the amelioration of arthritis.

Several compounds targeting effectors of small GTPases are already available, and some have been tested in clinical trials with successful results. In cancer clinical trials, blockage of the Raf/MEK/ERK pathway with compounds targeting Raf (BAY 43-9006, ISIS 5132) and MEK (PD184352, PD0325901 and ARRY-142886) have showed promising results\(^50\). In RA clinical trials, the use of VX-702 and RO-440-2257 as p38 inhibitors have showed only limited success\(^51\)\(^52\), and further analysis of the distinct contributions of Ras effector pathways to RA is urgently needed.
Direct targeting of small GTPases has also been investigated in different disease settings. We have shown in chapter 6 the possibility of using C-terminal peptides to interfere with specific small GTPase signaling in vivo for the treatment of CIA. In chapter 5 we used locked nucleic acid (LNA) as a tool to efficiently inhibit RasGRF1 expression in vitro. Compounds that interfere with small GTPase post-translational modification, such as farnesyl transferase inhibitors (FTIs), have received special attention in cancer settings. The FTIs Tipifarnib and lonafarnib are currently under clinical investigation with encouraging results in the treatment of hematological malignancies and breast cancer\(^{53}\). Although not yet tested in clinical trials, bacterial toxins have also been shown to target individual small GTPases. Clostridium difficile toxin A and B specifically glucosylate Rho proteins, while bacterial C3-like ADP-ribosyltransferase induces ADP-ribosylation of RhoA, both inhibiting in a specific manner small GTPase downstream signaling\(^{54,55}\). Maintenance or activation of small GTPases may also be interesting to modulate inflammation in RA. We have shown in chapter 3 that maintenance of Rap1 signaling ameliorates disease in CIA. 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8CPT-2Me-cAMP) is a cAMP analog that can selectively activate Epac, a Rap1 GEF\(^{56}\). Although not yet tested in a disease setting, it would be interesting to analyze the effect of this analogue in mice with CIA.

Finally, targeting cell surface receptors upstream of the small GTPases may allow specific modulation of GTPase activity, through the regulation of GEFs and GAPs. One such candidate may already be in the clinic. CTLA-4Ig (abatacept), which disrupts the interaction between the co-stimulatory CD28 molecule and CD80/86 on APCs, has shown clinical benefit in RA patients. In vitro, this compound has been shown to prevent T cell Rap1 inactivation by synovial fluid adherent cells\(^{57}\). Future work will need to determine how restored T cell Rap1 function may contribute to the clinical benefit of CTLA-4Ig therapy. In sum, the data presented in this thesis provides strong pre-clinical evidence that targeting Ras superfamily GTPases may be beneficial in the treatment of RA, a potential awaiting the development of compounds specifically targeting these enzymes in the clinic.
General discussion and summary

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