Targeting intracellular signaling pathways at the interface of T lymphocyte and innate immunity in immune-mediated inflammatory diseases
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Chapter 8

General Discussion
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Section I: The contribution of pro-angiogenic macrophage Tie2 signaling to pathology in rheumatoid arthritis

Macrophage Tie2 activation and inflammation
The tyrosine kinase receptor Tie2 drives angiogenesis and blood vessel remodeling via interactions with its ligands, angiopoietins (Ang). Whereas Ang-1 mediated Tie2 activation leads to blood vessel stabilisation and quiescence\(^1\), the role of Ang-2 is more context and cell-type dependent. Ang-2 can act as a natural inhibitor of Ang-1 induced Tie2 phosphorylation\(^2\). However in the absence of Ang-1, or when Ang-2 is present in high concentrations, Ang-2 can induce Tie2 phosphorylation and increase vascular permeability and vascular regression\(^3\)\(^4\). In endothelial cells, Ang-dependent Tie2 phosphorylation activates several downstream signaling pathways, such as phosphoinositide-3-kinase (PI3K)-protein kinase B (PKB)\(^5\)\(^6\), mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinases (ERK)\(^7\) and janus kinase (JAK)-signal transducer and activator of transcription (STAT)\(^8\). Moreover, numerous studies have demonstrated that Tie2, Ang-1 and Ang-2 are expressed on endothelial cells and fibroblast-like synoviocytes (FLS) in the synovium of RA and PsA patients\(^9\)\(^-\)\(^11\). In chapter 2 we examined the targets of Ang signaling in RA synovial tissue and the effects of Ang-2 neutralisation in murine CIA. Here, we identified synovial CD68\(^+\) and CD163\(^+\) macrophages in RA patients as the primary targets of Ang signaling leading to Tie2 activation. In contrast, only limited Tie2 activation was observed in VWF\(^+\) endothelial cells and CD55\(^+\) FLS. Differences in the regulation of Tie2 activation in the synovial tissue could be influenced by several synovial factors. First, restriction of Tie2 activation to macrophages might be a result of local Ang production being limited to neighbouring cells. Second, differences in cellular expression of the inhibitory receptor Tie1, might limit prominent Tie2 activation to synovial macrophages. Third, it is possible that macrophages differ from other synovial cell populations in their sensitivity or exposure to soluble inhibitory Tie1 splice variants in vivo\(^12\). Involvement of the myeloid compartment in the angiogenic process has gained recent attention. Tie2-expressing monocytes (TEMs) are a subset of proangiogenic monocytes, representing 2-7% of the total human peripheral blood mononuclear cells, which are essential for tumor neovascularisation, cancer progression and are actively recruited to solid tumors\(^13\)\(^-\)\(^16\). We observed, consistent with previous literature\(^15\), that Tie2 is expressed on human peripheral blood-derived monocytes and to a lesser extent on human macrophages. We observed no differences in the expression of macrophage Tie2 between healthy donors and RA patients. Importantly, we found that Tie2 expression is functionally expressed on human macrophages, as Ang-1 and Ang-2 synergised with TNF to induce IL-6 production. In the case of Ang-1, this effect is at least partially
mediated by NF-κB and MEK/ERK activation. Similar findings were seen by others in endothelial cells, where Ang-2 controls the responsiveness to inflammatory stimuli, such as TNF\(^{17}\). We also observed that the expression of other inflammatory mediators, such as macrophages inhibitory protein (MIP)-1\(\alpha\), is enhanced upon macrophage stimulation by Ang-1 or Ang-2, which synergised with either TNF or LPS. MIP-1\(\alpha\) is highly expressed in RA synovium and plays an essential role in animal models of RA\(^{18}\). Interestingly, besides promoting macrophage chemokine production Ang-2 is also able to suppress thrombospondin (TSP)-2 production. TSP-2 is an inhibitor of matrix-metalloproteinases (MMP) production and also blocks angiogenesis in vivo and inhibits migration of capillary endothelial cells in vitro\(^{19}\). On the other hand overexpression of TSP-2 inhibits angiogenesis, inflammation and lymphocytes accumulation in an animal model of RA\(^{20}\). As these observations suggests a specific role for Ang-2 signaling to macrophages in the pathology of RA, we used a neutralising humanised anti-Ang-2 antibody in CIA. In previous studies, this compound was shown to be effective in a murine cancer model\(^{21}\). Strikingly, we observed that Ang-2 neutralization significantly reduces disease severity, synovial inflammation (including macrophage cellularity), cartilage destruction and synovial neo-vascularisation and lymphangiogenesis in CIA. The protective effect of the neutralizing Ang-2 antibody was almost as effective as treatment with prednisolone, still one of the most potent anti-inflammatory drugs for many IMIDs. Furthermore, the effects we observed on reduced lymphangiogenesis by Ang-2 blockade is interesting as increased lymphangiogenesis is found in RA compared to OA patients\(^{22}\). Therapeutic modulation of synovial lymphangiogenesis could be important, as lymphatic vessels transport extracellular matrix components into the synovial fluid and tissue, and are involved in trafficking leukocytes to and from the lymph nodes and secondary lymphoid tissues. In conclusion, this study underscores that Ang-2, alone or in combination with TNF contributes to the inflammatory character of macrophages, and that in vivo, neutralization of Ang-2 is sufficient to reduce pathology in murine CIA.

Phenotyping polarized macrophages

Macrophages can broadly be classified into (classically activated) pro-inflammatory M1 or (alternatively activated) M2a wound-healing and M2b immunosuppressive macrophages\(^{23}\). Upon entering tissue, the fate of a monocyte differentiating into a macrophage is determined by a synergistic interaction between cytokines and TLR ligands present in the local environment\(^{23;24}\). For instance, IFN-\(\gamma\) and LPS give rise to a pro-inflammatory, classically activated M1 macrophage\(^{25}\) which is characterized by a IL-12\(^{\text{high}}\), IL23\(^{\text{high}}\), IL1B\(^{\text{high}}\) and IL-10\(^{\text{low}}\) phenotype and has elevated bactericidal and phagocytic capacities to defend the body against pathogens\(^{26}\). In contrast, IL-4 induces alternatively activated M2 macrophages which produce the cytokine IL-10 and are important in tissue repair, allergic reactions and defense against parasitic infections\(^{24;27;28}\). Other factors such as immune complexes, IL-10, TGF-\(\beta\) and
corticosteroids were also identified to skew into M2 profiles. GM-CSF and M-CSF induce macrophages with M1- and M2-like phenotypes, respectively. In order to facilitate the study of macrophage subsets in vivo, we aimed in chapter 3 to systematically validate phenotypic surface markers for the three main polarized macrophages subsets in humans. We validated CD80 and CD64 as markers for MΦ_{IFN-γ}, CD200R for MΦ_{IL-4}, and CD163 and CD16 for MΦ_{IL-10} in humans. The markers CD64 and CD16 were specifically upregulated by IFN-γ and by IL-10 respectively, and not by other polarizing factors and could therefore not be identified as universal markers for either M1 or M2 macrophages. Moreover, we observed differences in phenotypic marker expression between GM-CSF/M-CSF differentiation and the IFN-γ/IL-4/IL-10 polarization model which emphasizes the complexity of the macrophage phenotypic polarization model. In disease tissue, mixed macrophage populations, or macrophages with mixed phenotypes appear to be common. For example, in SpA an upregulation of CD163 macrophages has been observed compared to RA synovium, while CD68 is expressed at similar levels. For the future it is very important to further extend the research on the phenotypic validation of macrophages, especially at sites of inflammation, as this will contribute to our knowledge of differences in polarizing macrophages and their potential contribution to pathology in different IMIDs.

Another important characteristic of macrophages is their plasticity, defined as reversible adaptation to a changing microenvironment, which has been demonstrated both in vitro and in vivo. It would be interesting to study the effect of a changing microenvironment, upon anti-cytokine treatment for example, and the phenotypical effect on synovial macrophages. And finally, the most crucial and potential future therapeutic application is to identify whether the phenotypic makers play a functional role in macrophage biology. For example, CD200R ligation inhibits ERK and JNK signaling pathways and treatment with an agonistic antibody suppresses inflammation in a experimental autoimmune uveoretinitis. Also potential roles for CD163 in inhibiting T cell activation have been described. A main challenge for the future will be thus not only to identify macrophage phenotypic markers, but also their functional contributions to pathology in IMIDs in vivo.

Tie2 activation in polarized macrophages

Tie2 expression on myeloid cells was previously thought to be limited to TEMs, which have primarily immunosuppressive M2-like properties. In chapter 2 we found activated Tie2 expression on synovial macrophages, and moreover functional Tie2 on GM-CSF macrophages. Therefore in chapter 4 we wished to explore the relationship between macrophage polarization and Tie2 expression and function in detail. We differentiated macrophages in the presence of growth factors or cytokines into 3 main macrophage subsets: classically activated (pro-inflammatory M1: GM-CSF, IFN-γ), and alternatively activated wound healing (M2a: M-CSF, IL-4), and immunosuppressive
(M2c: IL-10) macrophages\textsuperscript{31,44}. Tie2 protein and the ratio of Tie2:Tie1 mRNA expression was found to be most highly upregulated in IL-10 and IFN-γ polarized macrophages raising the possibility that these two types of macrophages might be most responsive to Ang signaling via Tie2. Because Ang-2 had been reported to reinforce M2-like transcriptional programs of TEMs\textsuperscript{45} we wondered if Ang-1 or Ang-2 could influence macrophage polarization. However we didn’t find any effect of Ang-1 or Ang-2 on the expression of human macrophage phenotypic polarization markers we identified in chapter 3. While we observed an enhanced pro-inflammatory response upon Tie2 activation in GM-CSF differentiated macrophages (chapter 2), previous studies showed that Ang-2-dependent activation of TEMs induce a more M2-like gene profile with upregulation of cathepsin B, MMP-9, IL-10 and enhanced expansion of regulatory T cells\textsuperscript{45,46}. In contrast, we observed strong pro-inflammatory responses to both Ang-1 and Ang-2 by IFNγ-differentiated macrophages, which was observed only in synergism with TNF. More specifically we found an upregulation of genes encoding CXCL-3/-6/-8 chemokines,IL-6, and IL-12β. In both IL-10- and IFN-γ-differentiated macrophages, Ang-1 and Ang-2 cooperated with TNF to induce IL-6 production. On the other hand in IL-10 macrophages, we found slightly opposite functional effects of Ang-1 and Ang-2. While Ang-1 enhanced IL-10 production, Ang-2 suppressed both IL-10 and TSP-2 production. In both types of macrophages, Ang-1, and to a lesser extent Ang-2, enhanced TNF-dependent activation of ERK, p38, NF-kB and PKB signaling pathways. Independently of macrophage polarization conditions, Ang-1 and Ang-2 cooperated with TNF to induce chemokine secretion profiles which were capable of recruiting monocytes in vitro. IFN-γ-differentiated macrophages were most responsiveness to stimulation with Ang-1 and Ang-2. This could be due to intrinsic signalling capacities of these macrophages, or perhaps because these macrophages express the highest relative ratios of Tie2 to inhibitory Tie1 compared to other macrophage subsets\textsuperscript{47}. Overall, we can conclude from these studies that although macrophage polarization conditions can regulate Tie2 expression, Tie2 expression is not restricted to pro-inflammatory M1 nor immunoregulatory M2 macrophages. When expressed and activated, macrophage Tie2 signaling can cooperate with TNF to promote pro-inflammatory gene expression, again independently of macrophage polarization conditions.

Possible clinical significance of targeting Tie2 signaling in vivo

Numerous studies have shown that interference with Tie2 signaling potently inhibits pathology and angiogenesis in cancer and in IMIDs\textsuperscript{13,48-51}. Remarkably, depletion of TEMs even completely prevented the angiogenic phase of human gliomas in the mouse brain and caused widespread tumor necrosis and regression\textsuperscript{13}. Thus, in this setting, myeloid Tie2 expression may play a requisite immunosuppressive role. On the other hand, inducible overexpression of Tie2 in mice causes a psoriatic-like phenotype\textsuperscript{52}. Additionally, Ang-2 transgenic overexpression increases susceptibility to inflammatory reactions in
models of contact hypersensitivity and peritonitis\textsuperscript{53}. In vivo, continuous expression of Ang-2 enhances the mobilization and recruitment of myeloid cells\textsuperscript{54}. Moreover, Ang-2 neutralization in our study prevented pathology and bone destruction in CIA. The close correlation between angiogenesis and inflammation suggests that angiogenic factors such as Ang-1, Ang-2 and Tie2 trigger both angiogenic sprouting and the recruitment of inflammatory cells. In this case, targeting Ang-1 and Ang-2 might have the dual effect of dampening two processes which are crucial for the development and maintenance of many IMIDs. Before translating these observations into clinical application, a few remaining issues need to be addressed. First of all the question is, which angiopoietin should be targeted. We and others\textsuperscript{55-57} identified Ang-2 as a potential therapeutic candidate in cancer and in IMIDs. Further data supporting Ang-2 treatment in IMIDs is that Ang-2 levels are more than 10-fold increased compared to Ang-1 in RA synovium versus controls\textsuperscript{58}. Also, high levels of Ang-2, and not of Ang-1, strongly correlates with disease severity in active systemic lupus erythematosus patients\textsuperscript{59,60}. However, although our data indicates that targeting Ang-2 is protective in animal models of RA, similar studies have yet to be attempted neutralizing Ang-1. Further work is also needed to discern the relative effects of Tie2 signaling by myeloid and stromal (endothelial) cells in vivo.
SECTION II: The role of T-cell Rap1 GTPase in immune-mediated inflammatory diseases

Rap1 GTPases and their role in T-cell function

In chapter 5 and 6 we examined the role of constitutive Rap1 activation in T-cells in murine models of RA and multiple sclerosis (MS). Rap1 is a member of the Ras family of the small GTPases, which are small cytosolic proteins that couple extracellular stimuli to diverse cellular responses. T cell Rap1 is activated by signaling via the T cell receptor (TCR), chemokines and several adhesion proteins. Recently, a new membrane-bound protein, repulsive guidance molecule-a (RGM-a) has been identified to activate Rap1 in T-lymphocytes and was found to be involved in the pathogenesis of experimental autoimmune encephalitis (EAE), a murine model of MS. Stimulation of the TCR results in the activation of guanine nucleotide exchange factors (GEFs), which promote accumulation of Rap1 in an active GTP-bound form, while RapGAP1 activated by the T cell costimulatory protein CD28, catalyzes return of Rap1 to an inactive GDP-bound form. Rap1 inactivation by CD28 can be circumvented by CTLA4 ligation or interference with CD28 activation using soluble CTLA4 (Abatacept treatment). Active Rap1 plays a dominant role in the control of cell–cell and cell–matrix interactions by regulating the function of integrins and other adhesion molecules which are needed for T cell trafficking and adhesion to antigen-presenting cells. Moreover in certain conditions, Rap1 suppresses TCR-dependent ERK activation and IL-2 production, directly via blockade of Raf kinase activation or indirectly via diminishing TCR-dependent reactive oxygen species (ROS) production. A potential role for Rap1 in RA is suggested by the fact that Rap1 is inactivated in RA SF T cells. Indirect evidence of Rap1 inactivation is observed in RA synovial fluid T cells, which following TCR triggering, produce high ROS levels and T cell cytokine production. Mice with decreased T cell Rap1 function, by RapGAPI transgenic overexpression, lead to an accumulation of CD69high T-cells possibly reflecting an autoreactive phenotype. On the other hand activation of Rap1, either in mice deficient for the RapGAP Spa-1, or in mice overexpressing active Rap1 in the T cell compartment (Rap1E63 mice), leads to decreased T-cell proliferation and cytokine production both upon TCR-ligation and recall antigen challenge. Altogether, the studies described above suggest that maintaining T cell Rap1 function might be an interesting therapeutic approach in autoimmune diseases.

Sustained T-cell Rap1 signaling in RA

In chapter 5 we studied whether constitutively active Rap1 function in T cells influences inflammation and joint destruction in murine collagen-induced arthritis (CIA), using transgenic mice expressing the hypermorphic mutant of Rap1a (RapV12). We chose RapV12-transgenic mice because RapV12 levels in these T cells are equivalent.
to endogenous Rap1, RapV12 transgenic mice have no obvious alterations in T cell homeostasis, and finally, RapV12 can still cycle between active and inactive states, although at a very reduced rate. We show that, compared to wild type (WT) mice, disease incidence, inflammation, and cartilage and bone damage were significantly reduced in RapV12 mice. The protective effects of T cell Rap1 activation in CIA were not due to alterations in T cell homeostasis, T cell anergy or unresponsiveness as, consistent with previous studies, we didn’t observe changes in proinflammatory T cell cytokine production in healthy RapV12 versus WT mice. However, in vivo we observed a selective defect in TNF production by CD8+ T cells in RapV12 transgenic mice during the onset of arthritis, whereas other cytokines such as IL-2, IFN-γ, IL-18 and IL-10 were not affected. A second and interesting observation of this study was that Rap1 activity regulated the expression of the T cell costimulatory surface molecules inducible costimulator (ICOS) and CD40L. We observed a failure to upregulate these costimulatory proteins in T cells of RapV12 transgenic mice. Together with a reduced anti-collagen antibody production in vivo, this points to a defect in the generation of follicular T cell help needed to promote B cell immunoglobulin class switching. The suppression of T cell CD40L expression by active Rap1 may be clinically relevant as CD40L is frequently expressed by RA synovial T cells and importantly, anti-CD40L treatment prevents disease development in CIA. ICOS expression is also thought to contribute to the pathogenic T cell behavior in RA. ICOS expression is elevated in RA synovial T cells compared to disease controls and ICOS is required for optimal T cell activation and TNF production. In line with this, the defective TNF production in CD8+ T cells in RapV12-transgenic mice, could be a direct result of decreased ICOS expression. Another potential mechanism underlying the specific loss of CD8+ producing T cells in RapV12 transgenic mice during CIA is that a specific auto-reactive T cell population is being deleted or inactivated in these mice. It has been shown that under highly inflammatory conditions, RapV12-mediated integrin activation may prolong T cell contact with antigen-presenting cells (APCs), leading to activation-induced cell death (AICD). An alternative explanation is that this might be due to clonal exhaustion of autoimmune T cell clones, a phenomenon that has been observed in murine antigen-specific T cell populations during chronic viral infection. In summary, mice expressing RapV12 in the T cell compartment were protected from CIA, and this protective effect was associated with an almost complete absence of TNF production by CD8+ lymph node T cells during arthritis onset and a decrease in anti-collagen antibody production. Both these processes could be due to a diminished expression of the costimulatory molecules ICOS and CD40L in CD4+ and CD8+ T cells upon TCR/CD28 triggering of RapV12 transgenic mice. It remains to be determined whether RapV12 T cells also fail to induce ICOS and CD40L expression during CIA in vivo.
Sustained T-cell Rap1 signaling in an animal model of MS

To determine whether the effect of RapV12 to protect against autoimmune disease is specific for CIA or may be a more general effect of immune-mediated inflammatory diseases (IMID), we assessed the effect of T-cell RapV12 mutant expression in myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE). In this experimental setup, using 2D2 TCR and 2D2RapV12 double transgenic mouse, 85% of the total T cell pools are autoreactive T cells, which can be tracked and studied by their TCRs which are specific for the immunodominant epitope MOG\textsuperscript{86}. In chapter 6 we examined the qualitative and quantitative effects of sustained Rap1 in the autoreactive T cell population in EAE. Somewhat similar to the observations in CIA, we found that the autoreactive T cells in the double transgenic 2D2RapV12 animals are not anergic but rather hyperresponsive. We observed a proliferative advantage, increased IL-2 production and upregulation of ICOS in splenocytes of 2D2RapV12 mice during primary and secondary responses to MOG. Moreover, pro-inflammatory T cell cytokine production (TNF, IFN\textgamma and IL-17) in mice with sustained T cell Rap1 activation was also not affected. While T cell differentiation under homeostatic conditions was similar between both mice strains, an increased accumulation of effector and central memory, and a reciprocal decrease in naïve autoreactive T cells was observed in response to MOG priming in 2D2RapV12 mice. This increased MOG-induced proliferation of autoreactive T cells in the presence of sustained Rap1 could not be attributed to AICD. The enhanced T cell proliferative and survival capacities, might be attributed to ADAP, a TCR adaptor protein and Rap1 effector, which promotes antigen-dependent T cell-APC interactions\textsuperscript{87}. Comparable results have been observed in RapV12 transgenic mice whereby positive selection of thymocytes with low antigen affinity are promoted leading to a survival advantage \textsuperscript{76}. In line with the clinical data in CIA, we observed that RapV12 attenuates EAE, although we only observed a protective effect for survival, not for disease incidence and severity. As this modest clinical effect could not be explained by changes in qualitative responses (because auto-reactive T cell proliferation, cytokine production and survival are enhanced in mice expressing RapV12), we evaluated whether Rap1 affects the size of the autoreactive T cell pool. Interestingly, the MOG-autoreactive T cell pool was reduced by approximately 30% in 2D2RapV12 animals. Because a similar reduced effect on autoreactive tetramer positive cells was also observed in RapV12 transgenic mice, this suggests that Rap1 signaling affects central tolerance\textsuperscript{88}. A possible explanation for this observed effect is that sustained Rap1 function promotes negative selection of autoreactive T cells by strengthening thymocyte-APC interactions. However, more research is needed to investigate how Rap1 exactly affects positive and negative selection of T cells, and if integrin-dependent mechanisms are involved. Overall, we can conclude that maintained Rap1 signaling suppresses T cell mediated autoimmunity. In EAE, with a great pathogenic contribution of autoreactive T cells, Rap1 affects both qualitative and quantitative responses of the autoreactive T cell pool. On the other hand,
in CIA, a model more reliant on the pathogenic behavior of proinflammatory cytokines and anti-collagen antibodies, Rap1 inhibits at least partly CD8+ T cell function and is probably also involved in T-B cell interactions. The precise molecular mechanism of how Rap1 function modulates immunological processes in both disease models is unknown and remains to be determined. But our studies underscore that the biological effects of sustained Rap1 activation are diverse, not restricted to a specific T cell subpopulation, and are of potential therapeutic interest in the treatment of IMIDs.

Future therapeutic implications for targeting Ras family GTPases

Therapeutic modulation of specific intracellular signaling pathways has been identified as interesting targets to treat IMIDs. Some of these pathways control development, proliferation and activation of proinflammatory and autoreactive T cells. Ras and Rap1 GTPases are considered as key candidates, because they are activated by chemokines and TCR ligation and defects in these signaling pathways are associated with the pathobiology of autoimmune disease. The pathogenic character of synovial T cells in RA has been proposed to depend on constitutive activation of Ras proteins by synovial inflammatory cytokines in combination with CD28-dependent Rap1 inactivation, leading to ROS production and expression of activation markers which further stimulate macrophages. Multiple therapeutic strategies to interfere with small GTPase signaling in IMIDs have been suggested. First of all, GTPase signaling can be inhibited by peptides preventing GTPase membrane localization. Examples are farnesyltransferase inhibitors (FTI), which target the protein farnesyltransferase, thereby preventing activity of downstream effector Ras protein. A few compounds targeting farnesyltransferase, such as lonafarnib and tipifarnib, are currently being investigated in clinical trials. Secondly, enhancing Rap1 activity by pharmacologic modulation of GEFs, with EPAS as the first example, improves the adhesive and migratory capacity of progenitor cell populations. However T cells don't have any EPAC. And besides GEFs, modulation of GAP catalytic activity has been proposed as well, although until now no chemical compounds have been developed yet. On the other hand targeting GAP catalytic activity indirectly, for example with abatacept (CTLA4-Ig), has gained some attention. The successful treatment of RA by abatacept might at least in part be attributed to the restoration of Rap1 signaling in synovial T cells, mediated by CD28-CTLA4 interactions with RapGAPI. Finally, GTPases have many downstream effectors such as mitogen-activated protein kinases and phosphatidylinositol 3-kinases which play crucial roles in the pro-inflammatory behavior and prolonged survival of many inflammatory cell types, and are currently being studied for therapeutic exploitation.

Therapeutic intervention: IL-12/IL-23 inhibition in RA

In chapter 7 we described the results of the first clinical study with an oral IL-12/IL-23 inhibitor in the treatment of active RA. In this phase IIa, randomized, double-
blind, placebo-controlled clinical trial 29 patients with active RA were treated with either apilimod mesylate or placebo, in combination with methotrexate (3:1 ratio of apilimod- to placebo-treated patients). Apilimod mesylate is an orally-administered small molecule which inhibits IL-12/23 production at the transcriptional level by blocking the nuclear translocation of a transcription factor, c-Rel. Patients were treated with apilimod 100mg/day (or placebo) for a period of 4 weeks (stage 1, n=12) or 8 weeks (stage 2, n=10); in stage 3, patients (n=7) were treated with apilimod 200mg/day (or placebo) with an optional extension to 12 weeks. The final outcomes were safety, tolerability, pharmacokinetics, and efficacy of apilimod treatment. Apilimod was safe and well tolerated at a dosage of 100mg/day; only mild adverse events were observed. However, when administered in 200mg/day all patients experienced headache and/or nausea. Overall, treatment with apilimod did not induce robust clinical improvement. Among apilimod-treated patients (100 mg/day), there was a small, but significant reduction in the Disease Activity Score in 28 joints (DAS28), moreover an American College of Rheumatology 20% improvement (ACR20) response was reached in only 1 out of 17 patients after 4 weeks and 2 out of 8 patients after 8 weeks treatment. Consistent with the clinical results, apilimod did not have an effect on the expression of CD68+ synovial macrophages, which previously was shown to be a sensitive biomarker for clinical response92,93.

There are several possible reasons why treatment with apilimod did not lead to clinical improvement in RA patients. First, because we didn’t observe a reduction in IL-12/IL-23 expression in the synovial tissue, our analyses suggests that insufficient levels of apilimod reach the site of inflammation to mediate a potential biologic effect. Second, the number of patients in this phase IIa study is relatively small to determine a “true” clinical benefit of apilimod. However, previous studies with similar study design92,94,95 indicate that our study size should have been sufficient to detect a relevant, robust change in the synovium after effective treatment. A third explanation for the lack of efficacy of apilimod could be the short treatment duration, although we previously showed that early changes in the synovium preceeds clinical improvement during effective antirheumatic treatment94,96,97. And finally, we need to take into consideration that IL-12/IL-23 are not good targets for the treatment of RA. Recent studies have indicated that IL-23 rather than IL-12 is the critical cytokine in mediating chronic inflammation. IL-23 rather than IL-12 proved to be essential for the development of experimental autoimmune disease in the joints98 and central nervous system99,100. Remarkably, IL-12-deficient p35−/− mice were more susceptible to CIA with 80% disease incidence compared to wild-type animals, while IL-23 gene-targeted mice did not develop clinical signs of disease and were completely resistant to the development of joint and bone pathology98. Disease resistance was correlated with an absence of IL-17-producing CD4+ T cells. On the other hand, IL-12-deficient p35−/− mice developed more IL-17-producing CD4+ T cells, as well
as elevated mRNA expression of proinflammatory TNF, IL-1β, IL-6, and IL-17 in affected tissues of diseased mice. Abovementioned studies, suggest that IL-12 is not critical for disease development and may even play an undefined protective role by suppressing IL-17 secretion. Finally, genetic association studies suggest that, in contrast to other IMIDs, IL-12 and IL-23 don’t play an important role in the pathogenesis of RA. SNPs coding for IL-12B and IL-23R have been identified as candidate genes associated with susceptibility for psoriasis\(^{101}\) and psoriatic arthritis\(^{102}\), while only the minor alleles of the IL23R polymorphisms (and not of IL-12B) were associated with increased predisposition to RA\(^ {103}\) and this association was modest compared to the association found in Crohn’s disease\(^ {104}\).

**Future implications of IL-12/IL-23 treatment**

While we and others\(^ {105}\) did not detect a clinical effect of the small molecule apilimod, targeting IL-12/IL-23 with monoclonal antibodies, has been shown to provide significant clinical benefit in immune-mediate inflammatory disease. Two human anti-p40 antibodies have been used therapeutically to date, ustekinumab and briakinumab. A very recent phase 2b clinical trial in Crohn’s disease\(^ {106}\) demonstrated that in patients who had an inadequate response to TNF antagonists, ustekinumab significantly increased response rate and remission. Moreover, ustekinumab showed to be effective in clearing psoriasis\(^ {107-109}\) and has been approved for moderate and severe plaque psoriasis in the United States, Canada and Europe. Other IL-23 pathway inhibitors in the pipeline include anti-p19 monoclonal antibody which interfere with IL-23 activity, as well as secukinumab, LY-2439821, and AMG-827, which exhibit their activity against IL-17 pathway.

**To conclude**, the first part of this thesis shows that interference with Tie2 signaling might have good therapeutic potential in the treatment of RA. However, further analysis of the unique contributions of macrophage Tie2 signaling and the effects of Ang-1 blockade in RA, is needed. In the second part we demonstrate that the biological effects of sustained Rap1 activation are diverse, not restricted to a specific T cell subpopulation, and are of potential therapeutic interest in the treatment of IMIDs. The successful treatment of RA by abatacept, might at least in part be attributed to the restoration of Rap1 signaling in synovial T cells, mediated by CD28-CTLA4 interactions with RapGAPI. Nevertheless, the exact molecular mechanism of how Rap1 function modulates immunological processes remains to be determined.
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