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# INTRODUCTION

#### Rheumatoid arthritis

Rheumatoid arthritis (RA) is a complex, chronic, autoimmune disease that affects approximately 1% of the population and has a higher incidence in women<sup>1</sup>. The primary manifestations are joint pain, stiffness and swelling, and when it is not adequately treated the synovial inflammation leads to erosion and destruction of cartilage, bone and periarticular structures. The lining of the synovium, which is adjacent to the joint space and is composed of differentiated macrophages and fibroblast-like synoviocytes (FLS), attaches to the joint at the bone-cartilage junction, and forms a destructive pannus. The synovium in patients with RA is characterized by cellular hyperplasia, prominent angiogenesis and an influx of inflammatory leukocytes, mainly consisting of T and B lymphocytes, macrophages and plasma cells<sup>2</sup>. These cells contribute to the perpetuation of inflammation by producing pro-inflammatory cytokines, such as interleukin (IL)- $1\beta$  and tumor necrosis factor (TNF)- $\alpha$ , that together with secreted chemokines, promote the infiltration and activation of more leukocytes into the joints. Locally expressed degradative enzymes, including matrix metalloproteinases (MMPs), digest the extracellular matrix and destroy the articular structures. Although the mechanisms involved in the initiation of RA are still unknown, increased knowledge on the pathogenesis of this disease has implicated several cell types as key players in autoimmune inflammation.

## T lymphocytes

One of the strongest pieces of evidence for the involvement of T cells in RA is the increased frequency of expression of specific major histocompatibility complex (MHC) alleles in patients with RA. Shared epitopes present on HLA-DR1 and HLA-DR4 alleles are thought to present arthritogenic peptides to lymphocytes, which might initiate RA3. The presence of the shared epitope might also influence the severity of disease, as the risk of extra-articular and erosive disease is greater in patients that have the genes, and is further increased by homozygosity4. A number of possible auto-antigens have been identified including citrullinated proteins, heavy chain binding proteins, human cartilage glycoprotein 39 (GP39), heat shock proteins and type II collagen5. In experimental arthritis models, T cell-dependence has also been demonstrated. Animal models of arthritis, such as collagen-induced arthritis (CIA) or adjuvant arthritis, are clearly T cell dependent and transfer of CD4+ T cells from sick animals into healthy recipients is enough to trigger the development of disease,

while CD4 depletion is able to diminish inflammation<sup>6,7</sup>. T cells are also important for the production of auto-antibodies, as they provide help for B cell activation. Ligation of CD154 (CD40L) on activated T cells with CD40 on B cells stimulates the last to proliferate, produce antibodies and switch isotype<sup>8</sup>.

Recently, Th17 cells characterized by the production of the highly inflammatory IL-17 cytokine have been implicated in RA. Several studies have described the presence of high levels of IL-17 in RA synovial fluid (SF)<sup>9;10</sup> and the spontaneous production of IL-17 by RA synovial tissue (ST) T cells<sup>11;12</sup>. Also, increased numbers of Th17 cells in RA SF and ST have been found when compared to healthy donor and disease controls<sup>11;12</sup>. However, different studies have shown the predominate presence of Th1, rather than Th17, cells in inflamed joints, raising the possibility that other cells in the synovium are responsible for local IL-17 production<sup>13;14</sup>.

The hypothesis that RA synovial T cells have defective TCR signaling cascades is still controversial. Synovial T cells are highly differentiated CD45RO+ T cells, and present markers of recent activation such as CD69 and HLA-DR<sup>15;16</sup>. However, chronic exposure to cytokines and/or oxidative stress has been proposed to downregulate TCRzeta expression<sup>17-19</sup> and displace linker for activation of T cells (LAT) from the plasma membrane<sup>20</sup>. In this case, T cells would no longer be able to respond to TCR ligation and would contribute to inflammation by TCR-independent mechanisms. In normal immune responses, regulatory T cells (Tregs) are important for the suppression of abnormal and excessive inflammation. However in RA, studies have showed that defective Treg function and/or resistance of effector RAT cells to Treg-mediated suppression may contribute to the persistence of chronic inflammation<sup>21;22</sup>.

Besides specific antigen-driven activation, T cells may also contribute to synovial inflammation via cell-contact interactions with neighboring macrophages, FLS and B cells, thereby promoting their activation. Activated T cells are able to induce macrophage TNF- $\alpha$ , IL-1 $\beta$  and MMP production via cell-cell contacts<sup>23-26</sup> and interaction with FLS induces IL-6, IL-8 and MMP-1 production<sup>27;28</sup>.

## **B** lymphocytes

The first clue that B cells may play an important role in autoimmunity came from the identification of antibodies specific for IgG in the blood of RA patients, known as rheumatoid factors (RF). Up to 75% of RA patients are seropositive for RF, and its presence predicts a more aggressive and destructive course of disease<sup>29</sup>. Anti-citrulinated protein antibodies (ACPA) have also been identified, and these can be detec-

ted years before clinically onset of inflammatory arthritis. ACPA are directed against epitopes in which the L-arginine amino acid has been postranslationally modified to L-citruline30. While RF can also be detected in other autoimmune diseases, systemic infections and even 5% of healthy individuals, the presence of ACPA is very specific to RA, so its detection is an important diagnostic tool in RA<sup>29</sup>.

In the synovium, lymphoid neogenesis can be found in one third of RA patients<sup>31</sup>. Up to 20% of these patients display lymphoid aggregates with germinal centers (GC)<sup>32</sup>. Although the presence of synovial lymphoid neogenesis is associated with the degree of synovial inflammation, it does not support antigen-driven clonal B cell expansion or antibody affinity maturation<sup>33;34</sup>. Additionally, no relation is found between synovial lymphoid neogenesis and the levels of RF or ACPA, so the relevance of these organized structures in the synovium to the severity of clinical signs and symptoms is uncertain.

Besides auto-antibody producers, it is thought that B cells in the joints play an important role in antigen presentation, as B cell depletion prevents the formation of ectopic germinal centers and impairs T cell activation<sup>35</sup>. It is therefore not surprising that B cell directed therapies, such as the anti-CD20 monoclonal antibody Rituximab, has shown promising results. This antibody depletes all B cell subsets, except plasma cells, which lack CD20 expression. Besides a possible role in diminishing antigen presentation, Rituximab may exert its effects by an indirect effect on antibody producing plasma cells as well<sup>36</sup>. Since B cells are precursors of plasma cells, B cell depletion may indirectly result in a decrease in numbers of the short-lived plasma cells. In agreement, treatment with anti-CD20 monoclonal antibody reduces the levels of serum RF and ACPA, inducing significant clinical benefit in patients with RA<sup>36,37</sup>.

## Macrophages

Macrophages are important players in joint inflammation. They are present in high numbers in the inflamed synovium and display signs of cellular activation, such as expression of MHC class II molecules. They are also important sources of proinflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , as well as chemokines and MMPs<sup>38</sup>. Moreover, a positive correlation has been found between the degree of synovial macrophage infiltration, TNF- $\alpha$  expression and clinical features, such as radiological progression of joint destruction<sup>39,40</sup>.

In the synovium, macrophages are important components in the recruitment and

activation of inflammatory cells. They produce chemokines that will attract other cells to the joint such as T and B cells, neutrophils and other macrophages, and secrete cytokines that will activate these newly arrived cells<sup>41</sup>. Additionally, cellular contacts between macrophages and T cells, FLS, and endothelial cells, constitute an important component of macrophage effector responses, even in the absence of antigen. Cell-cell contacts between macrophages and FLS elicits the production of IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-8<sup>42</sup>, while interaction with activated T cells can induce TNF- $\alpha$ , IL-1 $\beta$  and MMP production<sup>23-26</sup>. Importantly, ST macrophages are used as biomarkers of clinical response in RA clinical trials, as their numbers decrease in response to successful anti-rheumatic treatment<sup>43,44</sup>.

## Fibroblast-like Synoviocytes

In normal synovium, fibroblast-like synoviocytes (FLS) are mesenchymal cells that produce extracellular matrix and secrete hyaluronan and lubricin, key components of synovial fluid and important for joint lubrication<sup>45</sup>. However in RA, FLS in the synovial lining layer display numerous features of cellular activation that ultimately contribute to their aggressive and invasive behavior. In the lining, layer FLS increase in number, thickening the synovial lining layer into a hyperplastic tissue. In vitro, they grow in an anchorage-independent manner and lack contact inhibition<sup>46</sup>. Activated FLS contribute to the degradation of extracellular matrix. They attach to cartilage and release matrix degrading enzymes, particularly MMPs, allowing them to deeply invade the extracellular matrix<sup>45</sup>. Both MMP-1 and MMP-3 are elevated in the synovial fluid and serum of RA patients, and their production has been mainly attributed to FLS<sup>47;48</sup>.

In vitro studies have showed the capacity of RA FLS to divide more rapidly than cells from normal or osteoarthritic joints<sup>49</sup>. Different mechanisms have been proposed to explain the hyperplastic growth of FLS in RA synovium. Exposure to cytokines and growth factors in the synovium are thought to play a crucial role in this process. Furthermore, FLS have a high expression of transcription factors and molecules that regulate cell cycle, which might be involved in their increased proliferation. Proto-oncogene products of ras, myc, and others can be found abundantly in FLS, especially at sites of invasion into cartilage and bone<sup>50</sup>. Another mechanism that may underlie synovial hyperplasia is a decline of cells undergoing apoptosis. In this context, high levels of phosphorylated protein kinase B (PKB, also known

as Akt), are found in RA FLS51. PKB is an important protein in the phosphatidy-linositol 3-kinase (PI3K) signaling cascade, which in turn regulates processes such as cell growth, differentiation, survival and proliferation<sup>52</sup>. The fact that PKB levels can be further increased by TNF- $\alpha$  stimulation, together with the findings that RA synovium lacks expression of the tumor suppressor PTEN (PI3K inhibitor) at sites of invasive FLS growth<sup>53</sup>, might explain in part the impaired apoptosis associated with the proliferating synovium in RA.

Clearly, there are many pieces of evidence in RA pointing to a de-regulation of intracellular signaling pathways involved in inflammation, cell proliferation and survival. Targeting these pathways and restoring normal cellular behavior seems therefore an elegant and promising manner of reducing cellular inflammation and joint destruction. To accomplish this, a detailed examination of how signaling pathways might be de-regulated in RA is necessary.

# Small GTPases: Key regulators of cellular functions

The Ras superfamily of small GTPases constitutes a large group of structurally and functionally related proteins. They are important components of signal transduction pathways used by antigen receptors, costimulatory, cytokine and chemokine receptors to regulate the immune response. Small GTPases control fundamental biological processes including cell division, differentiation, shape changes, and survival. The Ras superfamily of proteins is divided into five major subfamilies on the basis of their sequence and functional similarities: Ras, Rho, Rab, Ran and Arf<sup>54</sup>.

Despite a high level of homology, members of the Ras GTPase superfamily display major differences in their signaling specificity. These functional differences can be explained in part by their different cellular localization, which is mainly determined by the hypervariable domain at their C-termini and specific effector domains, which bind to and regulate downstream signaling proteins<sup>55,56</sup>.

### Regulation of GTPase activation

Small GTPases are highly conserved throughout all eukaryotes, and their activity is regulated by common biochemical mechanisms. All Ras-related proteins cycle between an inactive guanosine diphosphate (GDP) and an active guanosine trip-

hosphate (GTP) bound form, operating as binary switches that control cell activation in response to environmental cues<sup>57</sup>. GTPases adopt different conformations when binding GTP vs. GDP. The active GTP-bound conformation allows GTPases to interact with downstream effectors and thereby initiate downstream signaling pathways, which regulate many important biological processes. Two main classes of regulatory proteins control this cycle: guanine nucleotide exchange factors (GEFs) promote the exchange of GDP for GTP, and GTPase-activating proteins (GAPs) stimulate the otherwise slow intrinsic GTPase activity, promoting the formation of the inactive GDP-bound configuration. Canonical mutations that affect the GTPase cycle lead to constitutively- active or dominant-negative molecules<sup>58</sup>. Mutations that abolish GT-Pase activity (e.g., glycine 12 to valine; G12V), such as RasV12 or RapV12, result in constitutive activation of the small GTPase, and mutations that affect interaction of the GTPase with its GEFs and effectors (e.g., threonine 17 to asparagine; T17N) result in dominant-negative molecules. Both activated and dominant-negative GTPases can dominantly perturb cellular processes in which the GTPases are involved.

#### The Ras subfamily of small GTPases

Members of the subfamily of Ras GTPases include the Ras proteins (H-, K-, and N-Ras), R-Ras, M-Ras, Rap, Ral and Rheb proteins. These have been recognized for their involvement in signal transduction cascades that regulate cell growth, proliferation, differentiation and survival, primarily through the modulation of gene expression<sup>59</sup>.

#### Ras GTPases

The Ras proteins are ubiquitously expressed 21-kDa proteins. The three homologues of Ras, H-Ras, K-Ras, and N-Ras, share a high degree of sequence homology (>85%), especially in the effector domain that directly couples them to downstream signaling proteins<sup>60</sup>. As all members of the Ras GTPase family, these proteins are regulated by GEFs and GAPs. The selectivity of GEFs in activating distinct Ras homologues, as well as the differential coupling of activating GEFs, such as Son-of-sevenless (Sos) and Ras guanine nucleotide-releasing factor (RasGRF) 1 to tyrosine kinase-dependent and G protein-coupled receptors, respectively, contributes to the signaling specificity of each Ras homologue. All three homologues are important

in activating intracellular downstream pathways such as mitogen-activated protein kinase (MAPK) cascades, PI3K and Ral family GTPases. However, genetic and detailed cell biology studies were able to demonstrate that differential subcellular localization of each Ras homologue confers to them distinct signaling properties<sup>61,62</sup>. Biochemical studies have demonstrated the different specificity of the three Ras homologues in activating downstream signaling pathways. While K-Ras was shown to be a more potent activator of Raf-1 and the downstream MAPK cascade than H-Ras, H-Ras seemed to be more efficient in activating PI3K<sup>63</sup>. Furthermore, targeting experiments have showed that whereas h-ras and n-ras single or double knock-out mice are completely viable, targeted disruption of the k-ras gene leads to lethality<sup>64-66</sup>. In addition, different ras genes are found mutated in different types of tumors.

#### Ras GTPase effectors and signaling

In the active GTP-bound conformation, Ras GTPases can activate a large panel of downstream effectors in response to diverse extracellular stimuli. Members of the Raf family, the PI3K and members of a family of exchange factors for the small GTPase Ral, e.g., RalGDS, have been established as Ras effectors.

One of the best studied effectors of Ras is the serine-threonine kinase Raf. Raf is involved in a signaling pathway where activation of the MAPK cascade culminates in the regulation of cell proliferation. In this signaling pathway, active Ras binds to and promotes the translocation of Raf to the plasma membrane, where additional phosphorylation events promote full Raf kinase activation<sup>67</sup>. Once active, Raf phosphorylates and activates MEK (MAPK/Erk kinase), a dual specificity tyrosine-threonine kinase, which in turn phosphorylates and activates the Erk1/2 MAPK. Activated ERK translocates to the nucleus, where it phosphorylates transcription factors involved in cell proliferation and differentiation<sup>59</sup>. In this way, signals arising from an extracellular growth factor or cytokine are transmitted from the cell surface to the nucleus, ultimately changing the activity of nuclear transcription factors.

#### Ras GTPases and tumors

Ras proteins have been the subject of intense research, partly because of their critical roles in human oncogenesis. A high frequency of ras mutations has been detected in a variety of tumors. The commonly occurring mutations (at codons 12, 13 and 61)

make the GTPase insensitive to the action of GAPs and thereby lock it in the GTP-bound, active state68. The highest incidence of ras mutations is found in pancreatic adenocarcinomas where almost 90% of the tumors are associated with a mutation in kras. Also, in 50% of colon, lung and thyroid tumors, mutations in ras genes have been observed<sup>68</sup>.

#### Ras GTPases and rheumatoid arthritis

In RA synovial tissue, abundant expression of Ras proteins can be found, predominantly in synovial lining cells attached to cartilage and bone at the site of joint destruction<sup>50</sup>. In systemic lupus erythematosus (SLE) T cells, de-regulated Ras expression has also been observed. In a subset of patients, Ras expression and function is reduced, while mice defficient for the Ras GEF Ras guanine nucleotide-releasing protein 1 (Ras GRP1) develop a spontaneous SLE-like disease<sup>69-71</sup>.

In arthritic synovium, point mutations in h-ras were initially described72. However, when assessing higher number of samples in later studies, no activating mutations could be found<sup>73</sup>. However, the relevance of Ras signaling pathways in synovial inflammation has been underscored by several studies. Activation of Ras effector pathways, including MAPK, PI3K, and nuclear factor-kappa B (NF-kB), is enhanced in RA compared to disease controls<sup>51,74,75</sup>. In vitro, over-expression of dominant-negative Ras has been shown to suppress RA FLS proliferation, IL-6 production and IL-1-induced ERK activation<sup>76</sup>. Moreover, RA FLS stably expressing a dominant negative version of c-Raf, which can bind to and interfere with signaling of Ras GTPases, have reduced MMP-1 and MMP-3 production<sup>77</sup>. In these cells c-Raf inhibition also decreases ERK and JNK phosphorylation as well as FLS invasiveness. Importantly, inhibition of Ras family function in vivo has been shown to be protective in experimental arthritis models<sup>76-78</sup>.

## Rap1 GTPase

In mammals there are two isoforms of Rap1, Rap1a and Rap1b, which are encoded by distinct genes but share 95% amino acid identity<sup>79</sup>. In T cells, Rap1 is transiently activated upon TCR ligation<sup>80</sup>, and like other GTP-binding proteins its activation is dependent on the action of GEFs. C3G, PDZ-GEF, exchange protein directly activated by cyclic AMP (EPAC) and calcium diacylglycerol regulated guanine nucleotide

exchange factor (CalDAG-GEF, RasGRP) are some of the GEFs identified for Rap1<sup>79</sup>. Two groups of GAPs regulate inactivation of Rap1, the RapGAP and the Spa1 families<sup>81</sup>. Members of the RapGAP family include RapGAP1A, RapGAP1B and RapGAP2. The Spa1 family of GAPs, consists of Spa1 and E6TP1.

#### Rap1 and the regulation of cell adhesion

One of the best characterized functions of Rap1 is the regulation of cell adhesion. Integrin activation<sup>82</sup>, cadherin-mediated adhesion<sup>83</sup> and cell-cell junction formation<sup>84</sup> are adhesion processes regulated by Rap1. Several studies have investigated the mechanisms by which Rap1 controls integrin activation. In its active state, bound to GTP, Rap1 can associate with RAPL (regulator of adhesion and cell polarization enriched in lymphoid tissues)85, which in turn, associates with and activates the serine-threonine kinase Mst1 (mammalian sterile twenty-like-1)86. This allows the spatial distribution of LFA1 to the leading edge, or to the immunological synapse, and integrin clustering85. Other Rap1 effectors include PKD1 (protein kinase D1)87 and RIAM (Rap1-interacting adaptor molecule)88. Association of active Rap1 with these effectors has also been demonstrated to induce cellular adhesion. In vitro studies have demonstrated that afadin (AF-6) can also act downstream of Rap1 activation, inhibiting endocytosis of E-cadherin, and allowing the maintenance of cellular junctions89. Many studies have tried to unravel the mechanism of regulation of integrins by Rap1, but it is not clear yet whether RAPL, PDK and RIAM control separate pathways that are required for Rap1-induced integrin activation, or whether they participate in the same pathway that leads to Mst1 function, and ultimately to integrin clustering.

Rap1 regulation of integrin activation has important consequences in the immune system, as effective T cell responses are critically dependent on appropriate T cell-antigen presenting cell (APC) interactions. The formation of a stable immunological synapse requires proper integrin activation. Upon TCR triggering Rap1 becomes rapidly activated and induces conformational changes in integrin structure, which increases their avidity (clustering) as well as ligand affinity, thereby potentiating T cell-APC interactions<sup>90,91</sup>. Ligation of chemokine receptors is also able to activate Rap1, which in turn enhances integrin function. This allows T cell trafficking and migration to lymphoid organs and sites of inflammation<sup>91,92</sup>.

#### Rap1 and the regulation of ROS production

In T lymphocytes, Rap1 activation is able to suppress Ras-dependent reactive oxygen species (ROS) production. ROS are proposed to act as important second messengers in T cell activation<sup>93</sup>. TCR triggering results in transient ROS production, and scavenging intracellular ROS with antioxidants has been shown to suppress TCR-induced NF-kB, AP-1 and IL-2 promoter transcription<sup>94</sup>. On the other hand, chronic oxidative stress can lead to constitutive activation of NF-kB-dependent inflammatory gene products<sup>95</sup>.

The intracellular production of ROS involves the activation of the small GTPase Ras and its downstream target Ral<sup>96</sup>. Simultaneous activation of Rap1 is able to attenuate ROS production, distally from Ral, and in a PI3K-dependent manner. When both Ras and Rap1 are transiently activated, limited ROS production is used as a second messenger, optimizing Ras-dependent activation of ERKs and transcription factors.

# Rap1 and rheumatoid arthritis

In a number of human autoimmune diseases, including RA97, multiple sclerosis (MS)<sup>98</sup> and SLE<sup>99</sup>, chronic oxidative stress triggered by ROS is thought to underlie the pathogenic T cell behavior. In RA, destructive proliferative synovitis has been related to oxidative stress<sup>100;101</sup>. In SF T lymphocytes, chronic oxidative stress derived from increased intracellular ROS production may induce constitutive activation of NF-kB-dependent gene transcription. This results in the upregulation of pro-inflammatory cytokines, which will contribute to the perpetuation of synovial joint inflammation<sup>102</sup>.

In T cells from the SF of RA patients, a high rate of endogenous ROS production correlates with a constitutive activation of Ras and an inhibition of Rap1 activation. In vitro experiments have showed that a restored redox balance could be achieved in RA SF T cells by introduction of a dominant-negative form of Ras%, indicating that deregulated Ras and Rap1 signaling underlies the chronic oxidative stress observed in RA SF T cells.

## Function of Rap1 in vivo

Genetic manipulation of Rap1 signaling has been of great importance to under-

stand the role of Rap1 in the regulation of the immune system. As mentioned before, Rap1 is an important mediator of integrin activation, and this is confirmed in Rap1 knockout mice, where T cell polarization and integrin-dependent adhesion is impaired  $^{103;104}$ . Previous in vitro studies have also suggested that Rap1 regulates positive and negative thymocyte selection  $^{105}$  and in agreement, transgenic expression of Spa1 renders animals with a defect in  $\alpha/\beta$  thymocyte development at the double negative stage  $^{106}$ .

An accumulating amount of evidence suggests Rap1 as a critical mediator of T cell responses. RapGAP1 transgenic mice display an age-dependent accumulation of activated T cells107. On the contrary, mice lacking Spa1 expression exhibit age-dependent defects in T cell responses B cell responses are also diminished in these animals due to reduced T helper cell function. Similarly, transgenic mice expressing the active Rap1E63 mutant show decreases in T helper and effector cell functions, although LFA-mediated adhesion is increased 109. In these mice the defective responses correlate with increased numbers and function of CD4+CD103+ regulatory T cells. Finally, RapV12 transgenic mice, expressing constitutive active Rap1 in the T cell compartment, show that increased Rap1 -dependent adhesion can enhance T cell function in conditions where TCR-MHC interactions are of low affinity 110.

## The Rho subfamily of small GTPases

Like Ras, Ras homologous (Rho) proteins also serve as key regulators of extracellular-stimulus-mediated signaling networks that regulate actin organization, cell cycle progression and gene expression<sup>111</sup>. Most studies on this family have been focused on three of the 22 mammalian Rho GTPases: Rac1, RhoA and Cdc42.

Rho GTPases have been shown to control cellular motility and polarity in migrating cells by regulating actin and myosin organization. In this context, RhoA was shown to promote actin stress fiber formation and focal adhesion assembly, Cdc42 actin microspikes and filopodium formation and Rac1 lamellipodium formation and membrane ruffling<sup>111</sup>. In this thesis we will focus on one of the Rho GTPases: Rac1.

#### Rac1 GTPase

Rac1 is a key protein in the regulation of cell migration, as well as in the adhesion of cells to the underlying protein matrix, or to other cells. It is ubiquitously expressed

and found activated at the leading edge of migrating cells<sup>112</sup>. Rac1 is activated by the action of distinct GEFs including Vav-1, Tiam1 and  $\beta$ -Pix<sup>113-115</sup>. Once bound to GTP, Rac1 can interact with target effector proteins, such as Pak and PI3K, and regulate several signaling pathways including JNK, p38, NF- $\kappa$ B and PKB, in different cell types<sup>116-120</sup>.

Genetic studies in mice have showed that rac1 deletion provokes embryonic lethality<sup>121</sup>, so conditional gene disruption has been used to unravel the role of Rac1 in different cell types. Rac1 is important for the optimal reconstitution of the hematopoietic system, having roles both in the engraftment and retention of hematopoietic stem cells (HSCs) in the bone marrow. This GTPase is essential for the entry of HSCs into cell cycle upon extracellular stimulation, as well as for their progression through S and G2/M phases<sup>122</sup>. In neutrophils, deficiency for Rac1 makes them defective in inflammatory recruitment in vivo, migration to chemotactic stimuli, and chemoattractant-mediated actin assembly 123. Upon monocyte activation, Rac1 is used to assemble the activated NADPH oxidase complex<sup>124</sup>, and its deficiency in macrophages leads to defects in cell spreading and membrane ruffling<sup>125</sup>. Rac1 activation is also of great importance in the formation of immunological synapses. Dendritic cells lacking expression of both Rac1 and Rac2 show defective cytoskeletal re-arrangements, migration and antigen presentation that, as a result, prevent adequate T cell priming<sup>126</sup>. Finally in B cells, Rac1, together with Rac2, plays an important role in BCR-induced activation, transducing BCR signals that control survival and cell cycle entry<sup>127</sup>.

Many of the pathways regulated by Rac1 seem to be involved in the inflammatory process that occurs in RA. In agreement, in vitro inhibition of Rac1 signaling is able to reduce FLS proliferation, invasiveness and JNK activation. In the same way, Rac1 has been found to regulate both in vitro and in vivo osteoclastogenesis and bone resorption<sup>128;129</sup>.

#### Outline of this thesis

In this thesis we analyse the involvement of distinct small GTPases in RA, and investigate the consequences of their activation or inhibition in vitro and in vivo, in a murine model for arthritis.

T cells from the synovial fluid (SF) of RA patients are believed to behave in a hyporesponsive manner and signaling abnormalities that impair T cell activation have been extensively described. In chapter 2 we perform single-cell analysis of these T cells to evaluate where de-regulated small GTPase function might impair TCR-dependent responses.

Previous studies in RA SF T cells have demonstrated a constitutively block in Rap1 activation. In chapter 3 we explore the consequences of Rap1 activation in vivo in a collagen-induced arthritis model. We induce arthritis in transgenic mice expressing a constitutively active form of Rap1 within the T cell lineage, and assess susceptibility and severity of arthritis.

Chapter 4 describes how the expression of inactivators of Rap1, the RapGAPs, is regulated upon T cell activation.

In chapter 5 we examine the expression of an important Ras GEF, RasGRF1, in RA synovial tissue, and evaluate the influence of RasGRF1 expression and function on RA FLS and synovial tissue MMP and pro-inflammatory cytokine production.

In chapter 6 we investigate the in vivo effects of blocking Rac1 signaling in arthritis.

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