Integration of phosphatidylinositol 3-kinase (PI3K) activity and inflammatory signaling pathways in autoimmune disease

Linda M. Hartkamp

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Integration of phosphatidylinositol 3-kinase (PI3K) activity and inflammatory signaling pathways in autoimmune disease

Integratie van phosphatidylinositol 3-kinase (PI3K) activiteit en signaalroutes betrokken bij ontsteking in auto-immuunziekten
(met een samenvatting in het Nederlands)

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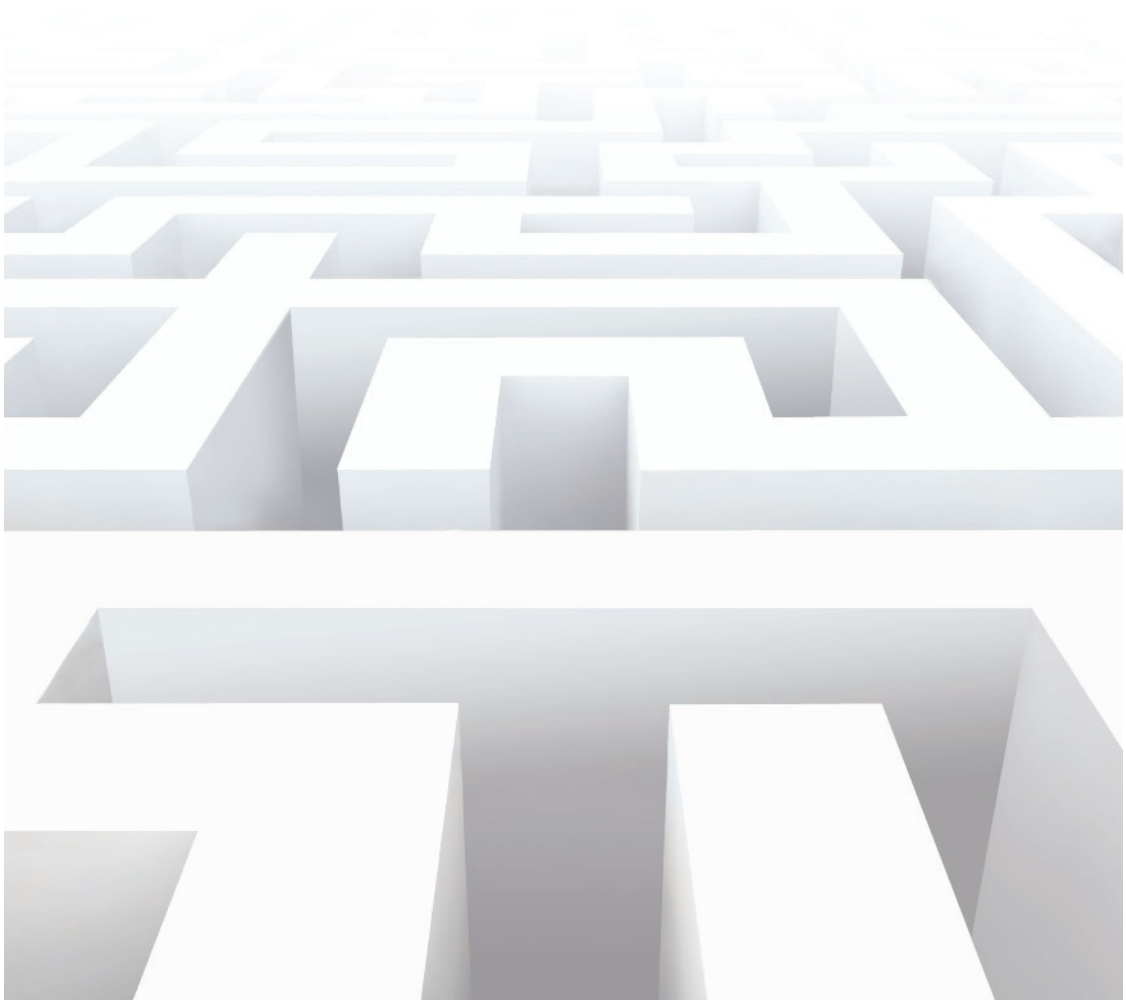

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General Introduction

Adapted from:

Bruton's tyrosine kinase in chronic inflammation: from pathophysiology to therapy

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Introduction

Autoimmune disorders arise from the dysregulation of the immune system and the subsequent abnormal immune response to self-antigens. Genetic factors and environmental factors contribute to the pathogenesis of autoimmune disorders, but the triggers that initiate disease remain to be elucidated. Rheumatoid arthritis (RA) is a chronic and progressive inflammatory disease characterized by the recruitment and accumulation of activated immune cells along with hyperplastic growth of intimal lining layer fibroblast-like synoviocytes (FLS) in the synovial compartment.[1-2] The initiation of RA likely involves antigen-presenting cell (APC) activation due to so-called dangers signals, and the recognition of autoantigens by B and T cells and their subsequent production of autoantibodies. Autoantibody formation can precede clinical signs and symptoms by years and this does not coincide with subclinical inflammation in the joint, suggesting that multiple pathological events (hits) are needed to develop chronic synovitis.3 When synovitis does occur and pro- inflammatory cytokines are produced, in combination with the formation of immune complexes, this can lead to the activation of synovium-infiltrating and tissue resident myeloid cells, like macrophages, monocytes, dendritic cells (DCs), neutrophils and mast cells. Eventually, synovitis leads to erosion of the joint surface, causing deformity and loss of function. The prevalence rate is approximately 1% of the population, with women affected three to five times as often as men. Commonly used animal models of RA include the murine collagen induced arthritis (CIA), adjuvant-induced arthritis (AIA) and K/BxN serum transfer models.[4-5]

Immune-mediated inflammatory diseases are characterized by deregulated processes in immune and stromal cells such as migration, survival, proliferation and differentiation. Phosphatidylinositol 3-kinases (PI3Ks) play a crucial role in these cellular processes, which include control of cell growth and survival, nutrient uptake, proliferation, migration and differentiation.[6-8] In the last 20 years our understanding of how lipid products are regulated and interact with downstream effectors to exert their biological roles, has developed rapidly. The contribution of PI3Ks in the development of several inflammatory and autoimmune diseases, such as type 1 diabetes, cancer, atherosclerosis and rheumatological autoimmune diseases, has generated a great interest in targeting these enzymes in the clinic.9-12 With a growing knowledge of the role of PI3K lipid products, and the downstream proteins they activate, targeting PI3K signaling is becoming of increasing interest. By blocking or inhibiting individual components of the PI3K signaling pathway, multiple cellular processes could be influenced and could provide novel targets for therapeutic interventions in RA and other immune-mediated inflammatory disorders.

PI3K signaling

The PI3K family can be separated into three distinct classes (class I, II and III). In this thesis we will focus on recent developments in understanding the mechanisms of class I PI3Ks signaling and downstream events. Class I PI3Ks are lipid kinases, subdivided into class IA and IB PI3Ks. Class IA PI3Ks are heterodimers consisting of catalytic subunits PI3Kα, β or δ, and an adaptor protein (p85α, p85β, p55γ, p55α or p50α). In contrast to class IA PI3Ks, the class IB PI3K p110γ operates downstream of heterotrimeric G-protein-coupled receptors (GPCRs), coupled by p101 or p84 regulatory subunits. However, recently it has been shown that most class I PI3K subunits can be activated by GPCRs, either directly through Gβγ protein subunits (in the case of p110β and p110γ) or indirectly, for example through Ras (via their Ras Binding Domain).[13-14] Thus, class IA PI3Ks may be more responsive to GPCR stimuli than initially anticipated, and 'classical' class IB PI3K can signal downstream of protein tyrosine kinases upon Ras activation.[13-14] All four PI3K p110 catalytic subunits (α/β/γ/δ) are ubiquitously expressed in mammalian tissue, and p110δ and p110γ expression is enriched in cells of hematopoietic origin.

The primary function of PI3Ks is to provide membrane targeting signals to mediate membrane recruitment of selected proteins. They catalyze the phosphorylation of the 3-position of the inositol headgroup of phosphatidylinositol to generate second messengers PtdIns*3P*, PtdIns(3,4)*P2*, PtdIns(3,5)*P2* and PtdIns(3,4,5)*P3* (PIP3). Together with the dephosphorylation product PtdIns(3,4)*P2*, these second messengers regulate the localization, conformation and function of multiple proteins by binding to their PH (pleckstrin homology) domain.[9] Cellular levels of PIP3 are tightly regulated by PI3K, but also by its phosphatases (SHIP-1, SHIP-2 and PTEN), which dephosphorylate PIP3. PTEN (Phosphatase and tensin homolog deleted from chromosome 10) is a 3'- specific PIP3 phosphatase which will lead to termination of PI3K signaling. SHIP (Src homology 2 domain-containing inositol-5-phosphatase) 1 and SHIP-2 hydrolyze the phosphate group at the 5-position. While PTEN is constitutively active, the regulatory role of SHIP is controlled by activation of receptors with an immunoreceptor tyrosine-based inhibition motif. When phosphorylated, these receptors form a docking site for the SH2 domains of SHIP proteins.[15-16]

PI3K inhibition

Studies by our group and others have identified a potential important role for PI3K signaling in maintaining cellular recruitment, activation, and survival in RA synovial tissue.[17] In situ hybridization studies of RA synovial tissue showed negligible expression of PTEN in the intimal lining layer, whereas the synovium of non-arthritic individuals

showed clear staining throughout the different cellular layers.[18] Furthermore activation of protein kinase B (PKB, also known as Akt) had been demonstrated to be elevated in RA versus non-RA tissue.[19] We have shown that phosphorylation of the PKB-downstream target FoxO4 in synovial macrophages, particularly in the synovial sublining, is significantly enhanced in RA. Additionally, PKB-dependent phosphorylation of FoxO1 in synovial FLS was observed.[20] Available data indicates that targeting PI3K signaling pathways may have therapeutic benefit in autoimmune diseases, and genetic deletion or pharmacological inhibition of PI3K p110 γ or δ catalytic isoforms is protective in various animal models of RA.[21-23] Modest responses of earlier-generation PI3K inhibitors led to a rapidly evolving development of PI3K isoform-selective inhibitors. Inhibition of PI3K-γ by both genetic and pharmacological approaches suppresses joint inflammation and destruction in 2 murine models for RA, CIA and anti-collagen II antibody- (αCII-) induced arthritis. In the CIA model PI3Kγ inhibition led to a significant reduction in paw swelling and reduced symptoms of joint inflammation and cartilage erosion. Similar observations were made in the αCII-induced arthritis model.[21] In the antigen-induced arthritis mouse model it was shown that both pharmacological and genetic inhibition of PI3Kγ resulted in reduced joint inflammation and reduced activation and migration of macrophages and neutrophils.[24] PI3Kδ mRNA and protein expression has been demonstrated to be higher in RA than in osteoarthritis synovium and inhibition of PI3Kδ resulted in inhibition of synoviocyte growth.[25] In the K/BxN serum transfer arthritis mouse model joint inflammation was attenuated in PI3Kδ and PI3Kγ KO mice, and a much stronger protection was observed when both isoforms were inactivated.[26] However the development of PI3K inhibitors for clinical use has been severely hampered by their toxicity and off target effects.[27] The envisioned advantage of the isoform-selective inhibitors is the potential of more focused toxicities compared with the pan-isoform inhibitors, achieving a more complete and reliable inhibition of kinase activity at lower doses. Despite therapeutic advances there are still concerns regarding compensatory feedback mechanisms, alternate pathway activation and potency issues. Perhaps going downstream of PI3K towards targeting effector proteins prevents these concerns and this would result in more robust and selective inhibition of kinase activity.[28-30]

The increase in concentration of second messengers PtdIns(3,4,5)*P3* and PtdIns(3,5)*P2* in the inner leaflet of the plasma membrane results in relocalization of effector proteins containing PH domains. It remains to be elucidated how engagement of PtdIns(3,4,5)*P3* /PtdIns(3,5)*P2* with the PH domain of effector proteins regulates effector function.[8] The PH containing downstream targets of PI3K include phosphoinositide-dependent

kinase (PDK), activators of Rho family GTPases and AGC kinases like protein kinase B (PKB, also known as Akt) and serum glucocorticoid kinase (SGK), and Tec family kinases. Many of the best-described effects of PI3K signaling on cellular activation, cell growth, proliferation and survival are mediated by a subgroup of the AGC protein kinases, namely PKB and SGK. PKB has been shown to modulate the activity of small GTPases, the cell death-inducer Bad, the TORC1 complex, GSK3β, and FoxO transcription factors. Furthermore, PKB has been reported to connect the PI3K pathway with components of another well-known pro-inflammatory pathway, the NFκB pathway, modulating inflammatory responses even more. Interestingly, it was recently found that SGK1 can exert similar functions as PKB.[31-32] Like PKB, SGK1 is activated downstream of PI3Ks, mediated by PDK1. Both kinases prefer to phosphorylate proteins at the consensus sequence R-X-R-X-X-(Serine/Threonine), where R stands for arginine and X for any amino acid. Hence, the majority of targets, including GSK3β, B-Raf and forkhead box (FoxO) transcription factors, are shared between SGK1 and PKB.[33-36] However more and more evidence is emerging that SGK1 and PKB can also phosphorylate distinct proteins and can have different functions *in* vivo.[33-37] SGK1 and its downstream target NDRG1 have been shown to be involved in the regulation of NFκB via phosphorylation of NFκB components, alluding to a potential role in inflammation and inflammatory cytokine production.[38-39] SGKs have been described to be involved in several diseases like cancer, hypertension, neuronal diseases, fibrotic diseases and ischemia.[40-41] In vivo, SGK1 has been shown to regulate T helper differentiation through involvement in Th1 and Th2 cell-fate polarization, and repression of the production of interferon-γ (IFN-γ).42 Furthermore it has been shown to be involved in the induction of pathogenic Th17 cells.[43-44]

Tec family kinases, in particular Btk, are being increasingly recognized for their role in inflammation as well. Btk is involved in the differentiation and activation of B cells and the activation of myeloid cell populations, suggesting that targeting Btk might be particularly useful in autoimmune diseases characterized by pathologic antibodies, macrophage activation and myeloid derived type I IFN responses.

Btk and the Tec kinase family

Btk, the best-known member of the Tec family kinases was identified as a new protein kinase, mutations in which caused X-linked agammaglobulinemia (XLA) in men and X-linked immunodeficiency (XID) in mice. The Tec family of kinases forms the second largest class of cytoplasmic protein tyrosine kinases after the Src family kinases (SFKs) and consists of 5 mammalian members: Btk, Bmx (bone marrow

kinase on the X-chromosome, also known as Etk), Itk (IL-2 inducible T-cell kinase), Rlk (resting lymphocyte kinase, also known as Txk) and Tec (tyrosine kinase expressed in hepatocellular carcinoma).[45] Most of the Tec family kinases are primarily expressed in the hematopoietic system, although both Tec and Bmx are also expressed in stromal tissues such as liver and endothelial cells, respectively.[45-47] Two characteristic features that set the Tec family kinases apart from SFKs are the presence of a pleckstrin homology (PH) domain, located at the N-terminus of the protein, and a proline-rich region which is part of the Tec homology (TH) domain. The PH domain is able to bind phospholipids and signaling proteins such as the βγ-subunits of heterotrimeric G proteins and protein kinase C, and is required for Tec family kinase membrane localization and molecular activation. The proline-rich region, which can interact with SFKs and other Src homology (SH) 3 domain-containing proteins, is implicated in autoregulation. In addition to the PH domain and the TH domain, Tec family kinases contain a phospho-tyrosine-binding SH2 domain, and SH3 and catalytic domains (SH1). Activation of Tec family kinases upon cell-surface receptor triggering, requires relocalization of the protein to the plasma membrane, which is mediated by the interaction of the PH domain with the lipid phosphatidylinositol (3,4,5)*P3*, formed by activated phosphatidylinositol-3 kinase. Subsequent phosphorylation by SFKs and autophosphorylation of tyrosine 223 result in the complete activation of Tec family kinases. Although translocation to the membrane seems to be a pre-requisite for activation, Btk, Itk and Rlk are also found in the nucleus upon cell activation.[46-48] In addition to the well-studied role of Tec family kinases in PLCγ activation and Ca^{2+} mobilization, they can also act downstream of numerous cell-surface receptors that influence a wide range of signaling pathways involved in proliferation, differentiation, apoptosis, cell migration, transcriptional regulation, cellular transformation and inflammation.[45-47]

Btk not only plays a critical role in B cell receptor (BCR) signaling in B cells but has also been shown to function downstream of CD40, affecting B cell activation and function.[49] Btk has also been demonstrated to directly interact with cytoplasmic Toll/IL-1 receptor (TIR) domains of most Toll-like receptors (TLRs) and can interact with downstream adaptors MYD88, TRIF and MYD88 adaptor-like protein (MAL; also known as TIRAP), and IL-1R-associated kinase 1 (IRAK1). Btk regulates signaling by TLR2, 3, 4, 7, 8 and 9 in macrophages, dendritic cells (DCs) and B cells, activating transcription factors like NF-κB and IRF3 ultimately leading to cytokine and type I IFN production.[50-59] Furthermore it has been shown that Btk signals downstream of Fc receptors, leading to increased cytokine production in myeloid cells and mast cells and degranulation in mast cells.[60-62]

Btk regulates production of many cytokines in myeloid cells. In DCs Btk has been reported to regulate type I IFN, IL12, IL18, and IL10.[56-57-63-64] In monocytes/macrophages pro-inflammatory cytokines like TNF, IL1 and IL6 and MMPs are affected by Btk.[52-60-65-67] As in monocytes/macrophages, IL6 and IL8 production is also regulated by Btk in platelets.[68-69] In mast cells, degranulation and TNF production are Btk-dependent.[61-62] The involvement of Btk in the production of these cytokines and inflammatory mediators suggests that targeting Btk could be beneficial in various immune-mediated inflammatory disorders.

Consequences of Btk mutations

Btk is required for B-cell development and function and is the only known member of the family that causes disease in humans. XLA is a primary immunodeficiency resulting from mutations in Btk. Over 1000 mutations have been described that render Btk non-functional or prevent protein expression. XLA is characterized by the complete absence of circulating B-cells due to a developmental block between pro- and pre-B-cell stages, and a severe reduction in serum immunoglobulin levels.[70-71] A similar, although considerably milder syndrome in the mouse, XID, is also caused by a mutation in Btk.[71] Apart from its role in B-cells it has also been shown to regulate monocyte, macrophage and DC function, mast cell and platelet activation and osteoclast differentiation.[62-72-76]

The role of Btk in autoimmune diseases

Btk KO and XID models combined with biological specimen from XLA patient form a powerful tool to examine the role of Btk under steady state and pathological conditions. In addition, the recent development of several covalent and non-covalent small molecule inhibitors specifically targeting Btk can contribute to our understanding of the functional role of Btk in several cell types and disease mechanisms. Below we will discuss the potential use of Btk inhibitors in several autoimmune diseases and their potential path towards clinical development.

Rheumatoid arthritis

Initial evidence that Btk might play a role in RA arose from observations that XID mice are resistant to inflammation and joint destruction in CIA.[77] This spurred further research and when small molecule inhibitors specifically targeting Btk were developed, they were studied in both in vivo and *in vitro* models of arthritis. LFM-A13 (leflunomide metabolite analogue alpha-cyano-beta-hydrxy-beta-methyl-N-[2,5-dibromophenyl]-propenamide) was one of the first rationally designed Btk selective inhibitors. LFM-A13 binds to the catalytic domain and inhibits Btk in a reversible manner. Even though

LFM-A13 has been described as a highly selective inhibitor targeting Btk, it has also been shown to target the activity of other kinases like JAK2 and the erythropoietin receptor. Despite its anti-leukemia activity and the lack of any major toxicity in pre-clinical studies, rather high doses are needed for a pharmacological effect which prohibited LFM-A13 to enter clinical development.[78-80]

Following up on the success of LFM-A13 several covalent and non-covalent small molecule inhibitors for Btk have been developed (Table 1).

One of the first new chemotypes of small molecule Btk inhibitor, CGI1746, stabilizes Btk in an inactive non-phosphorylated enzyme conformation and inhibits both auto- and trans-phosphorylation steps needed for Btk kinase activation. It was demonstrated that CGI1746 blocks BCR-mediated B cell proliferation and decreases autoantibody levels in murine CIA.[60] Pro-inflammatory cytokines like TNF, IL1β and IL6 induced by Fc-receptor triggering were inhibited in mouse macrophages and human monocytes. CGI1746 decreased cytokine levels in the joints and ameliorated disease in myeloid and FcγR-dependent autoantibody-induced arthritis. This study provided direct evidence that Btk inhibition could suppress experimental arthritis via effects on both B lymphocyte and myeloid cell populations.[60] Another reversible Btk inhibitor, RN486, potently and selectively inhibits Btk in human cell-based assays as well as animal models of arthritis. It was shown to efficiently block FcεR cross-linking-induced degranulation of mast cells, and BCR-induced B cell activation.[69-81] Furthermore RN486 inhibited inflammatory mediator production in response to multiple agonists relevant to inflammatory arthritis (i.e. FcγR, TLR and CD40 ligation) in human macrophages, as well as genes induced by RA patient synovial fluid. Importantly, RN486 was able to suppress spontaneous cytokine and matrix metalloproteinase production in RA synovial tissue explants.[65] Lastly, RN486 was shown to reduce levels of platelet p-PLCγ2 upon GPVI stimulation with convulxin and could block IL6 and IL8 production in a human platelet-FLS co-culture system. It displayed similar functional activity in animal models of arthritis like mouse CIA and rat AIA. RN486 inhibited both joint and systemic inflammation, reducing paw swelling and inflammatory markers in peripheral blood.[65-68-69-81]

Ibrutinib, or PCI-32765, was the first Btk inhibitor to advance into clinical trials for the treatment of B cell malignancies. Ibrutinib is a covalent, irreversible, Btk inhibitor, which binds the cysteine 481 residue in the ATP binding domain of the catalytic domain, preventing full activation of Btk. While ibrutinib was chosen for clinical development because of its high selectivity (IC_{50}: 0.5 nM), a small number of other

kinases are known to be targeted as well (for example, EGFR, HER2, Itk and Tec) albeit at higher IC_{50}'s. It still remains unknown as to what extent targeting these off-target kinases contributes to the efficacy and toxicity of ibrutinib. Ibrutinib was shown to reverse arthritic inflammation in murine CIA and prevented clinical arthritis in the collagen antibody-induced arthritis (CAIA) murine model. It inhibited infiltration of immune cells to the joints, bone resorption, cartilage destruction and inflammation. Ibrutinib was also demonstrated to be efficacious in cell-based assays, as it prevented BCR-activated B cell proliferation, inhibited cytokine induction by FcγR triggering and prevented degranulation of mast cells upon activation.[82-84]

A second compound entering clinical trials for B cell malignancies is ONO-4059. A phase I clinical trial for oral treatment of B cell lymphoma was started in 2012. This compound binds covalently to Btk and reversibly blocks BCR signaling and B cell proliferation. ONO-4059 was demonstrated to suppress production of inflammatory chemokines and cytokines by monocytes in a murine CIA model and prevented cartilage and bone destruction.[84-86]

While ibrutinib and ONO-4059 advanced quickly in clinical trials for hematological malignancies, 3 different small molecule inhibitors targeting Btk are now in first-stage clinical trials for RA. CC-292, also described as AVL-292, is similar to ibrutinib in that it covalently binds cysteine 481 in the kinase domain. It showed promising results in animal models of arthritis with a 95% reduction in clinical scores and a decrease in inflammation and cartilage and bone destruction. CC-292 is the first Btk inhibitor reported appropriate for use in a human clinical setting of autoimmune disease. Healthy volunteers were treated with 2mg/kg orally and the compound was shown to consistently engage all circulating Btk protein. This first trial provided rapid insight into safety, pharmacokinetics (PK), and pharmacodynamics (PD), and CC-292 has currently progressed into a phase 2a clinical trial. This study will test the clinical effectiveness and safety of an orally administered dose of CC-292 compared to placebo in patients on methotrexate (MTX) with active RA.[78-87-88]

GDC-0834, another Btk specific inhibitor, has been shown to ameliorate disease in the rat CIA model. This study suggested that a high level of inhibition of phosphorylation of Btk was required for it to affect inflammatory arthritis in rats.[89] A recent phase I study in healthy volunteers showed that the inhibitor is metabolized to an inactive metabolite in the liver via amide hydrolysis. It is currently undergoing further clinical development for RA.[86-90]

Finally, HM-71224 is an orally active and irreversible Btk inhibitor showing a strong efficacy in mice and rat CIA models. The first clinical trial was started to evaluate the safety, tolerability, PK, PD, and food effect of HM-71224 following single ascending doses (SAD) and multiple ascending doses (MAD) in healthy volunteers. An interim report demonstrated a well-tolerated safety profile in healthy volunteers and desirable PK and PD properties supporting sustained target inhibition. A phase 2 study in active RA patients is expected to be initiated soon.[86-91-92]

Compound	Irreversible/ reversible	IC50	Tested in animal models for	Clinical stage
CGI1746	**Reversible**	**1.9nM**	**RA**	**Preclinical**
RN486	**Reversible**	**4nM**	**RA, SLE**	**Preclinical**
Ibrutinib	**Irreversible**	**0.5nM**	**RA, SLE**	**Preclinical**
ONO-4059	**Reversible**	**2.2nM**	**RA**	**Preclinical**
CC-292	**Irreversible**	**5.9nM**	**RA**	**Phase IIa**
GDC-0834	**Reversible**	**5.9nM**	**RA**	**Phase I**
HM-71224	**Irreversible**	**2nM**	**RA**	**Phase I**
PF-06250112	**Reversible**	**0.5nM**	**SLE**	**Preclinical**

Table 1 Btk inhibitors in development for immune mediated inflammatory disorders

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease, which is characterized by autoantibodies recognizing nuclear self-antigens. Autoreactive T- and B-cells evade negative selection and, once activated, collaborate to produce autoantibodies, which culminate in immune complex deposition on tissues leading to organ damage. Deposition occurs most often in skin and kidneys (lupus nephritis), activating complement and promoting local inflammation by activating macrophages and DCs. Activation of plasmacytoid DCs (pDCs) leads to production of type I IFNs, major contributors to pathology in SLE. Various animal models of spontaneous lupus are commonly used and include the classical F1 hybrid of New Zealand Black and New Zealand White strains (NZB x NSW F1) and the MRL/lpr lupus-prone model.[86-93-94] Various Btk murine knock-out models have suggested that Btk plays a role in SLE. In SLE murine models Btk has been demonstrated to be required for the initial loss of tolerance to DNA and the ensuing production of anti-DNA antibodies.[95-96] Furthermore it has been shown that specific inhibition of Btk by RN486 can regulate TLR9 signaling in pDCs and thus inhibit type I IFN production.[51] In addition to *in vitro* studies, in vivo studies have also been conducted with RN486 in the NZB x NZW F1 model. The administration of RN486 completely blocked disease progression and this study

suggested that Btk inhibition may simultaneously target autoantibody producing cells and impact the effector function of autoantibodies on monocyte activation in SLE.[97] The results of this study were consistent with results of a study that used a different Btk inhibitor, PF-06250112. PF-06250112 binds residue cysteine 481 covalently but reversibly and was identified as a potent, orally bioavailable, small-molecule inhibitor of Btk. This study made use of the same mouse model (NZB x NZW F1) and showed that Btk inhibition prevented the development of proteinuria and reduced glomerular injury and inflammatory infiltrates. Furthermore it showed a significant reduction in serum IgG anti-dsDNA antibodies and complement deposition.[98] Ibrutinib also has been shown to be efficacious in animal models of SLE. Treatment of B6.Sle1 and B6.Sle1.Sle3 lupus-prone mice with ibrutinib resulted in decreased renal damage and lymphocyte infiltration. Moreover, a significant reduction in autoantibodies was shown. In the MRL/lpr mouse model, ibrutinib treatment resulted in reduced proteinuria and blood urea nitrogen and showed a trend towards improvement of glomerulonephritis.[82-99] The clinical therapeutic efficacies have not been studied in human SLE thus far and further evaluation of the potential of targeting Btk is of interest.

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) caused by an autoimmune response to self-antigens. Large, confluent plaques of demyelination are formed in the white and the gray matter. Even though MS is extensively studied, key aspects of MS etiology and pathophysiology still remain to be elucidated. Active tissue injury in all stages of MS is associated with inflammation, with the inflammatory infiltrates being composed of T lymphocytes, B lymphocytes and plasma cells. This leads to active demyelination and axonal or neuronal damage at sites of microglia activation and macrophage infiltration of the tissue. Experimental autoimmune encephalomyelitis (EAE) is the main animal model for MS and is also characterized by autoreactive T cells.[100-102] Although Btk inhibition has not been studied in EAE models, Btk-deficient XID mice develop less severe EAE.[70] In line with this, Btk inhibition might target and inhibit B-cell and macrophage activation which are both implicated in the pathogenesis of the disease. However, Btk targets TNF and type I IFN are hypothesized to play a dual role in MS. TNF has been described to be protective in lymphoid organs but pathogenic in the CNS during EAE. Furthermore, anti-TNF treatment has been associated with increased disease activity. In multiple autoimmune disorders like SLE and Sjögrens syndrome type I IFNs are increased and this signature suggests a possible involvement in these disorders, paradoxically, however, in MS treatment with type I IFN or its induction has proven beneficial.[103-107]

Thus both TNF and type I IFNs could have pleiotropic effects that depend on the timing, dosage and cell type and this should be taken into account when considering targeting Btk in MS.[108] As current treatments are only partially effective, studying the role of Btk in MS pathology could provide an alternative approach to therapeutic intervention.

Type 1 diabetes

Type 1 diabetes (T1D) is characterized by immune-mediated destruction of insulin-producing pancreatic β cells by autoreactive T cells. Autoantibodies are produced and pancreatic islets are infiltrated by inflammatory cells like macrophages and CD4+ T cells, leading to life-long dependence on exogenous insulin and an increased risk for kidney failure, heart disease, blindness, limb loss and episodes of hypo- and hyperglycemia. Both genetic and environmental factors play a role in the pathogenesis of T1D.[109-112] The majority of our knowledge on the pathogenesis and etiology of T1D comes from the study of spontaneous disease in the nonobese diabetic (NOD) mouse model. The NOD mouse model showed that also B cells have flaws in tolerance mechanisms.[112] B cells contribute to the disease by producing autoantibodies and activating autoreactive T cells. Elimination of B cells has proven successful at preventing disease, and Btk deficiency in NOD mice protects against T1D. Btk deficiency led to a failure to produce insulin autoantibodies by decreasing relative availability of mature autoreactive B cells.[113-114] This suggests that by targeting Btk, also T cell-mediated autoimmunity could be alleviated in T1D. However, one case report described the development of T1D in an XLA patient, implying that neither autoantibodies nor B cell function is absolutely required for T1D onset.[115] No studies examining pharmacological inhibition of Btk in T1D animal models have been reported thus far, and would represent the next step in examining the role of Btk in this disease.

Systemic Sclerosis

Systemic sclerosis (SSc), or scleroderma, is an autoimmune disease, which is characterized by vasculopathy, immune dysfunction and fibrosis leading to multi-organ failure. Heterogeneity is caused by variable expression of these three pathological features which makes early diagnosis challenging. Historically, scleroderma research has been focused on the altered function of fibroblasts. Excessive extracellular matrix and collagen deposition by fibroblast activation and activation of Th2 cells were believed to play a prominent role. However, recent findings suggest a complex interplay between immune cells, endothelial cells and fibroblasts indicating an important role for immune cells in the pathogenesis of SSc. Genetic association studies indeed revealed that among the most highly associated susceptibility markers, genes encoding immune

signaling molecules like T bet, IRF5 and STAT4 were included.[116] Recently, pDCs have also been shown to play a prominent role in SSc pathology. These cells have the unique ability to trigger both the innate and the adaptive immune system, are key producers of type I IFNs, and in SSc, produce high levels of CXCL4, which might directly promote fibrosis.[117] Autoantibodies are commonly observed in SSc patients, although there is debate as to whether they contribute to disease or are an epiphenomenon. There are several animal models known for this disease, including the bleomycin-induced fibrosis and the tight skin-1 (Tsk-1) mouse models.[118-120] As mentioned above, specific inhibition of Btk by RN486 can regulate TLR9 signaling in pDCs and thus inhibit type I IFN production.[51] Therefore, one could speculate Btk inhibition would also prove beneficial in the treatment of SSc, but such studies have not yet been reported.

Sjögren's syndrome

Primary Sjögren's syndrome (pSS) is considered to be the second most common systemic autoimmune disease after RA. pSS is characterized by the infiltration of immune cells in the exocrine glands, leading to ocular and mouth dryness but also includes arthralgia and fatigue as frequent disabling symptoms. Approximately one third of the patients also develop systemic complications such as arthritis, lung interstitial disease, neurological manifestations or tubular nephropathy. This latter group is at a higher risk for lymphoma development, possibly driven by ongoing B cell proliferation and/or stimulation. Although the underlying etiology has yet to be fully elucidated, in the past decade major advances have been made in understanding the pathogenesis of pSS. T cells have been shown to play a major role in the pathogenesis, and the innate system seems to be involved in early stages of disease through the production of type I IFN. Besides their role in autoantibody production, B cells are now recognized to have multiple roles in pSS pathophysiology and might play a central role in the development of the disease. There also appears to be a crucial interplay between T cells, DCs and exocrine gland epithelial cells. Various mouse models exist for pSS. The earliest models were strains that develop disease spontaneously (NZB x NZW F1 and MRL/lpr mice also possess SLE features), but transgenic (Tg) overexpression of many immune-related genes (as observed in BAFF Tg, TGFβ Tg or IL12 Tg mice) and genetic deficiencies can lead to the disease phenotype as well. Induced models include ro-peptide-induced or murine CMV-induced pSS.[121-125] Btk KO or inhibition has yet to be studied in the context of pSS. However, based on the ability of Btk inhibition to decrease autoantibody production and inhibit myeloid type I IFN responses in other disease models, a rationale to study the role of Btk in pSS is readily apparent.

Concluding remarks

Extensive research has established a critical role for PI3K and its downstream effectors in several disease-relevant signaling pathways making this signaling pathway an appealing drug discovery target. By targeting downstream effectors, compensatory feedback mechanisms, alternate pathway activation and potency issues could be avoided and this could result in more robust and selective inhibition of kinase activity. In particular, the current small-molecule Btk inhibitors have demonstrated potent and selective Btk inhibition in pre-clinical studies and some inhibitors have propelled the clinical development of such molecules towards clinical trials in RA patients. Future studies on the effectiveness of pharmacological inhibition of these and other components of the PI3K pathway in autoimmune diseases could further broaden the understanding of the disease mechanisms as well as lead to the well-desired broadening of therapeutic armamentarium. In this thesis we aim to study the role of the PI3K signaling pathway and downstream effectors in chronic inflammation.

In **chapter 2** we look at the role of individual PI3K isoforms in human macrophages. In **chapter 3** we examine the role of downstream target FoxO1 in the survival of RA FLS. In **chapters 4 and 5** we determine the functional role of downstream effector Btk in RA synovial biopsies and human macrophages. We study the role of SGK in human macrophages and RA FLS in **chapter 6**.

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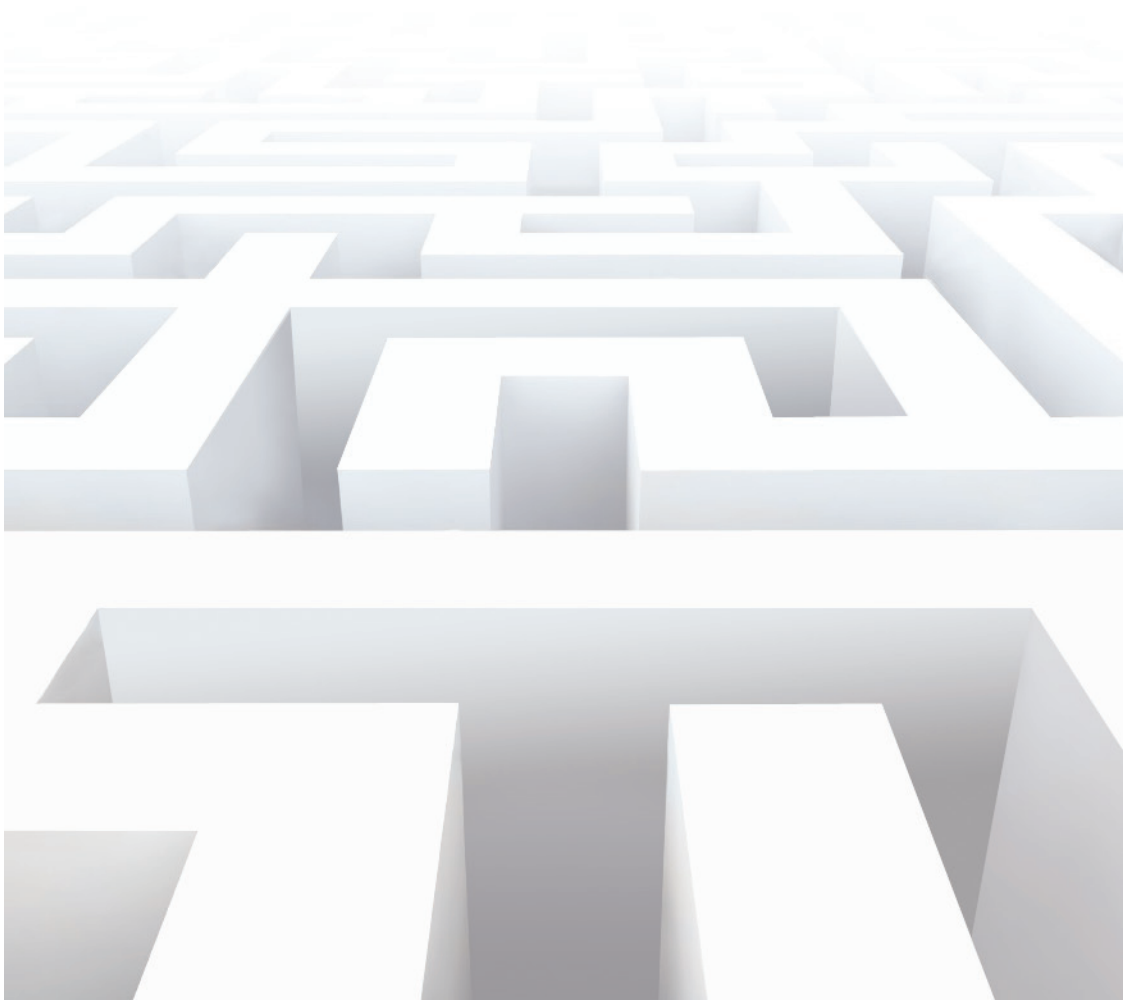

2

LY294002 induced inhibition of macrophage activation and survival is not class I PI3K dependent

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ABSTRACT

Class I phosphatidylinositol 3-kinases (PI3Ks) are important membrane-bound lipid kinases which, via downstream effectors like protein kinase B (PKB), mediate fundamental cellular processes such as cell growth, viability and cytokine production. The role of PI3K in cell viability and functional processes has largely been established utilizing the well-known pan-PI3K inhibitor LY294002, although more specific PI3K isoform-specific inhibitors have been recently generated. We now have the opportunity to study the role of specific class I PI3Ks in more detail in human macrophages with p110-selective inhibitors in regard to macrophage activation and survival. Here, we demonstrate that class I PI3Ks have little influence on macrophage activation, although LPS-induced TNF and IL10 production could be partially affected by PI3Kδ inhibition. Additionally, unlike LY294002, specific inhibition of class I PI3Ks failed to induce macrophage apoptosis, concomitant with their inability to regulate macrophage expression of cell survival-related genes that were sensitive to LY294002. We show that many of the effects of LY294002 on macrophage gene expression can be attributed to off-target effects on BET bromodomain family epigenetic regulatory proteins. Our results suggest that many of the effects of LY294002 on macrophage survival and activation ascribed to class I PI3K activity are PI3K-independent.

Introduction

Phosphatidylinositol 3-kinases (PI3Ks) are a family of intracellular lipid kinases, which are involved in fundamental cellular processes such as cell growth, proliferation, differentiation, cytokine production, cell viability and intracellular trafficking.[1-5] The PI3K family can be separated into three distinct classes (class I, II and III), of which class I PI3Ks are the best studied in immunology. Class I PI3Ks are subdivided in class IA and IB PI3Ks. Class IA PI3Ks consist of 110 kDa catalytic subunits (p110α, p110β or p110δ) and smaller regulatory subunits, which are activated downstream of protein tyrosine kinase-coupled receptors. In contrast, the class IB PI3K, p110γ, is activated downstream of G-protein coupled receptors (GPCRs). However, recently it has been shown that most class I PI3K subunits can be activated by GPCRs, either directly through Gβγ protein subunits (in the case of p110β and p110γ) or indirectly, for example through Ras GTPases.[2-4;6;7]

For many years, the general approach to show involvement of PI3Ks in biological systems was based upon the usage of the pan-PI3K inhibitor LY294002. LY294002 is known to inhibit class I PI3Ks, but this compound also inhibits mTOR (mammalian target of rapamycin) and DNA-PK (DNA dependent protein kinase), enzymes having significant homology to class I PI3Ks in their catalytic kinase domains. mTOR and DNA-PK are inhibited to the same extent by LY294002 as PI3K.[8-10] Extremely high concentrations of LY294002, up to 50x IC50, have been used historically to study involvement of class I PI3Ks, but several other protein kinases, such as GSK3, CK2, PLK1, PIM1, PIM3, and HIPK2 are inhibited at these concentrations as well.[11] PI3K-independent effects have been reported by a number of studies by making use of the PI3K-inactive analogue LY303511. Furthermore LY294002 has been shown not only to inhibit kinase function but is also capable of inhibiting BET family bromodomain proteins, epigenetic “reader” proteins which couple newly acetylated histones to transcription factors needed for gene expression.[12;13] Also, the data obtained with pan-PI3K inhibitor wortmannin should be considered carefully. For class I PI3K the IC50 is 1-5 nM, but in higher concentrations other kinases like mTOR, DNA-PK, ATM and polo-like kinases are also inhibited.[8;14-16] The recent generation of more specific PI3K inhibitors has allowed for more selective inhibition of the enzymatic activity of each of the four class I p110 isoforms.[17;18]

Studies with these inhibitors, and the generation of PI3K isoform-specific knock-out mice has shed new light as to how PI3K signaling pathways contribute to inflammation. Macrophages from PI3Kγ-null mice show impaired migration towards chemokines like MCP1, RANTES and CSF-1 at sites of inflammation.[19-21] In the antigen-induced arthritis mouse model it was shown that both pharmacological and genetic inhibition

of PI3Kγ resulted in reduced joint inflammation and reduced activation and migration of macrophages and neutrophils.[22] Also, in murine models of atherosclerosis, PI3Kγ knockout mice are protected from disease, coinciding with diminished macrophage activation.[23] Furthermore, the PI3K signaling pathway is suggested to be important in macrophage survival and activation, as PI3K-dependent activation of protein kinase B (PKB, also known as Akt) promotes rheumatoid arthritis (RA) synovial macrophage survival by upregulating anti-apoptotic Bcl-2 family protein Mcl-1 protein expression.[24;25] Paradoxically, it has been reported that activation of PI3K upon binding of pattern recognition receptors by pathogen-associated molecular patterns, like lipopolysaccharide (LPS), dampens the production of pro-inflammatory cytokines in myeloid cells.[26-31] In this study we used 3 isoform-specific inhibitors to inhibit class I PI3Ks and examined the effects of treatment with these inhibitors, in comparison to LY294002, on human macrophage activation and survival.

MATERIALS AND METHODS

PI3K inhibitors and other stimulatory reagents

The following inhibitors were used at approximately 5x IC_{50}: a dual p110α/β and mTOR inhibitor, PI103 (100nM, from EMD Chemicals), a p110δ inhibitor, D-000 (1.5 μM, from Labotest), a p110γ inhibitor, AS605240 (40nM, Echelon Biosciences Inc.), the pan-PI3K inhibitor LY294002 (7.5 and 40 μM, 5x and 27x IC_{50} respectively) (Calbiochem), PKB inhibitor VIII (10 μM, EMD Chemicals) and JQ1 (0.5 μM, Cayman Chemicals). LPS (*E. Coli* 0111:B4, Sigma-Aldrich) was used at 1 μg/ml to stimulate macrophages for western blot analysis and cytokine production.

Cell culture

Monocytes were isolated from buffy coats (Sanquin) of healthy donors using Lymphoprep (AXIS-SHIELD) density gradient centrifugation followed by Standard Isotone Percoll gradient centrifugation (Amersham). Monocytes were plated in Iscove-modified Dulbecco medium (IMDM, Invitrogen), supplemented with 1% fetal bovine serum (FBS) for 30 minutes at 37°C. Non-adherent cells were removed, and monocytes were cultured for 7 days in IMDM containing 10% FBS, 100μg/ml gentamycin and 5ng/ml GM-CSF (Tebu Bio) prior to use in experiments. Purity of monocytes and differentiation of monocytes to macrophages was confirmed by labelling of cells with PE-conjugated CD14, PE-conjugated CD86, FITC-conjugated CD64, PE- conjugated CD163 and APC-conjugated CD200R and FACS analysis (FACSCalibur, BD Biosciences).

RA patient FLS (passages 4-9) were isolated from synovial biopsies of patients fulfilling the American College of Rheumatology/European League Against Rheumatism revised criteria for RA, and cultured as previously described.[32;33] All patients provided prior written informed consent, and these studies were approved by the Medical Ethics Committee of the Academic Medical Center, Amsterdam, The Netherlands. Negative selection was used to obtain purified unstimulated T lymphocyte populations from healthy donors (Dynal bead kits, Invitrogen).

Gene expression profiling

Following 24h of treatment, total RNA was extracted from macrophages using an RNeasy kit (Qiagen) and DNAse treatment. RNA concentration and purity was determined with a Nanodrop spectrophotometer (Nanodrop Technologies). cDNA was synthesized from 800 ng of RNA using an RT2 First Strand Kit (SABiosciences) and the expression of genes was analyzed using RT2 ProfilerTM PCR arrays PAHS-058 and PAHS-012 (SABiosciences) according to the manufacturer's instructions. After PCR amplification, threshold values were manually equalized for all samples and the threshold cycle (Ct) determined for each analyzed gene. Relative expression of each gene was calculated using StepOne Software v2.1 (Applied Biosystems) and Microsoft Excel spreadsheet software (Microsoft) and corrected for the expression of the housekeeping gene HPRT.

Western blot analysis and nuclear extraction

Cells were lysed in 1x Laemmli buffer and protein content was quantitated using a BCA protein Assay Kit (Pierce). Equivalent amounts of lysate were resolved by electrophoresis on 4-12% gradient PAGEr EX gels (Lonza), and analysed by western blotting using primary antibodies recognizing phospho-PKB Ser473 (Cell signalling) and tubulin (Sigma-Aldrich), followed by development with IRDye-680-labeled anti-rabbit or IRDye-800-labeled anti-mouse immunoglobulin secondary antibodies (LI-COR Biosciences) and visualization using an Odyssey infrared imaging system (LI-COR Biosciences). Nuclear fractions were prepared as described previously and NFκB p65 DNA-binding activity was determined using a TransAM ELISA (Active Motif).[34]

Cytokine production in macrophages

Macrophages were left unstimulated or stimulated for 24 h with 1 μg/ml LPS. Prior to the stimulations, cells were pre-incubated for 1 hour with the PI3K inhibitors. Cell-free supernatants were harvested and IL6, IL8, TNF and IL10 production was measured using PeliKine Compact ELISA kits (Sanquin Reagents) as per the manufacturer's instructions.

Apoptosis analysis

Immediately prior to apoptosis analysis, cells were harvested, washed in AnnexinV buffer (10 mM HEPES, 150 mM KCl, 1 mM MgCl2, and 1.3 mM CaCl2) and incubated in FITC-labeled AnnexinV (IQ Products) for 30 minutes. Propidium iodide (PI, Sigma) was added right before the cells were analyzed by flow cytometry. Apoptosis was defined as cells staining single positive for AnnexinV or double positive for AnnexinV/PI.

Statistical analyses

Statistical analysis was performed using Windows Graphpad Prism 5 (GraphPad Software, Inc.). Flow cytometry and ELISA results were expressed as the mean ± SEM. Potential differences between samples were analyzed by Student's t-test. P-values ≤ 0.05 were considered significant.

RESULTS

Expression of class I PI3K isoforms and PI3K signaling pathway-related genes in isolated cell populations

In order to obtain a better understanding of the PI3K signaling pathways operative in human macrophages, we performed quantitative PCR arrays of PI3K signaling components in GM-CSF-differentiated healthy donor buffy coat-derived macrophages. For comparison, we also isolated monocytes and T lymphocytes from the same donors (n=3), as well as FLS from RA patient synovial tissue (n=4). All class I PI3K catalytic isoforms were similarly expressed in macrophages (Fig 1). Expression of the PI3K negative regulatory gene PTEN was highest in monocytes. Intriguingly, PTEN expression was sharply down-regulated during monocyte differentiation into macrophages. PKB (also known as Akt) isoforms were expressed in all of the various cell types tested, whereas AKT3 (PKB3) was expressed at highest levels in T cells.

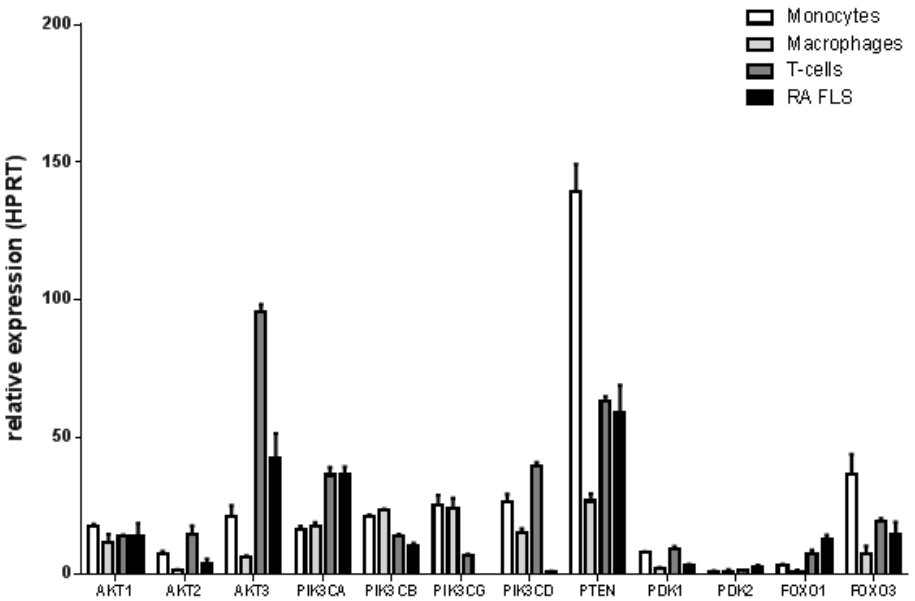


Figure 1. Expression of class I PI3K isoforms and PI3K-related genes in isolated cell populations.
Total RNA was extracted from healthy donor buffy coat monocytes, GM-CSF –differentiated macrophages, T cells (n = 3 donors for each), and RA FLS (n = 4), reverse transcribed and expression of PI3K isoforms and PI3K-related genes was monitored by qPCR array. Data are presented as mean ±SEM expression relative to HPRT, relative quantity (R.Q.).

Phosphorylation of PKB is induced by TLR4 triggering and this is dependent upon class I PI3Ks in macrophages

As has been reported previously TLR4, triggering activates PI3K signaling and phosphorylates its downstream target PKB.[35] Human macrophages were stimulated with 1 µg/ml LPS for the indicated time-points, showing a peak of activation of PKB after 15 minutes stimulation (Fig 2A), indicating the PI3K pathway is activated upon triggering human macrophages with LPS. This phosphorylation event could be blocked by pretreating the cells with the PKB inhibitor VIII (Fig 2B).

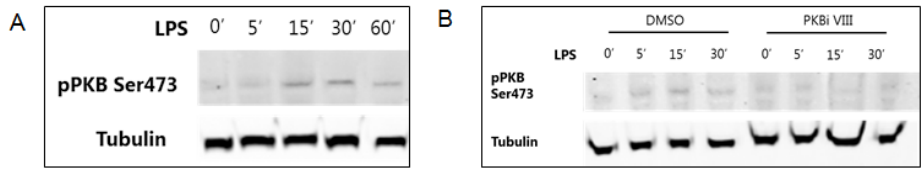


Figure 2. Phosphorylation of PKB is induced by TLR4 triggering and this is dependent on class I PI3Ks in macrophages. (A) Human healthy donor macrophages were stimulated for the indicated timepoints with LPS (1 µg/mL). Protein extracts were prepared and analyzed by western blotting with antibodies recognizing phospho-(p)PKB and tubulin (Tub). (B) Human healthy donor macrophages were left untreated (med) or were treated with 100nM PI103 (α/β inhibitor), 40nM AS605240 (γ inhibitor), 1.5 µM D000 (δ inhibitor), 7.5 µM LY294002 (pan), 40 µM LY294002 or 10 µM PKB inhibitor VIII (PKBi) for 1 hour before 15 min stimulation with LPS (1 µg/mL). Protein extracts were prepared and analyzed by western blotting with antibodies recognizing phospho-(p)PKB and tubulin (Tub).

TNF and IL10, but not IL6 and IL8, production is partially dependent on class I PI3Ks in human macrophages

To determine the relative contribution of specific class I PI3K catalytic subunits to human macrophage activation, we examined the effects of specific inhibitors and LY294002 on TLR-induced cytokine production. Macrophages were either left unstimulated or stimulated with LPS in the absence or presence of PI3K isoform selective inhibitors for 20 hours. LPS-induced IL6 and IL8 production in macrophages was unaffected by each of the specific PI3K inhibitors, as well as LY294002, although IL6 production was significantly reduced ($P < 0.01$) at higher concentrations of LY294002 generally used in the literature (Fig 3A, upper left panel). PKB inhibition also had no effect on LPS-induced IL6 production, but significantly enhanced ($P < 0.01$) production of IL8 (Fig 3A, upper panels). LPS-induced TNF production was inhibited by approximately 75% ($P < 0.005$) by LY294002 at both concentrations tested (Fig 3A, lower left panel), but could only be partially attributed to the involvement of specific class I PI3K catalytic subunits. Of the specific inhibitors tested, potential involvement of only PI3Kδ was observed, as treatment with D-000 reduced TNF production by approximately 40% ($P < 0.01$) (Fig 3A, lower left panel). Similarly, LY294002 almost completely inhibited LPS-induced IL10

production ($P < 0.005$), and again, of the class I PI3K-specific inhibitors, only D-000 displayed an, albeit small, inhibitory effect ($P < 0.05$). However, potential involvement of PI3K-dependent involvement in LPS-induced IL10 production was also suggested by the effects of PKB inhibition, which effectively suppressed IL10 production to levels observed with LY294002 ($P < 0.005$). As interactions of PI3K signaling with NFκB have been previously reported,[36-38] we examined if effects on NFκB activation could explain the effects we observed on LPS-induced cytokine expression. However, no effects of PI3K isoform-selective inhibition or LY294002 were observed on NFκB p65 DNA-binding following macrophage LPS stimulation. Our results suggest that, with the exception of potential small contributions of PI3Kδ to LPS-induced TNF and IL10 production, the effects of LY294002 on macrophage cytokine production are largely PI3K-independent.

Class I PI3K inhibition does not affect cell viability in human macrophages

PI3K-dependent activation of PKB is reported to promote RA synovial macrophage survival by upregulating anti-apoptotic Bcl-2 family protein Mcl-1 protein expression.[24;25] To assess the role of individual class I PI3K isoforms on cellular survival we looked at the effects of their selective inhibitors on macrophage apoptosis. Representative dot plots with our staining strategy are shown in Fig 4A. Macrophages were left untreated or exposed for 24 hours to PI3K isoform-selective inhibitors or LY294002. Interestingly, we observed no effect on cell viability when we treated macrophages for 24 hours with class I PI3K isoform-selective inhibitors, individually or in combination with all isoform-selective inhibitors (Fig 4B). Treatment with the pan-PI3K inhibitor LY294002 at 7.5 μM, a comparable dosage in regards to IC50 as used for PI3K isoform-selective inhibitors, also failed to induce apoptosis in macrophages (Fig 4B). However, macrophages treated with LY294002 at 40μM underwent a strong and significant ($P < 0.01$) apoptotic response, comparable to published studies.[25] This observation could not be reproduced when we used similarly high concentrations of PI3K isoform-selective inhibitors (data not shown). To further elucidate the role of individual class I PI3Ks on macrophage viability we determined the mRNA expression levels of 84 apoptosis-related genes following macrophage incubation with selective and non-selective PI3K inhibitors. In this qualitative screening, only genes that were regulated more than twofold were considered for further analysis. (Fig 4C) Only a small number of genes were regulated by PI3K isoform-selective inhibitors: 1 gene (*LTA*) was regulated by the α/β inhibitor PI103 and the γ inhibitor AS605240 and 3 genes (*LTA, CD70 and TP73*) were regulated by the δ inhibitor. Slightly more genes were affected by inhibition with LY294002 at 7.5 μM, 5 genes in total. In contrast to what was published in RA synovial macrophages[24], we observe an upregulation of MCL1 gene expression upon inhibition by LY294002 at

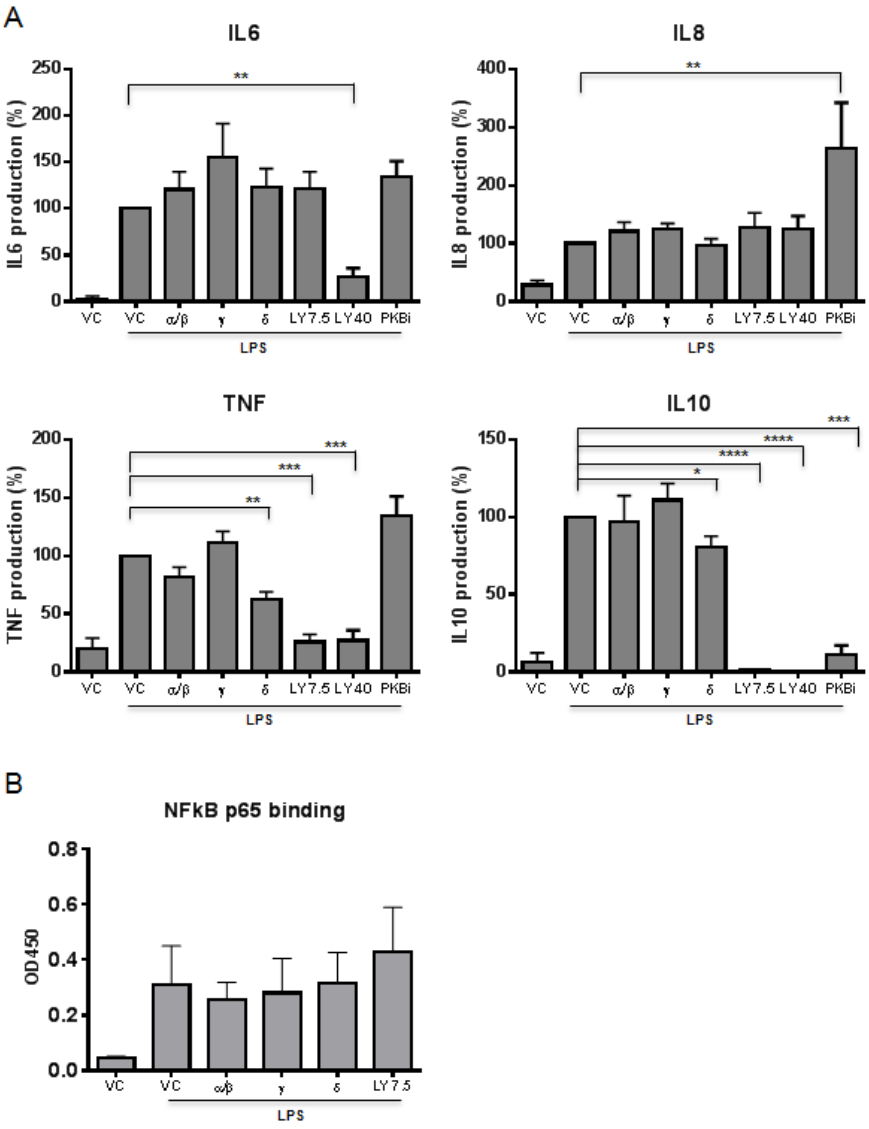
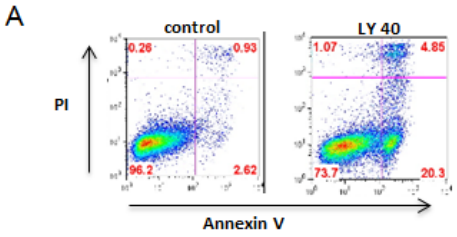
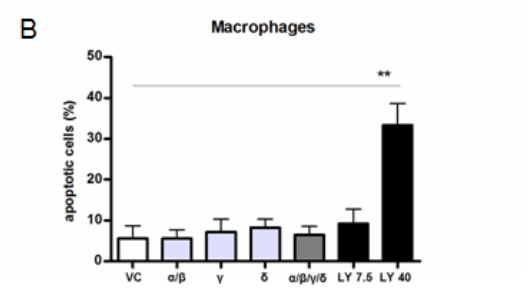
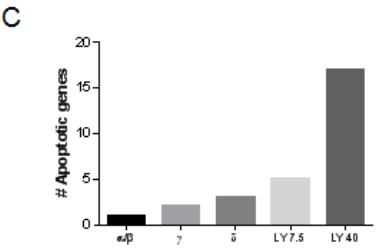


Figure 3. TNF and IL10, but not IL6 and IL8, production is partially dependent on class I PI3Ks in human macrophages. (A) Analysis of IL6, IL8, TNF and IL10 production in supernatants of macrophages either left unstimulated or stimulated with LPS (1μg/ml,) for 24 hours, in the absence or presence of carrier DMSO or 100nM PI103 (α/β inhibitor), 40nM AS605240 (γ inhibitor), 1.5 μM D000 (δ inhibitor), 7.5 μM LY294002 (pan-inhibitor), 40 μM LY294002 or 10 μM PKB inhibitor VIII (PKBi) as assessed by ELISA. Bars represent the means and SEM of 6 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001 versus cells exposed to LPS. (B) Human macrophages were left unstimulated or were stimulated with 1 μg/mL LPS with or without 100nM PI103 (α/β inhibitor), 40nM AS605240 (γ inhibitor), 1.5 μM D000 (δ inhibitor), 7.5 μM LY294002 (pan-inhibitor) for 1 hour, nuclear fractions were extracted, and levels of p65 NF-κB binding were determined using an ELISA-based DNA-binding assay. Results are presented as the mean ± SEM OD450 in p65 DNA-binding from four independent experiments.







α/β	γ	δ	LY 7.5	LY 40
LTA	LTA	LTA	*BCL2L11 (Bim)* **	BCL2 **
		CD70	*CD40LG*	*BCL2L11 (BIM)* ****
		TP73	*MCL1*	NAIP ****
			TNFRSF9 *	BIRC3 **
			CD70 *	CARD6 ***
				CASP5 **
				CRADD *
				GADD45A **
				HRK **
				RIPK2 *
				TNF *
				TNFRSF10A *
				TNFRSF25
				TNFRSF9 *
				CD70 *
				TNFSF8 ***
				TP73 **

Figure 4. Class I PI3Ks do not affect cell viability in human macrophages. Immediately prior to apoptosis analysis, cells were harvested, washed in AnnexinV buffer and incubated in FITC-labeled AnnexinV for 30 min. Propidium iodide (PI) was added right before the cells were analyzed by flow cytometry. Apoptosis was defined as cells staining single positive for AnnexinV or double positive for AnnexinV/PI. (A) Representative dot blots with our gating strategy, and a quantification thereof (n=6) in (B). (C) Macrophages were left untreated or were treated for 24 hours with PI3K isoform-selective inhibitors or the pan-PI3K inhibitor LY294002. The expression of 84 apoptosis-related genes was quantitatively analysed using RT2 ProfilerTM PCR arrays. Relative expression was calculated using StepOne Software v2.1 (Applied Biosystems) and Microsoft Excel spreadsheet software (Microsoft) and corrected for the expression of housekeeping gene HPRT. Only genes that were regulated more than two-fold were considered for further analysis. Gene in italic is upregulated, gene underlined is downregulated. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001.

7.5 μM in healthy donor macrophages. In line with the strong and significant ($P < 0.01$) apoptotic response we observe in macrophages treated with LY294002 in a higher concentration, 17 genes involved in apoptosis were regulated by LY294002 40 μM, of which 3 were also regulated by LY2940002 at 7.5 μM (Fig 4C).

BET inhibition is responsible for a subset of human macrophage survival effects observed with LY294002

LY294002 has recently been shown to exhibit dual PI3K and BET bromodomain inhibitory capacities resulting in suppression of cytokine and chemokine production in human PBMCs, and many genes targeted by LY294002 have been shown to be modulated by chemical analogues of LY294002 that do not inhibit class I PI3Ks.[39-41] We hypothesized that the effects of high concentrations of LY294002 on survival-related gene expression in macrophages may be due to off-target effects on BET family proteins.[40] Therefore, we compared the expression of 84 apoptosis-related genes from macrophages treated with LY294002 at 40 μM to macrophages treated with JQ1, a specific BET inhibitor.[41] Initial comparison of the effects of the two compounds on macrophage gene expression suggested that although a number of unique genes were differentially regulated, LY24002 and JQ1 had similar effects on many genes (Fig 5A). For instance, both significantly downregulated expression of *NAIP, TNFSF8,* and *CASP5*, while similar trends were observed in the regulation of *BCL2L11, BCL2*, and *CARD6* (Fig 5B). In contrast, only LY294002 significantly regulated expression of the known FoxO target *GADD45A*, while only JQ1 significantly regulated expression of *FAS* and *TNSF10*. Paired global analysis of the data sets demonstrated a high and significant degree of similarity ($R = 0.7583$, $P < 0.0001$) in the overall influence of LY294002 and JQ1 on macrophage gene expression (Fig 5C). These findings provide a molecular basis for how LY294002 can regulate macrophage gene expression independently of PI3K signaling.

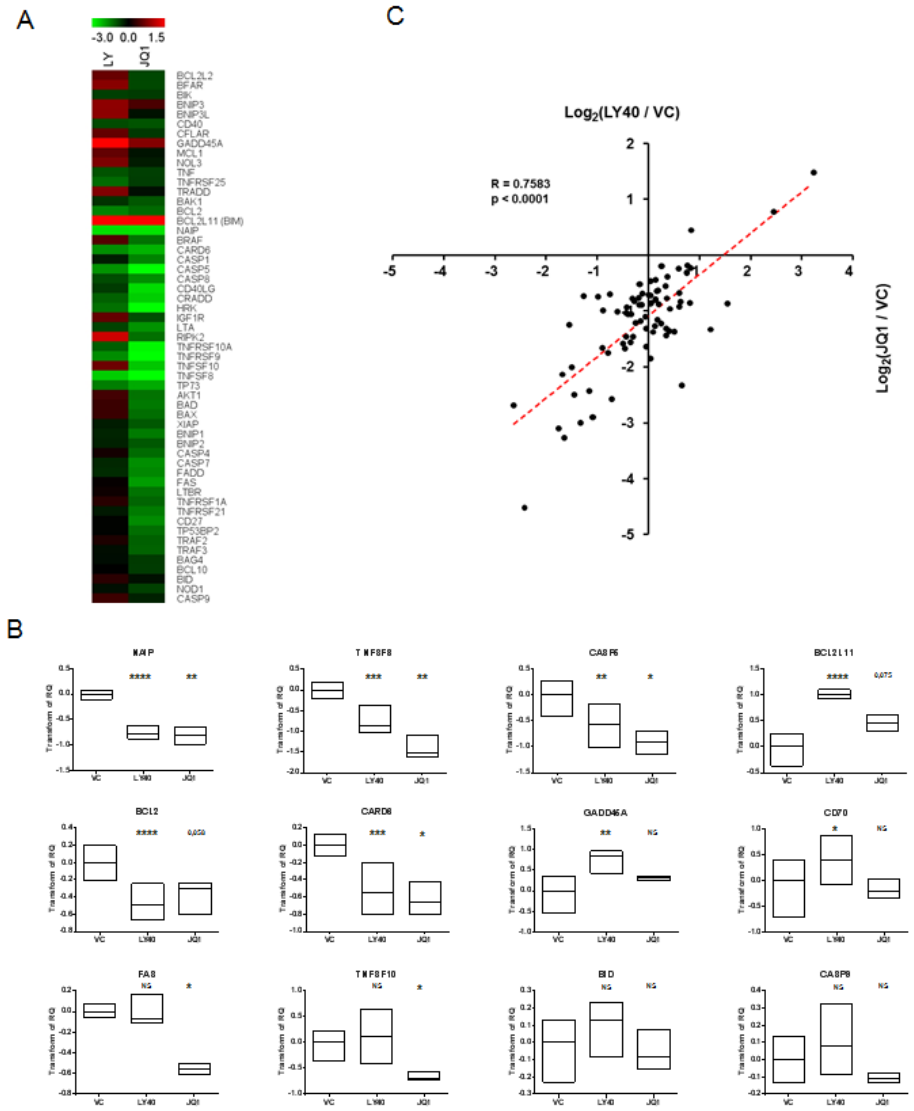


Figure 5. Effects observed with LY294002 on cell viability in human macrophages is partly dependent on bromodomain inhibition Healthy donor human macrophages were left untreated or treated with 40 μM LY294002 or 1 μM JQ1 for 24 h. Total RNA was extracted, reverse transcribed and gene expression analysed by low-density qPCR gene array. (A) Heatmap analysis of mRNA levels of IL1β induced genes significantly regulated by LY40 or JQ1. (B) Fold changes in expression of 84 genes were log transformed and correlation between mean values for I-BET and JQ1 analysed by Pearson correlation. Graphs of expression levels of specific selected genes are shown in (C). *p<0.05, **p<0.01, ***p<0.001.

Discussion

In the last decade our understanding of the basic framework of PI3K signaling has developed rapidly by making use of genetic approaches and pharmacological tools. PI3Ks and their downstream effectors have been implicated in multiple cellular processes such as control of cell growth and survival, nutrient uptake, proliferation, migration and differentiation.[42] PI3Ks, or namely their deregulated activation, are involved in the development of inflammatory and autoimmune diseases, such as diabetes, cancer, atherosclerosis and several rheumatological autoimmune diseases.[43-45] With a growing knowledge of the role of these lipid kinases and their downstream targets, targeted therapies of key intracellular signaling pathways become increasingly interesting. By blocking or inhibiting individual components of the PI3K signaling pathway, several cellular processes could be influenced and provide novel targets for therapeutic interventions.

For many years, the pan-PI3K inhibitor LY294002 has been used to show involvement of PI3Ks in multiple inflammatory processes. While initially proving valuable as a research tool, increasing appreciation of its off-target effects has complicated strategies to target PI3K signaling pathways in the clinic.[8;10;11] Given the widespread use of LY294002 in attempts to understanding PI3K involvement in health and disease, as evidenced by over 6800 PubMed reference hits, and the contributions of LY294002-dependent studies in advancing the design of more selective PI3K inhibitors, the contributions of this compound cannot be underestimated. However, the increasing number of studies reporting PI3K-independent effects of LY294002 would suggest that a reassessment of PI3K involvement in chronic inflammatory diseases such as RA, as a therapeutic target, is warranted.

In this study we show that LPS-induced production of pro-inflammatory cytokines IL6 and IL8 is not PI3K-dependent. Genetic deletion or pharmacological inhibition of PI3Kγ was reported to diminish macrophage activation as shown by lower production of nitric oxide (NO), IL1β and IL6 in a murine model of arthritis.[22] Also, in mast cells pro-inflammatory cytokines were shown to be PI3K-dependent as FCεRI-mediated release of IL6 and TNF were significantly reduced in mast cells derived from PI3Kδ KO mice or mast cells treated with a PI3Kδ inhibitor (IC87114).[46] Conversely it was reported that activation of PI3K upon binding of pattern recognition receptors by pathogen-associated molecular patterns, like LPS, dampens the production of pro-inflammatory cytokines in myeloid cells, suggesting that the PI3K pathway is a negative inflammatory feedback regulator.[26-31] In our study none of the isoform-selective inhibitors or LY294002

at 5x IC50 showed a positive or negative regulatory effect on IL6 or IL8. LPS-induced TNF production could only be partially ascribed to the involvement of specific class I PI3K catalytic subunits. Of the specific inhibitors tested, potential involvement of only PI3Kδ was observed. LY294002 did inhibit TNF up to 75%, however TNF production was not affected by PKBi, possibly suggesting off-target effects by LY294002. Likewise, LY294002 almost completely inhibited LPS-induced IL10 production, and again, only the class I PI3K-specific inhibitor D-000 exhibited an, albeit small, inhibitory effect. However, potential involvement of PI3K-dependent involvement in LPS-induced IL10 production was further suggested by the effects of PKB inhibition, which effectively suppressed IL10 production to levels observed with LY294002. As interactions of PI3K signaling with NFκB have been previously reported[36-38], we examined if effects on NFκB activation could explain effects we observed on LPS-induced cytokine expression. However, no effects of PI3K isoform-selective inhibition or LY294002 were observed on NFκB p65 DNA-binding following macrophage LPS stimulation. Our results suggest that, with the exception of potential small contributions of PI3Kδ to LPS-induced TNF and IL10 production, the effects of LY294002 on macrophage cytokine production are largely PI3K-independent.

Another possibility is that effects of LY294002 on LPS-induced cytokine production in macrophages is secondary to apoptotic effects. The PI3K signaling pathway is suggested to be important in macrophage survival and activation, as PI3K-dependent activation of PKB promotes RA synovial macrophage survival by upregulating anti-apoptotic Bcl-2 family protein Mcl-1 protein expression.[24;25] However, we demonstrated that macrophage cell survival is PI3K-independent as PI3K isoform selective inhibitors did not affect cell viability. Moreover, when combining all PI3K isoform selective inhibitors no effect on cell viability was observed. Cell viability was only affected by LY294002 at high concentrations. This was also reflected by the effects seen on expression levels of apoptosis related genes. In line with the strong apoptotic response we observed in macrophages treated with LY294002 in a higher concentration, 17 genes involved in apoptosis are affected by LY294002 at 40 μM. In contrast to what was published in RA synovial macrophages[24], we observe an upregulation of MCL1 gene expression upon inhibition by LY294002 at 7.5 μM in healthy donor macrophages.

The effects seen by LY294002 could possibly be explained by the observation that the compound also possesses BET bromodomain inhibitory capacity.[12] A well-known BET inhibitor, JQ1 has been described to affect LPS-induced IL6, TNF and IL10 production, but not IL8 production in murine macrophages.[39] Possibly, effects seen with LY294002

at high concentrations are PI3K-independent and could be explained by the BET inhibitory capacity of LY294002. The fact that IL8 is not affected could be pointing towards selectivity in BET bromodomain inhibition. Indeed, we show that a broad analysis of genes regulated by LY294002 and JQ1 in human macrophages clearly shows a strong and significant overlap in gene expression patterns. Not all genes regulated by LY294002 were also regulated by JQ1, consistent with LY294002 having other well-known off-targets including mTOR and DNA-PK.[8;10;15] Conversely, not all JQ1 targets were also regulated by LY294002. This is likely due to the observation that while JQ1 binds to both bromodomains of BET family members, LY analogues show preferential binding to one of the two bromodomains.[40] However, the data presented here provide a molecular basis for the effects of LY294002 on macrophage gene expression. Within the limitations of our study, we find that the previously ascribed effects of LY294002 on macrophage activation and survival cannot be reproduced with specific PI3K catalytic subunit inhibitors, perhaps suggesting that targeting enzymes downstream of PI3K may be more effective strategies in the treatment of chronic autoimmune diseases such as RA.

Acknowledgments

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Authorship

Contributions: L.M.H., P.K., and S.K. contributed to research design, performed experiments, analyzed data and contributed to writing the paper; I.E. van E. performed experiments and analyzed data; P.P.T. and K.A.R. designed research, analyzed and interpreted data, and contributed to writing the manuscript.

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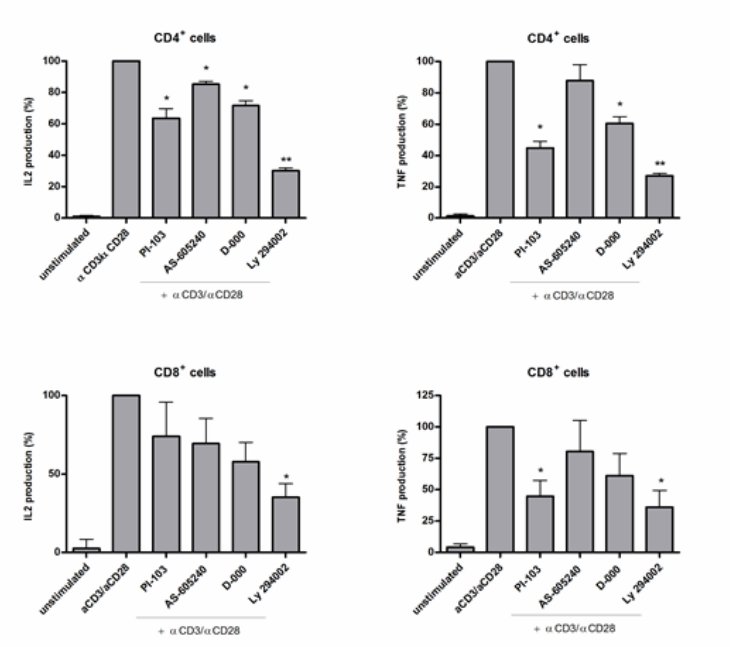
SUPPLEMENTAL

PI3K isoform specific inhibition affects functional responses in human T-cells

To study the involvement of PI3K p110 isoforms in cytokine production and migration in T cells, human peripheral blood T-cells were stimulated and incubated with isoform-specific PI3K inhibitors and the pan-PI3K inhibitor LY294002. (Supplemental figure 1) In stimulated CD4+ T cells, inhibition of all class I PI3K significantly suppressed production of the Th1 cytokines, IL2 and TNF ($P < 0.05$), although we observed a stronger effect of almost 70% inhibition after treatment with LY294002 ($P < 0.01$). A trend towards decreased IL2 and TNF production was observed when CD8+ T-cells were treated with the isoform-specific inhibitors. Again, in the presence of LY294002, reduction of IL2 and TNF production was more pronounced ($P < 0.05$) compared to isoform-specific inhibition.



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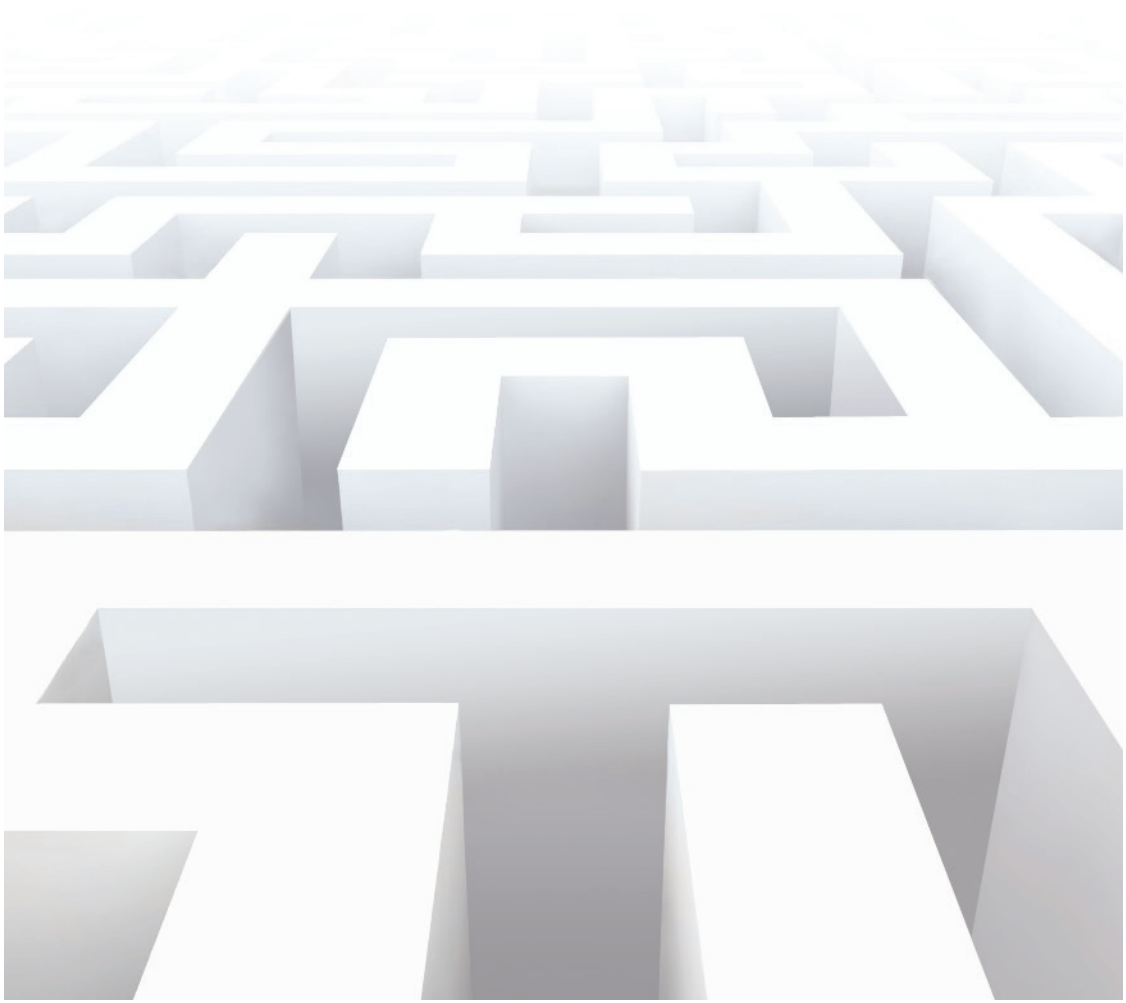

3

JNK-dependent downregulation of FoxO1 is required to promote the survival of fibroblast-like synoviocytes in rheumatoid arthritis

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ABSTRACT

Background: Forkhead box O (FoxO) transcription factors integrate environmental signals to modulate cell proliferation and survival, and alterations in FoxO function have been reported in rheumatoid arthritis (RA).

Objectives: To examine the relationship between inflammation and FoxO expression in RA, and to analyze the mechanisms and biological consequences of FoxO regulation in RA fibroblast-like synoviocytes (FLS).

Methods: RNA was isolated from RA patient and healthy donor (HD) peripheral blood and RA synovial tissue. Expression of FoxO1, FoxO3a and FoxO4 was measured by qPCR. FoxO1 DNA binding, expression and mRNA stability in RA FLS were measured by ELISA-based assays, immunoblotting and qPCR. FLS were transduced with adenovirus encoding constitutively active FoxO1 (FoxO1ADA) or transfected with siRNA targeting FoxO1 to examine the effects on cell viability and gene expression.

Results: FoxO1 mRNA levels were reduced in RA patient peripheral blood compared to HD, and RA synovial tissue FoxO1 expression negatively correlated with disease activity. RA FLS stimulation with IL-1β or TNF caused rapid downregulation of FoxO1. This effect was independent of protein kinase B (PKB), but dependent upon c-Jun N-terminal kinase (JNK)-mediated acceleration of FoxO1 mRNA degradation. FoxO1ADA overexpression in RA FLS induced apoptosis associated with altered expression of genes regulating cell cycle and survival, including BIM, p27Kip1, and Bcl-XL.

Conclusions: Our findings identify JNK-dependent modulation of mRNA stability as an important PKB-independent mechanism underlying FoxO1 regulation by cytokines, and suggest that reduced FoxO1 expression is required to promote FLS survival in RA.

Introduction

Aberrant regulation of genes and pathways involved in cellular proliferation and survival makes pivotal contributions to development and perpetuation of inflammation in chronic immune-mediated inflammatory diseases (IMIDs), including systemic lupus erythematosus (SLE), type I diabetes and rheumatoid arthritis (RA).[1;2] In RA, macrophages and stromal fibroblast-like synoviocytes (FLS) display increased resistance to apoptotic stimuli, which promotes their accumulation in the inflamed joint, as well as release of products responsible for bone and cartilage damage.[3;4] Because of their ability to promote cellular survival and proliferation, and evidence of their enhanced activation in RA synovial tissue, phosphatidylinositol 3-kinases (PI3Ks) have emerged as potential therapeutic targets.[5;6] Protein kinase B (PKB, also known as Akt), a downstream target of PI3K, modulates cell fate choices through multiple mechanisms, among which is regulation of forkhead box O (FoxO) transcription factors. FoxO transcriptional targets include genes regulating cellular metabolism, response to oxidative stress, proliferation and apoptosis. FoxO-dependent gene expression profiles are cell type- and stimulus-specific, and unique combinations of environmental signals trigger transcriptional programs which either promote cell cycle arrest and survival in response to stress, or induce apoptosis.[7;8]

The activity of three ubiquitously expressed FoxO family members, FoxO1, FoxO3a and FoxO4, is regulated by a complex interplay between phosphorylation, acetylation, ubiquitilation and other post-translational modifications.[9] PKB-mediated FoxO phosphorylation disrupts FoxO interaction with target DNA sequences and facilitates nuclear export, leading to FoxO inactivation. Reciprocally, FoxO phosphorylation by mitogen-activated protein kinases (MAPKs) promotes FoxO nuclear retention and transcriptional activity. Also, reversible FoxO acetylation modulates FoxO DNA-binding affinity and gene target specificity.[9] FoxO proteins are regulated not only through posttranslational mechanisms, but also transcriptionally. PI3K-PKB signaling downregulates FoxO1 levels in B cells and fibroblasts,[10;11] and involvement of other signaling pathways in the regulation of FoxO expression has recently been reported.[12;13] Persistent activation of the PI3K-PKB axis in the inflamed synovium, observed both in animal arthritis models and in RA synovial tissue,[14-16] might contribute to RA pathology partly through the inactivation of FoxO transcription factors. PKB-inactivated FoxO proteins are detected in RA synovial tissue, and synovial macrophages express elevated levels of inactive FoxO4 compared with disease controls.[17] Peripheral blood mononuclear cells (PBMCs) of RA patients express lower levels of FoxO1 compared to healthy individuals,[18] and hypermethylation of the FoxO1 gene has been reported

in RA FLS.[19] In another IMID, reduced FoxO4 expression has been reported in colonic epithelial cells of patients with inflammatory bowel disease compared with healthy controls.[20] The importance of FoxO proteins in maintaining immune system homeostasis is further supported by animal studies. FoxO3a-deficient mice develop spontaneous autoimmunity due to persistent T-cell proliferation and survival,[21] while deletion of both FoxO1 and FoxO3a in T-cells leads to defective regulatory T cell development and multifocal inflammatory disease.[22] Finally, mice lacking FoxO4 develop more severe mucosal inflammation compared to wild-type mice in a colitis model.[20] Despite this, the molecular mechanisms leading to changes in FoxO expression and function in chronic IMIDs have not been characterized. The present study was undertaken to understand the relationships between FoxO expression and disease activity in RA, to identify mechanisms underlying inflammation-mediated regulation of FoxO proteins, and to assess the biological consequences of alterations in FoxO function in RA FLS.

MATERIALS AND METHODS

Subjects, peripheral blood and synovial tissue samples

Peripheral blood (PB) was collected from a previously described cohort of 10 methotrexate-naïve RA patients and 15 healthy individuals.[23] Synovial biopsy specimens were obtained by arthroscopy from actively inflamed joints of 19 RA patients fulfilling the 1987 revised criteria of the American College of Rheumatology[24] as described before,[25] embedded in TissueTek OCT (Miles Diagnostics, Elkhart, IN), snap-frozen and stored in liquid nitrogen. The study was approved by the institutional review board and all patients provided written informed consent. Clinical characteristics of patients are detailed in Table 1.

Characteristic	Median (range)
Age (y)	60 (38-67)
Male:female (n)	6:13
Disease duration (months)	86.5 (1-409)
ESR (mm/h)	19 (4-119)
CRP (mg/l)	9.1 (1-122.6)
DAS28	4.64 (1.87-7.49)

Table 1 Clinical features of RA patients (n = 19) included in the study.
*ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = disease activity score 28.

Cell culture and stimulations

FLS were isolated from synovial biopsies of RA patients and cultured as previously described.[26] After overnight culture in medium containing 1% fetal bovine serum (Invitrogen, Breda, The Netherlands) FLS between passages 4 and 9 were stimulated with IL-1β, PDGF (both from R&D Systems, Minneapolis, MN), TNF (Biosource International, Camarillo, CA) or LPS (Sigma-Aldrich). Activation of signaling pathways was blocked using PKB inhibitor-VIII, SB203580, U0126, SP600125, AS601245 or JNK inhibitor-IX (all from Calbiochem/EMD Millipore, Billerica, MA). Transcription was blocked by actinomycin D (Sigma-Aldrich). Q-VD-OPh (R&D Systems) was used to inhibit caspase activity.

mRNA expression analyses

RNA extraction from PB and large-scale expression profiling by cDNA microarrays was described previously.[23] RNA was isolated from FLS and synovial tissue biopsies as described before,[27;28] and mRNA expression levels were determined by quantitative (q) PCR or by RT2 Profiler PCR Arrays (SABiosciences-Qiagen, Frederick, MD) as described in detail in the supplementary methods.

Protein extraction, immunoblotting and FoxO1 DNA-binding

Protein extracts prepared by lysis in Laemmli's buffer were resolved by electrophoresis on 4-12% Bis-Tris SDS-NuPAGE gels (Invitrogen) and analyzed by immunoblotting as described in the supplementary methods. Nuclear fractions were prepared as described before,[28] and FoxO1 DNA-binding activity was determined using a TransAM ELISA (Active Motif, Carlsbad, CA).

Overexpression and knockdown of FoxO1

Control adenoviruses encoding GFP (provided by Dr SW Tas, our institute)[29] and adenoviruses encoding constitutively active FoxO1 mutant (FoxO1ADA, provided by Dr. D Accili, Columbia University, New York)[30] were amplified and titrated in transcomplemental HEK-293 cells and purified with cesium chloride gradient ultracentrifugation as described previously.[31] To silence FoxO1 expression, FLS were transfected with control or FoxO1-specific siRNAs (Dharmacon, Schwerte, Germany) using the DharmaFECT-1 transfection reagent (Dharmacon).

Measurements of cell viability and apoptosis

Cell viability was determined by MTT reduction assay as described previously.[28] Induction of apoptosis was assessed using Cell Death Detection ELISA (Roche Diagnostics, Mannheim, Germany).

Statistical analyses

Data are presented as mean±SEM unless otherwise indicated. The Spearman's correlation coefficient was used for correlation analyses. An overall Kruskall-Wallis test and Dunns' post-hoc test were used for analyzing sets of data requiring multiple comparisons. The Mann-Whitney U test was used for all other comparisons. p values ≤0.05 were considered statistically significant.

RESULTS

FoxO1 expression in RA synovial tissue negatively correlates with clinical parameters and IL-6 expression.

A recent study demonstrating selective reduction of FoxO1 levels in RA PBMCs suggested that alterations in FoxO1 expression might be involved in RA pathology.[18] Here, we compared peripheral blood FoxO1 and FoxO3a mRNA expression in 10 methotrexate-naive RA patients and 15 healthy donors (HD) previously subjected to global gene expression profiling.[23] While no differences were observed between HD and RA patients in FoxO3a expression, FoxO1 levels were significantly reduced in RA patients compared to HD (figure 1A,B). Analysis of mRNA obtained from RA patient synovial biopsies revealed significant negative correlations between synovial FoxO1 mRNA levels and patient C-reactive protein (CRP) serum levels (figure 1C; R=-0.771, P=0.0008), erythrocyte sedimentation rate (ESR) (figure 1D; R=-0.739, P=0.0003), and 28-joint disease activity score (DAS28) (figure 1E, R=-0.575, P=0.01). A strong negative relationship was also noted between synovial FoxO1 expression and local expression of interleukin (IL)-6 (figure 1F: R=-0.628, P=0.004).

Inflammatory stimulation rapidly downregulates FoxO1 expression in RA FLS

These initial data raised the possibility that during inflammation in RA, FoxO proteins may be regulated not only by PKB-dependent phosphorylation, but also through changes in FoxO expression. To test this possibility we stimulated RA FLS, which express high levels of FoxO1 compared to other cell types present in the synovial tissue,[17] with IL-1β, TNF and LPS for 24 h and analyzed expression of FoxO family members. Both IL-

1β and TNF caused an 80% downregulation of FoxO1 mRNA expression ($p<0.01$), while LPS treatment led to only a modest reduction (figure 2A). Inflammatory stimulation failed to modulate FoxO3a expression, while FoxO4 levels, expressed at lower levels in FLS than synovial macrophages,[17] were significantly affected only by TNF (figure 2B,C). FoxO1 transcript levels were reduced by 25% following 2 h stimulation, and decreased further over time (figure 2D). Consistent with changes at the mRNA level, exposure to IL-1β caused a downregulation of FoxO1 protein levels (figure 2E), as well as a reduction in the amount of active FoxO1. A ~50% inhibition of FoxO1 DNA binding was observed within 4 h after FLS exposure to IL-1β ($p<0.05$), and this effect was maintained at later time points (figure 2F). These results indicate that FoxO1 expression and functional activity is rapidly reduced in IL-1β-stimulated RA FLS.

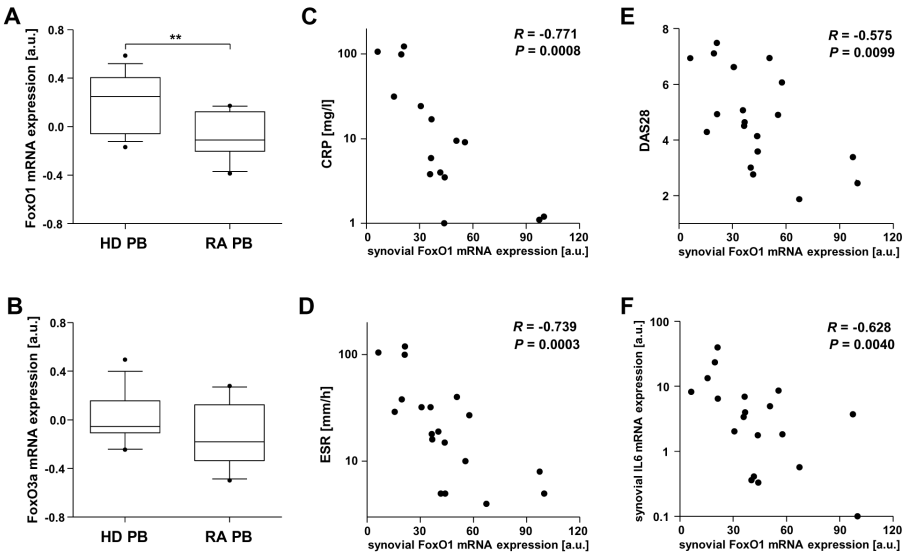


Figure 1. FoxO1 mRNA levels are reduced in RA peripheral blood and FoxO1 expression in RA synovial tissue negative correlates with disease activity.

Total RNA was extracted from peripheral blood (PB) from 15 healthy donors (HD) and 10 methotrexate-naïve RA patients, and expression levels of (A) FoxO1 and (B) FoxO3a expression were retrieved from the results of gene array analysis.[23] Data are presented as boxplots where the boxes represent the 25th and 75th percentiles, the line within the box denote the median value, and the lines outside of the box mark the 10th and 90th percentiles. **p<0.01, Mann-Whitney U test. (C-F) mRNA was isolated from synovial tissue specimens from 19 RA patients, cDNA was synthesized and expression of FoxO1 and IL-6 determined by qPCR. Correlations of relative FoxO1 expression with (C) C-reactive protein (CRP) levels, (D) erythrocyte sedimentation rate (ESR), (E) 28-joint disease activity score (DAS28) and (F) IL-6 levels were calculated using Spearman's rank correlation coefficient. Circles indicate individual patient values, and Spearman R and P values are indicated in each graph.

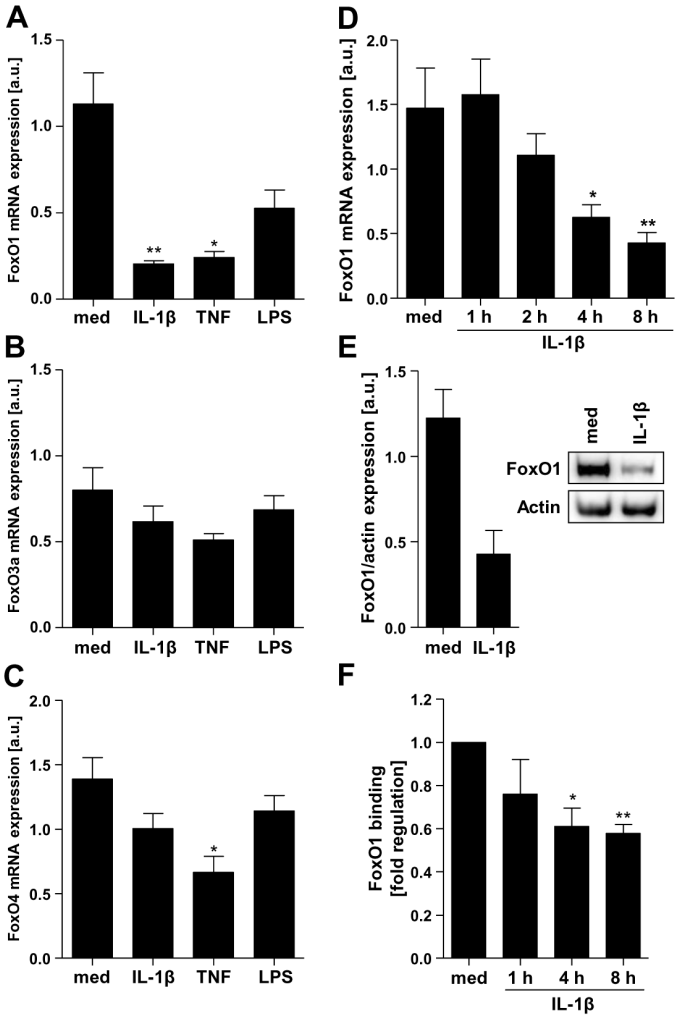


Figure 2. Inflammatory stimulation reduces FoxO1 expression and activity in RA FLS.
(A) RA FLS (n = 4) were either left unstimulated in medium (med) or were stimulated with 1 ng/ml IL-1β (A), 10 ng/ml TNF (B) or 1 μg/ml LPS (C) for 24 h, total RNA was extracted, reverse transcribed and changes in FoxO1, FoxO3a, or FoxO4 mRNA accumulation were monitored by qPCR. Data are presented as mean±SEM relative FoxO1 expression. (D) Alternatively, RA FLS (n = 6) were left untreated (med) or were stimulated with IL-1β (1 ng/ml) for the indicated time (h), and FoxO1 mRNA expression analyzed as above. The graph represents mean±SEM relative FoxO1 expression. (E) RA FLS (n = 3) were left untreated (med) or were stimulated with IL-1β (1 ng/ml) for 24 h, protein extracts were prepared and analyzed by immunoblotting with anti-FoxO1 and anti-actin antibodies. Signal intensity was then quantified and the results of densitometric analysis are shown as mean±SEM FoxO1 expression relative to actin (left panel). Additionally, one experiment representative of 3 independent experiments is shown (right panel). (F) RA FLS were stimulated as in (B), nuclear fractions were extracted and levels of active FoxO1 were determined using a TransAM transcription factor DNA binding assay. Data represent the mean ±SEM fold change in FoxO1 DNA binding of 4 to 6 independent experiments. *p<0.05, **p<0.01, Kruskal-Wallis test followed by Dunns' multiple comparison analysis.

PKB-independent, JNK-mediated acceleration of FoxO1 mRNA degradation is responsible for the downregulation of FoxO1 expression by cytokines in RA FLS

We next analyzed the potential involvement of signaling and regulatory pathways typically activated by cytokines in RA FLS to identify the molecular mechnism(s) underlying FoxO1 downregulation by IL-1β. Because FoxO transcription factors are capable of regulating their own expression,[10] we reasoned that the observed reduction of FoxO1 levels might be secondary to PKB-dependent FoxO1 inactivation. RA FLS were stimulated with either IL-1β or with platelet-derived growth factor (PDGF) in the absence or presence of a PKB inhibitor (PKBi). Compared to PDGF, IL-1β caused only a minor induction of PKB phosphorylation, consistent with other studies (figure 3A),[32] and PKBi did not rescue FoxO1 expression in the presence of IL-1β (figure 3B). Strikingly, while PDGF stimulation also reduced FoxO1 expression ($p<0.05$) (figure 3B), inhibition of PKB activity failed to prevent this effect, despite blocking PKB phosphorylation (figure 3A) and inactivation of FoxO1 DNA-binding activity (figure 3C). Together, these results indicated that IL-1β mediates FoxO1 downregulation at the mRNA level independently of PI3K-PKB signaling. We next tested whether cytokine-mediated FoxO1 downregulation is dependent on MAPK signaling pathways. While inhibition of p38 or extracellular signal-regulated kinase (ERK) activity failed to significantly affect FoxO1 expression levels in IL-1β-stimulated FLS, JNK inhibition almost completely prevented IL-1β-dependent reduction of FoxO1 mRNA levels ($p<0.05$) (figure 3D). Similarly, JNKi rescued RA FLS FoxO1 DNA-binding activity and protein expression following IL-1β stimulation (figure 3E,F). The dose-dependent and specific inhibition of c-Jun phosphorylation by JNKi was confirmed by immunoblotting (figure 3F,G). Use of a second pan-JNKi, but not a JNK2/3-selective inhibitor, also rescued FoxO1 mRNA expression, indirectly suggesting that JNK1 may have a predominant role in regulation of FoxO1 (supplementary figure 1).

Analysis of 3' untranslated regions (UTRs) revealed the presence of AU-rich elements (ARE) in the transcripts of FoxO family members. To determine if inflammatory cytokines modulated FoxO1 mRNA stability, we analyzed degradation of FoxO transcripts after blocking transcription with actinomycin D (ActD). A comparison of mRNA stability of FoxO transcription factors in the presence of IL-1β revealed that the FoxO1 transcript was comparatively unstable, with a half-life of approximately 2h. In contrast, half-lives of FoxO3a and FoxO4 were longer than 4 h (figure 4A). Additionally, FoxO1 mRNA degradation was accelerated when RA FLS were exposed to IL-1β, compared to unstimulated cells (figure 4B). Treatment of RA FLS with JNKi completely prevented IL-1β-induced acceleration of FoxO1 mRNA degradation (figure 4C), indicating that IL-1β-

mediated FoxO1 downregulation is regulated by the JNK pathway through modulation of FoxO1 mRNA stability.

FoxO1 activation modulates expression of pro- and anti-apoptotic genes and induces apoptosis in RA FLS

To examine the effect of rescuing FoxO1 expression and function on RA FLS activation and survival, we transduced RA FLS with an adenoviral vector encoding constitutively active FoxO1 (FoxO1ADA). Overexpression and biological activity of FoxO1ADA were confirmed by immunoblotting and FoxO1 DNA-binding assay, respectively (figure 5A,B). FoxO1ADA reduced FLS viability by approximately 50% ($p<0.05$) (figure 5C), associated with elevated levels of cytosolic histone-complexed DNA fragments, a hallmark of apoptotic cell death (figure 5D). FoxO1-induced apoptosis was fully dependent on caspase activation (figure 5D). In FoxO1ADA-transduced cells, we observed altered expression of genes involved in regulation of apoptosis and cell cycle progression, many of which are known FoxO targets.[8] Namely, FoxO1ADA reduced the mRNA levels of the Bcl-2-like protein Bcl-XL, and enhanced expression of BIM and the cell cycle inhibitor p27Kip1 compared to control GFP-transduced cells (figure 5E). FoxO1ADA also moderately increased mRNA expression of MnSOD in unstimulated FLS and prevented the induction of another Bcl-2 family member, Bfl-1/A1, while leaving FLIP expression unaffected (figure 5E). Changes in Bcl-XL, p27Kip1 and BIM expression following FoxO1ADA overexpression were confirmed at the protein level (figure 5F), and, reciprocally, Bcl-XL protein levels were increased in FLS after siRNA-mediated FoxO1 knockdown (figure 5G). However, FoxO1-silencing failed to influence effects of JNKi on Bcl-XL expression, indicating that FoxO1 inactivation is required but not sufficient to regulate Bcl-XL under inflammatory conditions (supplementary figure 2). Together, these data indicate that inflammatory cytokines promote the survival of RA FLS in part via reducing the expression and activity of FoxO1.

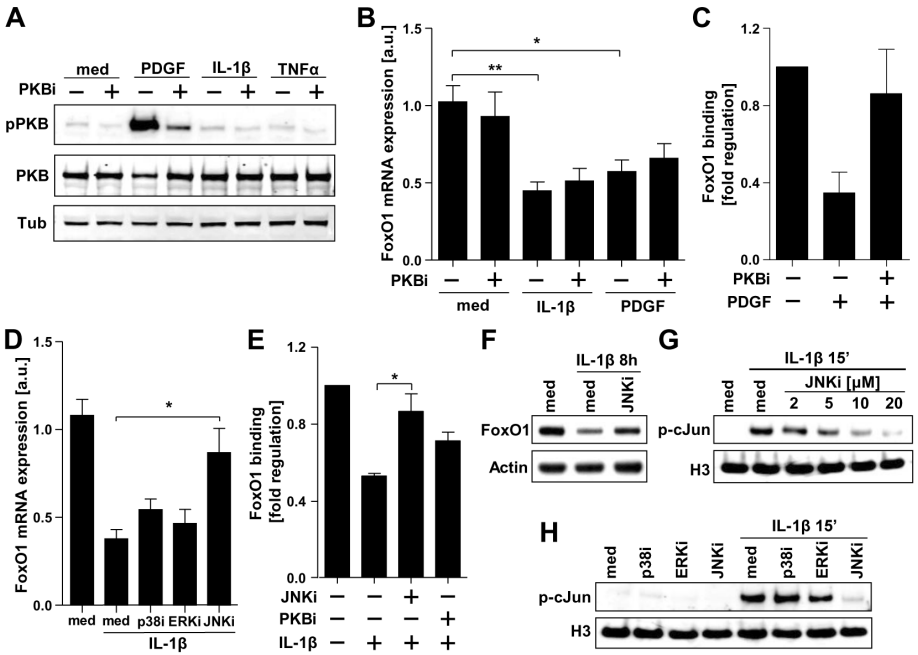
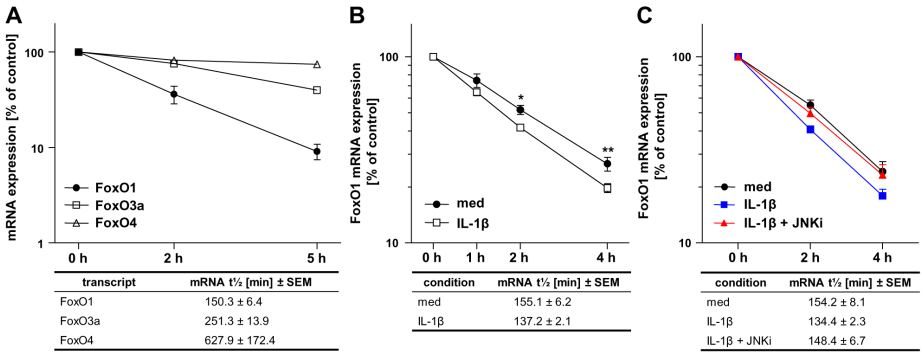


Figure 3. c-Jun N-terminal kinase (JNK) mediates downregulation of FoxO1 expression by IL-1β independently of PI3K-PKB signaling.

(A) RA FLS were left untreated (med) or were treated with 10 μM PKB inhibitor VIII (PKBi) for 30 min prior to stimulation with IL-1β (1 ng/ml), TNF (10 ng/ml) or PDGF (10 ng/ml) for 30 min. Protein extracts were prepared and analyzed by immunoblotting with antibodies recognizing phospho-(p)PKB, PKB and tubulin (Tub). (B) RA FLS (n = 7) were left unstimulated (med) or were incubated with 10 μM PKBi for 30 min followed by stimulation with 1 ng/ml IL-1β or 10 ng/ml PDGF for 4 h. RNA was extracted, cDNA was synthesized and changes in FoxO1 mRNA levels were assessed by qPCR. Data are presented as the mean±SEM relative FoxO1 expression. (C) RA FLS were left untreated (med) or were stimulated 1 ng/ml IL-1β in with or without PKB inhibitor VIII (PKBi, 10 μM) for 2 h, nuclear fractions were extracted and levels of active FoxO1 were determined using an ELISA-based DNA-binding assay. Results are presented as the mean ±SEM fold change in FoxO1 DNA binding of 3 independent experiments. (D) RA FLS (n = 6) were left unstimulated (med) or were treated with SB203580 (p38i, 10 μM), U0126 (ERKi, 10 μM) or SP600125 (JNKi, 20 μM) for 30 min and then stimulated with 1 ng/ml IL-1β for 4 h. FoxO1 expression was analyzed by qPCR and presented as the mean±SEM relative FoxO1 expression. (E) RA FLS were left untreated (med) or were treated with SP600125 (JNKi, 20 μM) or PKB inhibitor VIII (PKBi, 10 μM) for 30 min prior to stimulation with IL-1β (1 ng/ml) for 8 h, nuclear extracts were collected and FoxO1 DNA binding activity was measured as in C. Data represent the mean±SEM fold change in FoxO1 DNA binding of 4 independent experiments. (F) RA FLS were treated with IL-1β (1 ng/ml) in the absence or presence of SP600125 (JNKi, 20 μM) for 8 h. Protein extracts were prepared and protein expression of FoxO1 and acting was analyzed by immunoblotting. (G) Alternatively, RA FLS were incubated with increasing concentrations of JNKi (2-20 μM) or (H) with p38i (10 μM), ERKi (10 μM) or JNKi (20 μM) for 30 min before stimulation with 1 ng/ml IL-1β for 15 min. Cells were then lysed and protein extracts were subjected to analysis by immunoblotting with anti-phospho(p) c-Jun or anti-histone (H)3 antibodies. *p<0.05, **p<0.01, Kruskal-Wallis test followed by Dunns' multiple comparison analysis. All immunoblotting analyses show a representative of at least 3 independent experiments.



transcript	mRNA t½ [min] ± SEM
FoxO1	150.3 ± 6.4
FoxO3a	251.3 ± 13.9
FoxO4	627.9 ± 172.4

condition	mRNA t½ [min] ± SEM
med	155.1 ± 6.2
IL-1β	137.2 ± 2.1

condition	mRNA t½ [min] ± SEM
med	154.2 ± 8.1
IL-1β	134.4 ± 2.3
IL-1β + JNKi	148.4 ± 6.7

Figure 4. IL-1β-mediated reduction of FoxO1 mRNA stability is mediated via the c-Jun N-terminal kinase (JNK).

For mRNA stability studies RA FLS were left unstimulated or were stimulated with IL-1β (1 ng/ml) for 2 h before 10 µg/ml actinomycin D (ActD) was added to block transcription. RNA was extracted at indicated time points from the start of ActD treatment (0 h) and the rates of (A) FoxO1, FoxO3a and FoxO4 mRNA degradation in the presence of IL-1β (n = 4) or (B) the rates of FoxO1 mRNA decay in the absence and presence of IL-1β (n = 6) were analyzed by qPCR. Expression values for the 0 h time point were normalised to 100%, and remaining values were presented as the mean ±SEM percentage of mRNA levels compared with controls. *p<0.05; **p<0.01, Mann-Whitney U test. (C) RA FLS were either left unstimulated (med) or were stimulated with 1 ng/ml IL-1β for 2 h in the presence or absence 20 µM JNKi. Transcription was blocked with 10 ng/ml ActD and changes in FoxO1 mRNA stability analyzed as above. The results of 4 independent experiments are depicted as the mean percentage ±SEM of FoxO1 transcript levels compared to the time point 0 h. (A-C) For each individual experiment the mRNA half-life was estimated using linear regression and tabular presentation of the mean mRNA half-life±SEM [min] for each experimental condition is included under the graphs.

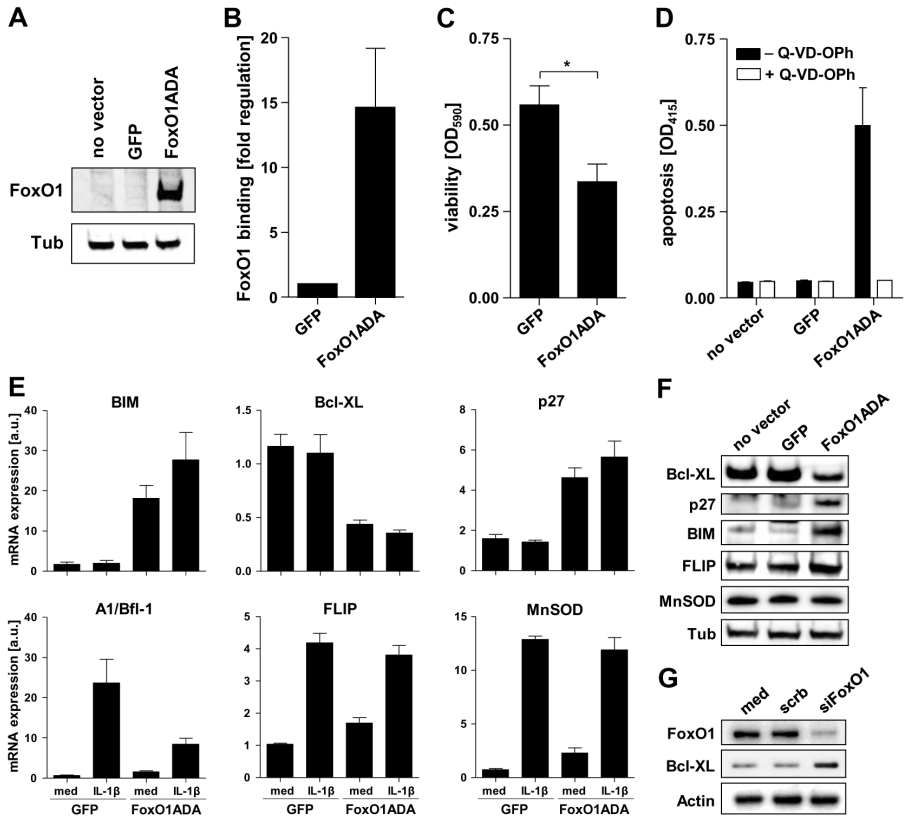


Figure 5. Constitutive activation of FoxO1 modulates expression of pro- and anti-apoptotic genes in RA FLS and induces cell death.

FLS were transduced with the control GFP-encoding or the FoxO1ADA-encoding adenoviral vector at a multiplicity of infection of 500 for 48 h and serum-starved in medium containing 1% FBS for another 24 h prior to further processing. (A) Transduced cells were lysed and protein extracts were analyzed by Western blotting with antibodies specific for FoxO1 and tubulin (Tub). (B) Nuclear fractions were extracted and transcriptional activity of FoxO1 was assessed using an ELISA-based DNA binding assay. The data are shown as the mean±SEM fold change in FoxO1 DNA binding of 3 independent experiments. Viability and induction of apoptosis in RA FLS transduced with GFP or FoxO1ADA-expressing vectors were determined using a MTT reduction assay (C) and Cell Death ELISA (D), respectively. Apoptosis was assessed in the absence or presence of the pan-caspase inhibitor Q-VD-OPh (20 μM). Data are presented as mean optical densities ± SEM at 590 nm (C) and 415 nm (D) and show results of 5 and 3 independent experiments, respectively. *p<0.05, Mann-Whitney U test. (E) Transduced FLS (n = 4) were cultured for 24 h in medium containing 1% FBS, and then were either left unstimulated (med) or stimulated with 1 ng/ml IL-1β for 4 h. Total RNA was then extracted, reverse transcribed, and relative mRNA expression levels of BIM, Bcl-XL, p27Kip1, A1/Bfl-1, FLIP and MnSOD were determined by quantitative PCR using a customized RT2 Profiler™ PCR Array. Data are shown as mean±SEM relative mRNA expression compared to unstimulated cells transduced with the control GFP-expressing vector. (F) Protein extracts from unstimulated RA FLS transduced with GFP or FoxO1ADA vectors were analyzed by immunoblotting with antibodies specific for Bcl-XL, p27Kip1, BIM, FLIP, MnSOD and tubulin (Tub). RA FLS were left untreated (med) or were transfected with control non-targeting siRNA (scrb, 20 nM) or siRNA specific for FoxO1 (siFoxO, 20 nM). After 48 h cells were lysed and expression levels of FoxO1, Bxl-XL and actin were analyzed by immunoblotting. All immunoblotting analyses show a representative of at least 3 independent experiments.

Discussion

FoxO transcription factors integrate growth, inflammatory and stress signals to control cell fate decisions. In the absence of growth factors, cytokines and antigen receptor signaling, all of which activate the PI3K-PKB pathway, FoxO family members localize to the nucleus to modulate expression of genes regulating cell cycle progression, proliferation and survival. Genes involved in cellular inflammatory responses are also direct FoxO targets,[8] and studies in animals lacking FoxO proteins have provided evidence for essential roles of FoxO transcriptional activity in the immune system.[33] In RA, reduced expression and PKB-mediated inactivation of FoxO family members has been reported,[17;18] and in SLE FoxO1 expression in PBMCs negatively correlates with disease severity,[18] indicating FoxO involvement in protection against autoimmune processes. Here, we show that both peripheral and synovial FoxO1 expression is strongly associated with pathology in RA, as synovial FoxO1 expression negatively correlates with clinical parameters of disease activity and local IL-6 expression. FoxO1 expression is rapidly reduced after inflammatory stimulation of RA FLS, and we identify JNK-mediated acceleration of FoxO1 mRNA degradation as a novel mechanism regulating FoxO1 expression.

Initial observations in murine B cells, which downregulate FoxO1 mRNA expression in response to B cell receptor activation, indicated that the PI3K-PKB pathway is at least partly responsible for this effect.[11] The promoter region of FoxO1 contains a conserved FoxO-binding motif that is indispensable for FoxO1 transcriptional induction by itself and by FoxO3a. Growth factor-mediated activation of PI3K/PKB signaling and FoxO nuclear exclusion repress the expression of FoxO1 in human fibroblasts, demonstrating that FoxO activity is required for maintaining FoxO1 expression in some cell populations.[10] However, we find that PKB inhibition fails to influence FoxO1 expression in IL-1β or PDGF-stimulated RA FLS. Moreover, overexpression of FoxO1ADA did not induce endogenous FoxO1 expression (data not shown), arguing against a positive feedback loop mode of FoxO regulation in RA FLS. Instead, we show that the FoxO1 transcript is relatively unstable compared to other FoxO family members and that degradation of FoxO1 mRNA is significantly accelerated upon IL-1β stimulation in a JNK-dependent fashion. Although JNK signaling is generally associated with mRNA stabilization, as observed in the case of several cytokines and growth factors,[34;35] a destabilizing role for JNK has also been reported for a small number of transcripts.[36;37] The latter effects have been associated with JNK-dependent induction of ARE-binding proteins, such as TTP and AUF1. Although we observed rapid, JNK-dependent increases in TTP protein levels in IL-1β-stimulated RA FLS, silencing of TTP expression with siRNA failed to prevent IL-

1β-induced downregulation of FoxO1 mRNA levels (data not shown). Interestingly, JNK can also directly regulate one member of the FoxO family, as FoxO4 phosphorylation by JNK results in nuclear translocation and transcriptional activation in cells subjected to oxidative stress.[38] While endoplasmic reticulum stress has also been reported to induce FoxO1 activation via a JNK-dependent pathway,[39] FoxO1 is not subject to phosphorylation by JNK, suggesting that JNK affects FoxO signaling through multiple direct and indirect mechanisms in a context-dependent manner.[40]

Notably, JNK signaling in RA synovial tissue is activated early in the course of disease.[27] JNK activation in several cell types, including FLS, contributes to pathology in part through cellular production of inflammatory cytokines and MMPs.[41] The strong negative association between FoxO1 expression and disease severity observed in our study supports a model in which the inflammatory processes in RA synovial tissue induce JNK-dependent FoxO1 downregulation in FLS, promoting FLS accumulation and persistence. In line with this possibility, we find that overexpression of FoxO1ADA induces apoptosis of RA FLS associated with altered expression profiles of two Bcl-2 family members known to be directly or indirectly regulated by FoxO proteins, pro-apoptotic BIM and anti-apoptotic Bcl-XL.[8] Of these, Bcl-XL is known to be elevated in the intimal lining layer in RA synovial tissue compared to OA.[42] Reduced FoxO1 expression might also promote FLS proliferation through downregulation of the cell cycle inhibitor p27Kip1, as well as sensitize FLS to oxidative stress and prevent DNA repair processes through downregulation of MnSOD.[8] RA FLS express lower levels of MnSOD mRNA compared to OA FLS,[43] which may contribute to the accumulation of oxidative stress-induced mutations in tumor suppressor genes, such as p53, detected in RA FLS and synovial tissue.[44] It is unlikely however that FoxO1 downregulation is sufficient by itself to promote RA FLS survival and proliferation. For example, the Bcl-XL gene locus is also sensitive to the JNK-dependent AP-1 transcription factor.[45;46] In preliminary experiments, we were unable to enhance RA FLS proliferation or survival in the presence of JNKi by first silencing FoxO1 (data not shown), consistent with the convergence of other JNK-dependent pathways, as well as functional redundancies amongst FoxO family members.[47]

Collectively, the data presented here identify a novel mechanism regulating FoxO-dependent transcriptional programs, modulation of FoxO1 mRNA stability, which represents an additional layer in a complex network responsible for fine-tuning of FoxO activity. Recent studies in animal arthritis models have suggested that therapeutic targeting of PI3K signaling might be beneficial in RA.[48-50] Although the contributions of

FoxO activity to the anti-arthritic effects of PI3K inhibitors have not been characterized, at least some effects of PI3K blockade can be attributed to cellular processes regulated by FoxO proteins.[5;32] Our findings raise the possibility that targeting PI3Ks in RA might be partly hampered by JNK signaling and the subsequent lack of FoxO1 expression in cells exposed to inflammatory cytokines, at least in regard to PI3K inhibitor effects on cell proliferation and survival. Instead, therapeutic strategies directed against JNK, or upstream MAPK kinases (MKK)-4 and -7, may be more beneficial, as they would not only suppress inflammatory activation of immune and stromal cells,[41] but also restore the capacity of FoxO1 to limit RA FLS proliferation and survival.

Acknowledgements

We would like to thank Dr. O Korchynskyi for assistance with amplification and titration of adenoviral vectors, as well as Dr. MW Tang and Dr. DM Gerlag for providing us with patient material and clinical information. We would also like to thank Ms. Beatriz Malvar Fernandez for technical support.

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SUPPLEMENTAL MATERIALS AND METHODS

mRNA expression analysis by qPCR

Equivalent amounts of RNA extracted from RA FLS or synovial tissue biopsies were reverse transcribed using a First-Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania), and relative expression of FoxO1, FoxO3a, FoxO4, and IL-6 was determined using Fast SybrGreen PCR Master Mix (Applied Biosystems, Foster City, CA). qPCR reactions were performed on a StepOne Plus Real-Time PCR System (Applied Biosystems,) in duplicate, and specific amplification of PCR products was confirmed by analysis of dissociation curve for each pair of primers. Relative gene expression was calculated using StepOne Software v2.1 (Applied Biosystems) and expressed as the ratio between the gene of interest and expression of 18S and/or GAPDH. Sequences of the primers used in the study are listed in the table below:

FoxO1	forw	TCTTCCTCCTGAGTCTGGGTAATT
	rev	CAGAGAGCTACCAAGGATTCATGA
FoxO3a	forw	TCTACGAGTGGATGGTGCGTT
	rev	CGACTATGCAGTGACAGGTTGTG
FoxO4	forw	TGGAGAACCTGGAGTGTGACA
	rev	AAGCTTCCAGGCATGACTCAG
IL-6	forw	GACAGCCACTCACCTCTTCA
	rev	CCTCTTTGCTGCTTTCACAC
18S	forw	CGGCTACCACATCCAAGGAA
	rev	GCTGGAATTACCGCGGCT
GAPDH	forw	GCCAGCCGAGCCACATC
	rev	TGACCAGGCGCCCAATAC

mRNA expression profiling by qPCR arrays

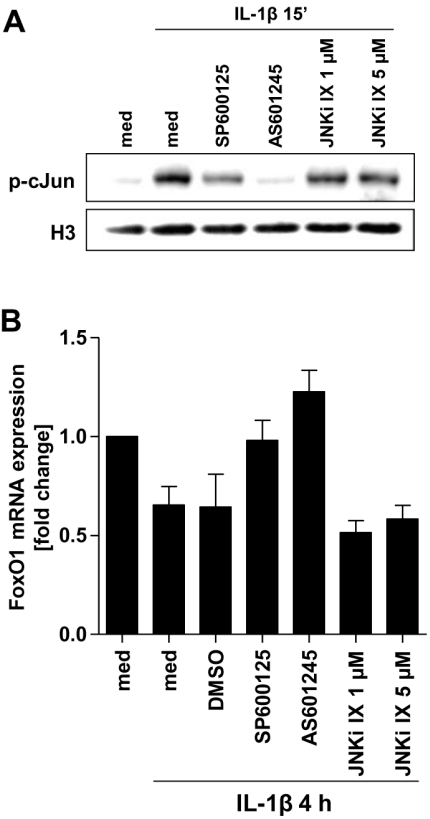
After 4 h stimulation with IL-1β total RNA was extracted from RA FLS transduced with control GFP-encoding adenovirus or adenovirus encoding FoxO1ADA using an RNeasy mini kit (Qiagen, Venlo, The Netherlands). cDNA was synthesized from 1 µg of RNA with an RT2 First Strand Kit (SABiosciences-Qiagen, Frederick, MD) and expression of 84 genes involved in the regulation of inflammation and cell survival was analyzed using a customized RT2 Profiler™ PCR Array set (SABiosciences-Qiagen) according to the manufacturer's instructions. After PCR amplification, threshold values were equalized for all samples and the threshold cycle (Ct) determined for each analyzed gene. Relative expression of each gene was calculated using StepOne Software v2.1 (Applied Biosystems) and corrected for the mean expression of three housekeeping genes (B2M, HPRT1 and RPL13A).

Immunoblotting

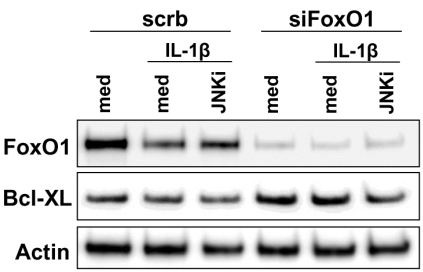
After electrophoresis proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) using a wet transfer apparatus (Invitrogen). Membranes were then blocked in TBS containing 2% milk and 0.05% Tween-20 (both from Bio-Rad Laboratories) and probed overnight at 4°C with antibodies recognizing FoxO1, tubulin (both from Sigma-Aldrich), phospho-(p)PKB, PKB, p-cJun, histone 3 (all from Cell Signaling Technology, Beverly, MA), p27Kip1, Bcl-XL, MnSOD (all from BD Transduction Laboratories), BIM and FLIP (both from Enzo Life Sciences, Farmingdale, NY). After extensive washing membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit Ig antibodies (Dako, Glostrup, Denmark), and proteins visualized using a Lumi Light detection kit (Roche Diagnostics, Mannheim, Germany) or a SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL).

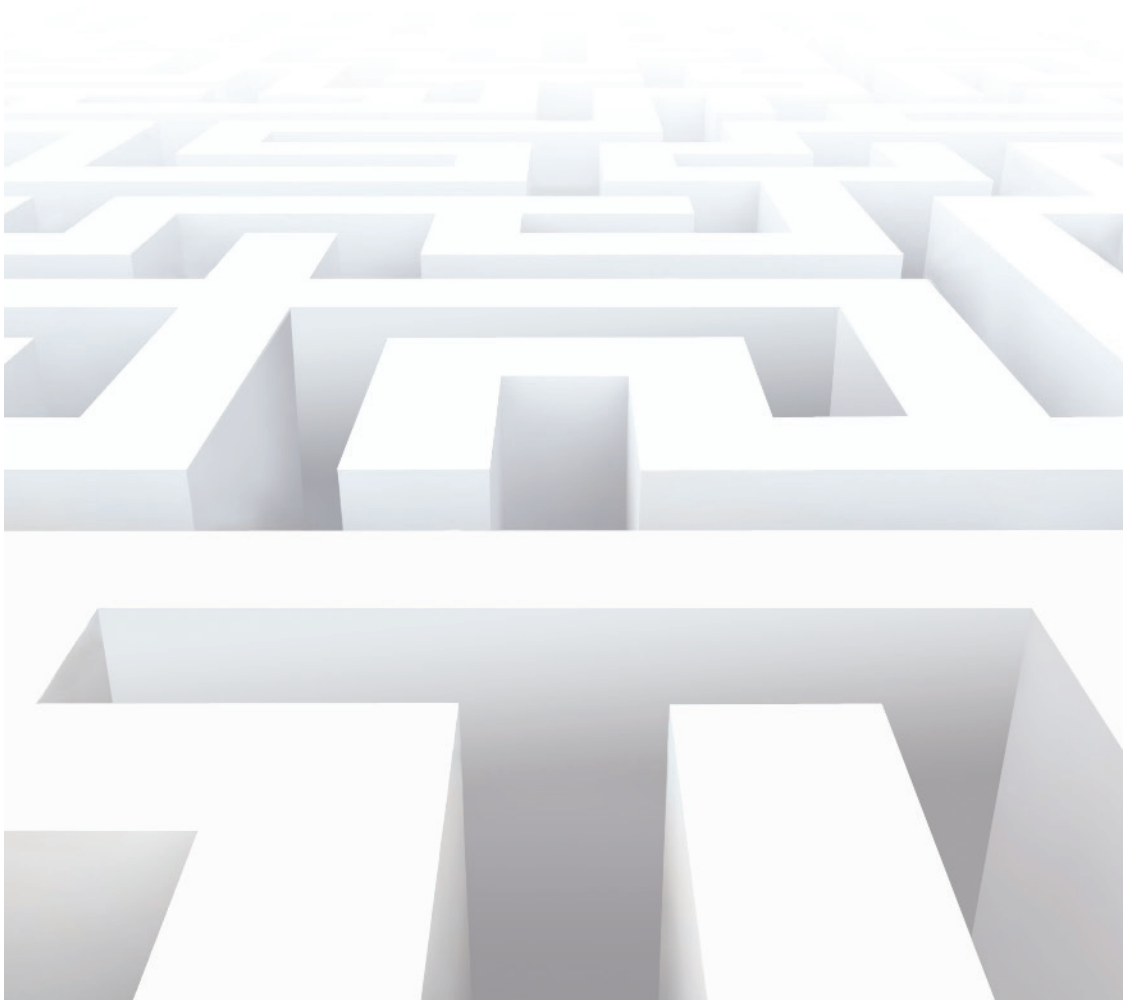
SUPPLEMENTAL DATA

Supplementary figure 1.
IL-1β-induced downregulation of FoxO1 in RA FLS is predominantly mediated by JNK1.
(A) RA FLS were incubated with pan-JNK inhibitors SP600125 (20 μM), AS601245 (50 μM), or with a JNK2/3-selective inhibitor (N-(3-Cyano-4,5,6,7-tetrahydro-1-benzothien-2-yl)-1-naphthamide, JNKi IX, 1 or 5 μM) for 30 min before stimulation with 1 ng/ml IL-1β for 15 min. Cells were then lysed and protein extracts were subjected to analysis by immunoblotting with anti-phospho(p) c-Jun or anti-histone (H)3 antibodies. A representative of 2 independent experiments is shown. Selective JNK2/3 inhibition partly prevented IL-1β-induced c-Jun phosphorylation, indicating that in RA FLS both JNK1 and JNK2 are activated by IL-1β. (B) RA FLS (n = 4) were incubated with the same concentrations of JNK inhibitors as in (A) followed by stimulation with IL-1β (1 ng/ml) for 4 h. RNA was extracted and changes in FoxO1 mRNA expression were analyzed by qPCR. The graph represents mean±SEM fold change in FoxO1 mRNA expression. While both pan-JNK inhibitors prevented FoxO1 downregulation by IL-1β, the JNK2/3-selective JNKi IX had no effect on FoxO1 mRNA levels, indicating that IL-1β reduces FoxO1 expression predominantly through the JNK1 pathway.



Supplementary figure 2.
Silencing of FoxO1 expression in RA FLS upregulates Bcl-XL, but fails to influence the effects of JNKi on Bcl-XL expression.
RA FLS were transfected with control non-targeting siRNA (scrb, 20 nM) or siRNA specific for FoxO1 (siFoxO1, 20 nM). After 48 h cells were stimulated for 8 h with 1 ng/ml IL-1β in the absence or presence of SP600125 (JNKi, 20 μM). Protein extracts were prepared and analyzed by immunoblotting with antibodies recognizing FoxO1, Bxl-XL and actin. A representative of 4 independent experiments is shown.





4

Btk inhibition suppresses agonist-induced human macrophage activation and inflammatory gene expression in RA synovial tissue explants

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ABSTRACT

Objectives: Bruton's tyrosine kinase (Btk) is required for B lymphocyte and myeloid cell contributions to pathology in murine models of arthritis. Here, we examined the potential contributions of synovial Btk expression and activation to inflammation in rheumatoid arthritis (RA).

Materials and methods: Btk was detected by immunohistochemistry and digital image analysis in synovial tissue from biological-naïve RA (n=16) and psoriatic arthritis (PsA) (n=12) patients. Cell populations expressing Btk were identified by immunofluorescent double labelling confocal microscopy, quantitative (q-) PCR and immunoblotting. The effects of a Btk-specific inhibitor, RN486, on gene expression in human macrophages and RA synovial tissue explants (n=8) were assessed by q-PCR, ELISA and single-plex assays.

Results: Btk was expressed at equivalent levels in RA and PsA synovial tissue, restricted to B lymphocytes, monocytes, macrophages and mast cells. RN486 significantly inhibited macrophage IL-6 production induced by Fc receptor and CD40 ligation. RN486 also reduced mRNA expression of overlapping gene sets induced by IgG, CD40 ligand (CD40L), and RA synovial fluid, and significantly suppressed macrophage production of CD40L-induced IL-8, TNF, MMP-1 and MMP-10, LPS-induced MMP-1, MMP-7, and MMP-10 production, and spontaneous production of IL-6, PDGF, CXCL-9, and MMP-1l by RA synovial explants.

Conclusion: Btk is expressed equivalently in RA and PsA synovial tissue, primarily in macrophages. Btk activity is needed to drive macrophage activation in response to multiple agonists relevant to inflammatory arthritis, and.promotes RA synovial tissue cytokine and MMP production. Pharmacological targeting of Btk may be of therapeutic benefit in the treatment of RA and other inflammatory diseases.

Introduction

Recent studies have identified a potentially important role for phosphatidylinositol 3-kinase (PI3K) signalling in maintaining cellular recruitment, activation, and survival in rheumatoid arthritis (RA) synovial tissue.[1] The primary function of PI3K family members is to provide membrane targeting signals which mediate recruitment of selected proteins to the plasma membrane. PI3Ks catalyze the phosphorylation of phosphatidylinositol (PI) to generate the second messengers PI(3)*P*, PI(3,4)*P2*, PI(3,5)*P2* and PI(3,4,5)*P3*, which recruit and regulate the function of pleckstrin homology (PH) domain-containing proteins.[1] Genetic deletion of either the γ or δ isoforms of the PI3K p110 catalytic subunit, or treatment with specific inhibitors of these PI3K isoforms, has been shown to be protective in murine models of arthritis, associated with reduced chemotactic responses, cytokine production, and survival of immune cells.[2-4]

Protein kinase B (PKB) and Tec family kinases represent two downstream targets of PI3K signalling which have been best characterized within the context of RA.*1* PKB is a serine/threonine kinase which regulates mammalian target of rapamycin (mTOR) and forkhead box O (FoxO) transcription factors. Levels of activated, phosphorylated (p-) PKB are elevated in RA versus non-RA synovial tissue, and PKB-dependent phosphorylation of FoxO4 in synovial macrophages, particularly in the synovial sublining, is significantly enhanced in RA.[5;6] Tec family kinases include Tec, Bruton's tyrosine kinase (Btk), Itk, Bmx, and Rlk.[7] Complete activation of Tec family kinases upon cell-surface receptor triggering requires PI3K-dependent relocalization of the protein to the plasma membrane, subsequent phosphorylation by a Src family kinase, and finally autophosphorylation.[8] In addition to the well-studied role of Tec family kinases in PLCγ activation and Ca^{2+} mobilization they can also act downstream of numerous cell-surface receptors that influence a wide range of signalling pathways involved in cellular proliferation, differentiation, apoptosis, cell migration, and inflammatory gene transcription.[7]

Inactivating mutations in the gene encoding Btk cause X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (XID) in mice. Btk is absolutely required for human B cell development and function, and XLA is characterized by the complete absence of circulating B cells due to a block between pro- and pre-B-cell stages, and a severe reduction in serum immunoglobulin levels.[9] A similar, although milder syndrome is observed in XID mice.[10] Apart from its role in B-cells, where Btk mediates antigen receptor, CD40 and chemokine receptor signalling responses, Btk has also been shown to regulate activation of monocytes, macrophages, mast cells

and platelets, and osteoclast differentiation.[11-17] Btk deficiency in myeloid lineages leads to poor inflammatory responses, and XID mice develop less severe experimental autoimmune encephalitis, DSS-induced colitis, and carrageenan-induced acute edema.[18]

A potential role for Btk in RA was first suggested by the finding that XID mice are resistant to collagen induced arthritis (CIA).[19] Subsequent pharmacological development of specific Btk inhibitors and their assessment in experimental rodent models of RA has demonstrated that Btk activity in both B cell and myeloid cell populations contributes to disease.[20-25] However, while this preclinical evidence suggests a therapeutic potential in targeting Btk in RA, little is known about the expression or function of Btk in RA synovial tissue. Here, we examined the expression of Btk in RA and psoriatic arthritis (PsA) synovial tissue, and the effects of a specific Btk inhibitor on gene expression in human macrophages and RA synovial explant cultures.

MATERIALS AND METHODS

Patients, immunohistochemical analysis and confocal microscopy

Synovial biopsies were obtained by needle arthroscopy as previously described from clinically active joints of RA (n=16) and PsA (n=12) patients.[26] RA and PsA patients fulfilled the 1987 American College of Rheumatology criteria for RA and the CASPAR classification criteria for PsA.[27;28] None of the patients had been previously treated with biologicals. Patient clinical characteristics are detailed in table 1. Synovial biopsies were also obtained from a second independent cohort of 7 RA patients and 7 osteoarthritis (OA) patients (see supplemental table 1 for patient clinical characteristics). All patients gave their written informed consent prior to study inclusion, and this study was approved by the Medical Ethics Committee of the Academic Medical Center, University of Amsterdam. Immunohistochemical analysis, as previously described, and confocal microscopy techniques are detailed in Supplemental Materials and Methods.[29;30]

Cell isolation, tissue culture, adenoviral transduction, measurement of cytokine production, measurement of mRNA expression and gene expression profiling

Detailed protocols of isolation, culture, and differentiation of lymphocyte, myeloid, and FLS cells, adenoviral transduction of macrophages, measurement of cytokine production, RNA extraction, cDNA synthesis, quantitative PCR and gene expression profiling are provided in Supplemental Materials and Methods.

Characteristic	RA (n=16)	PsA (n=12)	*P*
Age (y)	53 (38-71)	56 (24-85)	0.87
Male:female (n)	8:8	3:9	0.25
Disease duration: months	89 (1-169)	52 (0-276)	0.60
RF positive: n/total (%)	12/14 (86)	1/11 (9)	0.000
ACPA positive: n/total (%)	8/14 (57)	0/11 (0)	0.003
ESR (mm/h)	28 (4-112)	11 (2-47)	0.09
CRP (mg/l)	15.6 (3-144)	4.4 (1-33.9)	0.027
DAS28	6.23 (3.53-8.15)	4.21 (1.96-7.03)	0.006
Receiving MTX: n/total (%)	15/16 (94)	6/6 (50)	0.023

Table 1. Clinical features of RA and PsA patients included in the study.
*ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = disease activity score 28; RF = rheumatoid factor; ACPA = anti-cyclic citrullinated peptide antibody; MTX = methotrexate

Macrophage stimulation

Cell culture grade Anti-Biotin MACSiBead™ Particles (Miltenyi Biotec) were loaded with biotinylated IgG1 (Biolegend) according to the manufacturer's instructions (30µg biotinylated primary antibody per 1 x 10^8 bead particles). Macrophages were treated with either vehicle control or RN486 (1µM) for 1 h, and then either left unstimulated or stimulated with IgG beads (1:1 bead:cell ratio), CD40L (1µg/ml, R&D Systems), TNF (10 ng/ml, Biosource), LPS (10-1000 ng/ml, Sigma Aldrich) or 10% RA patient synovial fluid (RA SF, pooled from 5 RA patients) for 4-24 h.

Statistical analysis

Data are presented as mean±SEM unless otherwise indicated. The Mann-Whitney *U* test was used for comparing synovial Btk expression between diagnostic groups. The Spearman's correlation coefficient was used for correlation analyses. Analyte concentration data were analyzed using one and two–sided Student's t-tests, as appropriate. *P* values < 0.05 were considered statistically significant.

RESULTS

Btk is expressed in synovial tissue of patients with inflammatory arthritis

We first examined the expression of Btk in RA and PsA synovial tissue. Specific staining of antibodies recognizing Btk was observed in RA synovial tissue, while no staining was observed with irrelevant control antibodies (figure 1A). In both RA and PsA synovial tissue, Btk was most readily apparent in cellular infiltrates of the synovial sublining (figure 1B). Equivalent expression levels of Btk were observed in RA and PsA synovial tissue, as detected by digital image analysis (figure 1C). Similar levels of Btk expression were also observed in an independent analysis of RA and OA patients (figure 1D). No relationship was observed between Btk expression levels and patient clinical characteristics (serum C-reactive protein levels, erythrocyte sedimentation rate, DAS28 score, and ACPA- or RF-positivity) (data not shown). To study the relationship between Btk expression and synovial cellular composition, sections were stained with antibodies against specific markers for T-cells, B-cells, FLS, macrophages, endothelial cells and mast cells. We found a positive correlation between Btk expression levels and numbers of $CD68^+$ synovial macrophages ($R=0.6324$, $p<0.01$), $CD163^+$ macrophages ($R=0.7275$, $p<0.005$) and $CD3^+$ T-cells ($R=0.7903$, $p<0.001$) (figure 1E), but no correlation with $CD22^+$ B-cells (figure 1E), $CD55^+$ FLS, or vWF^+ endothelial cells in RA synovial tissue (supplemental table 3). In PsA synovial tissue we did not observe any significant correlations with the expression of cellular markers (figure 1E and supplemental table 3), although all cellular markers were detected at similar levels in RA and PsA synovial tissue (supplemental table 4). These data demonstrate that Btk is expressed in the synovial tissue of arthritis patients, and suggest that at least in RA, synovial Btk expression correlates with myeloid and T cell infiltration.

Btk is expressed in RA synovial myeloid cells and B lymphocytes

Immunofluorescent double labelling confocal microscopy was performed to identify which cell types express Btk in RA synovial tissue. Btk expression colocalized with $CD22^+$ B-cells, $CD68^+$ macrophages and MCT^+ mast cells (Spearman colocalization coefficient 0.461, 0.674 and 0.506, respectively), but not $CD3^+$ T cells, vWF^+ endothelial cells, or $CD55^+$ FLS (figure 2A and supplemental figure 1). Btk also colocalized with $CD68^+$ and $CD163^+$ macrophages in PsA synovial tissue (supplemental figure 2). Immunoblotting experiments performed on lysates from purified B cells, T cells, monocytes, and GM-CSF-differentiated macrophages derived from healthy donor peripheral blood, as well as RA FLS, confirmed that Btk expression was restricted to B cells and myeloid cells (figure 2B). Itk protein expression was restricted to T cells, and Bmx protein was detected in all

cell populations except for RA FLS (figure 2C). Similar results were obtained examining mRNA expression of Btk, with equivalent levels of Btk mRNA observed in both freshly isolated monocytes and differentiated macrophages (figure 2D). Itk was detected in T cells, while Bmx mRNA was detected at low levels in all cell types compared to positive control K562 cells (figure 2D), possibly indicating Bmx antibody cross-reactivity with other Tec family kinases (figure 2C). Similar results, as well as Btk expression levels, were observed following analysis of RA and PsA peripheral blood-derived macrophages differentiated in GM-CSF, and healthy donor macrophages differentiated in either 10% RA or 10% PsA SF (supplemental figure 3).

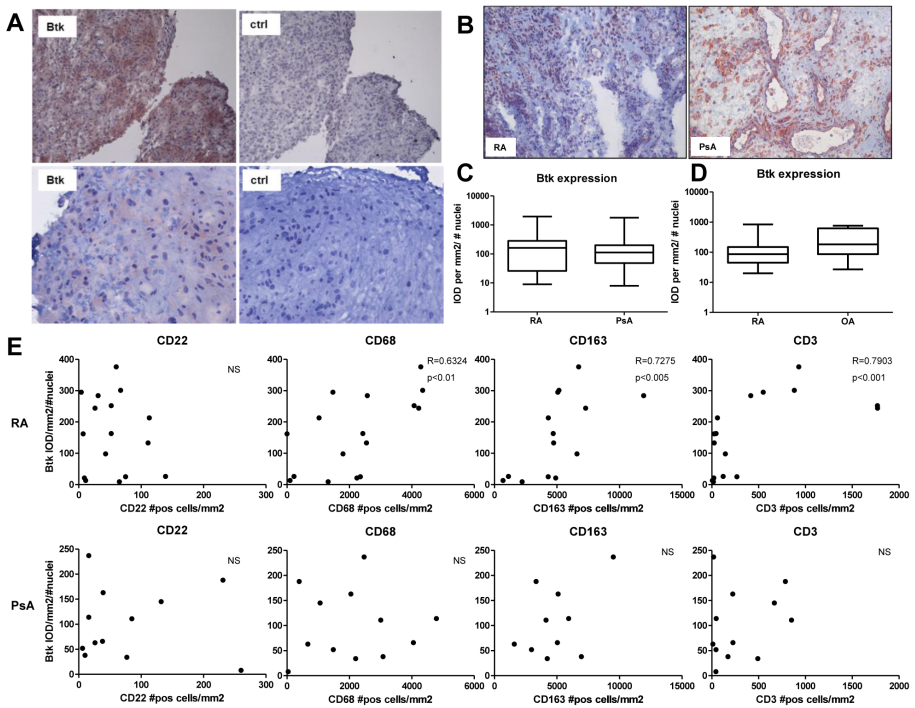

Figure 1. Btk expression in rheumatoid arthritis, psoriatic arthritis and osteoarthritis synovial tissue.
Tissue sections from patients with rheumatoid arthritis (RA) and psoriatic arthritis (PsA) were stained with irrelevant control antibodies or with antibodies against Btk, CD3, CD22 and CD68. (A) Representative staining of RA synovial tissue with Btk and control (ctrl) antibodies (magnification upper panel x200, bottom panel x400). (B) Representative stainings of RA and PsA synovial tissue with Btk antibodies (magnification x200). (C) Quantitative analysis of Btk expression in RA (n = 16) and PsA (n = 12) synovial tissue. (D) Quantitative analysis of Btk expression in RA (n=7) and osteoarthritis (OA, n=7) patient synovial tissue. Values are expressed as integrated optical density (IOD) per mm2 per number of nuclei calculated by computer-assited digital imgae analysis. Data are presented as boxplots where the boxes represent the 25th and 75th percentiles, the line within the box denote the median value, and the lines outside of the box mark the 10th and 90th percentiles. (E) Correlation of Btk expression and number of cells/mm2 expressing CD22, CD68, CD163 and CD3 in RA (upper panels) and PsA (lower panels) synovial tissue. Correlations were calculated using Spearman's rank correlation coefficient. Circles indicate individual patient values, and Spearman R and P values are indicated in each graph. NS, not significant.

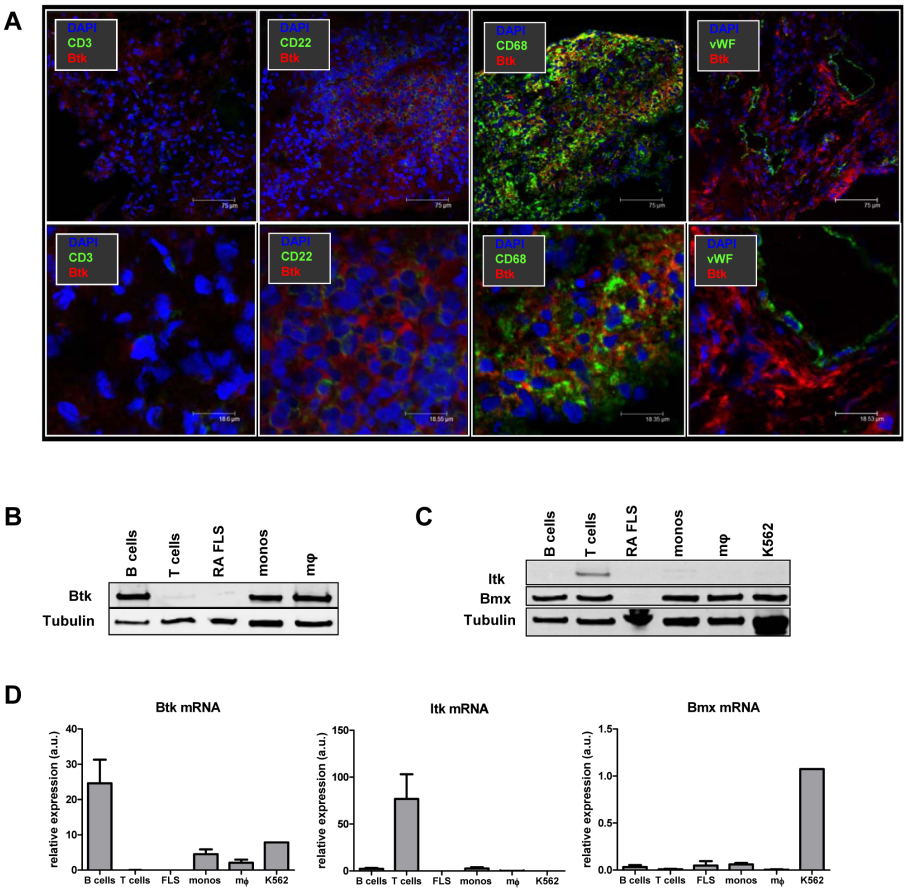

Figure 2. Btk expression in specific cell types in rheumatoid arthritis synovial tissue.
(A) Representative immunofluorescent double-staining of RA synovial tissue with anti-Btk antibody (red), CD3, CD22, CD68 and vWF cell-specific markers (green), DAPI (blue) (magnification x400). (B) Protein extracts were prepared from healthy donor peripheral blood B cells, T cells, buffy coat monocytes (monos) and GM-CSF –differentiated macrophages (MΦ), RA FLS, and K562 cells, and analyzed by immunoblotting with antibodies recognizing (B) Btk and tubulin or (C) Itk, Bmx, and tubulin. Results are representative of three independent experiments. (D) Total RNA was extracted from healthy donor peripheral blood B cells, T cells, buffy coat monocytes (monos) and GM-CSF –differentiated macrophages (MΦ) (n = 4 donors for each), RA FLS (n = 3), and K562 cells, reverse transcribed and expression of Btk, Itk, and Bmx mRNA was monitored by qPCR. Data are presented as mean ±SEM expression relative to GAPDH, arbitrary units (a.u.).

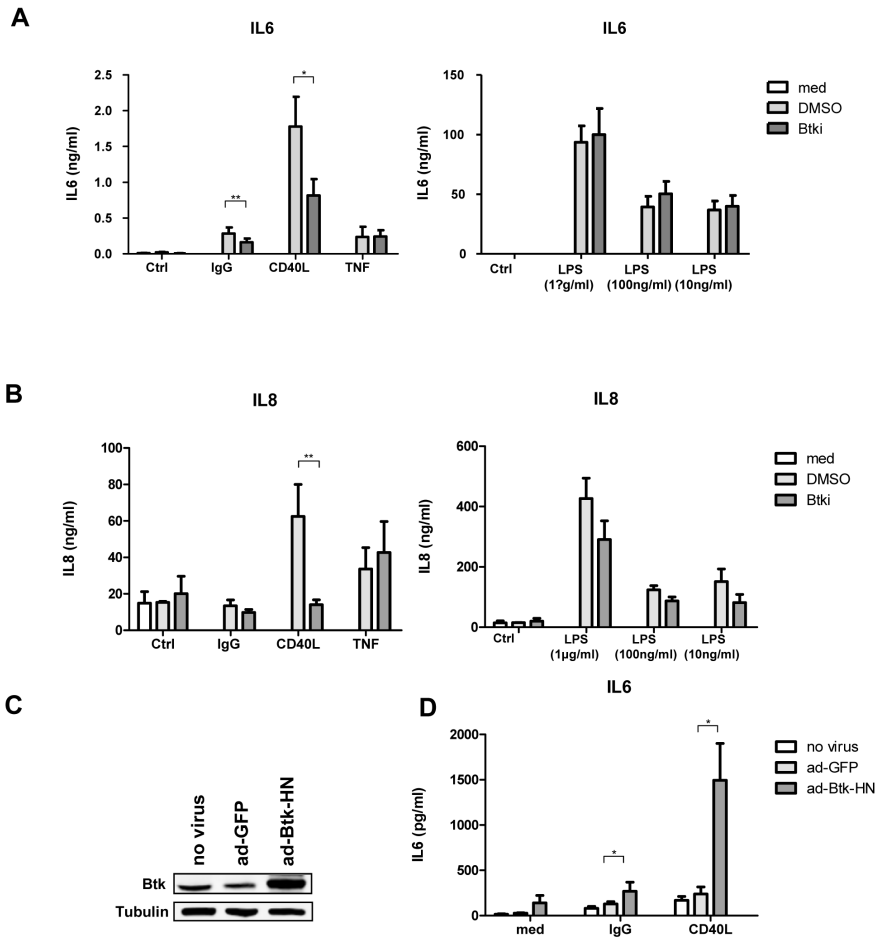

Figure 3. Btk inhibitor RN486 suppresses macrophage production of cytokines in response to IgG and CD40L stimulation.

Analysis of (A) IL-6 and (B) IL-8 production in supernatants of macrophages stimulated with medium alone (Ctrl), IgG beads (1:1 ratio), CD40L (1µg/ml), TNF (10 ng/ml) and LPS (1µg/ml, 100ng/ml, 10ng/ml) for 24 hours, in the absence (med, performed only with unstimulated cells) or presence of carrier DMSO or RN486 (Btki, 1 µM) as assessed by ELISA. Bars represent the means and SEM of 7 independent experiments. *P < 0.05, **P < 0.01 versus cells exposed to DMSO. (C) Immunoblotting of lysates obtained from control macrophages (no virus) and macrophages transduced with adenoviruses encoding control GFP (ad-GFP) or Btk (ad-Btk-HN). (D) Analysis of IL-6 production in supernatants of control macrophages (no virus), and macrophages transduced with GFP (ad-GFP) or Btk (ad-Btk-HN) stimulated with medium (med) alone, IgG beads or CD40L as in A above. Bars represent the means and SEM of 6 independent experiments. *P < 0.05 compared to cells transduced with GFP alone.

Inhibition of Btk selectively prevents inflammatory mediator induction by macrophage agonists

To determine the potential contributions of macrophage Btk activation to inflammation in RA, we stimulated macrophages with IgG, CD40L, TNF, or LPS, all agonists relevant to macrophage activation in RA. Of these, Btk involvement has previously been reported in B cell CD40 signalling, and monocyte Fc receptor and TLR signalling. Experiments were performed in the absence or presence of saturating concentrations of a specific Btk inhibitor, RN486.[22] In the absence of stimulating agonists, neither carrier solvent (DMSO) nor RN486 had any effect on basal production of IL-6 (figure 3A) or other secreted products tested (see below) compared to macrophages in medium alone. In parallel, we compared the effects of carrier solvent and RN486 on agonist-induced IL-6 production. IgG-coated beads, CD40L, TNF and LPS (figure 3A) all induced IL-6 production in human macrophages derived from healthy donor (n=6) monocytes, as determined by ELISA. In the presence of RN486, macrophage IL-6 production in response to Fc receptor (40% inhibition, $p<0.01$) and CD40 stimulation (50%, $p<0.05$), but not TNF was significantly inhibited (figure 3A). RN486 failed to influence LPS-induced IL-6 production at any of the LPS concentrations tested (figure 3A). CD40L, TNF, and LPS all induced macrophage IL-8 production (figure 3B), but only IL-8 produced in response to CD40L was blocked in the presence of RN486 ($p<0.01$) (figure 3B). Macrophage transduction with adenoviruses encoding Btk, but not GFP, increased cellular expression of Btk (figure 3C) and increased macrophage IL-6 production in response to IgG ($p<0.05$) and CD40L stimulation ($p<0.05$) (figure 3D).

To obtain a broader assessment of Btk-dependent macrophage gene expression which might be relevant to RA, we stimulated macrophages (n=3 independent donors) with IgG beads, CD40L or 10% RA SF in the absence or presence of RN486 and determined expression levels of 238 genes involved in the regulation of innate immune responses, extracellular matrix metabolism, cell adhesion, inflammation and angiogenesis. In this qualitative screening, only genes which were up-regulated two-fold or more in three independent experiments were considered for further analysis (supplemental tables 5-7). Each stimulus generated an unique but overlapping profile of induced genes, and only four genes involved in IL-1 signalling, *IL1B, IL36B, IL1RN,* and *IL36RN*, were induced by all three stimuli (figure 4A). In each of the three experiments, RN486 inhibited by more than 2-fold 12 of 21 genes induced by IgG, 11 of 52 genes induced by CD40L, and 6 of 25 genes induced by 10% RA SF (figure 4B). These putative Btk-regulated genes included ones that were uniquely inhibited in response to a specific agonist, such as *IL1B* (IgG stimulation), *CXCL9* (CD40L), and *CCR3* (RA SF), as well as those inhibited

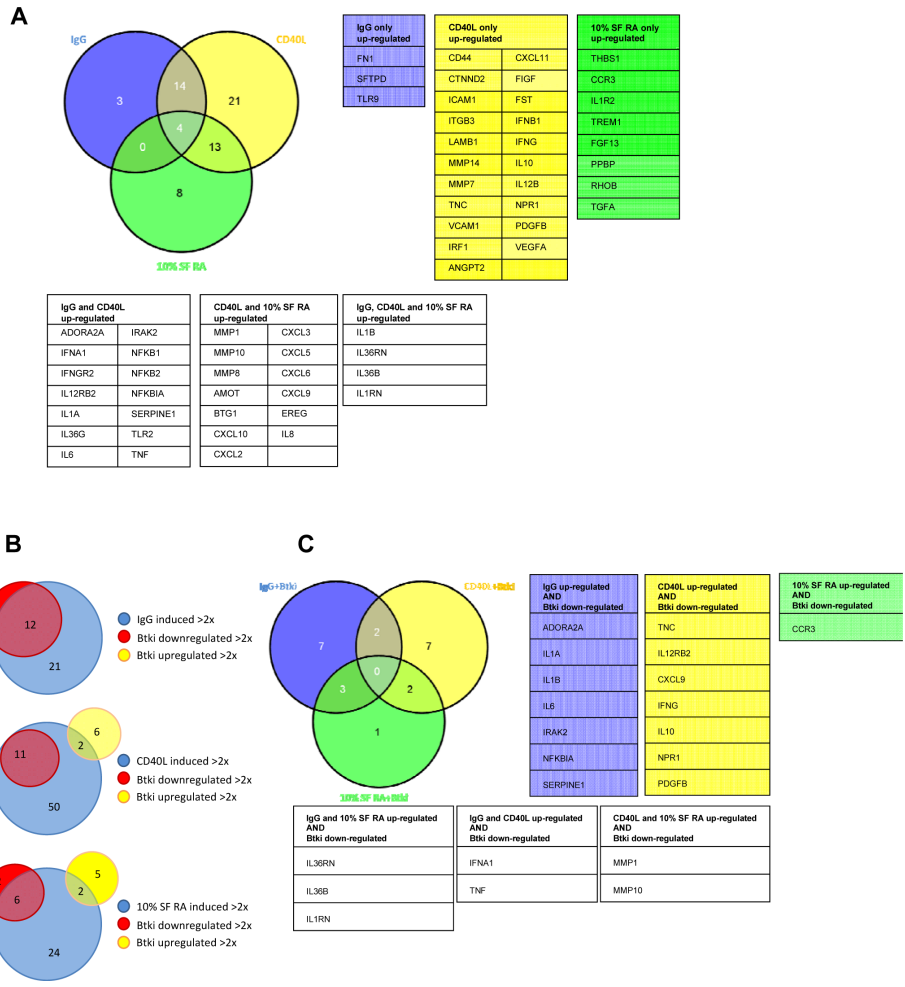

Figure 4. Identification of genes regulated by IgG, CD40L and RA synovial fluid stimulation of human macrophages and their regulation by Btk.

(A) Venn diagram of genes that are induced by more than 2-fold in three independent experiments in macrophages obtained from different donors stimulated for four hours with IgG beads (1:1 ratio), CD40L (1µg/ml), 10% RA synovial fluid (SF RA, pooled from 5 RA patients) compared to medium alone. Genes induced by specific or groups of stimuli are listed. (B) Diagrams showing overlap in genes that are more the 2-fold up regulated by indicated stimuli and more than 2-fold up- or down-regulated upon Btk inhibition. (C) Venn diagram of IgG, CD40L and SF RA –inducible genes that were suppressed by more than 2-fold by RN486 (Btki, 1 µM) in three independent experiments in human macrophages compared to DMSO alone.

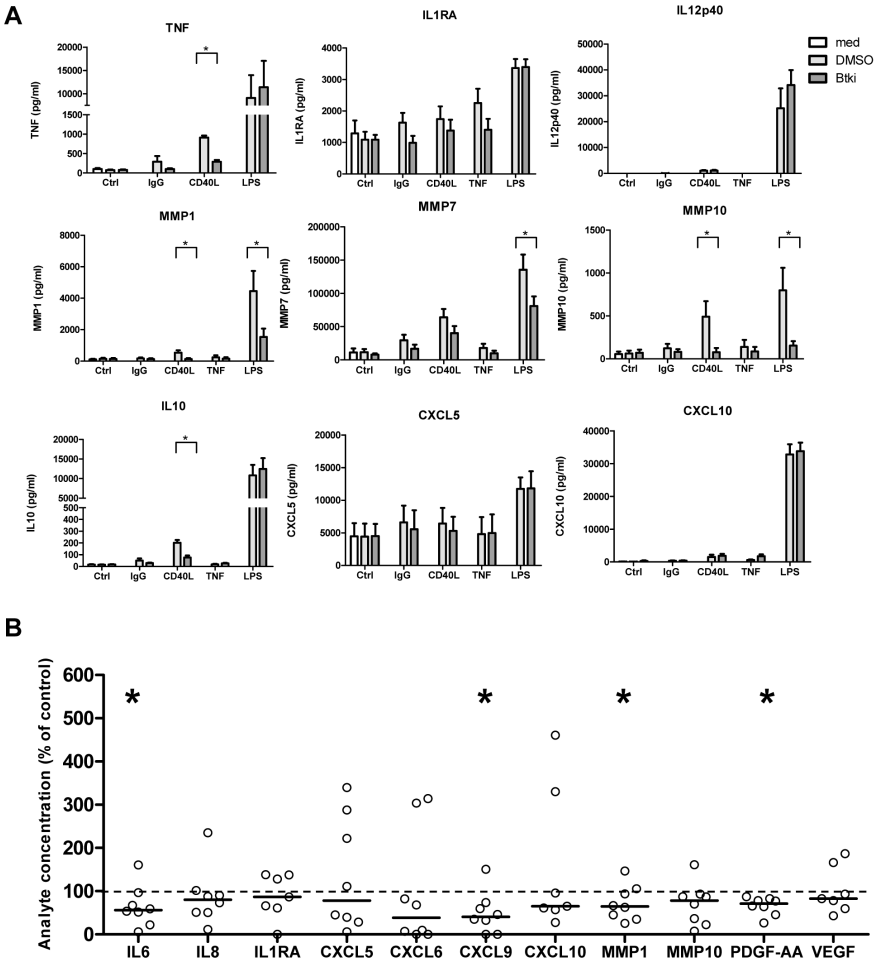

Figure 5. Effects of RN486 on the production of inflammatory mediators by human macrophages and RA patient synovial tissue explants.

(A) Analysis of indicated cytokines, soluble receptors, matrix metalloproteinases and chemokines in supernatants of macrophages stimulated with medium alone (Ctrl), IgG beads (1:1 ratio), CD40L (1µg/ml), TNFα (10 ng/ml) and LPS (1µg/ml) for 24 hours, in the absence (med, performed for unstimulated cells only) or presence of carrier DMSO or RN486 (Btki, 1 µM) as assessed by single-plex assays. Supernatants assessed here are the same as those used for measuring IL-6 and IL-8 in Figure 3. Bars represent the means and SEM of 7 independent experiments. *P < 0.05, **P < 0.01 versus cells exposed to DMSO. (B) Analysis of indicated cytokines, soluble receptors, matrix metalloproteinases and chemokines in supernatants of RA patient (n = 8) synovial tissue explants cultured for 48 hours in the absence (carrier DMSO only) or presence of RN486 (1 µM) as assessed by ELISA (IL-6 and IL-8) or single-plex assays. Data for each patient was adjusted for biopsy weight and was normalized to 100 for tissue exposed to carrier only, and data expressed as % analyte concentration of RN486-treated tissue compared to control. Each circle represents data from an individual patient and bars represent the median values. * P<0.05 compared to control samples.

in response to multiple stimuli, such as IL-1 signalling components, *TNF, MMP1* and *MMP10* (figure 4C).

Btk inhibition blocks CD40-induced inflammatory mediator production in macrophages and RA synovial explants

To independently validate these findings, we assessed macrophage culture supernatants for candidate secreted proteins. Of the analytes assessed, RN486 failed to significantly influence their induction by either IgG or TNF (figure 5A). However, CD40L-induced macrophage production of TNF, MMP-1, MMP-10 (all $p<0.05$) and IL-10 ($p<0.01$) was significantly inhibited by RN486, while production of IL-1RA, IL-12p40, CXCL5, CXCL10, and MMP-7 was unaffected (figure 5A). Surprisingly, RN486 significantly reduced levels of LPS-induced MMP-1, MMP-7, and MMP-10 (all $p<0.05$). IL-1α, IL-1β, IFNγ, VEGF, and CXCL-11 were not produced by macrophages at detectable levels in these experiments. To determine if the observed contributions of Btk to macrophage activation might also be relevant to expression of inflammatory mediators in RA synovial tissue, we cultured RA patient (n=8) synovial explants in the absence and presence of RN486. RN486 significantly decreased synovial explant production of IL-6 ($p<0.01$), PDGF-AA ($p<0.01$), CXCL-9 ($p<0.05$), and MMP-1 ($p<0.05$), but not IL-1RA, VEGF, CXCL-5, CXCL-6, CXCL-10 and MMP-10 (figure 5B), while supernatant concentrations of IL-10, TNF, IL-12p40 and MMP-7 were below the detection limits of the assay (data not shown).

Discussion

Btk plays not only critical roles in B cell development and antigen-dependent activation of mature B lymphocytes, but also in the inflammatory activation of myeloid lineage cells.[10;31] Accordingly, pharmacological interference with Btk signalling protects against pathology in both B cell-dependent and myeloid cell-dependent models of experimental arthritis. In murine (CIA), a model critically dependent upon B cell activation and autoantibody production, prophylactic or therapeutic administration of Btk inhibitors reduces autoantibody production, synovial inflammation, and cartilage and bone destruction.[20-23;25] Btk inhibitors similarly protect against synovial inflammation and joint destruction in murine autoantibody-induced arthritis, and rat adjuvant-induced arthritis, models in which myeloid cells are primary contributors to pathology.[20;22;25] Here, we demonstrate that Btk is expressed and contributes to the maintenance of inflammation in the synovium of patients with established RA.

Btk is expressed at equivalent levels in the synovium of patients with RA and PsA, and consistent with known physiological expression patterns, we observe that Btk protein is expressed in B cells, macrophages and mast cells in RA synovial tissue. Btk was also readily detected in synovial macrophages of PsA patients, but Btk expression levels did not correlate with synovial macrophage numbers in PsA, possibly due to the smaller size of this cohort. Although LPS has been reported to induce Btk expression in RA FLS *in vitro* when microRNA-346 induction is suppressed, we were unable to detect Btk expression in RA FLS *in vivo* or *in vitro*.[32] Thus, based on cellular prevalence, our data indicate that macrophages would be the most prominent direct synovial cell population targeted by eventual application of Btk inhibitors to the treatment of RA. In other tissue compartments, such as lymph nodes and bone marrow, B cells and osteoclasts are likely to represent additional potential targets.[14;17]

To examine potential contributions of macrophage Btk signalling to inflammation, we assessed the effect of a highly specific Btk inhibitor, RN486, on macrophage responses to agonists relevant to their activation in inflammatory arthritis. RN486 displays a more than one hundred-fold inhibitory selectivity for Btk over other kinases, including Tec family members, prevents pathology in murine CIA and rat AIA, and suppresses FcγR-mediated TNF production in human monocytes.[22] This latter observation might be particularly relevant to a role for Btk in the pathology of RA, as RF and ACPA immune complex activation of FcγRIIa can promote macrophage TNF production.[33-35] While we observed that RN486 could significantly but partially suppress IgG-induced IL-6 production by human macrophages, only trends toward reduced TNF and MMP-7 production were observed. These results are consistent with previous observations that FcγR signalling in mature human macrophages displays a less stringent requirement for Btk involvement than in human monocytes or murine macrophages.[20;25]

Activation of toll-like receptor (TLR)s expressed by synovial macrophages and FLS are thought to make important contributions to pathology in RA.[36] Studies using monocytes and macrophages from XLA patients, XID mice, and treatment of healthy donor human and wild-type murine macrophages with Btk siRNA and Btk inhibitors have provided conflicting interpretations as to whether Btk is required[37-39] or dispensable[20;22;25;40] for mediating TLR2 and TLR4-induced inflammatory cytokine production. These differences might be explained by different requirements for Btk signalling during myeloid differentiation and between human and mouse, as in the case of FcγR signalling. However, it is also noteworthy that in the above-described studies, readouts for cellular activation were largely limited to IL-6, IL-8 and TNF production.

In our studies, we failed to observe an effect of RN486 on LPS-induced production of these cytokines. However, we do find that RN486 selectively and significantly inhibits macrophage production of MMP-1, MMP-7, and MMP-10 in response to LPS.

CD40, responding to stimulation by CD40L during cell-cell contact, activates many cell populations found in RA synovial tissue, including lymphocytes, macrophages, dendritic cells, and FLS, and therapeutic strategies specifically targeting CD40 expressed by myeloid cells has shown to be effective in CIA.[41;42] Potential contributions of CD40 to pathology in RA are underscored by identification of SNP variants in the CD40 gene locus, and in genes encoding signalling proteins downstream of CD40, that constitute an extended risk pathway.[43;44] For these reasons, we were intrigued by recent data indicating that coupling of TLR signalling to Btk in macrophages occurs not at the cellular surface membrane, but in endosomal compartments following TLR internalization and requires Btk association with major histocompatability class II proteins via CD40.[39] As CD40 signalling in endothelial cells has been reported to require endosomal internalization, and CD40 regulates Btk activity in B cells, we considered whether Btk inhibition may also influence CD40-dependent activation of macrophages.[45;46] Indeed, we find that RN486 significantly reduces CD40L-induced IL-6, IL-8, MMP-1, and MMP-10 production, a profile which overlaps with but is distinct from the effect of RN486 on LPS-induced gene expression in macrophages. Treatment of RA synovial explants with RN486 reduced spontaneous production of gene products associated with macrophage Btk involvement in response to CD40L (IL-6, IL-8, MMP-1, PDGF-B) and unidentified components of RA SF (CXCL-9), possibly indicating that these are predominant pathways driving macrophage Btk activation in RA synovial tissue. The abundant expression of Btk in RA and PsA synovial macrophages, the pro-inflammatory involvement of Btk in macrophage responses to multiple stimuli, and the strong association between synovial macrophages and disease activity[47] as well as the consistent relationship between reductions in macrophages and clinical improvement after effective therapy in RA[48] suggest that continued development of Btk inhibitors may be of potential therapeutic benefit in the treatment of RA, as well as other forms of inflammatory arthritis in which macrophage activation contributes to pathology.

Acknowledgements and affiliations

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Competing interests

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Authorship

Contribution: L.M.H. contributed to research design, performed experiments, analyzed data and contributed to writing the paper; I.E. van E. performed experiments and analyzed data; M.W.T. obtained, recorded, analyzed and interpreted all clinical records and materials, J.H., J.S.F, M.S., J.W., S.N., J.D., P.P.T. and K.A.R. designed research, analyzed and interpreted data, and contributed to writing the manuscript.

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SUPPLEMENTAL MATERIALS AND METHODS

Immunohistochemistry and digital imaging analysis

Serial sections from 6 different TissueTek-embedded biopsy samples per patient were cut with a cryostat (5µm), fixed with acetone and endogenous peroxidase activity blocked with 0.3% hydrogen peroxide in 0.1% sodium azide/phosphate buffered saline. Sections were stained overnight at 4°C with antibodies against Btk (Sigma-Aldrich), CD3 (BD), CD22 (Bioconnect), CD55 (Bioconnect), CD68 (Dako), vWF (Dako), MCT (Dako). Equivalent concentrations of irrelevant control rabbit (anti- fluorescein isothiocyanate (FITC)) or mouse monoclonal antibodies were used as negative controls. Sections were then washed and incubated with swine anti-rabbit horseradish peroxidise (HRP)-conjugated antibody (Dako) or goat anti-mouse-HRP (Dako), followed by incubation with biotinylated tyramide and streptavidin-HRP and development with amino-ethylcarbazole (AEC, Vector Laboratories). Sections were subsequently counterstained using Gill's hematoxylin (Klinipath) and mounted in Kaiser's glycerol gelatine (Merck). Stained sections were analyzed by computer-assisted image analysis using the Qwin analysis system (Leica) as previously described in detail. Values of integrated optical densities (IOD)/mm^2 were obtained and corrected for the total number of nuclei/mm2. For quantitative analysis of cell type-specific markers data were presented as number of positive cells/mm^2.

Confocal microscopy

Sections were incubated overnight at 4°C with primary antibodies, followed by incubation with alexa-488 conjugated swine anti-mouse and alexa-594 conjugated goat anti-rabbit antibodies (Molecular Probe). Subsequently, slides were stained with DAPI nuclear staining and mounted in Vectashield and analyzed with confocal microscopy (Leica TCS-SP2 mounted on an inverted microscope). Results were assessed with Huygens image restoration software.

Cell isolation and tissue culture

Mononuclear cells were isolated from healthy donor buffy coats (Sanquin), or the peripheral blood of RA or PsA patients using Lymphoprep (AXIS-SHIELD) density gradient centrifugation followed. Negative selection was used to obtain purified unstimulated B and T lymphocyte populations (Dynal bead kits, Invitrogen). Monocytes were further isolated by Standard Isotone Percoll gradient centrifugation (Amersham) and plated in Iscove modified Dulbecco medium (IMDM, Invitrogen), supplemented with 1% fetal bovine serum (FBS) for 30 minutes at 37°C. Non-adherent cells were

removed, and monocytes cultured for 7 days in IMDM containing 10% FBS, 100µg/ml gentamycin and 5ng/ml GM-CSF (R&D), or 10% pooled synovial fluid (SF) from 5 RA or PsA patients . Intact synovial biopsies from RA patients (n=8, see supplemental table 2) were cultured for 48 hours in complete DMEM supplemented with 10% FBS in the absence or presence of RN486 (1µM). Cell-free tissue culture supernatants were harvested for cytokine analysis. RA patient FLS (passages 4-9) were obtained from patient arthroscopic material and cultured as previously described.[6]

Measurement of mRNA expression and gene expression profiling

Total RNA was extracted from cells using a GenElute RNA isolation kit (Sigma-Aldrich) and RNA concentration and purity was determined with a Nanodrop spectrophotometer (Nanodrop Technologies). RNA was reverse transcribed using a First-Strand cDNA synthesis kit (Fermentas). Quantitative (q) PCR was performed on a CFX96 (Bio-Rad) using Power SybrGreen PCR Master Mix (Applied Biosystems). For quantitative analysis of Tec family mRNA expression, the following primers (all from Invitrogen) were used: Btk forward, AGACTGCTGAACACATTGCG; Btk reverse, GCTCATCTGCTTTCTCATGC; Itk forward, CCCGAATCAAATGTGTTGAG; Itk reverse, TCTCACGATCTGGAGCAAAC; Bmx forward, GACTTGAACCTTCCCAGCTC; Bmx reverse, TCTCTGTCCACCAAGCAGTT; GAPDH forward, GCCAGCCGAGCCACATC; GAPDH reverse, TGACCAGGCGCCCAATAC. Specific PCR product amplification was confirmed by dissociation curve analysis for each primer pair. For gene expression profiling, total RNA was extracted from macrophages (RNeasy kit, Qiagen) (n=3 donors) and DNAse treatment. cDNA was synthesized from 800 ng of RNA using an RT^2 First Strand Kit (SABiosciences) and the expression of genes was analyzed using RT^2 Profiler^{TM} PCR array sets (PAHS-013, PAHS-052, PAHS-072, SABiosciences). After PCR amplification, thresholds values were manually equalized for all samples and the threshold cycle (Ct) determined for each analyzed gene. Relative expression of each gene was calculated using StepOne Software v2.1 (Applied Biosystems) and Microsoft Excel spreadsheet software (Microsoft) and corrected for the expression of housekeeping gene GAPDH.

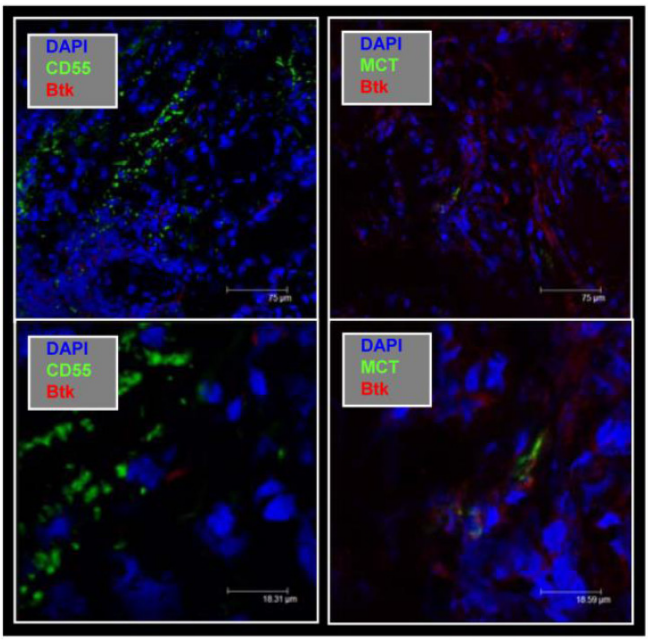
Adenoviral trandsuction of macrophages

Adenoviruses encoding control GFP (provided by Dr S. W. Tas, AMC, Amsterdam, The Netherlands) and 6xHN epitope-tagged Btk (Signagen, Rockville, MD) were amplified and titrated in HEK-293 packaging cells. Virus-containing supernatant was collected and macrophages were transduced at a multiplicity of infection of 200 for 48 hours.

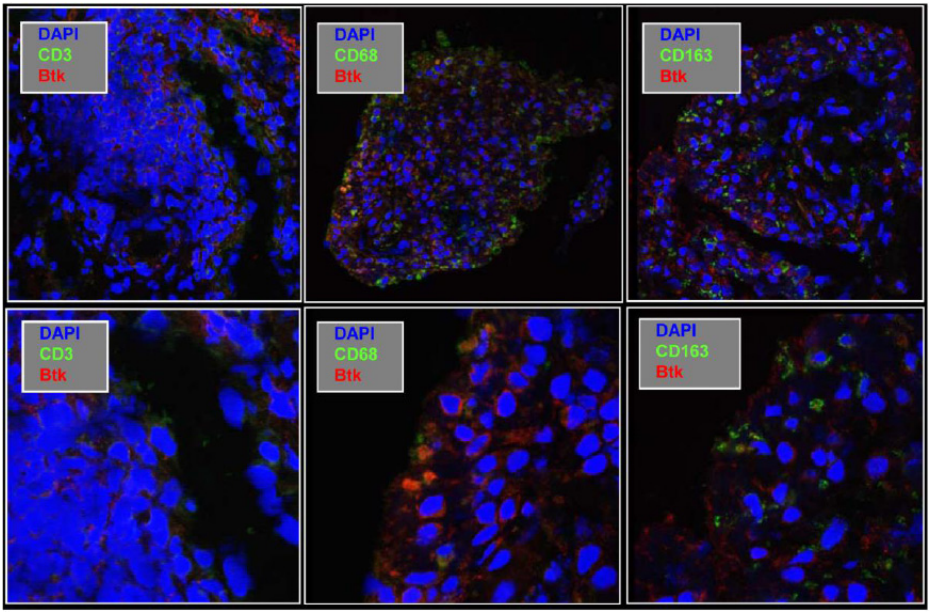
Measurement of cytokine production

Cell-free supernatants were harvested and IL-6, IL-8 and TNF production was measured using PeliKine Compact ELISA kits (Sanquin Reagents) as per the manufacturer's instructions. All other analytes were measured using Bio-plex Suspension Array System (Bio-Rad). Concentrations of analytes in synovial biopsy explant supernatants were corrected for biopsy weight.

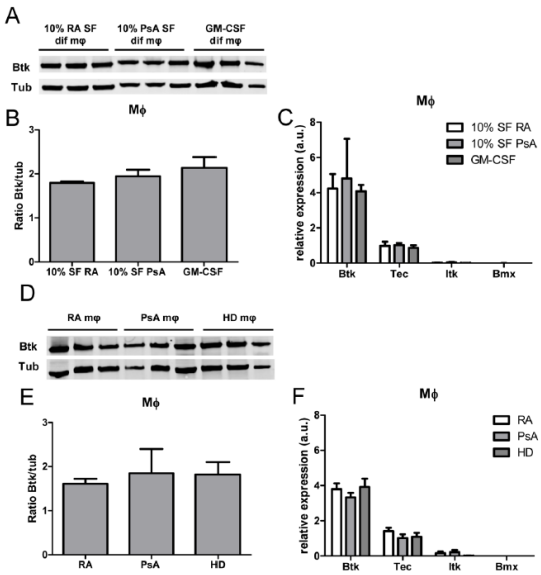
SUPPLEMENTAL DATA



Supplemental figure 1. Btk expression in specific cell types in rheumatoid arthritis synovial tissue. Representative immunofluorescent double-staining of RA synovial tissue with anti-Btk antibody (red), CD55, and mast cell tryptase (MCT) cell-specific markers (green), and DAPI (blue) (magnification x400).



Supplemental Figure 2. Btk expression in specific cell types in psoriatic arthritis synovial tissue. Representative immunofluorescent double-staining of PsA synovial tissue with anti-Btk antibody (red), CD3, CD68, and CD163 (green) and DAPI (blue) (magnification x400).



Supplemental Figure 3. Btk expression in patient-derived macrophages and synovial fluid-differentiated macrophages. (A) Protein extracts were prepared from healthy donor buffy coat-derived macrophages (MΦ) (n=3 donors) differentiated in 10% RA synovial fluid (SF) pooled from 5 patients, 10% pooled PsA SF or GM-CSF, and analyzed by immunoblotting with antibodies recognizing Btk and tubulin (Tub). (B) Quantitative analysis of Btk protein expression relative to tubulin (tub) in A as determined by densitometry. (C) Total RNA was extracted from macrophages (n=3 donors), reverse transcribed and expression of Btk, Tec, Itk, and Bmx mRNA was monitored by qPCR. Data are presented as mean ±SEM expression relative to GAPDH, arbitrary units (a.u.). (D) Protein extracts were prepared from RA or PsA patient peripheral blood monocytes or healthy donor (HD) buffy coat monocytes (n=3 each) differentiated in GM-CSF, and analyzed by immunoblotting with antibodies recognizing Btk and tubulin (Tub). (E) Quantitative analysis of Btk protein expression relative to tubulin (tub) in D as determined by densitometry. (C) Total RNA was extracted from macrophages derived from RA patients, PsA patients or HD as in D (n=3 donors or patients each), reverse transcribed and expression of Btk, Tec, Itk, and Bmx mRNA was monitored by qPCR. Data are presented as mean ±SEM expression relative to GAPDH, arbitrary units (a.u.).

Characteristic	RA (n=7)	OA (n=7)
Age (y)	53 (43-64)	55 (43-67)
Male:female (n)	1:6	2:5
Disease duration: months	94 (1-409)	37 (2-240)
RF positive: n (%)	5 (71)	ND
ACPA positive: n (%)	4 (57)	ND
ESR (mm/h)	18 (5-126)	19 (8-38)
CRP (mg/l)	5.9 (1-88)	8 (3-112)
DAS28	4.51 (2.45-6.62)	ND
Receiving MTX: n (%)	29%	ND

Supplemental Table 1. Clinical features of RA and OA patients included in the study.
*ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = disease activity score 28, RF=rheumatoid factor, ACPA=anti-cyclic citrullinated peptide antibody; MTX=methotrexate, ND = not determined

Characteristic	(n=8)
Age (y)	65 (50-72)
Male:female (n)	5:3
Disease duration	297 (12-681)
RF positive	3
ACPA positive	4
ESR (mm/h)	33 (12-43)
CRP (mg/l)	31.3 (3.4-129)
DAS28	3.64 (2.73-5.54)

Supplemental Table 2. Clinical features of RA patients included in studies of synovial biopsy explants.
*ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = disease activity score 28, RF=rheumatoid factor, ACPA=anti-cyclic citrullinated peptide antibody. 1 patient was being treated with NSAIDs only, 2 with MTX, 2 with NSAIDs and MTX, 2 with MTX and anti-TNF, and 1 with NSAIDs, MTX, corticosteroids and anti-TNF.

	RA	PsA
CD3	R=0.7903 p<0.001	R=0.2098 p=0.5128
CD22	R=-0.07211 p=0.7907	R=0.04203 p=0.8968
CD55	R=0.4571 p=0.0867	R=0.2238 p=0.4845
CD68	R=0.6324 p<0.01	R=0.2098 p=0.5128
CD163	R=0.7275 p<0.005	R=0.08392 p=0.7954
vWF	R=0.4464 p=0.0953	R=0.2448 p=0.4433

Supplemental Table 3. Correlation of Btk expression and number of cells/mm2 expressing cell markers in RA and PsA synovial tissue.

* Correlations were calculated using Spearman's rank correlation coefficient.

	RA (n=16)	PsA (n=12)	p
CD3	Mean: 439.7 SEM: 149.8	Mean: 297.9 SEM: 91.1	0.9076
CD22	Mean: 54.1 SEM: 10.2	Mean: 78.0 SEM: 25.0	0.8164
CD55	Mean: 715.5 SEM: 237.7	Mean: 755.3 SEM: 175.3	0.4792
CD68	Mean: 2188.6 SEM: 370.24	Mean: 2107.3 SEM: 423.0	0.9076
CD163	Mean: 4977.8 SEM: 746.0	Mean: 4059.5 SEM: 798.5	0.3681
vWF	Mean: 657.1 SEM: 109.1	Mean: 736.7 SEM: 191.9	0.9611

Supplemental Table 4. Mean and SEM of number of positive cells/mm2 for each cellular marker in the RA and PsA cohort.

* The Mann-Whitney U test was used for comparing cellular marker expression between diagnostic groups.

	Btki		CD40L		CD40L +Btki		10% SF RA		10% SF RA +Btki	
Gene symbol	Fold induction	SD	Fold induction	SD	Fold induction	SD	Fold induction	SD	Fold induction	SD
ADAMTS1	0,20	0,15	1,00	0,11	1,16	0,55	0,87	1,05	2,51	2,33
ADAMTS13	1,27	0,45	1,33	0,64	1,50	0,26	0,37	0,29	0,93	0,43
ADAMTS8	0,17	0,28	0,74	0,36	0,82	0,25	0,30	0,03	0,14	0,04
CD44	0,84	0,02	2,87	0,37	2,92	0,38	0,81	0,12	0,92	0,03
CDH1	0,94	0,40	1,71	0,92	1,06	0,61	0,30	0,11	0,29	0,14
CNTN1	0,07	0,19	0,13	0,18	0,23	0,17	0,29	0,19	0,04	0,04
COL11A1	0,29	0,10	0,96	0,95	5,94	6,45	0,97	1,13	0,91	1,14
COL12A1	2,67	0,44	0,46	0,45	0,67	0,88	0,29	0,19	0,24	0,19
COL14A1	0,57	0,18	0,45	0,26	0,66	0,30	0,23	0,04	0,22	0,12
COL15A1	0,60	0,07	0,25	0,01	0,34	0,05	0,27	0,12	0,38	0,06
COL16A1	3,38	0,50	0,42	0,32	0,65	0,60	0,30	0,17	0,25	0,18
COL1A1	0,87	0,17	1,74	1,02	1,34	0,27	0,58	0,32	0,83	0,18
COL4A2	0,71	0,10	1,02	0,09	0,96	0,18	1,35	0,18	1,05	0,14
COL5A1	3,83	0,77	0,45	0,45	0,61	0,96	0,29	0,19	0,24	0,19
COL6A1	0,97	0,19	1,99	0,28	1,70	0,78	0,87	0,29	1,22	0,65
COL6A2	0,66	0,24	1,30	1,69	1,34	2,13	0,55	0,37	0,81	0,64
COL7A1	0,80	0,54	1,36	0,78	0,35	0,31	0,10	0,03	0,39	0,45
COL8A1	0,35	0,17	0,84	0,19	0,67	0,28	0,44	0,10	0,53	0,15
VCAN	0,85	0,15	1,30	0,44	1,44	0,37	1,68	0,86	1,71	0,98
CTGF	1,24	0,86	0,91	0,48	3,77	0,23	0,33	0,22	0,06	0,05
CTNNA1	0,72	0,08	0,98	0,16	0,99	0,09	0,85	0,15	0,90	0,10
CTNNB1	0,68	0,05	1,02	0,27	1,00	0,24	1,73	0,38	1,63	0,22
CTNND1	1,04	0,06	1,14	0,12	1,22	0,10	0,56	0,11	0,76	0,05
CTNND2	2,53	0,57	2,78	2,34	2,60	1,34	0,21	0,22	0,27	0,15
ECM1	0,74	0,03	1,02	0,32	0,95	0,16	0,76	0,18	0,92	0,14
FN1	0,51	0,24	1,20	0,45	1,36	0,49	0,61	0,24	0,72	0,28
HAS1	0,23	0,11	0,29	0,32	0,92	1,32	0,18	0,19	0,31	0,37
ICAM1	1,01	0,12	7,66	0,81	6,63	0,44	0,59	0,07	0,56	0,07
ITGA1	0,66	0,32	1,50	0,56	1,49	0,88	0,76	0,38	1,60	0,09
ITGA2	0,64	0,15	1,05	0,29	0,95	0,13	0,44	0,21	0,64	0,35
ITGA3	0,94	0,24	1,03	0,64	0,73	0,20	0,41	0,17	0,63	0,08
ITGA4	0,60	0,10	0,67	0,22	0,67	0,19	0,49	0,09	0,56	0,06
ITGA5	0,92	0,11	1,33	0,09	1,23	0,10	0,71	0,11	0,78	0,03
ITGA6	0,85	0,22	1,26	0,29	1,10	0,14	0,90	0,18	0,97	0,18
ITGA7	1,95	0,49	0,78	0,83	0,43	0,37	0,53	0,39	0,98	0,71
ITGA8	0,24	0,67	0,14	0,10	0,63	1,00	0,38	0,32	0,04	0,04
ITGAL	0,93	0,08	0,85	0,11	0,89	0,23	0,48	0,08	0,60	0,05
ITGAM	1,06	0,11	0,68	0,07	0,78	0,19	0,54	0,02	0,64	0,09
ITGAV	0,94	0,05	1,87	0,32	1,69	0,27	0,79	0,11	0,90	0,07
ITGB1	0,72	0,05	1,00	0,26	0,96	0,21	0,91	0,25	1,01	0,19
ITGB2	0,87	0,08	0,98	0,02	0,99	0,12	0,69	0,04	0,77	0,06
ITGB3	0,67	0,35	2,60	0,91	2,00	0,57	1,46	0,67	1,21	0,41
ITGB4	1,19	0,39	0,35	0,05	0,78	0,21	0,39	0,13	0,53	0,10
ITGB5	0,97	0,09	0,98	0,17	1,08	0,08	0,60	0,18	0,81	0,07
KAL1	0,75	0,14	0,72	0,64	0,68	0,57	0,54	0,23	0,75	0,29
LAMA1	0,60	0,20	0,43	0,25	0,34	0,19	1,93	2,03	2,04	2,68
LAMA2	0,94	0,16	1,48	0,23	1,11	0,24	0,96	0,38	1,48	0,49
LAMA3	0,82	0,06	1,34	0,40	1,31	0,19	0,52	0,03	0,50	0,13
LAMB1	1,16	0,20	3,43	1,44	3,42	0,82	0,76	0,50	1,10	0,14
LAMB3	0,51	0,03	1,47	0,27	1,24	0,15	1,66	0,38	1,56	0,21
LAMC1	0,87	0,29	1,14	0,28	0,91	0,18	0,81	0,24	0,92	0,30
MMP1	0,62	0,41	28,38	27,72	5,17	5,05	6,45	3,58	1,02	0,47
MMP10	0,73	1,20	17,91	8,05	7,14	3,94	8,26	5,97	1,56	1,02
MMP11	1,32	4,45	1,59	1,24	2,95	1,03	0,63	0,63	0,76	0,35
MMP12	7,63	7,99	0,71	0,17	0,60	0,05	0,89	0,21	0,84	0,34
MMP13	3,79	0,74	0,46	0,45	0,67	0,88	0,29	0,19	0,24	0,19
MMP14	1,03	0,29	2,76	0,63	3,28	0,18	0,47	0,16	0,54	0,12
MMP15	0,27	0,50	1,95	0,66	1,65	0,43	0,12	0,10	0,09	0,11
MMP16	1,38	0,84	0,38	0,54	0,67	0,88	0,29	0,19	0,24	0,19
MMP2	0,66	0,15	1,00	0,58	0,94	0,53	0,62	0,21	0,70	0,28
MMP3	1,94	1,26	0,44	0,47	0,67	0,88	0,48	0,26	0,43	0,29
MMP7	2,07	1,33	27,79	9,74	20,33	7,96	0,56	0,14	0,66	0,34
MMP8	1,69	0,27	2,34	0,54	1,92	0,48	3,72	1,91	1,93	0,81
MMP9	0,80	0,21	1,36	0,81	1,33	1,08	0,69	0,20	0,94	0,24
NCAM1	1,11	0,37	1,11	0,27	1,02	0,04	0,57	0,24	0,69	0,14
PECAM1	1,45	0,25	0,73	0,15	0,69	0,13	0,88	0,17	0,83	0,09
SELE	3,95	0,88	0,40	0,45	0,65	0,78	0,29	0,19	0,24	0,19
SELL	0,89	0,29	0,97	0,05	0,97	0,07	0,57	0,24	0,55	0,18
SELP	0,19	0,23	0,41	1,34	0,74	2,15	0,33	0,12	0,24	0,17
SGCE	0,59	0,70	0,67	0,42	0,94	0,39	0,28	0,08	0,37	0,23
SPARC	1,03	0,04	0,88	0,46	1,04	0,26	0,59	0,23	0,98	0,24
SPG7	0,90	0,13	1,27	0,17	1,28	0,09	0,50	0,09	0,70	0,12
SPP1	0,90	0,26	1,07	0,39	1,19	0,45	1,90	0,27	1,31	0,08
TGFBI	0,71	0,12	0,80	0,13	0,70	0,13	1,28	0,14	1,21	0,12
THBS1	0,73	0,32	0,58	0,15	0,52	0,18	22,45	6,98	43,93	16,33

THBS2	1,31	0,21	0,65	0,15	0,92	0,22	1,03	0,46	1,42	0,22
THBS3	1,12	0,28	0,80	0,24	0,91	0,12	0,40	0,12	0,79	0,13
TIMP1	1,04	0,19	1,28	0,08	1,48	0,47	1,81	0,63	1,57	0,73
TIMP2	1,03	0,15	0,90	0,09	0,92	0,07	0,68	0,05	0,83	0,03
TIMP3	2,51	0,90	1,85	1,26	0,94	0,35	0,43	0,34	0,37	0,35
CLEC3B	1,14	0,13	0,25	0,02	0,32	0,09	0,52	0,10	0,63	0,13
TNC	0,36	0,11	45,36	26,38	21,44	5,55	1,38	0,36	0,84	0,10
VCAM1	1,07	0,41	6,03	2,89	5,93	0,83	n.d.		n.d.	
VTN	0,74	0,18	1,14	0,36	1,20	0,44	0,73	0,11	0,92	0,25

Supplemental Table 5. Regulation of extracellular matrix and adhesion molecule gene expression by RN486 in macrophages.

Macrophages were left untreated or treated with RN486 (Btki, 1 µM) for 30 minutes prior to stimulation with medium alone, CD40L (1µg/ml) or 10% RA patient synovial fluid (SF RA) for 4 hours. Total RNA was extracted, reverse-transcribed and changes in gene expression analyzed by low density qPCR array (Qiagen, PAHS 013C). Genes amplifying after cycle 33 are noted in orange. Data were normalized to the expression of 5 housekeeping genes (B2M, HPRT1, RPL13A, GAPDH and ACTB) and are presented as mean fold induction compared to untreated unstimulated cells ± standard deviation (SD) of 3 independent experiments. n.d., not detectable.

	Btki only		IgG		IgG+Btki		CD40L		CD40L+Btki		10% SF RA		RA+Btki	
Gene Symbol	Fold Induction	SD	Fold Induction	SD	Fold Induction	SD	Fold Induction	SD	Fold Induction	SD	Fold Induction	SD	Fold Induction	SD
ADORA2A	0,67	0,19	5,17	1,00	2,02	0,80	173,01	0,33	121,57	14,39	1,55	0,19	0,70	0,17
C5	1,18	0,15	1,14	0,22	1,14	0,15	0,48	0,13	0,57	0,04	0,54	0,11	0,98	0,19
C8A	1,25	0,43	1,53	0,51	1,41	0,59	0,19	1,86	0,80	1,52	0,83	0,79	0,23	0,30
CAMP	0,92	0,20	0,93	0,18	0,79	0,14	0,65	0,30	0,69	0,23	0,84	0,11	0,89	0,18
CASP1	0,98	0,26	1,22	0,29	1,03	0,33	1,66	0,06	1,47	0,11	0,69	0,04	0,71	0,05
CASP4	1,00	0,25	1,24	0,27	1,05	0,31	1,42	0,05	1,30	0,19	0,80	0,06	0,97	0,12
CCL2	0,84	0,18	1,93	0,21	1,40	0,42	1,74	0,42	1,44	0,26	1,06	0,85	1,34	0,73
CCR3	1,16	0,15	1,42	0,13	1,25	0,23	1,83	1,13	1,45	1,85	4,03	2,61	1,32	1,96
CD14	1,25	0,28	1,42	0,20	1,23	0,31	0,82	0,17	0,80	0,15	0,56	0,35	0,77	0,27
CD1D	0,59	0,26	0,50	0,31	2,57	2,08	0,42	0,91	0,70	0,81	0,56	0,49	0,52	0,47
CD55	1,23	0,38	1,56	0,37	1,32	0,45	1,73	0,05	1,34	0,06	1,63	0,02	1,83	0,22
CHUK	0,97	0,31	1,40	0,44	1,09	0,39	1,11	0,23	0,99	0,17	1,05	0,16	1,18	0,11
COLEC12	1,13	0,21	1,04	0,16	1,02	0,21	0,57	0,09	0,61	0,04	0,81	0,10	0,85	0,13
CRP	2,85	2,09	1,02	0,63	1,13	0,76	0,19	1,67	0,84	1,32	0,52	0,79	0,47	0,62
CXCR4	1,34	0,23	0,97	0,10	1,08	0,14	0,97	0,19	0,89	0,11	0,85	0,20	1,03	0,30
CYBB	1,18	0,28	1,37	0,29	1,20	0,33	1,03	0,17	0,98	0,15	0,68	0,03	0,79	0,06
DEFB4A	0,75	1,17	1,59	3,28	0,43	1,20	0,46	1,67	0,84	1,32	0,84	0,97	0,76	0,96
DMBT1	1,47	0,42	1,36	0,41	1,68	0,55	1,11	0,58	7,13	7,94	0,90	2,48	0,81	1,78
FN1	1,18	0,47	2,10	1,33	1,30	0,53	1,03	0,22	1,09	0,37	0,70	0,40	0,84	0,27
HMOX1	1,12	0,37	1,55	0,57	1,39	0,52	0,47	0,16	0,54	0,11	0,68	0,12	0,92	0,04
IFNA1	1,42	0,49	4,87	1,60	2,05	1,52	2,17	0,17	0,80	0,16	0,63	0,25	0,57	0,31
IFNB1	1,89	1,07	1,22	0,31	1,47	0,34	0,73	2,17	1,03	1,49	0,53	0,79	0,11	0,06
IFNGR1	1,31	0,26	1,23	0,18	1,25	0,27	0,98	0,09	0,96	0,07	1,06	0,13	1,60	0,25
IFNGR2	1,04	0,16	2,15	0,37	1,34	0,34	3,78	0,08	3,93	0,82	0,57	0,15	0,71	0,17
IKBKB	1,04	0,20	1,36	0,26	1,18	0,25	1,33	0,04	1,11	0,12	0,71	0,10	0,90	0,14
IL10	1,15	0,26	0,73	0,15	1,14	0,43	1,79	0,16	0,92	0,26	0,56	0,17	0,33	0,05
IL12RB2	0,69	0,29	3,06	1,73	2,23	1,03	2,62	0,36	0,85	0,22	0,57	0,22	0,39	0,14
IL1A	0,85	0,22	5,37	1,51	2,09	0,64	45,32	0,51	27,88	9,34	1,85	0,23	1,72	0,25
IL1B	0,97	0,44	6,77	2,40	2,21	0,96	211,64	0,36	125,81	11,22	4,21	0,20	3,68	1,41
IL1F10	1,99	1,03	2,76	1,41	2,08	1,17	0,46	1,67	0,84	1,32	0,51	0,79	0,47	0,62
IL36RN	0,71	0,34	2,70	3,53	0,85	0,54	5,74	0,41	5,72	2,46	7,02	0,20	2,50	0,84
IL36A	1,33	0,47	1,46	0,64	1,31	0,47	0,40	4,22	0,93	3,13	0,55	0,79	0,47	0,62
IL37	1,26	0,30	1,42	0,47	1,34	0,37	1,10	0,17	1,60	0,92	0,77	0,31	2,09	1,51
IL36B	1,11	1,96	2,36	5,66	0,82	2,00	2,16	0,27	2,58	1,13	3,24	0,16	1,25	0,26
IL36G	1,62	0,53	3,54	1,80	1,78	0,22	34,64	0,31	23,54	12,72	n.d.		n.d.	
IL1R1	1,59	0,50	1,01	0,28	1,36	0,44	0,32	0,19	0,33	0,04	1,00	0,17	1,52	0,32
IL1R2	0,99	0,42	1,32	0,53	0,97	0,41	1,08	0,48	0,50	0,21	7,21	0,68	6,63	6,46
IL1RAP	0,98	0,21	1,71	0,29	1,18	0,29	0,76	0,01	0,75	0,10	1,64	0,14	2,01	0,79
IL1RAPL2	0,49	0,72	0,71	1,07	0,93	0,95	1,00	0,36	4,40	3,73	0,11	1,74	0,77	1,29
IL1RL2	0,88	0,15	1,04	0,28	0,96	0,14	0,24	0,42	0,55	0,48	1,49	0,32	0,95	0,17
IL1RN	0,78	0,15	2,31	1,31	0,84	0,14	6,65	0,04	6,06	0,55	7,14	0,22	2,48	0,69
IL6	1,12	1,29	8,35	3,87	3,76	2,51	129,29	0,08	75,13	24,67	0,75	0,09	1,03	0,27
IRAK1	1,35	0,18	1,83	0,33	1,54	0,45	0,99	0,14	0,99	0,13	0,89	0,21	1,10	0,35
IRAK2	0,94	0,17	5,63	1,10	2,11	0,69	11,70	0,34	8,91	1,38	1,57	0,53	1,08	0,47
IRF1	1,26	0,21	1,81	0,30	1,52	0,37	3,37	0,08	3,76	0,71	0,75	0,19	1,44	0,53
LALBA	2,15	0,74	1,82	0,54	1,69	0,57	0,11	4,56	0,84	3,62	0,52	0,79	0,47	0,62
LBP	1,33	0,41	0,97	0,17	1,37	0,46	0,14	1,79	0,83	1,43	0,47	0,65	0,44	0,54
LTF	1,12	0,87	0,93	0,45	0,85	0,84	0,71	0,41	1,07	0,08	0,49	0,09	0,66	0,13
LY96	1,07	0,35	1,25	0,29	1,14	0,40	0,63	0,06	0,59	0,05	0,74	0,07	0,75	0,08
LYZ	1,20	0,46	1,21	0,39	1,10	0,42	0,92	0,03	0,95	0,06	0,70	0,19	0,88	0,20
MAPK14	1,22	0,25	1,46	0,31	1,28	0,34	0,98	0,15	0,99	0,11	0,60	0,13	0,96	0,16
MAPK8	1,31	0,28	1,40	0,26	1,34	0,36	1,16	0,25	0,96	0,12	0,76	0,14	1,21	0,26
MIF	0,94	0,12	1,03	0,13	0,89	0,13	0,93	0,15	1,05	0,13	0,83	0,18	0,82	0,16
MYD88	1,19	0,29	1,70	0,39	1,36	0,49	1,13	0,21	1,10	0,22	0,87	0,05	0,98	0,19
NCF4	1,09	0,29	1,18	0,29	1,07	0,30	0,70	0,04	0,70	0,06	0,54	0,08	0,78	0,09

NFKB1	0,94	0,20	2,64	0,52	1,57	0,54	7,12	0,05	6,20	0,55	1,04	0,04	0,86	0,05
NFKB2	1,50	0,26	2,71	0,50	1,86	0,32	2,71	0,02	2,22	0,39	0,63	0,19	1,05	0,30
NFKBIA	0,84	0,13	4,69	0,97	2,08	0,48	9,58	0,09	8,86	0,83	1,02	0,06	0,87	0,10
NLRC4	0,90	0,20	0,80	0,17	0,79	0,18	0,49	0,18	0,47	0,10	0,91	0,08	1,41	0,26
NOS2	1,46	0,32	1,79	0,55	1,78	0,39	0,36	0,53	0,25	0,10	0,52	0,42	0,42	0,37
PGLYRP1	1,87	0,65	1,63	0,58	1,91	0,73	0,66	0,74	0,39	0,26	0,17	0,73	0,25	0,15
PGLYRP2	1,33	0,53	1,09	0,27	1,54	0,70	0,89	1,67	0,83	1,33	0,52	0,79	0,04	0,04
PGLYRP3	1,57	0,62	1,41	0,52	1,54	0,64	0,98	0,32	1,34	0,38	0,72	0,37	0,61	0,28
PPBP	0,82	0,90	1,04	1,08	0,92	0,91	1,30	0,18	1,04	0,31	2,76	0,50	4,32	2,53
PROC	1,14	0,19	1,73	0,72	1,08	0,10	0,13	0,21	0,30	0,22	0,26	0,20	0,49	0,07
PTAFR	1,18	0,27	1,33	0,28	1,13	0,31	1,79	0,20	1,83	0,39	0,52	0,15	1,08	0,08
S100A12	0,81	0,15	0,86	0,14	0,79	0,09	1,59	0,33	1,29	0,38	0,40	0,42	0,47	0,25
SERPINA1	1,06	0,23	1,22	0,22	1,00	0,22	1,19	0,07	1,23	0,13	0,68	0,13	0,88	0,17
SERPINE1	0,82	0,25	3,87	1,36	1,41	0,52	25,51	0,30	14,20	1,56	1,34	0,30	1,57	0,85
SFTPD	1,74	0,46	2,02	0,96	1,63	0,46	1,30	0,28	1,52	0,43	1,53	0,42	1,25	0,75
TGFB1	1,22	0,24	1,57	0,30	1,30	0,33	0,89	0,07	0,94	0,13	0,60	0,15	0,78	0,21
TLR1	0,95	0,36	1,34	0,26	1,15	0,25	1,88	0,19	1,72	0,37	0,68	0,26	1,03	0,24
TLR10	0,39	2,25	1,51	10,22	0,73	4,67	1,67	0,33	1,26	0,49	0,30	0,16	0,46	0,24
TLR2	1,01	0,14	2,75	0,53	1,58	0,35	3,42	0,08	2,94	0,34	1,04	0,29	1,05	0,37
TLR3	1,51	0,35	0,98	0,15	1,38	0,32	0,46	0,37	0,41	0,11	0,60	0,36	0,98	0,15
TLR4	1,25	0,23	1,03	0,10	1,16	0,23	0,52	0,18	0,62	0,10	1,01	0,19	1,20	0,34
TLR6	1,15	0,15	1,65	0,28	1,47	0,32	1,06	0,11	1,03	0,20	0,77	0,22	1,00	0,20
TLR8	1,25	0,23	0,85	0,19	0,97	0,12	0,66	0,07	0,69	0,09	0,61	0,09	0,93	0,12
TLR9	1,29	0,22	2,84	0,80	2,19	0,42	0,97	0,40	0,81	0,44	1,24	0,94	0,74	0,62
TNF	0,82	0,20	3,86	0,77	1,60	0,47	12,73	0,48	8,36	2,42	0,37	0,44	0,36	0,24
TNFRSF1A	0,94	0,19	0,93	0,12	0,88	0,16	0,75	0,21	0,84	0,18	0,65	0,23	0,92	0,23
TOLLIP	0,92	0,20	1,21	0,24	1,02	0,29	0,89	0,13	0,79	0,12	0,76	0,03	0,81	0,04
TRAF6	1,24	0,25	1,44	0,29	1,34	0,35	1,13	0,19	0,95	0,10	0,93	0,13	1,22	0,16
TREM1	0,71	0,25	1,48	0,31	0,94	0,06	1,43	0,03	1,21	0,17	2,54	0,23	1,93	0,47

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Supplemental Table 6. Regulation of innate and adaptive immune response gene expression by RN486 in macrophages.

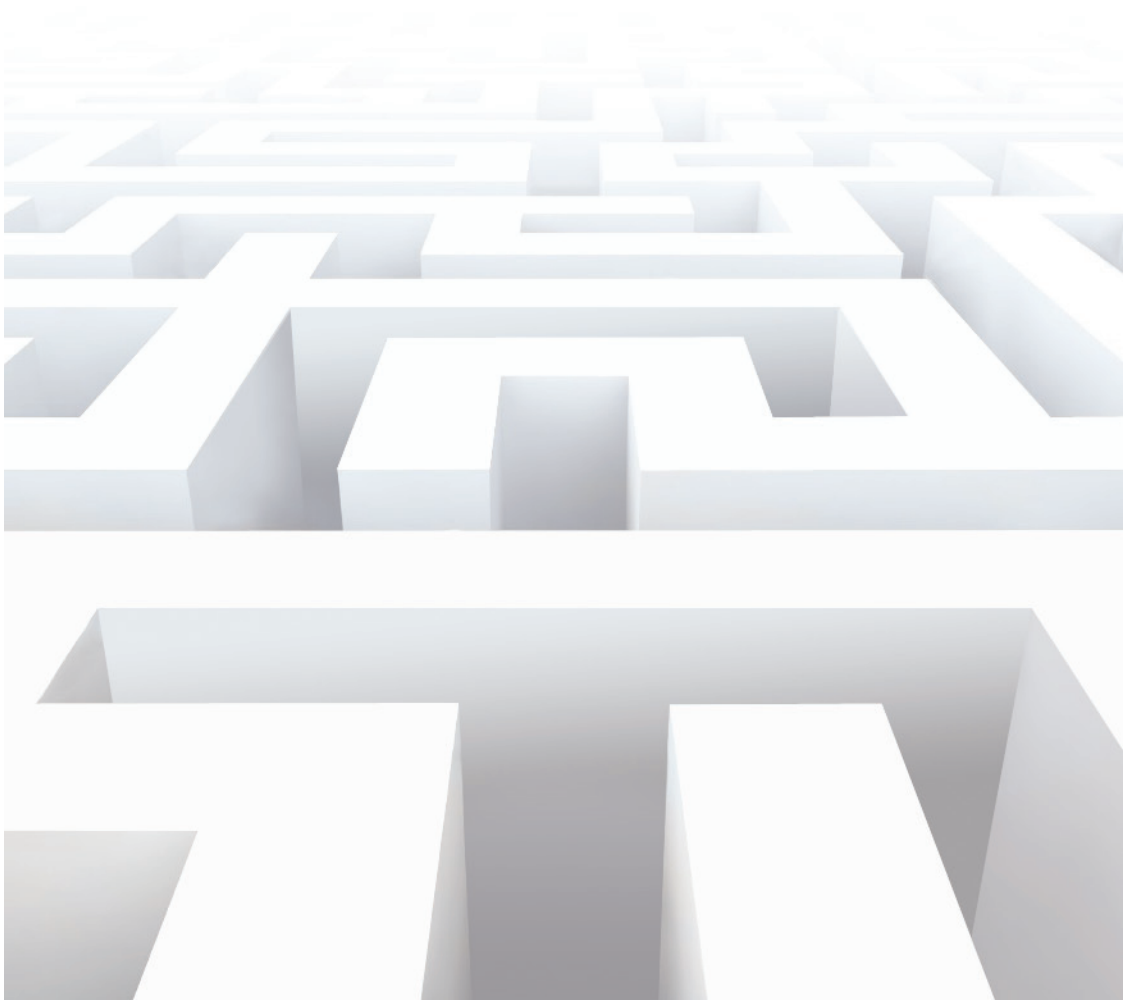
Macrophages were left untreated or treated with RN486 (Btki, 1 µM) for 30 minutes prior to stimulation with medium alone, CD40L (1µg/ml) or 10% RA patient synovial fluid (SF RA) for 4 hours. Total RNA was extracted, reverse-transcribed and changes in gene expression analyzed by low density qPCR array (Qiagen, PAHS 052C). Genes amplifying after cycle 33 are noted in orange. Data were normalized to the expression of 5 housekeeping genes (B2M, HPRT1, RPL13A, GAPDH and ACTB) and are presented as mean fold induction compared to untreated unstimulated cells ± standard deviation (SD) of 3 independent experiments. n.d., not detectable.

	Btki		CD40L		CD40L+Btki		10% SF RA		10% SF RA+Btki	
Gene symbol	Fold induction	SD	Fold induction	SD	Fold induction	SD	Fold induction	SD	Fold induction	SD
AGGF1	0,82	0,09	0,66	0,08	0,69	0,17	0,91	0,18	1,21	0,11
AMOT	1,64	0,93	3,34	2,91	2,43	1,57	3,09	3,05	1,56	1,38
ANG	1,08	0,06	0,73	0,06	0,62	0,08	0,52	0,06	0,72	0,09
ANGPT1	3,45	0,43	1,51	1,84	0,99	1,12	0,49	0,50	0,40	0,38
ANGPT2	1,24	0,22	4,90	1,53	2,92	0,13	0,64	0,33	1,14	0,47
ANGPTL1	1,64	0,94	1,92	1,04	0,50	0,18	0,50	0,30	0,59	0,20
BAI1	1,26	0,42	1,14	0,38	1,25	0,67	0,47	0,10	0,67	0,33
BMP2	4,86	0,70	1,67	1,01	1,47	0,46	0,11	0,05	0,40	0,38
BTG1	0,86	0,11	2,70	0,52	2,27	0,36	2,13	0,49	2,05	0,22
CCL15	3,98	0,54	1,68	1,94	1,09	0,80	0,49	0,50	0,40	0,38
CCL2	0,57	0,15	1,84	0,45	1,64	0,46	1,19	0,42	1,57	0,79
CD55	0,95	0,16	1,76	0,20	1,50	0,02	1,66	0,19	2,03	0,23
CD59	0,94	0,19	1,13	0,13	0,96	0,16	0,71	0,15	0,83	0,13
CHGA	1,05	2,40	0,46	0,21	0,47	0,28	0,31	0,15	0,26	0,16
COL18A1	1,32	0,15	0,55	0,24	0,42	0,08	0,29	0,02	0,35	0,11
COL4A3	1,28	1,37	1,72	0,33	3,25	0,45	1,90	0,81	3,65	1,80
CSF3	5,78	1,47	1,68	2,05	1,20	2,38	0,32	0,28	0,32	0,37
CXCL10	0,19	0,08	35,24	16,16	61,30	29,55	2,13	0,58	2,85	1,27
CXCL11	0,20	0,09	20,04	5,90	31,53	15,55	0,39	0,21	0,38	0,33
CXCL12	0,49	0,30	0,45	0,56	0,66	0,28	0,84	0,81	1,35	1,36
CXCL13	0,90	0,30	1,47	1,58	1,15	0,81	0,49	0,50	0,34	0,26
CXCL14	1,70	1,14	4,96	7,39	0,43	0,37	0,13	0,10	0,10	0,01
CXCL2	1,00	0,52	4,54	1,40	3,26	1,09	6,41	1,11	6,25	1,33
CXCL3	1,28	0,43	13,88	3,06	8,58	2,43	15,35	1,29	13,37	3,13
CXCL5	2,27	2,00	7,76	,16	5,87	2,19	9,34	6,68	10,35	6,56
CXCL6	1,78	0,15	5,45	3,29	4,13	3,13	2,11	0,92	3,84	2,00
CXCL9	0,08	0,06	13,03	25,99	5,31	6,11	2,01	2,03	1,19	0,48
TYMP	1,04	0,16	1,97	0,62	1,73	0,34	0,77	0,18	1,11	0,36
EDIL3	1,66	4,78	1,25	1,52	0,91	0,86	0,49	0,50	0,36	0,42
EREG	0,47	0,13	11,53	3,75	5,85	0,89	12,05	5,55	8,53	2,79
FGF1	1,66	1,33	1,46	1,68	0,92	1,06	0,49	0,50	0,40	0,38

FGF13	1,34	0,56	0,86	0,26	1,47	0,70	2,98	1,63	3,49	2,72
FGF2	1,20	0,30	0,38	0,19	0,25	0,05	0,20	0,13	0,50	0,24
FGFBP1	1,10	1,37	5,19	4,95	2,67	1,52	0,49	0,30	2,72	2,96
FIGF	1,62	0,47	4,35	1,52	2,39	0,15	0,80	0,35	1,43	0,47
FN1	0,55	0,25	1,05	0,40	1,30	0,49	0,64	0,35	0,71	0,27
FST	1,05	0,88	13,66	6,97	17,73	3,62	0,39	0,23	0,34	0,13
GRN	1,11	0,13	1,08	0,04	1,06	0,15	0,63	0,11	0,87	0,10
GRP	4,40	0,80	0,91	1,51	0,65	0,85	0,39	0,41	0,32	0,31
HGF	0,82	0,22	0,42	0,10	0,49	0,07	0,73	0,27	0,85	0,28
IFNA1	0,91	0,29	3,72	1,52	1,16	0,36	0,55	0,22	0,19	0,16
IFNB1	3,56	0,24	2,63	3,31	2,10	1,64	0,53	0,59	0,44	0,45
IFNG	0,54	0,28	4,01	2,48	1,55	0,88	0,71	0,31	0,80	0,21
IL10	0,81	0,13	2,37	0,44	1,14	0,23	0,55	0,13	0,55	0,05
IL12A	2,34	1,58	1,84	0,87	1,95	0,43	1,31	0,64	0,42	0,22
IL12B	1,68	2,48	6273,47	3587,06	8583,71	4616,64	0,38	0,05	0,25	0,11
IL17F	1,37	3,18	1,74	3,49	1,09	2,18	0,44	0,53	0,37	0,41
IL6	1,51	1,28	225,00	303,57	139,60	181,02	1,12	0,82	1,96	2,84
IL8	0,75	0,47	59,34	12,69	37,75	12,22	3,63	1,32	2,77	1,13
KITLG	0,67	0,13	1,93	0,73	2,14	0,82	0,81	0,47	1,50	0,54
KLK3	0,80	3,71	0,38	0,16	0,38	0,17	0,44	0,53	0,09	0,06
LEP	0,43	0,42	0,41	0,29	0,48	0,66	1,06	1,51	1,09	1,83
MDK	1,03	0,22	0,89	0,11	0,99	0,50	0,63	0,41	0,60	0,35
FOXO4	0,87	0,14	0,65	0,10	0,77	0,13	0,75	0,24	0,97	0,19
NPPB	0,80	4,73	1,64	0,39	2,95	0,89	0,18	0,20	0,12	0,06
NPR1	1,11	1,54	4,29	2,53	1,00	0,07	1,33	0,33	2,13	0,60
PDGFB	1,23	0,48	7,99	3,15	3,47	0,50	0,26	0,06	0,36	0,08
PDGFD	0,91	1,95	1,65	0,47	0,76	0,43	1,11	0,88	1,25	0,68
PF4	0,99	0,17	1,61	0,47	1,22	0,03	1,31	0,67	1,72	0,27
PGF	5,93	4,45	0,90	0,15	0,73	0,15	0,42	0,83	0,75	1,03
PLG	4,74	2,93	1,51	1,84	1,07	1,04	0,11	0,04	0,40	0,38
PPBP	2,03	1,46	1,28	0,26	1,13	0,35	2,96	1,37	4,87	2,87
PRL	1,53	0,89	1,60	1,50	1,10	0,85	0,49	0,50	0,44	0,36
PROK1	0,92	6,75	0,82	0,17	1,53	0,68	0,67	0,24	0,57	0,16
PTN	1,27	0,91	1,54	4,00	1,04	2,40	0,40	0,38	0,33	0,30
RHOB	0,54	0,07	0,89	0,19	0,71	0,16	3,20	0,83	2,46	0,33
RNH1	1,09	0,10	0,92	0,12	0,91	0,13	0,63	0,22	0,73	0,10
RUNX1	0,85	0,10	1,68	0,06	1,25	0,29	1,17	0,08	1,52	0,29
SERPINC1	0,89	0,45	1,83	0,36	2,45	0,19	0,26	0,36	0,51	0,30
SERPINE1	0,64	0,19	24,82	12,61	14,14	1,77	1,37	0,37	1,56	0,73
SERPINF1	1,14	0,20	0,89	0,15	0,93	0,19	0,59	0,13	0,81	0,20
SPINK5	4,07	0,58	1,51	1,84	1,48	0,86	1,11	1,19	0,91	0,92
STAB1	0,75	0,25	0,99	0,41	0,80	0,33	0,54	0,08	0,89	0,33
TGFA	1,02	0,23	0,50	0,18	0,57	0,21	3,28	1,46	2,74	0,50
TGFB1	0,95	0,13	1,04	0,07	1,01	0,14	0,56	0,06	0,79	0,18
THBS1	0,94	0,43	0,68	0,12	0,70	0,11	29,63	12,88	65,15	22,95
TIE1	2,48	1,03	1,87	0,98	0,57	0,21	0,50	0,21	0,93	0,11
TIMP1	1,04	0,20	1,63	0,36	1,59	0,46	1,63	0,36	1,67	0,82
TIMP2	1,10	0,13	0,99	0,05	0,90	0,09	0,67	0,09	0,94	0,05
TIMP3	2,75	0,98	1,68	1,73	1,07	1,04	0,49	0,50	0,40	0,38
TNF	0,73	0,13	18,32	5,56	9,11	1,75	0,37	0,04	0,38	0,21
TNNI2	1,25	0,19	0,64	0,13	0,63	0,12	0,71	0,15	0,82	0,14
TNNI3	0,90	2,03	0,88	0,54	0,91	0,04	0,85	0,42	3,72	1,35
VEGFA	1,10	0,14	2,59	0,73	1,76	0,57	1,47	0,23	1,85	0,76

Supplemental Table 7. Regulation of angiogenic growth factor and angiogenesis gene expression by RN486 in macrophages.

Macrophages were left untreated or treated with RN486 (Btki, 1 µM) for 30 minutes prior to stimulation with medium alone, CD40L (1µg/ml) or 10% RA patient synovial fluid (SF RA) for 4 hours. Total RNA was extracted, reverse-transcribed and changes in gene expression analyzed by low density qPCR array (Qiagen, PAHS 072C). Genes amplifying after cycle 33 are noted in orange. Data were normalized to the expression of 5 housekeeping genes (B2M, HPRT1, RPL13A, GAPDH and ACTB) and are presented as mean fold induction compared to untreated unstimulated cells ± standard deviation (SD) of 3 independent experiments.



5

Bruton's tyrosine kinase plays distinct roles in coupling CD40 and FcγR stimulation to cytokine production in macrophages

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ABSTRACT

We have previously shown that Btk is expressed in rheumatoid arthritis (RA) synovial tissue, especially in macrophages, and *in vitro* regulates macrophage production of inflammatory mediators in response to multiple ligands relevant to RA. The objective of this study was to elucidate the molecular mechanisms by which Btk regulates macrophage activation and contributes to pathology in RA. Btk phosphorylation was detected via Phos-Tag SDS-PAGE and immunoblottting. IL6 production and NFκB DNA-binding was measured by ELISA and NFκB Trans-AM experiments respectively. In contrast to B cells, Btk was already activated in in macrophages that were in steady state, and Btk inhibitors exerted their functional effects without modifying Btk phosphorylation. In macrophages derived from XLA patients lacking functional Btk, IL6 production was increased compared to healthy donor macrophages and this was not due to increased NFκB binding. IgG-induced NFκB binding was Btk kinase-dependent, while CD40L-induced NFκB binding was independent of Btk kinase activity. Btk activity drives IL6 production in macrophages and this can only be partially explained by activation of NFκB, as Btk also drives macrophage cytokine production by an NFκB-independent route not requiring Btk catalytic activity.

Introduction

Tec family kinases, in particular Btk, are being increasingly recognized for their role in several disease-relevant signaling pathways in B- and myeloid cells, making it an appealing drug discovery target. Small-molecule Btk inhibitors have been demonstrated to potently and selectively inhibit Btk in pre-clinical studies and some inhibitors have propelled the clinical development of such molecules towards clinical trials in RA patients.[1-3] Tec family kinases are the second largest class of cytoplasmic protein tyrosine kinase, consisting of 5 mammalian members: Btk, Bmx (bone marrow kinase on the X-chromosome, also known as Etk), Itk (IL-2 inducible T-cell kinase), Rlk (resting lymphocyte kinase, also known as Txk) and Tec (tyrosine kinase expressed in hepatocellular carcinoma). Activation of Tec family kinases upon cell-surface receptor triggering, requires relocalization of the protein to the plasma membrane, which is mediated by the interaction of the PH domain with the lipid phosphatidylinositol (3,4,5)P3, formed by activated phosphatidylinositol-3 kinase (PI3K). Subsequent phosphorylation by Src family kinases (SFKs) and autophosphorylation of tyrosine 223 results in the complete activation of Tec family kinases.[2;4;5] However, Btk has also been reported to be post-translationally modified at other residues. Phosphorylation of Btk at serine 180 by PKCβ results in relocalization to the cytoplasm, thereby inhibiting its function.[6] Similarly, Pin1-mediated phosphorylation of Btk at serine 21 and serine 115 results in negative regulation of Btk.[7] Dual phosphorylation of Btk by PKB can also result in inhibition of its function as PKB-dependent phosphorylation of serine 51 and threonine 495 can provide binding motifs for 14-3-3ζ preventing translocation of Btk to the nucleus.[8;9]

Btk was first identified as the critical kinase for B-cell development and function and is the only known member of the family that causes disease in humans. Mutations in the Btk gene give rise to X-linked agammaglobulinemia (XLA) in men and X-linked immunodeficiency (XID) in mice. Over 1000 mutations have been described that render Btk non-functional or prevent protein expression. XLA is characterized by the complete absence of circulating B-cells due to a developmental block between pro- and pre-B-cell stages, and a severe reduction in serum immunoglobulin levels. A similar, although less severe syndrome in mice, XID, is also caused by a mutation in Btk. In the first years of life, male patients with XLA have an increased susceptibility to recurrent bacterial infections.[10;11] Apart from its role in B cells, where Btk mediates antigen receptor, CD40 and chemokine receptor signaling responses, Btk has also been shown to regulate activation of monocytes, macrophages, dendritic cells (DCs), mast cells and platelets, and osteoclast differentiation.[12-17] It has been shown that Btk signals downstream of Fc

receptors, leading to increased cytokine production in myeloid cells and mast cells and degranulation in mast cells.[13;18;19] Btk has also been identified to regulate signaling by TLR2, 3, 4, 7, 8 and 9 in macrophages, DCs and B cells, activating transcription factors like NF-κB and IRF3 ultimately leading to cytokine and type I IFN production.[20-29] Although there seems to be a functional linkage between Btk and TLR signaling, contradicting reports exist on inflammatory responses by Btk-deficient cells and/or animal models. Btk deficiency in myeloid lineages leads to poor inflammatory responses, and XID mice develop less severe experimental autoimmune encephalitis, DSS-induced colitis, and carrageenan-induced acute edema.[10] XID mice are also reported to be resistant to collagen-induced arthritis.[30] Moreover, in response to systemic LPS treatment XID mice produce less TNF and IL1 and isolated XID macrophages and neutrophils are less responsive to inflammatory stimuli.[3;10] However, in a different study opposing observations have been made in Btk-deficient murine B-cells and macrophages, where TLR4 triggering resulted in increased production of pro-inflammatory cytokines TNF and IL6.[31;32] Also, myeloid cells derived from XLA patients show increased production of inflammatory cytokine in response to LPS.[33;34] These contradictory results warrant the pursuit of a more complete understanding of the molecular mechanisms behind Btk-regulated macrophage activation.

We have shown in **chapter 4** that Btk is expressed in RA synovial tissue, especially in macrophages, and pharmacological inhibition of Btk regulates production of inflammatory mediators by macrophage in response to multiple ligands relevant to RA *in* vitro.[35] The objective of this study was to elucidate the molecular mechanisms by which Btk regulates macrophage activation and contributes to pathology in RA.

MATERIALS AND METHODS

Cell culture

Monocytes were isolated from buffy coats (Sanquin) of healthy donors or XLA patients as previously described.[35] XLA patient samples were obtained from the Academic Medical Center Amsterdam and University Medical Center Utrecht and written informed consent was obtained from both parents/legal representatives and the studies were approved by the ethics committee of the Academic Medical Centre of Amsterdam and the Local Institutional Review Board of the University Medical Center Utrecht. Monocytes were isolated using Lymphoprep (AXIS-SHIELD) density gradient centrifugation followed by Standard Isotone Percoll gradient centrifugation

(Amersham). Monocytes were plated in Iscove-modified Dulbecco medium (IMDM, Invitrogen), supplemented with 1% fetal bovine serum (FBS) for 30 minutes at 37°C, non-adherent cells removed, and monocytes were then cultured for 7 days in IMDM containing 10% FBS, 100μg/ml gentamycin and 5ng/ml GM-CSF (Tebu Bio) prior to use in experiments. The B-cell line Namalwa clone V3M was cultured in Dulbecco's modified Eagle Medium (DMEM, Gibco), supplemented with 10% FBS.

Macrophage and B-cell stimulation

Cell culture grade anti-biotin MACSiBead particles (MiltenyiBiotec) were loaded with biotinylated IgG1 or IgM (BioLegend) according to the manufacturer's instructions (30 mg biotinylated primary antibody per 1×10^8 bead particles). Macrophages were treated with either vehicle control or Btk inhibitor (Btki, PCI32765, 1μM, Selleckchem) for 1 hour, and then either left unstimulated or stimulated with IgG beads (1:1 bead:cell ratio), CD40L (1 μg/mL, R&D Systems), TNF (10ng/ml, Biosource) or LPS (1μg/ml, Sigma-Aldrich) for the indicated time-points. Namalwa clone V3M cells were treated with either vehicle control or Btki (PCI32765, 1μM) for 1 hour, and then either left unstimulated or stimulated with IgM beads (1:1 bead:cell ratio) for the indicated time-points.

Western blot analysis and nuclear extraction

Cells were lysed in 1x Laemmli buffer and protein content quantitated using a BCA protein Assay Kit (Pierce). Equivalent amounts of lysate were resolved by electrophoresis on 7.5% Bis-Tris SDS PAGE gels, either containing 25μM Phos-Tag and 50 μM MnCl2 or not. This method is used to detect protein phosphorylation. The polyacrylamide-bound dinuclear Mn2+ complex (Mn2+-Phos-tag) enhances the mobility shifts of phosphorylated forms of proteins.[36] Gels were analyzed by western blotting using a primary antibody recognizing Btk (Sigma-Aldrich), followed by development with IRDye-680-labeled anti-rabbit immunoglobulin secondary antibodies (LI-COR Biosciences) and visualization using an Odyssey infrared imaging system (LI-COR Biosciences). Nuclear fractions were prepared as described previously and NFκB p50 and p65 DNA-binding activity was determined using a TransAM ELISA (Active Motif).[37]

Cytokine production

Macrophages were left unstimulated or stimulated for 20 h with 1 μg/ml CD40L, IgG beads (1:1 bead:cell ratio), TNF (10 ng/ml) or LPS (1 μg/ml). Cell-free supernatants were harvested and IL6 was measured using PeliKine Compact ELISA kits (Sanquin Reagents) as per the manufacturer's instructions.

Statistical analyses

For *in vitro* experiments, statistical analysis was performed using Windows Graphpad Prism 5 (GraphPad Software, Inc.). ELISA results were expressed as the mean ± SEM. Potential differences between samples were analyzed by Student's t-test. P-values ≤ 0.05 were considered significant.

RESULTS

Btk is already activated in steady-state in human macrophages

We have previously demonstrated (**chapter 4** of this thesis) that IgG and CD40L induce Btk-dependent expression of various genes in human macrophages and that pharmacological inhibition of Btk regulates macrophage production of IL6 in response to multiple ligands. Furthermore, Btk was reported to be phosphorylated on

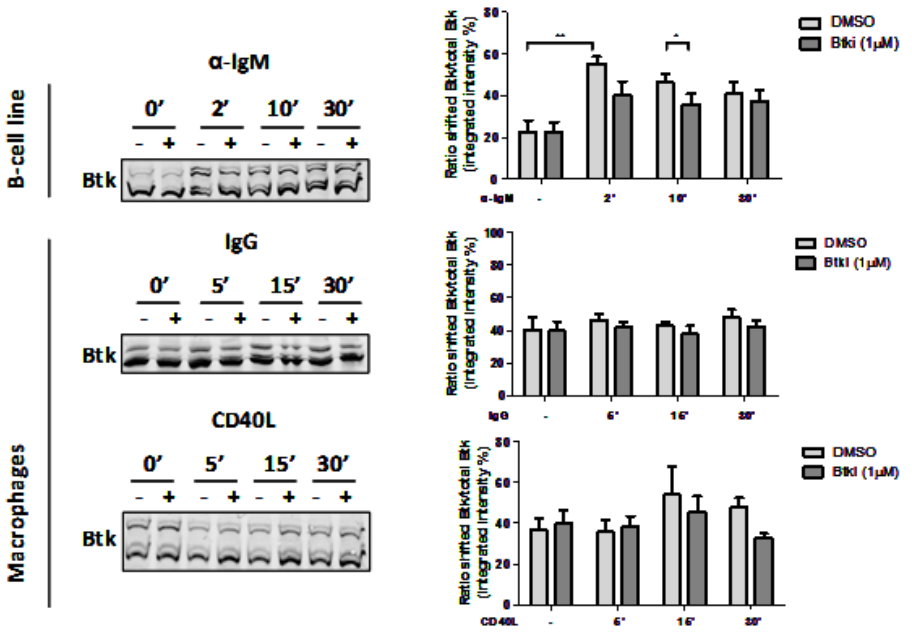

Figure 1. Btk is already activated in the steady-state in human macrophages. (A) Namalwa clone V3M B-cells and human healthy donor macrophages were stimulated for the indicated time-points with anti-IgM (B-cells) or IgG and CD40L (macrophages). Protein extracts were prepared and analyzed by Phos-Tag SDS PAGE and western blotting with antibodies recognizing Btk. The ratio of shifted versus total Btk was quantified using Li-Cor ImageStudio Lite Odyssey software and depicted as integrated density. Bars represent the means and SEM of 6 independent experiments. *P < 0.05

tyrosine-551 and tyrosine-223 in murine macrophages and human monocytes upon stimulation by immune complexes.[18] We either left cells unstimulated or stimulated with IgG complexes or CD40L. As a positive control we used a B-cell line, Namalwa clone V3M, left unstimulated or stimulated with anti-IgM antibodies. Measuring the phosphorylation-dependent shift of Btk by Phos-Tag SDS PAGE and western blotting we observed that in contrast to B cells, Btk was already activated in the steady state in human macrophages. (Fig 1A) Approximately 20% of total Btk protein was shifted in the Namalwa B-cell line in steady state. B-cell receptor triggering resulted in a significant ($P < 0.05$) phosphorylation-dependent shift and this shift could be inhibited upon pharmacological inhibition of Btk. Remarkably, in macrophages 40% of total Btk was shifted in the steady state, and this was not increased upon stimulation with either CD40L or IgG (Fig 1B).

Non-functional Btk promotes IL6 production in human macrophages

To determine the consequence of non-functional Btk on human macrophage activation, we examined cytokine production following various stimuli in both healthy donor macrophages and macrophages lacking functional Btk, derived from XLA patients (Fig 2). Macrophages were either left unstimulated or stimulated with IgG, CD40L, TNF or LPS for 20 hours. In line with our previous observations that both TNF- and LPS-induced IL6 production is not Btk-dependent (**chapter 4**), TNF and LPS-induced IL6 production in XLA macrophages was similar to the production observed in HD macrophages. However, in macrophages derived from XLA patients lacking functional Btk, IL6 production was increased upon Fc receptor triggering and CD40 ligation compared to healthy donor macrophages, seemingly contradicting our initial findings with specific Btki.

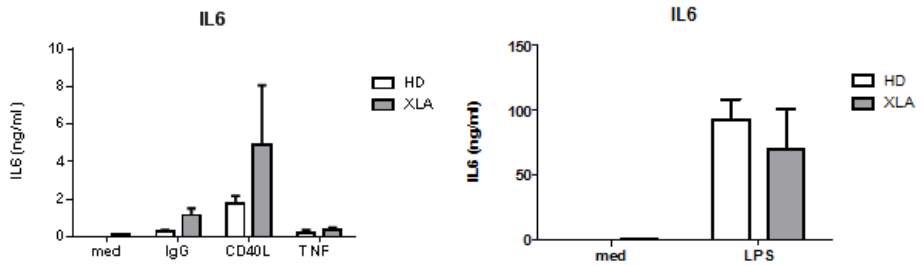

Figure 2. Non-functional Btk enhances agonist-dependent IL6 production in human macrophages. Analysis of IL6 production in supernatants of healthy donor (HD) macrophages and macrophages derived from XLA patients (XLA) either left unstimulated or stimulated with IgG beads (1:1 ratio bead:cell), CD40L (1 µg/ml), TNF (10ng/ml) or LPS (1µg/ml,) for 20 hours as assessed by ELISA. Bars represent the means and SEM of 4 independent experiments.

IgG-induced NF-κB binding is Btk kinase-dependent

To examine if this increased IL6 production was due to an increase in NF-κB activation, we determined NF-κB DNA-binding ability comparing healthy donor macrophages to XLA patient macrophages (Fig. 3A). We stimulated macrophages for 4 hours with IgG complexes and measured DNA-binding of classical NF-κB p50 and p65 subunits. IgG-induced p50 binding was not different between healthy donor macrophages and macrophages derived from XLA patients. In contrast, p65 binding was significantly ($P < 0.05$) reduced in macrophages derived from XLA patients by approximately 75%. To confirm these findings we determined DNA-binding of p50 and p65 subunits in healthy donor macrophages treated with a specific Btk inhibitor, PCI32765 (Fig. 3B). In line with our findings in XLA macrophages, IgG-induced p65 DNA-binding was significantly reduced by approximately 50% ($P < 0.05$), suggesting IgG-induced NF-κB binding is Btk kinase-dependent, but is not involved in the increased IL6 production observed in XLA patient macrophages.

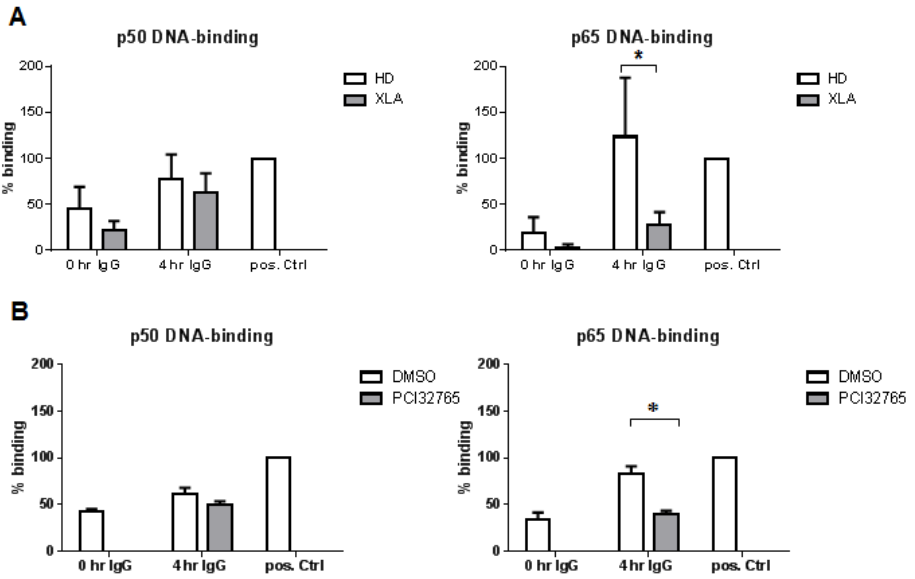

Figure 3. IgG-induced NF-κB DNA-binding is Btk kinase-dependent. (A) Healthy donor macrophages (HD) or macrophages derived from XLA patients (XLA) were left unstimulated or were stimulated with IgG beads (1:1 ratio bead:cell) for 4 hours, nuclear fractions were extracted, and levels of p50 and p65 NF-κB DNA-binding were determined using an ELISA-based assay. (B) Healthy donor macrophages were treated with 1 μM PCI32765 or vehicle control (DMSO) prior to stimulation with IgG beads (1:1 ratio bead:cell) for 4 hours, nuclear fractions were extracted, and levels of p50 and p65 NF-κB DNA-binding were determined using an ELISA-based assay. Results are presented as the mean ± SEM OD450 from 3 independent experiments. *$P < 0.05$

CD40L-induced NF-κB binding is independent of Btk kinase function

As IgG-induced NF-κB binding could not explain the observed increase in IL6 production in macrophages derived from XLA patients, we examined the DNA-binding capacity of NF-κB subunits following stimulation, again comparing healthy donor macrophages to XLA patient macrophages (Fig. 4A). We stimulated macrophages for 4 hours with CD40L and measured DNA binding of NF-κB p50 and p65 subunits. In line with the previous results we found no difference in p50 DNA=binding between healthy donor macrophages and XLA macrophages. However, in contrast to IgG-induced p65 binding CD40L-induced p65 DNA-binding was not dependent on expression of functional Btk. Similar results were obtained with healthy donor macrophages when Btk was pharmacologically inhibited, indicating that CD40L-induced NF-κB binding is independent of Btk kinase activity (Fig. 4B).

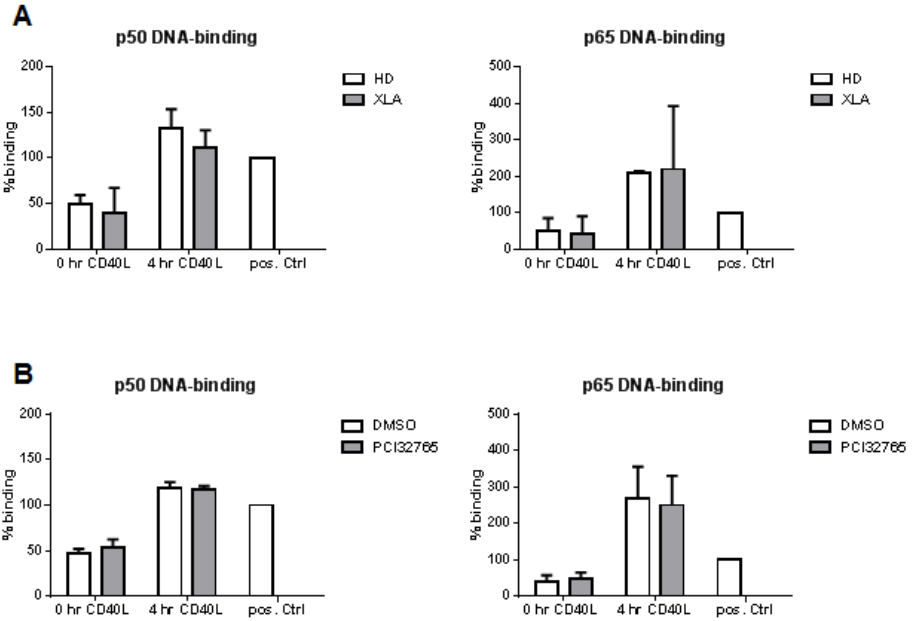

Figure 4. CD40L-induced NF-κB DNA-binding is independent of Btk kinase function. (A) healthy donor macrophages (HD) or macrophages derived from XLA patients (XLA) were left unstimulated or were stimulated with CD40L (1μg/ml) for 4 hours, nuclear fractions were extracted, and levels of p50 and p65 NF-κB binding were determined using an ELISA-based assay. (B) Healthy donor macrophages were treated with 1 μM PCI32765 or vehicle control (DMSO) prior to stimulation with CD40L (1μg/ml) for 4 hours, nuclear fractions were extracted, and levels of p50 and p65 NF-κB DNA-binding were determined using an ELISA-based assay. Results are presented as the mean ± SEM OD450 from 3 independent experiments. *P < 0.05

Discussion

Btk has attracted considerable interest as a drug target in autoimmune diseases and with this study we extended our study of the role of Btk in human macrophages to determine the mechanism of action of Btk inhibition. Although we find functional effects of Btk inhibition in macrophages (**chapter 4**), we show here that Btk inhibition does not affect Btk global phosphorylation in human macrophages. However global phosphorylation entails phosphorylation of classic activating tyrosine phosphor-sites T551 and T223 as well as inhibitory phosphor-sites like serine 180, serine 21, serine 115, serine 51 and threonine 495, possibly obscuring any effects of Btki on stimulus-induced tyrosine phosphorylation. Remarkably, we find that a larger percentage of Btk is phosphorylated in steady state conditions than B-cells, possibly indicating that tonic Btk post-translational modifications support Btk signaling in macrophages independently of auto-tyrosine phophorylation.

To determine the mechanism by which Btk inhibition affects functional outcomes in macrophages we made use of macrophages from XLA patients. These macrophages lack functional Btk protein and are useful tools to study Btk function. To our surprise we show that macrophages from XLA patients, lacking functional Btk, produce more IL6 in response to various agonists. This is in line with reports of increased pro-inflammatory cytokine production upon TLR triggering in murine and human myeloid cells lacking Btk expression. However we don't see this increase upon LPS stimulation in our experiments, perhaps indicating that differentiation status might influence this effect. Furthermore we show that the increased IL6 production is not NF-κB dependent, as binding of NF-κB subunits remains unchanged (upon CD40 ligation) or inhibited (upon Fc receptor triggering) between XLA and healthy donor macrophages. The increase in IL6 production could possibly be explained by activation of other transcription factors, such as NFAT (nuclear factor of T-cells). NFAT has been shown to contribute to innate immunity and to regulate IL6, IL10, IL12 and TNF expression in macrophages.[38]

Additionally NFAT was recently shown to be activated downstream of Btk, potentiating TNF production in macrophages.[20] Possibly Btk can activate NFAT independent of its kinase function. Future experiments using a kinase-inactive Btk could provide more insight into this mechanism. Another indication that Btk has a role apart from its kinase function and might play a role as an adaptor molecule is the observation that CD40L-induced p65 DNA-binding, in contrast to IgG-induced p65-binding, is kinase independent. Kinase-independent mechanisms of Btk signaling have already been shown in B-cells, where B cell receptor-triggered B-cells harboring a kinase-inactive

Btk can induce the activation of NF-κB.[39] Noncatalytic functions have also been described for the Tec family member Bmx, as genetic replacement of wild-type Bmx by catalytically inactive Bmx did not protect mice from experimental arthritis, whereas Bmx deletion did.[40] Finally, Btk has been shown to be part of signaling complexes located at the endosome.[41;42] Perhaps this 'non-canonical' route of signaling plays a role in macrophage activation via noncatalytic functions of Btk. Future studies are necessary to establish the importance of kinase-independent functions of Btk, and determine the mechanism of action of Btk inhibition in macrophages.

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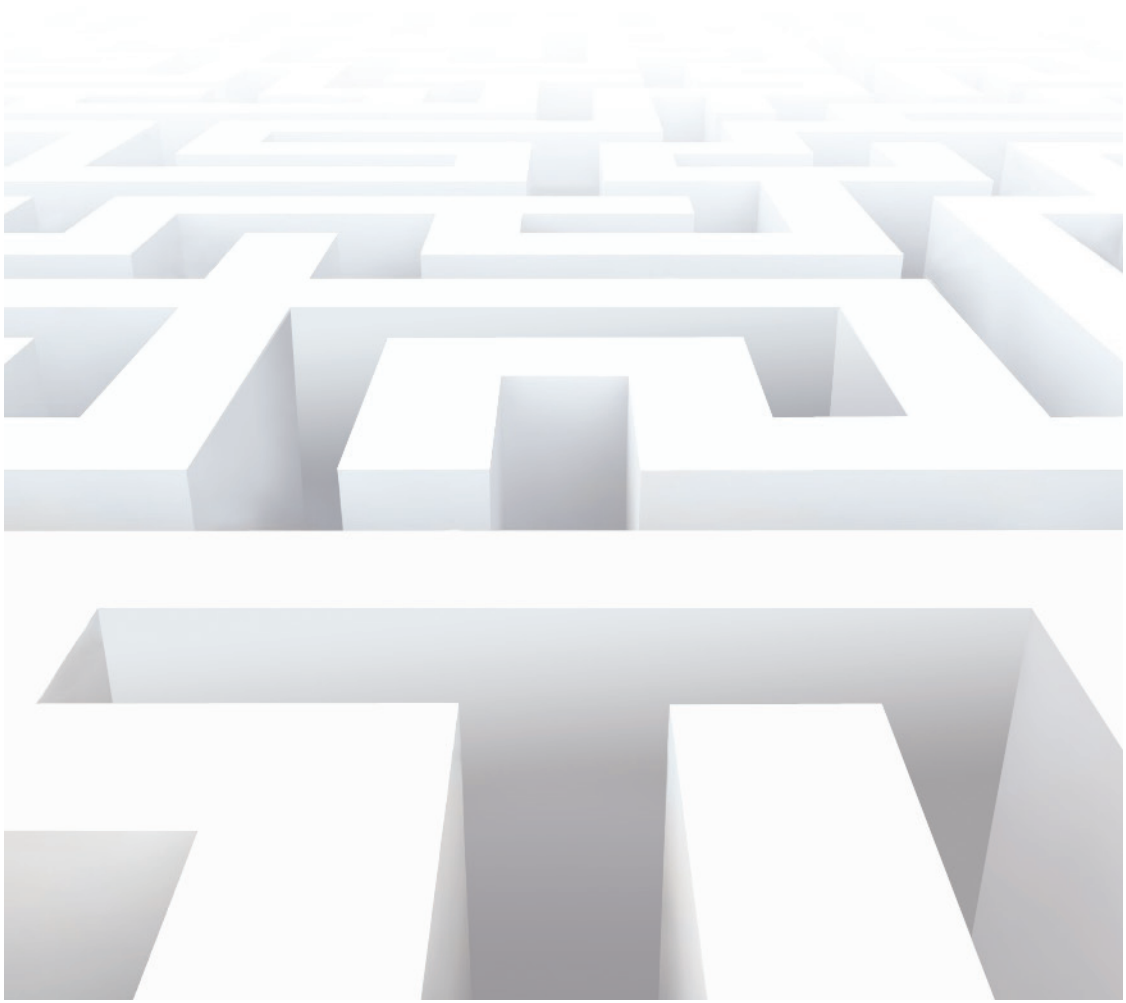

6

The PI3K-dependent AGC kinase SGK1 regulates inflammatory macrophage and rheumatoid arthritis fibroblast-like synoviocyte activation

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ABSTRACT

Phosphatidylinositol 3-kinase (PI3K) –dependent activation of protein kinase B (PKB), and subsequent PKB phosphorylation and inactivation of FoxO transcription factors, critically regulates cellular proliferation and survival. Constitutive activation of the PI3K/PKB pathway, and inactivation of FoxO proteins is observed in rheumatoid arthritis (RA) patient synovial tissue, and rescue of FoxO1 activity induces RA fibroblast-like synoviocyte (FLS) apoptosis. However, serum and glucocorticoid kinase (SGK), an AGC family kinase closely related to PKB, can also target the same residues of FoxO proteins. This raises the possibility that specific inhibition of SGK1 may have therapeutic potential, circumventing-off target effects of targeting the central node kinase PKB. Here, we sought to determine the relative contributions of PKB and SGK to RA FLS survival and activation and inflammatory activation of human macrophages. PKB and SGK isoform expression was detected by qRT-PCR in isolated cell populations. Effects of specific PKB and SGK inhibitors (PKBi and SGKi, respectively) on cellular metabolic activity and IL6 production was assessed by MTT assay and ELISA, respectively. The influence of PKBi and SGKi on expression of IL1β-responsive genes in RA FLS and human macrophages was determined using low density qPCR arrays. All PKB isoforms (AKT1, AKT2, and AKT3) were expressed in all cell populations, with AKT3 being expressed most highly in T cells. We observed expression of SGK1 and SGK3, but not SGK2, in all cell types tested, with SGK1 being most highly expressed in human macrophages. PKBi, but not SGKi, significantly decreased FLS mitochondrial activity. In contrast, only SGKi significantly reduced IL-1β-induced IL-6 production. Examining 84 genes induced by IL-1β in RA FLS, we found that PKBi suppressed transcription of CCL7, CXCL6 CXCL9, CXCL10, CXCL11, IL1RN, and MMP13, but enhanced IL23A and TNF expression. SGKi suppressed CXCL10, BCL2A1, CCL5, CSF3, IL6, MMP3, MMP7 and VCAM1 expression, and enhanced EREG expression. In human macrophages we found that PKBi suppressed transcription of IL36A and PGLYRP1, while SGKi suppressed IL6, TNF, IL36RN and SERPINE1. Our results provide the first evidence for a role of SGK in the inflammatory activation of RA FLS and human macrophages, and suggest that targeting distinct PI3K-dependent AGC kinases can preferentially modulate specific components of cellular activation relevant to pathology in RA.

Introduction

In rheumatoid arthritis (RA), macrophages and stromal fibroblast-like synoviocytes (FLS) are characterized by increased resistance to apoptotic stimuli, which promotes their accumulation and retention in the inflamed joint, as well as the release of products responsible for bone and cartilage damage.[1;2] Because of their ability to promote cellular survival and proliferation, and evidence of their enhanced activation in RA synovial tissue, the intracellular signaling phosphatidylinositol 3-kinase (PI3K) family of proteins has emerged as a potential therapeutic target.[3] PI3K family members regulate the recruitment and activity of pleckstrin homology (PH) domain containing proteins to the plasma membrane by catalyzing the phosphorylation of phosphatidylinositol (PI) and generating second messenger PI(3,4,5)P3 (PIP3). Prominent targets of PI3K signaling include protein kinase B (PKB, also known as Akt), and serum and glucocorticoid kinase (SGK).[3] PKB modulates cell fate choices through multiple mechanisms, among which regulation of forkhead box O (FoxO) transcription factors plays a central role. FoxO transcriptional targets include genes regulating cellular metabolism, response to oxidative stress, proliferation and apoptosis.4 FoxO (FoxO1, FoxO3a, and FoxO4) activity is regulated by phosphorylation, acetylation, and other post-translational modifications.[4] PKB-mediated FoxO phosphorylation disrupts FoxO interaction with target DNA sequences and facilitates nuclear export, ultimately leading to FoxO inactivation. SGK1 can mimic PBK by phosphorylating FoxO proteins at the same sites.[5;6]

The SGK family of protein kinases are members of the protein A, G, and C (AGC) family of serine/threonine kinases. The family comprises 60 members, including PKB, S6K, PDK1 and SGK. The SGK family consist of 3 members, SGK1, SGK2 and SGK3, of which multiple isoforms exist, adding even more complexity to the system.[7] SGK1 was first described as an immediate early gene transcriptionally induced by serum and glucocorticoids, but is now recognized to be transcriptionally activated downstream of many other agonists, including hormones (progesterone, gonadotropins),[8-11] growth factors (like TGF-β and PDGF),[12;13] cytokines (i.e. IL6)[14;15] and various cellular stressors like cell shrinkage, ischemic injury and excessive glucose concentrations.[16-19] Increased SGK1 expression is observed in diabetes, organ rejection, and wound healing and fibrosing conditions such as glomerulonephritis, liver cirrhosis, lung fibrosis, Crohn's disease and cardiac fibrosis.[20;21] SGK1 transcription is inhibited by heparin, dietary iron, mutations in the gene encoding methyl-CpG-binding protein 2, nucleosides and the peptide nephrilin.[22-24] Under basal conditions SGK1 expression is strictly regulated and kept low by rapid poly-ubiquitination and degradation by the proteasome. As SGK1

degradation is mediated by E3 ligase Nedd4-2, which is also subject to SGK1-mediated phosphorylation, SGK1 induces its own degradation via a negative feedback loop.[25-27] Next to transcriptional regulation, SGK1 is regulated by posttranslational modification, typically by phosphorylation and de-phosphorylation events. Amongst AGC family kinases, SGK1 shares the greatest homology with the PKB family and its catalytic domain is most similar to PKB.[28] Like PKB, SGK1 is activated downstream of PI3Ks, mediated by PDK1. Optimal activation requires phosphorylation of both threonine 256 in the activation loop by PDK1, and phosphorylation of serine 422 in the hydrophobic motif by a kinase termed PDK2/H motif kinase, that has yet to be identified. However, unlike PKB, association with the cell membrane is not a prerequisite for activation of SGK1 as SGK1 does not have a PH domain and does not require the presence or activation by the second messenger PIP3. Instead, PIP3 triggers a signaling cascade that activates the kinase responsible for SGK1 phosphorylation in the hydrophobic motif. Phosphorylation of serine 422 facilitates SGK1 binding to the PDK1-interacting fragment (PIF) binding pocket and thus promotes phosphorylation by PDK1.[20;29-31] A few studies have also implicated mTORC2 and BMK1 in activation of SGK1, but not much is known about these PI3K-independent mechanisms.[28;30] In line with the high degree of homology in their catalytic domains, PKB and SGK show considerable similarity in their substrate specificity. Both kinases prefer to phosphorylate proteins at the consensus sequence R-X-R-X-X-(serine/threonine), where R stands for arginine and X for any amino acid. Hence, the majority of targets, including GSK3β, B-Raf and FoxO transcription factors are shared between SGK1 and PKB. However, a few subtle differences exist. PKB has a preference for a serine residue in the consensus motif and requires the presence of a bulky hydrophobic amino acid immediately COOH-terminal to the phosphorylation site, whereas SGK prefers a threonine in the consensus site and the bulky hydrophobic residue is less critical.[32] This raises the possibility that SGK1 phosphorylates the same targets as PKB at the same, or different residues, and that SGK might have targets that are not PKB substrates. Hitherto, the only known targets exclusively phosphorylated by SGK1 are NDRG1 and NDRG2.[33;34] Functional specificity could also be achieved by differences in activating pathways or differences in cellular localization, as in contrast to PKB, SGK does not require membrane tethering by PIP3 and can localize to several subcellular compartments including mitochondria.[28]

SGKs have shown to be involved in several diseases like tumor growth, hypertension, neuronal diseases, fibrotic diseases, ischemia and diabetes.[20] However the role of SGK1 in rheumatic disorders has yet to be examined. Improper retention and accumulation of activated immune cells play an important role in perpetuation of inflammation in RA.

SGK1 has been demonstrated to regulate macrophage recruitment and activation in a pulmonary arterial hypertension model in rats.[35] Furthermore, SGK1 has been shown to regulate T helper differentiation and recent studies show that SGK1 is involved in Th1 and Th2 cell-fate polarization, and represses the production of interferon-γ (IFN-γ).[20] SGK1 has been shown to be involved in the induction of pathogenic Th17 cells, critical to the development of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (MS).[6;36;37] In mice SGK1 has also been shown to directly influence insulin sensitivity.[38] Moreover, SGK1 and its downstream target NDRG1 have been shown to be involved in the regulation of NFκB via phosphorylation of IKKβ, possibly signifying a role in inflammatory cytokine production.[39;40]

Even though SGK1 is highly homologous to PKB, and there's a considerable overlap in substrate specificity, more and more evidence is emerging that SGK1 and PKB phosphorylate distinct proteins and can have different functions in vivo.[41] Two specific SGK1 inhibitors have been developed, EMD638683 and GSK650394, showing efficacy in *in vitro* and in vivo in hypertension and cancer animal models however none have been tested in the clinic. Here we examined the potential contribution of SGK1 to inflammation in human macrophages and rheumatoid arthritis (RA) synovial fibroblasts.

MATERIALS AND METHODS

Inhibitors and stimulatory reagents

The following inhibitors were used: PKB inhibitor VIII (10 μM, EMD Chemicals) and SGK1 inhibitor (10 μM, GSK650394, Bio-Techne). IL1β (R&D Systems) was used at 1ng/ml for RA FLS and 10 ng/ml to stimulate macrophages for western blot analysis and cytokine production.

Cell culture

Monocytes were isolated from buffy coats (Sanquin) of healthy donors using Lymphoprep (AXIS-SHIELD) density gradient centrifugation followed by Standard Isotone Percoll gradient centrifugation (Amersham). Monocytes were plated in Iscove-modified Dulbecco medium (IMDM, Invitrogen), supplemented with 1% fetal bovine serum (FBS) for 30 minutes at 37°C, non-adherent cells were removed, and monocytes were cultured for 7 days in IMDM containing 10% FBS, 100μg/ml gentamycin and 5ng/ml GM-CSF (Tebu Bio) prior to use in experiments. The purity of monocytes and differentiation of monocytes into macrophages was confirmed by labelling of cells

with PE-conjugated CD14, PE-conjugated CD86, FITC-conjugated CD64, PE-conjugated CD163 and APC-conjugated CD200R antibodies and FACS analysis (FACSCalibur, BD Biosciences). RA patient FLS (passages 4-9) were isolated from synovial biopsies of patients fulfilling the American College of Rheumatology /European League Against Rheumatism revised criteria for RA, and cultured as previously described.[42;43] All patients provided prior written informed consent, and these studies were approved by the Medical Ethics Committee of the Academic Medical Center, Amsterdam, The Netherlands. Negative selection was used to obtain purified unstimulated T lymphocyte populations from healthy donors (Dynal bead kits, Invitrogen).

Measurements of mitochondrial activity and viability

Mitochondrial activity and viability was determined by MTT reduction assay as described previously.[44]

Gene expression profiling

Following 4 hours of stimulation with or without inhibitors, total RNA was extracted from RA FLS and macrophages using an RNeasy kit (Qiagen) and DNAse treatment. RNA concentration and purity was determined with a Nanodrop spectrophotometer (Nanodrop Technologies). cDNA was synthesized from 800 ng of RNA using an RT2 First Strand Kit (SABiosciences) and the expression of genes was analyzed using RT2 ProfilerTM PCR arrays (SABiosciences) according to the manufacturer's instructions. After PCR amplification, thresholds values were manually equalized for all samples and the threshold cycle (Ct) determined for each analyzed gene. Relative expression of each gene was calculated using StepOne Software v2.1 (Applied Biosystems) and Microsoft Excel spreadsheet software (Microsoft) and corrected for the expression of the housekeeping genes GAPDH and RPL13A.

Western blot analysis

Cells were lysed in 1x Laemmli buffer and protein content quantitated using a BCA protein Assay Kit (Pierce). Equivalent amounts of lysate were resolved by electrophoresis on 7.5% Bis Tris SDS PAGE gels, either containing 25µM Phos-Tag and 50 µM MnCl2 or not. This method is used to detect protein phosphorylation. The polyacrylamide-bound dinuclear Mn2+ complex (Mn2+-Phos-Tag) enhances the mobility shifts of phosphorylated forms of proteins. Gels were analyzed by western blotting using primary antibodies recognizing SGK1 (Cell signaling, clone D27C11) and tubulin (Sigma-Aldrich), followed by development with IRDye-680-labeled anti-rabbit or IRDye-800-labeled anti-mouse immunoglobulin secondary antibodies (LI-COR Biosciences) and

visualization using an Odyssey infrared imaging system (LI-COR Biosciences).

Cytokine production in RA FLS

RA FLS were left unstimulated or stimulated for 24 hours with 1 ng/ml IL1β. Prior to the stimulations, cells were pre-incubated for 1 hour with the indicated inhibitors. Cell-free supernatants were harvested and IL6 was measured using PeliKine Compact ELISA kits (Sanquin Reagents) as per the manufacturer's instructions.

Statistical analyses

For *in vitro* experiments, statistical analysis was performed using Windows Graphpad Prism 5 (GraphPad Software, Inc.). ELISA results were expressed as the mean ± SEM. Potential differences between samples were analyzed by Student's t-test. P-values ≤ 0.05 were considered significant.

RESULTS

Expression profile of SGK and PBK isoforms

In order to obtain a complete profile of PKB and SGK isoforms expressed in various human cell populations relevant to RA, we performed quantitative PCRs. Purified monocytes, T-cells and GM-CSF-differentiated macrophages derived from 3 individual healthy donor buffy coats, as well as RA FLS (n=4) were tested. All PKB (AKT) isoforms were present in all cell types, with AKT3 being expressed most highly in T cells (Fig 1). We observed expression of SGK1 and SGK3, but not SGK2, in all cell types tested, with SGK1 being most highly expressed in human macrophages.

Pharmacological inhibition of PKB, but not SGK decreases FLS mitochondrial activity

To be able to study the functional role of PKB and SGK1 we first assessed the effects of specific PKB (Akt inhibitor VIII) and SGK1 (GSK 650394) inhibitors on metabolic activity/viability by MTT assay. We left the cells untreated or treated them for 24 hours with either PKBi or SGKi in the absence or presence of either IL1β or PDGF. Pharmacological inhibition of PKB decreased FLS mitochondrial activity (Fig 2).[41] In both IL1β and PDGF stimulated cells PKB inhibition significantly reduced metabolic activity by approximately 40% ($P < 0.01$ and $P < 0.05$ respectively). Inhibition seen by PKBi in unstimulated cells was not significant, quite possibly due to the relatively small number of donors (n=6). This is in line with the reported role of PKB in cell cycle progression and proliferation. Surprisingly however, inhibition of SGK1 (SGKi) showed no effect on metabolic activity, suggesting a distinct role for SGK in RA FLS.

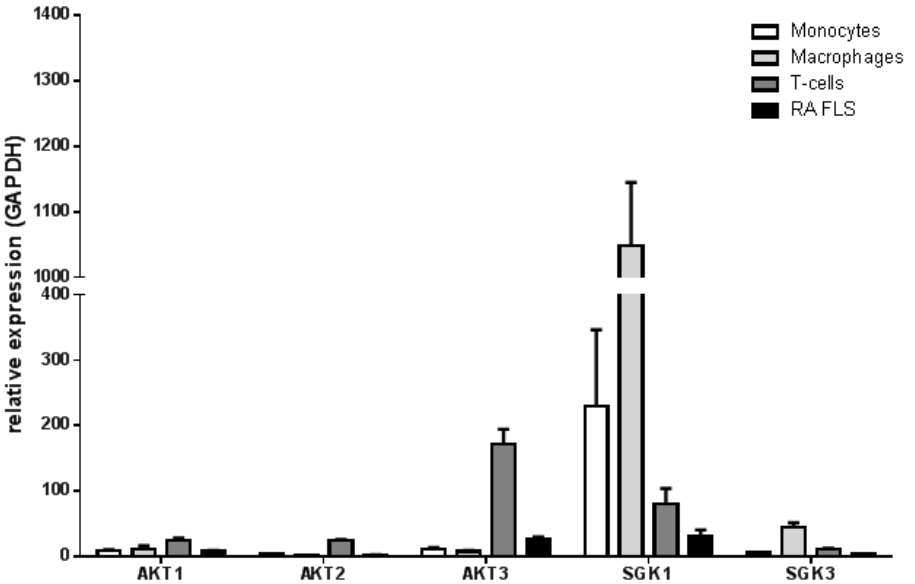


Figure 1. Expression profile of SGK and PBK isoforms. Total RNA was extracted from healthy donor buffy coat monocytes, GM-CSF –differentiated macrophages, T cells (n = 3 donors for each), and RA FLS (n = 4), reverse transcribed and expression of PKB and SGK isoforms was monitored by qPCR array. Data are presented as mean ± SEM expression relative to GAPDH (relative expression, a.u.).

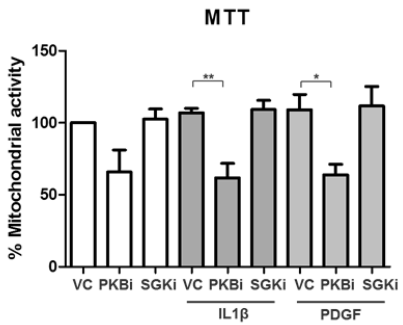
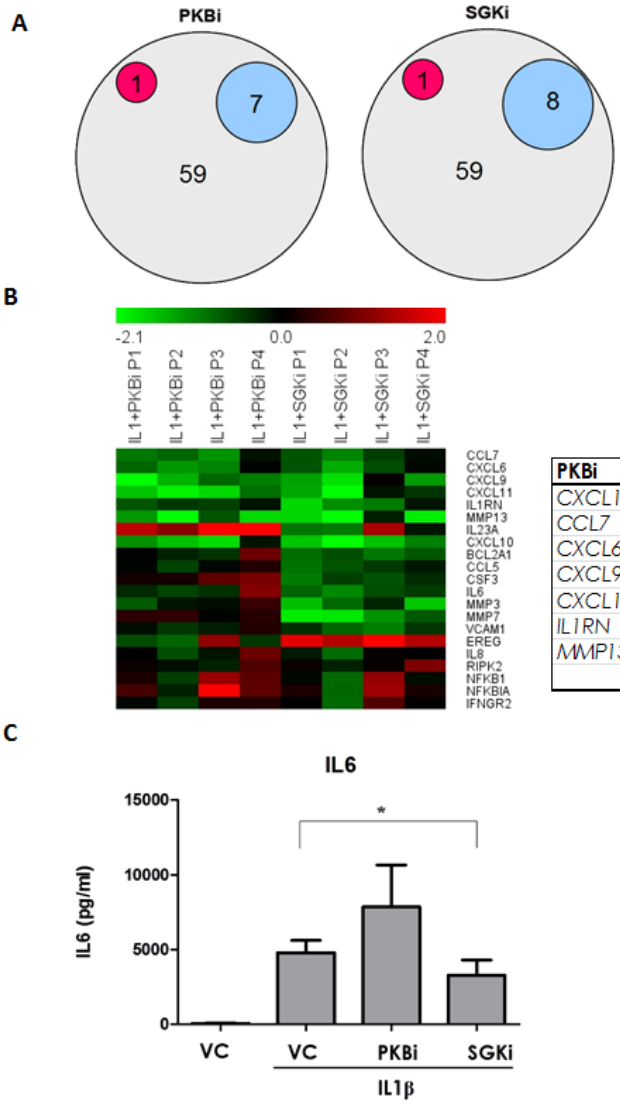


Figure 2. Pharmacological inhibition of PKB, but not SGK decreases RA FLS mitochondrial activity. Mitochondrial activity and viability in RA FLS (n=6) left untreated or treated with PKB inhibitor VIII (PKBi, 10 μM) or SGK1 inhibitor (SGKi, 10 μM) followed by stimulation with IL1β (1 ng/ml) was determined using a MTT reduction assay.



PKBi	SGKi
CXCL10	*CXCL10*
CCL7	*BCL2A1*
CXCL6	*CCL5*
CXCL9	*CSF3*
CXCL11	*IL6*
IL1RN	*MMP3,*
MMP13	*MMP7*
	VCAM1

Figure 3. The AGC kinases PKB and SGK differentially regulate inflammatory gene expression in RA FLS. RA FLS were untreated or were treated with PKBi or SGKi prior to stimulation for 4 hr with IL1β. The expression of 84 inflammatory genes was quantitatively analyzed using RT2 ProfilerTM PCR arrays. Relative expression was calculated using StepOne Software v2.1 (Applied Biosystems) and Microsoft Excel spreadsheet software (Microsoft) and corrected for the expression of housekeeping gene GAPDH and RPL13A. (A) Venn diagram of IL1β-inducible genes that were significantly up (red circle)- or down (blue circle)-regulated by either PKBi or SGKi. (B) Heatmap analysis of mRNA levels of IL1β induced genes significantly regulated by PKBi or SGKi and table showing overlap in genes that are more the two-fold up-regulated by IL1β and significantly ($P < 0.05$) downregulated by either PKBi or SGK1i. (C) Analysis of IL6 production in supernatants of RA FLS either left unstimulated or stimulated with IL1β (1ng/ml,) for 24 hours, in the absence or presence of carrier DMSO (VC) or 10μM PKBi or 10 μM SGKi as assessed by ELISA. Bars represent the means and SEM of 3 independent experiments. *$P < 0.05$

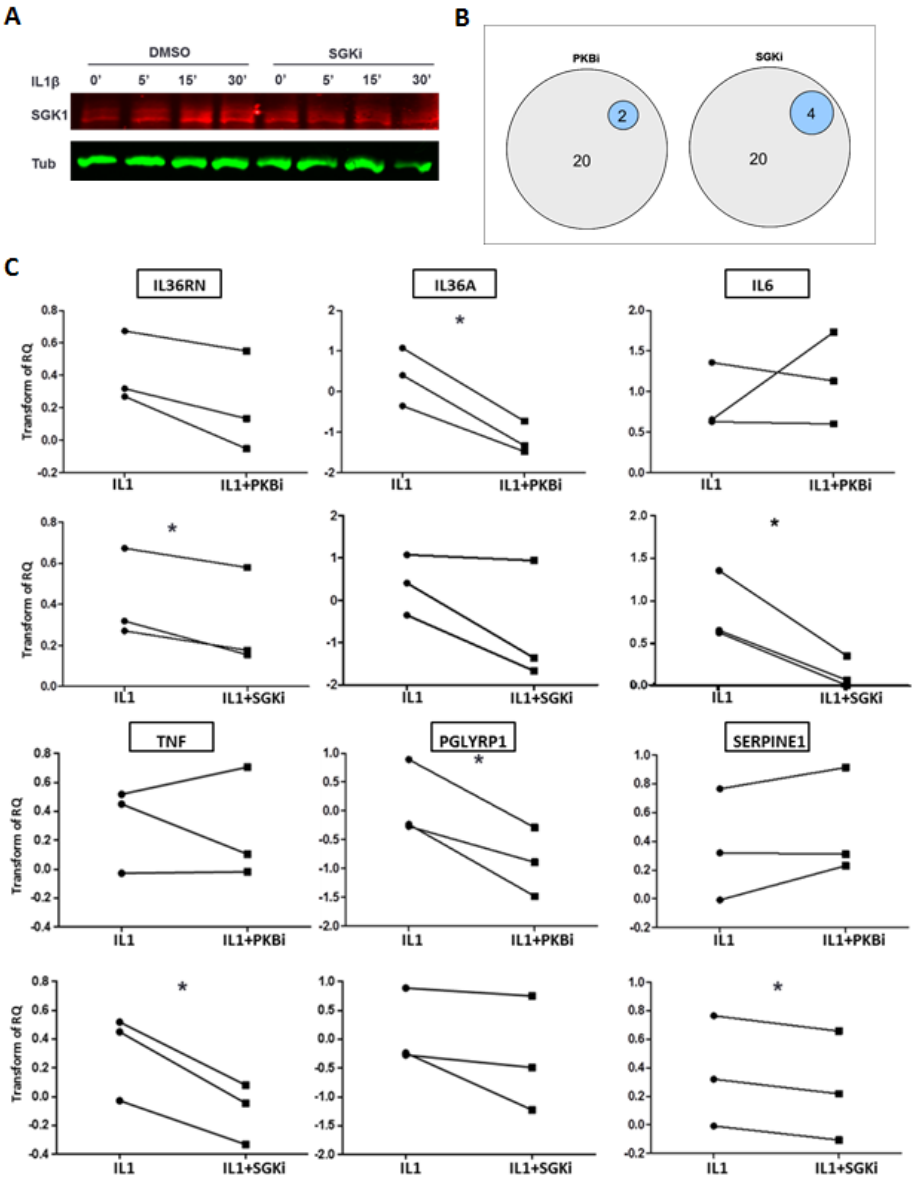


Figure 4. IL1β induces phosphorylation of SGK1 in human macrophages and this can be inhibited by SGKi. (A) Human healthy donor macrophages were stimulated for the indicated time points with IL1β (1 ng/mL) and treated with carrier (DMSO) or SGKi. Protein extracts were prepared and analyzed by western blotting with antibodies recognizing SGK1 and tubulin (Tub). (B and C) Human healthy donor macrophages (n=3) were untreated or were treated for with PKBi or SGKi prior to stimulation for 4 hours with IL1β. The expression of 84 inflammatory genes was quantitatively analyzed using RT2 ProfilerTM PCR arrays. Relative expression was calculated using StepOne Software v2.1 (Applied Biosystems) and Microsoft Excel spreadsheet software (Microsoft) and corrected for the expression of housekeeping gene GAPDH and RPL13A. Venn diagram of IL1β-inducible genes that were significantly down-regulated (blue circle) by either PKBi or SGKi (B) and log-transformed relative quantity (RQ) of indicated genes affected by PKBi or SGKi (C). *P < 0.05

The AGC kinases PKB and SGK differentially regulate inflammatory gene expression in RA FLS

To elucidate the functional consequences of pharmacological inhibition of PKB and SGK1 on inflammatory gene expression we determined expression levels of genes involved in inflammation in RA FLS. In this qualitative screening, only genes that were more than 2-fold upregulated by IL1β and significantly regulated by either PKBi or SGKi were considered for further analysis. Examining 84 genes induced by IL1β in RA FLS, we found that PKBi significantly suppressed transcription of *CCL7, CXCL6, CXCL9, IL1RN, MMP13 (P < 0.05), and CXCL11* (P < 0.01), but enhanced *IL23A* (P < 0.05) expression. SGKi enhanced EREG expression (P < 0.05), but suppressed *BCL2A1* (P < 0.01), *CCL5, CSF3, IL6, MMP3, MMP7* and *VCAM1* (P < 0.05) expression. Surprisingly, we found only one gene significantly suppressed by both inhibitors, *CXCL10* (PKBi P< 0.05 and SGKi P < 0.01). (Fig 3A) To confirm our findings on the selectivity of our inhibitors we looked at IL6 production upon stimulation by IL1β. SGKi significantly (P < 0.05) reduced IL1β-induced IL-6 production by approximately 30% in RA FLS (n=3) as measured by ELISA, whereas PKBi (10μM) did not. (Fig 3B)

IL1β-induces phosphorylation of SGK1 in human macrophages and this can be inhibited by SGKi

As macrophages play an important role in maintaining and promoting inflammation in RA, and SGK1 expression is highest in human macrophages, we examined the functional responses of PKB and SGK1 inhibition in macrophages. Measuring phosphorylation-dependent shift of SGK1 by western blotting, we observed that IL1β stimulation activated SGK1, with the peak of activation observed after 15 minutes of stimulation. IL1β-induced SGK1 phosphorylation was blocked by pharmacological inhibition of SGK1. (Fig 4A) Expression levels of genes involved in inflammation were determined by qualitative PCR arrays. We either left macrophages unstimulated or stimulated them with IL1β in the absence or presence of PKBi or SGK1i for 4 hours. In this qualitative screening, only genes that were upregulated 2-fold or more by IL1β and significantly regulated by either PKB or SGK inhibition were considered for further analysis. (Fig 4B) Examining 84 genes in human macrophages (n=3) we found that pharmacological inhibition of PKB significantly suppressed transcription of *IL36A* and *PGLYRP1* (P < 0.05), and in line with our previous data in RA FLS, SGK inhibition suppressed *IL6, TNF, IL36RN* and *SERPINE1* significantly (P < 0.05). IL6 and TNF were suppressed more than 2-fold, whereas IL36RN and SERPINE1 were only minimally (1.2 fold and 1.1 fold respectively) affected. (Fig 4C)

Discussion

Improper retention, survival and accumulation of activated immune cells and stromal FLS is thought to contribute to the perpetuation of inflammation in RA.[1;2] PI3K family members regulate cellular activation and survival, in large part through activation of downstream PKB, leading to inactivation of master FoxO transcription factors.[3] However, SGK, an AGC kinase related to PKB, can also target the same residues of FoxO proteins.[5] The development of highly specific inhibitors of PI3K family members and their success in animal models of RA heralded substantial excitement and preclinical efforts towards eventually targeting PI3K in the clinic.[3] However, work by our group (**chapter 2** of this thesis) and others has suggested that these compounds are largely ineffective in modifying the activation or survival of macrophages or FLS, two key cell populations promoting inflammation and joint destruction in RA synovial tissue.[45-49] This might be due to the limiting expression of PTEN, a negative regulator of PI3K signaling, in these cells, and as indicated in **chapter 3** of this thesis, modulation of FoxO proteins by PI3K/PKB independent pathways. Therefore, targeting of specific protein kinases downstream of, or working in parallel with, PI3K may result in the development of more effective treatment strategies.

SGKs have shown to be involved in several diseases like tumor growth, hypertension, neuronal diseases, fibrotic diseases, ischemia and diabetes.[20] Despite the clear homology to PKB and the substantial overlap in substrate specificity, more and more evidence is emerging that SGK1 and PKB activate distinct proteins and can have different functional outcomes in vivo.[28;32] Given the role of PKB as a critical signaling node in several processes like metabolism, survival, proliferation, and cell growth (exemplified by embryonic lethality of PKB KO mice) downstream of a plethora of cellular stimuli, more specific inhibition by targeting only SGK1 may have therapeutic potential, circumventing a multitude of (unwanted) side effects by targeting PKB. Here, we sought to determine the relative contributions of PKB and SGK to RA FLS survival and inflammatory activation in human macrophages. We show that all PKB isoforms are present in all cell types tested, while only SGK1 and SGK3, but not SGK2, were detected. We found highest expression of SGK1 in human macrophages. As expected, pharmacological inhibition of PKB decreased FLS mitochondrial activity signifying the crucial role of PKB in cell cycle progression and proliferation.[41] Surprisingly however, inhibition of SGK1 showed no effect on metabolic activity, suggesting a distinct role for SGK in RA FLS. This effect could be cell type-specific as SGK1 inhibition in cancer model systems does affect cell cycle and viability, although a different SGK1 inhibitor was used in this study.[50] In contrast to PKB, SGK is contributing to IL1β-induced IL6 production in

RA FLS, as IL6 production is inhibited upon specific targeting of SGK1. Intriguingly IL6 is one of many agonists leading to activation of SGK1 transcription, perhaps indicating a positive feedback loop to regulate its own activation.[15] However the mechanisms underlying SGK1 regulation of IL6 transcription remain unknown and require additional research. Furthermore we show that IL1β-induced inflammatory gene expression in RA FLS is affected by both PKBi and SGKi. Unexpectedly, as there is substantial overlap in substrate specificity, we only found 1 overlapping gene affected by both inhibitors, CXCL10. In line with the lack of effect of SGKi on metabolic activity/viability we show that gene expression of pro-apoptotic BCL2A1 is inhibited by pharmacologic targeting of SGK1. As macrophages play an important role in maintaining and promoting inflammation in RA, and SGK1 expression is highest in human macrophages, we examined the functional responses of PKBi and SGK1i in macrophages. We show for the first time that IL1β activates SGK in human macrophages, and that SGK1 critically regulates TNF and IL6 gene expression upon inflammatory stimulation with IL1β. This is in line with previous findings that SGK1 modulates transcription of TNF in mouse macrophages.[51] Our results provide the first evidence for a role of SGK in the inflammatory activation of RA FLS and human macrophages, and suggest that targeting distinct PI3K-dependent AGC kinases can preferentially modulate specific components of cellular activation. Future studies will be needed to determine differential signaling via PKB and SGK and contributions of differential activation, localization and substrate usage will need to be addressed. Making use of genetic or pharmacological approaches in animal models of arthritis could further elucidate the role of SGK1 in the pathology of rheumatoid arthritis in the future.

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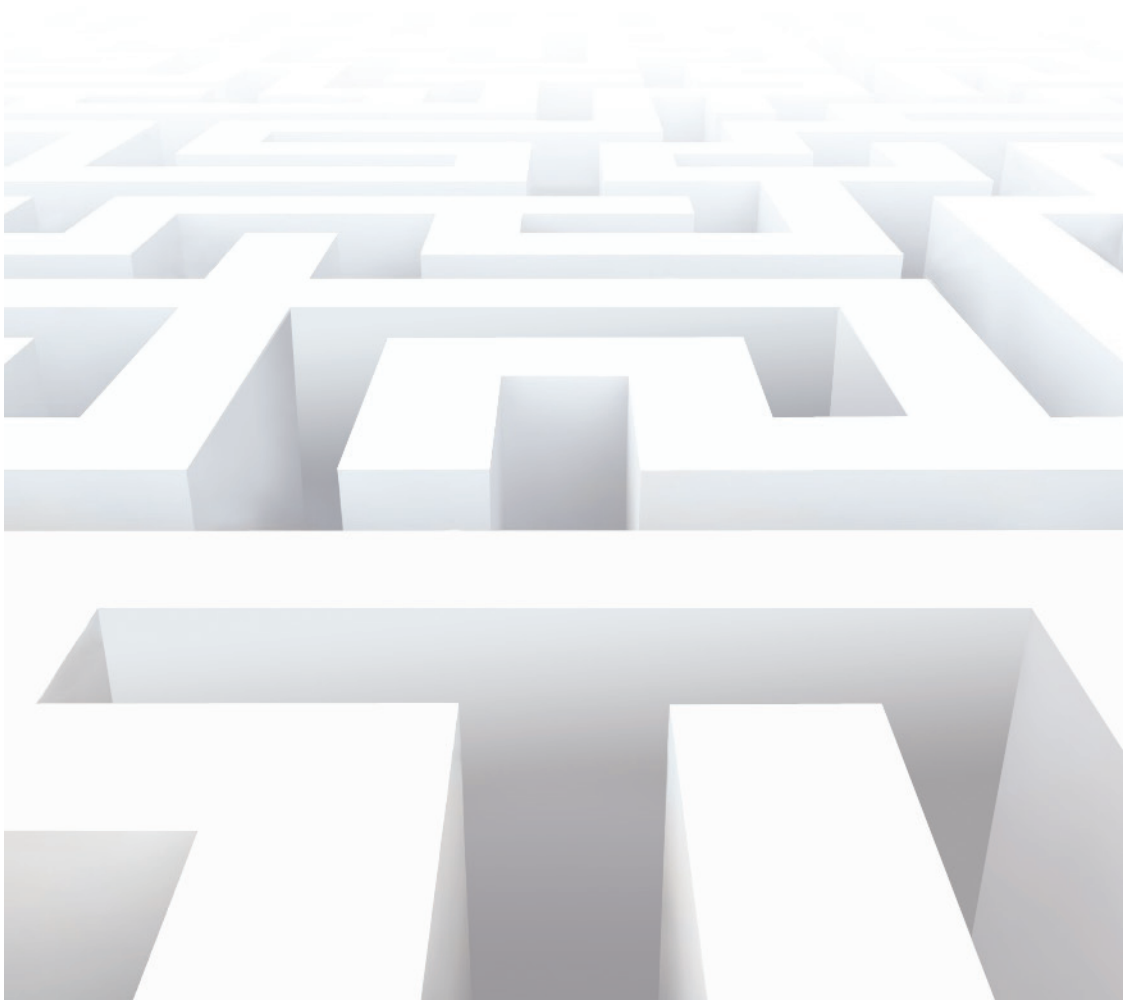

7

General Discussion

General Discussion

Phosphatidylinositol 3-kinases (PI3Ks) play a significant role in the development and perpetuation of several immune-mediated inflammatory disorders and autoimmune diseases. PI3Ks and their downstream effectors are involved in multiple cellular processes such as the control of cell growth and survival, cell metabolism, proliferation, migration and differentiation.[1-3] Class I PI3Ks are the most well-studied PI3Ks in immunology and consist of class IA (PI3Kα, β and δ) and class IB PI3Ks (PI3Kγ). Their main function is to provide membrane-docking signals to mediate recruitment of selected downstream signaling proteins. They catalyze the phosphorylation of the 3-position of the inositol headgroup of phosphatidylinositol (PtdIns) to generate the second messengers PtdIns*3P*, PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3 (PIP3). Together these second messengers regulate the localization, conformation and function of effector proteins by binding to their pleckstrin homology (PH) domain.[4] Cellular levels of PIP3 are tightly regulated by PI3K, but also by its phosphatases (SHIP-1, SHIP-2 and PTEN), which dephosphorylate PIP3.[5;6] The PH domain-containing downstream targets of PI3K include PtdIns-dependent kinase (PDK), activators of Rho family GTPases and AGC family kinases like protein kinase B (PKB, also known as Akt) and serum glucocorticoid kinase (SGK), and Tec family kinases. Many of the most-recognized effects of PI3K signaling are mediated by AGC protein kinases, like PKB and SGK. PKB has been shown to modulate the activity of small GTPases, the cell death-inducer Bad, the TORC1 complex, GSK3β, and FoxO transcription factors. Furthermore, PKB has been reported to connect the PI3K pathway with components of the pro-inflammatory NFκB pathway. [1;7;8] SGK1 has been reported to exert similar functions as PKB, and both of these kinases are activated downstream of PI3Ks, mediated by PDK1. Numerous targets, including GSK3β, B-Raf and forkhead box (FoxO) transcription factors, are shared between SGK1 and PKB. However SGK1 and PKB can also phosphorylate distinct proteins and can have different functions in vivo.[9-12] SGKs have been described to be involved in several diseases like cancer, hypertension, neuronal diseases, fibrotic diseases and ischemia.[13-16] Tec family kinases, in particular Btk, are increasingly recognized for their role in inflammation as well. Btk is involved in the differentiation and activation of B cells and the activation of myeloid cell populations, suggesting that targeting Btk might be particularly useful in autoimmune diseases characterized by pathologic antibodies, macrophage activation and myeloid derived type I IFN responses.[17;18]

For many years the pan-PI3K inhibitor LY294002 has been used to show involvement of PI3Ks in multiple cellular activation and survival processes. Although this compound initially proved valuable as a research tool, its broad targeting of all PI3K isoforms

and other off-target proteins has generated confusion in the literature regarding how targeting PI3K signaling pathways might be useful in the clinic. More specific PI3K isoform selective inhibitors have been developed over the years and isoform-selective involvement has been shown to regulate cellular activation and survival in multiple cell types and in animal models of human disease.[19-21] In **chapter 2** we show that, with the exception of potential small contributions of PI3Kδ to LPS-induced TNF and IL10 production, the effects of LY294002 on human macrophage cytokine production are largely PI3K-independent. Possibly, effects seen with LY294002 at high concentrations are PI3K-independent and can be explained by the BET bromodomain family-inhibitory capacity of LY294002.[22;23] This might also be the mechanism behind reported LY294002-dependent macrophage apoptosis. We demonstrated that macrophage cell survival is PI3K-independent, as PI3K isoform-selective inhibitors did not affect cell viability. Moreover, when combining all PI3K isoform-selective inhibitors no effect on cell viability was observed. Cell viability was only affected by LY294002 at high concentrations. When comparing effects of LY294002 and a well-known BET inhibitor JQ1, we observed a high degree of overlap in apoptotic genes affected, suggesting that the effects seen with LY294002 on apoptosis might be attributed to BET inhibition rather than inhibition of PI3Ks.

Despite the clear recognition that PI3K signaling is involved in a diverse array of immune-mediated inflammatory disorders, targeting PI3K itself doesn't seem to be the most effective therapeutic strategy. We need to gain more insight in the regulation and function of components of the PI3K signaling pathway and the role of crosstalk between signaling pathways in order to rationally design therapeutic strategies. Going downstream of PI3K to target specific effector proteins will shed light on the complex circuitry of PI3K signaling in chronic inflammation and identify new therapeutic targets. In **chapter 3** we show that synovial expression of the PI3K downstream target FoxO1 negatively correlates with clinical parameters of disease activity and local IL6 expression, indicating that synovial FoxO1 expression is strongly associated with pathology in RA. FoxO1 expression is rapidly reduced after inflammatory stimulation of RA FLS, and we demonstrate that c-Jun N-terminal kinase (JNK) mediates downregulation of FoxO1 expression by interleukin (IL)-1β independently of PI3K–PKB signaling. We identify JNK-mediated acceleration of FoxO1 mRNA degradation as a novel mechanism regulating FoxO1 expression. Although PI3K inhibitors display some anti-arthritic properties in animal models of RA, our findings raise the possibility that targeting PI3Ks in RA FLS might be partly hampered by JNK signaling. In **chapter 4 and 5** we study the role of another PI3K downstream effector, Btk, in RA synovial tissue and human macrophages. We examined the potential contributions of synovial Btk expression and activation

to inflammation in RA. We show that Btk is expressed equivalently in RA and PsA synovial tissue, primarily in macrophages. Btk activity is needed to drive macrophage activation in response to multiple agonists relevant to inflammatory arthritis, and promotes RA synovial tissue cytokine and MMP production.[27] The involvement of Btk in the production of these cytokines and inflammatory mediators suggests that targeting Btk could be beneficial in various immune-mediated inflammatory disorders. Btk inhibitors have already entered clinical trials in RA. Encouraging findings in animal models of SLE indicating clinical therapeutic efficacy suggest that Btk inhibition may be of therapeutic interest in other rheumatological diseases, but no direct studies of Btk in SLE patient primary cells have been reported. Similarly, a rationale to study the role of Btk in primary Sjögren's syndrome, systemic sclerosis, type I diabetes and multiple sclerosis is readily apparent and further evaluation of the potential of targeting Btk is of interest.[28-42] In **chapter 5** we extended our study of the role of Btk in the activation of human macrophages, attempting to determine the mechanism of action of Btk inhibition. Although we find functional effects of Btk inhibition in macrophages (**chapter 4**), we show in **chapter 5** that Btk inhibition does not affect global Btk phosphorylation in human macrophages. In our quest to determine the mechanism by which Btk inhibition affects functional outcomes in macrophages we made use of macrophages from XLA patients. These macrophages lack functional Btk protein and are useful tools to study Btk function.[43;44] To our surprise we show that macrophages from XLA patients, lacking functional Btk, produce more IL6 in response to various agonists. This increase is not NFκB-dependent, as binding of active NFκB subunits to target DNA sequences was similar between macrophages following CD40 ligation, yet inhibited upon Fc receptor triggering, when comparing macrophages derived from XLA patients and healthy donors. Kinase-independent mechanisms of Btk signaling have been reported, and possibly this plays a role in disparate, agonist-dependent effects of Btk inhibition on macrophage activation.[45-47] Btk family members Bmx and Tec provide additional, attractive targets to study. Both are also expressed in macrophages and could play a role in (functional) redundancy when targeting Btk. Similar to Btk, Bmx is reported to regulate TLR-induced IL6 production in human macrophages. In RA FLS, Bmx regulates LPS-induced IL6 and VEGF production. Furthermore, it has been reported that Bmx activates the STAT signaling pathway, which is implicated in RA pathogenesis.[48-50] Tec has been demonstrated to regulate the function and differentiation of various immune cells. In mast cells, Tec is part of the FcεRI signaling complex, and regulates mast cell effector function. Tec-knockout mice show impaired leukotriene C4 and IL4 production, and diminished GM-CSF, TNF and IL13 levels in mast cells. In monocytes Tec has been shown to mediate IL8 production upon LPS stimulation. Similar to Btk, Tec regulates

osteoclast differentiation and promotes survival through the M-CSF receptor signaling pathways in monocytes.[51-54] Although a role for Tec in immune-mediated disorders has yet to be identified, these findings suggest Tec could play a role in various disease mechanisms. In **chapter 6** we examine the role of another downstream target of PI3Ks, the AGC kinase family member SGK, and its role in inflammatory signalling in RA FLS and human macrophages. Although SGK has been shown to exert similar functions as PKB, more and more evidence is emerging that SGK1 and PKB activate distinct proteins, rendering SGK an attractive target for influencing inflammatory signalling. We demonstrate that SGK is expressed in all cell types tested, including RA FLS, with the highest expression in macrophages. We show that IL1β-induced inflammatory gene expression in RA FLS is affected by both PKBi and SGKi. Unexpectedly, as there is substantial overlap in substrate specificity, we only found 1 overlapping gene affected by both inhibitors, *CXCL10*. We show for the first time that IL1β activates SGK in human macrophages, and that SGK1 critically regulates TNF and IL6 gene expression upon inflammatory stimulation with IL1β. This is in line with our findings in RA FLS where, in contrast to PKB, SGK contributed to IL1β-induced IL6 production. Our results provide the first evidence for a role of SGK in the inflammatory activation of RA FLS and human macrophages, and suggest that targeting distinct PI3K-dependent AGC kinases can preferentially modulate specific components of cellular activation. It will be important to understand the molecular basis for the specific roles of different AGC kinases in inflammation downstream of PI3Ks. Future studies will be needed to determine differential signaling via PKB and SGK and contributions of differential activation, localization and substrate usage will need to be addressed. Making use of genetic or pharmacological approaches in various animal models could further elucidate the role of SGK1 in the pathology of immune-mediated inflammatory disorders.

Extensive research has established a critical role for PI3K and its downstream effectors in several disease-relevant signaling pathways making this signaling pathway an appealing drug discovery target. We show that by targeting downstream effectors, compensatory feedback mechanisms, alternate pathway activation and potency issues could be avoided and this could result in more robust and selective inhibition of kinase activity. We still need to gain more insight in the spatio-temporal regulation, substrate utilization, possible functional redundancy and potential binding partners of the downstream effector kinases. Future studies on the effectiveness of pharmacological inhibition of downstream effectors of the PI3K pathway in immune-mediated inflammatory disorders could further expand the understanding of the disease mechanisms and lead to the development of new therapeutic strategies.

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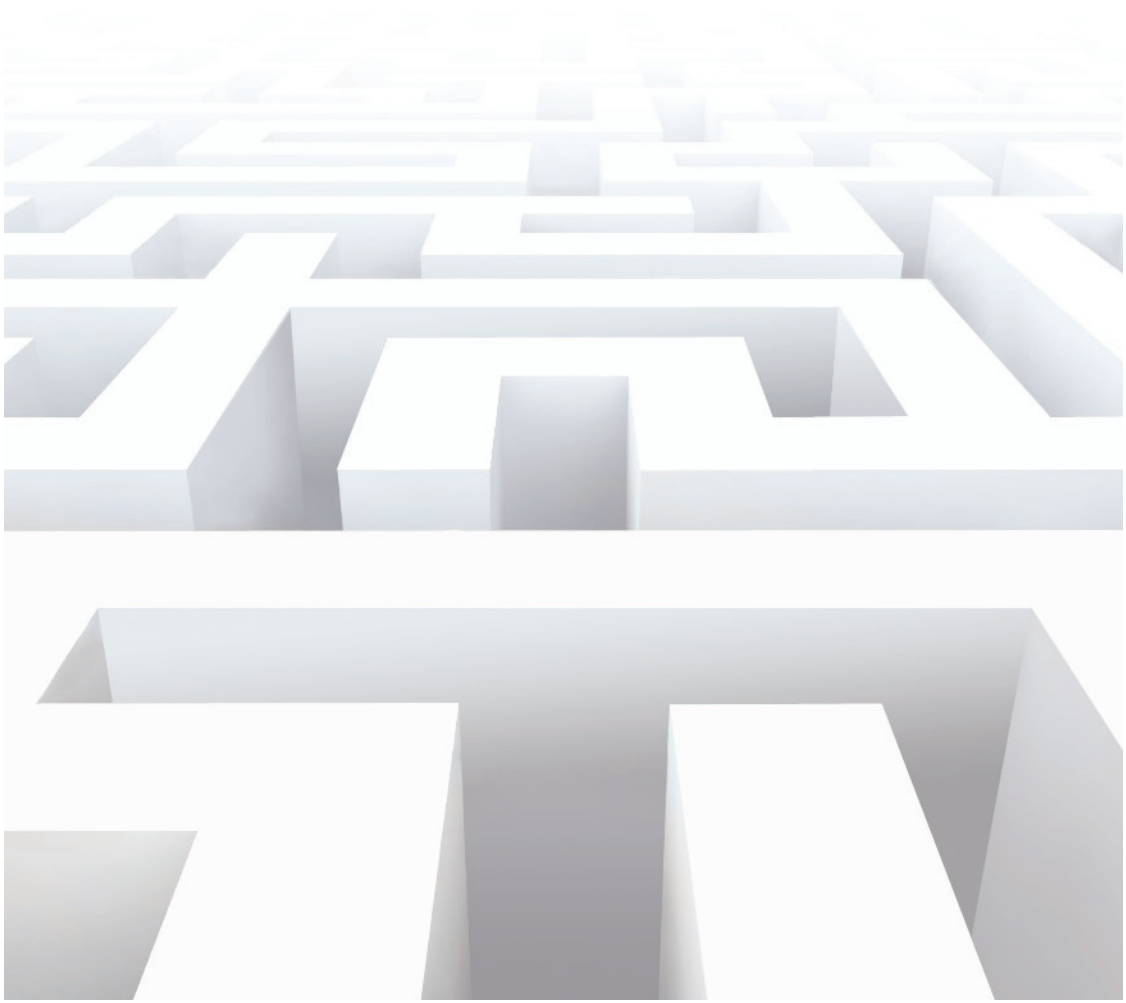

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Nederlandse samenvatting

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Dankwoord

Nederlandse samenvatting

Het immuunsysteem is cruciaal in de bescherming tegen indringers van buitenaf. Het is ontworpen om het lichaam te verdedigen tegen lichaamsvreemde stoffen zoals bacteriën, virussen en schimmels, maar is ook belangrijk bij het opruimen van niet-functionerende lichaamseigen cellen. Dit afweersysteem is ontzettend sterk, maar ook heel complex. De afweer bestaat uit verschillende verdedigingsmechanismen: de fysieke barrière, de aspecifieke afweer of aangeboren afweer en de specifieke of verworven afweer. Deze mechanismen zijn nauw met elkaar verbonden en werken veel samen. De fysieke barrière vormt een grens tussen het lichaam en de omgeving en kan er dus voor zorgen dat schadelijke stoffen niet in het lichaam terecht komen, een voorbeeld hiervan is de huid. De aspecifieke of aangeboren afweer richt zich niet specifiek op één ziekteverwekker, maar op alle lichaamsvreemde stoffen die in je lichaam voorkomen. Verschillende witte bloedcellen zijn belangrijk in deze vorm van afweer en vormen een snelle verdediging, die geen onderscheid maakt tussen de soorten indringers. Monocyten spelen een belangrijke rol in de aspecifieke afweer. Buiten de bloedbaan ontwikkelen zij zich tot macrofagen: een soort stofzuigers van het immuunsysteem die bacteriën, lichaamsvreemde cellen en beschadigde en dode cellen opnemen en opruimen. Het specifieke immuunsysteem is de afweer die je gedurende je leven ontwikkelt. Het systeem richt zich op één bepaalde ziekteverwekker, met behulp van gespecialiseerde afweercellen. Het grote voordeel van dit systeem is de ontwikkeling van geheugencellen die ervoor zorgen dat bij nieuw contact met dezelfde ziekteverwekker een snelle tegenaanval mogelijk is. Lymfocyten zijn de belangrijkste witte bloedcellen die betrokken zijn bij het specifieke immuunsysteem, zij herkennen een ziekteverwekker en kunnen snel een krachtig reageren. B-lymfocyten produceren bijvoorbeeld antilichamen en T-lymfocyten kunnen lichaamsvreemde of abnormale cellen doden. Wanneer er iets misgaat waardoor lichaamseigen cellen worden herkend en het immuunsysteem zich tegen het eigen lichaam keert, wordt er gesproken over een auto-immuunziekte. Reumatoïde artritis (RA) is één van de meest voorkomende auto-immuunziekten in de Westerse wereld. Alhoewel de exacte oorzaak nog niet bekend is, is wel duidelijk dat bij RA bestanddelen uit het gewricht door het immuunsysteem worden gezien als bedreiging en keert het immuunsysteem zich tegen lichaamseigen materiaal. Hierdoor ontstaat een ontsteking in het gewricht, waarbij verschillende immuuncellen zoals macrofagen, lymfocyten en gewrichtsspecifieke cellen (fibroblast-achtige synoviocyten, FLS) betrokken zijn. Wanneer deze auto-immuunziekte niet adequaat wordt behandeld ontstaat er onherstelbare schade wat gepaard gaat met pijn en invaliditeit. De medicijnen die voorhanden zijn hebben echter niet bij alle patiënten een afdoende effect. Onderzoek naar nieuwe behandelmethoden maar ook

naar de oorzaak van het ontstaan van auto-immuunziekten blijft dus nodig.

Het ontstekingsproces wordt gecontroleerd door cytokinen en andere regulerende kleine moleculen, ook wel ontstekingsmediatoren genoemd. Wanneer het ontstekingsproces wordt ontregeld, kan dit tot ernstige schade van het kraakbeen en bot in het gewricht leiden. Cellen maken gebruik van netwerken van met elkaar communicerende eiwitten. Communicatie vindt niet alleen plaats tussen cellen maar ook in cellen. De overdracht van signalen wordt signaaltransductie genoemd. De eiwitten communiceren met elkaar door aan elkaar te binden en elkaars activiteit te veranderen. Hierdoor kan het ene signaaleiwit een volgend signaaleiwit activeren en kan het signaal in de cel worden doorgegeven. In dit proefschrift wordt de rol van de PI3K signaaltransductie route in auto-immuunziekten bestudeerd.

Phosphatidylinositol 3-kinases (PI3K's) zijn belangrijke membraangebonden lipide kinases die via effectoren zoals proteïne kinase B (PKB), fundamentele cellulaire processen zoals celgroei, gereguleerde celdood en cytokineproductie reguleren. De rol van PI3K in gereguleerde celdood en functionele processen is grotendeels vastgesteld met behulp van de bekende pan-PI3K remmer LY294002. In de laatste jaren zijn er meer specifieke PI3K remmers gegenereerd. Met behulp van deze meer selectieve PI3K remmers kunnen we de rol van specifieke isovormen van PI3K in overleving en activatie van macrofagen onderzoeken. In **hoofdstuk 2** laten we zien dat klasse I PI3K's weinig invloed hebben op macrofaag activatie, hoewel LPS-geïnduceerde TNF en IL10 productie gedeeltelijk kan worden beïnvloed door remming van PI3Kδ. Bovendien leidt specifieke remming van PI3K isovormen, in tegenstelling tot remming met LY294002, niet tot gereguleerde celdood van macrofagen. Ook genen betrokken bij overleving en gereguleerde celdood worden niet gereguleerd door isovorm-specifieke remmers zoals dat wel het geval was met LY294002. We tonen aan dat veel van de effecten van LY294002 op macrofaag genexpressie kunnen worden toegeschreven aan off-target effecten op BET bromodomein eiwitten. Onze resultaten suggereren dat veel van de effecten van LY294002 op overleving en activering van macrofagen toegeschreven aan klasse I PI3K activiteit in feite PI3K-onafhankelijk zijn.

PI3K activeert PKB waarna regulatie van FoxO transcriptie factoren volgt. FoxO transcriptie factoren reguleren celdeling en overleving, en veranderingen in FoxO functie zijn beschreven in RA. In **hoofdstuk 3** onderzoeken we de relatie tussen ontsteking en FoxO expressie in RA en analyseren we de mechanismen en gevolgen van FoxO regulering in RA FLS. We laten zien dat synoviale expressie van PI3K effector

FoxO1 negatief correleert met klinische parameters van de ziekteactiviteit en lokale IL6 expressie, wat aangeeft dat synoviale FoxO1 expressie sterk wordt geassocieerd met de pathologie van RA. FoxO1 expressie wordt snel verminderd na inflammatoire stimulatie van RA FLS, en we laten zien dat downregulering van FoxO1 expressie door IL1β wordt veroorzaakt door c-Jun N-terminale kinase (JNK), onafhankelijk van PI3K-PKB signalering. We identificeren JNK-gemedieerde versnelling van FoxO1 mRNA degradatie als een nieuw mechanisme voor de regulatie van FoxO1 expressie. Hoewel PI3K remmers een aantal anti-artritis eigenschappen vertonen in diermodellen van RA, lijken onze bevindingen erop te wijzen dat de mogelijkheid PI3K te remmen in RA FLS gedeeltelijk zou kunnen worden belemmerd door JNK signalering.

In **hoofdstuk 4 en 5** bestuderen we de rol van een andere PI3K effector, Btk, in RA synoviaal weefsel en macrofagen. We onderzochten de potentiële bijdragen van Btk expressie en activatie aan ontsteking in het synovium in RA. We tonen aan dat Btk in even grote mate tot expressie komt in RA en PsA synoviaal weefsel, en dan voornamelijk in macrofagen. We tonen aan dat Btk activiteit nodig is voor macrofaag activatie na stimulatie met verschillende mediatoren relevant voor gewrichtsontsteking en dit leidt tot verhoogde cytokine en MMP productie in RA synoviaal weefsel. De betrokkenheid van Btk bij de productie van deze cytokinen en ontstekingsmediatoren suggereert dat het remmen van Btk bevorderlijk kan zijn bij verschillende auto-immuunziekten. In **hoofdstuk 5** verdiepen we ons onderzoek naar de rol van Btk in de activatie van macrofagen en proberen we het werkingsmechanisme van Btk remming te achterhalen. Hoewel we functionele effecten van remming van Btk zien in macrofagen (**hoofdstuk 4**), laten we in **hoofdstuk 5** zien dat Btk remming geen invloed heeft op de algehele fosforylering van Btk in macrofagen. We laten zien dat, in tegenstelling tot in B-cellen, Btk al geactiveerd is in macrofagen in homeostatische toestand. In onze zoektocht hebben we gebruik gemaakt van macrofagen van XLA patiënten. De macrofagen van deze patiënten missen functioneel Btk-eiwit en zijn nuttig om Btk functie te bestuderen. Tot onze verbazing zien we dat macrofagen van XLA patiënten meer IL6 produceren in reactie op diverse agonisten. Deze toename is niet NFκB-afhankelijk aangezien DNA binding van NFκB subunits vergelijkbaar is na CD40 ligatie, maar geremd na Fc-receptor activering, wanneer we macrofagen van XLA patiënten vergelijken met macrofagen van gezonde donoren. We laten zien dat NFκB binding na Fc-receptor activatie afhankelijk is van Btk kinase activiteit, terwijl CD40L-geïnduceerde NFκB binding onafhankelijk is van Btk kinase-activiteit. Dus Btk activiteit leidt tot IL6 productie in macrofagen maar dit kan slechts gedeeltelijk verklaard worden door activering van NFκB en wij suggereren dat Btk ook over kinase onafhankelijke capaciteiten beschikt.

In **hoofdstuk 6** onderzoeken we de rol van het AGC kinase familielid SGK, en haar rol in inflammatoire signaal transductie in RA FLS en macrofagen. Hoewel is aangetoond dat SGK soortgelijke functies als PKB uitoefent, is er steeds meer bewijs dat aantoont dat SGK1 en PKB verschillende eiwitten activeren, waardoor SGK een aantrekkelijk doelwit wordt voor het beïnvloeden van signaaltransductieroutes betrokken bij ontsteking. We tonen aan dat SGK aanwezig is in alle geteste celtypes, waaronder RA FLS, met de hoogste expressie in macrofagen. We tonen aan dat IL1β-geïnduceerde inflammatoire genexpressie in RA FLS wordt beïnvloed door zowel PKB remming als SGK remming. Onverwachts, aangezien er een aanzienlijke overlap is in substraatspecificiteit, is er maar 1 gen, CXCL10, waarvan de expressie wordt beïnvloed door beide remmers. We tonen voor de eerste keer aan dat IL1β SGK activeert in macrofagen, en dat SGK1 van groot belang is bij de regulatie van TNF en IL6 genexpressie. Dit is in lijn met onze bevindingen in RA FLS, waar, in tegenstelling tot PKB, SGK bijdraagt aan IL1β-geïnduceerde IL6 productie. Onze resultaten verschaffen het eerste bewijs voor een rol voor SGK in de inflammatoire activatie van RA FLS en macrofagen, en suggereren dat gericht beïnvloeden van afzonderlijke, PI3K-afhankelijke AGC kinases specifieke componenten van cellulaire activatie kan moduleren.

Uitgebreid onderzoek heeft een cruciale rol voor PI3K en haar effector eiwitten in verschillende ziekte-relevante signaaltransductieroutes aangetoond en dit maakt van deze signaleringsroute een aantrekkelijk doelwit voor therapie. We tonen aan dat door de interventie te richten op de effectoren, compensatiemechanismen kunnen worden vermeden en dit kan leiden tot robuustere en meer selectieve remming van kinaseactiviteit. We moeten nog meer inzicht krijgen in substraatgebruik, mogelijke functionele redundantie en potentiële bindingspartners van de downstream-effector kinases. Verder onderzoek naar de effectiviteit van farmacologische remming van effectoren van de PI3K signaaltransductieroute bij auto-immuunziekten kan kennis van de ziekte mechanismen vergroten en leiden tot de ontwikkeling van nieuwe therapeutische strategieën.

Curriculum Vitae

Linda Hartkamp was born on the 11th of March 1984 in Apeldoorn, the Netherlands. In 2002 she graduated from the Veluws College Walterbosch in Apeldoorn and started her bachelor education in Biomedical Sciences at Utrecht University. In 2005 she started the master program Biology of Disease at Utrecht University. As part of her master she performed her first internship at the Laboratory for Clinical Chemistry and Hematology at the UMC Utrecht under the supervision of prof. dr. PH. de Groot. During this internship she studied the role of β2-glycoprotein I and apolipoprotein receptor E2 in the antiphospholipid syndrome. As part of a science communication and education minor she performed her second internship at the Trimbos institute under the supervision of prof. dr. A.J. Waarlo, dr. R.J.M. Niesink and drs. R. Dekker on the science communication on multi-drug use. After obtaining her Master of Science degree in December 2007 she started as a research technician in 2008 at the Immunology department of the Wilhelmina children's hospital, Utrecht, the Netherlands in the lab of Jürgen Kuball and worked on anti-tumor properties of the γδ T-cell receptor. In 2009 she went to Melbourne, Australia, to work for 7 months as a research technician in the Virus assembly group of the Burnet Institute in the lab of Johnson Mak on structural reorganization of the HIV Gag polyprotein upon maturation. She travelled for the next 5 months through Australia, New Zealand and Borneo. In March 2010 she started her PhD at the department of Clinical Immunology and Rheumatology and the department of Experimental Immunology at the AMC Amsterdam under the supervision of prof. dr. Paul Peter Tak and dr. Kris Reedquist. In 2014 Kris Reedquist became associate professor at the UMC Utrecht and Linda joined him to finish her PhD at the department of Rheumatology and Clinical Immunology and the Laboratory of Translational Immunology at the UMC Utrecht. The results of her PhD research are described in this thesis.

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List of publications

1) Bruton's tyrosine kinase in chronic inflammation: from pathophysiology to therapy **Linda M. Hartkamp**, Timothy R. D. Radstake, Kris A. Reedquist, Accepted in Interferon, Cytokine and Mediator Research

2) Btk inhibition suppresses agonist-induced human macrophage activation and inflammatory gene expression in RA synovial tissue explants.
Hartkamp LM, Fine JS, van Es IE, Tang MW, Smith M, Woods J, Narula S, Demartino J, Tak PP, Reedquist KA. Ann Rheum Dis. 2014 Apr 24

3) JNK-dependent downregulation of FoxO1 is required to promote the survival of fibroblast-like synoviocytes in rheumatoid arthritis.
Grabiec AM, Angiolilli C, **Hartkamp LM**, van Baarsen LG, Tak PP, Reedquist KA. Ann Rheum Dis. 2014 May 8.

4) Tie2 signaling cooperates with TNF to promote the pro-inflammatory activation of human macrophages independently of macrophage functional phenotype.
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5) Redirecting αβ T cells against cancer cells by transfer of a broadly tumor-reactive γδT-cell receptor.
Marcu-Malina V, Heijhuurs S, van Buuren M, **Hartkamp L**, Strand S, Sebestyen Z, Scholten K, Martens A, Kuball J. Blood. 2011 Jul 7;118(1):50-9

6) Silencing the expression of Ras family GTPase homologues decreases inflammation and joint destruction in experimental arthritis.
de Launay D, Vreijling J, **Hartkamp LM**, Karpus ON, Abreu JR, van Maanen MA, Sanders ME, Grabiec AM, Hamann J, Ørum H, Vervoordeldonk MJ, Fluiter K, Tak PP, Reedquist KA. Am J Pathol. 2010 Dec;177(6)

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Geachte promotor prof. dr. Radstake, beste Tim, aan het eind van mijn promotietraject kwamen we naar Utrecht. Vanaf het eerste moment ben jij enthousiast geweest over mijn onderzoek en zag (en zie!) je vele mogelijkheden. Daarnaast ben je erg doelgericht en met jouw hulp konden we dit traject tot een goed einde brengen. Bedankt dat je mijn promotor wilde zijn en voor de kans om in 'mijn' Utrecht te kunnen promoveren. Geachte promotor prof. dr. Tak, beste Paul Peter, bedankt voor de mogelijkheid om op jouw afdeling mijn promotietraject te starten. Ook ben ik je dankbaar voor de vrijheid om mezelf te ontwikkelen binnen mijn promotieonderzoek en een zelfstandig onderzoeker te worden. Ondanks dat er niet veel tijd was om mijn werk persoonlijk te bespreken, ben ik dankbaar voor je kritische blik en waardevolle input bij onze artikelen.

En dan mijn co-promotor dr. Reedquist, beste Kris, je was mijn dagelijks begeleider en mentor en nu ben ik uitgegroeid tot een zelfstandig onderzoeker. Zonder jou was dit niet mogelijk geweest! Bedankt voor je begrip en geduld en de vrijheid om me te ontwikkelen, op zowel professioneel als persoonlijk vlak. 'Utreg' was ons 'stadsie' toen we in 020 zaten en nu heb ik door jouw management het thuisvoordeel tijdens mijn verdediging, wie had dat verwacht toen ik begon. Er is heel veel gebeurd tijdens mijn AIO periode, bedankt voor al het vertrouwen dat je me gegeven hebt.

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Lieve paranimfen, dank dat jullie naast mij willen staan tijdens mijn verdediging. Inge, jij was er bij vanaf het eerste jaar. Samen de meest efficiënte planning voor de week maken, buffy, de mooiste kleurenschema's voor de ELISA's, buffy, IHC kleuringen, buffy, western blots, buffy, buffy, buffy, en nog veel meer, wat hebben we veel samen gedaan. Vaak ging het goed, soms wat minder, maar…. chocola helpt altijd! Bedankt voor al je hulp in het lab en de gezelligheid daarbuiten! En is het nou pannenkoek, of misschien toch koekenpan?! Marjon, net op het moment dat het bij mij weer begon te kriebelen om te voetballen kwam ik jou tegen. En daar ben ik nog steeds blij mee! Niet alleen op het veld kunnen we elkaar goed vinden, maar ook daarbuiten. De vele dansavondjes, oude oorlogsdrama's, brakke zondagen en etentjes hebben mij door menig AIO dip heen geholpen. Kusjes!

Reedquist groep, Wendy, Debby, Marjolein, Sarah, Maartje en Daphne, jullie waren er toen ik begon. Bedankt voor al jullie hulp en uitleg, door jullie was ik al snel ingeburgerd. Alek, when I started you were already well on your way to finish your PhD. Thank you for your guidance in the lab and for the, many, discussions in- and outside the lab. It was a privilege to be your paranimf. And remember, eating is cheating! Nienke, bedankt voor je input en je hulp met de muis-gerelateerde zaken. And then the current members of the Reedquist lab, Samuel, Bea, Man Wai, Chiara and Pawel, thank you for your support and your help in the lab. It was and is a pleasure to work together. Iris, ook jij was eens onderdeel van de Reedquist groep en zo heb ik je leren kennen. Je tijd in onze groep was maar kort, maar gelukkig kon je aansluiten bij groep Dominique en bleef je op dezelfde afdeling. Samen zaten we in de borrelcommissie en hebben we vele vrijdagmiddag borrels gehouden. Ook buiten het werk wisten we mekaar te vinden, Utrecht onveilig maken, naar een festival, gezellig samen fietsen, en wat al niet meer. Dank voor alle gezellige momenten!

Ae-Ri, onze vriendschap begon toen we besloten samen naar een Keystone congres in Canada te gaan. Skiën deden we niet, maar we hebben ons prima vermaakt. De vakantie die we eraan vastplakte, maakte onze band nog sterker. Sinds die tijd heb ik er een lief vriendinnetje bij, met een geweldig luisterend oor. Ik vond het super om je op te zoeken in Philly en te zien hoe jij doorzette zelfs na tegenvallende resultaten. De bruiloft van Micha en jou was geweldig om bij te zijn en ik ben vereerd dat ik naast je mag staan wanneer jij promoveert. Dank voor alle ervaringen die we samen gedeeld hebben. Katinka, dank voor al je hulp in het lab en de gezellige thee en koekjes momenten, ik vind het heel leuk dat ik samen met jou naast Ae-Ri mag staan op haar dag.

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