Non-canonical NF-κB signaling in rheumatoid arthritis and beyond

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Citation for published version (APA):

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General discussion & summary
BACKGROUND
Rheumatoid arthritis (RA) is a complex, chronic, inflammatory autoimmune disease affecting the synovial joints. Current treatment regimens are quite effective in reducing synovial inflammation and preventing joint destruction, but a considerable percentage of RA patients does not respond sufficiently to these treatments. Therefore, it is necessary to expand the knowledge about the pathogenesis of disease and develop new treatment strategies. In this thesis we set out to investigate the role of non-canonical NF-κB signaling in RA synovial inflammation and asked the question whether NIK, the main kinase of this pathway, may be a new therapeutic target.

MAIN FINDINGS
Our studies show that NIK and subsequent non-canonical NF-κB signaling not only plays an important role in RA synovial inflammation and other inflammatory joint diseases, but also in tumor-associated angiogenesis.

In chapter 1, we presented the existing knowledge on several aspects of the pathogenesis of RA and synovial inflammation, focusing in particular on angiogenesis and ectopic lymphoid neogenesis (ELN). Furthermore, the two main NF-κB signal transduction pathways, the canonical and non-canonical NF-κB pathway, were described including their contribution to pro-inflammatory and regulatory processes.

In chapter 2, we summarized the current understanding of the role of the non-canonical NF-κB pathway in different cell types that are involved in the pathogenesis of RA, and elaborated on the overall contribution of non-canonical NF-κB signaling to synovial inflammation. Furthermore, we provided an overview of currently used drugs aimed at blocking the non-canonical NF-κB pathway and discussed future therapeutic compounds including specific pharmacological NIK inhibitors.

In chapter 3, we provided compelling evidence that the non-canonical NF-κB pathway, with its key mediator NIK, in endothelial cells (EC) regulates inflammation-induced and tumor-associated angiogenesis. NIK does not seem to be involved in angiogenesis during normal development, which is important in light of potential therapeutic applications since anti-angiogenic therapies should ideally not interfere with physiological angiogenesis. Although the exact underlying mechanisms by which the non-canonical NF-κB pathway regulates pathological angiogenesis remain to be elucidated, angiogenesis PCR array analysis provided some clues. Several pro-angiogenic genes, including ANGPT2, CD55, CSF3, CXCL5, CXCL6, CXCL11, CXCL12, FGF13, FN1, IL-6, IL-8, KITLG, PDGFB, PDGF, PGF, STAB1, and TGFA, are induced upon stimulation of the non-canonical NF-κB pathway. However, these genes do not point towards one or more distinct well-characterized pro-angiogenic pathways, suggesting that novel, as yet unknown mechanisms may also be involved. These may include matrix metalloproteinases (MMPs) and integrins that are important in sprouting angiogenesis. However, the potential interactions with non-canonical NF-κB signaling remain to be elucidated. The underlying mechanisms could be
investigated in more detail in vivo using the mouse matrigel plug assay. In this assay mice are subcutaneously injected with Matrigel containing growth factors like VEGF and/or other soluble pro-angiogenic factors that are subject to investigation (i.e. non-canonical NF-κB stimuli), in combination with specific inhibitors, such as pharmacological NIK inhibitors. An important advantage of this model is that it also allows studying CXCL12-mediated recruitment of EPC to the Matrigel plug (reviewed in [5]), which can provide important additional information as EPC are critical regulators of angiogenesis-mediated tumor progression and also contribute to angiogenesis in synovial inflammation. Performing these experiments in various specific knockout mice (i.e. for MMPs or integrins) may reveal the contribution of these factors to NIK-induced angiogenesis.

Since angiogenesis can already be observed in RA from the earliest phase of the disease, in chapter 4 we investigated the expression of NIK in synovial tissue of early arthritis patients and in autoantibody-positive individuals at risk for developing RA. We observed that NIK is already expressed in EC in the earliest phase of clinically manifest disease, which is associated both with systemic markers of disease activity (ESR and CRP) and with local disease activity (swelling of the joint and MRI scores) in DMARD-naive early arthritis patients, independent of the diagnosis. We provided clear evidence that NIK+ EC correlate with objective markers of local inflammation. Furthermore, our data indicate that the presence of NIK+ EC may be indicative of high angiogenic activity in the inflamed synovial tissue and may better correlate with disease activity than the total number of EC. Since angiogenesis may be regarded as a switch from acute to chronic inflammation, we propose that targeting the non-canonical NF-κB pathway and blocking NIK-induced angiogenesis could be particularly beneficial early in the disease process to prevent development of chronic synovial inflammation and autonomous disease progression. In clinical practice arthritis is examined primarily by physical examination, but nowadays it is common practice to complement this with ultrasonography. Using ultrasound, arthritis activity can be measured based on the presence of synovitis, joint effusion and power Doppler signal. Importantly, these parameters have been demonstrated to correlate significantly with the extent of angiogenesis inside the inflamed joint. Future studies should be performed to formally investigate the correlation between ultrasound findings and synovial NIK expression. If ultrasonographic findings reflect the presence of NIK+ EC in vivo, then ultrasonography might perhaps facilitate the choice for the optimal treatment strategy in future daily clinical practice if therapies become available targeting this pathway.

Because non-canonical NF-κB signaling is essential in normal lymphoid organogenesis and components of this pathway are highly expressed in EC in RA synovial inflammation, we hypothesized that NIK+ EC may contribute to the formation of tertiary lymphoid structures (TLS) in RA synovial tissue. Therefore, in chapter 5 we studied the presence and relationship between NIK+ EC, (pre)FDCs and ILC3 with TLS in RA synovial tissue. We found that not only FDCs, but also PDGFRβ+ perivascular preFDCs are abundantly present in RA synovial tissue containing TLS. In addition, we found positive correlations between NIK+ EC and...
the presence of PDGFRβ+ perivascular prefDCs and FDCs. This may suggest that, similar to their role in orchestrating lymph node and HEV formation and function 11, NIK+ EC also contribute to the formation of TLS in chronic inflammation. Recently, two interesting studies were published that are consistent with our hypothesis that NIK+ EC may be the orchestrators of TLS formation. In the first study it was shown that maintenance of ectopic tertiary stromal cell networks and conduits is mainly LTβ dependent 12. These data are in line with our findings that NIK is highly expressed in EC and PNAd+ HEVs inside the TLS in RA synovium and in human lymph nodes. Furthermore, it was demonstrated that EC-restricted LTβR signaling is required for normal lymph node and HEV formation and function 11. This further fuels our hypothesis that NIK-expressing EC are important orchestrators of TLS formation and may also be crucial for the development and maintenance of lymph nodes. Next, we demonstrated for the first time that ILC3/LTδ cells are present in human RA synovial tissue, but only in very low numbers. ILC comprise a family of developmentally related cells that are involved in immunity and in tissue development and remodelling 13. A specific subset of these cells termed ILC3 would be good candidates as LTβ-expressing cells and orchestrators of TLS formation in RA synovial tissue, because these cells are known to be the main regulators of normal lymphoid organogenesis 14. However, we could only find very few ILC3 in the inflamed synovial tissue via IF microscopy. Therefore, it is unlikely that these cells significantly contribute to the formation of TLS, which is in accordance with recent findings in a preclinical model of inflammatory bowel disease 15. There is still a need for more extensive studies to document the presence of the various ILC subtypes in the inflamed joint, for example studies using flow cytometry complementing studies that capture the spatial relationship between ILC and other cells, including information on the potential relation with TLS.

RA synovial inflammation is not only characterized by pro-inflammatory pathways, but regulatory mechanisms that may dampen the inflammatory response are also present 16. Examples of this are anti-inflammatory cytokines like IL-10, regulatory T cells and the immunoregulatory enzyme indoleamine-2,3-dioxygenase (IDO). Previous work from our group has established an important role for NIK in the expression of IDO in DC 17. Interestingly, it has also been suggested that non-canonical NF-κB signaling is required for AIRE expression in the thymus 18. Recently, AIRE protein has been detected in peripheral lymphoid organs, both in DC that also express tolerogenic molecules like IL-10 and IDO 19 and in distinct bone-marrow derived antigen presenting cells 20, suggesting that peripheral AIRE may play a complementary role in tolerance induction 21 and that non-canonical NF-κB signaling may be involved. Therefore, the objective of chapter 6 was to determine whether non-canonical NF-κB signaling contributes to peripheral AIRE expression in DC and whether extrathymic AIRE expressing cells (eTACs) are present in RA patients. After confirming that NIK, the main kinase of the non-canonical NF-κB pathway, is important for thymic AIRE expression, we showed that LTβR triggering does not result in AIRE expression in DC, whereas CD40-mediated signaling events do induce extrathymic AIRE expression in DC. Of note, we observed that LTβR stimulation of DC with LT or LIGHT...
did not result in non-canonical NF-κB activation, whereas stimulation of CD40 did result in p100/p52 processing. Preliminary studies indicate that CD40-induced AIRE expression is dependent on non-canonical NF-κB signaling. Next, we demonstrated that eTACs are present in RA synovial tissue, but only in very small numbers. Interestingly, these eTACs were exclusively found in tissues that contained TLS. Currently, synovial eTACs are further characterised for expression of classic DC markers. However, these cells may also be distinct antigen presenting cells derived from the bone marrow that are resistant to innate inflammatory stimuli and able to induce functional inactivation of CD4+ T cells 20. Additional studies should be performed to fully document the origin and nature of these eTACs in RA synovial inflammation, including the expression of MHC class II and co-stimulatory molecules. Another crucial experiment will be to determine the repertoire of auto-antigens that is expressed by these eTACs in the synovial tissue. The most elegant way to do this may be via laser capture microscopy and subsequent PCR analysis, but this may be difficult due to practical hurdles, including insufficient RNA quality after the IF staining protocol to detect AIRE expressing cells. Alternatively, a reverse approach of single cell analysis like Multi-Spectral Image Cytometry (MuSIC) and on-chip PCR may be used to fully characterise eTACs in RA synovial tissue 22. Detailed knowledge on the auto-antigen repertoire may teach us more about the functional role of eTACs in synovial inflammation. Other remaining questions are how eTACs in peripheral sites like RA synovial tissue are induced or retained, what the exact phenotype of these cells is and what their precise role is.

FUTURE PERSPECTIVES

The findings described in this thesis point towards a pivotal role of the non-canonical NF-κB pathway in synovial inflammation and more specifically in pathological angiogenesis (Figure 1). To obtain more evidence, we recommend performing arthritis and cancer models in EC-specific NIK knockout mice that could be generated by crossing floxed NIK mice with VE-Cadherin-Cre mice. Full NIK knockout mice are not lethal and given our results suggesting that NIK is only important in pathological angiogenesis and not in developmental angiogenesis, we expect that EC-specific NIK knockout mice will be viable. These mice could then be used to formally study the contribution of non-canonical NF-κB signaling in EC to inflammation and tumor progression/metastasis. In addition, different animal models of ELN could be performed in these mice to study the importance of NIK+ EC in this process 23-25. These mice would not only be useful in arthritis and cancer research, but also in other animal models of inflammatory diseases that involve EC such as atherosclerosis in which NIK may also be important since microvessels contribute to lesion growth and result in plaque instability leading to rupture and cardiovascular events such as CVA or myocardial infarction (Figure 2).

We performed in vitro, ex vivo and in vivo experiments to investigate the role of non-canonical NF-κB signaling in angiogenesis. Taken together, our results demonstrate that NIK is a key regulator of inflammation-induced and tumor-associated angiogenesis, whereas angiogenesis in development does not seem to be influenced by NIK. To gain
Inflamed synovial tissue

Non-canonical NF-κB signaling

CD40L
LT/LIGHT

T cell
B cell
Fibroblast

EC

CXCL12↑

Neoangiogenesis
EPC attraction?
Influx of immune cells/orchestration of ELN?

Figure 1. Schematic overview of the role non-canonical NF-κB signaling in endothelial cells in synovial inflammation. Non-canonical NF-κB signaling in endothelial cells is involved in pathological angiogenesis in synovial inflammation. The importance of this pathway in EC for EPC attraction and the orchestration of ELN is the subject of ongoing studies. bFGF = basic fibroblast growth factor; EC = endothelial cell; EPC = endothelial progenitor cells; ELN = ectopic lymphoid neogenesis; LT = lymphotoxin β; Mφ = macrophage.

Figure 2. NIK expression in human atherosclerotic plaques. NIK is expressed in vascular structures in tissue sections of atherosclerotic plaque from 2 atherosclerosis patients (Maracle et al., manuscript in preparation). Magnification 10x.

more insight into the exact underlying molecular mechanisms via which the non-canonical NF-κB pathway regulates pathological angiogenesis, ChIP sequencing experiments should be performed in human EC stimulated with non-canonical NF-κB pathway ligands such as LT, LIGHT, and CD40L using p52 specific antibodies to isolate the genomic regions of interest. Subsequently, genes identified through ChIP sequencing need to be verified at
the mRNA level by qPCR and at the protein level by Western blot and/or ELISA. It would also be interesting to investigate if hypoxia affects non-canonical NF-κB signaling in EC and whether VEGF stimulation and/or bevacizumab treatment of EC alters non-canonical NF-κB signaling.

In addition to these mechanistic studies in EC, it is useful to use in vitro systems mimicking the complex interactions between different cell types within the RA synovial tissue or tumor microenvironment. The Minitumor model is a 3 dimensional (3D) human spheroid-based system consisting of EC and fibroblasts in co-culture with breast cancer cells that has been successfully used to study tumour angiogenesis in vitro. In this model, the different cell types can be labeled with a fluorescent cell tracker dye prior to the culture and minitumor spheroids form endothelial capillary-like structures in a 3D collagen gel. The EC tubes are allowed to sprout in three dimensions and are supported by fibroblasts, which act as mural cells, and their growth is increased by the presence of cancer cells. This model allows independent manipulation of the different cell types, using common molecular techniques such as siRNA treatment, before incorporation into the model, and EC sprouts can be quantified. Taken together, this model would be ideal for studies of gene function in individual cell types and allows for the dissection of their roles in cell-cell interactions. Therefore, adaptation of this model to a model that can be used to study synovial inflammation is currently in progress in our laboratory. In this model, EC and RA fibroblast-like synoviocytes are co-cultured, but immune cells can be incorporated as well. We anticipate that this model will be very useful to identify underlying mechanisms or other factors that are involved in non-canonical NF-κB pathway regulated pathological angiogenesis in RA synovial inflammation (Figure 3). Furthermore, this model can be used to investigate the effects of pharmacological small molecule NIK inhibitors and other inhibitors of angiogenesis.

To obtain more knowledge on the role of NIK in extrathymic AIRE expression, we plan to examine AIRE (and IDO) expression in the arthritic joints of Nik−/− vs. Wt mice. The importance of the non-canonical NF-κB pathway in AIRE expression can be further investigated by crossing floxed Nik mice with Aire-Cre mice, which will allow studying whether non-canonical NF-κB signaling plays a direct role in both thymic and extrathymic AIRE expression. Next to detailed analysis of thymus, spleen and lymph nodes, it would be interesting to use these mice in an arthritis model to evaluate the effects of genetic NIK deletion on extrathymic AIRE expression.

To date, no specific peptide NIK inhibitors exist. However, the recent description of the crystal structure of NIK may facilitate the development of new potent peptide inhibitors that block the interaction between NIK and IKKα. Peptide inhibitors can be directed specifically to EC using a multimodular recombinant protein that specifically binds to cytokine-activated endothelium, which has been demonstrated to work very elegantly under inflammatory conditions in vitro, but remains to be tested in cancer treatment. A specific peptide NIK inhibitor would be a useful tool to investigate the role of the non-canonical NF-κB pathway both in vitro and in several animal models. Alternatively, local
gene therapeutic approaches could be used to inhibit NIK at the site of inflammation, for example using viral vectors that overexpress a kinase activity-deficient version of the protein. This approach has been successfully used to block NF-κB activity in arthritis and cancer models before 30. Of note, intra-articular gene therapy is currently being developed for clinical application in RA patients 31.

These studies could then open the way to the development of therapies that specifically target NIK to inhibit pathological angiogenesis. VEGF inhibition to block angiogenesis in patients is associated with side-effects, such as hypertension and thromboembolic events, which may limit its use in RA. Another disadvantage of current strategies aimed at inhibiting angiogenesis is that they affect both pathological and physiological angiogenesis. Based on our studies, targeting NIK and subsequent non-canonical NF-κB signaling in EC could overcome this problem. We anticipate that therapeutics designed to downregulate the level of NIK will not necessarily cause serious side effects. To increase clinical efficacy in RA, inhibition of NIK to target angiogenesis could also be used in addition to immunosuppressive treatment strategies which may result in a synergistic effect. Targeting NIK could also be combined with current anti-angiogenic cancer treatments such as bevacizumab to prevent or rescue the occurrence of resistance to the anti-angiogenic treatment. Indeed, preliminary results indicate that liver metastases of bevacizumab treated patients with colorectal cancer still contain NIK+ EC (Figure 4).
In conclusion, the studies described in this thesis reveal a new role of the NF-κB pathway in inflammation-induced and tumor-associated angiogenesis. In addition, this pathway may contribute to the formation of TLS in chronic inflammation. However, the contribution of the non-canonical NF-κB pathway to regulatory processes that also occur in synovial inflammation should be investigated in more detail to be able to better predict the net effect of therapies aimed at targeting the non-canonical NF-κB pathway. Additional preclinical studies with specific NIK inhibitors are warranted before clinical trials can be initiated. Our findings provide the rationale for further research on novel strategies to selectively inhibit non-canonical NF-κB activation in EC, which could result in reduced synovial inflammation and clinical improvement in a variety of diseases associated with pathological neovascularization including RA, cancer and atherosclerosis.

Figure 4. NIK colocalizes with endothelial cell marker CD31/CD34 in human colorectal cancer liver metastases. NIK is expressed in vascular structures in tissue sections of human colorectal cancer liver metastases from bevacizumab treated patients (Maracle et al., manuscript in preparation). Magnification 20x.
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