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

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Tertiary lymphoid structures in rheumatoid arthritis: NIK+ endothelial cells as central players

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ABSTRACT
Tertiary lymphoid structures (TLS) in chronic inflammation, including rheumatoid arthritis (RA) synovial tissue (ST), often contain high endothelial venules and follicular dendritic cells (FDC). Endothelial cell (EC)-specific lymphotoxin β (LTβ) receptor signaling is critical for the formation of lymph node and high endothelial venules. FDC arise from perivascular platelet-derived growth factor receptor β+ precursor cells (preFDC) that require specific group 3 innate lymphoid cells (ILC3) and LTβ for their expansion. Previously, we showed that RA ST contains EC that express NF-κB inducing kinase (NIK), which is pivotal in LTβ-induced non-canonical NF-κB signaling. We studied the relation between NIK+ EC, (pre)FDC and ILC3 with respect to TLS in RA ST. TLS+ tissues exhibited a significantly increased expression of genes involved in non-canonical NF-κB signaling, including NIK, and immunohistochemical analysis revealed that NIK was almost exclusively expressed by EC. ILC3 were present in human RA ST in very low numbers, but not differentially in TLS+ tissues. In contrast, TLS+ tissues contained significantly more NIK+ EC and perivascular platelet-derived growth factor receptor β+ preFDC, which correlated significantly with the quantity of FDC. We established a strong link between NIK+ EC, (pre)FDC and the presence of TLS, indicating that NIK+ EC may not only be important orchestrators of lymph node development, but also contribute to the formation of TLS in chronic inflammation.

Keywords
Tertiary lymphoid structures, NF-κB inducing kinase, endothelial cells, rheumatoid arthritis, ILC3, FDC
INTRODUCTION

Tertiary lymphoid structures (TLS) can be formed in peripheral tissues in various pathological conditions such as chronic inflammation, tumors, and graft rejection. TLS occur perivascular and sometimes resemble lymph node germinal centers, suggesting a link between TLS and autoimmune responses. Previously, our group has demonstrated that TLS are associated with more severe synovial and systemic inflammation in RA, but does not define a specific subset of disease. It is still not fully understood how TLS are induced and what their exact function is. The extent and pattern of lymphocyte infiltration in the chronically inflamed synovial tissue (ST) varies widely among RA patients. Small aggregates contain few PNAd+ HEV and B cells, but lack defined T-B compartmentalization and networks of follicular dendritic cells (FDC). Larger aggregates contain increased numbers of PNAd+ HEV and B cells, clear T-B compartmentalization and FDC networks. However, unlike germinal centers in lymph nodes, TLS are in direct contact with antigens and cytokines in the inflamed tissue. Normal lymphoid organogenesis requires lymphotoxin-β receptor (LTβR) signaling, which induces the expression of adhesion molecules, and subsequent production of chemokines such as CXCL12, CXCL13, CCL21, and CCL19 that are important in the formation of organized lymphoid structures. LTβR signaling is also thought to play a role in TLS, and involves both activation of the canonical and the non-canonical nuclear factor (NF)-κB pathways. The non-canonical pathway is dependent on stabilization and accumulation of NF-κB-inducing kinase (NIK), subsequent phosphorylation of IKKα, followed by processing of p100 to p52. Importantly, NIK- and IKKα-mediated non-canonical NF-κB signaling contributes significantly to lymphoid organ development, but has not yet been formally investigated in the context of TLS. In lymphoid organ development the main cell type responsible for the production of LTβ is the so-called lymphoid tissue inducer (LTi) cell. LTi cells are group 3 innate lymphoid cells (ILC3) that have been demonstrated to induce the expression of adhesion molecules and chemokines in stromal cells, which results in the attraction of lymphocytes and DCs during lymphoid organogenesis. In humans, ILC3 are characterized as lineage-negative cells that require the transcription factor RORC, both for development and function. In line with this, LTβ-expressing ILC3 would be good candidates responsible for the induction of TLS in chronic inflammation. However, in mouse models LTi cells/ILC3 are dispensable for the induction of TLS and data in humans are lacking. Also, in chronic (synovial) inflammation other well-documented sources of LTβ such as B cells, T cells, NK cells and DCs are present.

FDC not only play a key role in organizing the architecture of lymphoid organs, but they are also present in TLS in chronic inflammation. Recently, it was demonstrated that FDC arise from platelet-derived growth factor receptor beta (PDGFRβ)+ perivascular precursor cells (preFDC) whose expansion required both ILC3 and LTβ. This strategic location of preFDC near blood vessels may explain the perivascular formation of TLS at sites of inflammation. Interestingly, endothelial cell (EC)-specific LTβR signaling is critical for lymph node and HEV formation in mice, designating EC as important players in organizing
lymphoid tissue as well. Previous work from our group has established a clear link between TLS and synovial tissue vascularity. Furthermore, we have previously established that NIK-mediated non-canonical NF-κB signaling in human EC promotes pathological angiogenesis and induces the expression of CXCL12, an important chemokine for the attraction of immune cells. Based on these data, we hypothesize that NIK+ EC may play a key role in orchestrating TLS. Therefore, we set out to investigate the relation between the presence of TLS and NIK+ EC, (pre)FDC and ILC3 in the context of chronic inflammation, using RA as a prototype chronic inflammatory disease with localized (synovial) tissue inflammation.

**METHODS**

**Arthroscopic synovial biopsy and assessment of TLS**

Synovial tissue specimens were collected by arthroscopy, as previously described. All patients had active RA [DAS evaluated in 28 joints (DAS28) ≥3.2] and had not received previous treatment with biologics (see Table 1). The study was approved by the Medical Ethics Committee of the Academic Medical Center, University of Amsterdam; all patients gave written informed consent obtained according to the Declaration of Helsinki. Aggregates in the synovial tissue were counted and graded by size (score 1–3) by two blinded independent

<table>
<thead>
<tr>
<th>Table 1. Patient Characteristics.</th>
<th>TLS+ (n=15)</th>
<th>TLS- (n=25)</th>
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<tr>
<td><strong>Demographics</strong></td>
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<tr>
<td>Sex, female*</td>
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<td>16 (64)</td>
</tr>
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<td>49 (39-54)</td>
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<tr>
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<td>13 (52)</td>
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<td>NSAID use*</td>
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*Data are expressed as number (%). ´Data are expressed as median (interquartile range). Parameters are described as mean (SD), median (range) or number (n (%), as appropriate. ACPA, anticitrullinated protein antibodies; CRP, C-reactive protein; DAS28, disease activity score evaluated in 28 joints; TLS, Tertiary lymphoid structures; ESR, erythrocyte sedimentation rate; IgM-RF, IgM rheumatoid factor; NSAID, nonsteroidal anti-inflammatory drug.
observers in consensus (ARN, KPMvZ), as previously described. TLS+ tissues were defined as the presence of grade ≥2 aggregates and subclassified as described previously. Briefly, subclassification was based on the presence of CD21L+ FDC, which were counted in two different tissue sections 50μm apart to further minimize sampling error.

**Human lymph nodes**

Use of human lymph node tissue was approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam and was contingent on informed consent. Fetal lymph nodes were obtained from elective abortions and gestational age was determined by ultrasonic measurement of the skull or femur. Adult hepatic lymph nodes were obtained during multi organ donation procedures.

**Microarray**

Previously published microarray data was used to explore the gene expression levels of genes involved in the non-canonical NF-κB signaling cascade in TLS+ and TLS- synovial tissues of RA patients. In these studies we focused on the non-canonical pathway associated genes encoding NIK, p100/p52, and RelB, in relation to the canonical pathway associated genes encoding IKKβ, p65, p105/p50, and c-Rel. Data were either expressed as log2 mean or as ratio of mean non-canonical genes/canonical gene expression to determine relative non-canonical NF-κB activity.

**Immunohistochemical staining**

Frozen synovial tissue biopsies were cut in 5 μm sections and mounted on Star Frost adhesive glass slides (Knittelgläser, Braunschweig, Germany). Sections were fixed with acetone. Endogenous peroxidase activity was blocked with 0.3% H2O2 in 0.1% sodium azide in PBS for 20 minutes. Sections were stained with mouse monoclonal primary antibodies against NIK (sc-8417, Santa Cruz Biotechnology, Santa Cruz, CA), FDC-M1 (14-9968-80, Ebioscience, San Diego, CA), rabbit polyclonal primary antibodies against PDGFRβ (Sc-432, Santa Cruz Biotechnology), and the secondary antibodies goat-anti-mouse (p0447, DAKO, Glostrup, Denmark) or swine-anti-rabbit (p0399, DAKO, Glostrup, Denmark) and streptavidin labeled with horseradish peroxidase. Biotinylated tyramide was used for amplification, as previously described. As a negative control, isotype-matched immunoglobulins were applied to the sections instead of the primary antibody. Expression was scored semiquantitatively on a scale of 0 to 4 by 2 blinded researchers independently (ARN, KPMvZ). Semiquantitative scoring was employed since digital image analysis is less suitable for comparing tissue with TLS versus tissues without TLS, because information on the location of the cells in the tissue is lost.

**Immunofluorescence staining**

Frozen synovial tissue biopsies or lymph node tissues were cut in 5μm sections, fixed with acetone and incubated with the following: goat anti-CD31 (sc-1505, Santa Cruz Biotechnology) and anti-CD34 (sc-7045, Santa Cruz Biotechnology); mouse monoclonal
anti-NIK (sc-8417, Santa Cruz Biotechnology), FDC-M1 (14-9968-80, Ebioscience, San Diego, CA), rabbit polyclonal primary antibodies against PDGFRβ (Sc-432, Santa Cruz Biotechnology), rabbit anti-CD3 (1501-1, Epitomics, Burlingame, CA), rabbit anti-CD20 (1632-1, Epitomics), rat anti-ROR gamma (t) (14-6981, Ebioscience, San Diego, CA), rat anti-PNAd (clone MECA-79, Biolegend, San Diego, CA), goat anti-CXCL13 (AF801, R&D systems). After incubation with Alexa-594-conjugated goat anti-mouse or Alexa-488-conjugated goat anti-rabbit (a-11008, Molecular Probes Europe, Leiden, The Netherlands) or Alexa-488-conjugated swine anti-goat (ACI3404, Invitrogen, Breda, The Netherlands) or Alexa-488-conjugated rabbit anti-goat (11078, Molecular Probes Europe) or goat anti-rat-HRP (p0450, DAKO) followed with streptavidine-Alexa-594 (S-32356, Molecular Probes Europe) the slides were mounted with Vectashield containing DAPI (Brunschwig VC-H-1500, Amsterdam, The Netherlands). As a negative control, sections were incubated with isotype controls or PBS. The slides were analyzed using a Leica DMRA fluorescence microscope (Leica, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Version 7, Dutch Vision Components, Breda, The Netherlands).

Statistical analysis
Statistical analysis was performed by using Prism (Version 5, GraphPad Software, La Jolla, CA). The Mann-Whitney test was used (for IHC FDC-M1 and NIK) or an unpaired Student’s t-test was used (for IHC PDGFRβ). Data are shown as mean±SEM or median±IQR. Differences were considered statistically significant when P<0.05. Correlations were determined by Spearman correlation coefficient.

RESULTS
Increased non-canonical NF-κB signaling in RA synovial tissue containing TLS
First, we investigated whether non-canonical NF-κB signaling is differentially expressed in tissues containing TLS compared to tissues not containing TLS. TLS were defined as the presence of grade 2 or 3 aggregates, as described previously 3,7. For this, we used microarray data of synovial tissue biopsies from RA patients with established disease 29. Interestingly, gene expression of NIK, the main kinase of the non-canonical pathway, was significantly higher in RA ST with TLS (Fig. 1A). Furthermore, we investigated the expression of other non-canonical NF-κB pathway associated genes (p100/p52, RelB) compared to canonical NF-κB pathway associated genes (p65, IKKβ, p105/p50, c-Rel) in tissues with or without TLS. The calculated ratio of non-canonical NF-κB/canonical NF-κB pathway associated genes was significantly higher in tissues containing TLS (Fig. 1B), suggesting that non-canonical NF-κB signaling is more prevalent in RA ST containing TLS.

To confirm the microarray data, we performed immunohistochemistry with a specific NIK-antibody on RA ST without TLS (n=25) and with TLS (n=15) (Table 1). In all tissues NIK was highly expressed in the vasculature, both inside the TLS and in the adjacent tissue
(Fig. 1C), which is in line with our previous observations that NIK and activated phospho-IKKα are mainly expressed by EC in RA synovial tissue. Importantly, NIK protein expression was also significantly higher in tissues with TLS (TLS- 0.54 ± 0.19 vs. TLS+ 1.53 ± 0.32; P=0.0027) (Fig. 1D).

NIK is expressed in HEV in RA synovial tissue and in lymph nodes

Since NIK was highly expressed in vessels within the TLS, we performed double immunofluorescence (IF) stainings to investigate whether NIK+ vessels also comprised HEV. First, we demonstrated that NIK is expressed by CD31/CD34+ blood vessels (Fig. 2A). Furthermore, NIK co-localized with the HEV-marker peripheral node addressin (PNAd) in RA ST. Of note, all PNAd+ HEV expressed NIK, but NIK was also expressed by PNAd- blood vessels (Fig. 2B). Quantification of double-stained tissue sections demonstrated that approximately 10% (9.4 ± 5.4%; n=7) of NIK+ EC in RA synovial tissue were also PNAd-positive and thus regarded to be HEV.

Figure 1. Expression of (non-)canonical NF-κB pathway genes and NIK protein in RA synovial tissue. Microarray analysis of synovial tissue from RA patients with established disease, with or without TLS. A, relative gene expression of NIK B, the ratio of non-canonical/canonical NF-κB associated genes. Data are shown as mean of canonical (IKKβ, RelA/p65, p105/50, c-rel) vs. non-canonical NF-κB (NIK, p100/p52, RelB) associated genes TLS+ (n=4) and TLS- (n=8) RA synovial tissues. Data are shown as mean+SEM: *P<0.05. C, IHC staining of NIK in TLS+ (left panel, dotted line indicates TLS) and TLS- (right panel) RA synovial tissues. Original magnification left panel x100, right panel x200. D, Semiquantitative scoring (scale 0-4) of IHC NIK in TLS+ (n=15) and TLS- (n=25) RA synovial tissues, 2 independent experiments. Representative pictures are shown. Data are shown as mean+SEM: **P<0.01.
The non-canonical NF-κB pathway induces production of chemokines such as CXCL13, CCL21, and CCL19 in normal lymphoid neogenesis 14. CXCL13 is also expressed by EC in salivary glands of patients with Sjögren's syndrome 31, indicating that CXCL13 can also be expressed by EC under inflammatory conditions. Therefore, we investigated whether the non-canonical NF-κB regulated chemokine CXCL13 is also expressed in EC in RA ST containing TLS. CXCL13 was highly expressed in NIK+ EC in RA ST (Fig. 2C-D), supporting our hypothesis that NIK+ ECs may be important orchestrators of TLS, for instance via expression of CXCL13 or other chemokines that may attract B cells.

LTβR signaling is crucial for lymphoid organogenesis 9 and EC have been demonstrated to be instrumental in this process as well 26. However, it has not been investigated before if
stabilized NIK is also selectively present in EC in lymph nodes. Therefore, we investigated
NIK expression in lymph nodes using IF microscopy (Fig. 2E). Interestingly, NIK is highly
expressed in vascular structures in human lymph nodes, both in mesenteric lymph nodes
of 20 weeks gestation and in adult hepatic lymph nodes. In addition, double IF microscopy
demonstrated that NIK is expressed both in all PNAd+ HEV and in PNAd− blood vessels in
these lymph nodes (Fig. 2F).

**ILC3 are present in RA synovial tissue, but in very low numbers**

In RA synovial tissue a subset of B cells and T cells, mainly located in the aggregates,
express LTβR. We confirmed this and established that synovial tissues with TLS indeed
contain significantly more LTβ expressing B and T cells in our cohorts. However, LTi
cells (ILC3), key regulators of lymph node development (reviewed in [32]), are also a potential
source of LTβR that may induce non-canonical NF-κB signaling in stromal cells, including
EC. Therefore, we visualized ILC3 in RA ST by performing IF microscopy and quantified
the number of ILC3 as determined by CD3 RORC+ cells. Interestingly, we could for the
first time detect ILC3 in RA ST (Fig. 3A). However, these cells were only present in very
small numbers and we did not observe a clear correlation with TLS, since TLS+ tissues
contained only very low numbers of ILC3 in a few patients (3 of 15 patients; 1-2 ILC3/slide),
whereas TLS− tissues only contained ILC3 in 1 of 25 patients, in similar sized biopsies. Also,
these cells did not have a close spatial relationship to NIK+ HEV. In contrast, developing
lymph nodes contained a very high number of ILC3 (Fig. 3B). The very low number of
ILC3 in RA ST suggests that these cells are probably not the main orchestrators of TLS in
chronic inflammation.

Figure 3. ILC3 are present in RA synovial tissue and developing lymph nodes.
IF staining on ILC3 in A, RA synovial tissue containing TLS. CD3 RORC+ ILC3 are indicated by the white arrows. CD3 (red) RORC (green) and nuclei (blue). Original magnification x200. As a control, B, IF staining on ILC3 in adult popliteal lymph node. CD3 RORC+ ILC3 are indicated by the white arrows. CD3 (red) RORC (green) and nuclei (blue). Original magnification x200. Representative pictures of all analysed RA synovial tissues (n=40) and developing popliteal lymph nodes (n=3) are shown, 2 independent experiments.
PDGFRβ+ preFDC and FDC are preferentially present in TLS+ tissues

Recently, it was demonstrated that FDC arise from PDGFRβ+ perivascular precursor cells (preFDC) [25]. Furthermore, FDC and their precursors produce FDC-M1 that may opsonize apoptotic B cells and link these cells to phagocytes [25,33]. We have previously demonstrated that FDC are present in synovial tissues of some of the RA patients (mainly TLS+ tissues) [5]. Therefore, we investigated the expression of the FDC marker FDC-M1 and PDGFRβ in TLS+ and TLS− RA ST. Interestingly, PDGFRβ expression was mainly observed in a perivascular pattern, suggesting that these PDGFRβ+ cells are indeed preFDC (Fig. 4A). Furthermore, FDC-M1 expression was mainly observed inside the TLS in RA ST containing TLS (Fig. 4B). PDGFRβ+ cells were significantly more present in TLS+ tissues (TLS− 1.44 ± 0.24 vs. TLS+ 2.53 ± 0.26; P=0.005) (Fig. 4C) and FDC-M1 expression was also significantly higher in these tissues (TLS− 0.96 ± 0.20 vs. TLS+ 2.6 ± 0.03; P=0.0002) (Fig. 4D). Of note, TLS+ tissues that contained CD21L+ FDC (3 out of 15; 20% of TLS+ ST) exhibited the highest FDC-M1 expression (see Fig 4G; light grey circles).

Double immunofluorescence stainings for NIK with PDGFRβ and PDGFRβ with FDC-M1 confirmed that PDGFRβ+ cells are localized around NIK+ EC and that some of these cells already start to express FDC-M1 (Figure 4E). However, the majority of FDC-M1+ cells has downregulated PDGFRβ (Figure 4F), which is in line with previous findings that describe loss of PDGFRβ expression as perivascular preFDC progress and develop into mature FDC [25]. Finally, we examined whether NIK+ EC, perivascular PDGFRβ+ preFDC and FDC in RA ST showed a clear correlation. We observed a significant positive correlation between all markers; NIK vs. PDGFRβ (r=0.4177; P=0.0073) (Fig 4G), NIK vs. FDC-M1 (r=0.5406; P=0.0003) (Fig. 4H), FDC-M1 vs. PDGFRβ (r=0.5989; P<0.0001) (Fig. 4I). This may indicate that NIK+ EC, perivascular preFDC and FDC are all important mediators in the orchestration of TLS in chronic inflammation.

DISCUSSION

TLS occur in many types of chronic inflammation, but the exact role of these structures and the factors that initiate or orchestrate this process are largely unknown. Increasing evidence suggests that activated stromal cells are critically involved in the formation of TLS (reviewed in [34]). Given the crucial role of non-canonical NF-κB signaling in stromal cells in normal lymphoid organogenesis [9,12], we hypothesized that the non-canonical NF-κB pathway, with its key regulator NIK, also plays an important role in the formation of TLS in RA ST. We investigated whether active non-canonical NF-κB signaling in EC and the presence of certain cell types such as LTβ-producing ILC3 and perivascular (pre)FDC that may orchestrate TLS are related to the presence of TLS in chronic inflammation, using RA as a prototype immune-mediated inflammatory disease. First, we demonstrated that NIK and non-canonical NF-κB-associated genes are higher expressed in RA ST with TLS, compared to RA ST without TLS. Next, we confirmed by IHC that NIK is highly expressed...
Figure 4. FDC and PDGFRβ+ preFDC are present in RA synovial tissue and correlate with TLS. IHC staining on RA synovial tissue with and without TLS. A, PDGFRβ, and B, FDC-M1. Representative pictures of TLS+ (n=15; left panels) and TLS (n=25; right panels) RA synovial tissues are shown. Original magnification x100. Semiquantitative scoring on a scale of 0-4 of IHC stainings of C, PDGFRβ, and D, FDC-M1. Immunofluorescence staining on RA synovial tissue E, PDGFRβ (red) with NIK (green) and nuclei (blue). Original magnification x630. F, PDGFRβ (red) with FDC-M1 (green) and nuclei (blue). Original magnification x630. Correlation of semiquantitative IHC scores between G, NIK and PDGFRβ (r=0.4177; P=0.0073) H, NIK and FDC-M1 (r=0.5406; P=0.0003) and I, PDGFRβ and FDC-M1 (r=0.5989; P<0.0001). CD21L+ FDC containing TLS+ ST are depicted in light grey circles. For panels C-D data are shown as mean±SEM: **P<0.01, ***P<0.001. For panels G-I correlations were determined by Spearman correlation coefficient.
in TLS+ tissues and established that NIK is mainly expressed by EC. A subset of these NIK+ EC can be classified as PNAd+ HEV. Interestingly, we showed that all PNAd+ HEV express NIK, not only in RA ST but also in developing and adult lymph nodes, which has not been demonstrated before. These results are in line with a recent report demonstrating that EC-restricted LTβR signaling is required for normal lymph node and HEV formation and function [26]. Importantly, our data also indicate that LTβ-induced non-canonical NF-κB signaling in EC may be involved in the formation of TLS. However, to date the most important source of LTβ in the context of TLS remained enigmatic. LTi cells (ILC3), that orchestrate lymphoid organ development (reviewed in [32]) are able to produce high amounts of LTβ [19] and would therefore be good candidates for inducing TLS in chronic inflammatory conditions. We demonstrate for the first time that ILC3 are present in human RA ST. To our knowledge, this is also the first time that LTi cells or ILC3 are described in human chronically inflamed tissue containing TLS. However, we could only detect very small numbers of these cells in the inflamed synovial tissue. Since TLS formation in RA ST probably is a very dynamic process and may vary over time in the same patient [5], we investigated whether ILC3 are present both in tissues with TLS and tissues without TLS to exclude the possibility that the presence of ILC3 precedes TLS formation, as the exact kinetics of this process are still largely unknown [35]. We did not observe a clear correlation of these cells with the presence or absence of TLS. Therefore, it seems unlikely that these cells play an important role in TLS formation. This finding is consistent with the observation that LTi cells or ILC3 are not required for the development of TLS in the inflamed colon and thyroid [22,36]. This does not exclude the possibility that other LTβ producing ILC populations [17], which may also be present in the inflamed synovial tissue, contribute to TLS. Yet, in the inflamed synovium the abundantly present B and T cells are more likely to be the source of LTβ to induce TLS formation [5,6,29], whereas in lymph node development LTi cells are the main providers of LTβ [9]. Also, in analogy to lymph nodes, dendritic cells (DC) that produce LTβ may induce non-canonical NF-κB signaling in HEV to control lymphocyte entry to the chronically inflamed tissue [37] and may thus contribute to TLS formation. Alternatively, non-canonical NF-κB signaling may be induced in EC via other stimuli such as CD40L or LIGHT (TNFSF14) expressed by T cells under inflammatory conditions [38-40].

Although TLS formation and lymph node development share many aspects, including chemokine dependent and L-selectin-PNAd interaction-mediated lymphocyte homing, also clear differences between lymph nodes and TLS exist (reviewed in [41,42]). In TLS formation in chronic inflammation, local proinflammatory cytokines may for instance enhance hyaluronan expression on EC which would make EC receptive for CD44-mediated rolling of leukocytes leading up to integrin-dependent transendothelial migration of these cells [43]. Certain immune cells, including CD4+CD25+ Tregs, may be more dependent on this mechanism for homing, which could also account for some of the differences in the type of immune cells that accumulate in TLS compared to lymph nodes [41,44].

FDC are crucial for microarchitecture maintenance of germinal centers in lymph nodes and TLS in RA ST [7,24], and LTβR signaling is required for their differentiation [45,46]. In
addition, FDC are located at perivascular sites, and together with the endothelium secrete the chemokine CXCL13 that is essential for lymph node initiation. Previously, expression of CXCL13 in RA ST containing TLS was observed in FDC, whereas in Sjögrens syndrome CXCL13 is mainly expressed by EC. In the present study, we show that CXCL13 is also expressed by EC in RA ST containing TLS. These data may indicate that, NIK+ EC not only express the pro-angiogenic and immune cell attracting chemokine CXCL12, but also produce the non-canonical NF-κB regulated chemokine CXCL13, which plays a crucial role in the entry of B cells into lymphoid structures that may further amplify this process.

Recently, it was established that FDC emerge from ubiquitous perivascular precursors that resemble pericytes. These PDGFRβ+ perivascular preFDC are strategically located near blood vessels, which may explain the development of organized lymphoid structures at sites of chronic inflammation that is also characterized by angiogenesis. In this study, we report that not only FDC, but also PDGFRβ+ perivascular preFDC are abundant in RA ST containing TLS and are localized around NIK+ EC. In addition, we found positive correlations between NIK+ EC and the presence of PDGFRβ+ perivascular preFDC and FDC-M1+ FDC. This may indicate that, similar to their role in lymph node and HEV formation and function, NIK+ EC are central players in the orchestration of TLS in chronic inflammation. We hypothesize that LTβ-expressing immune cells induce non-canonical NF-κB signaling in EC resulting in the differentiation of EC into HEV. These specialized EC express chemokines, such as CXCL12 and CXCL13, that recruit more immune cells resulting in TLS. This hypothesis also fits with a study showing that maintenance of ectopic tertiary stromal cell networks and conduits is mainly LTβ dependent. Future experimental studies should address the role of LTβR-induced non-canonical NF-κB signaling in EC in this process, for example by studying TLS formation in mice that selectively lack LTβR or NIK in EC. These experimental studies could also provide clear answers with respect to the timing of the sequence of events that lead to TLS formation, which could be either sequential or take place simultaneously in a self-perpetuating loop.

Interestingly, inhibition of LTβR signaling via administration of a LTβR-immunoglobulin fusion protein has been proven beneficial in a preclinical model of arthritis and inhibited/reversed development of HEV and FDC networks in a preclinical model of Sjogren's syndrome, resulting in a significant reduction of TLS and improved salivary gland function. However, efficacy data of these therapies in humans are lacking. Our studies do not provide answers to the function of TLS in chronic inflammation. Consequently, future studies should also be aimed at further unravelling the role of TLS in chronic inflammation.

In conclusion, our results indicate that NIK+ EC and perivascular (pre)FDC are abundantly present in RA synovial tissue containing TLS and that these cell types may be more important orchestrators of TLS than LTi cells or ILC3. We propose that NIK+ EC and HEV are induced in synovial inflammation under the influence of LTβ-producer immune cells and may act as central players in TLS formation. Subsequently, perivascular PDGFRβ+ preFDC are activated and differentiate into FDC that further contribute to the orchestration of TLS.
AUTHOR CONTRIBUTIONS
A.R.N. contributed to the study design, all experiments, data analysis, and writing of the manuscript. K.P.M.Z. performed and collected data from immunohistochemistry and immunofluorescence experiments. L.G.M.B. performed microarray analysis and contributed to drafting of the manuscript. C.X.M, B.H., N.P., and M.R.H. performed and collected data from immunofluorescence experiments. T.C. contributed to and provided materials for immunofluorescence experiments, contributed to study design, discussion, and drafting of the manuscript. P.P.T. contributed to study design, drafting of the manuscript and provided advice and discussion. S.W.T. was responsible for project conceptualization, experimental design, writing of the manuscript and funding of the project.
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