IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Tertiary Lymphoid Structures in Rheumatoid Arthritis

NF- κ B—Inducing Kinase—Positive Endothelial Cells as Central Players

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Tertiary lymphoid structures (TLSs) in chronic inflammation, including rheumatoid arthritis (RA) synovial tissue (ST), often contain high endothelial venules and follicular dendritic cells (FDCs). Endothelial cell (EC)—specific lymphotoxin β (LT β) receptor signaling is critical for the formation of lymph nodes and high endothelial venules. FDCs arise from perivascular platelet-derived growth factor receptor β^+ precursor cells (preFDCs) that require specific group 3 innate lymphoid cells (ILC3s) and LT β for their expansion. Previously, we showed that RA ST contains ECs that express NF- κ B—inducing kinase (NIK), which is pivotal in LT β -induced noncanonical NF- κ B signaling. We studied the relation between NIK⁺ ECs, (pre)FDCs, and ILC3s with respect to TLSs in RA ST. TLS⁺ tissues exhibited a significantly increased expression of genes involved in noncanonical NF- κ B signaling, including NIK, and immunohistochemical analysis revealed that NIK was almost exclusively expressed by ECs. ILC3s were present in human RA ST in very low numbers, but not differentially in TLS⁺ tissues. In contrast, TLS⁺ tissues contained significantly more NIK⁺ ECs and perivascular platelet-derived growth factor receptor β^+ preFDCs, which correlated significantly with the quantity of FDCs. We established a strong link between NIK⁺ ECs, (pre)FDCs, and the presence of TLSs, indicating that NIK⁺ ECs may not only be important orchestrators of lymph node development but also contribute to the formation of TLSs in chronic inflammation. (*Am J Pathol* 2015, 185: 1935–1943; <http://dx.doi.org/10.1016/j.ajpath.2015.03.012>)

Tertiary lymphoid structures (TLSs) can be formed in peripheral tissues in various pathological conditions such as chronic inflammation, tumors, and graft rejection.¹ TLSs occur perivascularly and sometimes resemble lymph node germinal centers, suggesting a link between TLSs and autoimmune responses.^{2–4}

Previously, our group has demonstrated that TLSs are associated with more severe synovial and systemic inflammation in rheumatoid arthritis (RA)³ but do not define a specific subset of disease.⁵ The mechanisms for induction of TLSs and their exact function are poorly understood. The extent and pattern of lymphocyte infiltration in the chronically

inflamed synovial tissue (ST) varies widely among RA patients.⁶ Small aggregates contain few peripheral node addressin (PNAd)⁺ high endothelial venule (HEV) and B cells but lack defined T-B compartmentalization and networks of follicular dendritic cells (FDCs). Larger aggregates contain increased numbers of PNAd⁺ HEV and B cells, clear T-B compartmentalization, and FDC networks.⁷ However,

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unlike germinal centers in lymph nodes, TLSs are in direct contact with antigens and cytokines in the inflamed tissue.⁸

Normal lymphoid organogenesis requires lymphotoxin-β receptor (LTβR) signaling,⁹ which induces the expression of adhesion molecules, and subsequent production of chemokines such as CXCL12, CXCL13, chemokine ligand 21, and chemokine ligand 19 that are important in the formation of organized lymphoid structures.^{10,11} LTβR signaling also plays a role in TLSs and involves activation of both the canonical and the noncanonical NF-κB pathways.¹² The noncanonical pathway depends on stabilization and accumulation of NF-κB-inducing kinase (NIK) and subsequent phosphorylation of IκB kinase α, followed by processing of p100 to p52.¹³ Importantly, NIK- and IκB kinase α-mediated noncanonical NF-κB signaling contributes significantly to lymphoid organ development^{14,15} 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.¹⁶ LTi cells are group 3 innate lymphoid cells (ILC3s)¹⁷ that induce the expression of adhesion molecules and chemokines in stromal cells,¹⁸ which results in the attraction of lymphocytes and dendritic cells during lymphoid organogenesis.¹⁹ In humans, ILC3s are characterized as lineage-negative cells that require the transcription factor RORC, for both development and function.¹⁷ Consistently, LTβ-expressing ILC3s would be good candidates responsible for the induction of TLSs in chronic inflammation. However, in mouse models, ILC3 is dispensable for the induction of TLS,^{20–22} and data in humans are lacking. Also, in chronic synovial inflammation, other well-documented sources of LTβ such as B, T, natural killer, and dendritic cells are present.^{6,23}

FDCs not only play a key role in organizing the architecture of lymphoid organs but also are present in TLSs in chronic inflammation.²⁴ Recently, it was demonstrated that FDCs arise from platelet-derived growth factor receptor β (PDGFRβ)⁺ perivascular precursor cells (preFDCs) whose expansion required both ILC3 and LTβ.²⁵ This strategic location of preFDCs near blood vessels may explain the perivascular formation of TLSs at inflammation sites. Interestingly, endothelial cell (EC)-specific LTβR signaling is critical for lymph node and HEV formation in mice,²⁶ designating ECs as important players in organizing lymphoid tissue as well. Previous work from our group has established a clear link between TLSs and ST vascularity.⁵ Furthermore, we have previously established that NIK-mediated noncanonical NF-κB signaling in human ECs promotes pathological angiogenesis and induces the expression of CXCL12, an important chemokine for the attraction of immune cells.²⁷ On the basis of these data, we hypothesize that NIK⁺ EC may play a key role in orchestrating TLSs. Therefore, we investigated the relation between the presence of TLSs and NIK⁺ ECs, preFDCs, and ILC3s in the context of chronic inflammation by using RA as a prototype chronic inflammatory disease with localized ST inflammation.

Table 1 Patient Characteristics

Parameter	TLS ⁺ (n = 15)	TLS [−] (n = 25)
Demographics		
Sex, female*	8 (53)	16 (64)
Age, years [†]	49 (39–54)	55 (44–67)
Disease status		
Disease duration, years [†]	3.5 (1–11)	3.0 (1–9)
IgM-RF positive*	11 (73)	14 (56)
ACPA positive*	7 (47)	13 (52)
IgM-RF and ACPA, both positive*	7 (47)	10 (40)
ESR, mm/hour [†]	30 (22–55)	25 (15–39)
CRP, mg/L [†]	11 (6–28)	12 (3–23)
DAS28 [†]	5.8 (4.7–6.8)	5.5 (4.7–6.3)
Drug treatments*		
Methotrexate	8 (53)	16 (64)
Corticosteroid	3 (20)	6 (24)
NSAID	10 (67)	11 (44)

*Data are expressed as number (%).

[†]Data are expressed as median (interquartile range).

ACPA, anti-citrullinated protein antibodies; CRP, c-reactive protein; DAS28, disease activity score evaluated in 28 joints; ESR, erythrocyte sedimentation rate; IgM-RF, IgM rheumatoid factor; NSAID, nonsteroidal anti-inflammatory drug; TLS, tertiary lymphoid structures.

Materials and Methods

Arthroscopic Synovial Biopsy and Assessment of TLS

ST specimens were collected by means of arthroscopy, as previously described.²⁸ All patients had active RA (disease activity score evaluated in 28 joints ≥3.2) and had not received previous treatment with biologics (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 ST were counted and graded by size (score of 1 to 3) by two blinded independent observers in consensus (A.R.N. and K.P.M.v.Z.), as previously described.^{3,7} TLS⁺ tissues were defined as the presence of aggregates of grade 2 or higher and subclassified as described previously.⁵ Briefly, subclassification was based on the presence of CD21L⁺ FDCs, which were counted in two different tissue sections 50 μm 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 means of ultrasonic measurement of the skull or femur. Adult hepatic lymph nodes were obtained during multiorgan donation procedures.

Microarray

Previously published microarray data were used to explore the gene expression levels of genes involved in the noncanonical NF- κ B signaling cascade in TLS positive and negative STs of RA patients.²⁹ In these studies, we focused on the noncanonical pathway—associated genes encoding NIK, p100/p52, and RelB in relation to the canonical pathway—associated genes encoding I κ B kinase β , p65, p105/p50, and c-Rel. Data were expressed either as log2 mean or as ratio of mean noncanonical to canonical gene expression to determine relative noncanonical NF- κ B activity.

Immunohistochemical Staining

Frozen ST biopsy samples were cut in 5- μ m 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 phosphate-buffered saline 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 PDGFR β (sc-432; Santa Cruz Biotechnology), and the secondary antibodies goat—anti-mouse (p0447; Dako, Glostrup, Denmark) or swine—anti-rabbit (p0399; Dako) and streptavidin labeled with horseradish peroxidase. Biotinylated tyramide was used for amplification, as previously described.³⁰ 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 two blinded researchers independently (A.R.N. and K.P.M.v.Z.). Semiquantitative scoring was used because digital image analysis is less suitable for comparing tissue with TLS versus tissue without TLS because information on the location of the cells in the tissue is lost.

Immunofluorescence Staining

Frozen ST biopsy samples or lymph node tissues were cut in 5- μ m 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), rabbit polyclonal primary antibodies against PDGFR β (sc-432; Santa Cruz Biotechnology), rabbit anti-CD3 (1501-1; Epitomics, Burlingame, CA), rabbit anti-CD20 (1632-1; Epitomics), rat anti-RORgamma (t) (14-6981; eBioscience, San Diego, CA), rat anti-PNAd (clone MECA-79; BioLegend, San Diego, CA), and goat anti-CXCL13 (AF801; R&D Systems, Minneapolis, MN). After incubation with Alexa-594—conjugated goat anti-mouse or Alexa-488—conjugated goat anti-rabbit (a-11008; Molecular Probes Europe, Leiden, the Netherlands), Alexa-488—conjugated swine anti-goat (ACI3404; Invitrogen,

Breda, the Netherlands), Alexa-488—conjugated rabbit anti-goat (11078; Molecular Probes Europe), or goat anti—rat-horseradish peroxidase (p0450; Dako) followed with streptavidine—Alexa-594 (S-32356; Molecular Probes Europe), the slides were mounted with Vectashield containing DAPI (VC-H-1500; Brunschwig Chemie, Amsterdam, the Netherlands). As a negative control, sections were incubated with isotype controls or phosphate-buffered saline. The slides were analyzed using a fluorescence microscope (DMRA; Leica, Wetzlar, Germany) coupled to a charge-coupled device camera and Image-Pro Plus software version 7 (Dutch Vision Components, Breda, the Netherlands).

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA). The Mann-Whitney test was used (for immunohistochemical FDC-M1 and NIK), or an unpaired Student's *t*-test was used (for immunohistochemical PDGFR β). Data are shown as means $\pm$ SEM or median $\pm$ interquartile range. Differences were considered statistically significant for $P < 0.05$. Correlations were determined by using a Spearman correlation coefficient.

Results

RA ST Containing TLSs Has Increased Noncanonical NF- κ B Signaling

First, we investigated whether noncanonical NF- κ B signaling is differentially expressed in tissues containing TLSs compared with that in tissues without TLSs. TLSs were defined as the presence of grade 2 or 3 aggregates, as described previously.^{3,7} For this investigation, we used microarray data of ST biopsy samples from RA patients with established disease.²⁹ Interestingly, gene expression of NIK, the main kinase of the noncanonical pathway, was significantly higher in RA ST with TLSs (Figure 1A). Furthermore, we investigated the expression of other noncanonical NF- κ B pathway—associated genes (p100/p52; *NFKB2*, *RELB*) compared with canonical NF- κ B pathway—associated genes (p65; *RELA*, I κ B kinase β ; *IKBKB*, p105/p50; *NFKB1*, c-Rel; *REL*) in tissues with or without TLSs. The calculated ratio of noncanonical to canonical NF- κ B pathway—associated genes was significantly higher in tissues containing TLSs (Figure 1B), suggesting that noncanonical NF- κ B signaling is more prevalent in RA ST containing TLSs.

To confirm the microarray data, we performed immunohistochemical analysis with a specific NIK-antibody on RA ST without TLSs ($n = 25$) and with TLSs ($n = 15$) (Table 1). In all tissues, NIK was highly expressed in the vasculature, both inside the TLSs and in the adjacent tissue (Figure 1C), which is consistent with our previous observations that NIK and activated phospho—I κ B kinase α are mainly expressed by ECs in RA ST.²⁷ Importantly, NIK protein expression was also significantly higher in tissues with TLSs (TLS[−] 0.54 ± 0.19 versus TLS⁺ 1.53 ± 0.32 ; $P = 0.0027$) (Figure 1D).

Figure 1 Expression of noncanonical NF- κ B pathway genes and NF- κ B-inducing kinase (NIK) protein in rheumatoid arthritis (RA) synovial tissue (ST). Microarray analysis was performed in ST from RA patients with established disease, with or without tertiary lymphoid structures (TLSs). **A:** Bar graph shows relative gene expression of NIK. **B:** Bar graph shows the ratio of noncanonical (NC) to canonical (C) NF- κ B-associated genes. Data are shown as means of canonical (I κ B kinase β , *RELA*/p65, p105/50, c-Rel) versus noncanonical (NIK, p100/p52, *REL*B) NF- κ B-associated genes in TLS⁺ ($n = 4$) and TLS⁻ ($n = 8$) RA STs. **C:** Images show immunohistochemical staining of NIK in TLS⁺ (left panel; dotted line indicates TLS) and TLS⁻ (right panel) RA STs. **D:** Bar graph shows semiquantitative scoring on a scale of 0 to 4 of immunohistochemical NIK in TLS⁺ ($n = 15$) and TLS⁻ ($n = 25$) RA STs; two independent experiments were performed. Data are shown as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$. Original magnification: $\times 100$ (left panel, C); $\times 200$ (right panel, C).

NIK Is Expressed in HEVs in RA ST and in Lymph Nodes

Because NIK was highly expressed in vessels within the TLSs, we performed double immunofluorescence (IF) staining to investigate whether NIK⁺ vessels also comprised HEVs. First, we demonstrated that NIK is expressed by CD31/CD34⁺ blood vessels (Figure 2A). Furthermore, NIK

colocalized with the HEV marker PNAd in RA ST. Of note, all PNAd⁺ HEVs expressed NIK, but NIK was also expressed by PNAd⁻ blood vessels (Figure 2B). Quantification of double-stained tissue sections demonstrated that approximately 10% ($9.4\% \pm 5.4\%$; $n = 7$) of NIK⁺ ECs in RA ST were also PNAd-positive and thus regarded to be HEVs.

The noncanonical NF- κ B pathway induces production of chemokines such as CXCL13, chemokine ligand 21, and chemokine ligand 19 in normal lymphoid neogenesis.¹⁴ CXCL13 is also expressed by ECs in salivary glands of patients with Sjögren syndrome,³¹ indicating that CXCL13 can also be expressed by ECs under inflammatory conditions. Therefore, we investigated whether the noncanonical NF- κ B-regulated chemokine CXCL13 is also expressed in ECs in RA ST containing TLSs. CXCL13 was highly expressed in NIK⁺ ECs in RA ST (Figure 2, C and D), supporting our hypothesis that NIK⁺ ECs may be important orchestrators of TLSs, for instance via expression of CXCL13 or other chemokines that may attract B cells.

LT β R signaling is crucial for lymphoid organogenesis,⁹ and ECs have been shown to be instrumental in this process as well.²⁶ However, whether stabilized NIK is also selectively present in ECs in lymph nodes remains unknown. Therefore, we investigated NIK expression in lymph nodes by using IF microscopy (Figure 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⁺ HEVs and in PNAd⁻ blood vessels in these lymph nodes (Figure 2F).

ILC3s Are Present in RA ST but in Very Low Numbers

In RA ST, a subset of B and T cells, mainly located in the aggregates, express LT β .⁶ We confirmed this idea and established that STs with TLSs contain significantly more LT β expressing B and T cells in our cohorts.^{3,29} However, LT β i cells (ILC3), key regulators of lymph node development,³² are also a potential source of LT β ¹⁹ that may induce noncanonical NF- κ B signaling in stromal cells, including ECs. Therefore, we visualized ILC3s in RA ST by performing IF microscopy and quantified the number of ILC3s as determined by CD3⁻ RORC⁺ cells.

Interestingly, we could for the first time detect ILC3s in RA ST (Figure 3A). However, these cells were present only in very small numbers, and we did not observe a clear correlation with TLS because TLS⁺ tissues contained only very low numbers of ILC3s in a few patients (3 of 15 patients; one or two ILC3s per slide), whereas TLS⁻ tissues contained ILC3s in only 1 of 25 patients in biopsy samples of a similar size. Also, these cells did not have a close spatial relationship to NIK⁺ HEVs. In contrast, developing lymph nodes contained a very high number of ILC3s (Figure 3B). The very low number of ILC3s in RA ST

Figure 2 NF- κ B-inducing kinase (NIK) expression by endothelial cells (ECs) and high endothelial venules (HEVs) in rheumatoid arthritis (RA) synovial tissue (ST) versus normal lymph nodes. Images show immunofluorescence (IF) staining on RA ST containing tertiary lymphoid structures (TLSs). **A:** NIK is shown in red, with blood vessel markers CD31/CD34 in green and nuclei in blue in RA ST. **B:** NIK is shown in red, with peripheral node addressin (PNAAd)/MECA-79 in green and nuclei in blue in RA ST containing TLSs. **C:** CXCL13 is shown in red and CD20 in blue. **D:** CXCL13 is shown in red, with NIK in green and nuclei in blue in RA ST. Representative images of RA ST ($n = 15$) are shown. **E:** Images show IF staining of NIK (red) in mesenteric lymph nodes of 20 weeks' gestation (**left panel**) and in adult hepatic lymph nodes (**right panel**). **F:** Images show IF staining of NIK (red), with PNAAd (green) and nuclei (blue) in adult hepatic lymph node. Representative images of mesenteric ($n = 3$) and hepatic lymph nodes ($n = 3$) are shown. Original magnification: $\times 250$.

suggests that these cells are probably not the main orchestrators of TLSs in chronic inflammation.

PDGFR β^+ preFDCs and FDCs Are Preferentially Present in TLS $^+$ Tissues

Recently, it was shown that FDCs arise from PDGFR β^+ preFDCs.²⁵ Furthermore, FDCs 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 FDCs are present in STs (mainly TLS $^+$ tissues) of some RA patients.⁵ Therefore, we investigated the expression of the FDC marker FDC-M1 and PDGFR β in TLS $^+$ and TLS $^-$ RA ST. Interestingly, PDGFR β expression was mainly observed in a perivascular pattern, suggesting that these PDGFR β^+ cells are preFDCs (Figure 4A). Furthermore, FDC-M1 expression was mainly observed inside the TLSs in RA ST containing TLSs (Figure 4B). PDGFR β^+ cells were significantly more present in TLS $^+$ tissues (TLS $^-$ 1.44 ± 0.24 versus TLS $^+$ 2.53 ± 0.26 ; $P = 0.005$) (Figure 4C), and FDC-M1 expression was also significantly higher in these tissues (TLS $^-$ 0.96 ± 0.20 versus TLS $^+$ 2.6 ± 0.03 ; $P = 0.0002$) (Figure 4D). Of note, TLS $^+$ tissues that contained CD21L $^+$ FDCs (3 of 15; 20% of TLS $^+$ ST) exhibited the highest FDC-M1 expression (Figure 4G).

Double IF staining for NIK with PDGFR β and PDGFR β with FDC-M1 confirmed that PDGFR β^+ cells are localized around NIK $^+$ ECs and that some of these cells already start to express FDC-M1 (Figure 4E). However, the majority of FDC-M1 $^+$ cells had down-regulated PDGFR β (Figure 4F), which is consistent with previous findings that describe loss of PDGFR β expression as preFDCs progress and develop into mature FDCs.²⁵ Finally, we examined whether NIK $^+$ ECs, perivascular PDGFR β^+ preFDCs, and FDCs in RA ST showed a clear correlation. We observed a significant positive correlation between all markers: NIK versus PDGFR β ($r = 0.4177$; $P = 0.0073$) (Figure 4G), NIK versus FDC-M1 ($r = 0.5406$; $P = 0.0003$) (Figure 4H), and FDC-M1 versus PDGFR β ($r = 0.5989$; $P < 0.0001$) (Figure 4I). This finding may indicate that NIK $^+$ ECs, preFDCs, and FDCs are all important mediators in the orchestration of TLSs in chronic inflammation.

Discussion

TLSs 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 TLSs.³⁴ Given the crucial role of noncanonical NF- κ B signaling in stromal cells in normal

Figure 3 Group 3 innate lymphoid cells (ILC3s) are present in rheumatoid arthritis (RA) synovial tissue (ST) and developing lymph nodes. CD3⁺ RORC⁺ ILC3s are indicated by the white arrows. CD3 is shown in red, RORC in green, and nuclei in blue. **A:** Images show immunofluorescence (IF) staining on ILC3s in RA ST containing tertiary lymphoid structures (TLSs). **B:** As a control, IF staining was performed on ILC3s in adult popliteal lymph node. Representative images of all analyzed RA STs ($n = 40$) and developing popliteal lymph nodes ($n = 3$) are shown; two independent experiments were performed. Original magnification: $\times 200$.

lymphoid organogenesis,^{9,12} we hypothesized that the non-canonical NF- κ B pathway, with its key regulator NIK, also plays an important role in the formation of TLSs in RA ST. We investigated whether active noncanonical NF- κ B signaling in ECs and the presence of certain cell types such as LT β -producing ILC3 and preFDCs that may orchestrate TLSs are related to the presence of TLSs in chronic inflammation by using RA as a prototype immune-mediated inflammatory disease.

First, we demonstrated that NIK and noncanonical NF- κ B-associated genes are expressed at a higher level in RA ST with TLSs than in RA ST without TLSs. Next, we confirmed by means of immunohistochemical analysis that NIK is highly expressed in TLS⁺ tissues and established that NIK is mainly expressed by ECs. A subset of these NIK⁺ ECs can be classified as PNAd⁺ HEVs. Interestingly, we showed that all PNAd⁺ HEVs express NIK, not only in RA ST but also in developing and adult lymph nodes, which had not been demonstrated before. These results are consistent with those in a recent report demonstrating that EC-restricted LT β R signaling is required for normal lymph node and HEV formation and function.²⁶ Importantly, our data also indicate that LT β -induced noncanonical NF- κ B signaling in ECs may be involved in the formation of TLSs. However, to date, the most important source of LT β in the context of TLSs has remained enigmatic.

LTi cells (ILC3) that orchestrate lymphoid organ development³² are able to produce high amounts of LT β ¹⁹ and would therefore be good candidates for inducing TLSs in chronic inflammatory conditions. We demonstrate for the first time that ILC3s 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 TLSs. However, we could detect only very small numbers of these cells in the inflamed ST.

Because TLS formation in RA ST probably is a very dynamic process and may vary over time in the same

patient,⁵ we investigated whether ILC3s are present in tissues with TLSs and tissues without TLSs to exclude the possibility that the presence of ILC3s 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 TLSs. 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 TLSs in the inflamed colon and thyroid.^{22,36} However, the possibility that other LT β -producing innate lymphoid cell populations,¹⁷ which may also be present in the inflamed ST, contribute to TLSs cannot be excluded. However, in the inflamed synovium, the abundantly present B and T cells are more likely to be the source of LT β to induce TLS formation,^{3,6,29} whereas in lymph node development LTi cells are the main providers of LT β .⁹ Also, in analogy to lymph nodes, dendritic cells that produce LT β may induce noncanonical NF- κ B signaling in HEVs to control lymphocyte entry to the chronically inflamed tissue³⁷ and may thus contribute to TLS formation. Alternatively, noncanonical NF- κ B signaling may be induced in ECs via other stimuli such as CD40L or LIGHT (tumor necrosis factor superfamily member 14) expressed by T cells under inflammatory conditions.^{38–40}

Although TLS formation and lymph node development share many aspects, including chemokine-dependent and PNAd L-selectin interaction-mediated lymphocyte homing, clear differences between lymph nodes and TLSs exist.^{41,42} In TLS formation in chronic inflammation, local proinflammatory cytokines may, for instance, enhance hyaluronan expression on ECs, which would make ECs receptive for CD44-mediated rolling of leukocytes leading up to integrin-dependent transendothelial migration of these cells.⁴³ Certain immune cells, including CD4⁺ CD25⁺ regulatory T cells, may be more dependent on this mechanism for homing, which could also account for some of the

Figure 4 Follicular dendritic cells (FDCs) and platelet-derived growth factor receptor β (PDGFR β)⁺ perivascular precursor cells (preFDCs) are present in rheumatoid arthritis (RA) synovial tissue (ST) and correlate with tertiary lymphoid structures (TLSs). **A and B:** Immunohistochemical (IHC) staining on RA ST with and without TLSs. PDGFR β (**A**) and FDC-M1 (**B**) are shown. Representative images of TLS⁺ ($n = 15$; **left panels**) and TLS⁻ ($n = 25$; **right panels**) RA STs are shown. **C and D:** Bar graphs show semiquantitative scoring on a scale of 0 to 4 of IHC staining of PDGFR β (**C**) and FDC-M1 (**D**). **E and F:** Images show immunofluorescence staining on RA ST, with PDGFR β in red (**E and F**), NF- κ B-inducing kinase (NIK) in green (**E**), FDC-M1 in green (**F**), and nuclei in blue (**E and F**). **G–I:** Graphs show correlation of semiquantitative IHC scores between NIK and PDGFR β ($r = 0.4177$, $P = 0.0073$; **G**), NIK and FDC-M1 ($r = 0.5406$, $P = 0.0003$; **H**), and FDC-M1 and PDGFR β ($r = 0.5989$, $P < 0.0001$; **I**). CD21L⁺ FDCs containing TLS⁺ STs are depicted in light gray circles. Correlations were determined by means of a Spearman correlation coefficient. Data are shown as means $\pm$ SEM (**C and D**). ** $P < 0.01$, *** $P < 0.001$. Original magnification: $\times 100$ (**A and B**); $\times 630$ (**E and F**).

differences in the types of immune cells that accumulate in TLSs compared with those in lymph nodes.^{41,44}

FDCs are crucial for microarchitecture maintenance of germinal centers in lymph nodes and TLSs in RA ST,^{7,24} and LT β R signaling is required for their differentiation.^{45,46} In addition, FDCs are located at perivascular sites and together with the endothelium secrete the chemokine CXCL13 that is essential for lymph node initiation.⁴⁷ Previously, expression of CXCL13 in RA ST containing TLSs was observed in FDCs,⁴⁸ whereas in Sjögren syndrome CXCL13 is mainly expressed by ECs.³¹ Here we show that CXCL13 is also expressed by ECs in RA ST containing TLSs. These data may indicate that NIK⁺ ECs not only express the proangiogenic and immune-cell-attracting chemokine CXCL12²⁷ but also produce the noncanonical NF- κ B-regulated chemokine CXCL13, which plays a crucial role in the entry of B cells into lymphoid structures⁴⁹ that may further amplify this process.

Recently, it was established that FDCs emerge from ubiquitous perivascular precursors that resemble pericytes.²⁵ These PDGFR β ⁺ preFDCs 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. Here, we report that not only FDCs but also PDGFR β ⁺ preFDCs are abundant in RA ST containing TLSs and are localized

around NIK⁺ ECs. In addition, we found positive correlations between NIK⁺ ECs and the presence of PDGFR β ⁺ preFDCs and FDC-M1⁺ FDCs. This finding may indicate that, similar to their role in lymph node and HEV formation and function,²⁶ NIK⁺ ECs are central players in the orchestration of TLSs in chronic inflammation. We hypothesize that LT β -expressing immune cells induce non-canonical NF- κ B signaling in ECs, resulting in the differentiation of ECs into HEVs. These specialized ECs express chemokines, such as CXCL12 and CXCL13, which recruit more immune cells, resulting in TLSs. This hypothesis also fits with a study showing that maintenance of ectopic tertiary stromal cell networks and conduits is mainly LT β dependent.⁵⁰

Future experimental studies should address the role of LT β R-induced noncanonical NF- κ B signaling in ECs in this process, for example by studying TLS formation in mice that selectively lack LT β R or NIK in ECs.²⁶ These experimental studies could also provide clear answers with respect to the timing of the sequence of events that leads to TLS formation, which either could be sequential or take place simultaneously in a self-perpetuating loop.

Interestingly, inhibition of LT β R signaling via administration of an LT β R-immunoglobulin fusion protein has been proved beneficial in a preclinical model of arthritis⁵¹ and inhibited or reversed development of HEV and FDC

networks in a preclinical model of Sjögren syndrome, resulting in a significant reduction of TLSs and improved salivary gland function.⁵² However, efficacy data of these therapies in humans are lacking. Our studies do not provide answers to the function of TLSs in chronic inflammation. Consequently, future studies should also be aimed at further unraveling the role of TLSs in chronic inflammation.

Conclusions

Our results indicate that NIK⁺ ECs and preFDCs are abundantly present in RA ST containing TLSs and that these cell types may be more important orchestrators of TLSs than LTi cells or ILC3. We propose that NIK⁺ ECs and HEVs are induced in synovial inflammation under the influence of LTβ-producing immune cells and may act as central players in TLS formation. Subsequently, perivascular PDGFRβ⁺ preFDCs are activated and differentiate into FDCs that further contribute to the orchestration of TLSs.

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References

1. Aloisi F, Pujol-Borrell R: Lymphoid neogenesis in chronic inflammatory diseases. *Nat Rev Immunol* 2006, 6:205–217
2. Weyand CM, Goronzy JJ: Ectopic germinal center formation in rheumatoid synovitis. *Ann N Y Acad Sci* 2003, 987:140–149
3. Thurlings RM, Wijbrandts CA, Mebius RE, Cantaert T, Dinant HJ, van der Pouw-Kraan TC, Verweij CL, Baeten D, Tak PP: Synovial lymphoid neogenesis does not define a specific clinical rheumatoid arthritis phenotype. *Arthritis Rheum* 2008, 58:1582–1589
4. Humby F, Bombardieri M, Manzo A, Kelly S, Blades MC, Kirkham B, Spencer J, Pitzalis C: Ectopic lymphoid structures support ongoing production of class-switched autoantibodies in rheumatoid synovium. *PLoS Med* 2009, 6:e1
5. van de Sande MG, Thurlings RM, Boumans MJ, Wijbrandts CA, Modesti MG, Gerlag DM, Tak PP: Presence of lymphocyte aggregates in the synovium of patients with early arthritis in relationship to

diagnosis and outcome: is it a constant feature over time? *Ann Rheum Dis* 2011, 70:700–703

6. Takemura S, Braun A, Crowson C, Kurtin PJ, Cofield RH, O'Fallon WM, Goronzy JJ, Weyand CM: Lymphoid neogenesis in rheumatoid synovitis. *J Immunol* 2001, 167:1072–1080
7. Manzo A, Paoletti S, Carulli M, Blades MC, Barone F, Yanni G, Fitzgerald O, Bresnahan B, Caporali R, Montecucco C, Uguccioni M, Pitzalis C: Systematic microanatomical analysis of CXCL13 and CCL21 in situ production and progressive lymphoid organization in rheumatoid synovitis. *Eur J Immunol* 2005, 35:1347–1359
8. Corsiero E, Bombardieri M, Manzo A, Bugatti S, Uguccioni M, Pitzalis C: Role of lymphoid chemokines in the development of functional ectopic lymphoid structures in rheumatic autoimmune diseases. *Immunol Lett* 2012, 145:62–67
9. Mebius RE: Organogenesis of lymphoid tissues [Erratum appeared in *Nat Rev Immunol* 2003, 3:509]. *Nat Rev Immunol* 2003, 3:292–303
10. Luther SA, Bidgol A, Hargreaves DC, Schmidt A, Xu Y, Paniyadi J, Matloubian M, Cyster JG: Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *J Immunol* 2002, 169:424–433
11. Drayton DL, Ying X, Lee J, Lesslauer W, Ruddle NH: Ectopic LT alpha beta directs lymphoid organ neogenesis with concomitant expression of peripheral node addressin and a HEV-restricted sulfo-transferase. *J Exp Med* 2003, 197:1153–1163
12. Bonizzi G, Karin M: The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* 2004, 25:280–288
13. Sun SC: The noncanonical NF-kappaB pathway. *Immunol Rev* 2012, 246:125–140
14. Drayton DL, Bonizzi G, Ying X, Liao S, Karin M, Ruddle NH: I kappa B kinase complex alpha kinase activity controls chemokine and high endothelial venule gene expression in lymph nodes and nasal-associated lymphoid tissue. *J Immunol* 2004, 173:6161–6168
15. Yin L, Wu L, Wesche H, Arthur CD, White JM, Goeddel DV, Schreiber RD: Defective lymphotoxin-beta receptor-induced NF-kappaB transcriptional activity in NIK-deficient mice. *Science* 2001, 291:2162–2165
16. Spits H, Cupedo T: Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu Rev Immunol* 2012, 30:647–675
17. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, Koyasu S, Locksley RM, McKenzie AN, Mebius RE, Powrie F, Vivier E: Innate lymphoid cells—a proposal for uniform nomenclature. *Nat Rev Immunol* 2013, 13:145–149
18. Cherrier M, Eberl G: The development of LTi cells. *Curr Opin Immunol* 2012, 24:178–183
19. Cupedo T, Crellin NK, Papazian N, Rombouts EJ, Weijer K, Grogan JL, Fibbe WE, Cornelissen JJ, Spits H: Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nat Immunol* 2009, 10:66–74
20. Lochner M, Ohnmacht C, Presley L, Bruhns P, Si-Tahar M, Sawa S, Eberl G: Microbiota-induced tertiary lymphoid tissues aggravate inflammatory disease in the absence of RORgamma t and LTi cells. *J Exp Med* 2011, 208:125–134
21. Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa S, Gruss P: Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* 1999, 397:702–706
22. Marinkovic T, Garin A, Yokota Y, Fu YX, Ruddle NH, Furtado GC, Lira SA: Interaction of mature CD3+CD4+ T cells with dendritic cells triggers the development of tertiary lymphoid structures in the thyroid. *J Clin Invest* 2006, 116:2622–2632
23. Ware CF, Crowe PD, Grayson MH, Androlewicz MJ, Browning JL: Expression of surface lymphotoxin and tumor necrosis factor on activated T, B, and natural killer cells. *J Immunol* 1992, 149:3881–3888

24. Aguzzi A, Kranich J, Krautler NJ: Follicular dendritic cells: origin, phenotype, and function in health and disease. *Trends Immunol* 2014, 35:105–113
25. Krautler NJ, Kana V, Kranich J, Tian Y, Perera D, Lemm D, Schwarz P, Armulik A, Browning JL, Tallquist M, Buch T, Oliveira-Martins JB, Zhu C, Hermann M, Wagner U, Brink R, Heikenwalder M, Aguzzi A: Follicular dendritic cells emerge from ubiquitous perivascular precursors. *Cell* 2012, 150:194–206
26. Onder L, Danuser R, Scandella E, Firmer S, Chai Q, Hehlhans T, Stein JV, Ludewig B: Endothelial cell-specific lymphotoxin-beta receptor signaling is critical for lymph node and high endothelial venule formation. *J Exp Med* 2013, 210:465–473
27. Noort AR, van Zoest KP, Weijers EM, Koolwijk P, Maracle CX, Novack DV, Siemerink MJ, Schlingemann RO, Tak PP, Tas SW: NF-kappaB-inducing kinase is a key regulator of inflammation-induced and tumour-associated angiogenesis. *J Pathol* 2014, 234:375–385
28. Gerlag DM, Tak PP: How to perform and analyse synovial biopsies. *Best Pract Res Clin Rheumatol* 2009, 23:221–232
29. Timmer TC, Baltus B, Vondenhoff M, Huizinga TW, Tak PP, Verweij CL, Mebius RE, van der Pouw Kraan TC: Inflammation and ectopic lymphoid structures in rheumatoid arthritis synovial tissues dissected by genomics technology: identification of the interleukin-7 signaling pathway in tissues with lymphoid neogenesis. *Arthritis Rheum* 2007, 56:2492–2502
30. Smeets TJ, Barg EC, Kraan MC, Smith MD, Breedveld FC, Tak PP: Analysis of the cell infiltrate and expression of proinflammatory cytokines and matrix metalloproteinases in arthroscopic synovial biopsies: comparison with synovial samples from patients with end stage, destructive rheumatoid arthritis. *Ann Rheum Dis* 2003, 62: 635–638
31. Amft N, Curnow SJ, Scheel-Toellner D, Devadas A, Oates J, Crocker J, Hamburger J, Ainsworth J, Mathews J, Salmon M, Bowman SJ, Buckley CD: Ectopic expression of the B cell-attracting chemokine BCA-1 (CXCL13) on endothelial cells and within lymphoid follicles contributes to the establishment of germinal center-like structures in Sjögren's syndrome. *Arthritis Rheum* 2001, 44: 2633–2641
32. Eberl G, Littman DR: The role of the nuclear hormone receptor RORgamma in the development of lymph nodes and Peyer's patches. *Immunol Rev* 2003, 195:81–90
33. Kranich J, Krautler NJ, Heinen E, Polymenidou M, Bridel C, Schildknecht A, Huber C, Kosco-Vilbois MH, Zinkernagel R, Miele G, Aguzzi A: Follicular dendritic cells control engulfment of apoptotic bodies by secreting Mfge8. *J Exp Med* 2008, 205:1293–1302
34. Kain MJ, Owens BM: Stromal cell regulation of homeostatic and inflammatory lymphoid organogenesis. *Immunology* 2013, 140: 12–21
35. Manzo A, Bombardieri M, Humby F, Pitzalis C: Secondary and ectopic lymphoid tissue responses in rheumatoid arthritis: from inflammation to autoimmunity and tissue damage/remodeling. *Immunol Rev* 2010, 233:267–285
36. Lochner M: Tertiary lymphoid tissues in the colon: friend and foe. *Gut Microbes* 2011, 2:193–197
37. Moussion C, Girard JP: Dendritic cells control lymphocyte entry to lymph nodes through high endothelial venules. *Nature* 2011, 479: 542–546
38. Kang YM, Zhang X, Wagner UG, Yang H, Beckenbaugh RD, Kurtin PJ, Goronzy JJ, Weyand CM: CD8 T cells are required for the formation of ectopic germinal centers in rheumatoid synovitis. *J Exp Med* 2002, 195:1325–1336
39. Mach F, Schönbeck U, Sukhova GK, Bourcier T, Bonnefoy JY, Pober JS, Libby P: Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis. *Proc Natl Acad Sci U S A* 1997, 94:1931–1936
40. Lee Y, Chin RK, Christiansen P, Sun Y, Tumanov AV, Wang J, Chervonsky AV, Fu YX: Recruitment and activation of naive T cells in the islets by lymphotoxin beta receptor-dependent tertiary lymphoid structure. *Immunity* 2006, 25:499–509
41. Pitzalis C, Jones GW, Bombardieri M, Jones SA: Ectopic lymphoid-like structures in infection, cancer and autoimmunity. *Nat Rev Immunol* 2014, 14:447–462
42. Neyt K, Perros F, GeurtsvanKessel CH, Hammad H, Lambrecht BN: Tertiary lymphoid organs in infection and autoimmunity. *Trends Immunol* 2012, 33:297–305
43. Mohamadizadeh M, DeGrendele H, Arizpe H, Estess P, Siegelman M: Proinflammatory stimuli regulate endothelial hyaluronan expression and CD44/HA-dependent primary adhesion. *J Clin Invest* 1998, 101: 97–108
44. Ohmichi Y, Hirakawa J, Imai Y, Fukuda M, Kawashima H: Essential role of peripheral node addressin in lymphocyte homing to nasal-associated lymphoid tissues and allergic immune responses. *J Exp Med* 2011, 208:1015–1025
45. Fu YX, Huang G, Wang Y, Chaplin DD: B lymphocytes induce the formation of follicular dendritic cell clusters in a lymphotoxin alpha-dependent fashion. *J Exp Med* 1998, 187:1009–1018
46. Gonzalez M, Mackay F, Browning JL, Kosco-Vilbois MH, Noelle RJ: The sequential role of lymphotoxin and B cells in the development of splenic follicles. *J Exp Med* 1998, 187:997–1007
47. van de Pavert SA, Olivier BJ, Govers G, Vondenhoff MF, Greuter M, Beke P, Kusser K, Höpken UE, Lipp M, Niederreither K, Blomhoff R, Sitnik K, Agace WW, Randall TD, de Jonge WJ, Mebius RE: Chemokine CXCL13 is essential for lymph node initiation and is induced by retinoic acid and neuronal stimulation. *Nat Immunol* 2009, 10:1193–1199
48. Shi K, Hayashida K, Kaneko M, Hashimoto J, Tomita T, Lipsky PE, Yoshikawa H, Ochi T: Lymphoid chemokine B cell-attracting chemokine-1 (CXCL13) is expressed in germinal center of ectopic lymphoid follicles within the synovium of chronic arthritis patients. *J Immunol* 2001, 166:650–655
49. Kanemitsu N, Ebisuno Y, Tanaka T, Otani K, Hayasaka H, Kaisho T, Akira S, Katagiri K, Kinashi T, Fujita N, Tsuruo T, Miyasaka M: CXCL13 is an arrest chemokine for B cells in high endothelial venules. *Blood* 2005, 106:2613–2618
50. Link A, Hardie DL, Favre S, Britschgi MR, Adams DH, Sixt M, Cyster JG, Buckley CD, Luther SA: Association of T-zone reticular networks and conduits with ectopic lymphoid tissues in mice and humans. *Am J Pathol* 2011, 178:1662–1675
51. Fava RA, Notidis E, Hunt J, Szanya V, Ratcliffe N, Ngam-Ek A, De Fougères AR, Sprague A, Browning JL: A role for the lymphotoxin/LIGHT axis in the pathogenesis of murine collagen-induced arthritis. *J Immunol* 2003, 171:115–126
52. Gatumu MK, Skarstein K, Papandile A, Browning JL, Fava RA, Bolstad AI: Blockade of lymphotoxin-beta receptor signaling reduces aspects of Sjögren's syndrome in salivary glands of non-obese diabetic mice. *Arthritis Res Ther* 2009, 11:R24