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VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Host Lymphotoxin- β Receptor Signaling Is Crucial for Angiogenesis of Metanephric Tissue Transplanted into Lymphoid Sites

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The mouse lymph node (LN) can provide a niche to grow metanephric kidney to maturity. Here, we show that signaling through the lymphotoxin- β receptor (LT β R) is critical for kidney organogenesis both in the LN and the omentum. By transplanting kidney rudiments either in the LNs of mice undergoing LT β R antagonist treatment or in the omenta of *Ltbr* knockout (*Ltbr*^{-/-}) mice, the host LT β R signals were found to be crucial for obtaining a well-vascularized kidney graft. Indeed, defective LT β R signaling correlated with decreased expression of endothelial and angiogenic markers in kidney grafts as well as structural alterations. Because the number of glomerular endothelial cells expressing the LT β R target nuclear factor κ B-inducing kinase (NIK) decreased in the absence of a functional LT β R, it was speculated that an LT β R/NIK axis mediated the angiogenetic signals required for successful ectopic kidney organogenesis, given the established role of NIK in neovascularization. However, the transplantation of kidney rudiments in omenta of *Nik*^{-/-} mice revealed that NIK is dispensable for ectopic kidney vascular integration and maturation. Finally, defective LT β R signaling impaired compensatory glomerular adaptation to renal mass reduction, indicating that kidney regeneration approaches, besides whole kidney reconstruction, might benefit from the presence of LT β R signals. (*Am J Pathol* 2020, 190: 252–269; <https://doi.org/10.1016/j.ajpath.2019.08.018>)

Chronic and end-stage kidney disease impose a large burden on human health worldwide. Projected prevalence trend lines are not comforting,¹ suggesting an urgent need to explore new approaches to overcome limitations associated with traditional interventions. Dialysis—the most common method used to treat advanced kidney failure—can only replace 10% to 20% of kidney function and is associated with increased left ventricular mass, intramyocardial cell fibrosis, and capillary loss.^{2,3} Orthotopic kidney transplantation offers better quality of life and longer survivals,⁴ yet it is limited by organ shortage and the risks associated with immunosuppression, including increased vulnerability to infection and cancer, as well as organ toxicity.⁵

One novel solution to the shortage of human organs available for transplantation envisions growing new organs *in situ* via transplantation of developing anlagen from animal embryos.⁶ A few sites have been considered for renal anlage transplantation, and, paradoxically, the orthotopic

environment seems not to be the most appropriate. Indeed, transplantation of metanephric tissue into the adult mouse kidney does not lead to glomerular or tubular differentiation.⁷ Other sites have also been considered, including the anterior eye chamber,⁸ the omentum,⁹ the paraaortic area,¹⁰ and, recently, the lymph node (LN) has been identified as an attractive site for the maturation of mouse metanephroi into functional glomeruli and tubules.^{11,12}

Here, we show that host lymphotoxin- β receptor (LT β R) pathway generates angiogenic cues to support kidney organogenesis in both LNs and omenta. Our hypothesis that

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LT β R could affect kidney organogenesis in these sites sprang from the central role of LT β R in the development^{13,14} and homeostasis¹⁵ of lymphoid organs, as well as from our finding that glomeruli maturing in both LNs and omenta contained, during the early stages of engraftment, endothelial cells expressing the nuclear factor κ B-inducing kinase (NIK), a downstream target, among others, of LT β R.¹⁶ Given an established role for NIK in neo-vascularization,¹⁷ and the presence of host endothelial cells in ectopic kidney grafts, it was speculated that a host LT β R/NIK axis could allow successful kidney organogenesis. In accordance, defective LT β R signaling was found to be associated with decreased numbers of glomerular NIK-expressing endothelial cells, decreased absolute vascularization/angiogenesis, and structural alterations of the kidney grafts. However, kidney organogenesis was not affected in the absence of NIK. This result might suggest either the existence of different targets downstream of LT β R, leading to angiogenesis, or the activation of compensatory angiogenic pathways in the absence of NIK.

Finally, the absence of LT β R signals impaired compensatory glomerular adaptation to renal mass reduction, indicating that kidney regeneration approaches, besides whole kidney reconstruction, might benefit from the presence of LT β R signals.

Materials and Methods

Ethics Statement

Mice were purchased from Charles River Laboratories (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME), and bred and housed in the Division of Laboratory Animal Resources facility at the University of Pittsburgh. Experimental protocols followed NIH's *Guide for the Care and Use of Laboratory Animals*,¹⁸ and they were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Mixed sexes were used.

Tissues and Transplantations

E14 kidneys were retrieved from timed-pregnant green fluorescent protein (GFP)⁺ or wild-type C57BL/6 mice under a dissecting microscope. GFP⁺ kidneys served for transplantation of wild-type C57BL/6 LNs to investigate host-derived vascularization (CD31 stain) and expression of LT β R, erasmus university rotterdam—thymic reticulum antibody 7 (ER-TR7), LT- β , and NIK ($n = 6$) or to test the effect of LT β R-Fc treatment ($n = 6$) (see below). Wild-type kidneys served for transplantation of wild-type C57BL/6 LNs ($n = 3$) to analyze glomerular NIK expression. LN transplantation was performed as previously described,¹¹ and LN kidney grafts were collected 3 or 6 weeks after transplantation, depending on the experiment.

Alternatively, paired GFP⁺ E14 kidneys were transplanted in the omenta of wild-type or *Ltbr*^{-/-} C57BL/6

mice ($n = 10$ per group) or in the omenta of wild-type or *Nik*^{-/-} (*Map3k14*^{-/-}) C57BL/6 mice ($n = 5$ per group). Kidneys were split longitudinally in two pieces before transplantation. The kidney preparation was placed onto the exposed omentum, and the omentum was later folded onto itself. Growth Factor Reduced Matrigel (356231; Corning, Corning, NY) was used to glue the transplanted omenta. Intraomental kidney grafts from wild-type and *Ltbr*^{-/-} mice were collected at different time points (2 weeks: $n = 1$; 3 weeks: $n = 4$; 4 weeks: $n = 1$; 5 weeks: $n = 1$; 6 weeks: $n = 3$), whereas intraomental kidney grafts from wild-type and *Nik*^{-/-} mice were all collected 3 weeks after transplantation.

Wild-type or *Ltbr*^{-/-} C57BL/6 mice were also transplanted with GFP⁺ newborn (postnatal day 2) kidneys in their omenta ($n = 3$ per group). Half of a kidney was transplanted in each mouse as described in the paragraph above. One mouse from each group was sacrificed every week, starting at 2 weeks after transplantation up to the fourth week.

Images of kidney grafts were obtained with both the Olympus IX71 inverted fluorescence microscope (Center Valley, PA) and the Leica EZ4D stereomicroscope (Buffalo Grove, IL).

LT β R-Fc Treatments

To assess LT β R pathway contribution to kidney organogenesis inside the LN, recipient mice were treated intraperitoneally with 100 μ g of mouse LT β R-Fc fusion protein (71122; BPS Bioscience, San Diego, CA) or control immunoglobulin (PI31205; Pierce Biotechnology, Rockford, IL) 2 days before transplantation. Mice were further treated at day 8 and 15, and LNs were collected at day 22. LT β R-Fc concentration and exposure times were chosen based on previously published studies.^{19,20} LT β R blockage was confirmed by decreased glomerular NIK expression.

Stains and Quantifications

Engrafted LNs and omenta and mouse kidneys were fixed in 4% paraformaldehyde, embedded in optimal cutting temperature compound or paraffin, and cut into 4- to 7- μ m-thick sections for further analysis. Hematoxylin and eosin (H&E) staining was performed as described elsewhere.²¹ All other stains were performed as follows.

Fluorescent Immunohistochemistry Staining of Frozen Tissue Sections

Frozen sections were equilibrated at room temperature for 15 minutes, then rehydrated in phosphate-buffered saline (PBS) for 15 minutes, and permeabilized with cold 0.1% Triton X-100 for 10 minutes. After three washes in PBS supplemented with 0.05% Tween 20 (PBS-T), a solution of 3% bovine serum albumin was applied to the sections for 30 minutes to prevent nonspecific binding. Sections were then incubated with specific primary

antibodies at least 1 hour at room temperature or overnight at 4°C. After three washes in PBS-T, sections were incubated with Alexa Fluor 488 or 594 secondary antibodies (Thermo Fisher, Pittsburgh, PA) for 20 minutes. Sections were washed three times, and the nuclei were counterstained with Hoechst 33342 (Life Technologies, Frederick, MD). A solution of 50% glycerol in Hanks' balanced salt solution was used as mounting medium.

Fluorescent Immunocytochemistry Staining

CD45-depleted stromal cells from omenta were cytospun onto glass slides (see below for isolation), fixed with acetone for 8 minutes, and stained with specific antibodies according to the same protocol described for frozen sections.

Chromogenic Immunohistochemistry Staining of Paraffin-Embedded Tissue Sections

Paraffin sections were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidases were blocked with the use of 3% hydrogen peroxide for 10 minutes. Antigen unmasking was performed with 10 mmol/L sodium citrate buffer pH 6.0. Nonspecific binding was prevented by incubating the slides in 3% bovine serum albumin in PBS-T for 30 minutes. The slides were exposed to primary antibodies overnight at 4°C, incubated with biotinylated secondary antibody, and later with streptavidin-horseradish peroxidase (R.T.U. Vectastain Kit; Vector, Burlingame, CA). 3-Amino-9-ethylcarbazole (HK092-5KE; BioGenex, Fremont, CA) was used as a chromogen. Immunostained sections were counterstained with Mayer's hematoxylin (Dako, Carpinteria, CA) and mounted in permount mounting medium (SP15-500; Thermo Fisher).

A list of antibodies used is provided in Table 1. Isotype-matched antibodies were used as negative controls. Images were obtained with the Olympus IX71 inverted fluorescence microscope.

Fluorescent Quantifications

Areas of LN and intraomental kidney grafts were quantified by measuring pixel counts of GFP signal with the use of Photoshop CS5 (Adobe, San Jose, CA). The mean fluorescence intensity (MFI) of podoplanin (PDPLN) and the fluorescence intensities of CD31, CD105, and Ly-76 were measured among regions of interest drawn around the Bowman capsule with the use of ImageJ version 1.52a (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>), as previously described.²² Glomerular areas were quantified at the time of PDPLN MFI measurement. The corrected total glomerular fluorescence for CD31, CD105, and Ly-76 was later calculated with the following formula:

Integrated density – (area of selected glomerulus × mean fluorescence of background readings).

Length of lotus tetragonolobus lectin (LTL)⁺ tubules was obtained with ImageJ version 1.52a by drawing a line over the LTL⁺ stain (circular tubular structures were not

Table 1 List of Reagents Used in This Study

Antibody	Catalog number	Supplier
CD105	550546	BD Biosciences (San Jose, CA)
CD31	ab28364	Abcam (Cambridge, MA)
CD34	MA1-82073	ThermoFisher
CDH1	sc-7870	Santa Cruz Biotechnology (Dallas, TX)
CIV	1340-01	SouthernBiotech (Birmingham, AL)
EFNB2	ab131536	Abcam
ER-TR7	ab51824	Abcam
Ki-67	ab15580	Abcam
LT-β	ab64835	Abcam
LTβR	ab70063	Abcam
LTL	B-1325	Vector Laboratories (Burlingame, CA)
Ly-76	ab91113	Abcam
NIK	ab216409	Abcam
PAI-1	ab66705	Abcam
PDPLN	NBP1-90211	Novus Biologicals (Centennial, CO)

CDH1, cadherin 1; CIV, collagen IV; EFNB2, ephrin B2; ER-TR7, erasmus university rotterdam–thymic reticulum antibody 7; LT-β, lymphotoxin-β; LTβR, lymphotoxin-β receptor; LTL, lotus tetragonolobus lectin; NIK, nuclear factor κB–inducing kinase; PAI-1, plasminogen activator inhibitor 1; PDPLN, podoplanin.

considered) and later converting the obtained numbers into microns. Glomeruli and tubules from at least five sections per sample were considered. ER-TR7 MFI was measured in 6 sections per sample with the use of ImageJ version 1.52a. The number of NIK⁺ cells in LN and intraomental grafts was obtained by analyzing glomeruli from 7 to 13 microscopic fields depending on the experiment. The number of plasminogen activator inhibitor 1 (PAI-1)⁺ tubules in LNs and intraomental grafts was obtained by analyzing 12 to 20 microscopic fields.

Histologic Quantifications

Quantitative measurement of glomerular diameters was performed by taking the maximum diameter from the hilum to the end of the Bowman capsule of 18 H&E-stained glomeruli derived from three sections per mouse, using the measurement tool in cellSens Standard 1.9 software (Olympus). Ki-67⁺ nuclei were counted in at least five random sites within the cortex across two separate stained sections per mouse. The number of Ki-67⁺ nuclei was finally expressed as a percentage of the total number of nuclei.

All sections were randomly selected and analyzed under blinded conditions (M.G.F., B.H.).

Stromal Cell Isolation and Flow Cytometry

Stromal cells were isolated from GFP⁺ C57BL/6 mouse mesenteric LNs (*n* = 10) or omenta (*n* = 10) according to the method described by Turley laboratory.²³ Briefly, tissues were collected and digested with an enzyme mix comprised of RPMI 1640 medium that contained 0.8 mg/mL dispase (17105-041; Gibco, Gaithersburg, MD), 0.2 mg/mL collagenase P (11213857001; Sigma-Aldrich, St. Louis, MO),

and 1 mg/mL DNase I (89836; Thermo Fisher). After digestion, anti-CD45 immunomagnetic beads (130-052-301; Miltenyi Biotec, Auburn, CA) were used to deplete hematopoietic cells from the bulk population. CD45-depleted stromal cells were then stained with Sytox Blue (S11348; Molecular Probes, Eugene, OR), phycoerythrin-cyanine 7 CD45 (552848; BD Biosciences, Bedford, MA), phycoerythrin Podoplanin (127407; BioLegend, San Diego, CA), and peridinin chlorophyll protein/cyanine 5.5 CD31 (102419; BioLegend). Flow cytometric analysis was performed with a BD FACS Aria cytometer and FlowJo software version 9.6 (TreeStar, Ashland, OR), according to standard procedures. Isotype antibodies were used as negative controls.

Molecular Analysis

RNA isolation was performed with the RNeasy Mini Kit (74106; Qiagen, Germantown, MD) according to the manufacturer's instructions (including the optional on-column DNase digestion). RNA purity and concentration were measured with a NanoDrop 2000/c spectrophotometer (Thermo Fisher). cDNAs were synthesized with the RT² First Strand Kit (330401; Qiagen), and expression of 84 genes modulating the biological processes of angiogenesis were measured with the Mouse Angiogenesis RT² Profiler PCR Array (PAMM-024Z; Qiagen) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA), according to the manufacturer's

Table 2 List of Primer Sequences Used in This Study

Gene ID	Sequence	NCBI reference
<i>Angpt1</i>	F: 5'-AGTGCACAGAGCAGTACAA-3' R: 5'-TGGGCCATCTCCGACTTCAT-3'	NM_009640.4
<i>EfnA1</i>	F: 5'-TGTACCGTGGAGAAGCCTGT-3' R: 5'-CCTCACGGAACCTTGGGATTTGA-3'	NM_010107.4
<i>EfnB2</i>	F: 5'-CAAATGGGTCTTTGGAGGGC-3' R: 5'-ACGCTCTGTTGGACCGTGAT-3'	NM_010111.5
<i>Eng</i>	F: 5'-GCCCATTAACCTGCTGTTTG-3' R: 5'-CACAGCCGACTCTTTCTGC-3'	NM_007932.2
<i>Jag1</i>	F: 5'-AGTCTTCTGCTCGCCCTGCTC-3' R: 5'-CGGACTCCACCACAACAGTTCC-3'	NM_013822.5
<i>Nos3</i>	F: 5'-AACCTGTGGCTGTGGAACAAC-3' R: 5'-GGGAAGTGATGTCCAGGAAGT-3'	NM_008713.4
<i>Pecam1</i>	F: 5'-GTGGAAGTGTCCTCCCTTGAG-3' R: 5'-GGAGCCTTCCGTTCTTAGGGT-3'	NM_008816.3
<i>Pgf</i>	F: 5'-CAGGTCTAGCTGGGTGG-3' R: 5'-CGTTGAAAGGCACCATTCC-3'	NM_008827.3
<i>Ptk2</i>	F: 5'-CGGACACATGCAGTCTCTGT-3' R: 5'-CCTTCTCCAATACAGCGTCCA-3'	NM_007982.2
<i>Serpine1</i>	F: 5'-CAACCGACAGAGACAATCCT-3' R: 5'-TGAAGGCGTCTCTTCCCACT-3'	NM_008871.2
<i>Tbx1</i>	F: 5'-CCAAGGCAGGCAGACGAAT-3' R: 5'-TAGTGTACTCGGCCAGGTGT-3'	NM_011532.2
<i>Tek</i>	F: 5'-CCGGCTTAGTTCTCTGTGGA-3' R: 5'-GCAATGCAGGTGAGGGATGT-3'	NM_013690.3
<i>Tgfa</i>	F: 5'-CTCTGGGTACGTGGGTGTTC-3' R: 5'-CACCCTCACAGTGTTTGCG-3'	NM_031199.4
<i>Tgfb3</i>	F: 5'-GATCACCACAACCCACACCT-3' R: 5'-CCAGGTTGCGGAAGCAGTAA-3'	NM_009368.3
<i>Tie1</i>	F: 5'-TTGGCGACTGGAGCAATACA-3' R: 5'-GAGACGGAACCTACCACAGC-3'	NM_011587.2
<i>Vegfa</i>	F: 5'-GATTGAGACCCTGGTGACATC-3' R: 5'-CATCTCTCCTATGTGCTGGCT-3'	NM_001287056.1
<i>Gusb</i>	F: 5'-TGGCTGGGTGTGGTATGAAC-3' R: 5'-GGTGACCTCCCTCATGTTCC-3'	NM_010368.2
<i>Hsp90ab1</i>	F: 5'-TGCTAAGTCTGGCACGAAGG-3' R: 5'-ACACCAAAC TGCCGATCAT-3'	NM_008302.3

Primer sequences were designed with National Center for Biotechnology Information's (NCBI) Primer-BLAST (Basic Local Alignment Search Tool; <https://www.ncbi.nlm.nih.gov/tools/primer-blast>, last accessed April 27, 2018). The NCBI Reference sequences that were entered in the Primer-BLAST submission form are indicated for each gene and were retrieved from NCBI's Nucleotide database (<https://www.ncbi.nlm.nih.gov/refseq>, last accessed April 27, 2018). F, forward; R, reverse.

instructions. The C_T values from all plates and wells were then exported from the real-time PCR instrument and formatted into a new Excel spreadsheet (Microsoft, Redman, WA) (Supplemental Table S1). The data were uploaded in the RT² Profiler PCR Array Data Analysis Webportal (<https://dataanalysis.qiagen.com/pcr/arrayanalysis.php>, last accessed April 4, 2018) and analyzed. *Gusb* and *Hsp90ab1* were automatically selected as optimal housekeeping genes. Genes with fold changes >2 and P values < 0.05 were later validated with real-time PCR. Primers were designed with NCBI's Primer-Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>, last accessed April 27, 2018). Primers bridged an exon–exon junction or spanned introns. Free online Beacon Designer software version 7.9 (<http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1>, last accessed April 27, 2018) was used to evaluate possible secondary structures such as dimers or hairpins.

To check the primer specificity, before performing real-time PCR, conventional PCR was performed, and amplicons were separated on agarose gels. A melting curve analysis was performed after each real-time PCR to further confirm that each assay produced a single, specific product. Primers sequences are given in Table 2.

Unilateral Nx

Wild-type ($n = 3$) and *Ltbr*^{−/−} ($n = 4$) C57BL/6 mice underwent unilateral nephrectomy (Nx). After exposing the left kidney, the renal artery, vein, and ureter were tied off. The kidney was finally excised distal to the ligatures.

Statistical Analysis

Statistical analysis was performed with the unpaired t -test ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$,

Figure 1 Vascularization of glomeruli engrafted in the mouse lymph node (LN) might be driven by host lymphotoxin- β receptor (LT β R)/nuclear factor κ B-inducing kinase (NIK) axis. **A: Left column:** Frozen sections of a representative green fluorescent protein (GFP)⁺ metanephric (embryonic day 14) kidney graft 3 weeks after transplantation. Merged GFP signal with Hoechst stain (blue; **top panel**), merged GFP signal with CD31 (endothelial cell) stain (red; **middle panel**), and merged CD31 stain (red) with Hoechst stain (blue; **bottom panel**). **Boxed and circled areas** are shown at higher magnification to the right and demonstrate the presence of a host (GFP[−]) CD31⁺ cell in an engrafted glomerulus. **Right panel:** Cartoon depicting the localization of glomerular endothelial cells (image adapted from <http://what-when-how.com/acp-medicine/glomerular-diseases-part-1>, last accessed June 1, 2019). **B:** Representative immunofluorescence stain for CD34 (endothelial cell marker, red) and NIK (green) in an engrafted wild-type (GFP[−]) glomerulus 3 weeks after transplantation. **Arrows** point to double positive cells. **C:** Representative immunofluorescence stain for LT β R (red), erasmus university rotterdam—thymic reticulum antibody 7 (ER-TR7; LN fibroblastic reticular cells, red), or lymphotoxin β (LT- β , red) in engrafted GFP⁺ glomeruli 3 weeks after transplantation. Data presented in this figure are representative of one technical replicate of a minimum of three technical replicates per sample. $n = 3$. Scale bars: 50 μ m (**A, left column**); 25 μ m (**A, right column, B, and C**).

**** $P < 0.0001$). Statistical power analysis was also performed to assess error rates for tubule length measurements. For all studies, the power of a two-tailed t -test was >0.9 , given an α level of 0.05 (5%). A volcano plot of RT² Profiler PCR Array data was used to identify genes differentially expressed in kidneys engrafted in *Ltbr*^{-/-} versus wild-type omenta. Correlation between RT² Profiler PCR Array and real-time PCR was calculated by linear regression and Pearson correlation. Both SABiosciences' data analysis (Frederick, MD) and GraphPad Prism 7 software (GraphPad Inc., San Diego, CA) were used.

Results

Vascularization of Glomeruli Engrafted in the Mouse LN Might Be Driven by Host LTβR/NIK Axis

With the use of GFP-expressing mouse metanephric kidneys, successful nephron maturation inside a host mouse LN has been demonstrated.^{11,12} The LN-engrafted nephrons could rely on a remarkable vascular network originating from both host- and graft-derived vascular cells.^{11,12} Even more impressive were the data of human fetal kidney engraftment inside the LN, which clearly indicated that,

Figure 2 Treatment with lymphotoxin- β receptor antagonist (LT β R-Fc) impaired expansion of kidneys engrafted in lymph node (LN) and caused a decrease in nuclear factor κ B-inducing kinase (NIK)-expressing glomerular cells. **A:** Schematic representation of LN transplantation assay with the course of mouse treatments. Green K stands for green fluorescent protein (GFP)⁺ metanephric (embryonic day 14) kidney. **B:** Merged fluorescence/bright-field images of whole mount kidney-bearing LNs isolated from either control Ig— or LT β R-Fc—treated mice (GFP⁺ areas indicate kidney engraftment). **C:** Bar graph showing fold changes of GFP intensity (pixel counts obtained with Adobe Photoshop version CS5) in LNs as in **B** (blue bars: Control Ig; green bars: LT β R-Fc). **D:** Immunofluorescence stain for NIK (red) in glomeruli of engrafted kidneys as in **B**. **E:** Line graph showing the percentage of NIK-expressing cells in engrafted glomeruli as in **B**. Glomeruli were divided into five groups based on the number of NIK-expressing cells (0; 1 to 3; 4 to 6; 7 to 9; or 10 positive cells/glomerulus). Data presented in **D** and **E** are representative of one technical replicate of a minimum of three technical replicates per sample. $n = 3$ per group (**D** and **E**). ** $P < 0.01$ LT β R-Fc versus control Ig (all glomeruli were considered regardless of the frequency of NIK-expressing cells). Scale bars: 500 μ m (**B**); 25 μ m (**D**).

although the donor and host endothelial cells could coexist in the early stages of engraftment, over time the grafts were entirely re-vascularized by host cells.²⁴

Figure 1A shows abundance of host (GFP⁺) endothelial cells (CD31⁺) in the interstitium of a GFP⁺ mouse metanephric kidney 3 weeks after transplantation in the LN, as

well as the presence of a host endothelial cell in an engrafted glomerulus. Most glomerular endothelial cells (CD34⁺) expressed NIK (Figure 1B). Of interest, the number of NIK⁺ cells and/or NIK fluorescence intensity were significantly decreased in 6-week engrafted glomeruli (Supplemental Figure S1, A and B), suggesting that NIK expression could

Figure 3 Treatment with lymphotoxin-β receptor antagonist (LTβR-Fc) affected lymph node (LN) kidney graft architecture and vasculature. **A:** Representative immunofluorescence stains for podoplanin (PDPLN; podocytes and lymphatic endothelia, red), lotus tetragonolobus lectin (LTL; proximal tubules, red), CD31 (endothelial cells, red), CD105 (newly formed vessels, red), and Ly-76 (erythrocytes, red) on serial frozen sections of kidney grafts isolated from either Control Ig- or LTβR-Fc-treated mice. **Insets** show a representative glomerulus for each group. **B:** Bar graph showing PDPLN mean fluorescence intensity (MFI; plum bars) in glomeruli of kidney grafts as in **A**, measured with ImageJ version 1.52a (each bar represents a glomerulus). Gray bars indicate the area of each glomerulus considered (ImageJ). **C:** Scatter dot plot with bar graphs ± SD showing the length of LTL⁺ tubules in kidney grafts as in **A**, measured across sections, using ImageJ. Each dot represents a tubule. **D–F:** Scatter dot plot with bar graphs ± SD presenting the corrected fluorescence intensities of glomerular (CTGF) CD31, CD105, and Ly-76 in kidney grafts as in **A**, measured with ImageJ. Each dot represents a glomerulus. Data presented in this figure are representative of one technical replicate of a minimum of three technical replicates per sample. *n* = 3 per group. **P* < 0.05, *****P* < 0.0001. Power.t.test = 1, sig.level = 0.05 (**C**). Scale bars: 100 μm (**A**).

mark activated host endothelial cells during the early stages of kidney vascular integration in the host LN.

NIK is activated in response to stimulation of tumor necrosis factor receptor superfamily members such as LT β R.²⁵ LT β R is widely expressed on the three major CD45⁺ LN stromal cell populations—fibroblastic reticular cells (FRCs), lymphatic endothelial cells, and blood endothelial cells—and is important for several adult LN functions.¹⁵ LT β R signals on FRCs regulates LN vascular endothelial growth factor levels and, in turn, LN endothelial cell

proliferation.²⁶ Likewise, LT β R triggering on LN endothelial cells affects vasculature development.²⁷ We therefore hypothesized that host LT β R/NIK signaling facilitated glomerular vascularization inside the LN. In accordance with our hypothesis, engrafted glomeruli were found to be embedded in a host LT β R reactive cellular scaffold, compatible with ER-TR7⁺ FRCs (Figure 1C). Intraglomerular LT β R expression could also be observed, which, together with a rare intraglomerular expression of the membrane-anchored LT β R ligand LT- β (Figure 1C),

Figure 4 Metanephric kidney engraftment in omentum: similarity with the lymph node (LN) system. **A:** Frozen section of a representative green fluorescent protein (GFP)⁺ glomerulus 3 weeks after transplantation in the omentum. Merged GFP signal with Hoechst stain (blue; **left panel**), merged GFP signal with CD31 (endothelial cell) stain (red; **middle panel**), and merged CD31 stain (red) with Hoechst stain (blue; **right panel**). **Circled areas** are shown at higher magnification below and demonstrate the presence of a host (GFP[−]) CD31⁺ cell in the engrafted glomerulus. **B:** Merged GFP signal with CD31 (red; **left column**) or nuclear factor κ B-inducing kinase (NIK; red; **right column**) stain in serial sections of GFP⁺ kidney graft in the omentum (3 weeks after transplantation). **Top row** shows expressions of CD31 and NIK in glomeruli; **bottom row**, expressions of CD31 and NIK in a vessel. **Arrows** point to a host (GFP[−]) CD31⁺/NIK⁺ cell. **C:** Flow cytometric profiles of CD45⁺ cells isolated from LN (**left panel**) or omentum (**right panel**). From the podoplanin (PDPLN) and CD31 expression, distinct stromal cell populations can be identified: fibroblastic reticular cells (FRCs; PDPLN⁺/CD31[−]), lymphatic endothelial cells (LECs; PDPLN⁺/CD31⁺), and blood endothelial cells (BECs; PDPLN[−]/CD31⁺). **D:** Representative immunofluorescence stain for lymphotoxin- β receptor (LT β R; red) and PDPLN (green) on CD45⁺ omental cells. **White asterisks** indicate LT β R⁺/PDPLN[−] cells and **yellow asterisks** indicate LT β R⁺/PDPLN⁺ cells. Nuclei were counterstained with Hoechst (blue). Data presented in **A** and **B** are representative of one of three technical replicates per sample. $n = 3$ (**A** and **B**). Flow cytometric profiles and LT β R/PDPLN stain were generated by pooling cells from 10 mice. Scale bars: 25 μ m (**A**); 50 μ m (**B**); 100 μ m (**D**). Cy, cyanine; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Figure 5 Defective lymphotoxin- β receptor (LT β R) signaling in the omentum resulted in reduced kidney graft expansion. **A:** Merged fluorescence/bright-field (left panels) or dissecting scope (right panels) images of whole mount omenta from wild-type (WT) or *Ltbr*^{-/-} mice 2 to 6 weeks after transplantation of green fluorescent protein (GFP)⁺ metanephric (embryonic day 14) kidneys. Grafts from 3 mice are shown at 6 weeks after transplantation in comparison with an adult mouse kidney. Red arrows point to the engrafted kidneys in WT or *Ltbr*^{-/-} mice. **B:** Bar graph showing fold changes of GFP intensity (pixel counts obtained with Adobe Photoshop version CS5) in transplanted omenta as in **A** (blue bars: WT; green bars: *Ltbr*^{-/-}). Dotted lines separate the samples based on the time (weeks) elapsed from transplantation. *n* = 10 per group; 2 weeks: *n* = 1 per group; 3 weeks: *n* = 4 per group; 4 weeks: *n* = 1 per group; 5 weeks: *n* = 1 per group; 6 weeks: *n* = 3 per group. Scale bars: 500 μ m (main images); 250 μ m (enlargements).

strengthened the idea that the LT β R/NIK axis could play a role in glomerular vascularization inside the LN.

Inhibition of LT β R Signaling Decreases Glomerular Vascularization/Angiogenesis in the LN

To determine the role of LT β R signaling on kidney engraftment in the LN, 2 days before metanephric kidney transplantation, recipient mice were treated with a

recombinant LT β R-Fc fusion protein—which antagonized LT β R-mediated effects by engaging soluble and cell surface forms of LT β R ligands—or control Ig (day 1). Mice were treated again at day 8 and 15, and LNs were collected at day 22 (Figure 2A). LT β R-Fc treatment resulted in reduced expansion of transplanted GFP⁺ metanephric kidneys (Figure 2, B and C) and decreased numbers of NIK⁺ glomerular cells (Figure 2D). Indeed, although glomeruli devoid of NIK⁺ cells could be observed in both

experimental groups, the number of such glomeruli was doubled in grafts from LT β R-Fc–treated mice (Figure 2E). Moreover, although both the groups had similar percentages of glomeruli that contained one to three NIK⁺ cells, the number of glomeruli with four to six NIK⁺ cells was drastically reduced after LT β R-Fc treatment (Figure 2E). Overall, glomeruli engrafted in LT β R-Fc–treated mice had a mean of 1.48 ± 0.25 NIK⁺ cells, whereas glomeruli engrafted in control mice had a mean of 2.88 ± 0.33 NIK⁺ cells ($P = 0.001$).

When graft maturity was investigated through immunofluorescence staining for PDPLN to highlight the glomeruli, or LTL to visualize the proximal tubules, no obvious structural differences could be found between treated and control mice (Figure 3A). Further analysis confirmed that neither significant changes in total surface area of glomeruli nor significant changes in PDPLN expression had occurred (Figure 3B). Conversely, LT β R-Fc treatment resulted in decreased average LTL⁺ tubule length, calculated in cross sections (Figure 3C). The extent of vascularization/angiogenesis was also evaluated by staining for CD31 and the neo-angiogenic marker CD105 (Figure 3A). The intra-glomerular CD31 and CD105 fluorescence intensities were quantified, and a significantly weaker intensity was found for both markers in glomeruli of LT β R-Fc–treated mice (Figure 3, D and E). Nevertheless, no significant difference in glomerular blood perfusion was observed (Ly-76 stain), although fewer erythrocytes were located within blood vessels of treated grafts (Figure 3, A and F). Thus, LT β R signaling might mediate vascularization/angiogenesis of the kidneys transplanted in the LN.

Glomerular Vascularization/Angiogenesis Is Decreased in Kidneys Engrafted in the *Ltbr*^{-/-} Omentum

The LT β R ligands include the heterotrimeric LT $\alpha 1\beta 2$ (LT $\alpha 1\beta 2$)²⁸ and LT-related inducible ligand.²⁹ Although LT $\alpha 1\beta 2$ binds uniquely to LT β R, LT-related inducible ligand may also interact with herpesvirus entry mediator.³⁰ To rule out the possibility that impaired graft angiogenesis resulted from LT β R-Fc interference with signaling pathways other than those mediated by LT β R, the outcome of kidney transplantation in an LT β R defective environment, such as that offered by the *Ltbr*^{-/-} mouse, was investigated. In addition, this approach allowed excluding the possibility that the observed effects could be due to LT β R/herpesvirus entry mediator inhibition on donor kidney cells or other cell types present in the donor tissue at the time of transplantation, including stromal and hematopoietic cells. Because *Ltbr*^{-/-} mice do not have LNs,¹³ the omentum was used as an alternative site for transplantation. Similarly to the LN, the omentum supported the maturation of GFP-expressing metanephric kidneys. Intraomental kidney grafts contained host-derived (GFP⁻) glomerular endothelial cells (CD31⁺) (Figure 4A) as well as NIK-expressing endothelial cells (CD31⁺) in renal glomeruli and blood

vessels (Figure 4B). We therefore hypothesized that LN and omentum supported kidney organogenesis through a similar cellular/molecular mechanism. Our hypothesis was championed by the finding that both sites shared similar FRC/lymphatic endothelial cell/blood endothelial cell flow cytometric profiles (Figure 4C). Moreover, LT β R⁺/PDPLN⁺ and LT β R⁺/PDPLN⁻ cell subsets could be identified in omental cell suspensions, indicating the existence of FRCs and blood endothelial cells that mediated LT signals in this site as well (Figure 4D).

Consistent with our previous findings (Figure 2, B and C), expansion of GFP⁺ metanephric kidneys was affected in the *Ltbr*^{-/-} omentum. Not only did the grafts expand less over a 6-week period (Figure 5), but they also were significantly less vascularized (Figure 6A). The glomerular CD31, CD105, and Ly-76 fluorescence intensities were quantified; significant changes could be observed at every time point analyzed (Figure 6B). Of note, no significant changes in Ly-76 expression had been observed in LN kidney grafts isolated from mice undergoing LT β R-Fc treatment with respect to their control counterparts (Figure 3F). The cause for this different outcome remains unknown.

CD31 and CD105 fluorescence intensities were also compared over time (Figure 6, C and D). CD31 expression was stable up to 4 weeks in glomeruli engrafted in the wild-type omentum and decreased afterward (Figure 6C). Conversely, CD31 expression began falling soon after the second week of transplantation in glomeruli engrafted in the *Ltbr*^{-/-} omentum (Figure 6C). CD105 expression had a similar trend in both experimental groups, with the highest expression registered at 2 weeks after transplantation (Figure 6D). At this stage, significant differences in NIK⁺ glomerular cell numbers could also be observed between the two kidney groups (Supplemental Figure S1C and Figure 6E). Indeed, the number of glomeruli devoid of NIK⁺ cells increased in the *Ltbr*^{-/-} omentum (Figure 6E). Moreover, although almost an equal number of glomeruli had either one to three or four to six NIK⁺ cells in the wild-type omentum, the number of glomeruli with one to three NIK⁺ cells increased in the *Ltbr*^{-/-} omentum, to the detriment of glomeruli with four to six NIK⁺ cells (Figure 6E). Finally, glomeruli with seven to nine NIK⁺ cells were more abundant in the wild-type omentum than in the *Ltbr*^{-/-} omentum, and glomeruli with up to ten NIK⁺ cells could only be observed in the wild-type omentum (Figure 6E). Overall, glomeruli engrafted in the *Ltbr*^{-/-} omentum had a mean of 2.04 ± 0.23 NIK⁺ cells, whereas glomeruli engrafted in the wild-type omentum had a mean of 4.04 ± 0.30 NIK⁺ cells ($P < 0.0001$). Over the time course, the number of glomeruli devoid of NIK⁺ cells progressively increased in both groups, and the number of NIK⁺ cells per positive glomerulus also decreased (Supplemental Figure S1, D and E). Four weeks after transplantation, no more than three NIK⁺ cells could be observed in glomeruli engrafted in the *Ltbr*^{-/-} omentum,

whereas a small percentage of glomeruli engrafted in the wild-type omentum had four to six NIK⁺ cells (Supplemental Figure S1, D and E). These results suggested a critical time window between 2 and 3 weeks after transplantation for LTβR/NIK actions on the vascular remodeling of the transplanted glomeruli.

Structural Changes in Kidneys Engrafted in the *Ltbr*^{-/-} Omentum

With respect to their control counterparts, the kidneys engrafted in the *Ltbr*^{-/-} omentum also showed structural changes overtime, with complete loss of glomeruli and tubules by 6 weeks (Figure 7A).

The time point of 2 weeks was specifically studied. Although no significant changes in total surface area of glomeruli were observed, highly significant changes affected PDPLN expression (Figure 7B) and LTL⁺ tubule length (Figure 7C).

Tubulointerstitial injury has been described as a mediator of progression of kidney disease.³¹ It was therefore studied whether the observed glomerular and tubular loss in later stages of engraftment could be due to the expansion of renal interstitial fibroblasts. To test this hypothesis, staining was performed for ER-TR7, which besides outlining the various compartments of peripheral lymphoid organs by detecting the reticular fibroblasts, also identifies interstitial fibroblasts in the kidney (Supplemental Figure S2A).³² Significant changes were not observed between the kidneys engrafted in the wild-type omentum versus those engrafted in the *Ltbr*^{-/-} omentum over the 6-week period (Supplemental Figure S2B). Moreover, in each group, ER-TR7 levels were quite stable until the fifth week, after which time they decreased (Supplemental Figure S2C). Thus, the precise mechanism of glomerular and tubular loss in late-stage grafts remains unknown.

Similar results were obtained when using newborn kidneys, instead of metanephric kidneys (Supplemental Figure S3), indicating that host-driven angiogenesis was uncoupled from the maturation stage of the transplanted tissue (ie, ongoing versus completed angiogenesis). Of interest, when using newborn kidneys, more pronounced structural changes were detected between the two mouse groups, starting as early as the second week after transplantation (Supplemental Figure S3).

Thus, these results pinpointed the importance of host LTβR signaling for successful vascularization/angiogenesis and maturation of the kidney grafts in lymphoid sites.

Reduced Expression of Angiogenesis-Related Genes in Kidneys Engrafted in the *Ltbr*^{-/-} Omentum

PCR array profiles of angiogenesis-related genes confirmed that host LTβR signaling sustains ectopic kidney angiogenesis. Indeed, when six distinct transplants engrafted either in the *Ltbr*^{-/-} omentum or in the wild-type omentum were compared (3 weeks after transplantation), several proangiogenic factors were found to be down-regulated in the absence of functional LTβR signaling (Figure 8 and Supplemental Figure S4). These factors included components of the angiopoietin–Tie signaling system (*Angpt1*, *Tek*, and *Tie1*); the epidermal growth factor signaling system (*Tgfa*); the Eph–ephrin signaling system (*Efnal* and *Efnb2*); the transforming growth factor β signaling system (*Eng* and *Tgfb3*); the vascular endothelial growth factor signaling system (*Pgf*); and targets shared among these systems (*Jag1*, *Nos3*, *Ptk2*, *Serpine1*, *Tbx1*).

Expression of *Efnb2* and *Serpine1* products was later analyzed in sections of 3-week intraomental kidney grafts. EFNB2 was found to be expressed in glomerular cells (presumably podocytes and parietal epithelial cells), and PAI-1, the product of *Serpine1*, was expressed in renal tubules (presumably proximal tubules) (Figure 9A). Although EFNB2 fluorescence intensity was similar in both groups, a significant lower number of tubules were positive for PAI-1 in the kidneys engrafted in the *Ltbr*^{-/-} omentum (Figure 9B). A similar trend could be observed in kidneys engrafted in LNs under LTβR-Fc treatment (Figure 9, C and D), confirming that a similar cellular/molecular mechanism drove kidney organogenesis in LNs and omenta. These results also suggested that, although the lymphoid microenvironment provided signaling cues that affected the vasculature of the maturing kidney, the kidney graft self-sustained by producing angiogenic molecules. Production of these molecules appeared to be facilitated by an environment that mediated LT signals.

Figure 6 Defective lymphotoxin-β receptor (LTβR) signaling in the omentum resulted in reduced kidney graft vascularization/angiogenesis and decreased numbers of nuclear factor κB–inducing kinase (NIK)-expressing glomerular cells. **A:** Representative immunofluorescence stains for CD31 (endothelial cells, red), CD105 (newly formed vessels, red), and Ly-76 (erythrocytes, red) on serial frozen sections of intraomental kidney grafts from wild-type (WT) or *Ltbr*^{-/-} mice over the course of 6 weeks. **B:** Scatter dot plot with bar graphs ± SD presenting the corrected fluorescence intensities of intraglomerular (CTGF) CD31, CD105, and Ly-76 in kidney grafts as in **A**, measured with ImageJ version 1.52a. Each dot represents a glomerulus. **C** and **D:** Line graphs ± SEM presenting CD31 and CD105 CTGFs over the time course (blue line: WT; green line: *Ltbr*^{-/-}). **E:** Line graph showing the percentage of NIK-expressing cells in glomeruli 2 weeks after transplantation in WT (blue line) or *Ltbr*^{-/-} (green line) omentum. Glomeruli were divided into five groups based on the number of NIK-expressing cells (0; 1 to 3; 4 to 6; 7 to 9; or 10 positive cells/glomerulus). Data presented in this figure are representative of one of three technical replicates per sample. *n* = 10 per group; 2 weeks: *n* = 1 per group; 3 weeks: *n* = 4 per group; 4 weeks: *n* = 1 per group; 5 weeks: *n* = 1 per group; 6 weeks: *n* = 3 per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 *Ltbr*^{-/-} versus WT (in **E**, all glomeruli were considered regardless of the frequency of NIK-expressing cells). Scale bars = 100 μm (**A**).

Host NIK Is Not Required for Ectopic Kidney Organogenesis

A significant reduction in the number of NIK-expressing glomerular cells had been observed in kidneys engrafted in both LNs of mice undergoing LT β R antagonist treatment (Figure 2, D and E) and omenta of *Ltbr*^{-/-} mice (Figure 6E and Supplemental Figure S1, C–E). To elucidate whether a functional NIK was required for successful ectopic kidney organogenesis, GFP⁺ metanephric kidneys were

transplanted in wild-type or *Nik*^{-/-} omenta. Significant differences in graft size, structure, vascularization, or angiogenesis were not observed at 3 weeks after transplantation (Supplemental Figure S5, A–K), indicating that, although NIK could be activated by LT β R signaling in this system, it was dispensable for ectopic kidney organogenesis. Moreover, NIK-expressing cells could still be detected in kidneys engrafted in the *Nik*^{-/-} omentum 3 weeks after transplantation (Supplemental Figure S6), although NIK fluorescence intensity was much lower than that observed in

Figure 7 Defective lymphotoxin- β receptor (LT β R) signaling in the omentum affected kidney graft architecture. **A:** Representative immunofluorescence stains for collagen IV (CIV; glomerular basement membranes and mesangial cells, red), podoplanin (PDPLN; podocytes and lymphatic endothelia, red), and lotus tetragonolobus lectin (LTL; proximal tubules, red) on serial frozen sections of intraomental kidney grafts from wild-type (WT) or *Ltbr*^{-/-} mice over the course of 6 weeks. **B:** Bar graph showing PDPLN mean fluorescence intensity (MFI; plum bars) in glomeruli 2 weeks after transplantation in WT or *Ltbr*^{-/-} omentum, measured with ImageJ version 1.52a (each bar represents a glomerulus). Gray bars indicate the area of each glomerulus considered (ImageJ). **C:** Scatter dot plot with bar graphs $\pm$ SD showing the length of LTL⁺ tubules 2 weeks after transplantation in WT or *Ltbr*^{-/-} omentum, measured across sections with ImageJ. Each dot represents a tubule. Power.t.test = 1, sig.level = 0.05. Data presented in this figure are representative of one of three technical replicates per sample. $n = 10$ per group; 2 weeks: $n = 1$ per group; 3 weeks: $n = 4$ per group; 4 weeks: $n = 1$ per group; 5 weeks: $n = 1$ per group; 6 weeks: $n = 3$ per group. **** $P < 0.0001$. Scale bars = 100 μ m (A).

kidneys engrafted in the wild-type omentum at the same stage. These cells were likely donor cells, which might have been trying to compensate for the missing host NIK function.

LTβR Plays a Role in Glomerular Adaptive Responses to Nx

Demonstration that LTβR signaling is important for adult kidney repair would strengthen our hypothesis that kidney rebuilding approaches might benefit from the presence of a microenvironment that mediates LTβR signals. To our knowledge, there are no studies that investigated LTβR physiological role in kidney development or during compensatory growth.

The structure of *Ltbr*^{-/-} kidneys versus wild-type kidneys was first compared. LTβR did not appear to be essential for kidney development during embryogenesis because *Ltbr*^{-/-} mice had normal kidneys with what appeared to be normal vascularization and structure (Supplemental Figure S7). This finding likely suggested differences between ectopic organogenesis and normal organogenesis. Indeed, although LTβR-angiogenic signals appeared to be important for ectopic kidney organogenesis, in normal kidney organogenesis (which occurs mainly through vasculogenesis), LTβR was not so crucial.

LTβR relative contribution to compensatory renal growth after removal of one kidney was therefore investigated to corroborate the idea that not only was LTβR signaling beneficial for glomerular vascularization/angiogenesis in

lymphoid sites, but it also might play a role in glomerular adaptation to renal mass reduction.

Contralateral compensatory renal growth and increased renal function follows contralateral Nx in experimental models.³³ Accordingly, although kidneys of wild-type and *Ltbr*^{-/-} mice had similar weights before surgery, *Ltbr*^{-/-} mice showed significantly lower weights of their remnant kidneys 10 days after Nx (Figure 10A). The contralateral kidneys in wild-type mice showed glomerular enlargement as assessed in H&E-stained sections (Figure 10B). These glomeruli often contained more erythrocytes, indicating an increase of renal blood flow. Differences in adaptive responses seemed to be confined to the glomerulus, because tubular epithelial cells from both mouse groups showed a similar widespread positivity for the Ki-67 proliferation marker (Figure 10C). These data indicated a more efficient glomerular cell response to unilateral Nx in the presence of functional LTβR signaling.

Discussion

LN and omenta can be used as bioreactors to grow organs. LN grafting is a unique approach pioneered by our laboratory,^{11,12,24,34,35} with no previous records in the literature. Conversely, the greater omentum has long been described as an ideal transplantation site mainly for its easy accessibility and large vascular network.³⁶ Nevertheless, to our knowledge, cellular and molecular mechanisms that allow tissue and organ development within these locations have not been explored yet.

Figure 8 Down-regulation of pro-angiogenic genes in kidneys engrafted in the lymphotoxin-β receptor (*Ltbr*^{-/-}) omentum. **A:** The volcano plot displays genes differentially expressed in the kidneys engrafted in the *Ltbr*^{-/-} omentum as compared with the kidneys engrafted in the wild-type (WT) omentum. Plotted along the x axis is the mean of log2 fold-change; along the y axis is the negative logarithm to the base 10 of the *P* values. The vertical dashed lines and the horizontal line reflect the filtering criteria (fold change ≥ 2.0 and *P* values ≤ 0.05). Green dots mark genes with decreased expression. **B:** Heatmap of the 14 statistically significant differentially expressed genes. **C:** Correlation between RT² Profiler PCR Array and real-time PCR data. Relative expression levels determined by RT² Profiler PCR Array (x axis; Qiagen) and real-time PCR (y axis) are plotted for a subset of 16 genes (*Ltbr*^{-/-} 3 versus WT 3). Solid line indicates linear regression; both the squared and the Pearson correlation coefficients are given. **D:** Representative scatter plot (*Ltbr*^{-/-} 3 versus WT 3) showing the gene expression patterns (presented as fold change) of selected genes by RT² Profiler PCR Array (PCR Array) and real-time PCR.

By transplanting kidney rudiments either in the LNs of mice undergoing LTβR antagonist treatment or in the omenta of *Ltbr*^{-/-} mice, it has been shown that host LTβR signals are crucial for obtaining a well-vascularized kidney graft. Indeed, defective host LTβR signaling correlated with decreased expression of endothelial and angiogenic markers in the transplanted tissues as well as structural alterations. Although the number of glomerular endothelial cells expressing the LTβR target NIK decreased in the absence of a functional LTβR, the results from the transplantation of kidney rudiments in the omenta of *Nik*^{-/-} mice indicated that host NIK is not required for successful ectopic kidney organogenesis.

NIK and subsequent non-canonical NF-κB signaling have been described to regulate both inflammation-induced and tumor-associated angiogenesis.¹⁷ From this perspective, kidney angiogenesis in lymphoid tissues shares features with pathologic angiogenesis. However, the progressive reduction in NIK-expressing cells that was observed in glomeruli engrafted in LNs and omenta seems to suggest a tight regulation of angiogenesis in the ectopic kidney grafts, whereby NIK likely elicits a transient signal. In the wild-type omentum, for example, NIK and the neoangiogenic marker CD105 shared a similar pattern, with decreased expressions in engrafted glomeruli starting as early as the second week after transplantation, a pattern different from that of the endothelial cell marker CD31, which was quite stable for 4 weeks. These data seem to suggest that NIK is required during the early stages of engraftment, coinciding with early angiogenesis and tissue vascular integration, but is no longer needed in later stages of kidney maturation. NIK-mediated signals, however, are not necessary for kidney organogenesis in lymphoid sites, because overall vascularization/angiogenesis was not affected in *Nik*^{-/-} recipients.

Besides activating non-canonical NF-κB pathway through NIK,¹⁶ LTβR could also trigger the canonical NF-κB route to stimulate endothelial cells.³⁷ Moreover, other angiogenic signals elicited by the donor tissue itself must not be underestimated, as indicated by the data of PAI-1 production by the kidney tubular cells. How host

Figure 9 Pro-angiogenic gene expression in kidneys engrafted in the omentum and lymph node (LN). **A:** Representative immunofluorescence stains for ephrin B2 (EFNB2; red) and plasminogen activator inhibitor-1 (PAI-1; red) on serial frozen sections of kidneys engrafted in wild-type (WT; top left panels) or lymphotoxin-β receptor knockout (*Ltbr*^{-/-}; bottom left panels) omenta (3 weeks after transplantation). Boxed areas are shown at higher magnification to the right. **B:** Scatter dot plot with bar graphs ± SD presenting the number of PAI-1⁺ tubules per microscopic field of sections stained as in **A**. **C:** Representative immunofluorescence stains for EFNB2 and PAI-1 (red) on serial frozen sections of LN kidney grafts isolated from either mice treated with Control Ig (top left panels) or LTβR-Fc (bottom left panels) (3 weeks after transplantation). Boxed areas are shown at higher magnification to the right. **D:** Scatter dot plot with bar graphs ± SD presenting the number of PAI-1⁺ tubules per microscopic field of sections stained as in **C**. Data presented in this figure are representative of one of three technical replicates per sample. *n* = 3 per group. ****P* < 0.001, *****P* < 0.0001. Scale bars: 100 μm (**A** and **C**). GFP, green fluorescent protein.

LT β R signals can influence donor tissue production of PAI-1 and possibly of other angiogenetic molecules remains unknown.

Kidney tissue integration after transplantation likely involves extensive vascular remodeling, whereby several players are involved, including endothelial cells, smooth muscle cells, and fibroblasts. To record these dynamic processes is technically demanding, but not impossible. Transplanting GFP⁺ tissue in a wild-type recipient has been an effective method for visualizing the engrafted tissue later on, but the use of mice with constitutive or inducible endogenous fluorescent reporters, in combination with tracer injections and/or mosaic genetic recombination,³⁸ would better allow characterizing the multicellular dynamics of angiogenesis and vascular remodeling in the transplanted tissues.

Three-dimensional reconstruction of the engrafted kidneys that was recently performed on human fetal kidneys engrafted in the LN²⁴ would also allow us to better quantify the differences in tubular elongation across the experimental groups. Indeed, the current adopted strategy of LTL⁺ tubule length quantification on two-dimensional sections cannot be considered accurate, because the apparent tubule length depends on the level at which the tissue was sectioned and observed. However, this strategy highlighted some

important changes in the absence of a functional LT β R signal that warrant future study.

Despite these limitations, the findings of host lymphoid stromal microenvironment contribution to ectopic kidney organogenesis seem to suggest implications for future development of new approaches to treat kidney disease. The renal sub-capsular space has served as the preferred site for kidney regeneration from embryonic metanephroi^{39,40} and recently kidney organoids.^{41–43} However, there is no convincing evidence that this approach can improve kidney function after a major injury. The inflammatory and fibrotic milieu of a host's diseased kidney could constitute an adverse environment for cell and tissue engraftment and ultimately function. Finally, clinical progress in renal sub-capsular transplantation has lagged behind other techniques because of the inelastic and tight nature of the human renal sub-capsular space.⁴⁴ Additional strategies to engineer functional kidneys include repopulating synthetic/decellularized scaffolds with kidney-specific cells and transplanting these constructs orthotopically.⁴⁵ Unfortunately, these approaches have often failed to recapitulate native kidney functions because of inadequate blood supply. Indeed, despite efforts to endow the scaffolds with an endothelium,⁴⁵ it is still not possible to generate vascularized and perfused renal tissues that produce urine and/or have

Figure 10 Lymphotoxin- β receptor (LT β R) played a role in renal responses to nephrectomy (Nx). **A:** Scatter dot plot with bar graph $\pm$ SD showing kidney weights before and after Nx (PreNx and PostNx, respectively) in both wild-type (WT) and *Ltbr*^{-/-} mice. Each dot represents a mouse. **B: Left panel:** Scatter dot plot with bar graph $\pm$ SD showing glomerular diameters in WT and *Ltbr*^{-/-} PostNx kidneys. Each dot represents a glomerulus. **Right panel:** Representative hematoxylin and eosin (H&E) stain in Sham, WT_PostNx, or *Ltbr*^{-/-}_PostNx kidney paraffin sections. **C: Left panel:** Scatter dot plot with bar graph $\pm$ SD showing the percentage of Ki-67⁺ tubular cell nuclei from WT and *Ltbr*^{-/-} PostNx kidneys. Each dot represents one stained section. **Right panel:** Representative immunohistochemistry for Ki-67 in WT and *Ltbr*^{-/-} PostNx kidney paraffin sections (3-amino-9-ethylcarbazole, red). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars: 50 μ m (B and C).

endocrine functions such as generation erythropoietin,⁴⁶ necessary for erythropoiesis.⁴⁷ Although reconstructing a fully functional kidney in the LN or the omentum for clinical purposes is challenging, stromal cells from these sites could be exploited to create an artificial scaffold for renal progenitor cells and thus provide an auxiliary aid during renal failure. A major concern about this approach might arise from the LT β R documented role in kidney disease. Renal biopsies from lupus nephritis and IgA nephropathy patients showed elevated LT/LT β R levels, and LT β R inhibition improved renal function in a murine lupus model.⁴⁸ LT β R-Ig was efficacious when given from week 3 to 7, whereas delaying the treatment until 5 weeks did not lead to significant results, highlighting a dynamic role for LT β R across different stages of disease.⁴⁸ LT β R signaling in lupus likely results from the formation of tertiary lymphoid organs. Unlike secondary lymphoid organs such as the LN, tertiary lymphoid organs arise in response to nonresolving inflammation over the course of several years. Therefore, although tertiary lymphoid organ-mediated LT β R signaling leads to a prolonged, deregulated, and maladaptive response in injured kidneys that involves active inflammation and repeated unsuccessful attempts to repair the tissue, secondary lymphoid organ-mediated LT β R signaling might be exploited to support kidney development. The finding that glomerular adaptive responses to loss of renal mass were impaired in the absence of a functional LT β R indicated that LT β R can participate to repair responses after injury, although we cannot rule out that these responses might become maladaptive over time.

In conclusion, although our results do not allow any unbiased conclusion on the precise cellular/molecular machineries that support kidney organogenesis in lymphoid sites, they point to an important function of the lymphoid stromal microenvironment in sustaining angiogenesis that warrants future investigation. Better understanding of the unique properties of this microenvironment might have implications for future engineering of functional organs.

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M.G.F. designed the study, performed most of the experiments, analyzed the data, prepared the figures, and wrote the manuscript; B.H. managed mouse colonies, performed the mouse surgeries, isolated omental stromal cells, and analyzed them by immunofluorescence; E.L. designed and supervised the study; all authors discussed the results and approved the final version of the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.08.018>.

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