

SUPPLEMENTAL MATERIALS AND METHODS

Immunohistofluorescence

Tonsils, FL-LN and non-invaded/invaded BM cryosections were transferred to adhesive coated slides, fixed with 4% PFA for 15min and saturated for one hour with blocking solution (PBS, 10% BSA, 4% donkey serum, 0.1% saponin). BM sections were incubated with anti-CXCL12 (mouse IgG1, R&D Systems) and anti-CD20 (rabbit, Abcam) antibodies, overnight at 4°C before staining with anti-mouse biotin and anti-rabbit Alexa-Fluor 488, for 45min at room temperature (RT). After washing, sections were then incubated with streptavidin Alexa-Fluor 555 for 1h at RT. Tonsils and FL-LN sections were first stained with mouse anti-CXCL12 and rat anti-podoplanin (Affymetrix) antibodies overnight at 4°C before incubation with anti-mouse biotin and anti-rat Alexa-Fluor 488 for 45min at RT (Figure S1). After washing, sections were incubated with streptavidin Alexa-Fluor 555 for 1h at RT, then saturated with 10% mouse serum for 1h and incubated overnight at 4°C with anti-CD21L (mouse IgM, Dako) antibody followed by secondary antibody anti-mouse IgM Alexa-Fluor 647 for 45min at RT. Secondary antibodies were purchased from Jackson ImmunoResearch. Finally, slides were mounted in Mowiol containing SytoxBlue (Invitrogen) and analyzed by confocal microscopy on a SP8 (Leica Microsystems). ImageJ software was used for image analysis.

Immunohistochemistry

Immunohistochemistry (IHC) was applied to a tissue microarray (TMA) encompassing 1.5 mm duplicate cores of 195 formalin-fixed, paraffin-embedded (FFPE) tissue specimens from 186 patients diagnosed with FL¹. 4 µm sections were cut and IHC was performed on a Ventana BenchMark XT platform (Ventana, AZ) using a mouse monoclonal antibody against pSTAT-6 (dilution 1:100, clone 18/P-Stat6, BD Biosciences) and a rabbit monoclonal antibody against CXCL12 (dilution 1:200, clone D8G6H, Cell Signaling technology). Staining was evaluated semi-quantitatively for both p-STAT6 (0 = negative, 1 = occasionally positive in follicles, 2 = frequently positive in follicles, as described²) and CXCL12 (0 = negative, 1 = equal to reactive GC, 2 = more positive cells than in reactive GC). Representative images were acquired with a Nikon DS-Fi1 camera connected to a Nikon Eclipse 80i microscope.

Cell signaling

ADSCs were treated or not with TNF/LT for 24hrs, then starved for 2hrs in RPMI-1% FCS before stimulation with IL-4 (5ng/mL) for 5 min. For flow cytometry analysis, the reaction was stopped by adding 4% PFA for 15 min. Fixed cells were permeabilized using 80% methanol, rehydrated with PBS-1% BSA, and stained with anti-pSTAT6 Alexa-Fluor 488 (BD Bioscience) before analysis by flow cytometry. Unstimulated cells were used as a control to obtain MFI ratios. For western blot experiments, ADSC pellets were centrifugated at 8000g for 10 min before freezing. Proteins were extracted using RIPA buffer with protease and phosphatase inhibitors (Roche) and sodium orthovanadate (2mM, Sigma). Proteins were separated by SDS-PAGE (4-12%), before being transferred to a PVDF membrane (Life Technologies). Following antibodies were used: rabbit anti-pSTAT6 (Tyr641) and anti-STAT6 (Cell Signaling Technology), and mouse anti- β -actin primary Abs (Sigma Aldrich) followed by anti-rabbit or anti-mouse HRP conjugated Abs (Santa Cruz Biotechnology) and ECL western blotting substrate (Pierce Thermo fisher Scientific).

To evaluate CXCL12 signaling, FL B cells were stimulated by bead-bound CXCL12-Fc. Briefly, 1mg Dynabeads (Invitrogen) were incubated with 10 μ g CXCL12-Fc (Creative Biomart) on a rotator for 4hrs at room temperature. Suspension was washed three times with PBS-0.05% Tween and resuspended at 15mg/mL of antibody-coupled beads. Purified FL-B cells were starved for 4hrs, treated or not for 30 min by Ibrutinib (Selleckchem, 10mM), and stimulated by beads alone or CXCL12-Fc coated beads at 30 μ g/mL for 60 min. Western blot was then performed as described using anti-pSYK (Tyr525/526), anti-phospho-ERK, anti-ERK (Cell Signaling Technology), anti-SYK (SantaCruz Biotechnology), and mouse anti- β -actin (Sigma) primary Abs followed by HRP-conjugated secondary Abs (Santa Cruz) and ECL western blotting substrate (Pierce).

Migration assay

Purified FL B cells were treated or not with Idelalisib/CAL101 (10 μ M, Cayman Chemicals), Ibrutinib (10 μ M) or AMD3100 (1 μ M, Sigma) 30min and were then added (10⁵ cells/100 μ L) in RPMI-1%FCS to the upper compartment of Transwell chambers with 5- μ m pore filters. Lower chambers contained RPMI-1%FCS supplemented or not with CXCL12 (100 ng/mL; R&D Systems). Migrated cells were collected after

4hrs and the absolute number of viable CD19^{pos}TOPRO-3^{neg} cells was evaluated by flow cytometry using FlowCount beads (Beckman Coulter).

Adhesion assay

For adhesion experiment, CXCL12-Fc (1µg/mL) was coated on plastic wells and ADSCs were electroporated (AMAXA) using a pool of two different CXCL12 siRNA (final concentration 20nM) or control siRNA (20nM, Life Technologies) and maintained in culture for 48h to generate a confluent cell monolayer. RT-QPCR and ELISA for CXCL12 were performed to check the knockdown efficiency. FL B cells were labeled with CFSE (2µM, Interchim), washed, and treated or not with AMD3100 (2µg/ml) for 30min. CFSE-labeled cells were then put on CXCL12-Fc coated plates or ADSC layer for 2hrs at 37°C. After washing in PBS, fluorescence was recorded on a Varioskan (Thermofisher). Adhesion percentage was calculated by comparing the residual fluorescence after adhesion/washing with the fluorescence of the input.

Gene Expression Profiling (GEP) study

CD19^{pos}IgD^{pos}CD38^{lo}CD27^{neg}CD10^{neg} naive B cells, CD19^{pos}IgD^{neg}CD38^{hi}CD10^{pos}CXCR4^{pos} centroblasts, CD19^{pos}IgD^{neg}CD38^{hi}CD10^{pos}CXCR4^{neg} centrocytes and CD19^{pos}IgD^{neg}CD38^{lo}CD27^{pos}CD10^{neg} memory B cells were sorted from 6 tonsil B-cell samples on a FACSAria cell sorter (BD Biosciences) with a purity > 98.5%. In addition, CD20^{hi}IgD^{neg}CD44^{lo}CD38^{pos}CD138^{neg} FL B cells were obtained from 10 FL LN. After RNA extraction, biotinylated cRNA were amplified and GEP was analyzed using GeneChip HG-U133 Plus 2.0 microarrays. Hybridization and raw data of expression signal intensities extraction were processed by the CIT platform (www.cit.ligue-cancer.net). Affymetrix CEL files were normalized by the Robust Multichip Averaging algorithm using GC content (GC-RMA) and Log2 transformation with Partek[®] Genomics Suite[™] software. Prefiltering of raw data was performed by filtering all probesets whose intensity was below the background (defined as less than log₂(20) in all sample) and probesets without gene symbol assignment. Gene Set Enrichment Analysis (GSEA) software was used to determine gene set specific enrichment in purified FL B compared to tonsil centrocytes of previously published IL-4 target gene signatures obtained from the Pathway Interaction Database (PID) and by treating normal B cells with IL-4^{3,4}.

REFERENCES

1. Kridel, R. *et al.* Cell of origin of transformed follicular lymphoma. *Blood* **126**, 2118-2127 (2015).
2. Boice, M. *et al.* Loss of the HVEM Tumor Suppressor in Lymphoma and Restoration by Modified CAR-T Cells. *Cell* **167**, 405-418 e413 (2016).
3. Lu, X. *et al.* Distinct IL-4-induced gene expression, proliferation, and intracellular signaling in germinal center B-cell-like and activated B-cell-like diffuse large-cell lymphomas. *Blood* **105**, 2924-2932 (2005).
4. Schaefer, C.F. *et al.* PID: the Pathway Interaction Database. *Nucleic acids research* **37**, D674-679 (2009).

Figure S1

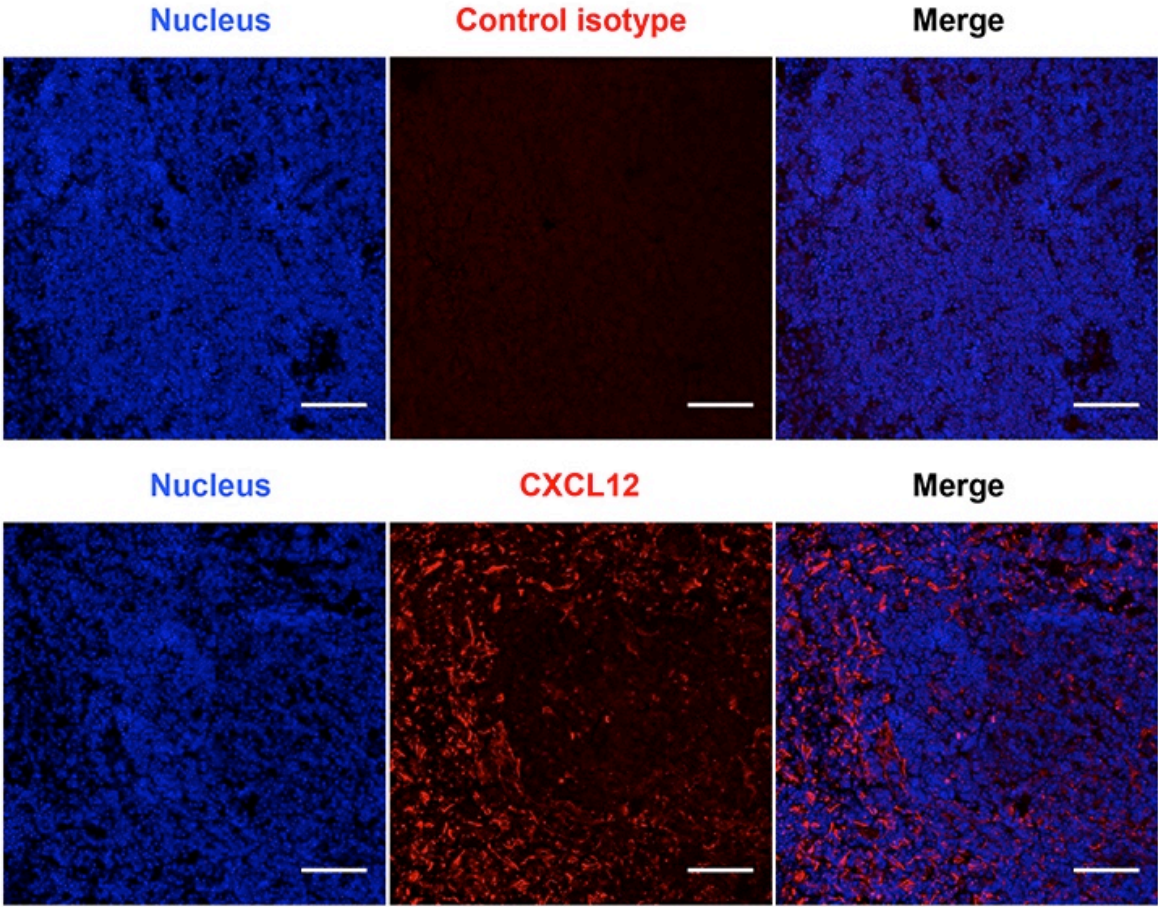


Figure S1: Validation of CXCL12 staining

FL LN serial sections were incubated with a mouse IgG1 control isotype (upper) or with a mouse IgG1 anti-CXCL12 antibody (lower) followed by appropriate secondary antibodies. Nuclei were counterstained with SytoxBlue. Scale bar, 100µm.

Figure S2

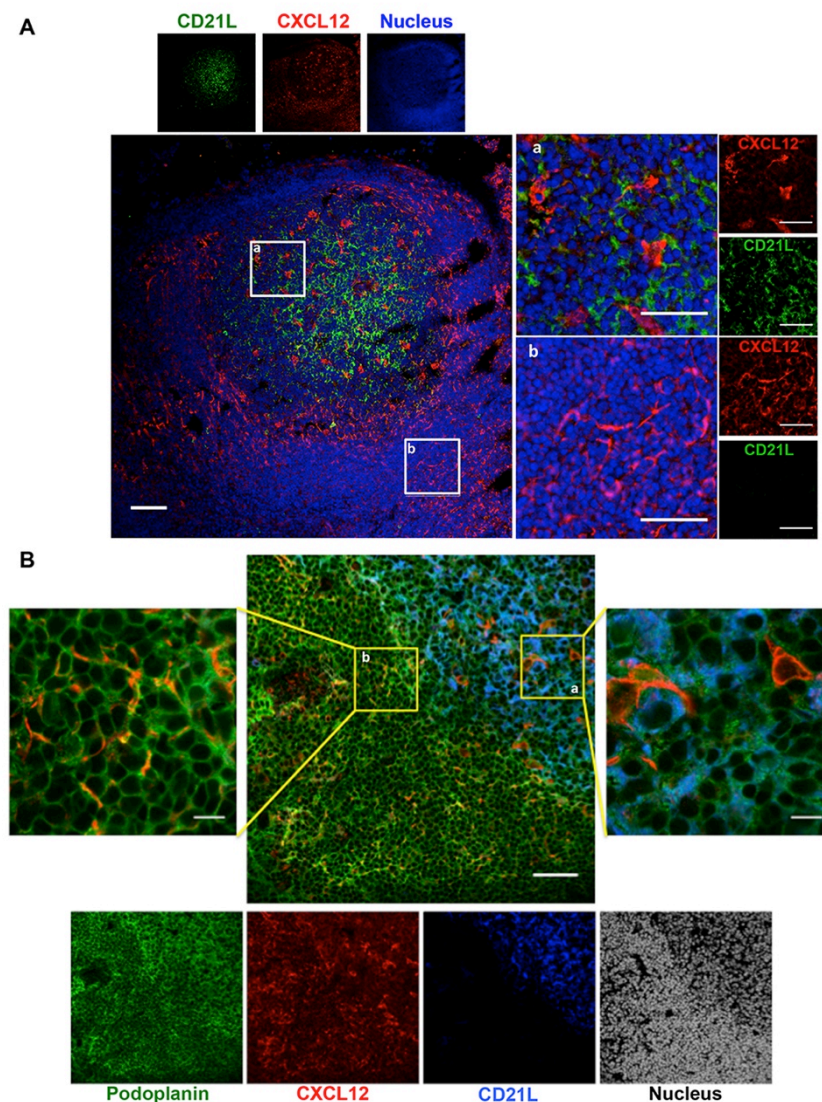


Figure S2: Expression of CXCL12 within tonsils

(A) Tonsil sections were stained with mouse IgM anti-CD21L, and mouse IgG1 anti-CXCL12 antibodies followed by appropriate secondary antibodies. Nuclei were counterstained with SytoxBlue. Scale bar, 100 μ m. Boxes indicate the areas magnified in right panels including intrafollicular zone (a) and extrafollicular zone (b) where scale bar represents 50 μ m. (B) Tonsil sections were stained with mouse IgM anti-CD21L, mouse IgG1 anti-CXCL12, and rat anti-podoplanin antibodies followed by appropriate secondary antibodies. Nuclei were counterstained with SytoxBlue. Scale bar, 100 μ m. Boxes indicate the areas magnified in right and left panels including intrafollicular zone (a) and extrafollicular zone (b) where scale bar represents 50 μ m.

Figure S3

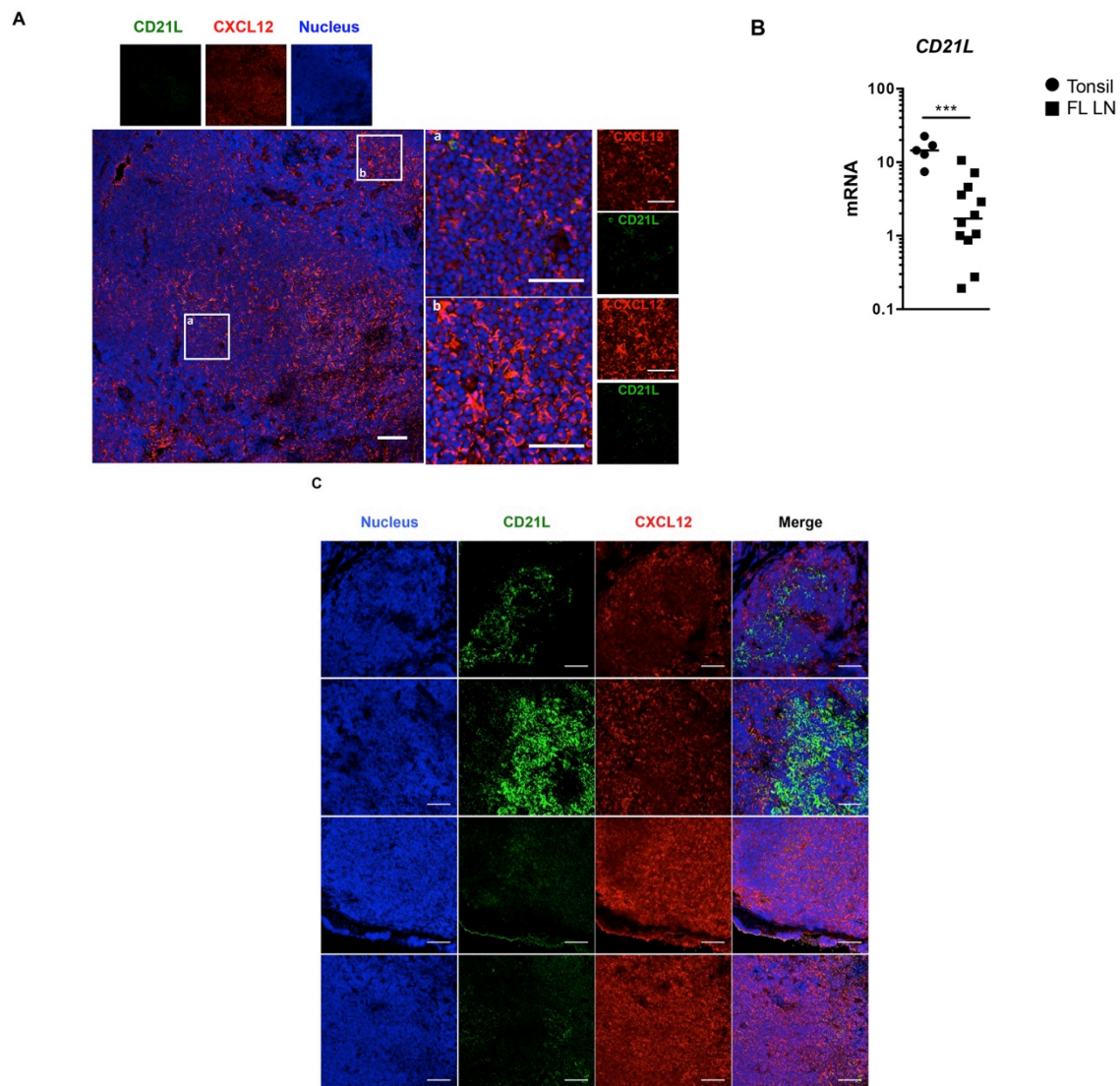


Figure S3: Expression of CXCL12 within FL LN niches

(A) FL LN sections were stained with mouse IgM anti-CD21L, and mouse IgG1 anti-CXCL12 antibodies followed by appropriate secondary antibodies. Nuclei were counterstained with SytoxBlue. Scale bar, 100 μ m. Boxes indicate the areas magnified in right panels including intrafollicular zone (a) and extrafollicular zone (b) where scale bar represents 50 μ m. (B) RNA was extracted from tonsils (n=5) and FL LN (n=12) sections and *CD21L* expression was quantified by RQ-PCR. *** P <0.001. (C) FL LN sections were stained with mouse IgM anti-CD21L, and mouse IgG1 anti-CXCL12 antibodies followed by appropriate secondary antibodies. Nuclei were counterstained with SytoxBlue. Scale bar, 100 μ m. Each line was obtained from a different FL patient.

Figure S4

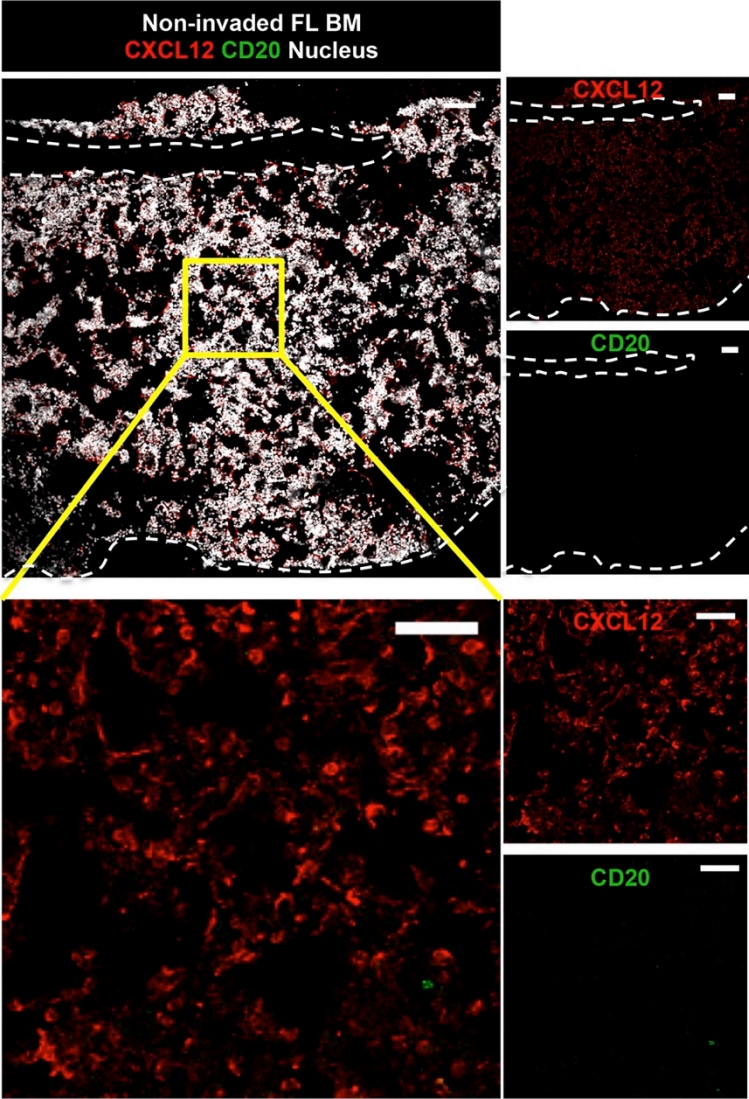


Figure S4: Expression of CXCL12 within non-invaded FL BM

Sections from non-invaded FL BM were stained with mouse IgG1 anti-CXCL12 and rabbit anti-CD20 antibodies followed by appropriate secondary antibodies. Nuclei were counterstained with SytoxBlue. Dotted lines indicate the bone. Scale bar, 100µm. Box indicates the areas magnified in lower panels where scale bar represents 50µm.

Figure S5

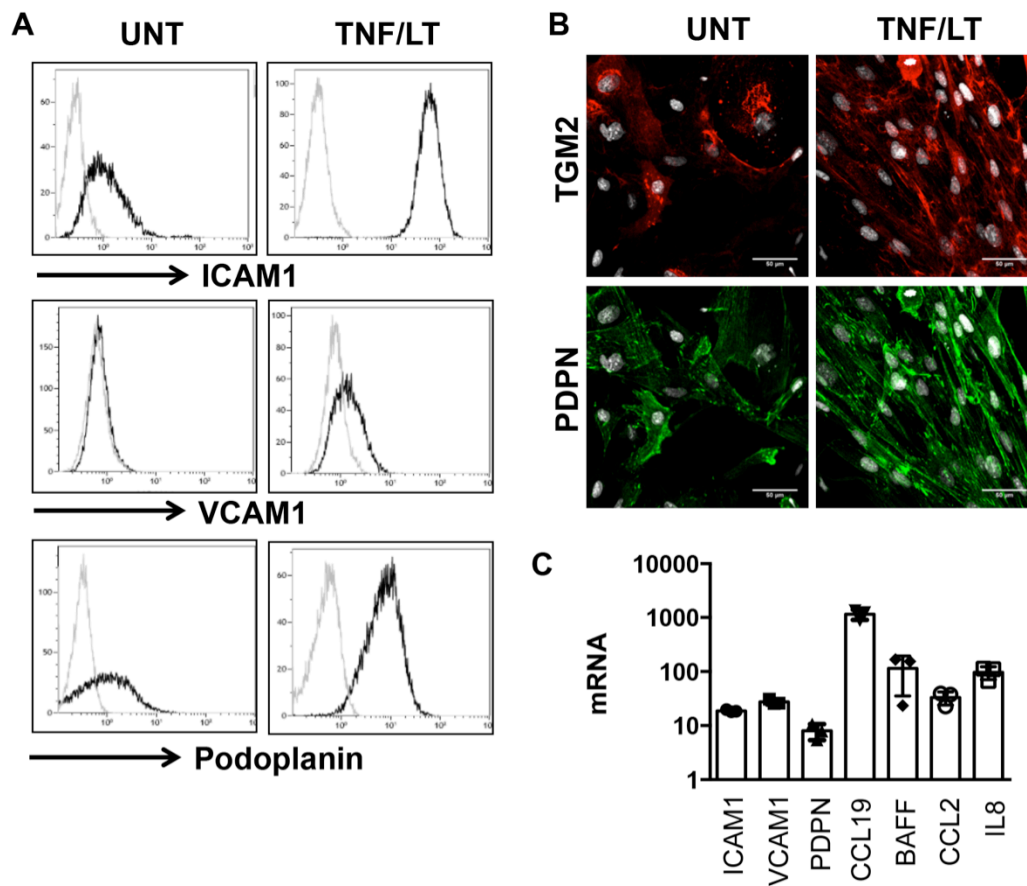


Figure S5: ADSC commitment towards lymphoid stroma differentiation in response to TNF/LT stimulation

(A) ADSCs were stimulated or not (UNT) with TNF/LT for 3 days before analysis of surface expression of ICAM-1/CD54, VCAM-1/CD106, and gp38/podoplanin by flow cytometry. Bold lines indicate specific staining and grey line control isotype. Shown is one representative experiment out of 3. **(B)** Transglutaminase (TGM2) and podoplanin (PDPN) networks were also analyzed by immunofluorescence using rat anti-podoplanin and mouse anti-transglutaminase antibodies followed by appropriate secondary antibodies. Nuclei were counterstained with SytoxBlue (white). Scale bar, 50 μm. Shown is one representative experiment out of 3. **(C)** Expression of *ICAM1*, *VCAM1*, *PDPN*, *CCL19*, *BAFF*, *CCL2*, and *IL8* was analyzed by RQ-PCR. The arbitrary value of 1 was assigned to untreated cells.

Figure S6

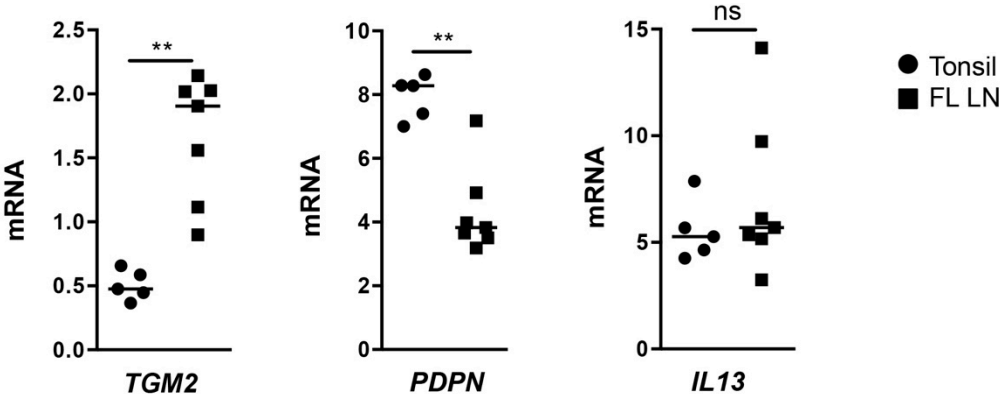


Figure S6: Analysis of lymphoid stroma-related marker expression in FL LN
RNA was extracted from tonsils (n=5) and FL LN (n=7) sections and *IL13*, *TGM2* and *PDPN* expression were quantified by RQ-PCR. ** $P < 0.01$.

Figure S7

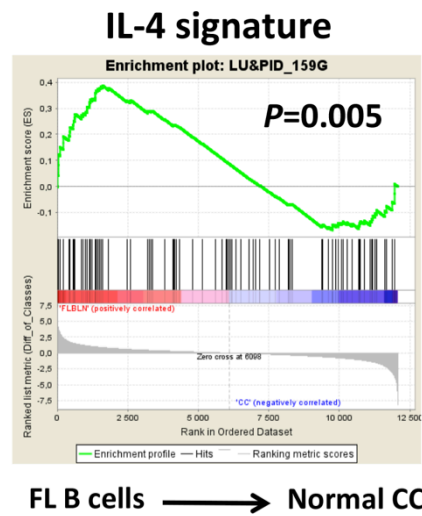


Figure S7: Analysis of IL-4 gene expression signature in FL B cells

The gene expression profile of $CD19^{pos}IgD^{neg}CD38^{hi}CD10^{pos}CXCR4^{neg}$ centrocytes (normal CC) sorted from tonsils ($n=6$) and $CD20^{hi}CD44^{lo}CD38^{pos}IgD^{neg}CD138^{neg}$ FL B cells ($n=10$) was analyzed using Affymetrix HG U133 Plus 2.0 microarrays and a GSEA analysis was performed using previously published IL-4 target gene signatures.

Figure S8

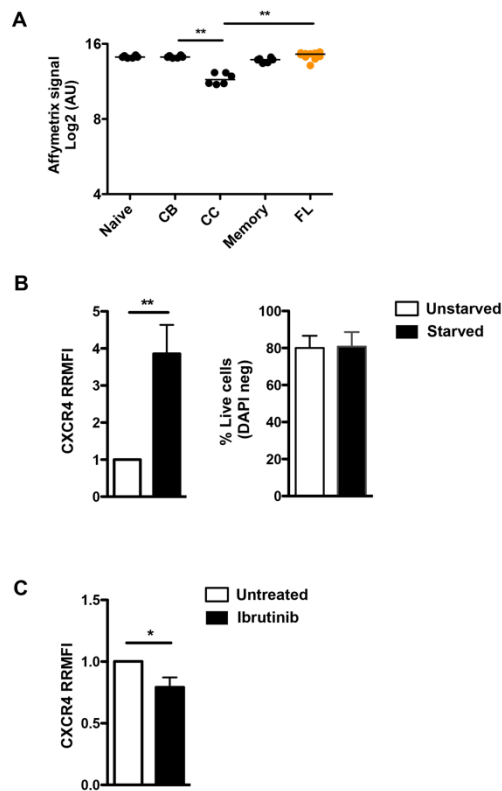


Figure S8: Modulation of CXCR4 expression in FL B cells

(A) Expression of CXCR4 (217028_at probeset) was evaluated in purified CD19^{pos}IgD^{pos}CD38^{lo}CD27^{neg}CD10^{neg} naive B cells, CD19^{pos}IgD^{neg}CD38^{hi}CD10^{pos} CXCR4^{pos} centroblasts (CB), CD19^{pos}IgD^{neg}CD38^{hi}CD10^{pos}CXCR4^{neg} centrocytes (CC), and CD19^{pos}IgD^{neg}CD38^{lo}CD27^{pos}CD10^{neg} memory B cells sorted from tonsils (n=6) and CD20^{hi}CD44^{lo}CD38^{pos}IgD^{neg}CD138^{neg} FL B cells (n=10) using Affymetrix HG U133 Plus 2.0 microarrays. Mann Whitney test was performed. **P<0.01. (B) Primary FL B cells were starved for 4h and CXCR4 expression and percentage of DAPI^{neg} viable cells were assessed using flow cytometry. For CXCR4 staining, the ratio of mean fluorescence intensity (RMFI) was calculated by comparison with the control isotype. The ratio of RMFI (RRMFI) was then defined by comparing the RMFI of starved *versus* unstarved B cells. Results are expressed as the mean +/- SD from 5 experiments. **P<0.01. (C) Primary FL B cells were starved and treated or not with Ibrutinib before quantification of CXCR4 expression. Results are expressed as the mean +/- SD from 6 experiments. *P<0.05.

Figure S9

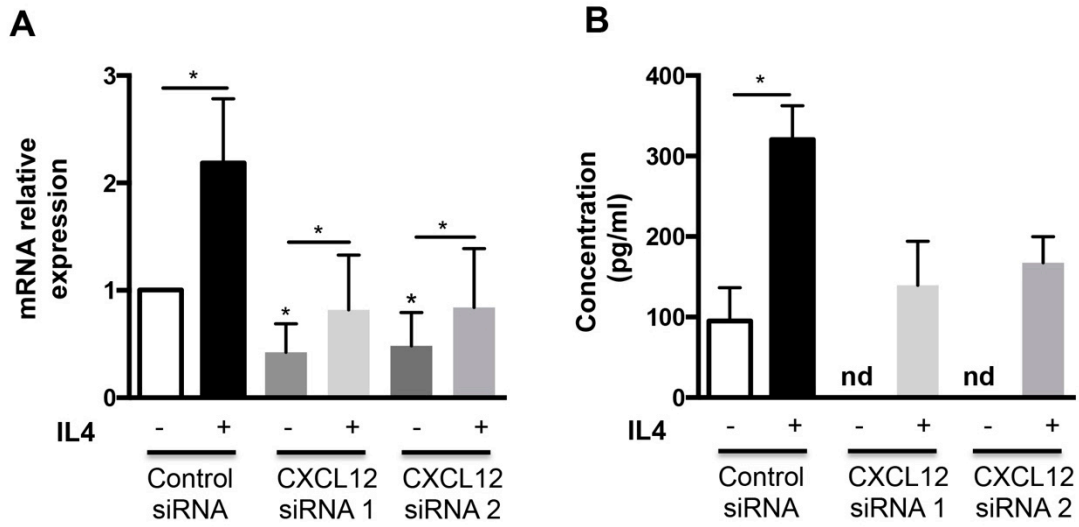


Figure S9: Validation of CXCL12 siRNA

ADSCs were transduced with control or two different CXCL12 siRNA (20nM) and maintained in culture for 48h in the presence or not of IL-4. CXCL12 expression was then quantified by RT-PCR (**A**) and ELISA (**B**). In PCR experiments, the arbitrary value of 1 was attributed to ADSCs transduced with control siRNA and not treated with IL-4. Results are expressed as the mean +/- SD from 6 (PCR) and 4 (ELISA) experiments. * $P < 0.05$, nd: not detected.

Figure S10

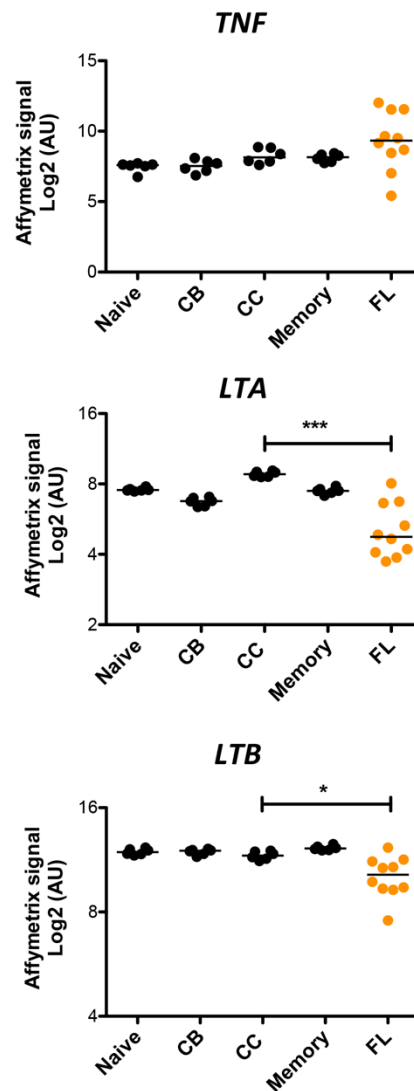


Figure S10: Expression of TNF family members in FL B cells

Expression of *TNF* (207113_s_at probeset), *LTA* (206975_at probeset), and *LTB* (207339_s_at probeset) was evaluated in purified CD19^{pos}IgD^{pos}CD38^{lo}CD27^{neg}CD10^{neg} naive B cells, CD19^{pos}IgD^{neg}CD38^{hi}CD10^{pos}CXCR4^{pos} centroblasts (CB), CD19^{pos}IgD^{neg}CD38^{hi}CD10^{pos}CXCR4^{neg} centrocytes (CC), and CD19^{pos}IgD^{neg}CD38^{lo}CD27^{pos}CD10^{neg} memory B cells sorted from tonsils (n=6) and CD20^{hi}CD44^{lo}CD38^{pos}IgD^{neg}CD138^{neg} FL B cells (n=10) using Affymetrix HG U133 Plus 2.0 microarrays. Mann Whitney test was performed. * $P < 0.05$, *** $P < 0.001$.

Figure S11

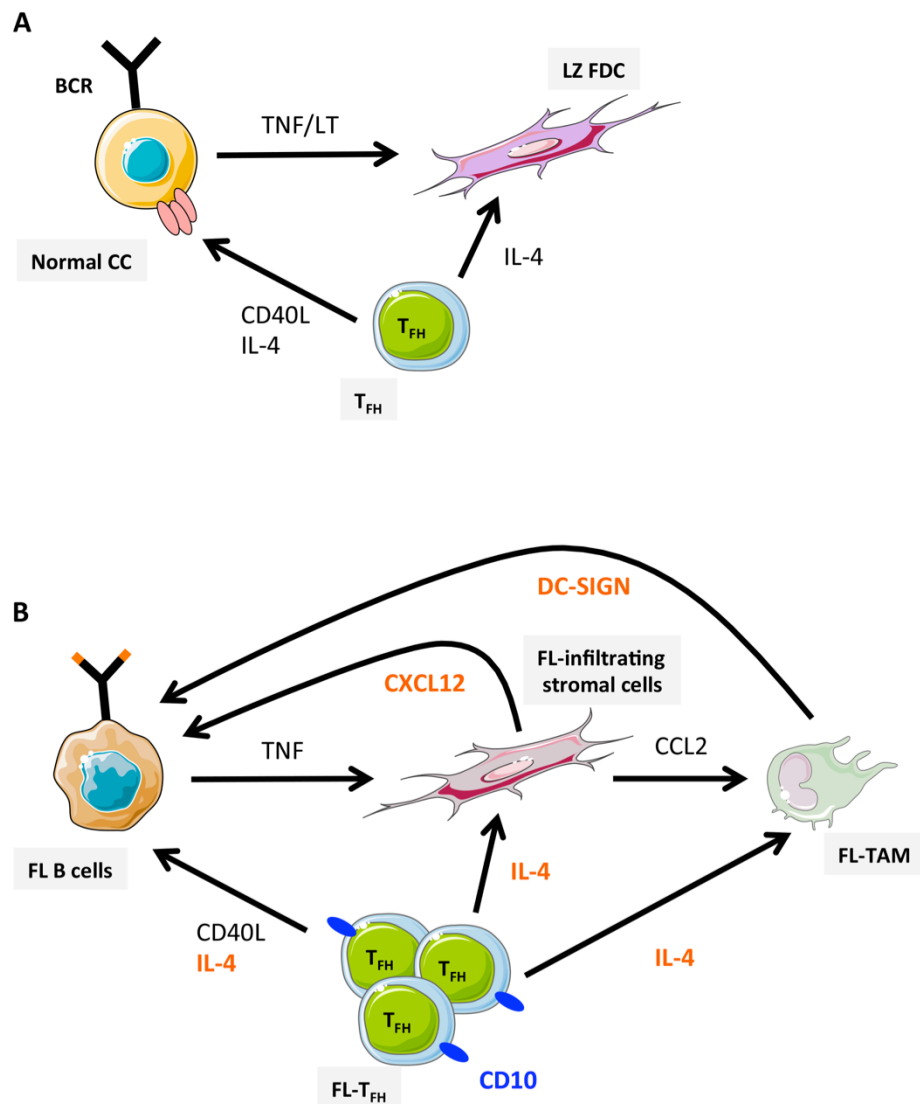


Figure S11: Model for T_{FH} -stroma crosstalk within FL cell niche

(A) Within normal germinal center, centrocytes (CC) produce TNF and LT that are mandatory for the differentiation of mature light zone follicular dendritic cells (LZ FDC). T_{FH} produce variable amounts of IL-4 and express CD40L that deliver survival signals to selected high-affinity B cells. (B) In FL cell niche, malignant B cells downregulate LT expression, in association with a loss of mature FDC, whereas a specific subset of $IL-4^{hi}CD10^{pos}$ T_{FH} is expanded. T_{FH} -derived IL-4 is involved in the direct activation of FL B cells, in the induction of DC-SIGN on tumor-associated macrophages and in the increase of CXCL12 production by FL-infiltrating stroma. Both DC-SIGN/FL-BCR and CXCL12/CXCR4 interactions have been shown to contribute to FL B cell activation.