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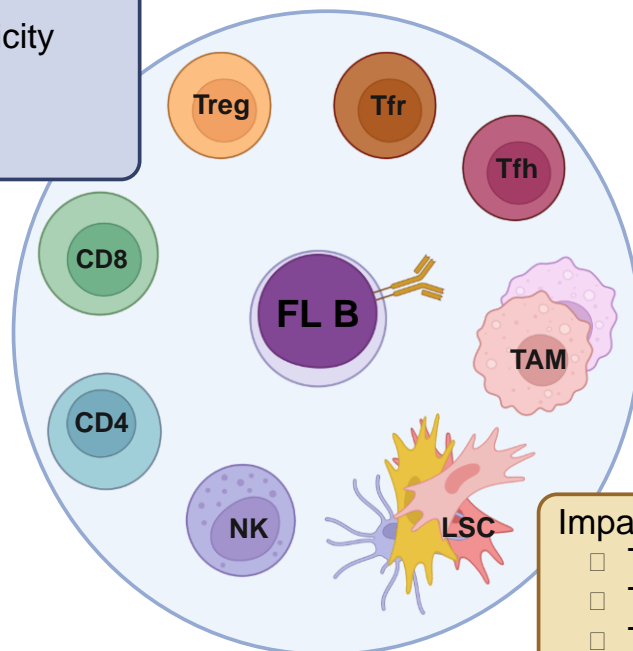


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Cell Crosstalk Within Lymphoma Tumor Microenvironment (TME): Follicular Lymphoma (FL) as a Paradigm

Determinants of FL-TME heterogeneity

- FL subtype
- Tumor genetics
- Tumor transcriptional plasticity
- Tumor localization
- Tumor kinetics



Impact of FL-TME heterogeneity

- Tumor growth & evolution
- Tumor relapse
- Treatment resistance/response
- Therapeutic target

Conclusions: The FL tumor niche is highly heterogeneous and relies on a bidirectional dynamic interplay between tumor B cells and their immune and stromal TME, resulting in tumor growth and providing predictive biomarkers and therapeutic targets.

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**Blood
Visual
Abstract**

**Cell crosstalk within lymphoma tumor microenvironment:
follicular lymphoma as a paradigm**

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Running Title: Follicular Lymphoma tumor microenvironment

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ABSTRACT

Follicular lymphoma (FL) is an indolent yet incurable germinal center B-cell lymphoma retaining a characteristic follicular architecture. FL tumor B cells are highly dependent on direct and indirect interactions with a specific and complex tumor microenvironment (TME). Great progress has been recently made in describing the heterogeneity and dynamics of FL-TME and in depicting how tumor clonal and functional heterogeneity rely on the integration of TME-related signals. Specifically, FL-TME is enriched for exhausted cytotoxic T cells, immunosuppressive regulatory T cells of various origins, and follicular helper T cells overexpressing B cell and TME reprogramming factors. FL stromal cells have also emerged as crucial determinants of tumor growth and remodeling, with a key role for deregulated chemokines and extracellular matrix composition. Finally, tumor associated macrophages play a dual function, contributing to FL cell phagocytosis and to FL cell survival through BCR long-lasting activation. The resulting tumor-permissive niches show additional layers of site-to-site and kinetic heterogeneity, which raise questions about the niche of FL-committed precursor cells supporting early lymphomagenesis, clonal evolution, relapse, and transformation. In turn, FL B cell genetic and non-genetic determinants drive the reprogramming of FL immune and stromal TME. Therefore, offering a functional picture of the dynamic crosstalk between FL cells and TME holds the promise of identifying the mechanisms of therapy resistance, stratifying patients, and developing new therapeutic approaches capable of eradicating FL disease in its different ecosystems.

Introduction

Follicular lymphoma (FL) is a B-cell neoplasm derived from germinal center (GC) B cells and involving primarily nodal sites.¹ Normal GC are transient and dynamic microanatomical structures dedicated to the antigen-driven expansion, selection, and differentiation of high-affinity B cells through a finely controlled process ultimately conferring long-term humoral immunity.² Recent studies have demonstrated that class-switching to downstream immunoglobulin isotypes IgG, IgE, and IgA essentially occurs early in pre-GC B cells.³ Within GC, B-cell fate results from the integration of signals received from the BCR and from T follicular helper cells (Tfh) and specialized lymphoid stromal cell (LSC) subsets. In particular follicular dendritic cells (FDC) organize a spatial segregation of the GC. In the dark zone (DZ), centroblasts proliferate and undergo random somatic hypermutation in immunoglobulin variable regions. In the light zone (LZ), high-affinity centrocytes (CC) capture antigen from FDC, thus receiving a BCR-dependent signal,⁴ and receive also survival and differentiation stimuli from cognate CD4^{pos}CXCR5^{pos}PD1^{hi}ICOS^{hi} Tfh. Selected CC recirculate to the DZ for additional cycles of proliferation and mutations or differentiate into memory B cells and plasma cells. Tfh help is counterbalanced by a subset of Foxp3^{pos} GC-resident T cells called T follicular regulatory T cells (Tfr), acting as a critical modulating force in GC B-cell selection and contributing to GC contraction.⁵ The vast majority of GC B cells are not positively selected and about half of them undergo apoptosis every 6h before being cleared by GC tingible-body macrophages.⁶ Finally, several subsets of non-GC LSC, collectively referred as fibroblastic reticular cells (FRC), sequentially regulate each step of B-cell activation within discrete lymph node (LN) three-dimensional areas by recruiting naïve B and T cells, transferring antigens to B cells, organizing activated B and T cell trafficking and interaction, and mediating plasmablast and plasma cell migration and survival.^{7,8} In turn, B cells impact both Tfh maturation and function through cognate antigen presentation and costimulatory signals⁹, and FDC and FRC differentiation and maintenance, in particular through the production of tumor necrosis factor- α (TNF) and lymphotoxin- α 1 β 2 (LT).¹⁰⁻¹³ All these cell subsets are present within FL LN and contribute to the organization of tumor-permissive niches while receiving FL B-cell derived signals modulating their functional properties. A rich ecosystem of anti-tumor immune cells is also detected, but displays altered phenotype and functions supporting immune escape mechanisms. The dynamic interplay between pro- and anti-tumor cell components, organized within a highly remodeled extracellular matrix (ECM),

structures the FL tumor microenvironment (TME), whose composition and organization has been largely associated with FL development, evolution, and prognosis, thus providing predictive biomarkers and putative therapeutic targets.

General FL niche organization

Histological examination of FL LN classically reveals a follicular growth pattern, where neoplastic follicles include malignant B cells, Tfh, Tfr, and a CD21^{pos} FDC network of varying intensity¹ (**Figure 1**). However, FL follicles display several altered features including a low proliferation rate, a loss of DZ/LZ polarization, a lack of tingible-body macrophages, and attenuated mantle zones. Moreover, unlike their normal counterpart, FL follicles are not confined to the LN cortex but are disseminated throughout chronically enlarged LN with a disrupted architecture. Surrounding extrafollicular areas are reduced and display a dense network of activated FRC and T cells with disseminated neoplastic CC. CD8^{pos} T cells are usually excluded from FL follicles.¹⁴ Subcapsular and medullary sinuses are typically obliterated. FL tissues contain numerous blood vessels and neovascular sprouts are associated with tumor-infiltrating macrophages (TAM) in extrafollicular areas.¹⁵ Beside conventional FL, early *in situ* lesions of FL, including *in situ* follicular neoplasia and duodenal-type FL, share with conventional FL a follicular pattern¹ and the t(14;18) translocation, the earliest event initiating FL in about 85% of cases by producing constitutive expression of the BCL2 antiapoptotic protein. In contrast, t(14;18)^{neg} CD23^{pos} FL were recently proposed as a distinct entity called follicle center lymphoma (FCL) and usually show diffuse growth pattern.¹⁶ In addition to BCL2 deregulation, FL is characterized by numerous recurrent genetic hits, particularly targeting the epigenetic machinery. The impact of FL genetic landscape on FL TME composition and organization, whose understanding has recently been boosted by detailed analysis of genetically-engineered FL mouse models, has only recently emerged as a major determinant of FL pathogenesis and clinical outcome.^{17,18}

Extracapsular extension is common in conventional FL with invasion of the adipose perinodal tissue by ectopic GC-like structures and formation of fibrous band in the extranodal tissue.⁷ Accordingly, FL is a disseminated disease with multiple nodal lesions and bone marrow (BM) involvement is about 70% of cases.¹⁹ Within invaded BM, tumor cells are organized as follicle-like structures displaying a predominant paratrabecular localization and including ectopic functional FDC and PD1^{hi} CD4^{pos} T cells. As a whole, FL is characterized by its capacity

to disseminate and the requirement of a follicle-like organization and cellular composition to support malignant B cell growth, underlying the key role of tumor/TME crosstalk in this neoplasia.

Protumoral versus anti-tumoral immune TME: T cells at the front

T cells are the major component of FL TME (**Figure 2**). FL T cells are enriched for Tfh, exhausted cytotoxic T cells, and specific subsets of Treg compared to non-malignant LN, creating a FL-specific T-cell signature.^{20–22} FL-Tfh are not only expanded but overexpress IL-4, CD40L, and IL-21, which contribute to malignant B-cell survival and may also favor the commitment of a small fraction of the B-cell clone to plasma cell differentiation,^{20,23–25} challenging the concept of FL B cells being uniformly “frozen” at the GC stage.¹⁷ In addition, FL-Tfh contribute indirectly to FL growth as demonstrated in several *in vitro* studies. In particular, FL-Tfh stimulate the capacity of FL B cells to respond to TAM-derived IL-15²⁶ and to BCR activation mediated by DC-SIGN-expressing TAM.²⁷ Indeed, N-glycosylation sites are introduced in FL BCR variable regions by somatic hypermutation and are occupied by unusual oligomannose glycans recognized by DC-SIGN, triggering antigen-independent BCR activation (see Freda Stevenson’s review in the same series). CD40L and IL-4 signaling also trigger the production of the Treg chemoattractant CCL22 by FL B cells, thereby promoting immune escape.²⁸ Finally, FL-Tfh-derived IL-4, TNF, and LT participate to the differentiation of FL cancer-associated fibroblasts (FL-CAF)²⁹ and to their overexpression of CXCL12, which is involved in B-cell recruitment, adhesion, and activation.³⁰ The major role of Tfh-derived IL-4 in FL pathogenesis is further supported by the demonstration that the gain-of-function phenotype of STAT6 mutations frequently found in FL is strictly dependent on the presence of IL-4.³¹ FL has been largely recognized as an immune-responsive neoplasia, infiltrated by cytotoxic CD8 and CD4 T cells, $\gamma\delta$ T cells, and NK cells, and responding to vaccination strategies and immunomodulatory agents. However, FL-infiltrating CD4 and CD8 T cell effectors are characterized by the co-expression of numerous immune checkpoint inhibitors, including LAG-3, TIM-3, and TIGIT.^{32–36} In contrast, PD-1 is not a relevant exhaustion marker in FL due to its high expression on fully functional Tfh²³ and Treg/Tfr.³⁷ In addition, intratumoral T cells lacking CD27 and CD28 co-stimulatory receptor expression are enriched in FL.³⁸ Besides reduced proliferation and cytokine production, FL T cells also exhibit an impaired LFA-1-dependent motility³⁹, and a defective capacity to form mature

immunological synapses with malignant B cells.⁴⁰ Expansion of immunosuppressive cells is another hallmark of FL TME with a strong amplification of regulatory T cells, including Treg and Tfr, that could both contribute to immune escape.^{37,41}

Importantly, FL T-cell polarization relies on a dynamic crosstalk with FL B cells as highlighted by the impact of FL genetics on T cell features (**Figure 2**). The loss of HVEM expression by somatic mutations in 40% of FL patients disrupts the inhibitory signal delivered to BTLA^{hi} Tfh, favoring their amplification, their capacity to deliver survival signal to normal and malignant GC B cells, and their overexpression of TNF/LT.²⁹ Mutations of HVEM are essentially mutually exclusive with gain-of-function mutations of the cysteine protease Cathepsin S (CTSS), which similarly result in increased tumor-supportive Tfh infiltration in both mouse models and FL patients by favoring MHC II-restricted antigen presentation.^{42,43} These data further underscore the central role of Tfh in FL pathogenesis. High expression of CD40, IL-6, and ICOSL by tumor B cells has also been implicated in Tfh recruitment, activation, and proliferation.^{44,45} Although FL B cells do not express PD-1 or TIGIT ligands^{34,46}, *in vitro* experiments identified CCL22, CD70, ICOSL, and TGF β overexpression by tumor B cells as drivers of T-cell reprogramming toward exhausted and regulatory phenotypes.^{28,37,47–49} More generally, MHC II silencing in FL B cells, frequently found in patients with genetic alterations in *CREBBP* or *EZH2*¹⁷, is associated with a reduced tumor infiltration by exhausted cytotoxic CD8 and CD4 T cells, suggesting that T-cell exhaustion in FL relies on persistent MHC II expression by tumor cells.²⁰ Interestingly, a recent evaluation of the TCR repertoire coupled with scRNAseq further argues for a contribution of tumor antigen-driven clonal expansion of cytotoxic CD8 T cells and Tfh/Treg in FL.²² This study also reveals that a substantial proportion of clonotypes are shared between FL-Tfr and FL-Tfh, identifying a FL-Tfh-to-FL-Tfr developmental trajectory under the continuous contact with malignant FL B cells. A similar process has recently been described in reactive lymphoid tissues for Tfh-descendant Tfr with dual T-cell suppressive/B-cell helper function⁵⁰, suggesting that FL co-opts an existing differentiation pathway to trigger the amplification of tumor-supportive T cells. Regarding other cytotoxic effectors, FL B cells were proposed to engage the inhibitory receptor BTLA on infiltrating $\gamma\delta$ T cells in HVEM wt patients, thereby blocking their proliferation.⁵¹ Similarly, overexpression of lectin-like transcript 1 (LLT1) by FL tumor cells dampens *in vitro* the function of CD161^{pos} NK cells.⁵² In conclusion, the FL T cell compartment displays heterogeneous phenotypes and functions collectively forming a protumoral immune niche

dependent on a bidirectional crosstalk with malignant B cells and providing the basis for immunotherapeutic interventions.

Stromal cells as organizers of FL TME

FL-CAF develop at the expense of normal LSC within infiltrated LN and display quantitative and qualitative alterations supporting LN remodeling and tumor growth (**Figure 3**). Data on FL stromal cells initially result from the analysis of *in vitro* expanded cells and from *in situ* low-throughput phenotypic characterization. However, the recent transcriptomic profiling of native FL-FRC and FL-FDC at bulk and single cell levels has paved the way to a better understanding of their heterogeneity, plasticity, mechanisms of polarization, and tumor-supportive function. First, both FL-FRC and FL-FDC overexpress CXCL12^{30,53}, directly impacting tumor B cell growth. In addition, heparinase, which increases the bioavailability of heparin-binding factors like CXCL12, is strongly expressed in about 50% of FL.⁵⁴ Within normal GC, CXCL12 expression is restricted to CD21^{neg} DZ FDC (also called CXCL12-expressing reticular cells, or CRC) whereas CD21^{pos} LZ FDC are CXCL12^{neg}.⁸ Within FL follicles, CXCL12 upregulation parallels the loss of LZ FDC markers, including CD21, CD23, and CD35, related to a lower expression of LT by malignant FL B cells compared to normal CC.^{30,55,56} Interestingly, CD23/IL4R α expression on LZ FDC was recently shown in mice to restrict IL-4 availability for normal LZ CC⁵⁷. Together with the expansion of IL4^{hi} FL-Tfh, the loss of CD23^{pos} FDC in FL could thus contribute to the increase of IL-4 bioavailability and function. All FL-LSC subsets also overexpress the chemokines CCL19/CCL21, that could contribute to the dissemination of CCR7^{pos} FL B cells, but also to the recruitment of immune TME.⁵³ Transcriptomic analyses identified a huge deregulation of genes involved in cell adhesion and ECM composition and organization in FL-LSC.^{53,58} Recently, elegant *in vitro* 3D models, integrating stromal cells and/or functionalized hydrogels, have been developed in order to capture the role of ECM and biomechanical forces in B-cell lymphomas. These pioneer works have proposed a specific role for the α 4 β 1 integrin signaling and shear stress in the stroma-dependent lymphoma B-cell growth⁵⁹ and drug resistance. A 3D alginate spheroid model cocapsulating LSC with lymphoma B cells and integrating a layer of ECM lining the inner wall of the capsules was proven efficient to sustain primary FL B cell survival *in vitro*.⁶⁰ Importantly, ECM remodeling could also impair the accessibility of tumor B cells to cytotoxic T cells, as described for solid cancer CAF. In addition, FL-CAF express the TIGIT ligands CD122 and CD155, as well as PGE2^{34,61}, that could both participate to the defective immune

response in FL niches. Beyond T cells, FL-CAF affect tumor-infiltrating myeloid cells. In particular, FL-CAF overexpress CCL2, involved in the recruitment of monocytes that could be then converted into pro-angiogenic and anti-inflammatory TAM as demonstrated by in vitro cocultures.⁶² As discussed before, FL-TAM contribute to FL B cell activation in particular by triggering weak but long-lasting BCR signalling through the crosslinking of oligomannosylated BCR by DC-SIGN.^{27,63} Furthermore, even if the phagocytosis of tumor B cells by TAM is involved in the clinical activity of anti-CD20 antibodies, FL B cells express huge amounts of the “don’t eat me” receptor CD47⁶⁴, inhibiting phagocytic and inflammatory functions of macrophages expressing signal regulatory protein α (SIRP α), found to display T-cell suppressive activity in FL.⁶⁵ Finally, FL-TAM express classical immune checkpoint inhibitors, like PD-L1⁶⁶, and the immunosuppressive enzymes indoleamine-2,3 dioxygenase (IDO)⁶⁷ and interleukin-4-induced gene 1 (IL4I1)⁶⁸, involved in the catabolism of tryptophan and phenylalanine, respectively. However, the impact of immunosuppressive myeloid cells on immune escape and drug resistance is less clear in FL than in DLBCL.

Malignant FL B cells play a central role in driving the reprogramming of FL stromal cells (**Figure 3**). In particular, B cell-derived TNF and LT have been involved in the upregulation of CCL2⁶² and TNF/LT synergize with TGF- β to induce CCL19 and CCL21 production by FL-CAF.⁵³ Gain-of-function mutations of the methyltransferase EZH2, found in 20-30% of FL patients, are associated with an early overexpression of LT and TNF by LZ CC in genetically-engineered murine models, resulting in a preferential interaction with FDC.⁶⁹ In agreement, the FDC network was found more expanded in EZH2 mutated FL patients. Thus, both HVEM and *EZH2* mutations result in FL stromal cell activation but by different mechanisms, including the overexpression of TNF/LT by Tfh versus FL B cells. Tfh also contribute to the polarization of FL-CAF, in particular by producing high amounts of IL-4, TNF, and LT.^{30,41} Interestingly, FL-Tfh overexpress CD40L and IFN γ , both of them being able to activate stromal cells and regulate their production of inflammatory chemokines or immunosuppressive factors.^{70,71} In turn, FL-CAF contribute to the polarization of FL-Tfh by favoring the early commitment of pre-Tfh cells into IL-4^{hi} producer cells, in a Notch and ICAM1/LFA1-dependent manner.⁷² As a whole, FL-CAF are engaged in a bidirectional crosstalk with tumor B cells and the other TME components and display pleiotropic activities.

Spatial and kinetic heterogeneity of FL TME

An important open question is to understand how the heterogeneity of FL B cells, Tfh, and CAF translates into the organization of distinct tumor niches reflecting coordinated spatial and kinetic heterogeneity and with different capacities to sustain disease progression and relapse. Spatial heterogeneity has emerged as an important determinant of disease heterogeneity in FL. First, the comparison of synchronous distant nodal sites reveals a unique correlation between site-to-site tumor cell subclonal, transcriptomic, and phenotypic dissimilarity and site-to-site variability in FL-Tfh abundance, confirming Tfh as a key player in FL lymphomagenesis.³³ Furthermore, analysis of paired LN and BM tumor lesions identifies specific features of BM FL B cells, including lower cytological grade, reduced proliferation, altered phenotypic, metabolic, and transcriptomic profiles, and divergent subclonal evolution compared to LN B cells.⁷³⁻⁷⁵ Regarding stromal cells, ectopic FRC-like and FDC-like cells are detectable within BM follicle-like structures, confirming the dependence of FL B cells on LSC.^{7,76} BM and LN FL-CAF share other common features, including upregulation of CCL2, CXCL12, and secreted protein acidic and rich in cysteine (SPARC), a matricellular protein involved in collagen deposition and organization.^{30,62,77} Even though they produce less LT than normal CC, the large number of GC-like B cells found in invaded FL BM leads to a local increase in TNF and LT levels, which may contribute to the induction of LSC commitment and CCL2 expression in BM stromal cells. In addition, as described in LN, IL4 and CXCL12 are correlated in FL BM, suggesting a contribution of FL immune TME to BM FL-CAF activation.³⁰ Although the CD4/CD8 T cell ratio is increased in FL-invaded BM, it remains lower than in FL LN^{76,78} and the presence of IL-4-producing Tfh-like cells in FL BM remains to be firmly established. Interestingly, BM stromal cells obtained from non-invaded FL BM show an intermediate gene expression profile between healthy donor BM stromal cells and FL-invaded BM stromal cells, suggesting a priming of BM stromal cells in the absence of direct contact with tumor B cells and expanded CD4 T cells.⁷⁵ This phenotype may be related to the production of extracellular vesicles by FL B cells that are internalized by BM stromal cells, increasing their capacity to support BM FL B cell survival and quiescence, at least in part through the upregulation of CXCL12.⁷⁵ This study further suggests that differences in LN versus BM stromal cell features support specific patterns of molecular interactions with malignant B cells and contribute to tumor spatial heterogeneity. To date, no study has been designed to evaluate whether, at the intra-tissular level, the dynamic continuum of transcriptional states that characterize FL B cells^{20,79} is related to the integration of various

signals from their surrounding TME, eventually corresponding to microanatomical areas supporting an additional layer of spatial heterogeneity. Collectively, these studies underscore the difficulty of capturing FL disease with a single biopsy.

In addition to spatial heterogeneity, the issue of kinetic heterogeneity is another key element with potential therapeutic implications. A first question pertains to the niche of FL committed precursor cells (CPC), which can be detected years before FL diagnosis, exhibit early driver genetic events, could be traced between paired pre-diagnostic blood and tumor biopsies, and are believed to be responsible for seeding FL relapses and transformation.¹⁷ These cells are virtually impossible to directly visualize or isolate due to their scarcity and lack of phenotypic markers but their specific localization and capability to integrate local microenvironment cues are an object of intense interest. In healthy individuals, FL precursor cells accumulate within GC as nonproliferative CC⁸⁰ and can also be detected in the BM.⁸¹ However, the niches where residual CPC remain post-treatment are completely unknown. Besides, data are missing regarding the impact of FL treatment on TME. Histologic transformation occurs in 8 to 15% of FL patients, usually into the GC B-cell subtype of diffuse large B-cell lymphoma (GCB-DLBCL)^{82,83}, in association with an increased proportion of large cells diffusely infiltrating LN and acquiring specific genetic features, an effacement of the follicular architecture, and a loss of FDC meshwork and Tfh infiltration, while TAM and CD8^{pos} T cell increase.⁸⁴ Several studies based on limited patient cohorts and/or targeted approaches have proposed variable TME-related markers predicting the transformation risk.^{85,86} Recently, a large whole-exome sequencing study has proposed a classifier predicting transformation from genetic features present at diagnosis.⁸⁷ Moreover, a thorough characterization of B cell/TME features at diagnosis identified a subgroup of FL patients with a B-cell profile related to memory B cells and an increased Tfh content as more prone to transformation⁸⁸, suggesting that B-cell/TME interplay may play a central role in FL evolution. Further studies are necessary to confirm the biological and clinical significance of these findings.

TME and non-conventional FL

Few studies have explored the composition and spatial organization of non-conventional FL. Duodenal-type FL, which share genetic alteration profile and nodular pattern with conventional FL, display a specific pro-inflammatory TME, with increased abundance of Th17

and activated T cells, and reduced FDC network, CCL21 expression, and Treg infiltration.⁸⁹⁻⁹¹ Interestingly, B cells from duodenal-type FL exhibit memory B cell features, including CCR6 expression, supporting the idea of an impact of TME features on FL B cell plasticity.^{90,92} Diffuse (14;18)^{neg} CD23^{pos} FCL show a gene expression profile enriched in T cell, NK, and dendritic cell signatures while tumor B cells produce very low amounts of LT in association with a lack of mature FDC.^{16,56} This specific TME composition may reflect how FCL B cells cope with the absence of the GC niche that characterize typical FL. Finally, when compared to t(14;18)^{pos} FL cases, t(14;18)^{neg} conventional FL (after excluding FCL and pediatric-type FL) show an enrichment for inflammatory signatures, including overexpression of NF-κB target genes and cytotoxic T-cell markers.⁹³ Importantly, N-glycosylation sites were detected less frequently in the immunoglobulin variable regions of these t(14;18)^{neg} FL that display a bias for *IGHV4-34* usage.⁹⁴ These features may indicate a responsiveness to autoantigens, unlike to DC-SIGN-dependent signaling, in this FL subgroup. Even if these non-typical FL entities are rare and heterogeneous, the demonstration that they have specific TME characteristics further underscores the role of tumor/TME bidirectional crosstalk in FL pathogenesis.

Clinical impact of B-cell/TME crosstalk

Since the pioneer study published in 2004⁹⁵, most prognostic biomarkers in FL reflect the immune TME composition and organization rather than the tumor genetic landscape. However, these studies have given contradictory conclusions, highlighting the significant influence of treatment regimens on the predictive value of individual biomarkers and the lack of validated tools to assess the composition, organization, and function of the FL TME. Nevertheless, they suggest that being heavily infiltrated by T cells in the presence of tumor cells retaining antigen-presenting capacities, even when associated with an immunosuppressive context, is of good prognosis.^{14,20,96-98} The role of local T-cell diversity, as could be studied directly or inferred from bulk RNASeq using appropriate bioinformatic tools,^{99,100} should be better evaluated in clinically-annotated patient series to conclude on its impact on patient outcome. Some cell types, such as Tfh/Tfr, stromal cells, or myeloid cell subsets, are challenging to explore using bulk transcriptomic data or conventional immunohistochemistry/immunohistofluorescence, the main technical approaches suitable for analyzing reproducibly large patient cohorts using FFPE samples. Conversely, advanced multiparametric strategies including scRNAseq approaches or flow cytometry/mass

cytometry on a limited number of samples have been instrumental in identifying TME complexity and plasticity and remain the only way to fully explore TME dynamics based on iterative tumor samples. Of note, the analysis of myeloid and stromal cell properties requires tissue enzymatic dissociation and is less advanced than the analysis of tumor-infiltrating lymphocytes. Finally, high-parameter spatial proteomic studies, either on tissue-microarrays (*i.e.* transferable to clinical application) or whole tissue sections (*i.e.* integrating intra-tumoral heterogeneity) are of utmost significance but require a huge work of standardization before moving to clinical practice, being currently better suited for expert analysis of clinical trials. The different spatial tools are detailed in another review of this series by Dr Andrea Radtke. As a whole, innovative technologies are essentially low-throughput and difficult to standardize at the pre-analytical, analytical, and data integration steps, but could produce new relevant cell signatures, currently essentially derived from peripheral blood or solid cancers, to develop better deconvolution strategies and transfer this knowledge to bulk RNAseq or targeted RNA/protein quantification, routinely proposed to study tumor heterogeneity. For example, we recently demonstrated that the frequency of exhausted cytotoxic T-cells is associated with clinical outcome using a deconvolution approach of bulk RNAseq data.²² Importantly, given the interplay between tumor genetics and TME, the mutational landscape should be analyzed when evaluating FL TME impact on clinical response, as reported for CTSS mutations and the response to standard immunochemotherapy.⁴³ Regarding stromal cells, FL-CAF-related ECM genes were recently proposed as markers of poor prognosis⁵⁸ but this study used transcriptomic data from patients treated with chemotherapy alone and should be reevaluated on patients treated with classical immunochemotherapy regimen. It is unclear to what extent peripheral blood can be used to identify soluble or cellular TME-related predictive biomarkers in FL. Nevertheless, it enables convenient patient follow-up, looking for the impact of therapeutic strategies on (re)circulating immune cells, in particular T cells.¹⁰¹

The combination of the immunomodulatory agent Lenalidomide to immunochemotherapy was proven effective in FL¹⁰² due to the capacity of Lenalidomide to induce early T-cell activation and reprogramming, and to restore long-term immune synapse formation *in vivo* in FL patients.¹⁰¹ Conversely, minimal responses to anti-PD1/anti-PDL1 antibodies have been obtained in FL. This may be related to the low expression of PD-L1 in FL, while PD-1 is expressed by various T-cell subsets with different functions, including not only exhausted T

cells but also fully functional Tfh and Treg/Tfr, requiring the targeting of other checkpoint inhibitors, like TIGIT or LAG-3, and/or of other suppressive mechanisms to achieve efficient anti-tumor T-cell activation. Recently, new immunotherapy strategies, such as tumor-targeting chimeric antigen receptor T cells (CAR-T) and bispecific antibodies (BsAb) redirecting endogenous T cells to the tumor have been introduced for the treatment of FL.¹⁰³ Despite the success of these novel treatments, clinical responses are heterogeneous and identifying biomarkers for response prediction requires new strategies, probably involving functional approaches to evaluate the capacity of tumor-infiltrating T cells to recognize and kill tumor B cells within tumor-supportive TME. As an example, ex vivo resistance to BsAb has been recently correlated to the frequency of Helios^{pos} Treg, data that should be prospectively validated in clinical cohorts¹⁰⁴. Similarly, resistance to lenalidomide and anti-CD20 association was found associated with a high percentage of pre-existing Treg.¹⁰¹ These data argue for considering Treg depletion and/or functional blockade in FL as recently described in solid cancers.¹⁰⁵

In addition to the direct activation of immune effectors by immunotherapy approaches, other therapeutic options initially designed to target B cells can reprogram the B-cell/TME crosstalk. EZH2 mutations found in 25% of FL patients and associated to a loss of MHC I and II expression¹⁰⁶ have been associated to a better outcome after CHOP (cyclophosphamide, doxorubicine, vincristine, prednisone)-based, unlike bendamustine-based, immunochemotherapy regimen in the GALLIUM clinical trial (NCT01332968).¹⁰⁷ The differential impact of the EZH2 mutational status is not due to a difference in the sensitivity of tumor B cells to chemotherapy. In contrast, unlike bendamustine, doxorubicine restores MHC I/II expression on EZH2-mutated tumor B cells, potentially promoting cytotoxic T cell response.¹⁰⁸ How EZH2 inhibitors currently evaluated in FL modulate immune and stromal TME polarization and functional interplay with tumor B cells remains to be explored but may be involved in the clinical response of EZH2 mutated and wild type patients. To date, only limited attempts have been made to design therapies targeting FL tumor-supportive niches. IL-4, which plays pleiotropic roles in FL B cell growth and is associated with Ibrutinib resistance in chronic lymphocytic leukemia¹⁰⁹, is a putative target. Moreover, interfering with BCR/DC-SIGN interaction¹¹⁰⁻¹¹² would also be relevant to block TME-dependent BCR activation. Finally, Tfh-derived signals could be abrogated by CAR-T cells targeting CXCR5, expressed on both B cells and Tfh¹¹³, or by CD19-targeting CAR-T cells delivering soluble

HVEM inside the tumor bed.²⁹ Besides their direct tumor supportive activities, CAF have been demonstrated in solid cancers to counteract the activity of immunotherapies, notably by maintaining anti-tumor T cells at the periphery of the tumor, by recruiting and activating Treg, and by inhibiting CD8 T cell and NK cell activity.¹¹⁴ Targeting CAF or CAF-derived ECM in FL may be an appealing strategy in the context of the development of novel immunotherapy approaches in this disease. Conversely, the huge expression of CCL21/19 by FL-CAF could contribute to the recruitment of anti-tumor immune effectors. The clinical impact of B-cell/TME crosstalk in FL deserves further evaluation through well-designed trials including thorough ancillary studies repositioning tumor cell functional heterogeneity within their various ecosystems.

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Authorship Contributions

All authors contribute writing the paper

Conflict-of-interest disclosure

The authors declare no other competing financial interest.

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FIGURE LEGENDS

Figure 1: *In situ* phenotyping of follicular lymphoma grade 1-2 on FFPE section using multiplex immunohistochemistry.

A) The normal architecture of the lymph node is effaced by neoplasia follicles of malignant B cells (CD20, clone L26, cyan) sustained by CD21^{pos} FDC (clone EP3093, magenta). Tumor cells strongly express BCL2 (clone SP66, green). Tumor-infiltrating T cells include a high number of CD4^{pos} T cells (red) in inter and intrafollicular areas whereas CD8^{pos} T cells (clone C8/144B, orange) are less numerous and usually observed at the follicle border.

B) Detection of Tfh and Tfr subpopulation in FL. Tfh cells are mostly observed in neoplastic follicles and co-express CD4 (red), PD1 (clone NAT105, yellow) and CXCR5 (clone D6L3C, magenta). High magnification of follicle area shows Tfr cells positive for CD4 (red), CXCR5 (magenta) and FOXP3 (clone EP340, green).

Figure 2: T-cell subsets within follicular lymphoma microenvironment

A) FL is characterized by a defective anti-tumor immune TME including amplified Treg/Tfr and exhausted CD4 and CD8 T cells expressing high amounts of immune checkpoint inhibitors and reduced CD27 and CD28 co-stimulatory receptors. Ligands of immune checkpoint inhibitors and immunosuppressive enzymes are mainly expressed by tumor-associated macrophages (TAM) and follicular dendritic cells (FDC). However, tumor B cells play a central role in the induction of the altered immune TME, by overexpressing CCL22, ICOSL, and TGF β , involved in T-cell exhaustion and Treg amplification. FL B cells also frequently display reduced MHC I and MHC II expression, thus abrogating immune recognition by cytotoxic T cells. In addition, the cytotoxic activity of TAM and NK cells is inhibited, in particular through CD47 and LLT1 expression by tumor B cells.

B) Tfh are expanded in FL and overexpress CD40L, IL-4, IL-21 and TNF/LT, thus contributing to tumor B cell survival and transcriptomic plasticity. FL-Tfh also favor B-cell/TAM crosstalk, trigger CXCL12 expression in FL cancer-associated fibroblasts (CAF), and increase CCL22 expression by tumor B cells. FL-Tfh could differentiate into FL-Tfr that contribute to tumor immune escape. FL B cells contribute to FL-Tfh amplification and reprogramming, notably by expressing CD40, ICOSL, and IL-6, and by losing HVEM expression thus alleviating the BTLA-mediated inhibitory signal on Tfh.

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Figure 3: Role of stromal cells within follicular lymphoma microenvironment

FL-CAF are central organizers of FL TME. They overexpress CXCL12, CCL19 and CCL21, contributing to tumor B cell recruitment and activation, and CCL2, involved in TAM recruitment. FL-CAF also show a deregulated expression of adhesion molecules and extracellular matrix components affecting B cell and immune cell migration. Finally, they contribute to the polarization of TAM and pre-Tfh.

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Figure 1

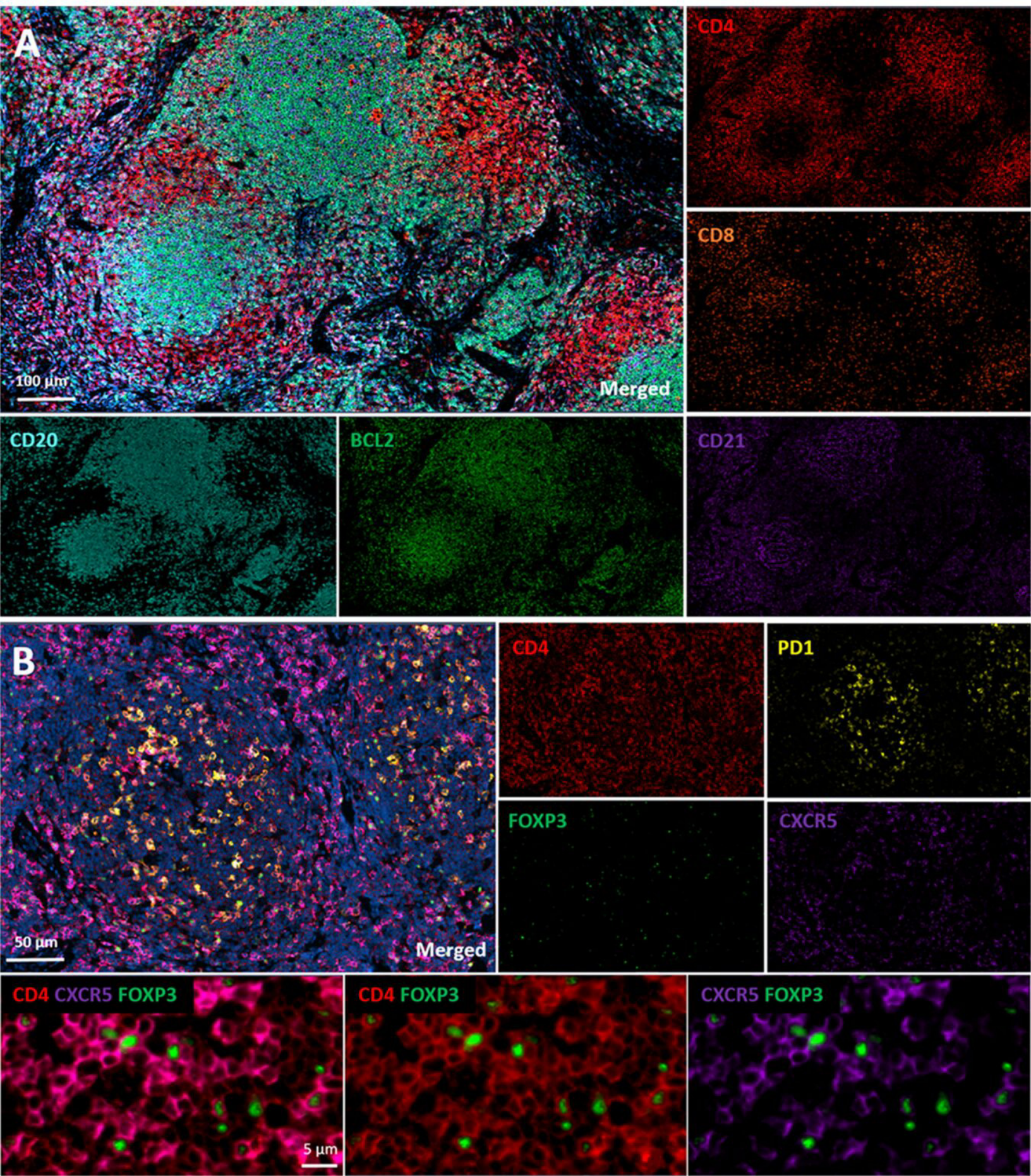
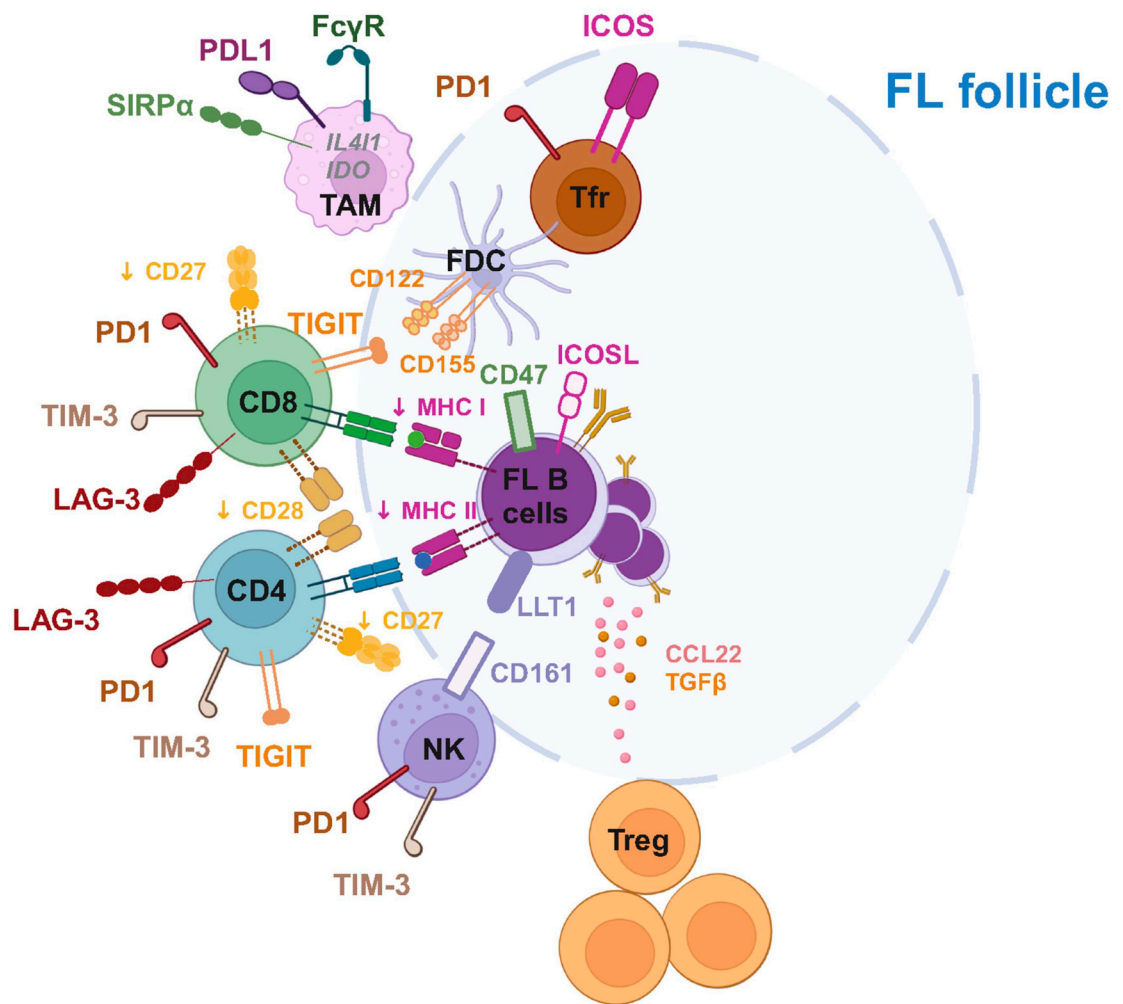


Figure 2

A



B

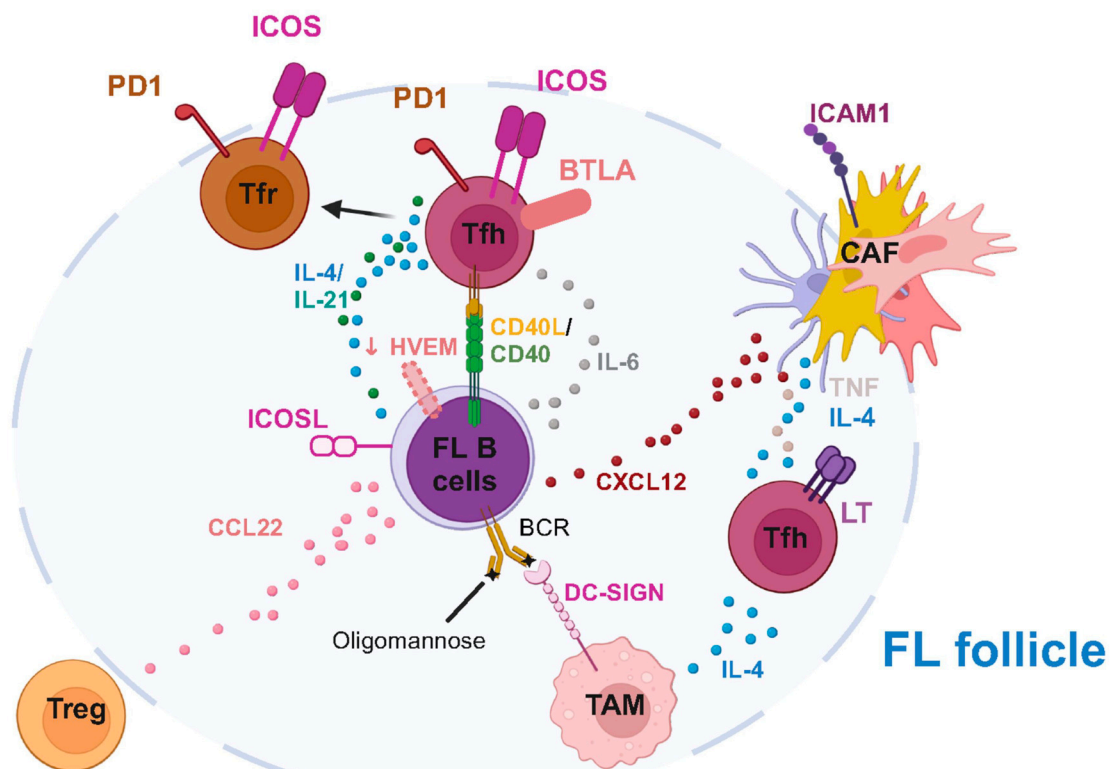


Figure 3

