CD10 delineates a subset of human IL-4 producing follicular helper T cells involved in the survival of follicular lymphoma B cells

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Running title: Follicular lymphoma-supporting T\textsubscript{FH} express CD10

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KEY POINTS

1. CD10 identifies a unique subset of fully functional GC-TFH that are activated and amplified within follicular lymphoma (FL) cell niche
2. FL CD10pos T<sub>FH</sub> specifically display an IL-4<sup>hi</sup>IFN-γ<sup>lo</sup> cytokine profile and encompass the malignant B cell-supportive T<sub>FH</sub> subset.

ABSTRACT

In follicular lymphoma (FL), follicular helper T cells (T<sub>FH</sub>) have been depicted as one of the main component of malignant B-cell niche and a promising therapeutic target. Although defined by their capacity to sustain FL B-cell growth together with specific gene expression and cytokine secretion profiles, FL-T<sub>FH</sub> constitute an heterogeneous cell population. However, specific markers reflecting such functional heterogeneity are still lacking. In this study, we demonstrate that CD10 identifies a subset of fully functional GC-T<sub>FH</sub> in normal secondary lymphoid organs. Importantly, this subset is amplified in the FL context, unlike in other B-cell lymphomas with a follicular growth pattern. Furthermore, whereas FL-T<sub>FH</sub> produce high levels of IL-21 and low levels of IL-17 irrespectively of their CD10 expression, CD10<sup>pos</sup> FL-T<sub>FH</sub> specifically exhibit an IL-4<sup>hi</sup>IFN-γ<sup>lo</sup>TNF-α<sup>hi</sup> cytokine profile associated with a high capacity to sustain directly and indirectly malignant B-cell survival. Altogether, our results highlight the important role of this novel functional subset in FL cell niche.
INTRODUCTION

Follicular lymphoma (FL) microenvironment is characterized by a strong infiltration of helper T (Th) cells displaying a complex phenotype, including an overexpression of both activation and exhaustion markers, and a specific gene expression profile (GEP), underlying altered T-cell activation, motility, and polarization\textsuperscript{1-5}. Recently, we demonstrated more precisely that genes related to follicular helper T cells (T\textsubscript{FH}), the specialized CD4\textsuperscript{pos} T cells involved in normal germinal center (GC) B-cell survival and differentiation\textsuperscript{6}, represent a significant part of FL-specific microenvironment signature and revealed their unique capacity to support malignant B-cell growth\textsuperscript{7,8}. FL-T\textsubscript{FH} are regarded as a promising therapeutic target in this still incurable disease\textsuperscript{9}. FL-T\textsubscript{FH} are characterized by a specific cytokine profile, combining overexpression of IL-4, IFN-\textgreek{g}, and TNF-\textgreek{a}, and decreased expression of Th17-related genes\textsuperscript{8}. However, specific markers associated with FL-T\textsubscript{FH} heterogeneity and identifying precisely the tumor-supportive FL-T\textsubscript{FH} subset are lacking.

In reactive lymphoid tissues, CD57 has been initially proposed as a marker of B-cell supportive GC-T\textsubscript{FH}\textsuperscript{10,11} but further GEP and functional studies revealed that CD57\textsuperscript{pos} and CD57\textsuperscript{neg} T\textsubscript{FH} are rather similar\textsuperscript{12}. Neuropilin-1 (Nrp-1) was also detected on a subset of T\textsubscript{FH} but no specific function could be attributed to Nrp-1\textsuperscript{pos} T\textsubscript{FH}\textsuperscript{13}. Interestingly, CD10, a marker of immature T and B cells, and GC B cells virtually absent on circulating mature T cells\textsuperscript{14} has been reported on a subset of poorly characterized CD5\textsuperscript{pos} T cells within reactive lymphoid hyperplasia (RLH), FL, and marginal zone lymphoma\textsuperscript{15}, as well as on malignant T\textsubscript{FH} in angioimmunoblastic T cell lymphoma\textsuperscript{16,17}. Such results raise the possibility that CD10 expression highlights a subset of TFH within normal and malignant lymph nodes (LN).

Combining GEP, histology, phenotype, and functional approaches, we demonstrate that CD10 expression is restricted to a unique subset of GC-T\textsubscript{FH}, specifically amplified in FL context. Moreover, CD10\textsuperscript{pos} FL-T\textsubscript{FH} exhibit a peculiar IL-4\textsuperscript{hi}IFN-\textgreek{g}\textsuperscript{lo} TNF-\textgreek{a}\textsuperscript{hi} cytokine profile associated to a strong capacity to sustain directly and indirectly malignant B-cell survival.
STUDY DESIGN
(For details see supplemental file)

Samples
Subjects were recruited under institutional review board approval and informed consent process according to the Declaration of Helsinki. Samples comprised LN obtained from patients with FL, nodular lymphocyte predominant Hodgkin’s lymphoma (NLPHL), and mantle cell lymphomas (MCL), tonsils collected from children undergoing routine tonsillectomy, and reactive LN with follicular hyperplasia. CD3\textsuperscript{pos}CD4\textsuperscript{pos}CXCR5\textsuperscript{hi}ICOS\textsuperscript{hi}CD25\textsuperscript{neg} T\textsubscript{FH}, CD10\textsuperscript{pos} T\textsubscript{FH}, and CD10\textsuperscript{neg} T\textsubscript{FH} were sorted using a FACSARia (BD Biosciences) (purity >98%). Tonsil and FL B cells were purified using the human B-cell isolation kit II (Miltenyi Biotec).

Phenotypic study
Membrane and intracellular staining were performed using standard flow cytometry techniques. Data were acquired on a CyAn\textsuperscript{®} ADP flow cytometer and analyzed using Kaluza software (Beckman Coulter). Tissue sections were used for single immunohistochemical (IHC; PD1), double IHC (CD10/PAX5) and double immunohistofluorescence (IHF) stainings (CD10/CD3, CD10/ICOS, CD10/CXCL13).

Microarray hybridization
GEP of 7 FL-T\textsubscript{FH} and 7 tonsil-T\textsubscript{FH} was analyzed using GeneChip HG-U133 Plus 2.0 microarrays (Affymetrix) and normalized using Partek software. Microarray data are registered to the Gene Expression Omnibus under accession number GSE66384.

FL B-cell anti-apoptotic assay
Purified FL malignant B cells were cultured alone, with an activation cocktail (CD40 ligand, IL-2, IL-4), or in the presence of purified CD10\textsuperscript{pos} or CD10\textsuperscript{neg} T\textsubscript{FH} (ratio 1:1). After 48 hours, B-cell apoptosis was assessed on CD20\textsuperscript{pos}CD2\textsuperscript{neg} B cells using active caspase-3 PE apoptosis kit (BD Biosciences).

Statistical analyses
Statistical analyses were performed with the GraphPad Prism software using non-parametric Wilcoxon test for matched pairs, or Mann Whitney U tests.
RESULTS AND DISCUSSION

Scattered PAX5\(^{\text{neg}}\)CD10\(^{\text{hi}}\) cells could be identified within neoplastic follicles in 16/19 FL samples and were characterized as CD3\(^{\text{pos}}\)CD4\(^{\text{pos}}\) T cells with a mature GC-TFH phenotype, \textit{i.e.} expressing high levels of CXCR5 and PD-1, together with ICOS and CXCL13 (Figure 1A-C). FL-infiltrating PD-1\(^{\text{neg}}\)CXCR5\(^{\text{neg}}\) non-TFH and PD-1\(^{\text{int}}\)CXCR5\(^{\text{nt}}\) pre-TFH, as well as blood CD4\(^{\text{pos}}\)CD45RA\(^{\text{neg}}\)CXCR5\(^{\text{pos}}\) T cells representing circulating memory FL-TFH did not express CD10 (Figure 1B and data not shown). To evaluate if CD10 expression on T FH was FL specific, we tested other B-cell lymphomas with a follicular growth pattern. Whereas cells expressing PD-1 were rarely detected in MCL samples, in agreement with a lack of PAX5\(^{\text{neg}}\)CD10\(^{\text{pos}}\) T cells, we confirmed the presence of numerous PD-1\(^{\text{pos}}\) cells forming rosettes around neoplastic cells in NLPHL but these cells were essentially CD10\(^{\text{neg}}\) (data not shown). The lack of MCL-infiltrating T FH and the absence of CD10 expression on PD-1\(^{\text{pos}}\) T cells in NLPHL microenvironment were confirmed by flow cytometry (data not shown). By contrast, we identified PAX5\(^{\text{neg}}\)CD10\(^{\text{pos}}\) T cells at a highly variable frequency within GC of all reactive lymphoid hyperplasia (RLH). These cells tended to concentrate at the periphery of GC and co-expressed CD3, ICOS, and CXCL13 (Figure S1A-B). To evaluate how CD10 expression could impact T FH function, we first sorted paired CD10\(^{\text{pos}}\) and CD10\(^{\text{neg}}\) T FH from tonsils and demonstrated similar expression of canonical T FH genes (Figure S1C). Moreover, CD10\(^{\text{pos}}\) and CD10\(^{\text{neg}}\) T FH displayed the same capacity to sustain immunoglobulin production by autologous purified B cells (Figure S1D). We next checked whether CD10\(^{\text{pos}}\) tonsil-T FH could be enriched for CD57- or Nrp-1-expressing cells (Figure S1E). As reported for Nrp-1\(^{13}\), CD10 was expressed by a higher proportion of CD57\(^{\text{pos}}\) than CD57\(^{\text{neg}}\) T FH (22.2% [6.1%-51.4%] \textit{versus} 15.6% [3.4%-35.2%], P<0.001, n=20). However, the expression of CD10 and Nrp-1 was mutually exclusive. Finally, CD10 was not expressed on CXCR5\(^{\text{hi}}\)ICOS\(^{\text{hi}}\)Foxp3\(^{\text{pos}}\)CD25\(^{\text{pos}}\) tonsil follicular regulatory T cells (T FR). Thus, CD10 identifies a distinct subset of fully functional GC-T FH in human RLH. Interestingly, conversely to \textit{NRP1} and \textit{CD57}, \textit{CD10/MME} was significantly upregulated in FL-T FH compared to tonsil-T FH, as revealed by GEP analysis. Accordingly, the percentage of CD10\(^{\text{pos}}\) T FH was increased in FL samples (6.17% [2.7%-22.9%]) compared to tonsils (1.56% [0.2%-10.1%], P< 0.001) (Figure 1D-E), raising the hypothesis that CD10\(^{\text{pos}}\) T FH expansion could play a specific role in FL pathogenesis.
CD10\textsuperscript{pos} FL-T\textsubscript{FH} were essentially Ki-67\textsuperscript{neg} but expressed high levels of HLA-DR indicating a non-proliferating but activated status (Figure S2). In addition, neither CD10\textsuperscript{pos} nor CD10\textsuperscript{neg} FL-T\textsubscript{FH} expressed TIM3 confirming that they represent fully activated and not exhausted T cells (data not shown). We next looked for the capacity of T\textsubscript{FH} to produce cytokines after \textit{in vitro} restimulation. Interestingly, CD10\textsuperscript{pos} tonsil-T\textsubscript{FH} produced similar levels of IL-21 and IFN-\gamma but significantly less IL-17A and more IL-4 than their CD10\textsuperscript{neg} counterpart, indicating that CD10 highlights a unique subset of T\textsubscript{FH} with specific functional properties (Figure 2A). Compared to tonsil-T\textsubscript{FH}, FL-T\textsubscript{FH} displayed an IL-21\textsuperscript{hi}IL-17\textsuperscript{lo} phenotype independently of their CD10 expression. However, CD10\textsuperscript{pos} FL-T\textsubscript{FH} exhibited a higher capacity to secrete IL-4 and a reduced capacity to secrete IFN-\gamma compared to CD10\textsuperscript{neg} FL-T\textsubscript{FH}. In agreement, IL-4-producing FL-T\textsubscript{FH} and IFN-\gamma-producing FL-T\textsubscript{FH} belonged to non-overlapping cell subsets (Figure 2B). These results prompted us to evaluate the direct role of CD10\textsuperscript{pos} versus CD10\textsuperscript{neg} FL-T\textsubscript{FH} on malignant FL B cells. CD10\textsuperscript{pos} FL-T\textsubscript{FH} were more efficient than their CD10\textsuperscript{neg} counterpart to support autologous malignant FL B-cell survival \textit{in vitro} (Figure 2C). Finally, the previously reported overexpression of TNFA by FL-T\textsubscript{FH}\textsuperscript{8} appeared uniformly supported by CD10\textsuperscript{pos} and CD10\textsuperscript{neg} FL-T\textsubscript{FH} subsets, containing both around 35% of TNF-\alpha–producing cells. Altogether, our data suggest that these two FL-T\textsubscript{FH} subsets may have different roles within FL cell niche. CD10\textsuperscript{pos} FL-T\textsubscript{FH} produce high amounts of IL-4 that could trigger B-cell activation, survival, and production of the Treg-recruiting chemokines CCL17 and CCL22\textsuperscript{8,18}. IL-4 also contributes to the polarization of tumor-associated macrophages\textsuperscript{19} that favor FL B-cell growth\textsuperscript{20,21}. In addition, CD10\textsuperscript{pos} FL-T\textsubscript{FH} exhibit a TNF\textsuperscript{hi}IFN\gamma\textsuperscript{lo} phenotype that could favor the induction and maintenance of a B-cell supportive lymphoid stroma network\textsuperscript{22}. Conversely, overexpression of IFN-\gamma by CD10\textsuperscript{neg} T\textsubscript{FH} could promote activation of cytotoxic CD8\textsuperscript{pos} T cells displaying efficient anti-tumor activity\textsuperscript{23} but also expression of stroma-derived indoleamine-2,3 dioxygenase that could inhibits not only T-cell but also malignant B-cell proliferation\textsuperscript{24}.

In conclusion, our study supports the current understanding of FL cell niche as an intricate network of cell interactions where each cell subset should be exquisitely characterized individually and in relationship with the other partners before being proposed as a biomarker or therapeutic target.
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AUTHORSHIP CONTRIBUTIONS
PAT designed and performed research, analyzed data, and wrote the paper. SH designed and performed research, analyzed data, and corrected the paper. CA, JM, MSB, RJ, JLP, CM, NM performed research. PG and KT supervised and designed research, analyzed data, and wrote the paper.

CONFLICTS OF INTEREST
SH was supported by the Novartis Foundation, Basel, Switzerland.
REFERENCES


**FIGURE LEGENDS**

**Figure 1: Characterization of CD10^{pos} T cells in B-cell lymphomas.**

(A) Double staining revealed the presence of scattered strong CD10^{pos} cells within FL neoplastic follicles. Numerous CD10^{pos} (brown) PAX5^{neg} (red) lymphocytes are evidenced (arrows). Original magnification: objective x20 (left panel) and x40 (right panel). (B) Representative characterization of ICOS, CD10, CXCR5 and PD1 staining by flow cytometry on CD3^{pos}CD4^{pos} viable cells from FL lymph nodes. (C) *In situ* characterization of CD10^{pos} T cells in reactive lymphoid hyperplasia (RLH) including reactive lymph nodes and tonsils, and B-cell lymphomas with follicular growth pattern (Mantle cell lymphoma, MCL; Nodular lymphocyte predominant Hodgkin lymphoma, NLPHL, and follicular lymphoma, FL). For each sample, the numbers of CD10^{pos}PAX5^{neg} and PD-1^{pos} cells found inside germinal centers were counted in 10 high power fields (x40), and the median of these 10 values was calculated. Data are expressed as the median [range] of the different samples tested. ND: not done. (D) Expression of MME/CD10 in CD3^{pos}CD4^{pos} CXCR5^{high}ICOS^{high}CD25^{neg} T_{FH} purified from 7 reactive tonsils and 7 FL samples (203435_s_at probeset of the GeneChip HG-U133 Plus 2.0 oligonucleotide arrays). ** P<0.01. (E) Frequency of CD10^{pos} T_{FH} among CD4^{pos} T cells in 15 FL LN and 25 reactive tonsil samples, evaluated by flow cytometry. *** P<0.001.

**Figure 2: Functional characterization of CD10^{pos} and CD10^{neg} FL-T_{FH}.**

(A) FL LN or reactive tonsil (Tons) cells were stimulated with PMA and ionomycin for 6 hours, and with brefeldin A for the last 4 hours of stimulation before intracytoplasmic detection of cytokines. The percentage of singlet viable CD10^{neg} (grey) or CD10^{pos} (white) viable T_{FH} producing IL-21, IL-17A, IL-4, and IFN-\(\gamma\) was determined. * P<0.05. ** P<0.01. (B) Representative plots of IFN-\(\gamma\)/IL-4, and CD10/TNF-\(\alpha\) staining on FL-T_{FH}. (C) Purified malignant FL B cells were cultured alone (Ø), with an activation cocktail (recombinant human CD40L, IL-2, and IL-4), or in the presence of autologous CD10^{neg} or CD10^{pos} T_{FH} at a 1:1 ratio for 48 hours. Representative plots of active caspase-3 staining gated on CD20^{pos} B cells (n=3).
### Figure 1

#### A

![Image of tissue samples with a circle and arrows indicating CD3<sup>+</sup>CD4<sup>+</sup> cells](image)

#### B

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#### D

![Graphs showing MME Affy signal (AU) and % CD10<sup>+</sup> TFH among CD4<sup>+</sup> T cells](image)

#### E

![Graphs showing % CD10<sup>+</sup> TFH among CD4<sup>+</sup> T cells in Tonsils and FL LN](image)

**Statistical Significance:**

- **D:** p < 0.01
- **E:** p < 0.001
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