



ORIGINAL ARTICLE

Monocytes and T cells cooperate to favor normal and follicular lymphoma B-cell growth: role of IL-15 and CD40L signaling

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Interleukin-15 (IL-15) has been extensively studied for its role in the survival and proliferation of NK and T cells through a unique mechanism of trans-presentation by producer cells. Conversely, whereas activated B cells have been described as IL-15-responding cells, the cellular and molecular context sustaining this effect remains unexplored. In this study, we found that, whereas human B cells could not respond to soluble IL-15, monocytes and lymphoid tissue-derived macrophages but not stromal cells efficiently trans-present IL-15 to normal B cells and cooperate with T-cell-derived CD40L to promote IL-15-dependent B-cell proliferation. Furthermore, CD40L signaling triggers a Src-independent upregulation of STAT5 expression and favors a Src-dependent phosphorylation of STAT5 in response to IL-15. In follicular lymphoma (FL), immunohistochemical studies reported a strong relationship between malignant B cells, infiltrating macrophages and T cells. We show here an overexpression of *IL-15* in purified tumor-associated macrophages, and *STAT5A* in purified tumor B cells. Moreover, FL B cells respond to IL-15 trans-presented by monocytes/macrophages, in particular, in the presence of CD40L-mediated signaling. This cooperation between IL-15 and CD40L reinforces the importance of tumor microenvironment and unravels a mechanism of FL growth that should be considered if using IL-15 as a drug in this disease.

Leukemia (2012) 26, 139–148; doi:10.1038/leu.2011.179; published online 26 July 2011

Keywords: B cells; follicular lymphoma; IL-15; macrophages; microenvironment

Introduction

Follicular lymphoma (FL) is the most frequent indolent lymphoma and results from the clonal expansion of germinal center (GC)-derived B cells that retain typical features of their normal counterpart.^{1,2} In particular, FL cells are strongly dependent on both B-cell receptor signaling and crosstalk with their supportive microenvironment, including specialized stromal cells, CD4^{pos} T cells and macrophages. In agreement, gene profiling analyses suggest that FL patient outcome is correlated with molecular signatures of non-malignant tumor-infiltrating cells.³ We previously demonstrated that bone marrow- and lymph node (LN)-derived stromal cells support FL B-cell migration and survival.⁴ Resident CD4^{pos} T cells are also supposed to have a crucial role in promoting malignant B-cell growth, especially through CD40-mediated signal,⁵ and we recently revealed that FL microenvironment is characterized by

a strong enrichment in follicular helper T cells (T_{FH}), the main CD40L-expressing T-cell subset.⁶ Finally, microarray gene expression studies, quantitative-PCR analyses and immunohistochemical experiments suggest that a high numbers of CD68^{pos} or CD163^{pos} tumor-associated macrophages (TAM) is of bad prognosis in FL patients treated with chemotherapy not associated with antibody-based immunotherapy.^{7–11} Moreover, in diffuse large B-cell lymphomas, the most frequent aggressive GC-derived lymphoma, monocytes deliver survival signals to malignant B cells at least in part through the production of B-cell activating factor (BAFF).¹² Nevertheless, how TAM favor FL B-cell growth *in situ* and what could be the cooperative role of neighboring T cells in this context have not been considered.

Interleukin-15 (IL-15), a pleiotropic cytokine mainly produced by monocytes/macrophages and mature dendritic cells,¹³ is a putative TAM-derived FL growth factor. In fact, whereas IL-15 has no stimulatory activity on resting B cells *in vitro*,¹⁴ several studies suggest that IL-15 synergizes with CpG oligonucleotides, B-cell receptor crosslinking or CD40 signaling to promote naive, GC and memory B-cell proliferation and differentiation.^{15–17} These data indicate that normal B cells should be considered as IL-15-responding cells, even if IL-15 producing cells as well as transduction signaling in this context have not been evaluated. Importantly, IL-15 has also been described as a growth factor in non-GC-derived mature B-cell neoplasias. In particular, paracrine IL-15 sustains survival and proliferation of chronic lymphocytic leukemia (CLL) B cells through a strong activation of both STAT5 and ERK1/2 pathways.^{18,19} Interestingly, the mitogenic and anti-apoptotic effects of IL-15 are enhanced when CLL B cells are pre-activated by CD40. These results are particularly relevant when considering that CLL cells, like FL cells, are strongly dependent on a specific microenvironment including CD4^{pos} T cells, stromal cells and myeloid cells.²⁰

These observations prompted us to speculate that monocytes/macrophages may support the growth of normal and FL B cells, and that IL-15 could be involved in this process. Given the role of stromal cells in lymphomagenesis, we also investigated their capacity to trans-present IL-15 to B cells. Finally, in agreement with the strong infiltration of FL tumors by T_{FH}, we have investigated the role and mechanism of T-cell-derived CD40 stimulation as an IL-15 cofactor.

Materials and methods

Cell samples

Subject recruitment followed institutional review board approval and written informed consent process according to the Declaration of Helsinki.

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Received 1 February 2011; revised 29 April 2011; accepted 6 June 2011; published online 26 July 2011

Primary cells were isolated from peripheral blood of adult healthy volunteers, from human tonsils collected from children undergoing routine tonsillectomy and from LN biopsies collected from patients with FL. Tonsil normal B cells and FL malignant B cells were purified as described.²¹ CD10^{pos} GC B cells were further selected from purified normal B cells using PC5-conjugated anti-CD10 monoclonal antibody (mAb, Beckman Coulter, Villepinte, France) and a FACSAria cell sorter (Becton Dickinson, San Diego, CA, USA). Tonsil T cells were purified using the Pan T Cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and peripheral blood NK cells using the StemSep Human NK Cell Enrichment Kit (StemCell Technologies, Vancouver, Canada). Peripheral blood monocytes were obtained by elutriation on a Beckman JE-6B apparatus (Beckman Coulter, Palo Alto, CA, USA) or using the Monocyte Isolation kit (Miltenyi Biotec), and culture experiments involving monocytes were performed in the presence of 20 ng/ml macrophage colony-stimulating factor (R&D Systems, Abingdon, UK). Tonsil- and FL-derived macrophages were cell sorted from CD19-depleted samples using PE-conjugated anti-CD36 mAb (BioLegend, San Diego, CA, USA). Purified cell fractions with at least 98% cell purity as evaluated by flow cytometry were used for further experiments. Tonsil macrophage depletion was performed using anti-CD36 mAb and goat anti-mouse IgG microbeads (Miltenyi Biotec).

B-cell growth assays

Cells were cultured at 7.5×10^5 cells/ml in RPMI1640 supplemented with 10% fetal calf serum, with or without 50 ng/ml IL-15 (R&D Systems) for specified time points. When indicated, purified B cells were simultaneously stimulated by a combination of 50 ng/ml CD40L and 5 μ g/ml enhancer polyhistidine mAb (R&D System), and/or cocultured with monocytes (1.5×10^5 /ml), or with autologous tonsil T cells (7.5×10^5 /ml) in the presence or absence of neutralizing anti-CD40L mAb or isotype-matched control (Becton Dickinson). B-cell growth was evaluated through the absolute quantification of TOPRO-3^{neg}CD19^{pos} viable B cells using TOPRO-3 (Invitrogen, Carlsbad, CA, USA), FITC-conjugated anti-CD19 mAb and FlowCount beads (Beckman Coulter). Flow cytometry analyses were performed on a FC500 flow cytometer (Beckman Coulter). Purified B cells were also cultured in 96-well plates with various combinations of monocytes, CD40L and IL-15 stimulation for 3 days before addition of 1 μ Ci/well tritiated thymidine (³H-TdR, Amersham, Pantin, France) for the last 16 h and quantification of the radioactivity on a scintillation analyzer.

B-cell proliferation and apoptosis

For proliferation assay, tonsil or FL cells were stained with 1 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) for 10 min at 37 °C before stimulation with various combinations of IL-15, CD40L and monocytes. After 3 days of culture, the absolute number of CFSE^{lo}TOPRO-3^{neg}CD19^{pos} viable proliferating B cells was determined by flow cytometry using FlowCount beads. B-cell apoptosis was analyzed using the PE-conjugated active caspase-3 apoptosis kit (Becton Dickinson) on selectively gated CD19^{pos} B cells.

Gene expression analysis

For real-time quantitative PCR (RQ-PCR), RNA was extracted using RNeasy Kit (Qiagen, Valencia, CA, USA), cDNA was generated using Superscript II reverse transcriptase (Invitrogen), and we used assay-on-demand primers and probes, and TaqMan

Universal Master Mix from Applied Biosystems (Foster City, CA, USA). Gene expression was measured using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) based on the $\Delta\Delta C_T$ calculation method. *ABL1* was previously determined as the appropriate internal standard gene for studying gene expression in purified B cells. In addition, we identified *ABL1*, *ELF1* and *GAPDH* as appropriate internal control genes with low variability among monocyte/macrophage samples using the TaqMan Express endogenous control plate (Applied Biosystems) and the geNorm software (<http://medgen.ugent.be/~jvdesomp/genorm/>).

For evaluating *STAT5A* expression, we used Affymetrix data of our previously described cohort of 16 purified FL B cells and 5 purified non-malignant B cells obtained from reactive LN.⁶ This cohort was further enriched by six samples from patients with newly diagnosed FL and five reactive LN samples that were used for CD19^{pos} B-cell purification, RNA extraction and qualification, biotinylated cRNA generation and hybridization to HUG133 plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA) exactly as described.⁶ Raw data were normalized with GC-RMA algorithm using Partek Genomics Suite (Partek Inc., St Louis, MO, USA).

IL-15/IL-15R α inhibition experiments

Monocytes were electroporated with 80 nM IL15, IL15RA or negative control small interfering RNA (Ambion, Foster City, CA, USA) using the Human Monocyte kit and the Y-01 program (Amaga AG, Köln, Germany) and were then cultured for 24 h before washing and addition of purified tonsil B cells. Electroporation efficiency was first measured by quantification of *IL15*, *IL15RA*, *ABL1*, *ELF1* and *GAPDH* mRNA by RQ-PCR. In addition, surface IL-15 and IL-15R α were detected using mAb to IL-15 (Diacclone, Besançon, France) and IL-15R α (eBioscience, San Diego, CA, USA) followed by staining with PE-conjugated goat anti-mouse secondary antibody (Beckman Coulter).

Analysis of STAT5 activation

For western blotting experiments, purified B cells were pre-incubated or not with CD40L and enhanced for 24 h in the presence of 10 μ M Src-kinase inhibitor PP2 (Merck Chemicals, Nottingham, UK) or dimethyl sulfoxide. Cells were then washed and left unstimulated for 5 h before direct IL-15 stimulation for 15 min. For coculture experiments, monocytes were pretreated with 500 IU/ml interferon (IFN) γ (R&D Systems) for 24 h before washing and incubation or not for 20 min with neutralizing anti-IL-15 mAb (10 μ g/ml, Diaclone) and anti-IL-15R α polyclonal Ab (1 mg/ml, R&D systems). Coculture with B cells was then initiated for 30 min and, when indicated, IL-15 was added for the last 15 min as a positive control. Cells were solubilised in lysis buffer and 30 μ g proteins were resolved by 10% SDS-PAGE before being transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Western blotting analysis was done using mouse anti-P-STAT5 (pY694, clone 47); mouse anti-STAT5 (clone 89, Becton Dickinson) and mouse anti- β -actin (Sigma, St Louis, MO, USA) primary Abs followed by secondary rabbit anti-mouse horseradish peroxidase-conjugated Ab. Visualization was performed using the enhanced chemiluminescence plus detection system (GE Healthcare, Little Chalfont, UK).

Statistical analysis

Statistical analyses were performed with GraphPad Prism software (GraphPad software, San Diego, CA, USA) using the

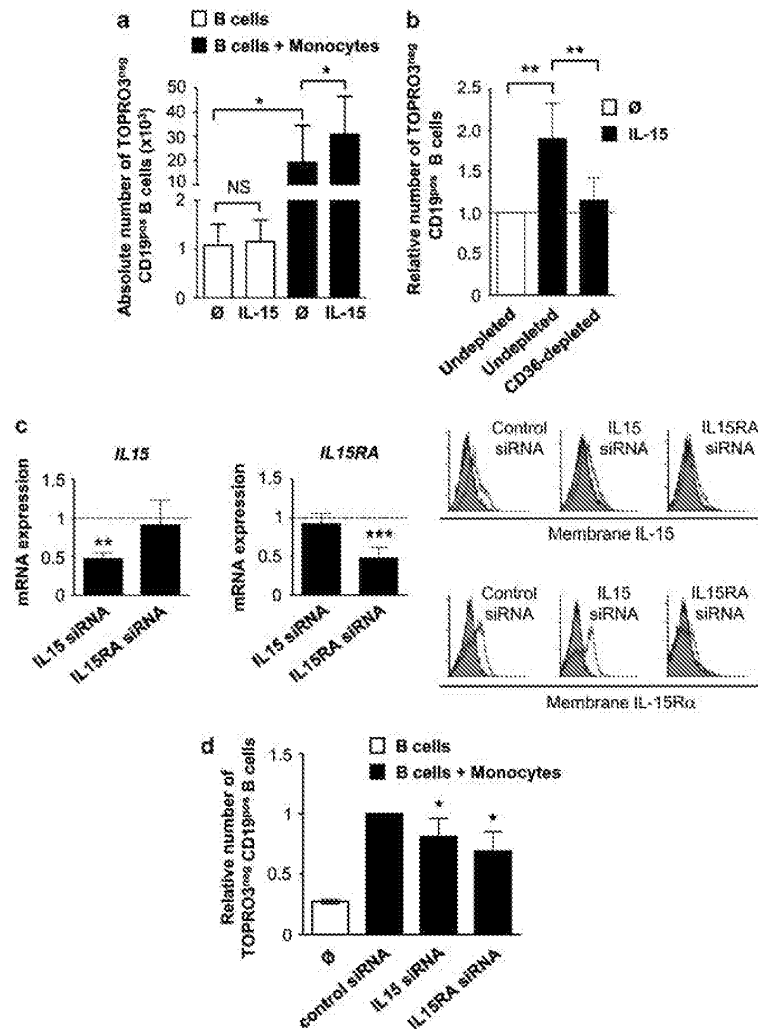


Figure 1 IL-15 is involved in the B-cell supportive effect of monocytes/macrophages. (a) Tonsil B cells were cultured alone or with purified monocytes in the presence or not (Ø) of IL-15. After 7 days, the number of viable B cells was quantified by flow cytometry using calibrated microbeads. Error bars represent s.d. from four independent experiments. (b) Whole tonsils (undepleted) or CD36-depleted tonsils were cultured for 4 days in the presence or absence of IL-15 before quantification of viable B cells. The arbitrary value of 1 was assigned to the number obtained with undepleted tonsils without IL-15. Error bars represent s.d. from six independent experiments. (c) *IL15* or *IL15RA* were targeted in monocytes with pre-designed small interfering RNA. Efficiency of gene silencing was validated 48 h after transfection at both mRNA (left) and protein (right) levels. RQ-PCR results were normalized to three housekeeping genes and compared with the level in monocytes transfected with control small interfering RNA, arbitrary assigned to 1. Data are expressed as the mean value $\pm$ s.d. from four independent experiments. For flow cytometry staining, black-filled histograms represent isotype-matched controls and shown is one representative of the three experiments. (d) Purified B cells were cultured alone or with monocytes transfected with indicated small interfering RNA, and the absolute number of viable B cells was evaluated after 4 days. The arbitrary value of 1 was assigned to the number obtained in coculture with monocytes transfected with control small interfering RNA. Error bars represent s.d. from four independent experiments. (* P <0.05; ** P <0.01; *** P <0.001).

Wilcoxon or Student's *t*-test for matched pairs or using Mann-Whitney *U*-test.

Results

Monocytes/macrophages efficiently trans-present IL-15 to B cells

Virtually all purified tonsil B cells died during short-term *in vitro* culture. Interestingly, circulating monocytes efficiently triggered

their survival, with an 18-fold increase in the number of viable B cells at day 7 (Figure 1a). Soluble IL-15 could not promote by itself the survival of purified B cells *in vitro*. However, IL-15 is known to be essentially trans-presented as a bioactive IL-15/IL-15R α complex by producing cells to neighboring cells bearing the IL-2/IL-15R β and the γ_c chains.^{22,23} In agreement, the protective effect of monocytes on B cells was significantly reinforced in the presence of exogenous IL-15, suggesting that resting B cells could directly respond to IL-15-presenting cells. To further evaluate the biological relevance of this process

within secondary lymphoid organs, we sought to identify a reliable marker for tonsil macrophage depletion. In fact, CD68 is predominantly an intracellular Ag, CD14 is poorly expressed on LN macrophages²⁴ and CD163 staining is too faint to allow an efficient Ab-based depletion. Conversely, the scavenger receptor CD36 is specifically and widely expressed on tonsil CD68^{pos} macrophages (Supplementary Figure S1). In agreement, purified CD36^{pos} cells exhibited a homogeneous CD68 expression whereas CD36-depleted cells contained no residual CD68^{pos} macrophages. Interestingly, IL-15 markedly enhanced the number of viable B cells when added on whole tonsil cell cultures, but this supportive effect was completely abolished when CD36^{pos} macrophages were selectively removed from tonsil cell suspensions (Figure 1b). We planned next to address the role of endogenous IL-15 in monocyte-driven B-cell survival. *IL15* and *IL15RA* were targeted by a gene-silencing approach leading to a $53 \pm 7\%$ and $54 \pm 10\%$ decrease in the corresponding gene expression, respectively (Figure 1c). These results were confirmed at the protein level by flow cytometry. *IL15* and *IL15RA* small interfering RNA exhibited no cross-reactivity, as revealed by the specific modulation of their respective mRNA. Conversely, targeting of *IL15RA* led to a substantial decrease in surface IL-15 expression confirming that membrane-bound IL-15 on monocytes was essentially loaded onto IL-15 α . The partial inhibition of IL-15 and IL-15 α expression significantly reduced the monocyte-dependent survival B cells ($20 \pm 12\%$ and $31 \pm 15\%$, respectively). Furthermore these effects are very similar to that previously reported for the blockade of BAFF signaling¹² (Figure 1d). Collectively, these results unequivocally demonstrated that, whereas B cells could not respond to soluble IL-15, both monocytes and lymphoid tissue macrophages could trans-present functional IL-15 to B cells as a part of their B-cell supportive capacity.

As LN-derived stromal cells support the survival of normal and FL B cells,^{4,21} we also checked their capacity to trigger a functional IL-15-dependent signal. We previously demonstrated that tumor necrosis factor- α (TNF- α) and lymphotoxin- α 1 β 2 (LT) reinforced the capacity of stromal cells to support B-cell survival.⁴ Interestingly, stimulation of stromal cells by TNF/LT strongly increased IL-15/IL-15 α expression (Supplementary Figure S2). However, whereas the combination of anti-IL-15 and anti-IL-15 α neutralizing mAb reduced $58 \pm 5\%$ of the survival effect of TNF/LT-pretreated LN stromal cells on NK cells, their capacity to sustain normal B-cell survival was not impaired. Same results were obtained for both B cells and NK cells using small interfering RNA strategy (data not shown). LN stromal cells could thus trans-present functional IL-15 but, conversely to monocytes/macrophages, this property was not involved in their B-cell supportive capacity.

T-cell-derived CD40L confers IL-15 sensitivity to B cells

IL-15 was previously described as a B-cell costimulatory agent in the presence of mitogenic signals.^{15,16} In agreement, IL-15 cooperated with CD40L to increase tonsil B-cell growth (Figure 2a). Interestingly, this was also the case for purified GC B cells, the normal counterpart of FL B cells (Supplementary Figure S3). Of note, IL-15 strengthened CD40L-dependent B-cell proliferation without any reduction of B-cell apoptosis compared with CD40L alone (data not shown). We then performed reconstitution experiments with autologous purified tonsil T cells that are known to contain a high percentage of CD4^{pos}CD40L^{pos} T_{FH} cells.⁶ As described for soluble CD40L molecule, coculture with autologous T cells increased B-cell growth, and IL-15 further reinforced this supportive effect

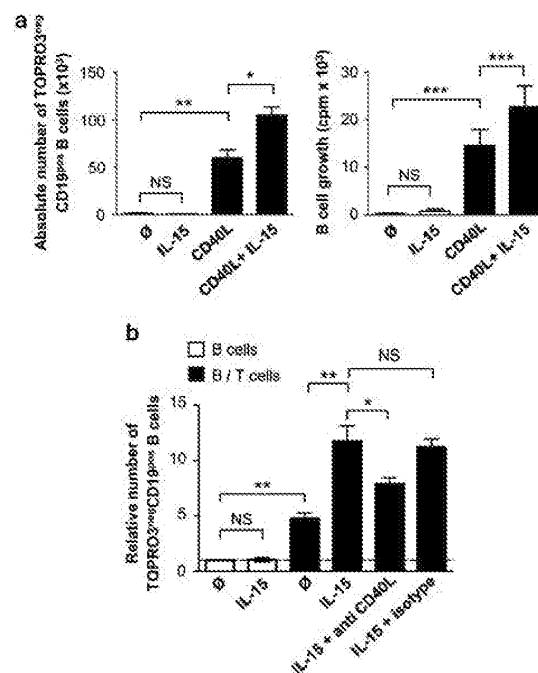


Figure 2 T-cell-derived CD40L promotes IL-15-dependent B-cell proliferation. (a) The growth of purified B cells in the presence or not ($\emptyset$) of CD40L and/or IL-15 was assessed by absolute quantification of viable B cells by flow cytometry at day 7 (left, $n=3$) and by incorporation of tritiated thymidine at day 3 (right, $n=6$). (b) Purified tonsil B cells were cultured with or without purified autologous tonsil T cells in the presence or not ($\emptyset$) of IL-15, neutralizing anti-CD40L mAb or unrelated isotype-matched control. After 4 days, the number of TOPRO-3^{neg} CD19^{pos} viable B cells was quantified by flow cytometry and compared with that of B cells cultured without any stimulation, arbitrary assigned to 1. Error bars represent s.d. from three independent experiments. (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; NS, not significant).

(Figure 2b). Moreover, a neutralizing mAb targeting CD40L reduced $54 \pm 12\%$ of this additional IL-15 mediated B-cell growth. Altogether, these results indicated that secondary lymphoid organ T cells conferred IL-15 sensitivity to B cells at least in part through CD40L signal.

We next decided to unravel the molecular mechanisms of this T-cell-based licensing. The increased sensitivity of CD40-activated B-CLL cells to mitogenic effects of IL-15 was previously associated with an increased expression of IL-15 α and IL-2R β .¹⁹ However, the level of IL-15 α was not affected by CD40 triggering in normal B cells at both mRNA and protein levels. In addition, the faint upregulation of IL-2R β and IL-2R γ was associated with a very limited increase in their capacity to bind exogenous IL-15 (Supplementary Figure S4). We thus studied the early signaling events triggered by CD40L and IL-15. First, whereas IL-15 induced only a weak STAT5 phosphorylation in resting B cells, CD40L-pre-activated B cells exhibited a strong STAT5 phosphorylation in response to IL-15 (Figure 3a). Recently, CD40L was shown to induce, in B cells, the expression and activation of c-Src tyrosine kinase that could associate with and phosphorylate STAT5a and STAT5b.^{25–27} We thus hypothesized that the cooperation of CD40L and IL-15 for the induction of P-STAT5 could be mediated through c-Src. Indeed, PP2, a selective inhibitor of c-Src, completely abolished

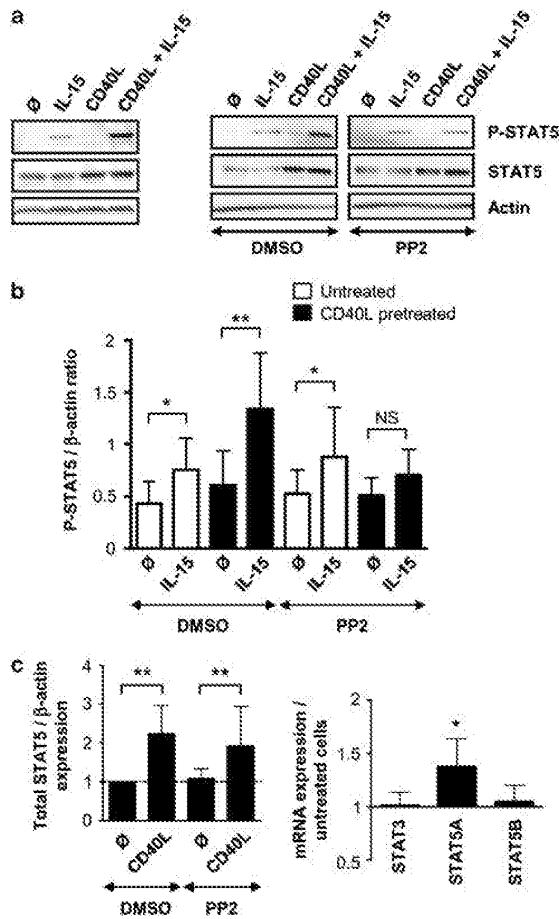


Figure 3 CD40L signaling favors STAT5 activation in B cells. (a) Purified tonsil B cells were pretreated or not with CD40L for 24 h with no inhibitor (left) or in the presence of the Src-kinase inhibitor PP2 or its solvent (dimethyl sulfoxide) (right). Cells were then serum starved and stimulated or not with IL-15 for 15 min. Proteins were reduced in SDS-PAGE, and STAT5, P-STAT5 and β -actin expression was determined by western blot. (b) P-STAT5 expression was quantified by densitometric scanning of western blot bands and normalized with β -actin as a loading control. Error bars represent s.d. from four independent experiments. (c) (Left) Purified tonsil B cells were pretreated or not ($\emptyset$) with CD40L for 24 h in the presence of PP2 or its solvent (dimethyl sulfoxide). Total STAT5 expression was then quantified and normalized with β -actin expression as a loading control. Error bars represent s.d. from four independent experiments. (Right) Purified tonsil B cells were treated or not for 24 h with CD40L before quantification of *STAT3*, *STAT5A* and *STAT5B* mRNA by RQ-PCR. Each sample was normalized to *ABL1* and compared with untreated cells. Error bars represent s.d. from six independent samples. (* $P < 0.05$; ** $P < 0.01$; NS, not significant).

the increase in IL-15-dependent STAT5 phosphorylation induced by CD40L prestimulation (Figures 3a and b). Interestingly, a deeper examination of STAT5 expression revealed that CD40L also promoted an IL-15-independent overexpression of total STAT5 in B cells, as evidenced after normalization to β -actin levels (Figure 3c). This increase was associated with a specific transcriptional upregulation of *STAT5A* mRNA, whereas *STAT5B* and *STAT3* expressions were not modified after CD40

engagement. Moreover, inhibition of Src kinase did not affect the induction of STAT5 expression by CD40L signaling. Overall, these results demonstrated that T-cell-derived CD40L increased B-cell response to IL-15 by two independent mechanisms, that is, the Src-independent IL-15-independent transcriptional upregulation of STAT5 expression and the Src-dependent increase of IL-15-driven STAT5 phosphorylation.

Monocyte-derived IL-15 and T-cell-derived CD40L cooperate to promote B-cell growth

To further confirm that monocytes/macrophages could trans-present IL-15 directly to B cells, we tried to explore endogenous IL-15 signaling during coculture. For that purpose, we used IFN- γ -activated monocytes that displayed a high expression of IL-15/IL-15R α , as previously reported²⁸ (Figure 4a). When resting B cells were cocultured with IFN- γ -activated monocytes expressing IL-15, we could not detect STAT5 phosphorylation whereas P-STAT5 was strongly induced by exogenous soluble IL-15 (Figure 4b). Importantly, P-STAT5 remained undetectable in purified monocytes stimulated *in vitro* with IL-15 in our conditions, suggesting that B cells are the only IL-15-responding cells in this coculture system (data not shown). Interestingly, when B cells were prestimulated by CD40L, transient coculture with IFN- γ -activated monocytes was sufficient *per se* to induce STAT5 phosphorylation and this STAT5 activation was completely abrogated by a combination of anti-IL-15 and anti-IL-15R α neutralizing mAb. Collectively, these results strongly argued for a direct trans-presentation of IL-15 to responding B cells by activated monocytes.

Within normal and FL LN, B cells are found in close interaction with both CD40L^{pos} T cells and IL-15-expressing macrophages making it mandatory to explore the potential synergy between these two signals for B-cell growth. We demonstrated that CD40L signaling greatly improved monocyte-dependent B-cell growth and that IL-15 further increased by twofold thymidine incorporation at day 3 in this tripartite culture system (Figure 4c). The same results were obtained using absolute viable B-cell count. To investigate how trans-presented IL-15 modulated B-cell proliferation and/or survival, we simultaneously evaluated the number of CFSE^{lo} proliferating B cells and the number of caspase-3^{pos} apoptotic B cells. Whereas CD40L/CD40L strongly promoted both B-cell proliferation and survival, IL-15 presented by monocytes further reinforced B-cell proliferation without additional anti-apoptotic effect.

IL-15 stimulates primary FL B-cell growth in the presence of monocytes and CD40L signaling

Malignant FL B cells are admixed with CD4^{pos}CD40L^{pos} T cells within invaded LN. Moreover, gene expression profiling of FL cell niche reveals a strong T_{FH} signature,⁶ and a preferential intrafollicular distribution of CD4^{pos} T cells was described as a predictor of worse prognosis.^{29,30} Finally, a high number of CD68^{pos} TAM predicts an unfavorable outcome in FL patients.^{7,30} These observations raise the hypothesis that monocyte-derived IL-15 and T-cell-derived CD40L could cooperate to promote malignant B-cell growth in FL.

In order to test this hypothesis, we first purified CD36^{pos} macrophages from tonsils and FL samples and compared them for IL-15 expression by RQ-PCR. FL TAM significantly overexpressed *IL-15* when compared with tonsil macrophages and with purified blood monocytes (Figure 5a). In parallel, microarray experiments were conducted on CD19^{pos} B cells purified from FL and reactive LNs and revealed an upregulation

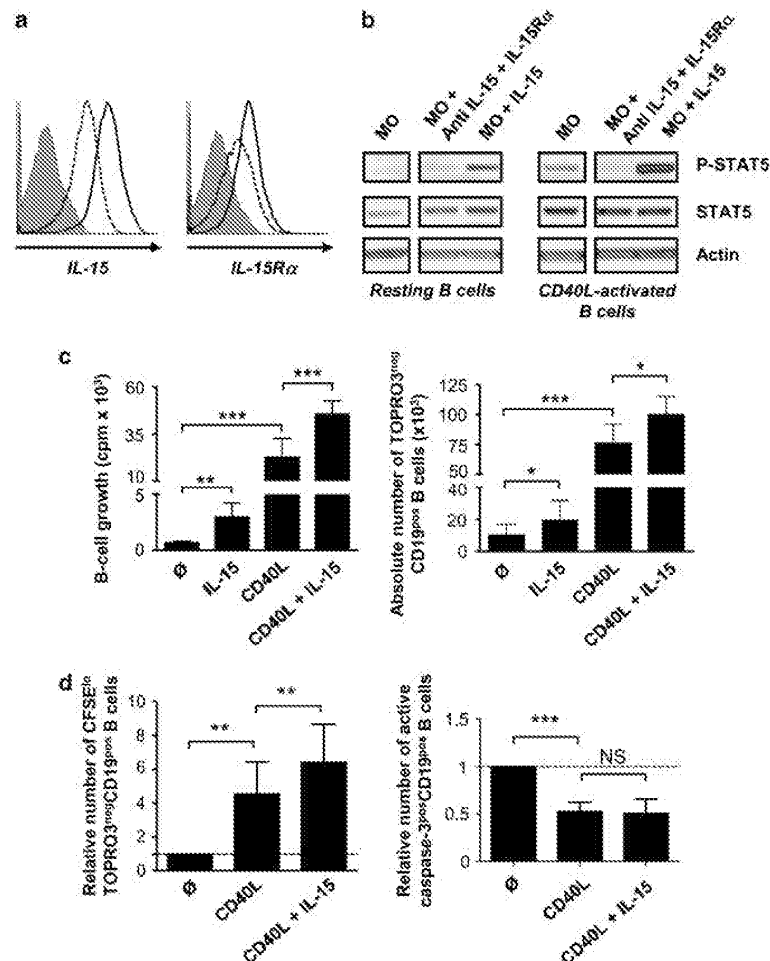


Figure 4 Monocyte-derived IL-15 and T-cell-derived CD40L cooperate to increase tonsil B-cell growth. (a) Monocytes were stimulated for 24 h with or without IFN γ before staining for IL-15 and IL-15R α expression. Filled histograms represent isotype-matched control, dashed lines untreated monocytes (MOs) and full lines IFN γ -treated MOs. Shown is one representative experiment out of three. (b) Purified MOs were pretreated with IFN γ and tonsil B cells were pretreated or not with CD40L for 24 h. Resting or CD40L-activated B cells were then cocultured with MOs for 15 min in the presence or not of neutralizing antibodies to IL-15 and IL-15R α or IL-15. STAT5, P-STAT5 and β -actin expressions were then determined by western blot. Shown is one representative from three independent experiments. (c) Purified tonsil B cells were cocultured with purified MOs in the presence or not ($\emptyset$) of IL-15 and CD40L and their growth was assessed by incorporation of tritiated thymidine at day 3 (left, $n=6$) and by absolute quantification of viable B cells by flow cytometry at day 7 (right, $n=4$). (d) (Left) Purified tonsil B cells were stained with CFSE and cultured with purified MOs in the presence or not ($\emptyset$) of IL-15 and CD40L. After 3 days, the number of CFSE^{lo}-proliferating viable B cells was determined by flow cytometry and compared with the number of B cells obtained in coculture with MOs without IL-15 and CD40L, arbitrary assigned to 1. Error bars represent s.d. from six independent experiments. (Right) Purified tonsil B cells were cultured with purified MOs in the presence or not ($\emptyset$) of IL-15 and CD40L. After 24 h, the number of apoptotic B cells was evaluated by active caspase-3 staining in the presence of calibrated microbeads, and compared with the number of apoptotic B cells cultured with MOs, arbitrary assigned to 1. Error bars represent s.d. from six independent experiments. (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; NS, not significant).

of STAT5A in malignant B cells. We confirmed this result by RQ-PCR using cell-sorted CD19^{pos}CD10^{pos} GC-derived B cells (Figure 5b). These data suggested that IL-15-overexpressing macrophages coexist with STAT5A-overexpressing B cells within FL cell niche.

We then tried to evaluate the functionality of IL-15 signaling in FL. Owing to the lower percentage of CD68^{pos} macrophages in the vast majority of FL samples compared with chronically inflamed tonsils,⁷ IL-15 alone had no *in vitro* supportive effect on FL B cells when tested on whole tissue culture (data not shown). We thus decided to undertake CD40L-mediated

proliferation assay as described above for normal B cells. Interestingly, we confirmed our previous study showing that a small but significant fraction of FL B cells were committed to cell division in response to CD40 triggering²¹ and shown that IL-15 strengthened this effect (Figure 5c). In addition, purified FL B cells exhibited strong similarities with purified normal B cells including a lack of response to free IL-15 that could be restored after CD40L stimulation without any upregulation of the expression of IL-15R α , IL-2R β or IL-2R γ (Supplementary Figure S3). Similarly, IL-15 promoted malignant B-cell proliferation in the presence of monocytes, and CD40L signaling further

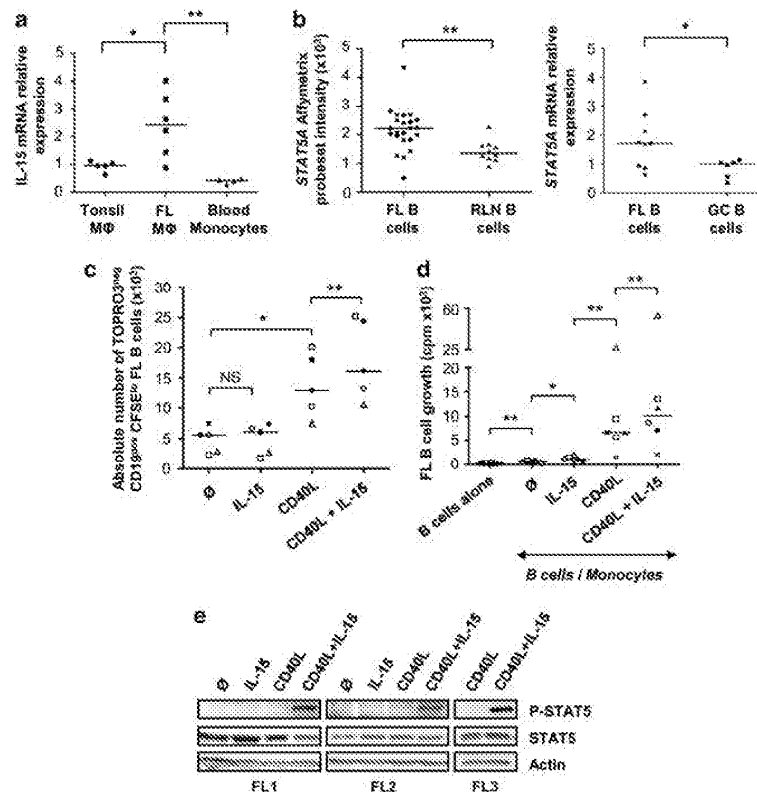


Figure 5 IL-15 pathway within FL cell niche. (a) *IL15* mRNA expression was measured by RQ-PCR in sorted tonsil ($n=5$) and FL ($n=6$) macrophages (MΦ), as well as in blood monocytes ($n=4$). Each sample was normalized to three housekeeping genes and the arbitrary value of 1 was assigned to the median expression in tonsil MΦ. (b) (Left) LN biopsies from 23 FL patients at diagnosis and 10 non-malignant inflamed LN were collected. CD19^{pos} B cells from FL (FL B) or reactive LNs (RLN B) were purified using anti-CD19 microbeads. RNAs were extracted and biotinylated cDNAs were amplified and hybridized to HGU133 Plus 2.0 arrays. Raw data were normalized using GC-RMA algorithm. (Right) STAT5A expression was determined by RQ-PCR in purified FL B cells ($n=9$; FL B cells) or CD19^{pos}CD10^{pos} GC-derived tonsil B cells ($n=6$; GC B cells). Each sample was normalized to *ABL1* and the arbitrary value of 1 was assigned to the median expression in GC B cells. (c) FL samples were stained with CFSE and cultured for 3 days in presence or not (Ø) of IL-15 and/or CD40L. The number of proliferating viable B cells was then determined by flow cytometry using calibrated microbeads. Each symbol corresponds to an individual patient sample. (d) Purified FL B cells were cultured alone or with elutriated monocytes in the presence or not (Ø) of IL-15 and CD40L, and tritiated thymidine incorporation was evaluated at day 3. Each symbol corresponds to an individual patient sample. (e) Purified FL B cells from three different patients were pre-treated or not with CD40L for 24 h before serum starvation and stimulation or not with IL-15 for 15 min. Proteins were then reduced in SDS-PAGE, and STAT5, P-STAT5 and β -actin expressions were determined by western blot. (* $P<0.05$; ** $P<0.01$; NS, not significant).

collaborated with monocytes to drive IL-15-dependent FL B-cell growth (Figure 5d). Finally, we checked STAT5 activation in purified FL cells by a western blotting approach that revealed a strong phosphorylation of STAT5 in response to IL-15 only in CD40L-prestimulated FL B cells (Figure 5e). Collectively, these results suggested that FL B cells could respond to IL-15 presented by monocytes/macrophages and that CD40L increased their sensitivity to IL-15 without inducing the expression of IL-15 receptor components.

Discussion

IL-15 has been extensively studied for its capacity to sustain differentiation, survival and proliferation of NK and T cells through a unique cell contact-dependent mechanism of trans-presentation involving the high affinity receptor IL-15R α that is associated with IL-15 at the surface of producer cells.¹³ The main objective of this study was to evaluate the capacity of

IL-15-presenting cells to support the growth of normal and malignant B cells and their possible cooperation with combinatorial signals occurring within secondary lymphoid organs, in particular the T-cell-dependent CD40/CD40L crosstalk.

Recent studies have demonstrated that IL-15 trans-presentation should work in conjunction with other environmental signals, such as CD27/CD70 and CD137/CD137L, to adequately support CD8^{pos} T-cell homeostasis.³¹ In addition, free IL-15 was previously shown to favor the growth of activated unlike resting B cells, even if no mechanistic study was provided.^{15,16} Moreover, ectopic expression of constitutively activated STAT5 in primary human B cells resulted in prolonged B-cell survival and proliferation only in the presence of CD40L signal or constitutive NF- κ B activation.³² Our detailed analysis of CD40L- and IL-15-driven signals provides clues to their respective roles in normal B cell behavior. Intriguingly, our study provides evidence that the observed synergy between IL-15 and CD40L in normal and FL B cells is based on a different mechanism from that reported in CLL B cells, which involved an

upregulation of IL-15R α expression.¹⁹ We demonstrated for the first time that CD40L drives an increase in the expression of total STAT5 in tonsil B cells. This unexpected result corroborates with the identification of a small population of activated tonsil GC B cells displaying an increased expression of total STAT5 and committed to memory cell fate. Moreover, activation of STAT5 was involved in the expansion of CD40L-stimulated B cells.³³ By contrast, we did not observe any upregulation of total STAT5 in malignant FL B cells following CD40 engagement but primary FL B cells expressed higher amount of STAT5A than reactive LNs B cells, a result further confirmed using cell-sorted CD19^{pos}CD10^{pos} GC-derived B cells. This result is in agreement with a strong infiltration of neoplastic follicles by CD40L^{pos} T_{FH} that could provide FL B cells with activation signals *in vivo*. Interestingly, both STAT5A overexpression and STAT5 activation were recently reported in FL samples.^{34,35}

Besides its effect on STAT5 expression, our work reveals that CD40L cooperates with IL-15 to favor STAT5 activation through a transduction pathway involving Src tyrosine kinase. Several other mechanisms could support the interconnection between IL-15 and CD40L signals. Indeed, cytokine-mediated STAT5 phosphorylation drives NF- κ B activation in the pro B-cell line Ba/F3 as well as in Hodgkin's lymphoma B cells.^{32,36} Moreover, non-phosphorylated STAT5 also favors NF- κ B activity proffering a model where unactivated STAT5 contributes to the regulation of lymphoid cell growth.³⁷ These results suggest that CD40L and IL-15 collaborate to support normal and lymphoma B-cell growth through a complex set of complementary transduction pathways.

An intriguing observation was that, in the presence of CD40L signaling, IL-15 essentially increases B-cell proliferation rather than B-cell survival. Interestingly, IL-15-mediated support of NK cell survival versus proliferation was recently demonstrated to be separable functions *in vivo*.³⁸ Similarly, in human intra-epithelial lymphocytes from patients with celiac disease, IL-15 drives cell proliferation and cell survival using independent signaling pathways.³⁹ Another IL-2 family member involved in B-cell proliferation and survival is IL-21, which displays positive or negative effects on B-cell fate depending on their activation status and developmental stage and on the presence of costimulatory signals.⁴⁰ IL-21 exhibits growth activity on multiple myeloma and Hodgkin's lymphoma whereas it promotes apoptosis of B cells from CLL, diffuse large B-cell lymphomas and FL through the preferential activation of STAT3. Of note, we never pointed out P-STAT3 in normal or FL B cells after exposure to IL-15 (data not shown).

With regard to IL-15 delivering cells, our work supports the hypothesis that IL-15 trans-presentation could have distinct functions when provided by different cell types. In particular, dendritic cell and macrophage subsets differentially regulate memory and effector CD8^{pos} T-cell differentiation, maintenance and proliferation *in vitro* and *in vivo*.^{31,38} In addition, stromal cells could differentially trans-present IL-15 depending on their origin and activation status. As an example, functional IL-15 is constitutively expressed on spleen-derived unlike skin-derived fibroblasts, and dermal fibroblasts could sustain IL-15-dependent T-cell proliferation only after stimulation by TNF.^{41,42} In our study, we demonstrate that LN-derived stromal cells could efficiently trans-present IL-15 to NK cells but not to B cells. In addition, even if bone marrow infiltration is a common feature in FL, resting bone marrow-derived mesenchymal stromal cells do not express membrane IL-15/IL-15R α complexes (data not shown). Finally, LN- and bone marrow-derived stromal cells do not provide B cells with functional IL-15 even after the concomitant upregulation of IL-15 and IL-15R α by TNF/LT

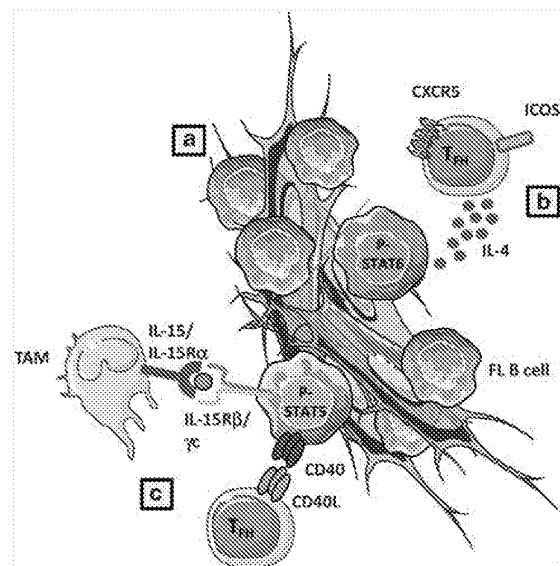


Figure 6 Model for cell interactions within FL malignant niche. (a) Stromal cells recruit and sustain the survival of malignant FL B cells. Their supportive properties are strongly influenced by the local cytokine context, in particular by TNF and LT that are overexpressed within FL microenvironment and promote stromal cell differentiation into functional FRC-like cells (4, 21). (b) FL LNs are characterized by a strong infiltration by CXCR5^{hi}ICOS^{hi}CD4^{pos} T_{fh}. FL T_{fh} specifically overexpress IL-4, which induces STAT6 activation in neighboring malignant B cells (6). (c) FL TAM overexpress IL-15 that triggers STAT5-dependent FL B cell activation. Interestingly, infiltrating T_{fh} could cooperate with this IL-15-mediated signal, in particular through a CD40L-dependent transcriptional induction of STAT5 that is found upregulated in malignant B cells. Figure prepared using tools from Servier Medical Art (<http://www.servier.fr/servier-medical-art>).

stimulation. We cannot exclude that follicular dendritic cells could trans-present IL-15 to normal and malignant GC-derived B cells *in vivo*, but fully functional human follicular dendritic cells could not be maintained *in vitro* thus preventing such evaluation.

Conversely, we demonstrated for the first time that purified TAM from patients with FL overexpressed IL15. Several factors could contribute to this induction. First, our previous study underlined an upregulation of IFNG in FL microenvironment,²¹ and IFN γ is an inducer of IL-15 in human monocytes.²⁸ Second, CD40L^{pos} T cells are one of the main non-malignant cell partners for FL B cells, and CD40 triggering increases IL-15R α expression on myeloid cells.⁴³ Finally, TNF is also upregulated within FL cell niche²¹ and initiates an interferon-mediated autocrine loop in primary macrophages associated to the expression of interferon-response genes, including STAT1.⁴⁴ Altogether, the promoters of both IL-15 and IL-15R α were shown to contain binding sites for IFN-response factors and NF- κ B, and corresponding stimuli were strongly expressed within chronically inflamed lymphoid organs and FL biopsies. IL-15 is thus a good candidate as a macrophage-derived FL growth factor that could contribute to the poor prognosis value of TAM in FL patients treated with chemotherapy. Interestingly, the presence of STAT1^{pos}CD68^{pos} macrophages in the vicinity of FL tumor cells was also associated with unfavorable outcome.⁴⁵ A recent study has underlined both the role of TAM in the engraftment of B-cell lymphomas in an elegant mouse xenograft model and their major role in anti-lymphoma activity of therapeutic

antibodies through phagocytosis of malignant B cells.⁴⁶ Additional transcriptomic and functional studies targeting tonsil-versus FL-derived macrophages are ongoing and will be very helpful to further unravel the complexity of normal versus lymphoma B-cell niches.

Collectively, our results provide new insights into the reciprocal role of macrophages and T cells in normal and malignant B-cell fate within secondary lymphoid organs. Importantly, FL constitutes a unique malignant setting where TAM overexpress IL-15, malignant cells overexpress STAT5 and tumor cell niche is heavily infiltrated by T_{FH} cells expressing an IL-15 costimulatory signal (Figure 6). The cooperation between the CD40L and the IL-15-mediated signals should be taken carefully into account when considering the pathogenesis of FL and the possibility to use IL-15 as a new drug to boost NK cell-dependent immunity in combination with therapeutic mAb.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Yannick Jacques (INSERM U892, Nantes) for helpful discussion, Christophe Ruaux for providing tonsil samples, the 'réseau clinique BREHAT' as well as the 'Centre de Ressources Biologiques-Santé' of Rennes. This work was supported by research grants from the Institut National du Cancer (INCa libre 2005; PL070 and INCa libre 2006; PL06-10), the Association pour le Développement de l'Hémo-Oncologie (ADHO) and the Ligue Régionale Contre le Cancer. GE was supported by a PhD studentship from the Association pour la Recherche contre le Cancer (ARC) and the Société Française d'Hématologie (SFH).

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Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)