Reduced lymphotoxin-beta production by tumour cells is associated with loss of follicular dendritic cell phenotype and diffuse growth in follicular lymphoma

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Abstract

Cytokine production is essential for follicular dendritic cell (FDC) maintenance and organization of germinal centres. In follicular lymphoma, FDCs are often disarrayed and may lack antigens indicative of terminal differentiation. We investigated the in situ distribution of cells producing lymphotoxin-beta (LTB), lymphotoxin-alpha (LTA), and tumour necrosis factor-alpha (TNFA) transcripts in human reactive lymph nodes and in follicular lymphomas with follicular or diffuse growth pattern. LTB was the cytokine most abundantly produced in germinal centres. LTB was present in nearly 90% of germinal centre cells whereas LTA and TNFA were detected in 30 and 50%, respectively. Moreover, the amount of LTB expressed in reactive germinal centre cells was 80-fold higher than that of LTA and 20-fold higher than that of TNFA. LTB-positive cells were more numerous in the germinal centre dark zone, whereas expression of the FDC proteins CD21, CD23, VCAM, and CXCL13 was more intense in the light zone. Tumour cells of follicular lymphomas produced less LTB than reactive germinal centre cells. The results of the in situ study were confirmed by RT-PCR; LTB was significantly more abundant in reactive lymph nodes than in follicular lymphoma, with the lowest values detected in predominantly diffuse follicular lymphoma. In neoplastic follicles, low production of LTB by tumour B cells was associated with weaker expression of CD21/CD23 by FDCs. Our findings detail for the first time the distribution of LTA-, LTB-, and TNFA-producing cells in human reactive germinal centres and in follicular lymphoma. They suggest the possibility that impaired tumour-cell LTB production may represent a determinant of FDC phenotype loss and for defective follicular organization in follicular lymphoma.

Keywords: lymphotoxin-beta lymphotoxin-alpha; TNF-alpha; lymph nodes; lymphoid hyperplasia; follicular lymphoma; follicular dendritic cell; in situ hybridization

Introduction

Organization of B cell follicles requires a mutually dependent collaboration of B cells and follicular dendritic cells (FDCs). While FDCs provide signals to sequester and maintain B cells within B cell follicles (CXCL13), B cells are essential for FDC maintenance by providing stimulation with tumour necrosis factor-alpha (TNFA) and lymphotoxin (LT) [1]. Mature FDCs derive from perivascular mural cells expressing platelet-derived growth factor receptor-beta and alpha smooth muscle actin. Perivascular mural cells also give rise to fibroblastic reticular cells (FRCs) and marginal reticular cells (MRCs) [2]. FDCs, FRCs, and MRCs have distinct morphologies and functions, but share common markers, and are probably strongly correlated [3].

Receptors for LT and TNF (LTβR and TNFR1) are highly expressed on FDC-precursors. Mice deficient in LTβR, TNFR1, or their ligands suffer from complex pathological phenotypes of lymphoid organs which may be devoid of FDCs [4–11]. It is well-established that LT and/or TNFα play a crucial role for maintenance of most FDC traits [12,13]; they
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consist of CXCL13 production [14–16], expression of ICAM1, VCAM1, and MadCAM1 [17,18], expression of complement receptors 1 and 2 (CR1 and CR2), and expression of Fc receptors for IgG, IgE, IgA, and IgM [18]. Inhibition of LT leads to the disappearance of multiple markers on FDCs. Inhibition of the TNFα pathway is also effective, but only in the absence of a strong antigenic response.

Most of the information concerning interactions between cytokines and FDCs were obtained in murine models or in in vitro studies. Until recently, visualization of cytokine-producing cells in tissue sections was extremely difficult. The development of RNA in situ hybridization (ISH) with the RNAscope technology has provided a major advance [19]. In fact, this technology is highly specific, and allows identification of cytokine-producing cells in tissue sections; moreover, the number of cytoplasmic dots per cell represents an approximate quantitative indication of the amount of cytokine RNA.

In the present study, we have investigated the tissue distribution of cells producing lymphotoxin-alpha (LTA), lymphotoxin-beta (LTB), and TNFA RNA in human reactive B cell follicles and in follicular B cell lymphomas (FL). Cytokine production was compared with expression of molecules indicative of FDC differentiation (CD21, CD23, VCAM, and CXCL13). Our findings indicate that there is a strict correlation between LTB production and FDC differentiation in reactive follicles and also in FL.

Materials and methods

Patients

Twenty-six lymph nodes, removed for diagnostic purpose at the Sant’Andrea Hospital of Rome, were investigated. Eleven cases (8M:3F; mean age 58 years) were diagnosed as reactive lymphadenitis (RL) with follicular hyperplasia. Lymph node site was: cervical (two), axillary (four), mediastinal (three), inguinal (one), and supraclavicular (one); the mean size of the lymph nodes was 1.75 cm. Eight cases were diagnosed as follicular lymphoma with predominantly follicular growth pattern (5M:3F; age-range 51–82 years (mean age 66 years), size range 1.8–3.8 cm (mean size 2.6 cm). Grading: G1/G2 (n = 8). Lymph node site: inguinal (n = 4), axillary (n = 2), mediastinal (n = 1), and cervical (n = 1). Seven cases were classified as predominantly diffuse FL (3M:4F) 49–68 years (mean age 58 years), lymph node size 2.5–4.5 cm (mean 3.43 cm). Grading: G1/G2 (n = 7). Lymph node site: inguinal (n = 6), cervical (n = 1). The study was performed in accordance with the Helsinki Declaration. Institutional Review Board approval was obtained (EC n° 168/SA/2003).

Tissue samples and immunostaining

Lymph nodes were formalin-fixed and paraffin-embedded (FFPE). Paraffin sections were immunostained for CD21 (clone 1F8), CD23 (clone MHM6), Bcl-2 (clone 124), CD10 (clone 56C6), Ki-67 (clone Mib-1) (Dako, Denmark), Stathmin (clone SP49; Spring Bioscience, Pleasanton, CA USA), VCAM (Clone VCAM1/843; Scytek Laboratories, Logan, UT, USA), and CXCL13 (Polyclonal Goat; R&D Systems, Minneapolis, MA, USA), using an automated immunostainer (Dako). The staining of follicular stroma and interfollicular fibroelastic stroma was graded quantitatively as previously described [20]: 0, absent; 1+ focal; 2+ extensive; 3+ diffuse.

Cytokine production

Cytokine mRNA production was investigated using two different techniques: RNA ISH with RNAscope technology (Advanced Cell Diagnostics, Milano, Italy), and real-time PCR (RT-PCR). RNAscope is an in situ enzymatic technique that also provides quantitative information; in fact, the number of dots per cell is directly proportional to the number of specific RNA molecules.

RNAscope

The RNAscope assay was applied to tissue paraffin sections using probes for LTA, LTB, and TNFA, as previously described [19]. In brief, FFPE tissue sections 2 μm thick were deparaffinized in xylene and then hydrated in an ethanol series. Hybridization was with target probes: Probe-Hs-LTA, Probe-Hs-LTB, and Probe-Hs-TNFA. The preamplifier, amplifier, label probe, and chromogenic detection procedures were performed according to the manufacturer’s instructions (RNAscope 2.0 HD Reagent Kit, Advanced Cell Diagnostics, Hayward, CA, USA).

RNAscope-stained tissue sections were digitalized at ×40 magnification using Aperio Scan Scope. Digital slides were used for determining percentage of positive cells and pixel of reactivity. For each lymph node, five different areas, measuring 17 000 μm² each, were selected within different regions (i.e. GC, mantle, interfollicular). The percentage of positive cells per area was then calculated by manually counting the total number of cells and the number of stained cells directly on the screen. The number of positive pixels was determined using the Aperio software Positive Pixel Count v9 Algorithm.

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RNA isolation and real-time PCR

Total RNA was extracted from paraffin sections of 25 lymph nodes (11 reactive, 8 nodular follicular lymphoma, and 6 diffuse follicular lymphoma) using High Pure miRNA Isolation kit (Roche Diagnostics, Monza, Italy). The quantity and quality of the RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA USA). One microgram of purified RNA was reverse transcribed into cDNA using iScript Select cDNA Synthesis Kit (BIO-RAD, Milan, Italy) according to the manufacturer’s protocol. For cytokine mRNA expression analysis, real-time PCR was performed using QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany) and the expression levels of cytokine were normalized to the housekeeping gene β-actin. Real-time PCR was performed on a 7500 Fast Real-Time PCR System (AB). Primer sequences used for real-time PCR analysis were: LTB fwd (5’- GAG GAC TGG TAA CGG AGA CG –3’); LTB rev (5’- GGG CTG AGA TCT GTT TCT GG-3’); ACTB fwd (5’- CGG TTC CGC TGC CCT GAG-3’); ACTB rev (5’- TGG AGT TGA AGG TAG TTT CGT GGA T-3’).

Results of RT-PCR performed in FFPE tissue were formed in triplicate.

Combined ISH and immunohistochemistry

RNAscope assay for LTB RNA was carried out on a FFPE section from a reactive lymph node as described above. After hybridization slides were immunostained for CD79a (clone JCB117, Dako) using Envision G2 System/AP, Rabbit/Mouse (Permanent Red) (Agilent, CA, United States) following the manufacturer’s instructions. Slides were mounted using Glycergel Mounting Medium (Dako).

Statistical analysis

The expression levels of LTB, LTA, and TNFA mRNA assessed by RNAscope or by RT-PCR were compared among the samples using Student’s t-test.

1p36 loss of heterozygosity (LOH)

1p36 LOH was assessed using five dinucleotide repeats (D1S2734, D1S199, D15508, D1S243, and D1S468). One of the primers in each pair was fluorescently labelled. Data analysis was performed using GeneScan software on a genetic analyzer ABI3100 (Applied Biosystems, Foster City, CA, USA). Samples were regarded as uninformative if the normal tissue was homozygous or if instability was present in neoplastic tissue. LOH of a locus was determined using the calculation of Ganzian et al [21]. Ratios less than 0.6 or greater than 1.67 were regarded as loss of the major or minor allele, respectively.

Results

LTB RNA is produced in B cell follicles of reactive lymph nodes

Cytokines known to be active in FDC differentiation (LTA, LTB, and TNFA) were investigated in paraffin sections of reactive lymph nodes. Cells producing cytokine RNAs were demonstrated with the RNAscope technology. Our findings provide evidence that LTB RNA is present mostly in reactive B cell follicles (Figure 1). At that site, cells positive for LTA RNA and TNFA RNA were significantly less numerous and contained fewer dots of reactivity (Figure 2). A more accurate estimate of the number of cytokine-positive cells was performed on digital slides (Table 1). It was found that almost 90% of GC cells were positive for LTB RNA, whereas cells producing LTA and/or TNFA RNA were significantly less numerous (30 and 50% reduction, respectively; p ≤ 0.001). This difference was more evident when cytokine RNAs were evaluated as pixels per area; in fact, LTB RNA produced by GC cells was 80-fold higher than that of LTA and 20-fold higher than that of TNFA (p < 0.001). These findings indicate that a large proportion of GC B cells are capable of producing LTA, LTB, and TNFA; nevertheless, the amount of LTB RNA produced by each single cell is much higher as compared with LTA and TNFA.

Most RNA-positive cells had the morphology of GC B cells; GC macrophages were negative for LTA, Italy). The hybridisation signals for each probe were evaluated in at least 100 interphase nuclei.
LTB, and TNFA. When GCs were compared with mantle zones, it was found that mantle cells were less efficient than GC cells in producing LTB RNA (Table 1). In fact, mantle cells stained for LTB RNA were less numerous (76 versus 88%; \( p = 0.137 \)) and produced 40% less LTB (81 131 pixels versus 129 372 pixels \( p = 0.039 \)) than GC cells. Mantle cells were also less effective in producing LTA RNA (611 pixels versus 1605 pixels \( p = 0.009 \)) and TNFA RNA (3306 pixels versus 6633 pixels \( p = 0.037 \)). The presence of GC cells positive for LTB RNA correlated with expression of CD21, CD23, VCAM, and CXCL13 by FDCs (Table 2). As expected, FDCs were diffusely and intensely positive for CD21/CD23, and were focally positive for VCAM and CXCL13. The percentage of GC cells positive for LTB RNA varied from 55 to 95% (mean 80 ± 13), and the number of pixels per cell varied from 442 to 1000 (743 ± 231). An interesting observation, made possible by the in situ study, was that cells positive for LTB RNA were not distributed homogeneously throughout the GC. In fact, cells positive for LTB RNA were more numerous in the basal dark zone of polarized GCs, where cell proliferation takes place (Figure 3). In contrast, expression of LTB induced molecules, such as CD21, CD23, VCAM, and CXCL13 was more prominent in the light zone, proximal to the sub-capsular sinus (Figure 3).

Production of LTB RNA in T-dependent interfollicular areas was significantly lower \( (p < 0.0001) \) as compared with B cell follicles (Table 1). At that site, LTB-positive pixels \( (17 975 ± 10 816) \) were 19% of those present in GCs. In Figure 3, a lymph node section was double-stained for LTB RNA (brown) and CD79a protein (red). It was confirmed that most LTB+ cells were of B cell origin (CD79a+), that they were polarized in GC, and that LTB+ cells were much less numerous in CD79a-negative interfollicular T cell areas. Stromal reticular cells of interfollicular areas were negative for CD21/CD23, and were occasionally positive for VCAM/CXCL13.

Figure 1. In situ hybridization for LTB RNA in a reactive lymph node using RNAscope technology. Reactivity, consisting of cytoplasmic brown dots, is present in the cytoplasm of RNA-producing cells, and is directly proportional to the number of RNA molecules. LTB RNA was mainly associated with cortical B cell follicles. GCs and mantle zones were both stained with higher levels in GCs.

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Figure 2. In situ hybridization for cytokines in a GC using RNAscope technology. LTB RNA (A), LTA RNA (B), and TNFA RNA (C) were all present, but LTB reactivity was much more pronounced. Large cells with clear cytoplasm and tingible bodies (GC macrophages) did not showed reactivity for TNFA RNA (C).
Table 1. LTA RNA, LTB RNA, and TNFA RNA in B cell follicles and in interfollicular areas of reactive lymph nodes*

| Case No. | LTA+ cells (%) | LTA+ pixels | LTB+ cells (%) | LTB+ pixels | TNFA+ cells (%) | TNFA+ pixels |
|----------|----------------|-------------|----------------|-------------|-----------------|--------------|
| **Germinai centre** | | | | | | |
| 1 | 23 | 857 ± 455 | 83 | 123 621 ± 50 123 | 48 | 7071 ± 3129 |
| 2 | 33 | 1503 ± 613 | 93 | 137 052 ± 64 044 | 34 | 2871 ± 1171 |
| 3 | 37 | 2209 ± 1166 | 95 | 140 043 ± 32 107 | 66 | 7978 ± 1041 |
| 4 | 30 | 1849 ± 685 | 80 | 116 592 ± 62 562 | 55 | 8613 ± 3882 |
| Mean± SD** | 31 ± 6 | 1605 ± 576 | 88 ± 7 | 129 327 ± 11 094 | 51 ± 13 | 6633 ± 2587 |
| **Mantle zone** | | | | | | |
| 1 | 10 | 370 ± 376 | 90 | 120 847 ± 9834 | 52 | 4531 ± 1407 |
| 2 | 16 | 524 ± 144 | 90 | 116 931 ± 43 960 | 24 | 1007 ± 326 |
| 3 | 17 | 863 ± 479 | 72 | 51 598 ± 81 715 | 54 | 4400 ± 1535 |
| 4 | 15 | 685 ± 333 | 50 | 35 149 ± 13 533 | 33 | 3227 ± 2394 |
| Mean±SD** | 15 ± 3 | 611 ± 212 | 76 ± 19 | 81 131 ± 44 142 | 41 ± 15 | 3306 ± 1646 |
| **Interfollicular area** | | | | | | |
| 1 | 14 | 356 ± 173 | 75 | 31 710 ± 10 500 | 19 | 2212 ± 774 |
| 2 | 6 | 519 ± 738 | 51 | 17 563 ± 10 677 | 10 | 868 ± 269 |
| 3 | 7 | 945 ± 324 | 30 | 5256 ± 4431 | 13 | 1156 ± 306 |
| 4 | 13 | 801 ± 541 | 41 | 17 370 ± 12 551 | 29 | 4059 ± 3570 |
| Mean±SD** | 10 ± 4 | 655 ± 266 | 49 ± 19 | 17 975 ± 10 816 | 18 ± 8 | 2051 ± 1442 |

Statistical analysis (Student's t-test): Germinai centre: LTB+ cells versus LTA+ cells p < 0.001; LTB+ pixels versus LTA+ pixels p < 0.001; TNFA+ cells versus LTA+ cells p = 0.001; TNFA+ pixels versus LTA+ pixels p = 0.005. Mantle zone: LTB+ cells versus LTA+ cells p < 0.001; LTB+ pixels versus LTA+ pixels p = 0.005; TNFA+ cells versus LTA+ cells p = 0.01; TNFA+ pixels versus LTA+ pixels p = 0.006; LTA+ cells versus TNFA+ cells in p = 0.009. Interfollicular area: LTB+ cells versus LTA+ cells p = 0.02; LTB+ pixels versus LTA+ pixels p = 0.009; LTA+ cells versus TNFA+ cells p = 0.01; TNFA+ cells versus LTA+ cells p = 0.01; TNFA+ pixels versus LTA+ pixels p = 0.07. Germinal Centre versus Mantle Zone: LTA+ cells p = 0.001; LTA+ pixels p = 0.009; TNFA+ cells p = 0.137; TNFA+ pixels p = 0.039; TNFA+ cells p = 0.176. Interfollicular Area versus Germinal centre: LTA+ cells p = 0.001; LTA+ pixels p = 0.012; TNFA+ cells p = 0.005; TNFA+ pixels p = 0.01; TNFA+ cells p = 0.003; TNFA+ pixels p = 0.01. *Paraffin sections of four reactive lymph nodes were stained for LTA, LTB, TNFA RNA using RNA scope technology. Stained sections were digitized using Aperio ScanScope. Percentage of stained cells and number of pixels (Aperio Positive Pixel Count v9 algorithm) were determined in areas measuring 17 000 μm² each. The value reported in the Table is the mean ± SD of five different measurements made in different follicles or in different regions of the same lymph node. **Mean ± SD of the four investigated cases.

**LTB RNA production in B cell follicular lymphomas**

Follicular B cell lymphomas are GC-derived malignant tumours, which may exhibit a **follicular** or a **diffuse** pattern of growth. The so-called ‘classical-type’ FL carries the t(14;18) translocation, expresses BCL2 protein in most cases, and generally has a

Table 2. Immunophenotype of FDCs and LTB RNA in germinal centres of reactive lymph nodes

| Case No. | Age/sex | CD21 | CD23 | VCAM | CXCL13 | + cells (%) | + pixels (n) | pixels/cell (n) |
|----------|---------|------|------|------|--------|-------------|--------------|----------------|
| 1 | 77/M | 3+ | 3+ | 3+ | 1+ | 83* | 123 621 ± 50 123* | 902** |
| 2 | 43/M | 3+ | 3+ | 3+ | 1+ | 78 | 61 841 ± 14 070 | 476 |
| 3 | 73/M | 2+ | 2+ | 1+ | 1+ | 93 | 137 052 ± 64 044 | 1000 |
| 4 | 32/F | 3+ | 3+ | 1+ | 1+ | 55 | 38 540 ± 24 996 | 602 |
| 5 | 81/M | 3+ | 3+ | 2+ | ND | ND | ND | ND |
| 6 | 74/M | 3+ | 3+ | 2+ | 1+ | 83 | 61 922 ± 22 160 | 442 |
| 7 | 63/F | 3+ | 3+ | 1+ | 2+ | 71 | 63 679 ± 16 271 | 624 |
| 8 | 77/F | 3+ | 3+ | 2+ | 1+ | 95 | 140 043 ± 52 107 | 909 |
| 9 | 71/M | 3+ | 3+ | 2+ | 3+ | 80 | 116 592 ± 62 562 | 988 |
| 10 | 8/M | 3+ | 3+ | 2+ | 3+ | 80 | 116 592 ± 62 562 | 988 |
| 11 | 34/M | 3+ | 3+ | 2+ | 3+ | 80 | 116 592 ± 62 562 | 988 |

*The immunostaining of FDCs was graded as previously described [20]; absent (0); focal (1+); extensive (2+); diffuse (3+).

*Mean percentage of LTB+ cells and mean number of LTB+ pixels (Aperio Positive Pixel Count v9 algorithm) determined in five different GC areas measuring 17 000 μm² each.

**Pixels per cell were calculated as total positive pixels/total number of stained cells.
predominantly follicular pattern of growth. Classical FL with a diffuse pattern of growth is extremely rare. More recently, a distinct subtype of FL has been described, the so-called ‘inguinal-type’ [22]. The latter is characterized by a predominantly diffuse pattern of growth, is usually BCL2-negative, and often carries the 1p36 deletion. In Table 3, we have investigated LTB RNA in follicular versus diffuse FL. It was found that tumour cells of predominantly follicular FL were less effective in producing LTB RNA than GC cells, but were more efficient than tumour cells of predominantly diffuse FL. In fact, the percentage of LTB+ cells in predominantly follicular FL (62%) and the number of LTB+ pixels were significantly lower than those of reactive GCs (p = 0.004 and p = 0.001). In predominantly diffuse FL, the levels of LTB RNA were the lowest; in fact, LTB+ cells were 14% and LTB+ pixels were 3,540 (96% less than GC and 91% less than predominantly follicular FL; p < 0.001) (Figure 4). In our series, no correlation was found between LTB production and tumour grade (G1/G2) or cell proliferation (% of Ki67+ cells).

Predominantly diffuse FL may still have limited follicular areas. Production of cytokine RNAs was investigated in follicular and diffuse areas of four cases of FL ‘inguinal-type’ (Table 4). It was found that production of LTB RNA was only three-fold higher in follicles as compared to diffuse areas. It is of interest that the small tumour-associated follicles were positive for CD10, BCL-6, and stathmin, but were negative or weakly positive for CD21 and CD23 (Figure 5). These findings seem to indicate that terminal differentiation of FDCs in the follicles of diffuse FL is to some extent impaired.

The data obtained with RNAscope technology indicate that there are significant differences in the levels of LTB RNA produced in reactive GCs, predominantly follicular FL and in predominantly diffuse FL. This observation was confirmed by the use of RT-PCR. cDNAs obtained from total RNA extracted from tissue sections of 11 reactive lymph nodes, 8 follicular FLs, and 6 diffuse FLs was tested for the presence of LTB by RT-PCR. The data shown in Figure 6 confirm that LTB RNA is more abundant in reactive lymph nodes than in predominantly follicular
Table 3. LTB RNA and FDCs in predominantly follicular and in predominantly diffuse follicular B cell lymphoma*

| Case | N | Age/sex | Lymph node site | Size (cm) | Grade | Ki67 (%) | BCL2# | 1p36 loss | LTB+ cells (%) | LTB+ pixels | LTB+ pixels/cell |
|------|---|---------|-----------------|-----------|-------|----------|-------|-----------|----------------|-------------|-----------------|
|      |   |         |                 |           |       |          |       |           |                |             |                 |
|      | 1 | 51/F    | Inguinal        | 1.8       | G2    | 10       | +     | ND        | 54             | 21 886 ± 11 138 | 257            |
|      | 2 | 53/M    | Axillary        | 2.5       | G2    | 40       | +     | ND        | 64             | 33 159 ± 22 146 | 299            |
|      | 3 | 67/F    | Inguinal        | 2.5       | G1    | 20       | +     | ND        | 75             | 60 813 ± 13 500 | 760            |
|      | 4 | 80/M    | Inguinal        | 3.8       | G2    | 30       | -     | ND        | 68             | 62 476 ± 40 999 | 625            |
|      | 5 | 54/M    | Cervical        | 3.0       | G2    | 20       | +     | ND        | 40             | 33 437 ± 9052  | 423            |
|      | 6 | 82/M    | Mediastinal     | 2.2       | G1    | <10      | +     | ND        | 68             | 35 017 ± 11 228 | 294            |
|      | 7 | 68/F    | Axillary        | 2.5       | G2    | 10       | +     | ND        | 65             | 28 890 ± 14 113 | 251            |
|      | 8 | 70/M    | Inguinal        | 2.5       | G2    | 20       | +     | ND        | 58             | 30 260 ± 8256  | 309            |
| Mean ± SD | |        |                 |           |       |          |       |           | 62 ± 11               | 38 242 ± 14 999 | 402 ± 190       |
|      | 1 | 53/M    | Inguinal        | 3.0       | G1    | <10      | -     | +         | 27             | 8044 ± 5832    | 223            |
|      | 2 | 68/F    | Inguinal        | 3.5       | G2    | <10      | -     | +         | 1512 ± 814     | 146           | 252            |
|      | 3 | 64/M    | Inguinal        | 3.5       | G2    | 20       | -     | +         | 730 ± 370      |              | 146            |
|      | 4 | 56/M    | Cervical        | 2.5       | G2    | 30       | +     | ND        | 40             | 750 ± 250      | 125            |
|      | 5 | 49/F    | Inguinal        | 2.5       | G2    | 10       | -     | -         | 33             | 9072 ± 5412    | 171            |
|      | 6 | 51/F    | Inguinal        | 4.5       | G2    | 30       | -     | +         | 9              | 2086 ± 840     | 116            |
|      | 7 | 68/F    | Inguinal        | 4.5       | G2    | <10      | -     | +         | 13             | 2587 ± 456     | 108            |
| Mean ± SD | |        |                 |           |       |          |       |           | 14 ± 12              | 3540 ± 3505   | 163 ± 56        |

Reactive lymph nodes (n = 8)*

|      | Mean ± SD | | | | | | | | | |
|------|-----------|---|---|---|---|---|---|---|---|---|
|      |           | 80 ± 13 | 92 912 ± 40 373 | 743 ± 231 |

Statistical analysis (Student t-Test): RL versus follicular FL: LTB+ cells p = 0.004; LTB+ pixels p = 0.001; LTB+ pixels/cell p = 0.003; RL versus diffuse FL: LTB+ cells p < 0.001; LTB+ pixels p < 0.001; LTB+ pixels/cell p < 0.001; follicular FL versus diffuse FL: LTB+ cells p < 0.001; LTB+ pixels p < 0.001; LTB+ pixels/cell p = 0.003.

*Paraffin sections were stained for LTB RNA using RNA scope technology. Stained sections were digitalized using Aperio ScanScope. The percentage of LTB+ cells and the mean number of LTB pixels were determined in five squared areas measuring 17 000 µm² each. Pixels per cell were calculated as total positive pixels/total positive number of stained cells.

#BCL2 expression was investigated by immunohistochemistry, using Dako clone 124. BCL2-negative cases were re-investigated by FISH which confirmed the absence of t(14;18).

1p36 Chromosomal loss was investigated by FISH and by LOH analyses.

Cases are detailed in Table 2.

Figure 4. In situ hybridization for LTB RNA using RNAscope technology in reactive GC, predominantly follicular FL, and predominantly diffuse FL. In reactive follicles, most GC B cells and mantle zone cells are positive for LTB RNA. In follicular FL, most tumour cells are positive. In diffuse FL, only rare cells are stained.
Table 4. LTB RNA and CD21/CD23 expression in follicular and diffuse areas of follicular lymphoma*

| Case No. | CD21 | CD23 | LTB+ pixels | CD21 | CD23 | LTB+ pixels |
|----------|------|------|-------------|------|------|-------------|
|          |      |      | follicles   |      |      | interfollicular areas |
| 1        | 1+   | 2+   | 21 886 ± 11 138** | 0    |      | 22 342 ± 8276** |
| 2        | 3+   | 2+   | 33 159 ± 22 146 | 0    |      | 2207 ± 1170 |
| 3        | 3+   |      | 60 813 ± 13 500 | 0    |      | 7096 ± 6411 |
| 4        | 3+   | 0    | 62 476 ± 40 999 | NE   |      | 17 159 ± 4409 |
| 5        | 2+   | NE   | 33 437 ± 9052  | 0    |      | 16 017 ± 3663 |
| 6        | 3+   | 1+   | 35 017 ± 11 228 | 1+   |      | 2748 ± 1543 |
| 7        | 1+   | 0    | 28 890 ± 14 113 | 0    |      | 11 305 ± 2497 |
| 8        | 3+   | 2+   | 30 260 ± 8256  | 0    |      | 5148 ± 2012  |
| Mean ± SD| 2.37 ± 0.92 | 1.17 ± 0.98 | 38 242 ± 14 999 | 0.14 ± 0.38 | 0.50 ± 0.55 | 10 503 ± 7414 |

|          |      |      | follicles   |      |      | Diffuse areas |
| 1        | 1+   | 0    | 26 539 ± 7794** | 1+   |      | NE 8044 ± 5832** |
| 2        | 1+   | 1+   | 7894 ± 14 189 | 0    |      | 1512 ± 814 |
| 3        | 0    | 0    | 2100 ± 1374  | 0    |      | 730 ± 370 |
| 4        | 1+   | 0    | ND           | 1+   |      | 750 ± 250° |
| 5        | 0    | 0    | 25157 ± 3924 | 0    |      | 9072 ± 5412 |
| 6        | 1+   | 0    | ND           | 1+   |      | 2086 ± 840° |
| 7        | 0    | NE   | ND           | 0    |      | 2587 ± 456° |
| Mean ± SD| 0.57 ± 0.53 | 0.17 ± 0.41 | 15 422 ± 12 281 | 0.43 ± 0.53 | 0.33 ± 0.58 | 3540 ± 3505 |

Statistical analysis (Student t-test):
Follicles versus interfollicular area of predominantly follicular FL: CD21 $p < 0.001$; CD23 $p = 0.09$; LTB+ pixels $p < 0.001$; Follicles versus diffuse areas of predominantly diffuse FL: CD21 $p = 0.31$; CD23 $p = 0.31$; LTB+ pixels $p = 0.08$; Follicles of predominantly follicular FL versus follicles of predominantly diffuse FL: CD21 $p < 0.001$; CD23 $p = 0.02$; LTB+ pixels $p = 0.01$; Interfollicular area of predominantly follicular FL versus interfollicular area of predominantly diffuse FL: CD21 $p = 0.13$; CD23 $p = 0.34$; LTB+ pixels $p = 0.02$.

NE, not evaluable because CD21 or CD23 were expressed by the lymphoid component of the tumour as well; ND, not determined.

*Paraffin sections were immunostained for CD21 and CD23. The staining intensity of FDCs was graded quantitatively as previously described [20]: absent (0); focal (1+); extensive (2+); diffuse (3+).

**Paraffin sections were stained for LTB RNA using RNA scope technology. Stained sections were digitalized using Aperio ScanScope. The mean numbers of LTB+ pixels were determined in five squared areas measuring 17 000 $\mu$m² each.

These values were excluded when follicles and diffuse areas were compared in predominantly diffuse FL.

Figure 5. Predominantly diffuse CD23+ B cell FL. Small CD10+/Stathmin+ B cell follicles are poorly stained for CD21/CD23. A positive control is provided by CD23 staining of tumour cells.
FL (p = 0.006). The lowest values of LTB RNA were observed in predominantly diffuse FL (RL versus diffuse FL p < 0.001; follicular FL versus diffuse FL p = 0.06).

Discussion

This is the first report describing the tissue distribution of cells producing LT and TNFA RNA in human lymphoid tissues. Our results show that cells producing LTB are mainly located in B cell follicles, and are more numerous than those producing LTA and TNFA. These observations are consistent with the notion that production of LTB RNA is constitutive in B-cells [23,24], whereas production of LTA and TNFA RNA is inducible [25]. It has to be emphasized that quantitative differences in cytokine RNAs do not necessarily reflect protein synthesis and release. In fact, it has been reported that TNFA RNA accumulates rapidly, but has a brief half-life. In contrast, LTA RNA accumulates more slowly, but persists much longer with a half-life longer than that of TNFA RNA [26]. Thus, RNA half-time and protein translation represent further regulatory checkpoints which might profoundly alter the levels of cytokine production in lymphoid tissues.

We provide evidence that proliferating B cells located in the basal dark zone of GCs contain large amounts of LTB RNA, and that light zone FDCs express high levels of molecules involved in FDC function. Indeed, differences in FDCs populating dark and light zone have already been reported. It was described that FDCs in the dark zone were half as dense as FDCs in the light zone [27], and that dark zone FDCs produce large amounts of CXCL12 [28]. These findings and our observations raise the possibility that the topographical organization of GCs in dark and light zones also affects FDC differentiation and function. In fact, B cell proliferation in the dark zone represents a signal that GC cells are efficiently stimulated and that productive antigen presentation is occurring. In this situation, it is necessary to optimize FDC function through induction of molecules involved in antigen trapping (CD21, CD23), cell-cell adhesion (VCAM), and B cell recruitment (CXCL13). Release of high levels of LTB by proliferating B cells of the dark zone might have this role. In agreement with this view, Mackay et al [7,12] demonstrated that inhibition of LTA/B in tissue cultures caused disappearance of multiple markers on FDCs within 1 day, and that inhibition of the TNF pathway was much less effective. The possibility that TNF and LT exert different actions on stromal cells is supported by an in vitro study [29] showing that TNF alone was able to induce a strong increase of adhesion molecules, but not of meshwork formation, whereas LT had the opposite effect. Thus, it seems likely that LT and TNF are both necessary to support FDC function, but with different roles.

Tumour B cells of predominantly follicular FL show a strict topographical and functional relationship with FDCs [30]; in fact, the latter are crucial for supporting tumour growth and survival [31,32]. Chang et al [20] investigated the immunophenotype of FDCs present in FL and found different patterns of antigen expression, depending on the pattern of growth (follicular versus diffuse). They reported that stromal reticular cells of diffuse FL showed only minimal immunophenotypic evidence of FDC-like differentiation, and that FDCs of follicular FL were characterized by reduced expression of FDC antigens as compared to reactive GC. Similar findings were reported in a more recent study, where it was shown that FDCs found in different types of lymphoma show reduced expression of several FDC antigens as compared with normal GCs [33]. We have confirmed and extended these observations. Moreover, the new information provided by our study is that there is a correlation between amounts of LTB RNA produced by tumour cells and levels of FDC differentiation of tumour-associated stromal reticular cells.

A purely diffuse pattern is rare in FL. More recently, a peculiar type of diffuse FL was proposed...
as a separate entity [22,34,35]. This tumour, known as ‘inguinal-type’, has distinctive traits consisting of frequent involvement of inguinal lymph nodes, absence of BCL2 translocation, frequent occurrence of 1p36 deletion, frequent expression of CD23 by tumour B cells, and a better prognosis [22,34,35]. Five of the seven cases of diffuse FL investigated in the present study had features of the ‘inguinal-type’ variant. We have found that these tumours produce low amounts of LTB RNA as compared with ‘classical’ predominantly follicular FL. It can be speculated that poor production of LTB by tumour cells of predominantly diffuse FL is responsible for defective differentiation of stromal reticular cells into FDCs, and hence for defective nodular organization.

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Author contributions statement

LR and ADN conceived experiments and designed the study. GP, CC, and SS carried out experiments. ADN, GP, LR, and EP analysed data. LR, ADN, and GP interpreted results and wrote the paper. All authors had final approval of the submitted and published versions.

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