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ORIGINAL ARTICLE

The interleukin (IL)-31/IL-31R axis contributes to tumor growth in human follicular lymphoma

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Interleukin (IL)-31A binds to an heterodimer composed of IL-31 receptor A (IL-31RA) and Oncostatin M Receptor (OSMR). The IL-31/IL-31R complex is involved in the pathogenesis of various skin diseases, including cutaneous T-cell lymphoma. No information is available on the relations between the IL-31/IL-31R complex and B-cell lymphoma. Here we have addressed this issue in follicular lymphoma (FL), a prototypic germinal center(GC)-derived B-cell malignancy. IL-31 enhanced primary FL cell proliferation through IL-31R-driven signal transducer and activator of transcription factor 1/3 (STAT1/3), extracellular signal–regulated kinase 1/2 (ERK1/2) and Akt phosphorylation. In contrast, GC B cells did not signal to IL-31 in spite of IL-31R expression. GC B cells expressed predominantly the inhibitory short IL-31RA isoform, whereas FL cells expressed predominantly the long signaling isoform. Moreover, GC B cells lacked expression of other IL-31RA isoforms potentially involved in the signaling pathway. IL-31 protein expression was significantly higher in surface membrane than in cytosol of both FL and GC B cells. IL-31 was detected in plasma membrane microvesicles from both cell types but not released in soluble form in culture supernatants. IL-31 and IL-31RA expression was higher in lymph nodes from FL patients with grade IIIa compared with grade I/II, suggesting a paracrine and/or autocrine role of IL-31/IL-31RA complex in tumor progression through microvesicle shedding.

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INTRODUCTION

Interleukin-31 (IL-31) is a member of the IL-6 cytokine superfamily originally identified as the product of activated T helper type 2 cells.[1] Additional cell types such as monocytes, macrophages, immature and mature dendritic cells and mast cells produce IL-31 following activation.[2–5] Increased IL-31 expression has been detected in inflamed tissues from patients with atopic dermatitis, bowel diseases, allergic asthma and rhinitis.[6–12]

The activity of IL-31 is mediated through an heterodimeric receptor composed of a gp130-like receptor chain, IL-31 receptor A (IL-31RA) and the Oncostatin M Receptor (OSMR).[13–15] Expression of IL-31RA mRNA is found in specific tissues, including the skin, testis, bone marrow, brain and thymus.[1,13,15] Furthermore, tumor cell lines derived from osteosarcoma, glioblastoma, melanoma and myelomonocytic leukemia express IL-31RA mRNA,[1,13–15] whereas OSMR is ubiquitously expressed in human tissues and organs.[13,15] Engagement of the IL-31R with IL-31 results in the activation of Janus-activated kinase 1 (JAK1) and, to a minor extent, of JAK2 followed by activation of signal transducer and activator of transcription factor 1 (STAT1)/3/5, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3′-kinase (PI3K) signaling pathways.[10,15–17] Co-expression of the IL-31RA and OSMR chains is essential for IL-31 signaling, because IL-31RA activates STAT phosphorylation while the activation of phosphatidylinositol 3′-kinase and mitogen-activated protein kinase pathways is mediated by OSMR.[18]

Different IL-31RA isoforms have been so far identified, of which a long signaling (745 aa residues) and a short non-signaling (560 aa residues) isoforms are the best characterized.[13,19,20]

Recent studies have linked IL-31 to human malignant lymphomas of T-cell lineage. It has been shown that malignant T-cell populations from cutaneous T-cell lymphoma produce IL-31, whose serum levels are increased in the same patients.[21,22] No information is so far available on the relationship between IL-31 and B-cell lymphoma.

Here we have investigated the expression and function of the IL-31/IL-31R complex in follicular lymphoma (FL) as a prototypic model of mature B-cell malignancy.[23,24]

MATERIALS AND METHODS

Patients and controls

Invaded lymph nodes from 25 FL patients (15 males and 10 females, age range: 46–61 years), biopsied for diagnostic purposes, were obtained from the San Martino Hospital-Istituto Scientifico Tumori Biobank (Genova, Italy). Diagnosis of FL was established according to the criteria of the Revised European-American Classification of Lymphoid Neoplasms.[23–25] Histopathological analysis showed that 11 FL patients had grade I; 7 FL patients grade II; and 7 FL patients grade III disease. All patients were studied at diagnosis and were untreated.

Lymph node mononuclear cells (MNCs) were isolated from FL patients as reported.[26] Staining for immunoglobulin (Ig) light chains showed that monoclonal B cells expressing either κ (10 of 25 cases) or λ (15 of 25 cases)

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light chain represented at least 85% of $CD19^+$ cells in all samples. FL MNCs were cryopreserved in a freezing solution composed of 50% RPMI 1640 (Sigma Chemical Co., St Louis, MO, USA), 40% fetal bovine serum (Sigma) and 10% dimethyl sulfoxide (Sigma). Cells were kept in liquid nitrogen until tested.

Ten tonsil samples surgically removed for localized inflammation and three peripheral blood samples from healthy donors were obtained following informed consent. The study was approved by the Institutional Review Board of Istituto Giannina Gaslini, Genova, Italy on 27 October 2005. Four reactive lymph nodes with follicular hyperplasia were obtained from San Martino Hospital-Istituto Scientifico Tumori Biobank (Genova, Italy).

Cell isolation and cultures

After thawing, lymphoma B cells were purified by removing: (i) residual normal B cells according to the expression of the Ig light chain not expressed by the malignant clone and (ii) other contaminant cell types (that is, $CD3^+$, $CD56^+$ and $CD14^+$ cells), through immunomagnetic bead manipulation (Miltenyi Biotec, Bergisch Gladbach, Germany). Tonsil B cells were isolated as reported[27] and enriched for germinal center (GC) B cells ($CD19^+$, $CD38^{high}$, $CD10^+$, $CD44^-$ cells) by centrifugation on a discontinuous (60–30%) Percoll (Pharmacia, Uppsala, Sweden) density gradient followed by depletion of $CD39^+$ naive and memory B cells.[28,29] B cells, purified as above contained consistently $>97\%$ GC B cells, with a centroblast/centrocytes ratio of approximately 3/1, as assessed by staining for the CD77 marker, specific for centroblast. All of the steps for GC B-cell isolation were performed at 4 °C to prevent spontaneous apoptosis. GC B cells were also isolated from the lymph nodes of individuals with reactive follicular hyperplasia as reported above.[28,29] In some experiments, non-B cells were isolated from FL patients ($n=4$) and tonsil MNCs ($n=4$) by negative selection with a CD20 mAb (Beckman Coulter, Brea, CA, USA) followed by immunomagnetic bead manipulation (Miltenyi Biotec). $CD4^+$ T cells were isolated from peripheral blood MNCs using anti-CD4 microbeads (Myltenyi Biotec) following the manufacturer's protocol and stimulated *in vitro* for 48 h with an anti-CD3 mAb (OKT3, coated overnight on 96-well plates).

The details for the U937, EA.hy926, DU145, T84 and K562 cell lines used in the study are reported in Supplementary Methods.

Antibodies for flow cytometry

The list of monoclonal antibodies (mAbs) used throughout the study is shown in Supplementary Methods. Cells were stained with fluorochrome-conjugated or unconjugated antibodies followed by secondary reagents. Isotype and fluorochrome-matched antibodies were tested as controls. Cells were run on a Gallios Instrument (Beckman Coulter), and data were analyzed using the Kaluza software (Beckman Coulter). Data were expressed as the percentage of positive cells.

Reverse transcriptase–PCR (RT-PCR)

Total RNA was isolated using the RNeasy kit (Qiagen, Milano, Italy) according to the manufacturer's instructions. RNA was assessed for integrity by gel electrophoresis and quantified by spectrophotometry (Nanodrop Products, Wilmington, DE, USA). One microgram of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Monza, Italy), according to the manufacturer's instructions.

The primer sequences for human IL-31, IL-31RA, OSMR, IL-31RA short and long isoforms and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA and the relative PCR conditions were as described.[1,19,30,31] Primers' sequences designed to identify the five different variants of IL-31RA mRNA are shown in Figure 3a (NCBI Unigene database),[20] together with the sequences of the primers for IL-31RA short and long isoforms. All the primers were purchased from TIB Molbiol (TIB MolBiol S.r.L., Genova, Italy). The amplified products were visualized by electrophoresis on a 2% agarose gels. mRNA expression levels of IL-31RA short and long isoforms were quantified by scanning densitometry using the VersaDoc instrument (BioRad Laboratories, Segrate, Italy). PCR reactions for each sample were performed at least twice.

Immunohistochemistry and immunofluorescence

Formalin-fixed and paraffin-embedded tissue sections from four tonsils with reactive follicular hyperplasia and four lymph nodes with FL were collected from the archives of the Human Pathology Section, Department of Health Sciences, University of Palermo, Palermo, Italy. The immunohistochemistry techniques are detailed in Supplementary Methods. In addition, 20 FL lymph node samples ($n=6$ grade I; $n=6$ grade II; $n=8$ grade III) were selected for tissue microarray (TMA) preparation. TMAs were used to investigate the expression of IL-31 and IL-31RA in relation to FL grading. See Supplementary Methods for TMA construction and immunofluorescence assay.

Cell signaling

Phosphorylation was investigated by flow cytometry in FL and tonsil GC B cells. See Supplementary Methods for detailed technique.

Cell proliferation and apoptosis

Purified FL cells were cultured in RPMI 1640 medium for 72 h with or without 20, 50 and 100 ng/ml hrIL-31 in combination or not with 100 ng/ml hrCD40L (Immunotools, Friesoythe, Germany) and tested for proliferation by overnight pulse with 0.5 μCi/well tritiated thymidine (^{3}H-TdR) or for apoptosis. See Supplementary Methods for detailed technique.

Enzyme-linked immunosorbent assay (ELISA)

IL-31 production was tested by ELISA in the supernatants of FL and tonsil and reactive lymph node GC B cells. See Supplementary Methods for detailed technique.

IL-31R internalization assay

FL and tonsil GC B cells were cultured at 37 °C for 15, 30, 60 min, 3 h and 6 h with or without 100 ng/ml hrIL-31 and then was investigated for IL-31RA and OSMR internalization. See Supplementary Methods for detailed technique.

Western blotting analysis

Total cell lysates were prepared using the Cell Extraction Buffer (Life Technologies) containing a protease inhibitor cocktail (Sigma) and analyzed by western blotting as previously described.[32] To obtain subcellular fractions, cells were processed with the Qproteome Cell Compartment kit according to the manufacturer's instructions (Qiagen) and resuspended with the above-mentioned lysis buffer. Additional details are provided in Supplementary Methods.

Microvesicles (MVs) purification, analysis and signaling activity on FL cells

MVs were isolated from culture supernatants of FL cells, tonsil GC B cells and the EA.hy926, Oci-Ly8 and K562 cell lines by differential centrifugation.[33,34] Additional technical details are provided in Supplementary Methods. The signaling activity of MVs was subsequently analyzed. FL cell suspensions from three patients (two with grade II and one with grade IIIa disease) were stimulated for 0 and 30 min with 100 ng/ml hrIL-31 or different dilutions (1:1, 1:2, 1:5) of MVs isolated from the Oci-LY8 and EA.hy926 cell lines. The signaling activity was evaluated as STAT-1 phosphorylation in target cells.

Statistical analysis

Data are reported in box plot in terms of medians, first and third quartiles and minimun and maximum values. For some experiments, data are represented in histograms as mean ± s.d. The Mann–Whitney *U* test was used to compare quantitative variables between the two groups of observation. All statistical tests were two tailed, and a P value <0.05 was considered statistically significant. Statistical analyses were performed using the Graph Pad Prism 5 software (La Jolla, CA, USA).

RESULTS

Characterization of IL-31 receptor in FL B cells

First, we investigated the expression of IL-31RA and OSMR mRNAs in malignant B cells purified from five invaded lymph nodes of FL patients. As shown in Figure 1a left panel, RT-PCR amplification products of the expected length (159 and 155 bp for IL-31RA and OSMR, respectively) were detected in FL cell suspensions, as well as in the U937 cell line tested as a positive control.[13] Flow cytometric analysis showed the expression of both IL-31RA

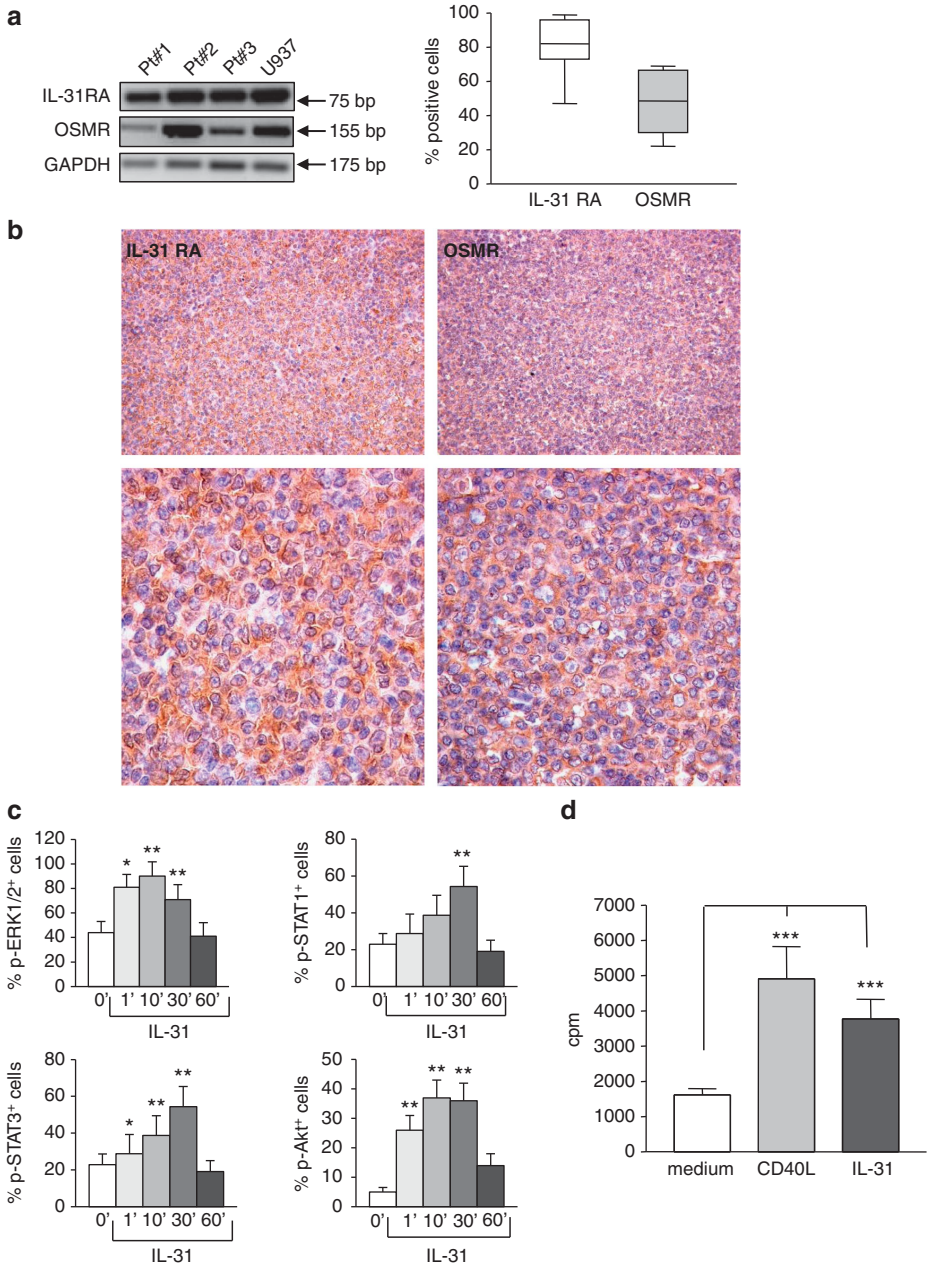


Figure 1. Expression and function of IL-31R in malignant FL cells. (**a**) Left panel. mRNA expression for IL-31RA, OSMR and GAPDH genes was detected by RT-PCR in FL cells isolated from patient (Pt) lymph nodes. The figure shows the results obtained with three FL cell suspensions out of the five tested. The U937 cell line was tested as a positive control. Right panel. Surface expression of IL-31RA and OSMR was determined on purified FL cells by flow cytometry. Results are expressed in box plot as the median percentage of positive cells, first and third quartiles and maximum and minimum values, from 25 different FL patients. (**b**) Immunohistochemical analysis of IL-31RA and OSMR in lymph node tissue section from FL patients (one representative staining out of the six performed). Original magnification, ×200 (upper panels) and ×630 (lower panels). (**c**) Purified FL cells were incubated for 0, 1, 10, 30 and 60 min with 100 ng/ml hrIL-31 and subjected to flow cytometric analysis using antibodies to p-Erk1/Erk2, -STAT1, -STAT3 and -Akt. Results are shown as the mean percentage of positive cells ± s.d. from six different experiments. p-Erk1/Erk2: $*P = 0.015$ at 1 min; $**P = 0.002$ at 10 min; $**P = 0.004$ at 30 min. p-STAT1: $**P = 0.008$ at 30 min. p-STAT3: $*P = 0.015$ at 1 min; $**P = 0.002$ at 10 min; $**P = 0.004$ at 30 min. p-Akt: $P = 0.004$ at 1 min; $**P = 0.004$ at 10 min; $**P = 0.004$ at 30 min. (**d**) Purified FL cells were cultured without (medium) or with 100 ng/ml hrIL-31 or 100 ng/ml hrCD40L for 72 h and tested for proliferation by ^{3}H-TdR incorporation. Results are the mean percentage of positive cells ± s.d. from 17 different experiments. $***P = 0.0001$ for hrCD40L and $***P = 0.0006$ for hrIL-31.

and OSMR proteins in FL cell suspensions (median percentage of IL-31RA$^+$ cells = 82.0, range 47–99; median percentage of OMSR$^+$ cells = 48.5, range 22–69; $n = 25$) (Figure 1a, right panel). A representative histogram for both IL-31RA and OSMR chains is shown in Supplementary Figure S1A. The U937 and T84 cell lines were tested as positive and negative[10] controls, respectively (Supplementary Figures S1B and C).

Expression of IL-31R was then assessed by immunohistochemistry in six lymph node samples from FL patients. In the representative experiment shown in Figure 1b, both IL-31RA and OSMR were diffusely detected in lymphomatous cells with a combined cytoplasmic and membrane staining pattern (Figure 1b, left and right panels, respectively).

The signaling pathway initiated by the binding of IL-31 to its receptor was then investigated in six different experiments. FL cells were incubated for different time intervals with hrIL-31 and then analyzed by flow cytometry using mAbs to phosphorylated (p) and non-p extracellular signal–regulated kinase 1 (Erk1)/Erk2, STAT1, STAT3 and Akt. As shown in Figure 1c, constitutive phosphorylation of all these molecules was detected in FL cells at time 0. Following incubation with hrIL-31, p-Erk1/Erk2, p-STAT1, p-STAT3 and p-Akt increased significantly, although with different kinetics. In particular, p-Erk1/Erk2, p-Akt and STAT-3 expression increased after 1 min, peaked between 10 and 30 min and returned to basal levels at 60 min. p-STAT1 increased significantly after 30 min of incubation and returned to baseline levels at 60 min (Figure 1c). One representative histogram for each phosphorylated molecule is shown in Supplementary Figure S2. In contrast, staining of FL cells incubated with hrIL-31 for the indicated times with mAbs to non-p Erk1/Erk2, STAT1, STAT3 and Akt did not reveal any change in the expression of these molecules compared with time 0 (not shown). These results were confirmed by western blotting analysis of primary FL cells incubated with hrIL-31. One representative experiment out of the three performed is shown in Supplementary Figure S3, where the densitometric values of the bands are shown.

Next, FL cells from 17 patients were cultured for 72 h without or with hrIL-31 or hrCD40L, which has been previously shown to induce proliferation of neoplastic B cells of GC origin.[35] As shown in Figure 1d, both IL-31 and CD40L significantly stimulated the proliferation of FL cells. No additive or synergistic effects on proliferation were found when FL cells were costimulated with hrCD40L and hrIL-31 (data not shown). Moreover, survival of lymphoma cells was not affected by stimulation with IL-31 in the presence or absence of CD40L (data not shown).

Characterization of IL-31 receptor in human GC B lymphocytes

IL-31RA and OSMR expression was next investigated in human GC B cells, which represent the normal counterpart of FL cells.[26] GC B cells purified from five tonsils were subjected to RT-PCR and flow cytometric analyses. IL-31RA and OSMR expression was detected at both mRNA (Figure 2a, left panel) and protein (Figure 2a, right panel) levels (median percentage of IL-31RA$^+$ cells = 77.0, range 52–94, median percentage of OSMR$^+$ cells = 63.5, range 40–84, $n = 7$) (a representative histogram for both the receptor chains is shown in Supplementary Figure S1B). In addition, both IL-31RA and OSMR were found to be expressed in tonsil tissue sections ($n = 6$) by scattered immunoreactive cells with lymphoid and monocytic morphology (Figure 2b, left and right panels, respectively).

The functional activity of IL-31R was next investigated by culturing five freshly isolated GC B-cell suspensions with hrIL-31 or medium for 1–60 min. AnnexinV staining showed that spontaneous apoptosis of GC B cells under these conditions was $< 5\%$ (data not shown). Moreover, no phosphorylation of Erk1/Erk2, STAT1, STAT3 and Akt was detected in GC B cells at any time and condition tested (data not shown).

In order to gain more insight into the differences in IL-31-driven signaling detected in normal GC B cells vs FL cells, we next investigated the internalization of the IL-31/IL31R complex in both cell types. Following incubation with hrIL-31, FL cells ($n = 4$) showed a significant reduction in surface expression of both IL-31RA and OSMR chains at 15 min, 30 min, 60 min and 3 h, indicative of ongoing internalization of both receptor chains. In contrast, GC B cells ($n = 4$) did not internalize IL-31RA or OSMR at any time tested, as assessed by flow cytometric analysis, following gating on AnnexinV$^-$ cells in order to exclude apoptotic cells (Figure 2c).

Previous studies have identified two different IL-31RA isoforms: a long one of 745 aa residues and a short one of 560 aa residues. IL-31R-driven signaling appears to be regulated by the ratio between the long and the short isoforms, the latter being considered as a dominant negative one.[19] Thus, we first analyzed the mRNA expression level of the previously described long and short IL-31RA isoforms in FL and GC B cells in comparison with those expressed in U937 and DU145 cells tested as controls.[19] FL cells expressed predominantly the long isoform (Pt: 73.5% vs 22.5%), similarly to the DU145 control cell line (long vs short: 60% vs 40%) (Figure 2d). In contrast, GC B cells expressed predominantly the short isoform (short vs long: 77.5% vs 22.5%), similarly to the U937 control cell line (short vs long: 62% vs 38%) (Figure 2d, representative samples out of the three tested are shown).

IL31R protein, however, can be translated from five different IL-31RA mRNA variants (Unigene Database from NCBI). The primers for the short isoform recognize all the five mRNA variants, whereas the primers for the long isoform do not discriminate among mRNA variants 1, 2 and 5, as indicated in Figure 3a. Thus we decided to get further insight on the predominant mRNA variant expressed by FL and GC B cells by designing a new set of primers described in Figure 3a. As shown in Figure 3b, IL-31R mRNA variants 1 and 2 codify for a long cytoplasmic region spanning from aa 580 to aa 764. These two variants differ because variant 2 lacks aa 1–21. IL-31R mRNA variants 3, 4 and 5 are translated into short isoforms; however, the products of variants 3 and 4 lack the terminal part of the cytoplasmic region, whereas the product of variant 5 lacks the terminal part of the extracellular region (Figure 3b). Variants 3 and 4 differ because variant 4 lacks aa 1–21, but both are devoid of the three tyrosine residues necessary for STAT activation.[20]

When the RNA from three FL and three GC B cells were tested with the panel of primers, the results indicated that the IL-31R mRNA variants expressed by neoplastic and normal cells were different (Figure 3c, where representative samples are shown). In particular, GC B cells did not express variants 1, 3, 4 and 5, expressing variant 2. Conversely, FL cells did not express variants 4 and 5, while expressed high level of variants 3 and 1.

The experiments on the expression of long and short isoforms and of the five mRNA variants strongly suggest that FL cells express IL-31R proteins with a complete extracellular region and a complete or incomplete cytoplasmic region. Conversely, GC B cells express IL-31R protein devoid of aa 1–21 but with a complete cytoplasmic region. These quantitative and qualitative differences may explain the lack of IL-31R-mediated signaling in GC B cells.

IL-31 expression in FL and GC B cells

The potential involvement of autocrine or paracrine loops related to the IL-31/IL-31R axis in FL cells was next investigated. To this end, IL-31 mRNA expression was first assessed in FL and GC B-cell fractions isolated from tonsil or reactive lymph nodes. As shown in Figure 4a, IL-31 mRNA was detected in FL cells, GC B cells and the Oci-Ly8 FL cell line, as well as in activated CD4$^+$ T cells tested as a positive control.[36]

Immunohistochemical analysis of four FL and six reactive lymph nodes showed a diffuse and intense expression of IL-31 in FL lymph nodes compared with reactive ones, in which the cytokine was detected more abundantly in the GC than in perifollicular areas (Figure 4b).

IL-31 protein secretion was next evaluated by ELISA in the supernatants from FL cells ($n = 18$), as well as from tonsil ($n = 10$) and reactive lymph node ($n = 4$) GC B cells that had been cultured for 24 h with hrCD40L and hrIL-4 to prevent spontaneous apoptosis.[28] The EA.hy926 cell line was tested as a positive control. IL-31 was detected in the supernatant from the latter cell line but not in the other supernatants tested (data not shown).

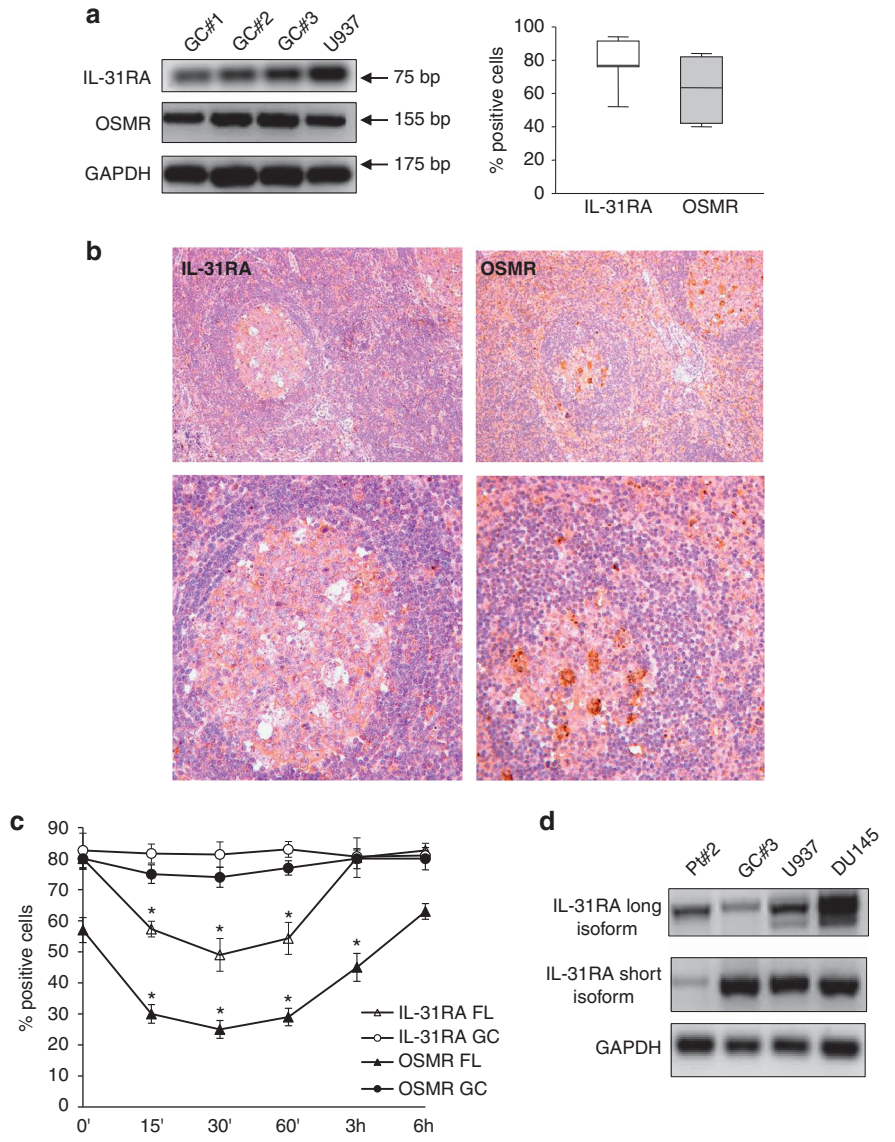


Figure 2. Expression and function of IL-31R in human GC B lymphocytes. (**a**) Left panel. GC B cells were purified from tonsil $CD19^+$ B cells and subjected to RT-PCR to determine mRNA expression levels for IL-31RA, OSMR and GAPDH genes. Three different GC B-cell suspensions out of the five tested are shown. The U937 cell line was tested as a positive control. Right panel. Surface expression of IL-31R was determined on purified GC B cells by flow cytometry. Results are expressed in box plot as the median percentage of positive cells, first and third quartiles and maximum and minimum values, from seven different GC B-cell suspensions. (**b**) Immunohistochemical analysis of IL-31RA and OSMR chains in tonsils tissue section (one representative staining out of the six performed). Original magnification, ×200 (upper panels) and ×630 (lower panels). (**c**) FL and GC B cells purified from four FL lymph nodes and four tonsils, respectively, were incubated with 100 ng/ml hrIL-31 for various time intervals (0 min, 15 min, 30 min, 1 h, 3 h, 6 h) and subsequently stained with antibodies to IL-31RA and OSMR. Results are the mean percentage of positive cells ± s.d. IL-31RA: $^*P = 0.05$ at 15, 30, 60. OSMR: $^*P = 0.05$ at 15 min, 30 min, 60 min, 3 h. (**d**) Expression of both short and long isoforms of IL-31RA mRNA levels in FL and tonsil GC B cells. One representative experiment out of the three performed for each cell fraction is shown. The DU145 and U937 cell lines were tested as controls.

Western blotting analysis of total cellular lysates from FL cells ($n = 3$), Oci-Ly8 cell line and tonsil ($n = 3$) and reactive ($n = 3$) lymph node GC B cells showed similar IL-31 protein expression levels, as assessed by densitometric analysis (Figure 4c). When cytosolic and membrane cellular fractions were separately analyzed, all samples showed significantly higher expression of membrane vs cytosolic IL-31, as indicated by densitometric analysis (Figure 4c).

The higher IL-31 expression detected by immunohistochemistry in FL vs reactive lymph nodes, together with the similar levels of cytokine detected by western blotting in purified FL and normal GC B cells, suggested that non-B cells present in the FL microenvironment contributed to IL-31 production. This latter hypothesis was also supported by the finding that such microenvironment is enriched for IL-4,[37] which is a well-known inducer of IL-31 expression.[38] To address this issue, we isolated non-B cells from FL and reactive lymph nodes by immunomagnetic bead manipulation and tested at the mRNA and protein levels by RT-PCR and western blotting, respectively. As shown in Figures 5a and b, all samples expressed IL-31 mRNA and protein; however, IL-31 protein expression was definitely higher in tumor vs reactive samples, indicating that non-B cells indeed contributed to the higher expression of the cytokine in FL lymph nodes.

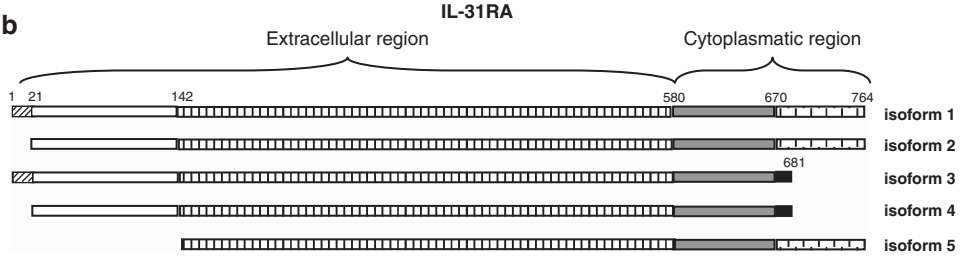
Immunofluorescence experiments were next performed to elucidate what non-B cell type(s) expressed IL-31 in FL vs reactive

a

primers	sequence 5'-3'	position variant 1	position variant 2	position variant 3	position variant 4	position variant 5
F short	GAC CAA GGT GGA GAA CAT TGG	1566-1586	1557-1577	1566-1586	1930-1950	1701-1721
R short	CTT CAG GTT TAG CTT ATC	2011-2028	2002-2019	2011-2028	2375-2392	2146-2163
F long	TGG AGT CCC TGA AAC GAA AG	1730-1749	1721-1740	1730-1749	2375-2392	1865-1884
R long	TTA GAC TTC TCC CTT GGT GTG C	2466-2487	2457-2478			2601-2622
F1	GCA ACT CAA GTT TTT CAC CAC G	204-225		204-225	204-225	
F2	CC TGG GCA CTG TGG ATG CTC	299-318	290-309	299-318	663-682	290-309
F3	GCC TGG CAG CTC TGC CAG C	338-356	329-347	338-356	702-720	
F4	AAA TTG ACT CAT CTG TGT TGG CC	1930-1952	1921-1943	1930-1952	2294-2316	2065-2087
R1	AAG TTG GGC AGG AAG ACA GAA T			2209-2230	2573-2594	
R2	GGT TTC ACA CGG AAA ATC TTA GG	661-683	652-674	661-683	1025-1047	796-818

primers		amplification product variant 1	amplification product variant 2	amplification product variant 3	amplification product variant 4	amplification product variant 5
A (F1 + R2)		479		479	843	
B (F2 + R2)		384	384	384	384	528
C (F3 + R2)		345	345	345	345	
D (F4 + R1)				300	300	
E (F4 + R long)		553	553			553

b



c

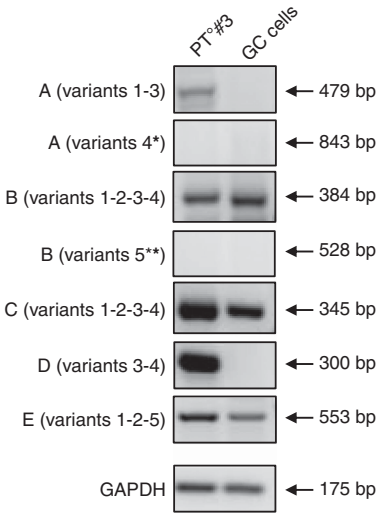


Figure 3. Expression of IL-31RA isoforms in FL and GC B cells. (**a**) Upper table: primers' sequences for IL-31RA mRNA variants and their position. Lower table: amplification products of primers in the five different IL-31RA variants. * or ** = amplification products with different bp for isoforms 4 and 5, respectively. (**b**) Schematic representation of the amino-acidic sequences for IL-31RA protein isoforms. (**c**) Patterns of amplification products obtained with the primers for the five different IL-31RA mRNA variants in three FL and three GC B-cell samples. One representative experiments out of the three performed is shown.

lymph nodes. As shown in Figure 5c, $CD4^+$ T cells and $CD68^+$ macrophages were found to express IL-31 in both types of lymph nodes, where $CD20^+$ B cells proved to be the major source of the cytokine with comparable expression in FL and reactive follicles. Of note, in reactive GCs, $CD68^+$ macrophages displayed IL-31 expression within tingible body vesicles, suggesting possible IL-31 uptake from GC B-cell removal, while FL-associated $CD68^+$ macrophages displayed a more homogeneous IL-31 expression pattern.

In vivo evidence for IL-31/IL-31RA involvement in FL growth

IL-31-driven stimulation of FL cell proliferation pointed to a role of the IL-31/IL-31R complex in FL progression. To gain more insight into this issue, we investigated IL-31 and IL-31RA protein expression in two TMAs from FL lymph nodes with different histological grading. The overall percentage of $IL\text{-}31^+$ cells was significantly higher in FL grade IIIa (median percentage of $IL\text{-}31^+$ cells = 85, range 50–90, $n = 8$) than FL grade I (median percentage of $IL\text{-}31^+$ cells = 26, range 10–60, $n = 6$) but not FL grade II (median percentage of of $IL\text{-}31^+$ cells = 60, range 50–70, $n = 6$) (Figure 6a and Supplementary Figure S4).

A statistically significant increment of $IL\text{-}31RA^+$ cells in FL grade IIIa (median percentage of $IL\text{-}31RA^+$ cells = 85, range 55–100, $n = 8$) compared with FL grade II (median median percentage of $IL\text{-}31RA^+$ cells = 40, range 10–80, $n = 6$) and FL grade I (median percentage of $IL\text{-}31RA^+$ cells = 25, range 5–50, $n = 6$) was also detected (Figure 6b and Supplementary Figure S4).

The increased expression of IL-31 and IL31RA in grade IIIa FL lymph nodes suggested the possible involvement of this complex in tumor progression, but this hypothesis was not supported by the failure to detected soluble IL-31 in FL cell supernatants. An alternative hypothesis was that the intercellular communication in FL lymph nodes might occur through release of IL-31 containing MVs.[39] Therefore we isolated plasma membrane MVs from grade IIIa FL cell suspensions ($n = 3$) and, for comparison, GC B-cell fractions ($n = 3$), the Oci-Ly8 FL cell line and the EA.hy926 cell line were tested as control. Dynamic light scattering analysis of MVs from all the above cell sources showed a bell-shaped size distribution profile, with a peak ranging from 334.5 ± 33.5 to 411.7 ± 21.6 nm and a polydispersity factor ranging from 0.155 ± 0.033 to 0.388 ± 0.062, indicative of homogeneous MV preparations (Figure 6c). AnnexinV staining confirmed the MV nature of the particles because of phosphatydylserine expression (data not shown).[40]

The presence of IL-31 in all MV preparations was next investigated by flow cytometry. As shown in Figure 6d, IL-31 was detected on MVs from all the samples analyzed (mean relative fluorescence intensity (MRFI) MVs FL: 3.9; MRFI MVs GC: 2.7; MRFI MVs Oci-Ly8:3.1;7. MRFI MVs EA.hy926: 7.2). These results are consistent with the hypothesis that IL-31 released by FL in MVs is involved in autocrine/paracrine loops supporting tumor growth. MVs isolated from FL patients with different grades ($n = 8$, 2 FL with grade I, 3 FL with grade II and 3 FL with grade III) did not differ for IL-31 content evaluated as MRFI (data not shown). In contrast, exosomes isolated from the Oci-Ly8 FL cell line and the EA.hy926 cell line, the latter tested as a control, did not contain IL-31 (data not shown).

We next investigated the signaling activity of MVs, isolated from the Oci-LY8 and Eahy.926 cell lines, on primary FL cells ($n = 3$). FL cells incubated for 30 min with or without the above MVs displayed a significant phosphorylation of STAT-1, indicating that MVs were biologically active (Figure 6e).

DISCUSSION

IL-31 is a recently discovered cytokine produced by different cell types, including T helper type 2 lymphocytes and mast cells, that has a role in the pathogenesis of cutaneous allergic diseases through itch stimulation[6,7,41] by binding to $IL\text{-}31RA^+$ sensory neurons.[36]

Limited information is available on the expression and/or function of the IL-31/IL-31R complex in malignant diseases. Different tumor cell lines have been reported to express IL-31 mRNA.[1,13–15] Furthermore, IL-31 was found to be produced by malignant T-cell populations from patients with cutaneous T-cell lymphomas,[22] with increased serum levels of the cytokine correlating to itch.[21] No information was so far available on the expression and the possible role of the IL-31/IL-31R complex in B-cell lymphomas.

Here we have addressed this issue by choosing FL as prototypic mature B-cell malignancy, as it represents the second most frequent B-cell lymphoma after diffuse large B-cell lymphoma.[23,24] In malignant B cells purified from FL patients IL-31R, composed of the IL-31RA and OSMR chains, was expressed both at mRNA and protein levels. Signal transduction studies showed that hrIL-31, upon binding to its receptor on primary FL cells, triggered phosphorylation of STAT1/3, ERK1/2 and Akt, similarly to that reported for other cell types.[10,16–18,20,30] Furthermore, hrIL-31 increased significantly proliferation of cultured FL cells over background levels. In contrast, GC B cells, although expressing IL-31RA and OSMR, did not signal in response to IL-31.

The different responsiveness of FL cells and GC B cells to IL-31 prompted additional studies addressing such issue. As ligand-induced internalization of signaling receptors usually correlates with initiation of signal transduction,[42] FL and GC B cells were cultured with hrIL-31 and tested at different times for the surface expression of IL-31RA and OSMR. FL cells displayed ligand-induced internalization of both IL-31RA and OSMR, whereas GC B cells did not, suggesting that such defect may be involved in lack of responsiveness of the latter cells to IL-31.

Previously it was shown that, despite co-expression of both IL-31R chains, only cell lines expressing predominantly the long isoform of IL-31RA signaled in response to IL-31 and that the short IL-31RA isoform operated as a dominant-negative receptor.[19] A similar dicotomy has been reported for other receptors such as those for prolactin, growth hormone and leptin, whose short isoforms 'silenced' the respective long isoforms.[43–45] Consistent with this background, signaling FL cells displayed prevalent expression of the long IL-31RA isoform, whereas non-signaling GC B cells expressed predominantly the short isoform.

Next, the expression of five IL-31RA mRNA variants was investigated in FL vs GC B cells. FL cells appeared to express mRNA variants 1, 2 and 3, whereas GC B cells appeared to express variant 2 only. Lack of mRNA variants translated into signaling IL-31RA isoforms might contribute to defective responsiveness of GC B cells to IL-31.

Experiments on lymph nodes from FL patients with different histological grading showed that the proportion of both IL-31$^+$ and IL31RA$^+$ FL cells was significantly higher in grade III compared with grade I/II FL. These results indicate that the progressive disruption of lymph node architecture is accompanied by increased expression of both IL-31 and IL-31RA. Additional experiments were performed to investigate whether other cell populations of the tumor microenvironment expressed IL-31. Both CD4 T cells and CD68 macrophages were found to express the cytokine.

In spite of IL-31 mRNA expression in FL cells as well as in GC B cells, soluble IL-31 was not detected in their culture supernatants. However, western blotting analysis of lysates from FL cells and GC B cells revealed the presence of IL-31 protein, with a significantly higher membrane vs cytosolic expression.

The majority of secretory proteins are externalized through the classical pathway, whereas others lacking hydrophobic signal sequence, such as IL-31, are released through different non-classical secretory pathways,[46] including MV shedding. MVs are small membrane vesicles shed from normal and malignant cells, either constitutively or following stimulation, that transport molecules involved in cell signalling and intercellular

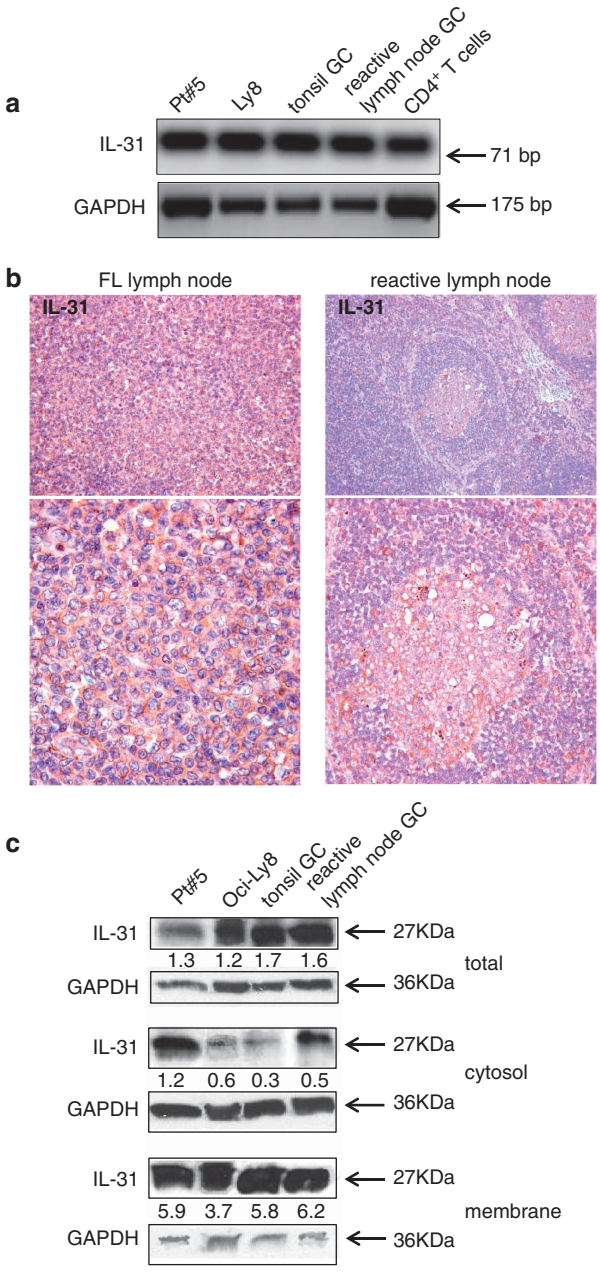


Figure 4. Expression of IL-31 in FL and GC B cells. (**a**) IL-31 and GAPDH mRNA expression were determined by RT-PCR in one FL patient (out of the three tested), Oci-Ly8 cell line, one tonsil and one reactive lymph node GC B-cell fractions (out of the three tested each) and one activated CD4$^+$ T-cell fraction (out of the three tested), tested as a positive control. (**b**) Immunohistochemical analysis of IL-31 in FL (one representative staining out of the six performed) and reactive (one representative staining out of the six performed) lymph node tissue sections. Original magnification, ×200 (upper panels) and ×630 (lower panels). (**c**) IL-31 protein expression was tested by western blotting analysis in FL cells (lane 1), Oci-Ly8 cell line (lane 2), tonsil (lane 3) and reactive lymph node (lane 4) GC B cells. One representative experiment out of the three performed for each cell fraction is shown. Total cytosolic and membrane cell lysates were subjected to western blotting with an antibody against hIL-31. GAPDH was used as a loading control. Numbers indicated the ratio between the intensity of the specific bands and that of the housekeeping protein bands, as assessed by densitometry. Membrane vs cytosolic fractions: $P < 0.0001$ for both FL and GC cells and Ly8 cell line; $P = 0.0002$ for reactive GC B cells.

communications.[39] MVs secreted from primary tumor cells mediate interactions with the tumor microenvironment and participate in the formation of metastatic niches.[39] Here we show that plasma membrane MVs from primary FL cells contained IL-31, and FL-derived MVs activated STAT-1 phosphorylation in primary FL cells. These data support the hypothesis that cell to cell transfer of the cytokine through MVs is involved in autocrine/paracrine loops promoting tumor growth.

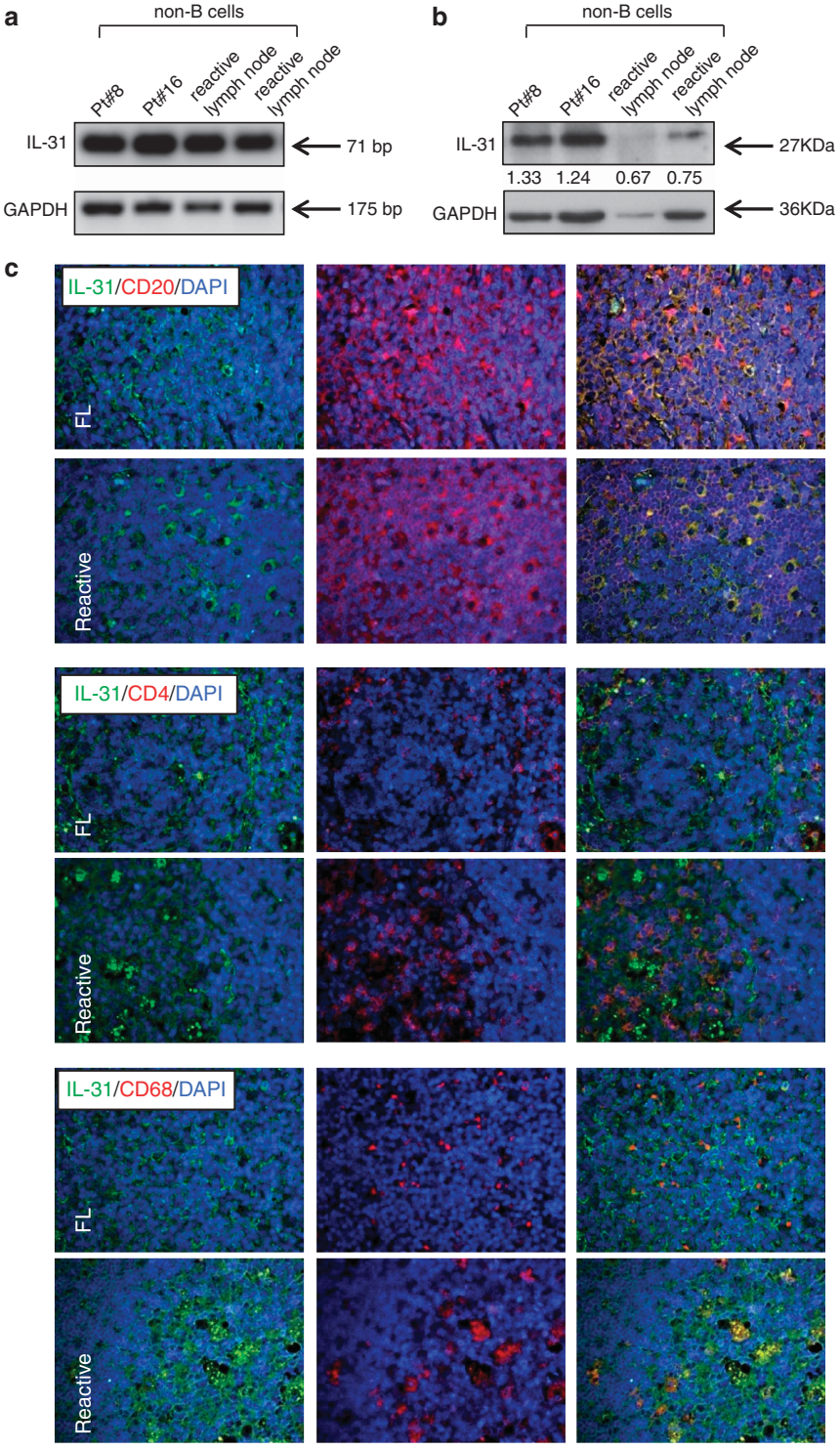


Figure 5. Expression of IL-31 in non-B cells from FL and reactive lymph nodes. (**a**) IL-31 and GAPDH mRNA expression were determined by RT-PCR in non-B cells from two FL patients (out of the four tested), two reactive lymph node (out of the four tested). (**b**) IL-31 protein expression was tested by western blotting analysis in non-B cells from FL (lanes 1 and 2) and reactive lymph node (lanes 3 and 4) GC B cells. Two experiments out of the four performed are shown. GAPDH was used as a loading control. Numbers indicated the ratio between the intensity of the specific bands and that of the housekeeping protein bands, as assessed by densitometry. (**c**) Double-marker immunofluorescence analysis of IL-31 (green) cells in combination with CD20 (red), CD4 (red) or CD68 (red) within neoplastic infiltrates of FL and reactive tissue. Original magnification, ×200. DAPI, 4,6-diamidino-2-phenylindole.

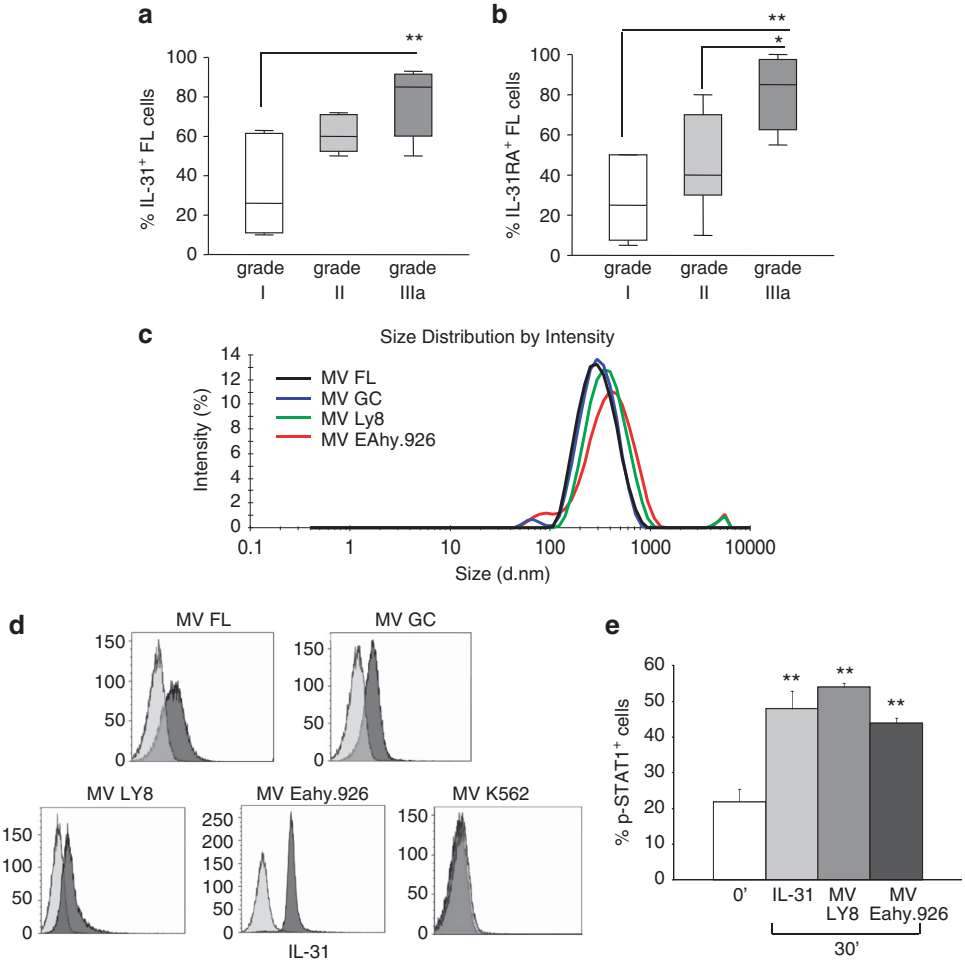


Figure 6. (**a**, **b**) IL-31/IL-31RA expression in relation to FL grading. Expression of IL-31 (**a**) and IL-31RA (**b**) in FL lymph node samples from patients with different histological grading was investigated by immunohistochemistry in TMA. Results are shown in box plot as the median percentage of positive cells, first and third quartiles and maximum and minimum values, from 20 different FL tissue sections. $^{**}P = 0.008$ for IL-31 grade I compared with grade III; $^{**}P = 0.002$ for IL-31RA grade I compared with grade III; $^{*}P = 0.014$ for IL-31RA grade II compared with grade III. (**c**, **d**) IL-31 expression in MVs from FL and GC B cells. (**c**) Dynamic light scattering analysis of MVs from FL cells (black line), GC B cells (blu line), Oci-LY8 (green line) and EA.hy926 cell lines (red line). One representative profile out of the three performed is shown. (**d**) Flow cytometric analysis of IL-31 content in MV preparations from FL cells, GC B cells (upper panels), LY8, EA.hy926 and K562 cell lines (lower panels). Grey histograms: IL-31 staining with a rabbit polyclonal antibody. Light grey histograms: rabbit serum control. One representative histogram for each MV preparation studied is shown. (**e**) Purified FL cells were incubated for 0 or 30 min with 100 ng/ml hrIL-31 or with MVs isolated from Oci-LY8 and EA.hy926 and subjected to flow cytometric analysis using an antibody to p-STAT1. Results are shown as the mean percentage of positive cells ± s.d. from three different experiments. $^{**}P = 0.002$ for MVs from both OCI-LY8 and EA.hy926.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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